Anti-diabetic and Anti-cancer Activities of Penta-O-galloyl-alpha-D-glucopyranose (alpha-PGG) and Its Derivative 6-chloro-6-deoxy-1,2,3,4-tetra-O-galloyl-alpha-D-glucopyranose (6Cl-TGQ)

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Yanyan Cao

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This dissertation titled Anti-diabetic and Anti-cancer Activities of Penta-O-galloyl-alpha-D-glucopyranose (alpha-PGG) and Its Derivative 6-chloro-6-deoxy-1,2,3,4-tetra-O-galloyl-alpha-D-glucopyranose (6Cl-TGQ)

by

YANYAN CAO

has been approved for

the Department of Biological Sciences

and the College of Arts and Sciences by

_________________________________

Xiaozhuo Chen
Associate Professor of Biomedical Sciences

_________________________________

Benjamin M. Ogles
Dean, College of Arts and Sciences
ABSTRACT

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Anti-diabetic and Anti-cancer Activities of Penta-O-galloyl-alpha-D-glucopyranose (alpha-PGG) and Its Derivative 6-chloro-6-deoxy-1,2,3,4-tetra-O-galloyl-alpha-D-glucopyranose (6Cl-TGQ) (256 pp.)

Director of Dissertation: Xiaozhuo Chen

Insulin receptor (IR) signaling is important for cell metabolism and proliferation. Dysfunction or dysregulation of the IR signaling results in metabolic syndromes or cancers. Previous studies show that Penta-O-galloyl-α-D-glucopyranose (α-PGG) binds IR α-subunit at a site different from insulin binding site and activates the IR signaling to trigger glucose uptake in 3T3-L1 adipocytes and reduce plasma glucose levels in diabetic mice. Interestingly, α-PGG was also found to inhibit adipogenesis, which is uncharacteristic of insulin function. Since α-PGG and insulin bind to different sites on IR, it was hypothesized that the IR activation signals produced by α-PGG may be different from that by insulin, enabling α-PGG to generate non-typical IR-mediated biological responses. To test this hypothesis, we compared the differences between α-PGG, 6Cl-TGQ (the derivative of α-PGG) and insulin in IR signaling activation rate and biological consequences. Both α-PGG and 6Cl-TGQ were slower and weaker than insulin in IR binding, IR signaling activation and early glucose uptake stimulation. Moreover, α-PGG and 6Cl-TGQ displayed biological functions different from insulin. Unlike insulin, α-PGG and 6Cl-TGQ inhibited glucose uptake and adipogenesis in 3T3-L1 preadipocytes with stronger inhibitory effects shown by α-PGG. Our further studies
indicated that α-PGG inhibited adipogenesis not through suppression of glucose uptake because addition of glucose did not reverse the adipogenesis inhibitory effects of α-PGG and α-PGG did not induce Wnt elevation and ERK activation at the middle stage of adipogenesis as glucose deprivation did. In addition to the differences in regulating adipogenesis, α-PGG and 6Cl-TGQ also behaved distinctly from insulin when treating cancer cells. While insulin promotes carcinogenesis, 6Cl-TGQ did not generate much effect and α-PGG induced cell cycle arrest and apoptosis in RKO cells. α-PGG induced apoptosis in RKO cells through activation of pro-apoptotic factors including p53, Bax and caspase 3. Importantly, the elevation of p53 and Bax by α-PGG was found to be mediated through the IR-MEK signaling pathway. This pathway has not previously been described for p53, Bax activation or apoptosis. Due to the insulin-unlike IR binding, α-PGG and its derivatives may offer a new strategy for the treatment of diabetes, obesity and cancer.

Approved: _____________________________________________________________

Xiaozhuo Chen

Associate Professor of Biomedical Sciences
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ABBREVIATIONS

3T3-L1: 3T3-Swiss albino is a cell line established from disaggregated Swiss mouse embryos and L1 is a continuous substrain of 3T3-Swiss albino developed through clonal isolation

6Cl-TGQ: 6-chloro-6-deoxy-1,2,3,4-tetra-O-galloyl-α-D-glucopyranose

ACC: acetyl-CoA carboxylase

Acrp30: adipocyte-related complement protein 30

AEMT: 3-(2-Aminoethyl)-5-((4-ethoxyphenyl)methylene)-2,4-thiazolidinedione

ADD1: adipocyte differentiation-dependent factor 1

AIF: apoptosis-inducing factor

AMPK: AMP-activated protein kinase

aP2: adipocyte-specific fatty acid binding protein

Apaf-1: apoptotic protease activating factor-1

α-PGG: Penta-O-galloyl-α-D-glucopyranose

BE: banaba extract

BMI: body mass index

Caspases: aspartate-specific cysterinyl proteases

CEA: serum carcinoembryonic antigen

C/EBP: CCAAT enhancer-binding protein

CHO: an immortalized cell line initiated from a biopsy of an ovary of an adult Chinese hamster
CHO-IR: Chinese hamster ovarian cancer cell line stably overexpressing wild type human insulin receptor

CREM: cAMP response element modulator

CS: calf serum

Cyt c: cytochrome c

Dex: dexamethasone

DFF: DNA fragmentation factor 45

DISC: death inducing signaling complex

DMEM: Dulbecco’s Modification of Eagle’s Medium

ECM: extracellular matrix

EndoG: endonuclease G

ER: endoplasmic reticulum

ERK: extracellular signal-regulated kinase

FADD: Fas-associated DD adapter protein

FAS: fatty acid synthase

FasL: Fas ligand

FBPase: fructose-1,6-bisphosphatase

FBS: fetal bovine serum

FHC: normal human colon epithelial cell

FFA: free fatty acids

G-6-Pase: glucose-6-phosphatase

GEFs: guanine nucleotide-exchange factors
**GH**: growth hormone

**GLP-1**: glucagon-like peptide-1

**GLUT**: facilitative Na⁺-independent sugar transporters

**GR**: glucocorticoid receptor

**GRB2**: growth receptor binding protein 2

**GS**: glycogen synthase

**GSK-3**: glycogen synthase kinase-3

**HPLC**: high performance liquid chromatography

**IBMX**: 3-isobutyl-1-methylxanthine

**IDDM**: insulin dependent diabetes mellitus

**IGF-1R**: insulin-like growth factor-1 receptor

**IP**: intraperitoneal

**IR**: insulin receptor

**IRR**: insulin receptor-related receptor

**KRP**: Krebs-Ringer phosphate buffer

**MAPK**: mitogen activated protein kinase

**MCE**: mitotic clonal expansion

**MCF-7**: human mammary gland (breast) adenocarcinoma cell line

**MCF-12A**: spontaneously immortalized non-tumorigenic human mammary gland epithelial cell

**MDI**: 3-isobutyl-1-methylxanthine, dexamethasone, insulin

**MEK**: MAP kinase kinase
**MEKK:** MAP kinase kinase kinase

**MKP:** mitogen-activated protein kinase phosphatase

**MMP:** matrix metallopeptidase

**mTOR:** mammalian target of rapamycin

**NIDDM:** non-insulin-dependent diabetes mellitus

**p70S6k:** 70 KDa ribosomal S6 kinase

**PC:** phosphatidylcholine

**PCD:** programmed cell death

**PDK:** 3-phosphoinositide-dependent protein kinase

**PEPCK:** phosphoenolpyruvate carboxykinase

**PI3K:** phosphatidylinositol 3-kinase

**PIP2:** phosphatidylinositol (4,5) bisphosphate

**PIP3:** phosphatidylinositol (3,4,5) triphosphate

**PKB or Akt:** Protein Kinase B

**PMA:** phorbol myristate acetate

**PP:** pancreatic polypeptide

**PPP:** Picropodophyllin

**PP1:** protein phosphatase-1

**PPARγ:** peroxisome proliferator activated receptor γ

**PTEN:** phosphatase with tensin homology

**PTPases:** protein tyrosine phosphatases

**PYY:** peptide tyrosine tyrosine
**Raf**: serine/threonine kinase

**RKO**: a poorly differentiated human colon carcinoma cell line containing wild-type p53

**RKO-E6**: a cell line generated from human colon carcinoma RKO by transfection with pCMV-E6 using Lipofectin, thus having decreased p53 levels and functions.

**RT**: reverse transcription

**RTK**: receptor tyrosine kinase

**RT-PCR**: real-time polymerase chain reaction

**SAPK/JNK**: c-Jun NH$_2$ terminal kinase

**SGLT**: Na$^+$-dependent glucose co-transporters

**SH2**: src homology 2

**SHIP2**: src homology 2 (SH2)-containing inositol 5'-phosphatase 2

**siRNA**: small interfering ribonucleic acid

**SMAC/DIABLO**: direct inhibitor of apoptosis binding protein

**SOS**: Son of sevenless

**SREBP1**: sterol regulatory element binding protein 1

**SREBPs**: sterol-regulatory element binding proteins

**TA**: tannic acid

**TBS**: tris buffered saline

**TNF**: tumor-necrosis factor

**TRAIL**: TNF-related apoptosis-inducing ligand

**UCP1**: uncoupling protein 1

**WGA**: wheat germ agglutinin

**XBP-1**: X-box-binding protein-1
INTRODUCTION

Insulin, Insulin Receptor and Insulin Receptor Signaling

Insulin

Insulin is a small peptide hormone produced in considerable amounts by the β cells in pancreatic islets of Langerhans. Insulin from different species varies only slightly in its primary structure and shares highly homologous sequences. Human insulin contains 51 α-amino acids which form 2 peptide chains, with Chain A having 21 amino acids and Chain B having 30 amino acids (Hua et al., 2008). Those two peptide chains are connected by two covalent disulfide bonds formed at A7-B7 and A20-B19 (Zhang, 1983). Besides, there is also an interior disulfide bond formed within Chain A bridging the amino acids A6 and A11 (Zhang, 1983). Those disulfide bonds help to maintain a relatively rigid α-helix secondary structure and tertiary structure of insulin. Acting in the tissues such as liver, muscle and adipose tissues, the main function of insulin is to regulate the absorption, conversion and storage of glucose to decrease plasma glucose level and maintain energy homeostasis. In addition, insulin, as an important growth factor, modulates cell growth and differentiation (Lee and Pilch, 1994).

Insulin receptor

Insulin receptor (IR) together with insulin-like growth factor-1 receptor (IGF-1R) and insulin receptor-related receptor (IRR), belongs to a subfamily of receptor tyrosine kinase (RTK) named Insulin receptor family (Patti and Kahn, 1998). Insulin receptor is highly homologous to its family members and can even form functional hybrids with
them. Unlike most membrane receptors, insulin receptor, instead of being monomeric or dimeric, is a 480 KDa heterotetrameric protein composed of two identical heterodimers, each of which contains one 135 KDa extracellular α-subunit and one 95 KDa transmembrane β-subunit (Fig. 1) (Lee and Pilch, 1994). The disulfide bonds inside insulin receptor function to stabilize the heterotetrameric structure by linking the α-subunits to the β-subunits to form heterodimers and at the same time coupling two heterodimers together to form heterotetramers (Finn et al., 1990). The 3 dimensional reconstruction of insulin receptor (Meyts and Whittaker, 2002) indicates that the two extracellular α subunits provide asymmetric binding sites for insulin to attach while the two transmembrane β subunits possess tyrosine kinase domains that are in a juxtaposition (close to each other in space) which allows tyrosine residues to be autophosphorylated and transphosphorylated upon insulin receptor activation by insulin binding (Luo et al., 1999). Insulin non-covalently binds to insulin receptor α subunits at specific sites with the help of electrostatic forces, hydrogen bonds, Van der Waals forces and hydrophobic forces. It was found that the binding of insulin and insulin receptor is mostly reversible and there are at least three binding affinity states (Gammeltoft, 1984). With more advanced techniques, recent studies revealed that the binding kinetics of insulin (wild type) with insulin receptor (wild type) fits a two-component sequential model (Whittaker and Whittaker, 2005). The receptor binding rate of insulin to its receptor is very high whereas the dissociation rate is quite low (Wanant and Quon, 2000). It was estimated that the high receptor binding affinity of insulin is at 0.03 nM and the low receptor binding affinity of insulin is at 0.4 nM (Whittaker and Whittaker, 2005). Binding of insulin with
insulin receptor causes the conformation of the receptor to change allosterically, which subsequently brings the tyrosine kinase domains of the receptor β subunits in adjacent to each other and results in the autophosphorylation of the receptor. Insulin receptors are widely distributed in all kinds of cells (Gammeltoft, 1984). And the receptors show tissue and species variations in insulin binding affinity, cooperativity and insulin recycle (Gammeltoft, 1984). The binding kinetics of insulin with insulin receptor enables insulin to actively modulate the receptor activity and the biological responses of the insulin target cells (Gammeltoft, 1984). Insulin receptor is the starting site of insulin signaling and its integrity is so important that when mutated or deleted, insulin signaling will be greatly impaired or even terminated. Defects of insulin receptor in one type tissue or multiple types of tissues can produce the pathology of type 2 diabetes (Saltiel and Kahn, 2001).

Figure 1. Schematic structure of insulin receptor. α indicates the α subunit while β indicates the β subunit of insulin receptor. The arrows point at the positions with one of the disulfide bonds, the transmembrane domain or the tyrosine kinase domain on the β subunit. This graph is adapted from (Meyts and Whittaker, 2002).
**Insulin receptor signaling**

The transmission of insulin signaling (Fig. 2) is initiated upon binding of insulin with insulin receptor, when insulin receptor undergoes a series of conformational changes and phosphorylates itself on the tyrosine residues of its transmembrane β subunits. The phosphorylated tyrosine residues serve as the docking sites and recruit several downstream substrates that have src homology 2 (SH2) domains (Morris et al., 2009; Yaoi et al., 2006). At least nine intracellular substrates of insulin/IGF-I receptor have been identified so far (Saltiel and Kahn, 2001). Among them, four belong to the insulin-receptor substrate (IRS) family: IRS-1, IRS-2, IRS-3 and IRS-4 (White, 1998). They are the major substrates of insulin receptor tyrosine kinase and, after being phosphorylated, take over most of the signaling relay task from the phosphorylated insulin receptor. Although IRS family proteins are highly homologous in sequence and structure, they are not redundant in function. Rather, they serve complementarily in insulin signaling transduction (Saltiel and Kahn, 2001). IRS-1 and IRS-2 are suggested by recent cell line and animal studies to play great roles in mediating insulin signaling, regulating carbohydrate metabolism and cell growth (Araki et al., 1994; Kido et al., 2000; Tamemoto et al., 1994; Withers et al., 1998). In contrast to IRS-1 and IRS-2, the functions of IRS-3 and IRS-4 are relatively unclear since IRS-3 and IRS-4 knockout mice show normal or close to normal growth and metabolism (Fantin et al., 2000). However, IRS-3 and IRS-4 are believed to have specific biological functions. There are some data suggesting that they may bring down IRS-2 levels, interrupt IRS-1 and IRS-2
phosphorylation and act as the negative regulators of other IRS proteins at several steps in insulin-like growth factor -1 (IGF-1) signaling pathway (Tsuruzoe et al., 2001).

**Insulin receptor signaling - PI3K/Akt pathway, the main metabolic pathway**

Phosphorylated IRS-1 and IRS-2 are in their active form and provide docking sites for multiple proteins with SH2 domains. Among them, a lipid kinase named phosphatidylinositol 3-kinase (PI3K) seems to be the predominant binding partner of the IRS proteins (Okada et al., 1994). PI3K is composed of a 85 KDa regulatory subunit and a 110 KDa catalytic subunit (Cheatham et al., 1994; Okada et al., 1994). The 85 KDa regulatory subunit is the phosphorylation target for the tyrosine kinase IRS while the 110 KDa catalytic subunit possesses innate kinase activity (Cheatham et al., 1994; Okada et al., 1994). Mediated by the phosphorylated tyrosine residues of the IRS proteins, PI3K is recruited to the plasma membrane of cells where its physiological substrate phosphatidylinositol (4,5) bisphosphate (PIP2) is located (Lizcano and Alessi, 2002). When in a juxtaposition with PI3K, PIP2 is phosphorylated by PI3K at position 3 of the inositol ring and is converted to phosphatidylinositol (3,4,5) triphosphate (PIP3) (Lizcano and Alessi, 2002). PIP3 regulates three major classes of signaling molecules: the TEC family tyrosine kinases, the guanine nucleotide-exchange factors (GEFs) of the Rho family GTPases, and the AGC family serine/threonine protein kinases (Jaffe and Hall, 2005; Mackay and Hall, 1998; Ziegler et al., 1993). Among them, one of the AGC kinases, 3-phosphoinositide-dependent protein kinase (PDK) is best characterized as being responsible for the phosphorylation and activation of several members of the AGC
kinase superfamily, including protein kinase B (PKB/Akt), p70 S6 kinase, and the two isoforms of atypical protein kinase C (PKC-ζ and PKC-λ) (Belham et al., 1999; Storz and Toker, 2002). Among the substrates of PDK, Akt is the one that has been paid most attention to for its central role in insulin signaling (Saltiel and Kahn, 2001). The activation of Akt requires both PIP3 and PDK1. The interaction of Akt with PIP3 brings Akt close to PDK1 and an unestablished PDK2 (Lizcano and Alessi, 2002). PDK1 and PDK2 phosphorylate Akt at Thr308 and Ser473 respectively (Lizcano and Alessi, 2002). Once phosphorylated, Akt is activated and dissociates from the plasma membrane (Lizcano and Alessi, 2002). A variety of cytosolic and nuclear substrates are then phosphorylated by Akt, generating numerous insulin-dependent bioactivities including glucose transport, glycogen synthesis, gluconeogenesis inhibition, protein synthesis and lipogenesis (Fig. 2).

Glucose is the most preferred building block and energy fuel for cells. Cells are able to take in glucose from extracellular environment. The glucose uptake processes involve the transport of glucose across plasma membranes and this occurs via integral transport proteins. These transporters comprise two structurally and functionally distinct groups: (i) the Na⁺-dependent glucose co-transporters (SGLT, members of a larger family of Na⁺-dependent transporters, gene name SLC5A); (ii) the facilitative Na⁺-independent glucose transporters (GLUT family, gene name SLC2A) (Joost and Thorens, 2001; Mueckler, 1994; Wright, 2001). SGLT promotes glucose transmembrane delivery through a secondary active transport mechanism whereas GLUT family facilitates thermodynamically downhill movements of glucose across the cytoplasmic membrane.
via a passive facilitative transport mechanism. Up till now, at least six Na⁺-dependent transporters, SGLT1 through SGLT6, have been identified (Wood and Trayhurn, 2003). They exist in the intestinal mucosa of small intestine (such as SGLT1), the proximal tubule of nephron (such as SGLT2) and other tissues to contribute to energy-dependent glucose reabsorption (Wright, 2001; Wright et al., 2007a). GLUT family has been receiving great attentions due to its functional importance. At least thirteen members have been classified into GLUT family, which are predicted to have typical twelve transmembrane regions with intracellularly located amino terminus and carboxyl terminus (Wood and Trayhurn, 2003). Based on the sequence homology comparison results, GLUT family members are classified into 3 classes: Class I: GLUT1-4; Class II: GLUT5, 7, 9 and 11; Class III: GLUT6, 8, 10, 12 and HMIT (Wood and Trayhurn, 2003). Among the three GLUT classes, Class I members are the best studied so far. GLUT1, which when absent gives rise to diseases such as Fanconi-Bickel syndrome, is ubiquitously expressed (Pascual et al., 2004). A particularly high level of GLUT1 expression has been recognized in mammalian erythrocytes and in endothelial cells lining the brain blood vessels (Dwyer and Pardridge, 1993; Zuo et al., 2003). Moderate levels of GLUT1 expression are observed in adipose tissue, muscle and liver (Zeller et al., 1994). GLUT3 is expressed particularly in neurons. Both GLUT1 and GLUT3 are constitutively expressed and continually transport glucose into cells at constant rates, maintaining the basal level glucose uptake. GLUT2 is expressed primarily in pancreatic β cells, liver, intestine and kidney (Watson and Pessin, 2001). In β cells, GLUT2 functions in the glucose-sensing system, while in liver it is expressed on the sinusoidal membrane of
hepatocytes and allows for the bi-directional transport of glucose under hormonal controls (Watson and Pessin, 2001; Wood and Trayhurn, 2003). GLUT2 is also found on the basolateral surface of the proximal renal tubules where it facilitates glucose and fructose transport and on intestinal epithelial cell membrane where it is responsible for the basolateral glucose delivery (Schaan et al., 2005). GLUT4 is the primary insulin-responsive glucose transporter that is predominantly localized in the adipose tissue and the striated muscle in heart and skeleton although it is also found in the brain (Vannucci et al., 1998). GLUT4, with the intrinsic activity regulated by insulin signaling, is responsible for the reduction in the postprandial rise of blood glucose levels (Niu et al., 2003; Somwar et al., 2002). GLUT4-null mice display insulin resistance, exhibit retarded growth, and show decreased longevity (Katz et al., 1995). Those GLUT4 knock-out mice also exhibit cardiac hypertrophy as well as severe reduction in adipose tissue mass (Katz et al., 1995). Muscle-specific GLUT4 knockout mice show severe insulin resistance and glucose intolerance from an early age (Zisman et al., 2000). Mice with adipose tissue-targeted GLUT4 reduction, although have normal growth status and adipose tissue deposits, exhibit glucose intolerance and hyperinsulinemia due to marked impairment in insulin sensitivity and glucose uptake activity in adipocytes, muscle and liver (Zisman et al., 2000). In contrast to the other GLUT family members, which are usually localized directly to the plasma membrane, GLUT4 is embraced by some specialized storage vesicles which at the basal state are cycling slowly between the plasma membrane and the intracellular compartments with the vast majority retaining within the cell interior (Bryant et al., 2002; Ploug and Ralston, 2002). Insulin triggers the translocation of those
specific GLUT4-containing vesicles from the cell interior to the plasma membrane by the means of largely increasing the rate of GLUT4 vesicle exocytosis and at the same time slightly decreasing the rate of GLUT4 internalization via endocytosis (Watson and Pessin, 2001). This eventually results in a rapid but reversible 10–20-fold increase in glucose transport (Kandror, 2003). Substantial data accumulated from multiple studies using various pharmacological inhibitors, dominant-interfering mutants, overexpression methods all consistently indicate that the activation of the type IA PI3 kinase and the subsequent generation of PI(3,4,5)P3 are essential for the insulin stimulation of GLUT4 translocation (Shepherd et al., 1998). Several other studies suggest a direct correlation between Akt function and insulin-stimulated GLUT4 translocation due to the observation that Akt activation results in persistent localization of GLUT4 to the plasma membrane and increased glucose transport whereby inactivation of Akt by dominant-negative Akt inhibits insulin-stimulated GLUT4 translocation to the plasma membrane (Czech and Corvera, 1999). Recent studies further indicate that Akt activation is required at a late stage of insulin-induced GLUT4 membrane translocation and facilitates the translocation probably through rearranging cytoskeleton (Brozinick et al., 2004; Imamura et al., 2003; van Dam et al., 2005). Besides the PI3K-Akt pathway, other signaling pathways such as the newly identified Crk-II/TC10 pathway are also involved in regulating GLUT4 membrane translocation (Ribon and Saltiel, 1997). However, the PI3K-Akt pathway is the most dominant GLUT4 translocation regulatory pathway (Gual et al., 2002).

Glycogen synthesis occurs in hepatocytes and muscle cells. Insulin stimulates glycogen accumulation through the activation of glycogen synthase (GS), a critical
enzyme in glycogen synthesis. GS is activated when dephosphorylated. Insulin promotes
the dephosphorylation of glycogen synthase through activation of protein phosphatase-1
(PP1) (Delibegovic et al., 2003; Syed and Khandelwal, 2000) together with inhibition of
kinases such as Akt and glycogen synthase kinase-3 (GSK-3) (Cross et al., 1995;
Mariappan et al., 2008).

The de novo synthesis of glucose (gluconeogenesis) happens generally in the liver
and the kidney with the liver being the major organ. Phosphoenolpyruvate carboxykinase
(PEPCK), fructose-1,6-bisphosphatase (FBPase) and glucose-6-phosphatase (G-6-Pase)
are the three critical enzymes in gluconeogenesis. Insulin potently inhibits
gluconeogenesis through stimulating the insulin receptor signaling especially the PI3K-
Akt signaling. It functions predominantly by suppressing the expression of the PEPCK
(Liao et al., 1998) and G-6-Pase genes (Barthel and Schmoll, 2003). Hepatic
gluconeogenesis is essential for the maintenance of normal blood glucose levels. In
patients with type 2 diabetes, a considerably increased rate of hepatic gluconeogenesis is
observed compared to the control group due to the lack of response of hepatocytes to
insulin (Basu et al., 2005).

The insulin-activated PI3K-Akt signaling also plays a role in protein synthesis. It
is known that the inactivation of GSK-3 by the PI3K-Akt signaling results in the
dephosphorylation of eIF2B at Ser535 (Wang et al., 2002). Together with additional
inputs, the dephosphorylation of eIF2B by the PI3K-Akt signaling activates eIF2B and
stimulates protein synthesis from amino acids (Wang et al., 2002). Besides, PI3K-Akt
signaling favors protein synthesis through the mammalian target of rapamycin (mTOR)
and p70S6k. mTOR is a member of the PI3K-related protein kinase family with the exceptional feature of acting primarily as a serine kinase (Raught et al., 2001). It relays the mitogenic signals from Akt to the downstream factors, regulates gene transcription and translation and coordinates cell growth in response to nutrition and hormones such as insulin. p70S6k has the similar function as mTOR. By phosphorylating the ribosomal S6 protein, p70S6k activates ribosome biosynthesis to promote the translation of the mRNAs with a 5’-terminal oligopyrimidine tract (Saltiel and Kahn, 2001). Since p70S6k directly regulates the protein synthesis machinery, it is not hard to understand that its activation is a functional requirement for insulin-mediated cell survival and growth in response to growth factors and stresses (Jonassen et al., 2004).

As a central signal molecule in insulin signaling pathway, Akt not only mediates glycogen synthesis and protein synthesis but also promotes fatty acid synthesis and inhibit lipid degradation. Fatty acid is synthesized primarily in the cytoplasm of hepatocytes and adipocytes. Akt drives forward lipogenesis at least partly by participating in the activation of sterol-regulatory element binding proteins (SREBPs), the major transcription factors that regulate gene expressions involved in fatty acid and cholesterol synthesis. Moreover, a DNA microarray study has identified several enzymes involved in fatty acid and cholesterol synthesis, such as pyruvate dehydrogenase, fatty acid synthase (FAS) and acetyl-CoA carboxylase, as targets for Akt regulation (Porstmann et al., 2005). In addition to promoting lipogenesis, insulin also strongly inhibits lipolysis primarily by inhibiting the hormone-sensitive lipase (Anthonsen et al., 1998). Through promoting
lipogenesis and inhibiting lipolysis, insulin largely increases lipid production and deposition.

**Insulin receptor signaling - phosphorylation cascade of MAP kinases, the mitogenic pathway**

Insulin is not just a potent metabolic hormone. It also functions as a growth factor, substantiating cell survival and promoting cell proliferation and differentiation. Similar to the other growth factors, insulin promotes mitogenesis and differentiation, at least in part, by stimulating mitogen-activated protein kinases (MAPKs). Insulin stimulates extracellular signal regulated kinase 1 and 2 (ERK1/2) phosphorylation up to 18 folds (Kayali et al., 2000). Like all other insulin-dependent signaling pathways, the insulin-initiated phosphorylation and activation of MAPKs originates from the interaction of insulin with its receptor. Activation of the insulin receptor by insulin results in tyrosine phosphorylation of its cellular substrates, including members of the IRS family of proteins and SHC (Fig. 2). Among those IR substrates, IRS-1 and SHC are most involved in MAPK stimulation (Fukunaga et al., 2000). Tyrosine-phosphorylated IRS1 or SHC interacts with growth receptor binding receptor 2 (Grb2), a small adaptor protein comprising one SH2 domain and two SH3 domains, by binding to its SH2 domain (Skolnik et al., 1993). Grb2 in turn recruits Son of sevenless (SOS) to the plasma membrane through the interaction between the SH3 domain of Grb2 and the proline-rich region of SOS. SOS is a ubiquitously expressed guanine nucleotide exchange protein. When recruited to the membrane, it activates Ras by catalyzing the conversion of the
GDP-bound inactive form of Ras to the GTP-bound active form of Ras (Margarit et al., 2003). In addition, the activation of Ras by insulin also requires the stimulation of SHPS-1 and SHP-2 (Saltiel and Kahn, 2001; Takada et al., 1998). SHPS-1 is a receptor-like protein that undergoes tyrosine phosphorylation at Tyr449 and Tyr473 in response to the stimulation of insulin and other mitogens (Takada et al., 1998). Through the phosphorylated tyrosine groups, SHPS-1 binds to SHP-2 and recruits SHP-2 from the cytosol to a site near the plasma membrane (Takada et al., 1998). SHPS-1 together with SHP-2 then recruits and phosphorylates SHC, which in turn binds and interacts with the Grb2-SOS complex, leading to the activation of Ras (Ling et al., 2005). Activated Ras operates as a molecular switch, triggering the sequential phosphorylation and activation of a serine/threonine protein kinase cascade that includes serine/threonine kinase (Raf, MAPKKK), MAP kinase kinase (MEK, MAPKK), extracellular signal-regulated kinase (ERK, MAPK), propagating a survival signal (Remacle-Bonnet et al., 2000). ERK, when phosphorylated and activated by MEK, can translocate into the nucleus where it regulates the transcriptional machinery by catalyzing the phosphorylation of transcription factors, leading to cell proliferation and differentiation (Boulton et al., 1991). Interestingly, the insulin-dependent activation of MAPK in adipocytes such as 3T3-L1 cells is mediated by the IRS-1/PI3K pathway instead of the Shc/Grb2/SOS pathway (Liu et al., 2000; Suga et al., 1997). This indicates that insulin-induced activation of MAPK is cell type specific and different types of cells employ different pathways to stimulate MAPKs (Suga et al., 1997). The cell type specificity of the pathways insulin chooses to stimulate the activation of MAPKs adds complexity to insulin signaling but at the same time provides a
mechanism for insulin to differentiate different types of cells and precisely regulate the growth of the whole body. Insulin-initiated signals are amplified greatly at almost every step along the MAPK cascade. Negative regulators are needed to attenuate or abrogate the signals when necessary. MAP kinase phosphatases (MKPs), for instance, are involved in the negative regulation of this insulin-stimulated MAPK activation pathway (Begum et al., 1996; Kusari et al., 1997). With both positive and negative regulatory machinery, MAPK pathway is under precise modulation, acting efficiently to regulate cell growth in response to insulin.
Figure 2. Insulin receptor signaling pathway diagram. Insulin receptor (IR) signaling pathway is mainly composed of two branch pathways: the IR – IRS – PI3K – PIP2 – PDK1/2 – Akt pathway and the IR – IRS or SHC – Grb2 – SOS – Ras – Raf – MEK – ERK pathway. The IR – PI3K – Akt pathway is mainly in charge of glucose homeostasis. The activation of the PI3K – Akt pathway by insulin and its analogs leads to glucose uptake, glycogen synthesis, gluconeogenesis, protein synthesis, lipid synthesis, lipolysis inhibition and cell growth. The IR – MEK – ERK pathway is conventionally considered as proliferative. Activation of the MAPK cascade by insulin and its analogs triggers cell proliferation and growth. However, recent studies start to shed light on the potential apoptotic consequence of MAPK signaling cascade activation. This diagram is adapted from Cell Signaling Technology (Cell Signaling Technology, 2009).
Negative regulators in insulin signaling pathways

It has been discussed above that insulin is potent in promoting glycogen, protein and lipid synthesis, inhibiting gluconeogenesis and lipolysis and at the same time stimulating glucose uptake through multiple insulin-dependent signaling pathways. Insulin acts as a stimulatory signal initiator in those pathways. To precisely regulate signaling pathways to generate appropriate cellular activities in response to environmental changes, not only positive stimulations are needed, but also negatively regulatory factors must be involved. And insulin-dependent signaling pathways are not exceptions. Negative regulators are necessary for the feedback inhibition and termination of insulin signaling. Generally, five different mechanisms are involved in the negative regulation of insulin signaling pathways: dissociation of insulin from its receptor, internalization of insulin receptors, direct blocking of molecular interactions, serine/threonine phosphorylation of insulin receptor and some IRS proteins, and the action of protein tyrosine phosphatases (PTPases).

Dissociation of insulin from its receptor is a direct way to terminate the continuous initiation of insulin signals. The dissociation of insulin from its receptor may happen because of the thermodynamic association/dissociation equivalence or due to the change of the affinity of insulin to its receptor as a result of possible conformation alternations. Internalization of insulin receptors is also a well-established feedback mechanism of cells by which they prevent themselves from being exposed to continuous insulin stimulation and protect themselves from possible harmful stimuli (Begum et al., 1985; Geiger et al., 1989). Those internalized insulin receptors undergo either
degradation or recycling during which those internalized receptors are redistributed to the cytoplasm membrane. Besides, physically blocking the interactions of signaling molecules is another method applied by cells to regulate the insulin receptor signaling. Those three negative regulatory mechanisms are undoubtedly essential for the regulation of insulin signaling. But perhaps the negative regulatory mechanisms arising from serine/threonine phosphorylation and protein tyrosine phosphatases action are even more important.

The change in phosphorylation state represents a widely-applied mechanism for controlling enzyme activities in cells. Insulin receptor and IRS proteins have tyrosine kinase activities and they all undergo serine phosphorylation in addition to tyrosine phosphorylation. Data suggest that this serine/threonine phosphorylation may act as a regulatory mechanism in insulin signaling pathways. Take IRS for example. Experimental results clearly show that serine/threonine phosphorylation of IRS can both positively and negatively regulate the tyrosine phosphorylation of IRS-1 and IRS-2 by IR (Greene and Garofalo, 2002). But it seems that most serine/threonine residues of IRS down-regulates insulin signaling when phosphorylated. For example, Ser24 (Greene et al., 2006), Ser302 (Werner et al., 2004), Ser307 (Gual et al., 2003), Ser312 (Greene et al., 2003), Ser332 (Lberman and Eldar-Finkelman, 2005), Ser408 (Liu et al., 2004), Ser612 (Gual et al., 2003; Mothe and Van Obberghen, 1996), Ser632 (Gual et al., 2003; Mothe and Van Obberghen, 1996), Ser636 (Bouzakri et al., 2003), Ser 662 (Mothe and Van Obberghen, 1996), Ser731 (Mothe and Van Obberghen, 1996), Ser789 (Qiao et al., 2002) and some additional serine residues in IRS-1 have been identified to play a key negative
modulating role in IRS-1 function and insulin action. And the inhibition or the termination of insulin signaling by the elevated serine/threonine phosphorylation of IRS occurs because serine/threonine phosphorylated IRS causes the dissociation of IRS from IR, the degradation of IRS proteins, and the blockage of the kinase activities of IRS (Paz et al., 1997). From above, serine/threonine phosphorylation is an important mechanism regulating IR signaling. And this mechanism is not restricted to IR signaling.

In addition, insulin action is also attenuated by protein tyrosine phosphatases (PTPases or PTPs), which directly catalyzes the dephosphorylation of the active (autophosphorylated) form of the insulin receptor, the cellular substrates of the insulin receptor kinase, such as IRS-1, and other phosphorylated proteins along the cellular cascade of insulin action. A number of PTPs, such as PTP1B, SH2-containing inositol 5’-phosphatase 2 (SHIP2), phosphatase with tensin homology (PTEN), TCPTP and TC45, have been identified and are considered to play a major role in regulating insulin signaling. PTP1B inhibits insulin signaling by interacting with the insulin-stimulated IR and dephosphorylating Tyr1162/Tyr1163 and Tyr972 in both of the IR β-subunits (Dadke et al., 2000; Galic et al., 2005; Romsicki et al., 2004). PTP-1B also maintains the IR in a dephosphorylated state during its biosynthesis, which further helps to attenuate insulin action (Romsicki et al., 2004). PTEN is capable of dephosphorylating PIP3 in vitro and down-regulating PIP3 levels in insulin-stimulated cells (Nakashima et al., 2000). In addition to PTP1B and PTEN, lipid phosphatase SHIP2 is also a physiologically critical regulator of insulin signaling, negatively regulating insulin-induced phosphorylation of Akt. Besides, other tyrosine phosphatases are also necessary for the precise regulation of
insulin signaling pathways and they are not redundant in function. For example, like PTP1B, TCPTP, TC45 also contribute to the negative regulation of IR signaling in vivo, recognizing the β-subunits of IR as a substrate (Galic et al., 2003).

**IGF-1R and IGF-1R Signaling**

It has long been noticed that IGF and IGF-1 receptor is highly homologous with insulin and insulin receptor respectively. Moreover, IGF-1 receptor signaling shares great similarity with IR signaling. In fact, it has been observed that IGF-1 may stimulate glucose transport through IGF-1R signaling as insulin does through IR signaling. Therefore, like IR signaling, IGF-1R signaling is also implicated in diabetes.

**IGF system**

Insulin-like growth factor (IGF) system is a complex regulatory network consisting of peptide hormones (IGF-1 and IGF-2), cell surface membrane receptors (IGF-1R and IGF-2R), circulating IGF binding proteins (IGFBPs: IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5, IGFBP-6) as well as the proteins involved in intracellular signaling distal to IGF-1R (IRS, Akt, Tor, S6 kinase, etc.) (Denley et al., 2005a).

IGF-1 is a single chain polypeptide with approximately 70 amino acids (Denley et al., 2005a). It has insulin-like short term metabolic effects and growth factor-like long term effects on cell proliferation and differentiation (Pollak et al., 2004). Most circulating IGF-1 is produced by the liver, but some is also synthesized in autocrine or paracrine-active organs other than the liver (Pollak et al., 2004). Regulation of IGF-1 production is
rather complex. Pituitary gland-derived growth hormone (GH) plays a dominant role in upregulating *igf1* gene expression. Similar to IGF-1, IGF-2 is a single chain polypeptide with about 67 amino acids and is expressed both in the liver and in extrahepatic sites (Pollak et al., 2004). But unlike IGF-1, IGF-2 is not tightly regulated by GH. Interestingly, both IGF-1 and IGF-2 share a common ancestry with insulin and have 45% amino acid homology with it (Laron, 2004). Moreover, IGF-1 and IGF-2 exhibit very close relationships with insulin in the structure of their receptors and the post-receptor signaling cascades. IGF-1R is the innate bioactive receptor for both IGF-1 and IGF-2. IR may bind to IGFs and IGF-1R may bind to insulin to a certain extent through cross interaction. However, the affinity of the cross binding of IR with IGFs and IGF-1R with insulin is about 500 fold lower than that of the binding of the ligands with their innate receptors (Table 1) (Denley et al., 2005a). Like IR, IGF-1R is a glycosylated transmembrane receptor tyrosine kinase composed of two extracellular alpha subunits (135 KDa) and two membrane spanning β subunits (95 KDa) (Czech and Corvera, 1999; Denley et al., 2005a). IGF-1 and IGF-2 reach IGF-1R via circulation delivery from remote sites or through local production. The local bioavailability of IGF-1 and IGF-2 for the receptors is regulated by IGF-2R, a 300 KDa protein which preferentially binds IGF-2 but lacks intracellular tyrosine kinase domain (Denley et al., 2005a). Meanwhile, the bioavailability of IGF-1 and IGF-2 for the receptors is also regulated by soluble IGFBPs, which have high affinity to IGFs and buffer the levels of IGFs through reversibly binding with IGFs to prevent them from interacting with IGF-1R while at the same time prolonging the half-life of IGFs (Denley et al., 2005a).
Table 1

**Binding Affinities of IGF-1, IGF-2 and Insulin for Receptors**

<table>
<thead>
<tr>
<th></th>
<th>Insulin (ED_{50} nM)</th>
<th>IGF-1 (ED_{50} nM)</th>
<th>IGF-2 (ED_{50} nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR-A</td>
<td>0.2</td>
<td>120</td>
<td>0.9</td>
</tr>
<tr>
<td>IR-B</td>
<td>0.3</td>
<td>366</td>
<td>11</td>
</tr>
<tr>
<td>IGF-1R</td>
<td>&gt;100</td>
<td>0.2</td>
<td>0.6</td>
</tr>
</tbody>
</table>

This table is adapted from (Denley et al., 2005a).

**IGF-1R signaling pathway**

IGF-1R, once bound with IGF-1 or IGF-2, undergoes conformational change which favors the activation of the intrinsic tyrosine kinase activity of the receptor. The activated receptor in turn initiates the signaling cascades which lead to a number of biological responses including cell survival, proliferation, growth, differentiation, migration and energy metabolism, and ultimately influence organism size, tumorigenesis and longevity (Fig. 3) (Baumann, 2002; Khandwala et al., 2000; Sonntag et al., 2005). The key signaling networks downstream of IGF-1R include the IRS-PI3K-Akt-GLUT4 pathway which stimulates glucose uptake in hepatocytes, adipocytes and muscle cells, the IRS-PI3K-Akt-mTOR-S6K pathway which relates to regulation of cell survival and mRNA translation, the IRS-SHC-GRB2-SOS-Ras-Raf-MAPK and the IRS-SHC-GRB2-SOS-Ras-MAPK-JNK pathways which mainly modulate cellular proliferation and differentiation (Fig. 3) (Laviola et al., 2007). Specifically, as a result of the binding of
IGF-1 or IGF-2 to IGF-1R and the subsequent activation of the receptor's intrinsic tyrosine kinase activities, the insulin receptor substrates, mainly IRS-1 and IRS-2, are phosphorylated on the tyrosine residues (Laviola et al., 2007). Tyrosine phosphorylated IRSs interact with cytosolic proteins bearing SH2 domains, such as PI3K (Laviola et al., 2007). PI3K activation transduces the functional effects of IGF-1, such as increased glucose transport and enhanced cell proliferation or growth (Laviola et al., 2007).

Alternative signaling pathways initiated upon IGF-1-receptor activation begin with the association of Grb2 (an adaptor protein) with SOS (a guanine exchange factor) and tyrosine phosphorylated SHC (Laviola et al., 2007). This then activates Ras, initiating a series of phosphorylation events involving MAP kinase kinase kinase (MAPKKK or MEKK), MEK, ERK and c-Jun NH2 terminal kinase (JNK) (Laviola et al., 2007; Lin et al., 2006). The IGF-induced MAPK phosphorylation cascades effectively modulate gene transcription through chromatin structure remodeling via histone 3 phosphorylation (Choi et al., 2005) and through regulation of transcriptional factors such as p90RSK (Ostrowski et al., 2000), c-Fos (Kidambi et al., 2009), CREB (Fernández et al., 2005), ATF (Recio and Merlino, 2002), PPARγ (Taketa et al., 2008), and Elk-1 (Uht et al., 2007). Different IGF-1R signaling pathways can communicate with each other, working synergistically or oppositely (Shelton et al., 2004; Zhang et al., 2004a). Noteworthily, these signaling pathways share great similarity with what are in the IR signaling and have a high frequency of cross-talk with the IR signaling. Like most signaling pathways, IGF-1R signaling is highly regulated. As indicated before, the accessibility of IGFs to IGF-1R is modulated by IGF-2R and IGFBPs (Denley et al., 2005a). The post-receptor activation of
the IGF-1R signaling is regulated by the same mechanisms as those in the IR signaling regulation, such as the dephosphorylation of signaling kinases by phosphotases and the phosphorylation of signaling molecules on the inhibitory serine/threonine residues. For example, suitable doses of IGF-1 activates Raf through phosphorylating Ser296 or Ser338 (Moelling et al., 2002; Subramaniam et al., 2005) whereas high doses of IGF-1 strongly suppresses Raf kinase activity via phosphorylating the Ser259 residue (Moelling et al., 2002; Subramaniam et al., 2005).
Figure 3. IGF-1R activation and its downstream signaling pathways. Insulin-like growth factor 1 (IGF-1) has insulin-like short term metabolic effects and growth factor-like long term effects on cell proliferation and differentiation. The binding of IGF-1 to IGF-1R activates the receptor's intrinsic tyrosine kinase activities, which initiates several signaling pathways including the IRS-PI3K-Akt-GLUT4 pathway which elevates glucose uptake in hepatocytes, adipocytes and muscle cells, the IRS-PI3K-Akt-mTOR-S6K pathway which relate to regulation of cell survival and mRNA translation, and the IRS-SHC-GRB2-SOS-Ras-Raf-MAPK or IRS-SHC-GRB2-SOS-Ras-MAPK-JNK pathway which mainly modulates cellular proliferation and differentiation. Recently studies revealed that prolonged activation of IGF-1R signaling (Stewart et al., 1999a) or MAPK (Goulet et al., 2005; Isoda et al., 2009) signaling may lead to apoptosis. This diagram is adapted from Cell Signaling Technology (Cell Signaling Technology, 2009) and (Korean UniGene Information, 2009).
Similarity and differences between IR and IGF-1R

Despite the significant structural and signaling cascade similarities between IGF-1, IGF-2 and insulin, results accumulated from some genetic studies indicate that each ligand can result in unique signaling outcomes and their physiological functions are distinct (Kim and Accili, 2002). For instance, IGF-2 is unable to compensate for the loss of IGF-1 activity and patients with an IGF-1 deficiency exhibit severe growth and mental retardation (Denley et al., 2005b; Walenkamp et al., 2005; Woods et al., 1997). Furthermore, cDNA microarrays analysis of mouse fibroblast NIH-3T3 cells indicates that more than half of the genes up-regulated by IGF-1 are associated with mitogenesis and differentiation, whereas none of the genes specifically up-regulated by insulin are associated with these processes, suggesting that IGF-1 is different from insulin in biological function and is a more potent activator of the mitogenic pathway than insulin (Dupont et al., 2001). Nevertheless, there is limited evidence from cell models suggesting that their signaling capabilities differ significantly. Thus, it remains unclear whether the different physiological roles of IR and IGF-1R result from extrinsic differences, such as tissue distribution, relative abundance and developmental regulation or arise from intrinsic differences in their abilities to activate distinct signaling pathways which have not yet been discovered (Kim and Accili, 2002).

Involvement of IGF-1R signaling in cancer, diabetes and obesity

Because IGFs, particularly through the IGF-1R-PI3K-Akt and IGF-1R-Ros-Raf-MAPK signaling, promote cell proliferation, they are considered to have a considerable
impact on cancer development (Holly, 2004; Pollak et al., 2004). For instance, IGF-1 stimulates cell cycle progression in breast cancer cells (Mawson et al., 2005), colorectal cancer cells (Probst-Hensch et al., 2001) and prostate cancer cells (Orio et al., 2002).

Meanwhile, IGFs, especially IGF-1, show intimate relationship with other pathological processes such as diabetes and obesity. IGF-1 helps to prevent and relieve both type 1 and type 2 diabetes. As evidence, mice with liver-specific igf1 gene deletion (Yakar et al., 2001) and transgenic mice with skeletal muscle-specific overexpression of a dominant-negative IGF-1R (Yakar et al., 2005) develop insulin resistance and type 2 diabetes secondary to the elevation of circulating growth hormone levels. Moreover, IGF-1 suppresses and delays the onset of type 1 diabetes in non-obese diabetic (NOD) mice (Kodama et al., 2004). Even more excitingly, IGF-1 have demonstrated its potential in lowering blood glucose and serum insulin levels and improving insulin resistance in subjects with type 2 diabetes in small-scale clinical trials (Moses, 2005). However, the mechanisms by which IGF-1 delays the onset of type 1 diabetes and improves insulin resistance have not been well-understood. It is possible that IGF-1 suppresses and delays the onset of type 1 diabetes through promotion of β cell development and survival via the IRS-2 signaling pathway (Withers et al., 1999), through maintenance of insulin secretion by affecting cellular ATP levels (Shepherd et al., 1998) and through amplification of the Th2-type response (Kodama et al., 2004). And it has been documented that IGF-1 improves insulin resistance and relieves type 2 diabetes through a certain mechanism possibly independent of IR activation (Haluzik et al., 2003; Moses, 2005). While showing some beneficial effects on both type 1 and type 2 diabetes, IGF-1, unfortunately,
deteriorates certain complications of diabetes, making the therapeutic role of IGF-1 in treating diabetes controversial. For instance, the excessive production of IGF-1 could be a major contributor to the development of diabetic glomerulosclerosis because it contributes to the altered extracellular matrix (ECM) turnover mediated by a reduction in matrix metallopeptidase (MMP)-2 activity (Lupia et al., 1999). Also, it is observed that upregulation of serum IGF-1 precedes and causes retinal deterioration in diabetic patients (Chantelau, 1998). It has not yet been evidenced that IGF-1 directly causes obesity. But the involvement of the IGF-1R signaling in adipogenesis has been identified. MDI cocktail (3-isobutyl-1-methylxanthine (IBMX), dexamethasone (Dex), insulin) triggers 3T3-L1 adipogenesis through the IR/IGF-1R signaling. It has also been observed that free IGF-1 concentrations are higher in obese subjects than in normal controls (Nam et al., 1997). It is likely that overnutrition and chronic hyperinsulinemia in obesity may increase circulating IGF-1 levels by insulin stimulation of IGF-1 production and suppression of hepatic IGFBP-1 and IGFBP-2 production (Nam et al., 1997; Tappy et al., 1993). And the upregulated IGF-1 might in turn promote obesity and obesity-related diabetes.

The Origin of Penta-O-galloyl-α-D-glucose and 6-Chloro-6-deoxy-1,2,3,4-tetra-O-galloyl-α-D-glucopyranose

Tea has long been widely consumed in Asian countries like China, Japan and Philippines, and is now becoming increasingly popular in western countries. Tea is good for health, helping reduce oxidant stress, enhance immune system, suppress the progression of tumors, and improve cardiovascular diseases and obesity (Crespy and
Williamson, 2004; Rizvi et al., 2005; Shimada et al., 2004). Epidemiologically, tea consumption has been suggested to prevent and improve type 2 diabetes and some kinds of herbal tea, such as cerassee tea (Bailey et al., 1985) and Xiaoke tea (Hale et al., 1989) has even traditionally been used as folk remedies for diabetes. Furthermore, the improvement of glucose metabolism in both diabetic and healthy subjects upon tea consumption has been verified by recent studies (Han, 2003; Hosoda et al., 2003; Tsuneki et al., 2004; Wu et al., 2004).

*Lagerstroemia speciosa L.*, known by its Tagalog name *banaba*, is a very popular drink in Philippines (Kakuda et al., 1996). Notably, it shows hypoglycemic effects and has been well-received by diabetic patients as a traditional remedy (Kakuda et al., 1996). Since 1996, interest has arisen in the identification of the banaba component(s) that exert these glucose-lowering effects. Preliminary studies have found that hot water extract obtained from banaba leaves, or banaba extract (BE) have beneficial effects on controlling blood glucose levels in genetically diabetic mice (Type II, KK-Ay), suggesting that the component(s) effective in the treatment of non-insulin-dependent type 2 diabetes are hydrolyzable (Kakuda et al., 1996; Suzuki et al., 1999). The observation of the insulin mimic activity of BE in triggering glucose uptake in 3T3-L1 cells further supports this supposition (Liu et al., 2001). Interestingly, unlike insulin, BE shows anti-obesity activity *in vitro* and *in vivo*. *In vitro*, it is unable to initiate adipocyte differentiation and even inhibits 3T3-L1 preadipocyte differentiation induced by the 3-isobutyl-1-methylxanthine, dexamethasone, insulin or MDI cocktail in a dose-dependent manner (Liu et al., 2001). *In vivo*, it promotes weight loss in obese female KK-Ay mice (Suzuki et al., 1999).
Moreover, according to some preliminary research stated by the BulkBanaba company, a dosage of 30 μg/ml of Banaba extract is effective in countering tumor cells (BulkBanaba, 2004). These observations raise a question whether the glucose uptake promoting effect and the anti-obesity, adipogenesis inhibitory and anti-cancer effects are exerted by the same component or different components in BE. Since BE is a mixture of compounds, this question was not answered by the time those phenomena were observed.

To target the critical components in BE, a series of separation and purification work was performed using HPLC (Liu et al., 2005). With glucose uptake assays and Western/Northern blot analyses as major tools and 3T3-L1 cells as a model, it has been found that the BE with tannin removed is devoid of both the glucose transport stimulatory activity and the adipocyte differentiation inhibitory activity (Liu et al., 2005). On the other hand, tannic acid (TA), a major component of tannins, possesses activities similar to BE (Liu et al., 2005).

Tannins are the polyphenolic secondary metabolites of higher plants with a molecular weight between 500 and 3000 Da (Baxter et al., 1997; Haslam et al., 1989; Khanbabaee and van Ree, 2001). They are broadly distributed and are considered to be part of the plant’s natural defense system against environmental stressors (Bennick, 2002). The antinutritive and toxic effects of tannins are well-known (Baxter et al., 1997; Bennick, 2002). Those biological functions of tannins depend largely on their protein-precipitating properties mediated via hydrophobic forces and hydrogen bonds (Baxter et al., 1997; Bennick, 2002). In addition to the originally identified antinutritive and toxic effects, tannins possess multiple bioactivities that are beneficial to human health, such as
immune modulatory activities (Chung et al., 1998), antioxidant activities (Hagerman et al., 1999; Fedeli et al., 2004), anticarcinogenic activities (Chung et al., 1998), and antimicrobial activities (Kolodziej et al., 1999; Chung et al., 1998).

Gallotannins are the simplest hydrolysable tannins that are composed of a central core of D-glucose whose hydroxyl groups are esterified with gallic acid(s) (Khanbabaee and van Ree, 2001). The anomeric center (1’-site) of the glycosidic residues of the gallotannins can be present in either an α- or a β- form. Most of the natural gallotannins that have a galloyl group at the anomeric center of the D-glucosyl residue exist in a β-configuration. Like most gallotannins, Penta-O-galloyl-D-Glucose (PGG), one of the simplest representative hydrolysable gallotannins, has two anomeric forms, α-configuration and β-configuration (Fig. 4). Penta-O-galloyl-β-D-glucose (β-PGG) can be found in many plants while Penta-O-galloyl-α-D-glucose (α-PGG) rarely occurs naturally. However, both α-PGG and β-PGG are available via a relatively simple chemical synthesis (Khanbabaee and Lo” tzerich, 1997). PGG derived from chemical synthesis is a mixture containing both anomers. Based on their chemical properties, the two anomers can be separated and purified using HPLC and crystallization.

β-PGG is one of the key intermediates in the biosynthesis of almost all hydrolysable polyphenols in plants. Functional studies of β-PGG have revealed the multifunctional characteristics of β-PGG. β-PGG strongly inhibits Na(+)K(+)−ATPase activity (Satoh et al., 1997), inactivates human placenta aldose reductase (Sawada et al., 1989), suppresses the formation of oxidative stress (Bhimani et al., 1993), decreases IL-8 expression in phorbol myristate acetate (PMA)-treated human monocytic U937 cells
through a mechanism involving IκBα degradation-dependent inhibition of NFκB (Oh et al., 2004), protects neuronal cells via induction of neuronal heme oxygenase-1 expression (Choi et al., 2002), and prevents tumor formation by inducing apoptosis (Huh et al., 2005; Pan et al., 1999), unfavouring mutation, and inhibiting tumor proliferation (Oh et al., 2001), angiogenesis (Huh et al., 2005; Lee et al., 2004) and metastasis (Ho et al., 2002).

Interestingly, an additional function of chemically synthesized β-PGG is characterized as it can stimulate glucose uptake in 3T3-L1 cells possibly via insulin signaling pathways (Li et al., 2005). Considering that β-PGG is one of the components of TA and both β-PGG and TA have similar glucose uptake stimulatory activity, it is believed that β-PGG is one of the components in TA that exert this effect.

Since α-PGG, the natural anomer of β-PGG, can hardly been found in natural sources, studies about α-PGG are largely delayed, leading to the lack of knowledge about the bioactivities of α-PGG. There is only one study reporting that α-PGG increases the adhesion between apposing phosphatidylcholine (PC) bilayers and when α-PGG is within a very narrow concentration range, collapses the interbilayer fluid space from about 15 Å to 5 Å (Huh et al., 1996). Seldom had there been any report about α-PGG since then until quite recently when the chemically synthesized α-PGG was found to possess glucose uptake stimulatory activity as β-PGG does and surprisingly, α-PGG is even more potent than β-PGG in triggering glucose uptake (Li et al., 2005). Because α-PGG shows better glucose transport stimulatory activity than β-PGG, a group of compounds based on the chemical structure of α-PGG were synthesized by Dr. Yulin Ren in Dr. Xiaozhuo Chen’s laboratory in collaboration with Dr. Klaus Himmeldirk, aiming at finding better anti-
diabetic or glucose transport promoting reagents. Among all the synthesized α-PGG analogs, a compound named 6-chloro-6-deoxy-1,2,3,4-tetra-O-galloyl-α-D-glucopyranose (6Cl-TGQ) was found to possess the best glucose uptake stimulatory activity (Ren et al., 2006).

![Diagrams of galloyl group, α-PGG, β-PGG, and 6Cl-TGQ](image)

**Figure 4.** Primary structures and computative 3D structures of PGG and their derivative 6Cl-TGQ. PGG consists of a central core of D-glucose, five of whose hydroxyl groups are esterified with galloyl groups. The anomeric carbon center of the glycosidic residues of PGG can be presented in either an α- or a β- form. 6Cl-TGQ is derived from α-PGG. The structure difference between 6Cl-TGQ and α-PGG resides in the side chain attached to the carbon of position 6. While α-PGG has an esterified galloyl group on carbon 6 of the glucose core, 6Cl-TGQ has a chloro group at the same position. This graph is adapted from (Ren et al., 2006).
SECTION I

ANTI-DIABETIC ACTIVITIES OF PENTA-\textit{O}-GALLOYL-\textit{\textalpha}D-
GLUCOPYRANOSE AND ITS DERIVATIVE 6-CHLORO-6-DEOXY-
1,2,3,4-TETRA-\textit{O}-GALLOYL-\textit{\textalpha}D-GLUCOPYRANOSE
INTRODUCTION

Diabetes Mellitus

Diabetes Mellitus, normally termed as diabetes, is a syndrome of metabolic disorders characterized by hyperglycemia (abnormally high blood glucose levels). Diabetes is resulted directly from defects in production, secretion and/or utilization of insulin, a key hormone regulating glucose and lipid homeostasis and energy balance. Hyperglycemia in diabetes leads to acute signs including excessive urine production (mellitus), thirst, blurred vision, weight loss, lethargy and change in body metabolism (Campbell, 1992; Hirsch, 1996). Chronic hyperglycemia causes damage to the eyes, kidneys, nerves, hearts and blood vessels and eventually leads to blindness, kidney failure, nerve damage, cardiovascular diseases, cancers and may finally threaten life (Steppan et al., 2001).

Diabetes has become more prevalent as an epidemic than ever before due to population aging, excessive food intake and sedentary life style in modern society. The number of people with diabetes in the world has already reached an alarming level, and a tremendous increase is still expected in the coming decades. International Diabetes Federation estimated in the year 2009 that more than 250 million people in the world have diabetes (International Diabetes Federation, 2009). And the number of people who have diabetes is expected to increase at a rate of a further 7 million people each year (International Diabetes Federation, 2009). Accordingly, the number of people suffering from diabetes would reach 390 million within the next 20 years. Diabetes happens more frequently in the developed countries such as the United States. Reported by American
Diabetes Association in 2009, there are 23.6 million children and adults in the United States, or 7.8% of the population, who have diabetes (American Diabetes Association, 2009). The high prevalence of diabetes calls for urgent attention and requires intensive research in the pathological causes and the therapeutic methods of diabetes.

Presently, diabetes is mainly classified into two types: type 1 diabetes and type 2 diabetes. However, there is no agreement on the standard nomenclature. For instance, various sources have defined type 3 diabetes or type 1.5 diabetes for the type 1 diabetes that shows insulin resistance or the type 2 diabetes that requires external insulin supplement. Besides, gestational diabetes has been defined as the glucose intolerance with the first onset during pregnancy. Nevertheless, type 1 diabetes and type 2 diabetes are the most recognized types of diabetes. Type 1 diabetes, formerly called Insulin-Dependent Diabetes Mellitus (IDDM) or juvenile diabetes, is characterized by insulin deficiency (1997; Lang et al., 2005). The deficiency of insulin production in type 1 diabetes is due to the destruction/loss of insulin-producing pancreatic β cells (Kapoor, 2008; Lang et al., 2005; Liadis et al., 2005). This β cell destruction is either immune-mediated or idiopathic. It is considered that the majority of type 1 diabetes is immune-mediated where β cell destruction is due to T-cell mediated autoimmune attack (Kapoor, 2008; Lang et al., 2005; Liadis et al., 2005; Rother, 2007). Thereby, type 1 diabetes has been widely accepted as a progressive autoimmune disease. The β cell loss in type 1 diabetes results in insulin deficiency, which consequently causes hyperglycemia. Individuals suffered from type 1 diabetes are insulin-dependent, relying on injection of insulin to maintain normal blood glucose levels, prevent ketosis and preserve life.
Although type 1 diabetes can develop at any age, it usually appears under the age of 30, from which it earns the name juvenile diabetes. It is estimated that approximately 0.12% or 340,000 people in the US have type 1 diabetes (WrongDisgnosis, 2009). And about 30,000 new cases of type 1 diabetes are reported annually (WrongDisgnosis, 2009). Type 2 diabetes was previously called non-insulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes. However, type 2 diabetes, although not frequently, is also being diagnosed within children and adolescents, especially among African Americans, American Indians, Asians/Pacific Islanders, and Hispanic/Latino Americans (National Diabetes Information Clearinghouse of National Institute of Diabetes and Digestive and Kidney Diseases, 2007). Like type 1 diabetes, type 2 diabetes is characterized by hyperglycemia. Unlike type 1 diabetes, which is known to be caused by insulin deficiency due to the dysfunction of pancreatic \( \beta \) cells, the direct cause of type 2 diabetes is mainly insulin resistance, the lack of response of insulin target cells to insulin and hyperinsulinemia is found during the early and mid stages of type 2 diabetes (Groop et al., 1993). The late stage of type 2 diabetes, however, often resembles type 1 diabetes when destruction of insulin-producing pancreatic \( \beta \) cells and lack of functional insulin secretion appear as pathological symptoms (Giddings et al., 1985). The exhaustion of pancreatic \( \beta \) cells in the late stage of type 2 diabetes is thought to be due to the long-term high demands of insulin production for compensating the insensitivity of insulin responsive cells (Escribano et al., 2009). The pathological progress of type 2 diabetes is usually slow but chronic, which means type 2 diabetes can be a lifelong disease. Patients with type 2 diabetes may encounter different problems at different stages of this disease.
and the symptoms appear at the late stage of type 2 diabetes resemble that of type 1
diabetes. Type 2 diabetes is much more prevalent than type 1 diabetes. It largely
outnumbers all other types of diabetes and has become an epidemic (Stumvoll et al.,
2005). About 90% of diabetic individuals have Type 2 diabetes (Fajans et al., 2001). The
global prevalence of type 2 diabetes mellitus in children, adolescents and adults is calling
for serious attention (Daly, 2003; Pinhas-Hamiel and Zeitler, 2005).

Although diabetes is a long-term serious condition, people with diabetes still have
the chance to live a long, healthy and happy life with the help of low sugar low calorie
diets, increased exercises, anti-diabetic drugs and even organ or stem cell transplantation
(Pavlakis and Khwaja, 2007). On the other hand, despite the availability of multiple anti-
diabetic treatments, there is, unfortunately, no absolute cure for diabetes presently.
Patients rely on constant medications and the situation of diabetes deteriorates with time.
As a result, research in diabetes pathology and development in diabetes therapeutics is
urgently demanded.

Generally, it is considered that the onset of type 1 and type 2 diabetes involves
both the genetic and the environmental factors and the relationship between the genetic
factors and the environmental factors is interwoven (Meier, 2008). Specifically, type 1
diabetes is normally considered as a T cell-mediated autoimmune disease where over-
activated T cells attack self cells in the pancreas (Lang et al., 2005; Liadis et al., 2005)
(Kapoor, 2008; Rother, 2007). Type 1 diabetes is often seen running in a family, which
indicates that genetic factors play an important role in the onset of the disease. In fact,
nearly 20 different genes have been found to be associated with the risk of type 1
diabetes, HLA region and the insulin gene for example (Awata et al., 1997; Caillat-Zucman et al., 1992; Dorman et al., 1995; Dubois-Laforge et al., 1997; Noble et al., 1996). The environmental factors that trigger the autoimmune attack remain largely unknown. It has been proposed that viral or bacterial infection (Filippi et al., 2009; Goldberg and Krause, 2009), chemical toxin exposure (Rydgren and Sandler, 2009), early-life inadequate food exposure (Luopajärvi et al., 2008) may be the triggers for the disease. However, these are only unproven hypotheses. In addition to the genetic and environmental factors, the progression of type 1 diabetes can also be attributed to body conditions such as aging, stress and certain diseases. For example, pancreatitis (Bluth et al., 2008), pancreatic adenocarcinoma (Syrigos et al., 1996), hypercholesterolemia (Zgibor et al., 2005) and hypertension (Zgibor et al., 2005) may directly or indirectly affect type 1 diabetes. Similar to type 1 diabetes, genetic and environmental factors are mediating type 2 diabetes. Diabetes-related human genome studies provide strong evidence supporting a genetic component to type 2 diabetes susceptibility as type 2 diabetes is associated with ethnicity and some human gene polymorphism or mutants have been identified to be associated with a Mendelian or mitochondrial pattern of inheritance of type 2 diabetes (Mercado et al., 2002; O'Rahilly et al., 2005; Silander et al., 2009; Takeuchi et al., 2009). It is worth noticing that the genetic variants associated with diseases like type 2 diabetes may display great variations across different populations (Adeyemo and Rotimi, 2009). Environmental factors such as pollution, obesity, energy-dense diets, insufficient physical activities are also considered to be playing a great role in type 2 diabetes and are maybe the major initial causes of type 2 diabetes (Amati et al.,
The modern society provides us with an “obesogenic” environment, excess foods of high calories combined with a sedentary lifestyle, causing a high risk of obesity. Since insulin resistance, or type 2 diabetes, goes hand in hand with obesity in most of the cases, our “obesogenic” life puts us at a high risk of getting type 2 diabetes as well. There is apparent discrepancy between type 2 diabetes and obesity since diabetes represents a state when body does not respond well to insulin, a potent hormone regulating carbohydrate metabolism and facilitating energy deposit, while obesity stands for a state of energy over-deposit. However, type 2 diabetes and obesity do have some complex connections at the molecular level, allowing them to be both the cause and the consequence of each other. In addition to the genetic factors and the environmental factors, type 2 diabetes, similar to type 1 diabetes, may also arise from aging, stress and some disorders, not necessarily the disorders of the insulin-responding cells or organs.

Numerous studies have found that the risk of type 2 diabetes gradually increases with age (Fontana, 2009). Increased β-cell apoptosis in type 2 diabetes is an important factor contributing to the progression of type 2 diabetes (Rhodes, 2005). Mitochondrial dysfunction can be another cause of the onset of type 2 diabetes. Mitochondria are organelles oxidizing fuels to generate energy. They play a significant role in regulating energy expenditure as well as insulin secretion by pancreatic β cells (Szabadkai and Duchen, 2009). Dysfunction of mitochondria due to the maternally inherited defects in mitochondrial DNA or the stress-induced damages of the mitochondrial components, such as mitochondrial inner membrane protein uncoupling protein-2 (UCP2), results in
the dysregulation of glucose homeostasis and imbalance of energy, and consequently leads to type 2 diabetes (Lowell and Shulman, 2005). The aberrant regulation of glucose homeostasis by brain is also suggested to play a role in the onset of type 2 diabetes (Schwartz and Porte, 2005). Besides, the same as type 1 diabetes, hypertension (Maheux et al., 1994), unhealthy lipid/cholesterol profile (high LDL, low HDL) (Kasim et al., 1988) and even cancer (Friberg et al., 2007; Katsumichi and Pour, 2007; Pothiwala et al., 2009) are thought to contribute to type 2 diabetes. Although numerous factors have been found to trigger the onset and drive the progress of type 2 diabetes, no conclusive agreement about the primary pathological cause of type 2 diabetes has been reached up till now. However, agreement does arrive at the point that type 2 diabetes represents a multifactorial, heterogeneous group of disorders and the pathogenesis of type 2 diabetes involves both the defect in insulin secretion and the progressive development of insulin resistance. Although the molecular basis for insulin resistance remains poorly understood, the disturbance in insulin receptor signaling pathway is considered to be responsible for insulin resistance (Hirayama et al., 1999; Parish and Petersen, 2005).

**Current Treatment of Diabetes**

Type 1 diabetes is mainly caused by insulin deficiency, which is the result of pancreatic β cell dysfunction. Those who develop type 1 diabetes are usually given insulin replacement via shots or pumps. Type 2 Diabetes which is characterized by insulin resistance rather than a true deficiency of insulin is more common than type 1 diabetes. For people who have type 2 diabetes, the levels of insulin in the blood are
similar to or even a little higher than in normal non-diabetic individuals, known as hyperinsulinemia (Weyer et al., 2000). However, insulin responsive cells in the body of patients with type 2 diabetes respond sluggishly to the insulin, resulting in hyperglycemia similar to that in type 1 diabetes. Although type 2 diabetes does not happen originally because of insulin deficiency, failure of pancreatic $\beta$ cell is often accompanied during the late stage of type 2 diabetes. Thus, occasionally insulin shots are applied to patients with type 2 diabetes. Insulin is a polypeptide hormone potent in controlling blood sugar level. However, unfortunately, the characteristics of insulin make it hard to be delivered. First, it cannot be orally administrated because it can be easily digested by enzymes residing in the digestion duct. As a result, injection is required for insulin administration. Second, because insulin is so potent that excessive insulin may cause temporary hypoglycemia or even shock, the dosage of insulin to be administrated needs to be carefully adjusted according to the real-time blood glucose level. Third, the biological turnover time of insulin is short with the half life ($t_{1/2}$) of only about 5-15 minutes (Dreyer et al., 1986; Mincu and Ionescu-Tirgoviste, 1980). Consequently, frequent administration of insulin is required. Fortunately, nowadays some new pharmaceutical forms of insulin and some new delivery methods have been developed to achieve controlled slow drug release. However, oral administration of insulin still cannot be operated up till now, remaining the biggest problem of insulin administration.

Fortunately, some oral glucose-lowering drugs have been developed, making diabetes treatment easier. The most common orally deliverable anti-diabetic drugs include insulin secretagogues, insulin response sensitizers and $\alpha$-glucosidase inhibitors
(Hsia and Davidson, 2002). Although most of them are not as potent as insulin, their discovery does contribute to the treatment of diabetes, especially to that of type 2 diabetes. At the early and the middle stages of type 2 diabetes, when no significant pancreatic β cell failure is resulted, drugs, such as sulfonylureas, that can stimulate pancreatic insulin secretion are good options for type 2 diabetes treatment (Yan et al., 2004). The main pathological cause of type 2 diabetes is insulin resistance instead of insulin deficiency. Accordingly, type 2 diabetes, especially the early and middle stages of type 2 diabetes, can be treated with insulin sensitizing drugs such as thiazolidinediones, the PPARγ agonists, to achieve better insulin action (Boden et al., 2005). Besides, biguanides may improve glucose tolerance through enhancing insulin-mediated glucose uptake and glucose utilization in peripheral tissues (Bailey, 1992) and α-glucosidase inhibitors alleviate diabetes by slowing and cutting down the absorption of carbohydrates from intestines and by reducing postprandial insulin levels (van de Laar et al., 2005).

Although those oral glucose-lowering agents are relatively effective and have advantages in delivery, they, like most drugs, have side effects. The major limitations of sulfonylureas include weight gain and hypoglycemia (Uwaifo and Ratner, 2005). The three major side effects that thiazolidinediones are particularly prone to have are weight gain, edema, and mild dilutional anaemia (O’Moore-Sullivan and Prins, 2002). The administration of biguanides can give rise to the serious side effect lactic acidosis (Stades et al., 2004). α-glucosidase inhibitors are associated with gastrointestinal discomfort such as abdominal pain, flatulence, diarrhea, meteorism and some other un-welcome side effects, like kidney dysfunction (Giorgino and Damato, 1995; Reuser and Wisselaar,
1994). Since the currently available therapies for diabetic patients are still far from satisfying, continuous efforts are being put in finding better diabetes therapeutic agents, orally deliverable, low toxicity products preferred. In recent years, scientists discovered that some gut peptides, such as ghrelin, pancreatic polypeptide (PP), peptide tyrosine tyrosine (PYY), amylin, glucagon-like peptide-1 (GLP-1) and oxyntomodulin, are regulating food intake and energy homeostasis (Cummings and Overduin, 2007; Karra and Batterham, 2009; Meier and Nauck, 2008; Murphy et al., 2006). Gut peptides and their analogs, agonists or antagonists are promising in treating diabetes. In addition to looking for anti-diabetic reagents from human body, people never stop searching for therapeutic reagents from herbs as herbs are a great source of bioactive products. Researchers in our laboratory are among the people who are searching for anti-diabetic drugs from herbs.

**Previous Diabetes-related Research in Our Laboratory**

Due to the worldwide high prevalence of type 2 diabetes and the unsatisfactory features of currently available therapies, researchers have been continuously searching for anti-diabetic agents from natural products. Our research aims at getting more informed about the pathological mechanisms of type 2 diabetes and finding natural products that are beneficial to diabetic patients.

The research in our laboratory initially focused on the confirmation of the biological effects of banaba extract as well as the isolation and characterization of novel anti-diabetic compounds from Banaba extract. We used both cultured cells such as 3T3-
L1 cells and diabetic/obese mice as models for these studies. Our data demonstrated that BE can significantly stimulate glucose uptake in 3T3-L1 adipocytes with an induction time and a dose-dependent response similar to those of insulin (Liu et al., 2001). These observations confirmed the previously known anti-diabetic effects of BE at the cellular level. In order to identify the effective component(s), BE components were separated by high performance liquid chromatography (HPLC) with water and acetonitrile as solvents on a reverse-phase column. Each fraction was examined for the glucose uptake stimulatory activity. We demonstrated that the BE with tannic acid removed is devoid of the glucose uptake stimulatory activity, while tannic acid, a major component of tannins, possesses the activity the same as BE (Liu et al., 2005). Our attentions thus switched from BE to TA. However, TA is still a mixture of compounds and the results of mechanism studies would not be convincing if using a mixture. So further efforts were made to separate the components in TA using HPLC. And finally, β-PGG was identified as one of the most effective components in TA (Li et al., 2005).

To further study its bioactivity, PGG was chemically synthesized and a mixture of α-PGG and β-PGG were obtained. Using the crystallization method, the two anomers were successfully separated. Both chemically synthesized α-PGG and β-PGG were tested for glucose uptake stimulatory activity in 3T3-L1 cells. And the two PGG anomers were found to exhibit glucose uptake stimulatory activity in 3T3-L1 cells in a dose-dependent manner (Li et al., 2005). Moreover, animal studies demonstrated that both α-PGG and β-PGG can help reduce blood glucose levels and improve glucose tolerance in diabetic and obese mice (Li et al., 2005). Compared to β-PGG, α-PGG is more potent in triggering
glucose uptake in 3T3-L1 cells and was carried on for insulin-like glucose uptake mechanism studies (Li et al., 2005). In the mechanism studies, it was found that IR inhibitor, PI3K inhibitor and GLUT4 inhibitor can completely abolish the glucose uptake induced by α-PGG (Li et al., 2005). Western blotting and enzyme activity analysis further identified that α-PGG can phosphorylate IR, activate PI3K, induce Akt phosphorylation, and stimulate GLUT4 translocation from cellular compartments to cytoplasm membrane (Li et al., 2005). Those results suggested that α-PGG may stimulate glucose uptake by activating the IR-PI3K-Akt-GLUT4 signaling pathway. Receptor binding studies provided direct evidence supporting that α-PGG can bind to IR (Li et al., 2005). Cross-linking study result further demonstrated that α-PGG may bind to IR at a site on the α-subunit of the receptor (Li et al., 2005). Interestingly, α-PGG was found to affect the binding of insulin to IR by reducing the maximum binding of insulin with IR without significantly altering the binding affinity between insulin and IR, suggesting that α-PGG may bind to a site different from insulin binding site (Li et al., 2005). Those receptor binding studies provided direct evidence supporting the argument that α-PGG stimulates glucose uptake by activating the IR signaling pathway. However, up till now, the exact binding site of α-PGG on IR has not been identified yet. Also, the IR binding affinity of α-PGG with IR has not been assessed and has not been compared with the affinity of insulin binding with insulin receptor.

More recently, an α-PGG derivative, 6Cl-TGQ was synthesized in our lab. The structural difference between 6Cl-TGQ and α-PGG is that the esterized galloy group at the 6th site of the glucose core is substituted with a chloride atom. Excitingly, 6Cl-TGQ
exhibited higher activity than α-PGG in triggering glucose uptake in 3T3-L1 adipocytes (Ren et al., 2006). On the other hand, both α-PGG and 6Cl-TGQ were not as potent as insulin in triggering glucose uptake as indicated by our preliminary results. Moreover, within the first 5 to 10 min of glucose uptake, the maximum glucose taken in by adipocytes induced by α-PGG or 6Cl-TGQ was lower than that by insulin. To explain the phenomena, we hypothesized that α-PGG, 6Cl-TGQ and insulin exhibit different receptor binding rate and affinity and the two compounds may either be slower in triggering glucose uptake or possess low efficacy in inducing glucose uptake. The following study comparing the timing of IR signaling activation by α-PGG, 6Cl-TGQ and insulin was carried out to test the hypotheses.
MATERIALS AND METHODS

Cell lines and culture conditions - Mouse 3T3-L1 preadipocyte cell line, Chinese hamster ovarian cancer cell line CHO were purchased from American Type Culture Collection (ATCC; Rockville, MD). CHO-IR cells, the CHO cells which stably overexpress wild type human insulin receptor were gifts from Dr. Alan Saltiel’s laboratory (Frattali et al., 1991). 3T3-L1 preadipocytes were cultured in Dulbecco’s Modification of Eagle’s Medium (DMEM) (Mediatech; Herndon, VA) containing 10% calf serum (CS) and 1% penicillin/streptomycin at 37°C in a 5% CO2 cell incubator. 3T3-L1 adipocytes were induced from 3T3-L1 preadipocytes and were cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in a 5% CO2 cell incubator. CHO and CHO-IR cells were cultured in DMEM with 10% CS and 1% penicillin/streptomycin at 37°C in a 5% CO2 cell incubator. CHO-IR cells were selected once every 3 months in DMEM containing 10% CS, 1% penicillin/streptomycin and 0.8 mg/ml G418 or neomycin sulfate (Mediatech; Herndon, VA).

3T3-L1 preadipocyte differentiation conditions - Mouse 3T3-L1 preadipocytes were cultured in 10% CS-containing DMEM until confluency. Medium was refreshed and cells were kept confluent for 2 days. Then cells were switched to 10% FBS-containing DMEM supplemented with 500 μM of 3-isobutyl-1-methylxanthine (Sigma-Aldrich; St. Louis, MO), 0.25 μM of dexamethasone (Sigma-Aldrich; St. Louis, MO) and 1 μg/ml of insulin (Sigma-Aldrich; St. Louis, MO) to induce differentiation. 48 hours after induction of differentiation, medium was replaced with fresh 10% FBS-containing DMEM supplemented with 1 μg/ml of insulin to continue driving differentiation. After
that, medium was replaced with fresh 10% FBS-containing DMEM every 48 hours until 3T3-L1 preadipocytes are fully differentiated to form adipocytes which was approximately 8 days after induction of differentiation.

**Compounds and compound treatment** - α-PGG and 6Cl-TGQ were synthesized by Dr. Yulin Ren and Dr. Klaus Himmeldirk in the Department of Chemistry and Biochemistry of Ohio University. Both α-PGG and 6Cl-TGQ were dissolved in ddH2O. Cells were treated with 30 μM of α-PGG or 6Cl-TGQ unless described otherwise.

**Glucose uptake assay** - The glucose uptake assay procedures were modified according to those that were previously used by other researchers (Cornelius et al., 1990; Li et al., 2005; Sakoda et al., 1999; Tafuri, 1996). In this assay, 3T3-L1 adipocytes were used 8 days after differentiation induction, when their glucose uptake ability had been well-established. Cells were first incubated with serum-free DMEM for 2 hours at 37 °C in a 5% CO2 cell incubator after being washed with serum-free DMEM twice. Then cells were switched to Krebs-Ringer phosphate (KRP) buffer, washed with KRP buffer for 3 times followed by incubation in the buffer for 30 min. After that, cells were treated with α-PGG, 6Cl-TGQ or insulin according to the experimental designs. Then, 1 μCi/ml 2-deoxy-D-(1-³H) glucose (Amersham Biosciences/GE Healthcare; Pittsburgh, PA) was added to initiate glucose uptake. After 10 min or other pre-determined lengths of time, buffer was aspirated and cells were washed with ice-cold PBS 3 times to terminate glucose uptake. Cells were then lysed with 1% triton X-100 (Sigma-Aldrich; St. Louis, MO). Finally, the radioactivity was determined by liquid scintillation spectrometry with a
Beckman Coulter LS 6400 Multi-Purpose Scintillation Counter (Beckman Coulter, Inc.; Fullerton, CA).

*Immunofluorescence confocal microscopy for GLUT4 translocation* - 3T3-L1 adipocytes growing on cover slips were incubated with serum-free DMEM for 2 hours at 37 °C in a 5% CO₂ cell incubator after being washed with serum-free DMEM twice. Then cells were switched to KRP buffer, washed with KRP buffer for 3 times followed by incubation in the buffer for 30 min. Then cells were treated with 30 μM of α-PGG, 30 μM of 6Cl-TGQ or 1 μM of insulin for 0 min, 5 min, 15 min, 30 min or 60 min. After treatment, buffer was aspirated and cells were washed with ice-cold PBS. Cells were fixed with ice-cold 100% methanol (VWR international; West Chester, PA) at -20°C for 20 min to immobilize cytoplasmic antigens. Then methanol was removed and the samples were air-dried. 0.3% H₂O₂ in methanol was added to incubate with the samples at room temperature for 10 min to block the endogenous peroxidase activity. After washing twice with PBS, cells were incubated with 4% paraformaldehyde (Fisher Scientific; Pittsburgh, PA) in PBS for 30 min at room temperature to fix cell surface antigens. Then, cells were blocked with 5% normal donkey serum in PBS with 0.2% Triton X-100 (Sigma-Aldrich; St. Louis, MO) and 1% DMSO (Sigma-Aldrich; St. Louis, MO) at room temperature for 50 min. When the blocking solution was removed, cells were incubated with the primary antibody anti-GLUT4 (Santa Cruz; Santa Cruz, CA) diluted at 1:200 in 7.5% donkey serum/PBS overnight at 4°C. After the overnight incubation, cells were washed twice with PBS and incubated with the secondary antibody (Fluorescein (FITC)-conjugated affinipure F(ad’))2 fragment Donkey anti-Mouse IgG)
(Jackson ImmunoResearch Laboratories; West Grove, PA) at a 1:100 dilution in 7.5% donkey serum/PBS at 37°C for 30 min. Finally, after washing with PBS 3 times, cells were mounted with a drop of Vectashied and visualized with a Zeiss LSM510 confocal microscope at wavelength of 488 nm for excitation and 520 nm for emission. The pictures were taken when the ocular lens was 10X and the objective lens was 100X.

*Total protein preparation* – CHO-IR cells of 60% confluency were incubated in serum-free DMEM for 2 hours before cells were incubated in KRP buffer for 30 min. After that, cells were treated with 30 μM of α-PGG, 30 μM of 6Cl-TGQ or 1 μM of insulin for 0 min, 1 min, 5 min, 15 min, 30 min or 60 min. Total proteins were isolated for western blot analysis right after cell treatment. To prepare total proteins, cells were washed with phosphate buffered saline (PBS) and lysed on ice with lysis buffer (10 mM Tris HCl pH 7.5, 1 mM EDTA, 1% triton X-100, 150 mM NaCl, 1 mM dithiothretol, 10% glycerol, 0.2 mM phenylmethysulphonyl fluoride and protease inhibitors). Cell lysate was subjected to sonication on ice at power level 3 with a 550 Sonic Dismembrator (Fisher Scientific; Pittsburgh, PA), 3 seconds each time for 3 times. The sonicated cell lysate was centrifuged at 14,000 g for 10 min at 4°C. Supernatant was collected. Protein concentrations were determined using the BCA protein assay kit (Pierce; Rockford, IL).

*Western blot analysis* - 50 μg of total protein isolated from CHO-IR cells was prepared in SDS sample buffer (Cell Signaling; Beverly, MA) and was loaded to 7.5% or 10% ready-to-use Tris-glycine polyacrylamide gels (Bio-Rad; Hercules, CA) which were soaked in electrode buffer (3 g/L tris, 14.4 g/L glycine, 1 g/L SDS). Proteins migrated in the electric field to be separated. After proteins were well-separated, they were
transferred to nitrocellulose membrane (Schleicher & Schuell; Dassel, Germany) when 110V electric field and transfer buffer (1.51 g/L tris, 7.2 g/L glycine, 20% methanol, 0.01% SDS) were present. One hour after initiation of protein transfer, nitrocellulose membrane with proteins was washed by tris buffered saline (TBS) supplemented with 0.1% Tween-20 (Sigma-Aldrich; St. Louis, MO) (TBST) and was blocked with 5% fat-free milk in TBST at room temperature for 1 hour. Then, target proteins were probed with specific primary antibodies (anti-phospho-IR/IGF-1R (Tyr1146) (Cell Signaling; Beverly, MA) and anti-phospho-Akt (Ser473) (Cell Signaling; Beverly, MA) overnight at 4°C. After a thorough wash with TBST, the nitrocellulose membrane with proteins was incubated with the secondary antibody strepavidin-conjugated goat-anti-rabbit (Cell Signaling; Beverly, MA) for 1 hour. Proteins were visualized by Lumi-Light western blotting substrates (Roche; Nutley, NJ). Non-specific bands or β-actin bands were used as controls. Each experiment was repeated at least twice.

Ligand-receptor binding with Biacore System - This experiment was carried out by our collaborator Dr. Thomas E. Wagner’s group.
RESULTS

α-PGG and 6Cl-TGQ were able to induce glucose uptake in 3T3-L1 adipocytes but the glucose uptake induced by α-PGG or 6Cl-TGQ during the first 10 minutes was lower compared with that by insulin - Increasing concentrations of α-PGG (5 μM, 10 μM, 20 μM, 30 μM, 40 μM, 60 μM, 80 μM), 6Cl-TGQ (5 μM, 10 μM, 20 μM, 30 μM, 40 μM, 60 μM, 80 μM) and insulin (0.01 μM, 0.1 μM, 0.25 μM, 0.5 μM, 0.75 μM, 1 μM) were added to 3T3-L1 adipocytes 15 min prior to addition of 2-deoxy-D-(1-3H) glucose. 10 min after addition of radioactive glucose, intracellular radioactivity which represents the amount of intracellular radioactive glucose was measured. The results indicated that α-PGG and 6Cl-TGQ induced glucose uptake in adipocytes as insulin did (Fig. 5). Moreover, α-PGG and 6Cl-TGQ induced glucose uptake with a dose-dependent pattern similar to insulin-induced glucose uptake (Fig. 5). With the increase of the concentrations of α-PGG and 6Cl-TGQ from 0 to around 30 μM and the increase of insulin concentrations from 0 to around 0.25 μM, the cumulative amount of glucose transported into cells during the first 10 min increased (Fig. 5). But the total amount of transported glucose in the first 10 min did not keep increasing with the increase of the compound or insulin concentrations. Instead, it reached a plateau when the concentrations of α-PGG and 6Cl-TGQ were over 30 μM and when insulin concentrations were higher than 0.25 μM (Fig. 5). Therefore, the lowest concentration at which α-PGG and 6Cl-TGQ generated maximum glucose uptake stimulatory activity was 30 μM and the lowest concentration for insulin to stimulate maximum glucose uptake was 0.25 μM (Fig. 5).
From the graph, the EC$_{50}$ for α-PGG and 6Cl-TGQ was around 15 μM while the EC$_{50}$ for insulin was approximately 0.05 μM (Fig. 5). Since the EC$_{50}$ of the compounds was about 100 times higher than the EC$_{50}$ of insulin, the potency of the compounds was lower than that of insulin. Furthermore, the cumulative glucose uptake triggered by α-PGG in the first 10 min was slightly less than that by 6Cl-TGQ (Fig. 5). And the cumulative glucose uptake triggered by either α-PGG or 6Cl-TGQ in the first 10 min was less compared to that by insulin (Fig. 5).

Figure 5. The glucose uptake in 3T3-L1 adipocytes induced by α-PGG or 6Cl-TGQ during the first 10 minutes was lower compared with that by insulin. 3T3-L1 adipocytes were treated with various concentrations of α-PGG (5 μM, 10 μM, 20 μM, 30 μM, 40 μM, 60 μM, 80 μM), 6Cl-TGQ (5 μM, 10 μM, 20 μM, 30 μM, 40 μM, 60 μM, 80 μM) or insulin (0.01 μM, 0.1 μM, 0.25 μM, 0.5 μM, 0.75 μM, 1 μM) 15 min prior to addition of 2-deoxy-D-(1-$^3$H) glucose. 10 min after addition of radioactive glucose, intracellular radioactivity was measured by scintillation counting. Each data point was an average of results from three independent experiments and presented as M±SD.
α-PGG and 6Cl-TGQ showed a slower rate, but not a lower efficacy in triggering glucose uptake in 3T3-L1 adipocytes compared to insulin - To find out the reason for the lower cumulative glucose uptake triggered by either α-PGG or 6Cl-TGQ in the first 10 min in 3T3-L1 adipocytes, a glucose uptake assay was carried out to compare the cumulative glucose uptake induced by α-PGG, 6Cl-TGQ or insulin during the first 60 min. 30 μM of α-PGG, 30 μM of 6Cl-TGQ or 1 μM of insulin was used to treat the cells 15 min prior to addition of 2-deoxy-D-(1-³H) glucose. The intracellular radioactivity was measured 1 min, 5 min, 10 min, 15 min, 20 min, 30 min and 60 min after addition of radioactive glucose. The data were expressed as the amount of glucose uptake relative to the cumulative glucose uptake induced by 1 μM of insulin during 60 min. As indicated by the result, the cumulative glucose uptake induced by α-PGG, 6Cl-TGQ or insulin during the first 60 min all increased over time (Fig. 6). As the accumulation of the intracellular radioactive glucose mediated by basal glucose uptake increased very slowly over time, the accumulation of intracellular radioactive glucose resulted from compound or insulin-induced glucose uptake increased relatively fast (Fig. 6). The glucose uptake time course of α-PGG, 6Cl-TGQ or insulin all exhibited two phases: the initial slow increase phase and the later fast increase phase (Fig. 6). Insulin induced the most amount of glucose uptake and α-PGG induced the least amount of glucose uptake throughout the entire time course (Fig. 6). The difference between α-PGG, 6Cl-TGQ and insulin-induced cumulative glucose uptake increased a little during the first 30 min and kept constant afterwards (Fig. 6). By 60 min, the relative difference between α-PGG, 6Cl-TGQ and insulin-induced cumulative glucose uptake had been very small (Fig. 6). This
pointed out that α-PGG and 6Cl-TGQ were slower in triggering glucose uptake in 3T3-L1 adipocytes compared to insulin. But their efficacy in triggering glucose uptake was very similar to that of insulin.

*Figure 6. α-PGG and 6Cl-TGQ showed a slower rate, but not a lower efficacy in triggering glucose uptake in 3T3-L1 adipocytes compared to insulin. 3T3-L1 adipocytes were treated with 30 μM of α-PGG, 30 μM of 6Cl-TGQ or 1 μM of insulin 15 min prior to addition of 2-deoxy-D-(1-³H) glucose. The intracellular radioactivity was measured 1 min, 5 min, 10 min, 15 min, 20 min, 30 min and 60 min after addition of radioactive glucose. The results were expressed as the cumulative amount of glucose uptake under a particular condition relative to the cumulative glucose uptake induced by 1 μM of insulin during 60 min. Each data point was an average of results from three independent experiments and presented as M±SD.*
GLUT4 translocation induced by α-PGG or 6Cl-TGQ was slower compared to that by insulin - GLUT4 is an insulin responsive glucose transporter which normally resides in cytosolic compartments but translocates to the cytoplasm membrane upon IR signaling activation (Bryant et al., 2002; Ploug and Ralston, 2002). 3T3-L1 adipocytes possess GLUT4 (Vannucci et al., 1998). On the 8th day of 3T3-L1 cell differentiation, cells were treated with 30 μM of α-PGG, 30 μM of 6Cl-TGQ or 1 μM of insulin in KRP buffer. 5 min, 15 min, 30 min and 60 min after treatment, cells were used for in situ immunostaining to visualize the subcellular localization of GLUT4. Fluorescence confocal microscopy revealed that insulin was able to induce significant GLUT4 translocation by 5 min while α-PGG and 6Cl-TGQ needed more time (30 min for α-PGG and 15 min for 6Cl-TGQ) to induce GLUT4 translocation (Fig.7). Therefore, GLUT4 translocation induced by the two compounds was slower than that by insulin.
Figure 7. GLUT4 translocation induced by α-PGG or 6Cl-TGQ was slower compared to that by insulin. 3T3-L1 adipocytes were treated with 30 μM of α-PGG, 30 μM of 6Cl-TGQ or 1 μM of insulin. 5 min, 15 min, 30 min and 60 min after treatment, cells were used for *in situ* immunostaining. The green lights indicated the subcellular localization of GLUT4. The pictures were taken when the ocular lens was 10X and the objective lens was 100X.

*α-PGG and 6Cl-TGQ induced a weaker activation of Akt compared to insulin -*

The activation of Akt, the signaling molecule in the IR signaling pathway, by α-PGG, 6Cl-TGQ or insulin was examined to try to find an explanation for the slower rate in triggering glucose uptake by the two compounds. CHO-IR cells, which stably overexpress human wild type IR were employed to represent an amplified IR signaling. CHO-IR cells were treated with 30 μM of α-PGG, 30 μM of 6Cl-TGQ or 1 μM of insulin. Total protein was isolated for western blot analysis 0 min, 1 min, 5 min, 15 min, 30 min and 60 min after treatment. Our results showed that α-PGG and 6Cl-TGQ were
able to induce Akt phosphorylation (Fig. 8), which is consistent with our previous findings (Li et al., 2005). Moreover, the level of phosphorylated Akt increased over time within the 60 min of induction by α-PGG, 6Cl-TGQ or insulin (Fig. 8). The pattern of Akt phosphorylation induced by α-PGG or 6Cl-TGQ resembled that by insulin (Fig. 8). However, Akt phosphorylation induced by the two compounds appeared weaker than that by insulin (Fig. 8). And the phosphorylation of Akt induced by α-PGG was the weakest among the three treatments (Fig. 8)
Figure 8. α-PGG and 6Cl-TGQ induced a weaker activation of Akt compared to insulin. CHO-IR cells were treated with 30 μM of α-PGG, 30 μM of 6Cl-TGQ or 1 μM of insulin. Total protein was isolated 0 min, 1 min, 5 min, 15 min, 30 min and 60 min after treatment for western blot analysis to detect phospho-Akt levels. Non-specific bands or β-actin bands were used as protein loading controls. The experiments were repeated. The results shown were representative and were reproducible.
α-PGG and 6Cl-TGQ induced a weaker activation of IR compared to insulin.

An activation study with CHO-IR cells was carried out in a way similar to the previous Akt activation study. Our western blot results indicated that α-PGG and 6Cl-TGQ were capable of inducing IR phosphorylation (Fig. 9). The pattern of IR phosphorylation induced by α-PGG or 6Cl-TGQ resembled that by insulin in that IR phosphorylation increased during the first 15 min of induction and gradually declined after 15 min (Fig. 9). On the other hand, similar to the situation in Akt activation, IR phosphorylation induced by the two compounds appeared weaker than that by insulin (Fig. 9). And the IR phosphorylation induced by α-PGG was the weakest among the three treatments (Fig. 9).
Figure 9. α-PGG and 6Cl-TGQ induced a weaker activation of IR compared to insulin. CHO-IR cells were treated with 30 μM of α-PGG, 30 μM of 6Cl-TGQ or 1 μM of insulin. Total protein was isolated 0 min, 1 min, 5 min, 15 min, 30 min and 60 min after treatment for western blot analysis to detect phospho-IR levels. Non-specific bands or β-actin bands were used as protein loading controls. The experiments were repeated. The results shown were representative and were reproducible.

The insulin receptor binding affinity of α-PGG or 6Cl-TGQ was lower than that of insulin - To illustrate the mechanism of the weak activation of the IR signaling and the slow induction of glucose transport by α-PGG and 6Cl-TGQ, Biacore System was applied by Dr. Thomas E. Wagner’s group to determine the IR binding constants of α-PGG and 6Cl-TGQ. The dissociation efficiency $K_d$, which may be determined by the Biacore System, is inversely proportional to receptor binding affinity. Accordingly to this in vitro binding assay, the $K_d$ of α-PGG was around 43 μM and the $K_d$ of 6Cl-TGQ was about 19 μM (Table 2). The $K_d$ of insulin, which was determined by another group, was approximately 1 nM (Table 2) (Wanant and Quon, 2000). Therefore, α-PGG and 6Cl-TGQ bound IR with a lower affinity than insulin. Based on the measured ligand-receptor association and dissociation constants, it was relatively easy for α-PGG to bind with IR
(k₁ = 1.01 x 10³), but α-PGG also tended to dissociate from IR very easily (k₋₁ = 0.438) (Table 2). It was very difficult for 6Cl-TGQ to bind with IR (k₁ = 127), but 6Cl-TGQ also dissociated from IR less easily (k₋₁ = 2.39 x 10⁻³) (Table 2). In comparison, insulin bound with IR very easily (k₁ = 1 x 10⁶), and it was very hard for insulin to dissociate from IR (k₋₁ = 1 x 10⁻⁴ to 4 x 10⁻⁴) (Table 2) (Wanant and Quon, 2000).

Table 2

*Insulin Receptor Binding Constants for α-PGG, 6Cl-TGQ and Insulin*

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Kₐ (k₁/k₋₁)</th>
<th>k₁ (M⁻¹S⁻¹)</th>
<th>k₋₁ (S⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-PGG</td>
<td>~ 43 μM</td>
<td>1.01 x 10³</td>
<td>0.438</td>
</tr>
<tr>
<td>6Cl-TGQ</td>
<td>~ 19 μM</td>
<td>127</td>
<td>0.00239</td>
</tr>
<tr>
<td>Insulin</td>
<td>~ 1 nM</td>
<td>1 x 10⁶*</td>
<td>0.0001-0.004 *</td>
</tr>
</tbody>
</table>

* (Wanant and Quon, 2000)
DISCUSSION

Diabetes mellitus is a prevalent metabolic disorder characterized by hyperglycemia. Diabetes is a serious disease. It not only affects the quality of life, causes morbidity, but also leads to mortality. Diabetes is generally classified into 2 types: type 1 diabetes which is due to insulin deficiency and type 2 diabetes which is mainly the result of insulin resistance (1997; Groop et al., 1993; Lang et al., 2005). Insulin is often administered to patients with type 1 diabetes. For type 2 diabetes, secretagogues, sensitizers and α-glucosidase inhibitors (Toeller, 1994) are available in addition to insulin (Hsia and Davidson, 2002). The marketed anti-diabetic drugs supplement insulin, augment insulin production, enhance insulin sensitivity or inhibit glucose absorption. Although various medications have been available for diabetes treatment, they are not satisfactory. Some of the medications generate serious adverse effects such as hypoglycemia (Dailey et al., 2009), weight gain (Buckingham and Hanna, 2008), gastrointestinal discomfort and so on (Hsia and Davidson, 2002). Therefore, continuous efforts have been made in novel anti-diabetic drug development. Herbs provide a natural source of medications. Some herbs such as banaba tea exhibits anti-diabetic activity (Kakuda et al., 1996). Previously, we successfully separated the components of banaba extract and identified that the tannic acid fraction of banaba extact retained the anti-diabetic activity (Liu et al., 2005). The subsequent work led us to find β-PGG as one of the bioactive anti-diabetic compounds in the tannic acid fraction (Li et al., 2005). The anomer of β-PGG, α-PGG was found to possess glucose transport stimulatory activity even higher than β-PGG (Li et al., 2005). Substantial data indicated that α-PGG bound
insulin receptor at a site different from insulin binding site but still were able to activate
the IR signaling and trigger glucose uptake (Li et al., 2005). 6Cl-TGQ, a synthesized
derivative of α-PGG, exhibited higher potential in triggering glucose uptake in 3T3-L1
adipocytes compared to α-PGG (Ren et al., 2006). Although it was documented that both
α-PGG (Li et al., 2005) and 6Cl-TGQ (Ren et al., 2006) were less potent than insulin in
triggering glucose uptake in 3T3-L1 adipocytes, it remained unknown whether α-PGG
and 6Cl-TGQ have lower or similar efficacy in triggering glucose uptake compared to
insulin. Also, the mechanism(s) by which 6Cl-TGQ induced glucose uptake had never
been studied before.

In the preliminary study, we compared the glucose uptake in 3T3-L1 adipocytes
induced by α-PGG, 6Cl-TGQ or insulin side by side (Fig. 5). α-PGG and 6Cl-TGQ have
lower potency in triggering glucose uptake in 3T3-L1 adipocytes compared to insulin
(Fig. 5). The EC50 for α-PGG and 6Cl-TGQ was around 15 μM while the EC50 for insulin
was approximately 0.05 μM (Fig. 5). When the dose-dependent glucose uptake induction
curves of α-PGG, 6Cl-TGQ and insulin were plotted in one graph with two scales so that
the concentrations at which α-PGG/6Cl-TGQ induced maximal glucose uptake
overlapped with the concentration at which insulin induced maximal glucose uptake, we
found that α-PGG and 6Cl-TGQ induced glucose uptake in a pattern similar to insulin-
induced glucose uptake (Fig. 5). This suggested that α-PGG, 6Cl-TGQ and insulin follow
a similar mechanism to induce glucose uptake in 3T3-L1 adipocytes. And it actually has
been reported that α-PGG induced glucose uptake through activating the IR-PI3K-Akt-
GLUT4 signaling pathway the same as insulin does (Li et al., 2005). Since α-PGG does
and 6Cl-TGQ is very likely to follow the same mechanism as insulin in triggering glucose uptake in 3T3-L1 adipocytes, the question arose as why the maximal glucose uptake triggered by α-PGG or 6Cl-TGQ was less than that by insulin (Fig. 5). We hypothesized that α-PGG, 6Cl-TGQ and insulin exhibit different receptor binding kinetics and the two compounds may either be slower in triggering glucose uptake or possess low efficacy in inducing glucose uptake. To test the hypothesis, compound action over time was studied. Glucose uptake assay with an extended assay time was used to measure the cumulative glucose uptake induced by α-PGG, 6Cl-TGQ or insulin over time. And it was found that the cumulative glucose uptake induced by α-PGG or 6Cl-TGQ was lower than that by insulin at all tested time points (Fig. 6). Interestingly, the difference between α-PGG, 6Cl-TGQ and insulin-induced cumulative glucose uptake increased a little during the first 30 min and kept constant afterwards (Fig. 6). By 60 min, the relative difference in cumulative glucose uptake between α-PGG, 6Cl-TGQ and insulin was very small (Fig. 6). This suggested that the glucose uptake rate difference between the three treatments only existed at the initial induction period. When time passed the initial induction period, the glucose uptake rate was almost the same for all the three treatments. In comparison with insulin, α-PGG, 6Cl-TGQ are slower in glucose uptake induction. But they have a similar efficacy in triggering glucose uptake as insulin. Accordingly, there should be an IR signaling activation threshold for glucose uptake induction. α-PGG and 6Cl-TGQ are both slower than insulin in inducing a threshold-level signaling activation. To test whether the reasoning is valid, insulin signaling activation by α-PGG, 6Cl-TGQ or insulin was studied and compared. GLUT4 is the
major insulin signaling responsive glucose transporter in 3T3-L1 adipocytes (Vannucci et al., 1998). GLUT4 is embedded in specialized storage vesicles the majority of which in the basal state are residing in the cell interior (Bryant et al., 2002; Ploug and Ralston, 2002). Insulin triggers the translocation of GLUT4-containing vesicles from the cell interior to the plasma membrane, which eventually results in a rapid and significant increase of glucose uptake (Kandror, 2003; Watson and Pessin, 2001). Thereby, GLUT4 translocation directly links IR signaling activation and glucose uptake. We used in situ immunoblot together with confocal microscopy to follow GLUT4 translocation induced by α-PGG, 6Cl-TGQ or insulin over time to verify the involvement of the IR signaling in the action of compounds and to confirm the association of the GLUT4 translocation rate with the glucose uptake rate. In our study, by 5 min of insulin treatment, a considerable amount of GLUT4 translocated to the cytoplasm membrane as indicated by the green light dots on the edge of cells (Fig. 7). In contrast, it took α-PGG around 30 min and 6Cl-TGQ around 15 min to induce a considerable GLUT4 translocation (Fig. 7). The amount of GLUT4 in the cytoplasm membrane correlated well with the rate of intracellular glucose accumulation (Fig. 6 & 7). Also, it was the first time that GLUT4 translocation induced by 6Cl-TGQ was observed (Fig. 7). After the correlation in speed was seen between GLUT4 translocation and glucose transport for α-PGG, 6Cl-TGQ and insulin treatments, we further examined the rate of IR and Akt phosphorylation induced by α-PGG, 6Cl-TGQ or insulin. Akt is a signaling molecule downstream of IR but upstream of GLUT4. Serine phosphorylation of Akt leads to the activation of this signaling molecule. Western blot analysis showed that Akt was phosphorylated by α-PGG, 6Cl-TGQ and
insulin (Fig. 8). For all the three treatments, the amount of phosphorylated Akt increased over time (Fig. 8). Throughout the entire course, insulin treatment resulted in the largest amount of phosphorylated Akt while \( \alpha \)-PGG treatment accumulated the least amount of phosphorylated Akt (Fig. 8). The phosphorylated Akt reached a high level within 1 min upon insulin treatment (Fig. 8). In contrast, \( \alpha \)-PGG and 6Cl-TGQ did not induce appreciable amount of phosphorylated Akt until 15 min and 5 min respectively (Fig. 8). In other words, it took \( \alpha \)-PGG and 6Cl-TGQ longer time to induce a threshold-level Akt phosphorylation compared to insulin. And this is consistent with the previous observations on glucose uptake and GLUT4 translocation (Fig. 6 & 7). IR, the known binding partner of insulin and \( \alpha \)-PGG (Li et al., 2005), is the starting point of the IR signaling. IR phosphorylation by \( \alpha \)-PGG, 6Cl-TGQ or insulin over time was examined. Our results showed that 6Cl-TGQ induced IR phosphorylation (Fig. 9). Therefore, 6Cl-TGQ, like \( \alpha \)-PGG and insulin, is able to induce the activation of the IR-Akt-GLUT4 signaling pathway and subsequently trigger glucose uptake (Fig. 6-9). Moreover, comparing the IR phosphorylation by \( \alpha \)-PGG, 6Cl-TGQ and insulin, it was found that although \( \alpha \)-PGG and 6Cl-TGQ induced the accumulation of phosphorylated IR in a pattern similar to insulin, the level of IR phosphorylation by the two compounds were lower than that by insulin (Fig. 9). Therefore, it is possible that the weakness in IR signaling activation by the compounds contributed to the delay in the accumulation of phosphorylated Akt, cytoplasm membrane GLUT4 and intracellular glucose. In order to reveal the reason for the weakness in IR signaling activation by the compounds, Biacore System was applied to measure the binding constants of \( \alpha \)-PGG and 6Cl-TGQ with IR.
Based on the association and dissociation constants measured by Dr. Thomas E. Wagner’s group with the Biacore System, it was relatively easy for α-PGG to bind with IR, but it was also very easy for α-PGG to dissociate from IR (Table 2). It was very difficult for 6Cl-TGQ to bind with IR, but 6Cl-TGQ also dissociated from IR less easily (Table 2). In comparison, insulin bound with IR very easily, and it was very hard for insulin to dissociate from IR (Table 2) (Wanant and Quon, 2000). The dissociation efficiency, which reflects ligand-receptor binding affinity, indicated that the IR binding affinity of insulin was 4000 and 2000 times higher than that of α-PGG and 6Cl-TGQ respectively (Table 2). All of the data point out that α-PGG and 6Cl-TGQ are slower in inducing GLUT4 translocation and glucose uptake in 3T3-L1 adipocytes because they bind with IR with a low affinity and initiate a weak activation of the IR-Akt signaling. It takes more time for the weak signaling to reach a threshold-level to generate significant biological responses. α-PGG and 6Cl-TGQ have a similar efficacy in inducing glucose uptake as insulin.
SECTION II

ANTI-ADIPOGENESIS ACTIVITIES OF PENTA-\textit{O}-GALLOYL-\textalpha-D-GLUCOPYRANOSE
INTRODUCTION

Obesity

“Obesity” is a term describing a pathological state in which body fat mass has far exceeded what is considered healthy. Being obese and being overweight are not the same. Generally, “obesity” represents an extremely overweight state and is considered to be a disease that needs proper treatment. People with obesity are at risk for serious health problems, including disability/morbidity, respiratory problems, cardiovascular diseases, osteoarthritis, certain types of cancers and type 2 diabetes. Multiple indices, like body mass index and waist circumference, have been used to determine whether an individual is overweight or obese (Mehta et al., 2009). Body Mass Index (BMI), defined as weight (kg) / height$^2$ (m$^2$), is the most common mathematical formula used as an overweight or obese index. A BMI of 30 or more is considered obese and a BMI between 25 to 29.9 is considered overweight. According to the Centers for Disease Control and Prevention 2008 statistic data, the number of overweight and obese Americans has been rising since last century and about 25% of the US population are obese (Centers for Disease Control and Prevention, 2008). Also, the prevalence of obesity is not restricted to the US. Rather, it has become global, spreading across the world from the western countries to the developing countries. To make the situation worse, the age for the onset of obesity is getting younger and younger. This unfavorable situation regarding obesity calls for serious public attention and appropriate solution. Intensive research related to obesity is urgently needed.
The susceptibility to obesity is partly determined by genetic factors. But genetic factors are not sufficient by themselves to generate obesity. They only provide a prerequisite and increase the probability of obesity onset. Obesity is a chronic metabolic disease resulting from a chronic disruption of the energy balance: the long-term imbalance of energy intake, energy expenditure, nutrient partitioning and adipogenesis. Basically, when energy intake chronically exceeds energy expenditure and when nutrient partitioning favors lipid formation and lipid storage, the amount and volume of fat cells is expanded. So in this sense, to express the obesity phenotype, an 'obesogenic' environment, as indicated before, is required in addition to the genetic prerequisite. Genetic factors are interwoven with environmental factors: with the presence of a genetic predisposition to obesity, the severity of the disease is largely determined by lifestyle and environmental conditions (Loos and Bouchard, 2003). Our highly industrialized society right provides this ‘obesogenic’ environment: lack of physical activity combined with easily accessible high-calorie foods, favoring the accumulation of fat in body. As a result, in the modern society, the tendency towards obesity is accelerated.

**Current Treatment of Obesity**

Most obese people are suggested to change their life style by increasing physical activity while limiting calorie intake. Medications are usually only used for those who have a BMI of 30 or higher and are suggested to be used together with diet and exercise. The anti-obesity medications currently approved by US FDA are sibutramine and orlistat (Lee et al., 2009). They work mostly by suppressing appetite. Surgery that decreases
stomach size or bypasses intestine may be an option for patients with a BMI of 40 or more (Baker et al., 2009). Both current medications and surgical treatments are not so satisfactory. Sibutramine increases blood pressure and heart rate (Bray, 2009). Orlistat increases lower bowel movement (Bray, 2009). Those marketed anti-obesity medicines require continuous usage. But people are not sure about the safety of long-term use of those drugs. Surgery is a great challenge for the body and brings life style change and nutritional problems (Baker et al., 2009). Therefore, continuous effort has been made to find alternative treatments for obesity. Some bioactive gut peptides such as ghrelin (Inui et al., 2004) and amylin (Karra and Batterham, 2009) stimulate hunger and increase nutrient absorption while other gut peptides such as PYY (Small and Bloom, 2005) and GLP-1 (Green et al., 2005) reduce appetite. The analogs of satiety-reducing peptides are good anti-obesity candidates. In addition to the recent progress in finding gut peptide analogs as promising anti-obesity drugs, scientists also find some weight control reagents from herbs (Hasani-Ranjbar et al., 2009). For instance, a major green tea polyphenol, (-)-epigallocatechin-3-gallate was found to inhibit obesity and metabolic syndrome (Bose et al., 2008). Our group is also aiming at finding better medications for overweight or obese individuals from natural herbal sources.

**Adipogenesis**

Obesity is characterized by adipocyte hypertrophy. They arise directly from a significant increase of adipose tissue mass, which is due to an elevation in both the number and the size of adipocytes. Adipocytes have traditionally been considered to be
the primary energy storage sites for the whole body. Energy is stored in adipocytes mainly in the form of triglycerides and free fatty acids (FFAs). And the storage process is promoted by insulin which markedly stimulates both glucose uptake and lipogenesis and at the same time inhibits lipolysis. When energy is excessive, energy is stored in adipocytes and when the body runs short of instant energy from glucose, the stored energy is mobilized by hormones such as epinephrine for usage. In this way, adipocytes successfully modulate the energy balance of the whole body. However, the balance is interrupted in type 2 diabetes and obesity when adipocytes are at a stressful state and fail to compensate for the continuously increasing catabolic load. Since adipocytes are essential for maintaining energy homeostasis and the dysfunction of adipocytes results in serious health problems including type 2 diabetes and obesity, it would be a good way to understand more about these diseases through studying adipocytes.

Adipocytes are connective tissue cells present in adipose tissue and specialized in storing energy as fat. They can respond to insulin and regulate glucose metabolism. Recently, they are also identified to be able to secret cytokines such as adiponectin, leptin, resistin, and TNFα, which adds importance to their role in regulating glucose and energy homeostasis. Interestingly, adipocytes are not born adipocytes. They originate from certain mesenchymal stem cells that can differentiate into two types of lipoblasts/preadipocytes, one giving rise to adipocytes (Rim et al., 2005). The differentiation process of adipocytes from preadipocytes is called adipogenesis, which occurs in both the prenatal and postnatal states (Rosen and Spiegelman, 2000). Most of our knowledge about adipogenesis comes from the cells derived from mice, such as
NIH3T3-L1 cells. Suggested by previous mouse adipocyte differentiation studies, adipogenesis requires the expression of receptors for receiving differentiation signals and is a cellular process involving a tightly-controlled program of transcriptional events (Fu et al., 2005). The transcriptional cascade in adipogenesis (Fig. 10) appears in three distinct temporal phases, namely the early stage, the middle stage and the end stage (Fu et al., 2005). Before starting the differentiation program, preadipocytes are preferred to be at a state of post-confluent and growth arrest due to contact inhibition, because in that state preadipocytes are sensitive to growth hormones and are ready to be guided to the early stage of differentiation. Once growth-arrested preadipocytes enter the early differentiation stage, CCAAT enhancer-binding protein (C/EBP) β and C/EBPδ undergo transient and rapid expression, initiating two rounds of synchronous cell division known as mitotic clonal expansion (MCE) which lasts for around 48 hours (Tang et al., 2003; Yeh et al., 1995; Zhang et al., 2004b). However, C/EBPβ and C/EBPδ still lack the DNA-binding ability until after a 14-hour lag, when they are hyperphosphorylated by GSK-3β that enters the nucleus (Tang et al., 2005). Concurrently, the insulin-stimulated transcription factor adipocyte differentiation-dependent factor 1 (ADD1, in mouse) or sterol regulatory element binding protein1 (SREBP1, in human) is expressed and translocates from the endoplasmic reticulum to the Golgi and then to the nucleus where it activates the transcription of a number of genes involved in the metabolism of cholesterol (such as LDL receptor and hydroxymethylglutaryl-CoA reductase) and the synthesis of fatty acids and triglycerides (Osborne, 2000). Passing the clonal expansion period, cells completely withdraw from cell cycle and move on to the 96-hour middle stage. During
that period, the expression of C/EBPα, an indispensable transcriptional activator of adipocyte genes are promoted by hyperphosphorylated C/EBPβ (P-C/EBPβ) and P-C/EBPδ, whereas the expression of PPARγ, a pleiotropic activator of terminal adipocyte differentiation, are promoted by P-C/EBPβ, P-C/EBPδ and ADD1/SREBP1 with ADD1/SREBP1 also activating PPARγ through promoting the production of endogenous PPARγ ligands (Christy et al., 1991; Fajas et al., 1997; Kim et al., 1998; Tang et al., 1999; Zhu et al., 1995). C/EBPα and PPARγ form a positive feedback loop and operate synergistically to promote the expression of multiple adipocyte-specific genes, such as adipocyte-specific fatty acid binding protein (aP2), fatty acid synthase (FAS), phosphoenolpyruvate carboxykinase (PEPCK), acetyl CoA carboxylase, GLUT4, adiponectin, resistin and leptin (Rosen and Spiegelman, 2000; Wu et al., 1999). The expression of those adipocyte-specific genes leads to the coming of the terminal differentiation stage of adipogenesis, when cells irreversibly become mature adipocytes which are characterized by abundant lipid droplets in cytoplasm. Although white adipocytes and brown adipocytes are morphologically and functionally distinct from mature brown adipocytes which preferentially express uncoupling protein 1 (UCP1) and PPARγ-coactivator 1, they share the same transcriptional regulators that are involved in their terminal differentiation (Puigserver et al., 1998; Rosen and Spiegelman, 2000; Ross et al., 1992).
Figure 10. Adipogenesis program. These transcriptional regulatory proteins are expressed in a cascade in which C/EBP\(\beta\) and C/EBP\(\delta\) are among the earliest seen. These two proteins induce the expression of C/EBP\(\alpha\) and PPAR\(\gamma\). PPAR\(\gamma\) can also be activated by ADD1/SREBP1 through inducing its expression as well as promoting the production of an endogenous PPAR\(\gamma\) ligand RXR\(\alpha\). C/EBP\(\alpha\) forms a positive feedback loop operates synergistically with PPAR\(\gamma\) to induce the expression of other adipocyte specific genes such as aP2, GLUT4 and leptin, maintaining the terminal differentiated state. This graph is drawn according to (Rosen and Spiegelman, 2000).

Unfortunately, the story of adipogenesis is a lot more complicated than what is known. Little is known about the commitment of pluripotent stem cells into lipoblasts and the factors that control the transition from preadipocytes to adipocytes are largely unknown too (Tong et al., 2000). As a result, continuous efforts have been made aming at elucidating the regulatory mechanisms of adipogenesis. Studies for MDI - induced 3T3-L1 cell differentiation indicate that three individual signaling pathways are involved in initiating the differentiation: the cAMP pathway, the glucocorticoid receptor (GR) pathway and the insulin/IGF-1 pathway (Rosen and Spiegelman, 2000). C/EBP\(\alpha\),
C/EBPβ, C/EBPδ, ADD1/SREBP1, and PPARγ are expressed as the consequent events of the activation of these three signaling pathways. Reasonably, any molecule in those signaling pathways relaying adipogenesis signals will affect adipogenesis. For instance, the MAPK kinase pathway is a potentially important signaling pathway regulating adipogenesis. Some in vitro experiments specifically show that phosphorylation of Thr188 in C/EBPβ by MAPK "primes" C/EBPβ for subsequent phosphorylation on Ser184 and Thr179 by GSK-3β to acquire the DNA-binding ability and the capability of transactivating the C/EBPα and PPARγ genes (Tang et al., 2005). There is other evidence indicating that overexpression of mitogen-activated protein kinase phosphatase (MKP) that can inactivate MAP kinases in insulin-responsive preadipocytes significantly blocks insulin-induced adipogenesis (Xu et al., 2003). Those data suggest a positive role of MAPKs in adipogenesis. On the contrary, MAPK activation achieved by constitutively overexpressing activated MEK1 inhibits PPARγ transcriptional activity, indicating a negative role of MAPKs in adipogenesis (Watanabe et al., 2003). Whether MAPKs perform a positive or a negative role in adipogenesis and lipogenesis are under debate. It is possible that MAPKs can act bifunctionally in these processes and some factors such as cell line, activation timing, activation sequence and activation extent contribute to the determination of the cumulative function of MAPKs in a certain adipogenesis event (Bost et al., 2005). In fact, there is some evidence suggesting that MAPK signaling is necessary for clonal expansion while it plays an inhibitory role in the subsequent differentiation stage and needs to be turned down for the differentiation process to continue. Similar to MAPKs, PI3K/Akt pathway also seems to play a bifunctional role in adipogenesis. It has
been reported that the activity of PI3K/Akt pathway rises during adipogenesis to regulate PPARγ activation and inhibition of PI3K by inhibitors blocks adipogenesis while interestingly, an opposite result has also been reported indicating that overexpression of PI3K results in a marked decrease in PPARγ activity (Kim and Chen, 2004; Watanabe et al., 2003; Xia and Serrero, 1999). So, the exact roles the kinases play in adipogenesis remain unclarified. It is possible that most of them have dual functions and can act as either activators or inhibitors in PPARγ activation and adipogenesis depending on the situation. In addition to kinases, Wnt signaling was found to be implicated in adipogenesis regulation. The Wnt signaling pathway is composed of the extracellular ligands (Wnts, 19 identified), seven transmembrane cytoplasm membrane receptors (Frizzled, 9 identified), the co-receptors (such as LRP), the intracellular signaling molecules (such as β-catenin) and the secreted antagonists (Schinner, 2009). Wnt signaling is branched into the canonical (β-catenin) pathway and the noncanonical pathway with the canonical pathway being better studied (Schinner, 2009). For the canonical pathway (Fig. 11), binding of Wnt ligands with the Frizzled receptor as well as the co-receptor inactivates GSK-3, the kinase which normally phosphorylates β-catenin (Schinner, 2009). Thereby, β-catenin becomes unphosphorylated and stabilized, and translocates to the nucleus to regulate transcription of Wnt target genes (Schinner, 2009). The Wnt signaling has established its role in embryogenesis and tumorigenesis (Schinner, 2009). Recent studies suggested that Wnt signaling is implicated in metabolic diseases (Christodoulides et al., 2006) and activation of Wnt signaling, especially Wnt10b signaling, inhibits adipogenesis (Bennett et al., 2002; Kennell and MacDougald, 2005;
Schinner, 2009). Overexpression of Wnt10b in mouse preadipocytes represses C/EBPα and PPARγ expression and keeps the preadipocytes undifferentiated both in vitro and in vivo (Ross et al., 2000). Also, transgenic mice overexpressing Wnt10b in white adipose tissue resist to diet-induced obesity (Longo et al., 2004) or genetically-proned obesity (Wright et al., 2007b). It has been revealed that activation of the Wnt canonical pathway inhibits adipogenesis by decreasing PPARγ mRNA expression whereas activation of the Wnt non-canonical pathway inhibits adipogenesis by causing the activation of histone methyltransferase SETDB1 which suppresses PPARγ activation (Takada et al., 2009). It is expected that more and more adipogenesis regulatory factors or signaling pathways will be identified as the research progresses.

Figure 11. Wnt canonical pathway. Binding of Wnt ligands with Frizzled receptor as well as the co-receptor inactivates GSK-3, the kinase which normally phosphorylates β-catenin. β-catenin becomes unphosphorylated and stabilized, and translocates to the nucleus to regulate transcription of Wnt target genes. This graph is adapted from (Schinner, 2009).
Link Between Diabetes and Obesity

Type 2 diabetes and obesity are bridged by factors that contribute to insulin deficiency and insulin resistance. The concepts of glucotoxicity, lipotoxicity, and cellular nutrient overload have been advanced previously to explain the pathogenesis of type 2 diabetes in obese individuals (Poitout and Robertson, 2002). According to the glucotoxicity hypothesis, long-term hyperglycemia in diabetic and obese subjects contributes to both insulin deficiency and insulin resistance, which in turn worsen the situation of diabetes (Buren et al., 2003; Maedler et al., 2002). In addition to the glucotoxicity hypothesis, lipotoxicity is another popular concept emerging to explain the connection between type 2 diabetes and obesity. In individuals who develop type 2 diabetes and/or obesity, fat cells tend to be enlarged and the storage capacity of those enlarged fat cells is exceeded and thus diminished. Lipids, including FFA, continuously overflow from the dysfunctional fat cells into muscle and liver and possibly pancreatic β cells, stimulating gluconeogenesis, exacerbating insulin resistance and impairing insulin secretion (DeFronzo, 2004). Although both visceral and subcutaneous adipose tissues are responsible for the release of lipids, visceral adipose tissue is the main site for lipid overflowing. PPARγ agonists such as thiazolidinediones, which can redistribute fat within the body by decreasing visceral and hepatic fat and increasing subcutaneous fat, have been shown to inhibit lipolysis, reduce plasma FFA and enhance adipocyte insulin sensitivity (DeFronzo, 2004). The mechanisms by which lipotoxicity contributes to type 2 diabetes and obesity are very complex. In fibroblasts, excessive lipids induce insulin resistance at least in part by activating MAPK, which when overactivated phosphorylates
insulin signaling molecules such as IRS-1 on serine residues and blocks insulin signaling (Usui et al., 1997). In skeletal muscle, the accumulation of fatty acids can lead to the accumulation of lipid peroxides, which may cause damage to mitochondria, resulting in energy imbalance, insulin resistance, type 2 diabetes and obesity (Schrauwen and Hesselink, 2004). In pancreatic β cells, excessive level of FFA up-regulates cAMP response element modulator (CREM)-17X repressors, suppressing insulin gene transcription and exacerbating β cell failure in type 2 diabetes (Zhou et al., 2003). And of course, there are many other mechanisms by which lipids harm insulin sensitivity.

Although nutrition overloading theories have explained to a certain extent the linking of excessive adiposity to insulin resistance and insulin secretory dysfunction, they fail to provide a universally acceptable and pathologically conclusive explanation (Tataranni and Ortega, 2005). Thus, new theories continue to emerge. Recent studies reveal that adipose tissue is not an inert tissue, but an active endocrine or even an immune organ, secreting biologically active protein factors known as adipokines (Lazar, 2005). Those adipocyte-derived factors modulate body metabolism and energy homeostasis in response to the nutritional status at physiological conditions. In the case of obesity, the expression of the anti-inflammatory and anti-diabetic factors such as adiponectin are down-regulated while the proinflammatory and diabetes-promoting adipokines, such as resistin, TNFα and IL-6 are overproduced and released from adipose tissue (Kharroubi et al., 2003). The presence of the diabetes-promoting adipokine profile leads to the development of insulin resistance and β cell dysfunction, which consequently results in type 2 diabetes (Kharroubi et al., 2003). Proinflammatory and diabetic
adipokines can block insulin secretion and impair insulin action by directly acting on pancreatic β cells, hepatocytes, muscle cells, and adipocytes through multiple mechanisms. For example, TNFα can mediate insulin resistance in diabetic/obese animals through its effects on tyrosine phosphorylation of IR and IRS-1 (Begum et al., 1996). Resistin can affect glucose tolerance and is related to whole-body and hepatic insulin resistance due to its effect in attenuating IR phosphorylation, IRS-1 phosphorylation, PI3K activation, PIP3 production, and Akt activation (Rajala et al., 2003; Steppan et al., 2001; Steppan et al., 2005). Since a variety of proinflammatory adipokines, for example TNFα and IL-6, are also produced by immune cells such as macrophage, to modulate the immune system, a new hypothesis has been proposed, suggesting that the immune system is involved in the process of type 2 diabetes and obesity and inflammation is a possible pathophysiological link between obesity and insulin resistance. This hypothesis is relatively convincing as obesity and insulin resistance seem to be positively associated with elevated markers of inflammation in most studies (Tatarani and Ortega, 2005). Indeed, there is a growing body of evidence indicating that obesity could be a chronic mild inflammatory state that leads to chronic activation of the innate immune system, causing progressive impairment of glucose tolerance and insulin action and ultimately resulting in type 2 diabetes (Tatarani and Ortega, 2005; Trayhurn, 2005). However, at this point, more efforts are still needed for better understanding of the mechanisms by which innate immunity links type 2 diabetes with obesity.
In addition to the theories involving adipokines and innate immunity, hypotheses implicated with subcellular stresses are also currently popular for the explanation of the association of type 2 diabetes with obesity. Among them, mitochondrial stress theory and endoplasmic reticulum (ER) stress theory are the most popular. Mitochondrion is the "energy factory" where most oxidative reactions take place. Mitochondrial function is normally required for glucose-stimulated insulin secretion from pancreatic β cells. They are, thereby, essential for metabolism and energy homeostasis. Loss of mitochondria and impairment of mitochondrial function have been observed in subjects that develop type 2 diabetes and/or obesity (Kelley et al., 2002; Ritov et al., 2005; Yang et al., 2000). Mitochondrial stress generated from hyperglycemia and hyperlipidemia causes mitochondrial dysfunction and reduces mitochondrial content. In malfunctional mitochondria, UCP-2 expression is elevated and mitochondrial fatty acid oxidation is decreased, leading to increased production of intracellular fatty acyl CoA and diacylglycerol (Lowell and Shulman, 2005). Excessive fatty acyl CoA and diacylglycerol activate PKC to initiate the activation of a serine kinase cascade which possibly involves IKK and JNK-1 (Lowell and Shulman, 2005). This leads to increased serine phosphorylation of IRS-1 and impaired insulin signaling and ultimately makes type 2 diabetes and obesity worse. Mitochondrial stress theory is well-received nowadays. But it is not the only stress theory for explaining the link of type 2 diabetes with obesity. ER stress theory is another popular stress theory recently developed. ER is essential for protein synthesis, protein post-translational modification and protein secretion. ER stress represents the state when ER homeostasis is disrupted and the unfolded or misfolded
proteins are accumulated (Hampton, 2000; Harding et al., 2002; Mori, 2000). Studies indicate that obesity can cause ER stress and ER stress in turn leads to the hyperactivation of JNK and the subsequent serine phosphorylation of IRS-1, resulting in the suppression of insulin signaling and development of type 2 diabetes and thus linking type 2 diabetes and obesity at the molecular, cellular, and organismal levels (Hotamisligil, 2005; Ozcan et al., 2004). This ER stress theory is further supported by an observation that mice, which are deficient in X-box-binding protein-1 (XBP-1), a transcription factor that modulates the ER stress response, develop insulin resistance (Ozcan et al., 2004). Nevertheless, like other theories, ER stress theory by itself cannot completely explain the link of type 2 diabetes with obesity.

In all, type 2 diabetes and obesity have been observed in clinical studies to be implicated with each other as twin diseases. To try to explain this phenomenon, multiple theories have emerged, including hyperglycemia theory, hyperlipidemia theory, inflammation theory, and organelle stress theories. Those theories are interwoven but none of them can give a conclusive explanation for the implication of type 2 diabetes and obesity. As a result, new theories continue to emerge. Combining all the theories together would help achieve a relatively good understanding about type 2 diabetes, obesity and their relationship.

Because diabetes, especially type 2 diabetes and obesity are implicated with each other, some medications for improving obesity have been proposed for type 2 diabetes treatment. For example, AMP-activated protein kinase (AMPK), acetyl-CoA carboxylase (ACC), adipocyte-related complement protein 30 (Acrp30), PPARα and PPARγ are
considered as potential therapeutic targets for preventing or reversing obesity-related diabetes (Moller, 2001). In fact, PPARγ agonists such as thiazolidinediones, which may render adipose tissue redistribution, have shown effects in improving insulin sensitivity and have become marketed anti-diabetic drugs (Boden et al., 2005).

**Previous Obesity-Related Research in Our Laboratory**

Like diabetes, obesity significantly affects the quality of life and the prevalence of obesity has reached an alarming level. On the other hand, currently the available therapies for obesity are quite limited. The anti-obesity medications are either not as effective, require continuous consumption, or bear severe adverse effects. Continuous efforts are demanded on the discovery and development of better anti-obesity agents. Since obesity is contributing to the onset and progression of type 2 diabetes, anti-diabetic drugs which also possess anti-obesity activity are highly desirable.

The research in our laboratory originally focused on the isolation and characterization of the anti-diabetic components from banaba extract. BE was found to stimulate glucose uptake in 3T3-L1 adipocytes like insulin does (Liu et al., 2001). However, unlike insulin, BE was not able to induce adipocyte differentiation with the presence of Dex and IBMX and it even inhibited 3T3-L1 preadipocytes differentiation induced by the MDI cocktail in a dose-dependent manner (Liu et al., 2001). With the help of HPLC, BE components were fractionated. Tannic acid was found to be the major bioactive component in BE. TA possesses both glucose uptake stimulatory activity and anti-adipogenesis activity (Liu et al., 2005). Using HPLC, TA was further fractionated.
And β-PGG was finally characterized as one of the effective components in TA, having not only the glucose uptake stimulatory activity but also the anti-adipogenesis activity (Li et al., 2005).

When synthesizing β-PGG, its anomer α-PGG co-existed as one of the end products. Both chemically synthesized α-PGG and β-PGG were tested for glucose uptake stimulatory activity and adipogenesis inhibitory activity in 3T3-L1 cells. And the two PGG anomers exhibited both activities in 3T3-L1 cells (Li et al., 2005; Liu, 2003). Consistently, in vivo studies showed that both α-PGG and β-PGG not only improved diabetic situations but caused loss of adipose tissue mass in diabetic and obese animals (Li et al., 2005; Liu, 2003). Moreover, α-PGG was less potent than β-PGG in inhibiting adipogenesis at low concentrations (1 μM to 30 μM) but had a similar potency to β-PGG at higher concentrations (30 μM to 50 μM) (Liu, 2003). Both α-PGG and β-PGG exhibited the strongest anti-adipogenic effect in 3T3-L1 preadipocytes when added to the MDI differentiation medium during the first two days of differentiation (Liu, 2003). Further studies indicated that when α-PGG or β-PGG was present in the medium upon induction of 3T3-L1 preadipocyte differentiation by MDI, only one cycle of mitotic clonal expansion was allowed to happen, while normally two cycles of clonal expansion are required for differentiation to occur (Liu, 2003). The preliminary PGG anti-adipogenetic activity mechanism studies revealed that PGG inhibited MDI-induced expression of C/EBPα and PPARγ and reversed MDI-suppressed expression of Pref-1 (Liu, 2003). It was suggested that those anti-adipogenesis effects may be mediated through MAPK-dependent activation of NFκB (Liu, 2003). However, our knowledge
about adipogenesis and adipogenesis inhibition is limited. What we know about the adipogenesis inhibition mechanisms of PGG may not represent the whole story. Further investigation is required.

6Cl-TGQ is derived from α-PGG (Ren et al., 2006). It has been observed that 6Cl-TGQ, like PGG, also possesses the adipogenesis inhibitory effects. But the adipogenesis inhibitory activity of 6Cl-TGQ was much lower in comparison with PGG. This finding reinitiated our interest in studying the mechanisms by which PGG, in particular α-PGG, inhibits adipogenesis, because α-PGG demonstrated strong activities in both the anti-diabetic aspect and the anti-adipogenesis aspect. Although α-PGG promoted glucose uptake in 3T3-L1 adipocytes, we surprisingly found that α-PGG inhibited glucose uptake in 3T3-L1 preadipocytes. It has been reported that adipogenesis of 3T3-L1 preadipocytes in the environment of low glucose (1 g/L) was inhibited (Wang and Tong, 2009). Therefore, we hypothesized that in addition to the previously identified MAPK-dependent activation of NFκB anti-adipogenesis mechanism, α-PGG may also inhibit adipogenesis through depriving glucose from preadipocytes. A study about the anti-adipogenesis mechanism of α-PGG was performed to test this hypothesis.
MATERIALS AND METHODS

Cell lines and culture conditions - Mouse 3T3-L1 preadipocyte cell line was purchased from American Type Culture Collection (ATCC; Rockville, MD). 3T3-L1 preadipocytes were cultured in DMEM (Mediatech; Herndon, VA) containing 10% CS and 1% penicillin/streptomycin at 37°C in a 5% CO₂ cell incubator. 3T3-L1 adipocytes were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin at 37°C in a 5% CO₂ cell incubator.

3T3-L1 preadipocyte differentiation conditions - Mouse 3T3-L1 preadipocytes were cultured in 10% CS-containing DMEM until confluency. Medium was refreshed and cells were kept confluent for 2 days. Then cells were switched to 10% FBS-containing DMEM supplemented with 500 μM of 3-isobutyl-1-methylxanthine, 0.25 μM of dexamethasone and 1 μg/ml of insulin to induce differentiation. 48 hours after induction of differentiation, medium was replaced with fresh 10% FBS-containing DMEM supplemented with 1 μg/ml of insulin to continue driving differentiation. After that, medium was replaced with fresh 10% FBS-containing DMEM every 48 hours until 3T3-L1 preadipocytes are fully differentiated to form adipocytes which was approximately 8 days after induction of differentiation.

Compounds and compound treatment - α-PGG was synthesized by Dr. Yulin Ren and Dr. Klaus Himmeldirk in the Department of Chemistry and Biochemistry of Ohio University. α-PGG was dissolved in ddH₂O before using. Cells were treated with 30 μM of α-PGG unless described otherwise.
**Glucose uptake assay** - The glucose uptake assay procedures were modified according to those that were previously used by other researchers (Cornelius et al., 1990; Li et al., 2005; Sakoda et al., 1999; Tafuri, 1996). In this assay, undifferentiated 3T3-L1 preadipocytes were used. Cells were first incubated with serum-free DMEM for 2 hours at 37°C in a 5% CO2 cell incubator after being washed with serum-free DMEM twice. Then cells were switched to KRP buffer, washed with KRP buffer for 3 times followed by incubation in the buffer for 30 min. After that, cells were treated for 15 min with mock (ddH2O), 30 μM α-PGG, MDI differentiation cocktail, or MDI with 30 μM α-PGG. Then, 1 μCi/ml 2-deoxy-D-(1-3H) glucose (Amersham Biosciences/GE Healthcare; Pittsburgh, PA) was added to initiate glucose uptake. 10 min after addition of 2-deoxy-D-(1-3H) glucose, buffer was aspirated and cells were washed with ice-cold PBS 3 times to terminate glucose uptake. Cells were then lysed with 1% triton X-100 (Sigma-Aldrich; St. Louis, MO). Finally, the radioactivity was determined by liquid scintillation spectrometry with a Beckman Coulter LS 6400 Multi-Purpose Scintillation Counter (Beckman Coulter, Inc.; Fullerton, CA).

**Oil Red O staining** - 3T3-L1 preadipocytes were cultured in DMEM with 10% CS and 1% penicillin/streptomycin in 24-well plates. 2 days after cells reached confluency, cells were induced to differentiate by MDI in 10% FBS-containing media. The media contained various concentrations of glucose and were supplemented with or without 30 μM α-PGG. 48 hours after initiation of differentiation, medium was replaced with fresh 10% FBS-containing DMEM supplemented with 1 μg/ml of insulin. Medium was then replaced with fresh 10% FBS-containing DMEM every other day till 8 days after
differentiation initiation when Oil Red O staining was performed. To perform Oil Red O staining, culture medium was removed. Cells were washed with PBS twice gently. Then 500 μl of Oil Red O solution (Chemicon/Millipore; Temecula, CA) was added to each well to stain the cells. Cells were incubated with Oil Red O solution for 15 min before the staining solution was carefully aspirated and the cells were washed 3 times with 1ml/well Wash Solution (Chemicon/Millipore; Temecula, CA). Cell pictures were taken using either a light-field microscope-coupled manual Nikon camera (Nikon; Japan) or a light-field microscope-coupled automated Nikon camera (ECLIPSE E600, Nikon; Japan) when the ocular lens was 10X and the objective lens was 20X. To extract the Oil Red O dye in the stained cells for quantification, 100 μl/well of Dye Extract Solution (Chemicon/Millipore; Temecula, CA) was added to cells followed by a 15-30 min shaking on a Barbital shaker or platform rocker at room temperature. Then, the extracted dye was transferred into cuvettes or 96-well plates and the absorbance was determined at 520 nm with an ELISA reader (SPECTRA max M2, Molecular Devices; Sunnyvale, CA).

Total protein preparation - 3T3-L1 preadipocytes were cultured in DMEM with 10% CS and 1% penicillin/streptomycin till 2 days after confluency. Then cells were induced to differentiate by MDI in 10% FBS containing media. The media contained either 0.5% or 4.5% glucose and were supplemented with or without 30 μM α-PGG. After 48 hours, cells were switched to DMEM containing 10% FBS and 1 μg/ml of insulin. 96 hours after differentiation initiation, total proteins were isolated for western blot analysis. To prepare total proteins, cells were washed with phosphate buffered saline (PBS) and lysed on ice with lysis buffer (10 mM Tris HCl pH 7.5, 1 mM EDTA, 1%
triton X-100, 150 mM NaCl, 1 mM dithiothretol, 10% glycerol, 0.2 mM phenylmethysulphonyl fluoride and protease inhibitors). Cell lysate was subjected to sonication on ice at power level 3 with a 550 Sonic Dismembrator (Fisher Scientific; Pittsburgh, PA), 3 seconds each time for 3 times. The sonicated cell lysate was centrifuged at 14,000 g for 10 min at 4°C. Supernatant was collected. Protein concentrations were determined using BCA protein assay kit (Pierce; Rockford, IL).

**Western blot analysis** - 50 µg of total protein was prepared in SDS sample buffer (Cell Signaling; Beverly, MA) and was loaded to 7.5% or 10% ready-to-use Tris-glycine polyacrylamide gels (Bio-Rad; Hercules, CA) which were soaked in electrode buffer (3 g/L tris, 14.4 g/L glycine, 1 g/L SDS). Proteins migrated in the electric field to be separated. After proteins were well-separated, they were transferred to nitrocellulose membrane (Schleicher & Schuell; Dassel, Germany) when 110V electric field and transfer buffer (1.51 g/L tris, 7.2 g/L glycine, 20% methanol, 0.01% SDS) were present. One hour after initiation of protein transfer, nitrocellulose membrane with proteins was washed by TBST and was blocked with 5% fat-free milk in TBST at room temperature for 1 hour. Then, target proteins were probed with specific primary antibodies overnight at 4°C. After a thorough wash with TBST, membrane was incubated with secondary antibodies for 1 hour. Proteins were visualized by Lumi-Light western blotting substrates (Roche; Nutley, NJ). Primary antibodies included those that are against: phospho-ERK1/2 (Thr202/Tyr204) (Cell Signaling; Beverly, MA), PPARγ (Santa Cruz; Santa Cruz, CA), and β-tubulin (Cell Signaling; Beverly, MA). Secondary antibodies used were HRP-conjugated goat-anti-mouse (Santa Cruz; Santa Cruz, CA) or strepavidin-
conjugated goat-anti-rabbit (Cell Signaling; Beverly, MA) depending upon the source of the primary antibodies. Each experiment was repeated at least once.

*Real time quantitative PCR* - Preadipocytes were cultured in 10% CS and 1% penicillin/streptomycin-containing DMEM till 2 days post confluency. Then cells were initiated to differentiate by MDI in 10% FBS-containing media. The media were glucose-free medium added with 0.5 g/L glucose, or glucose-free medium added with 4.5 g/L glucose, or DMEM, or DMEM supplemented with 30 μM of α-PGG, or DMEM supplemented with 30 μM of α-PGG and 18 g/L glucose. After 48 hours, cells were switched to DMEM containing 10% FBS and 1 μg/ml of insulin. 96 hours after initiation of differentiation, medium was replaced with fresh DMEM containing 10% FBS. 96 or 120 hours after initiation of differentiation, RNA was extracted using RNeasy total RNA extraction kit (Qiagen, Inc.; Valencia, CA) according to the manufacturer’s protocol. The quality of RNA was verified using 12% formaldehyde agarose gel electrophoresis and the concentration of the total RNA extracted was measured using spectrophotometer at 260 nm. 3 μg of the total extracted RNA was subjected to RT-PCR and cDNA was synthesized by the Effendorf Mastercycler gradient PCR machine (Effendorf; Westbury, NY) with the Bio-Rad iScript™ Select cDNA Synthesis kit (Bio-Rad; Hercules, CA) containing random primers and oligo dT mix. The cDNAs produced were used to specifically quantify the transcripts of interest using the instrument Bio-Rad iCycler (Bio-Rad; Hercules, CA). The used RT² PCR primer sets included the primers for mouse Wnt10b (SuperArray; Frederick, MD), mouse GLUT1 (SuperArray; Frederick, MD), mouse PPARγ (SuperArray; Frederick, MD), and mouse β-actin (SuperArray; Frederick,
MD). The Bio-Rad iQ™ SyBr Green Supermix kit (Bio-Rad; Hercules, CA) provided all other reagents necessary for the real-time PCR. The SyBr green dye offered in the Bio-Rad iQ™ SyBr Green Supermix kit intercalates with the double-stranded cDNA being formed during PCR, allowing an easy quantification of cDNA to indirectly quantify the original RNA transcripts present. For determining the transcript levels, ΔCt method was used.

Statistical analyses - All the assays described above were repeated more than once. The quantification assays were performed in triplicate and data were calculated and presented as mean ± standard deviation (M±SD). Glucose uptake assay data were analyzed by one-way ANOVA and Turkey’s post-hoc test. Significant level was set as p<0.05. ** indicated p<0.01; *** indicated p<0.001.
RESULTS

α-PGG inhibited glucose uptake in 3T3-L1 preadipocytes - Although α-PGG was known to stimulate glucose uptake in 3T3-L1 adipocytes, whether it stimulates or inhibits glucose uptake in 3T3-L1 preadipocytes remained unknown. In order to reveal the effects of α-PGG on glucose uptake in 3T3-L1 preadipocytes, a glucose uptake assay was performed. As shown by the assay, α-PGG, rather than promoting glucose uptake, inhibited glucose uptake in 3T3-L1 preadipocytes regardless of the absence (p<0.01) or presence (p<0.001) of MDI (Fig. 12). 30 μM of α-PGG was able to reduce glucose uptake in 3T3-L1 preadipocytes by 30-50% (Fig. 12). In addition, MDI treatment did not generate noticeable effect on glucose uptake in 3T3-L1 preadipocytes (Fig. 12).
Figure 12. α-PGG inhibited glucose uptake in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were treated with 30 μM of α-PGG 15 min prior to addition of 2-deoxy-D-(1-^{3}H) glucose. 10 min after addition of radioactive glucose, intracellular radioactivity was measured. Meanwhile, protein concentrations were determined. For each sample, the glucose uptake result was normalized with the correspondent protein concentration. Data were expressed as the glucose uptake activity of cells under a particular condition relative to the glucose uptake activity of mock-treated cells. Each data point was an average of results from three independent experiments and presented as M relative to mock-treated control ± SD. One-way ANOVA was used to determine the significance. ** indicated p<0.01; *** indicated p<0.001.

3T3-L1 cell adipogenesis was inhibited by glucose deprivation or α-PGG treatment - It has been reported that adipogenesis of 3T3-L1 preadipocytes in the environment of low glucose was inhibited (Wang and Tong, 2009). To verify the effects of low glucose and α-PGG in adipogenesis inhibition, 3T3-L1 preadipocytes were differentiated in 10% FBS-containing media with different concentrations of glucose with
the presence or absence of 30 μM of α-PGG. Oil Red O staining, which stains the intracellular lipid droplets, was used to stain the cells 8 days after differentiation initiation to determine the status of adipogenesis. Oil Red O staining demonstrated that with the decrease of the glucose concentration from 4.5 g/L (DMEM) to 0.45 g/L (1/10 DMEM plus 9/10 glucose-free medium), the ability of 3T3-L1 preadipocytes to differentiate to adipocytes decreased (Fig. 13). 3T3-L1 preadipocytes retained the full ability to differentiate to adipocytes in medium with 4.5 g/L glucose (DMEM) (Fig. 13). In the medium with 1.5 g/L glucose (1/3 DMEM plus 2/3 glucose-free medium), around 50% of the cells were able to differentiate to form adipocytes (Fig. 13). But when glucose level in the medium decreased to 0.9 g/L (1/5 DMEM plus 4/5 glucose-free medium) or lower (1/10 DMEM plus 9/10 glucose-free medium), almost no cells could differentiate (Fig. 13). These results matched very well with the reported effects of low glucose (1 g/L) on adipogenesis (Wang and Tong, 2009). When treating 3T3-L1 preadipocytes with 30 μM of α-PGG during differentiation induction, cells were unable to differentiate in medium with low concentrations of glucose and even in medium with 4.5 g/L glucose (DMEM) (Fig. 13). Considering that α-PGG could inhibit glucose uptake in 3T3-L1 preadipocytes, it was hypothesized that α-PGG may inhibit 3T3-L1 cell adipogenesis through inhibiting glucose uptake.
A

DMEM 10% FBS

1/3 DMEM 10% FBS + 2/3 glucose-free medium + 10% FBS + MDI

1/10 DMEM 10% FBS + 9/10 glucose-free medium 10% FBS + MDI

1/5 DMEM 10% FBS + 4/5 glucose-free medium 10% FBS + MDI

DMEM 10% FBS + MDI + 30 μM α-PGG

DMEM 10% FBS + MDI + 30 μM α-PGG
Figure 13. 3T3-L1 cell adipogenesis was inhibited by glucose deprivation or α-PGG treatment. 3T3-L1 preadipocytes were induced to differentiate by MDI in 10% FBS-containing media with different concentrations of glucose (4.5 g/L, 1.5 g/L, 0.9 g/L and 0.45 g/L) with the presence or absence of 30 μM of α-PGG. Undifferentiated cells were used as negative controls. Each condition was prepared in triplicate. 48 hours after differentiation induction, media were switched to fresh 10% FBS-containing DMEM supplemented with 1 μg/ml of insulin. After that, media were replaced with fresh 10% FBS-containing DMEM every 48 hours till 8 days after differentiation induction. Oil Red O staining was used to stain the cells to determine the status of adipogenesis. The lipid droplets were stained red. Cells with accumulated lipid droplets were adipocytes. (A) Light field microscopic pictures were taken for cells stained with Oil Red O when the ocular lens was 10X and the objective lens was 20X. Lipid droplets were indicated by red. (B) The Oil Red O dye in stained cells was extracted for quantification at 520 nm for each condition. Each data point was an average of results from three replicates and presented as M±SD.

Glucose supplement to glucose-free medium was able to increase adipogenesis in 3T3-L1 cells while glucose supplement was not able to reverse the adipogenesis inhibitory effects of α-PGG - Oil Red O staining was applied to study whether glucose supplement would reverse the adipogenesis inhibitory effects of low glucose and α-PGG. Our results indicated that addition of 0.5-1 g/L of glucose to glucose free-medium could
not fully support adipogenesis although adipogenesis improved as the increase of glucose concentration (Fig. 14). When 4.5 g/L glucose was added to glucose-free medium, 3T3-L1 cells fully differentiated (Fig. 14). For the situation with α-PGG, 30 µM of α-PGG almost completely inhibited 3T3-L1 cell adipogenesis even when 4.5 g/L of glucose was present in the differentiation medium (Fig. 14). This is consistent with our previous findings (Fig. 13). Unlike the situation of directly reducing glucose levels in the medium, glucose supplement was not reversing the adipogenesis inhibitory effects of α-PGG in 3T3-L1 cells (Fig. 14).
Glucose supplement to glucose-free medium was able to increase adipogenesis in 3T3-L1 cells while glucose supplement was not able to reverse the adipogenesis inhibitory effects of α-PGG. 3T3-L1 preadipocytes were induced to differentiate by MDI in 10% FBS-containing glucose-free media of various concentrations of glucose (0.5 g/L, 1 g/L and 4.5 g/L) or in 10% FBS-containing DMEM with the presence of 30 μM of α-PGG and different concentrations of additional glucose (0 g/L, 4.5 g/L and 18 g/L). Undifferentiated cells were used as negative controls. Cells induced to differentiate by MDI in 10% FBS-containing DMEM were used as positive controls. Each condition was prepared in triplicate. 48 hours after differentiation induction, media were switched to fresh 10% FBS-containing DMEM supplemented with 1 μg/ml of insulin. After that, media were replaced with fresh 10% FBS-containing DMEM every 48 hours till 8 days after differentiation induction. Oil Red O staining was used to stain the cells to determine the status of adipogenesis. The lipid droplets were stained red. Cells with accumulated lipid droplets were adipocytes. (A) Light field microscopic pictures were taken for cells stained with Oil Red O when the ocular lens was 10X and the objective lens was 20X. Lipid droplets were indicated by red. (B) The Oil Red O dye in stained cells was extracted for quantification at 520 nm for each condition. Each data point was an average of results from three replicates and presented as M±SD.

Figure 14. Glucose supplement to glucose-free medium was able to increase adipogenesis in 3T3-L1 cells while glucose supplement was not able to reverse the adipogenesis inhibitory effects of α-PGG. 3T3-L1 preadipocytes were induced to differentiate by MDI in 10% FBS-containing glucose-free media of various concentrations of glucose (0.5 g/L, 1 g/L and 4.5 g/L) or in 10% FBS-containing DMEM with the presence of 30 μM of α-PGG and different concentrations of additional glucose (0 g/L, 4.5 g/L and 18 g/L). Undifferentiated cells were used as negative controls. Cells induced to differentiate by MDI in 10% FBS-containing DMEM were used as positive controls. Each condition was prepared in triplicate. 48 hours after differentiation induction, media were switched to fresh 10% FBS-containing DMEM supplemented with 1 μg/ml of insulin. After that, media were replaced with fresh 10% FBS-containing DMEM every 48 hours till 8 days after differentiation induction. Oil Red O staining was used to stain the cells to determine the status of adipogenesis. The lipid droplets were stained red. Cells with accumulated lipid droplets were adipocytes. (A) Light field microscopic pictures were taken for cells stained with Oil Red O when the ocular lens was 10X and the objective lens was 20X. Lipid droplets were indicated by red. (B) The Oil Red O dye in stained cells was extracted for quantification at 520 nm for each condition. Each data point was an average of results from three replicates and presented as M±SD.

**Phosphorylation of MAPK in 3T3-L1 cells at adipogenesis middle stage was induced by low glucose condition during the initial stage of differentiation but not by α-PGG treatment at the early stage of differentiation** - In order to further compare the
effects of low glucose and α-PGG treatment on adipogenesis, 3T3-L1 preadipocytes were
induced to differentiate by MDI in media of different glucose concentrations and
supplemented with or without 30 μM of α-PGG. 96 hours after differentiation initiation,
total proteins were isolated for western blot analysis. MAPK phosphorylation at 96 h was
observed in cells that were subjected to low glucose condition (0.5 g/L glucose) at the
early stage of differentiation and no MAPK phosphorylation at 96 h was seen in cells that
were differentiated in glucose-free medium supplemented with normal concentration (4.5
g/L) of glucose (Fig. 15). On the contrary, MAPK phosphorylation at 96 h was not
detected in cells that were treated with 30 μM of α-PGG at the early stage of
differentiation (Fig. 15). PPARγ expression reflects the progression of adipogenesis.
During adipogenesis, PPARγ expression starts 48 hours and peaks 96 hours after
differentiation initiation (Liu, 2003). The expression of PPARγ was elevated 96 hours
after differentiation initiation under the normal differentiation condition (Fig. 15). PPARγ
expression level kept low in cells subjected to glucose-free differentiation medium added
with 0.5 g/L glucose or DMEM supplemented with 30 μM of α-PGG (Fig. 15).
Consistent with the results shown in Fig. 12, addition of 4.5 g/L glucose to glucose-free
differentiation medium prevented 3T3-L1 cells from being inhibited of adipogenesis as
indicated by the elevated PPARγ level 96 hours after differentiation initiation (Fig. 15).
However, supplementing high glucose (18 g/L) to DMEM with 30 μM of α-PGG did not
reverse the adipogenesis inhibitory effect of α-PGG in 3T3-L1 cells as indicated by the
low PPARγ level (Fig. 15).
Figure 15. Phosphorylation of MAPK in 3T3-L1 cells at adipogenesis middle stage was induced by low glucose condition during the initial stage of differentiation but not by α-PGG treatment at the early stage of differentiation. 3T3-L1 preadipocytes were induced to differentiate by MDI in media of different glucose concentrations and supplemented with or without 30 μM of α-PGG. 48 hours after differentiation induction, media were switched to fresh media with 10% FBS-containing DMEM supplemented with 1 μg/ml of insulin. 96 hours after differentiation initiation, total proteins were isolated for western blot analysis. Phospho-MAPK and PPARγ protein levels were determined to represent cell status. β-tubulin was used as a loading control.

<table>
<thead>
<tr>
<th>3T3-L1</th>
<th>96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM</td>
<td>+</td>
</tr>
<tr>
<td>MDI</td>
<td>-</td>
</tr>
<tr>
<td>Glucose free media</td>
<td>-</td>
</tr>
<tr>
<td>Glucose (g/L)</td>
<td>- 0.5 4.5 - 18</td>
</tr>
<tr>
<td>30 μM α-PGG</td>
<td>-</td>
</tr>
</tbody>
</table>

| Phospho-MAPK (Thr202/Tyr204) | 44/42 kd |
| PPAR-γ                       | 67 kd   |
| β-tubulin                    | 56 kd   |

### Table: Glucose Concentrations and β-tubulin Levels

<table>
<thead>
<tr>
<th>Glucose (g/L)</th>
<th>β-tubulin</th>
</tr>
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<tbody>
<tr>
<td>0.5</td>
<td>++</td>
</tr>
<tr>
<td>1</td>
<td>++ ++</td>
</tr>
<tr>
<td>4.5</td>
<td>+ +</td>
</tr>
<tr>
<td>18</td>
<td>+</td>
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Expression of GLUT1 in 3T3-L1 cells was not altered by both low glucose condition and α-PGG addition during the initial stage of differentiation whereas expression of Wnt10b was upregulated by low glucose condition but not by α-PGG supplement at the early stage of differentiation - Post-confluent 3T3-L1 preadipocytes were subjected to different differentiation conditions during the first 48 hours of differentiation. After that, regular differentiation procedures were carried out. Real-time PCR was applied to determine the effects of low glucose condition and α-PGG on...
GLUT1, PPARγ and Wnt10b expression during adipogenesis of 3T3-L1 preadipocytes. The result indicated that both the low glucose condition (0.5 g/L glucose) and 30 μM of α-PGG did not alter the expression level of GLUT1 96 and 120 hours after differentiation initiation (Fig. 16). Wnt10b expression was high in undifferentiated 3T3-L1 preadipocytes (Fig. 16). But the level of Wnt10b mRNA decreased 96 and 120 hours after the regular differentiation induction (Fig. 16). Interestingly, when 3T3-L1 preadipocytes were induced to differentiate in medium of only 0.5 g/L glucose, the expression level of Wnt10b remained almost as high as undifferentiated cells 96 and 120 hours after differentiation induction (Fig. 16). In contrast, when cells were induced to differentiate with the presence of 30 μM of α-PGG, the expression level of Wnt10b in cells was as low as that in the regularly differentiated cells 96 or 120 hours after differentiation initiation (Fig. 16).
Expression of GLUT1 in 3T3-L1 cells was not altered by both low glucose condition and α-PGG addition during the initial stage of differentiation whereas expression of Wnt10b was upregulated by low glucose condition but not by α-PGG supplement at the early stage of differentiation. 3T3-L1 preadipocytes were induced to differentiate by MDI in 10% FBS-containing glucose-free medium supplemented with 0.5 g/L glucose or in DMEM supplemented with 30 μM of α-PGG. Undifferentiated cells were used as negative controls while cells induced to differentiate by MDI in 10% FBS-containing DMEM were used as positive controls. 48 hours after differentiation induction, media were switched to fresh 10% FBS-containing DMEM supplemented with 1 μg/ml of insulin. 96 hours after differentiation initiation, media were replaced with fresh 10% FBS-containing DMEM. (A) 96 hours or (B)120 hours after differentiation initiation, RNA was isolated from cells. mRNA was reverse transcribed to cDNA for real-time PCR analysis of GLUT1, PPARγ and Wnt10b mRNA levels. Primers specific for GLUT1, PPARγ and Wnt10b cDNAs and iCycler iQ Real-time Detection System were applied in the analysis. β-actin levels were also measured from the same cDNA preparation to normalize the mRNA levels of GLUT1, PPARγ and Wnt10b, mRNA levels of GLUT1, PPARγ and Wnt10b were calculated based on the ΔCt method. SD was calculated accordingly. Each condition was run in two separate experiments. And in each experiment, cDNAs were measured in three quantitative PCR runs. The results shown were from one representative experiment and expressed as M±SD.
DISCUSSION

Obesity represents an extremely overweight state. Obesity has become prevalent across the world. According to the statistic data from the Centers for Disease Control and Prevention, more than 25% of the US population are obese (Centers for Disease Control and Prevention, 2008). Obesity is the result of chronic disruption of energy balance: the long-term imbalance of energy intake, energy expenditure, nutrient partitioning and adipogenesis. Both genetic and environmental factors contribute to the occurrence of obesity (Loos and Bouchard, 2003). Obesity not only causes morbidity, decreases the quality of life, but also increases the chance of diabetes, cardiovascular diseases, respiratory diseases, cancers and so on (Poitout and Robertson, 2002). Low-calorie diet and exercises are recommended for overweight or obese patients. Moreover, some anti-obesity medications are available for treating obesity. On the other hand, most of the currently available anti-obesity medications are not satisfactory. They either have severe side effects or are not as effective. Efforts have been made to search for better anti-obesity medications.

Adipogenesis is the differentiation of preadipocytes to adipocytes (Rosen and Spiegelman, 2000). It involves a tightly regulated transcriptional programming. 3T3-L1 cells are largely used for adipogenesis study. Although much knowledge about adipogenesis has been obtained from 3T3-L1 cells, adipogenesis is far from being fully understood. Since advanced adipogenesis contributes to obesity, inhibiting excessive adipogenesis could be a strategy for obesity treatment.
Our laboratory initially aimed at finding some anti-diabetic reagents from natural sources. Banaba extract, the tannic acid component of BE, the β-PGG from TA, and the synthesized α-PGG were sequentially found to activate the IR signaling to stimulate glucose uptake in 3T3-L1 adipocytes and reduce glucose levels in diabetic mice (Li et al., 2005; Liu et al., 2001; Liu, 2003; Liu et al., 2005). Unlike some anti-diabetic medications, BE, TA, β-PGG and α-PGG did not promote weight gain when treating diabetic animals (Li et al., 2005; Liu et al., 2001; Liu, 2003; Liu et al., 2005). Instead, they inhibited adipogenesis in 3T3-L1 cells (Li et al., 2005; Liu et al., 2001; Liu, 2003; Liu et al., 2005). This suggested that BE, TA, β-PGG and α-PGG are not PPARγ agonists. They do not improve insulin sensitivity by activating PPARγ. Rather, they mimic insulin to lower blood glucose level by working on the IR signaling directly (Li et al., 2005; Liu et al., 2001; Liu, 2003; Liu et al., 2005). While the anti-diabetic mechanisms of the extracts or compounds have been well-studied, the mechanisms by which BE, TA, β-PGG and α-PGG inhibit adipogenesis were not well-understood.

Because β-PGG and α-PGG are pure compounds, they were used in the anti-adipogenesis mechanism studies. It was found that PGG inhibited MDI-induced expression of PPARγ and C/EBPα and reversed MDI-suppressed expression of Pref-1 probably through MAPK-dependent activation of NFκB (Liu, 2003). However, the role of MAPK in adipogenesis was not well-established. What we know about the adipogenesis inhibition mechanisms of PGG may not represent the whole story. And in this study, we continued the mechanistic study for the adipogenesis inhibitory effect of PGG with the focus on α-
PGG because it showed significant effects in both diabetes treatment and adipogenesis inhibition.

\(\alpha\)-PGG has been known to activate the IR signaling pathway to stimulate glucose uptake in 3T3-L1 adipocytes (Li et al., 2005). The glucose uptake induced by \(\alpha\)-PGG is mediated by GLUT4, the insulin responsive glucose transporter (Li et al., 2005). GLUT4 exists predominantly in adipose tissue and striated muscle in heart and skeleton although it is also found in the brain (Vannucci et al., 1998). 3T3-L1 preadipocytes are embryonic fibroblast cells. GLUT4 is not expressed in 3T3-L1 preadipocytes until it is fully differentiated into adipocytes (Rosen and Spiegelman, 2000; Wu et al., 1999). Therefore, GLUT4-mediated glucose uptake lacks in 3T3-L1 preadipocytes. Using glucose uptake assay, we tested how \(\alpha\)-PGG influences glucose uptake in 3T3-L1 preadipocytes which lacks GLUT4. Interestingly, \(\alpha\)-PGG was found to inhibit glucose uptake in 3T3-L1 preadipocytes (Fig. 12). In contrast, MDI cocktail, which is normally used for inducing 3T3-L1 preadipocyte differentiation, did not affect glucose uptake in 3T3-L1 preadipocytes (Fig. 12). Because insulin is one of the components in MDI, the lack of effect of MDI on glucose uptake in 3T3-L1 preadipocytes supported the notion that 3T3-L1 preadipocytes do not express insulin responsive GLUT4. Therefore, \(\alpha\)-PGG did not inhibit glucose uptake in 3T3-L1 preadipocytes through the IR-GLUT4 pathway.

Glucose is the building block, energy source and signaling molecule of cells. It has been reported that adipogenesis of 3T3-L1 preadipocytes was inhibited in the environment of low glucose (1 g/L) (Wang and Tong, 2009). Considering that \(\alpha\)-PGG inhibited glucose uptake in 3T3-L1 preadipocytes regardless of the presence of MDI (Fig.
12), we hypothesized that \( \alpha \)-PGG may inhibit adipogenesis through reducing glucose uptake in 3T3-L1 preadipocytes. To test the hypothesis, the effect of glucose deprivation on adipogenesis and the effect of \( \alpha \)-PGG supplement on adipogenesis were compared side by side. Our results showed that the potential of 3T3-L1 preadipocytes to differentiate decreased as the glucose concentration in the differentiation induction medium declined from 4.5 g/L to 0.45 g/L (Fig. 13). When the concentration of glucose in the differentiation medium dropped below 0.9 g/L, almost no 3T3-L1 preadipocytes can differentiate (Fig. 13), which is consistent with the published results (Wang and Tong, 2009). \( \alpha \)-PGG showed an adipogenesis inhibitory effect in a dose-dependent manner (Liu, 2003). 30 \( \mu \)M of \( \alpha \)-PGG was seen to completely inhibit 3T3-L1 cell adipogenesis when added to the differentiation initiation media in which the glucose concentrations ranged from 0.45 g/L to 4.5 g/L (Fig. 13). PPAR\( \gamma \) level starts to increase 48 hours after differentiation initiation and peaks 96 hours after differentiation initiation in a regular MDI-induced adipogenesis process. The low level of PPAR\( \gamma \) expression indicates an undifferentiated status of cells. As shown in our subsequent results, PPAR\( \gamma \) expression levels remained low 96 and 120 hours after differentiation initiation for 3T3-L1 cells that were induced to differentiate in both the low glucose condition (0.5 g/L glucose) and the \( \alpha \)-PGG supplement condition (Fig. 16). This confirmed that cells were inhibited of differentiation under both conditions. At this point, it seemed that \( \alpha \)-PGG might inhibit adipogenesis in 3T3-L1 preadipocytes through reducing intracellular glucose levels. However, this was not firmly established.
Based on our hypothesis, it is possible that the adipogenesis inhibitory effect of α-PGG can be reversed by high concentrations of glucose. When testing whether glucose supplement may reverse the adipogenesis inhibitory effect of glucose deprivation, it was found that supplementing glucose to glucose-free medium not only protected cells from autophagy, but also enabled the cells to differentiate (Fig. 14). And the effect of glucose on adipogenesis was dose-dependent (Fig. 14). DMEM contains 4.5 g/L glucose. When 4.5 g/L glucose was supplemented to glucose-free medium, 3T3-L1 preadipocytes may fully differentiate as they were in DMEM (Fig. 14). In contrast, supplementing 4.5 g/L or 18 g/L external glucose to DMEM did not reverse the adipogenesis inhibitory effects of α-PGG (Fig. 14), although the increase of glucose uptake in cells was expected upon increasing environmental glucose concentrations. This suggested that α-PGG may either completely and irreversibly inhibit glucose uptake or inhibit adipogenesis through a mechanism other than glucose deprivation. Because α-PGG did not completely suppress glucose uptake in 3T3-L1 preadipocytes as shown by the glucose uptake assay (Fig. 12), it is more likely that α-PGG inhibits adipogenesis via a mechanism other than glucose deprivation. On the other hand, we cannot exclude the possibility that glucose-deprivation through glucose uptake inhibition also plays a minor role in α-PGG-suppressed adipogenesis.

MAPK is involved in the regulation of adipogenesis (Watanabe et al., 2003; Xu et al., 2003). Activation of MAPK signaling is necessary for clonal expansion while down-regulation of MAPK signaling is required for the subsequent differentiation process to continue. In our study, MAPK phosphorylation could not be detected 96 hours after
differentiation initiation if 3T3-L1 preadipocytes were subjected to differentiation in DMEM or glucose-free medium supplemented with 4.5 g/L glucose. However, when 3T3-L1 preadipocytes were induced to differentiate in medium with a low level of glucose (0.5 g/L), MAPK serine phosphorylation was detectable 96 hours after differentiation initiation (Fig. 15). Rather than being similar to the situation of low glucose, α-PGG treatment during the early stage of differentiation did not induce a detectable MAPK phosphorylation 96 hours after differentiation initiation (Fig. 15). This result supported that α-PGG inhibits adipogenesis mainly via a mechanism different from glucose deprivation.

Like MAPK signaling, Wnt signaling also regulates adipogenesis. Wnt signaling especially Wnt10b signaling inhibits adipogenesis (Bennett et al., 2002; Kennell and MacDougald, 2005; Schinner, 2009). Using Real-time PCR, we found that Wnt10b mRNA level was high in undifferentiated 3T3-L1 preadipocytes (Fig. 16). If cells were induced to differentiate in DMEM by MDI, Wnt10b mRNA level decreased 96 and 120 hours after differentiation initiation (Fig. 16). If the glucose level was low in the differentiation initiation medium, Wnt10b remained at a high level 96 and 120 hours after differentiation initiation (Fig. 16). In comparison, when α-PGG was supplemented to the differentiation initiation medium, Wnt10b mRNA in cells dropped to a level similar to that when 3T3-L1 preadipocytes were induced to differentiate in DMEM by MDI (Fig. 16). This again indicated that α-PGG and glucose deprivation did not follow the same mechanism to inhibit adipogenesis. In addition, GLUT1 is a ubiquitously expressed glucose transporter, which also exists on 3T3-L1 preadipocyte membrane (Pascual et al.,
By examining the level of GLUT1 expression, it was found that neither the low glucose condition nor the \(\alpha\)-PGG supplement condition during differentiation induction altered the expression of GLUT1 (Fig. 16), indicating that both glucose deprivation and \(\alpha\)-PGG did not inhibit adipogenesis by altering the level of GLUT1.

In all, 3T3-L1 preadipocyte adipogenesis is inhibited when cells are induced to differentiate by MDI under either the low glucose condition or the \(\alpha\)-PGG supplement condition. Although \(\alpha\)-PGG is able to decrease glucose uptake in 3T3-L1 preadipocytes, glucose deprivation is not likely to be the mechanism by which \(\alpha\)-PGG inhibits adipogenesis. In addition, it is the first time, to our best knowledge, that MAPK activation at the middle stage of adipogenesis and the resistance to MDI-induced Wnt10b down-regulation are observed in 3T3-L1 cells that are induced to differentiate by MDI under the low glucose condition.
SECTION III

ANTI-CANCER ACTIVITIES OF PENTA-O-GALLOYL-\(\alpha\)-D-
GLUCOPYRANOSE
INTRODUCTION

Cancer

Cancer or malignant neoplasm is a class of diseases in which the growth of a group of cells is out of control. Normally, cell growth is tightly controlled and the balance of cell proliferation and cell death is well-maintained in multicellular organisms through cell-cell and cell-environment communications. In the case of cancer, malignant cells ignore the growth arrest or apoptotic signals and undergo division beyond the normal limits. When the growth of the malignant cells reaches a certain extent, they invade and destruct the adjacent tissues and sometimes metastasize to remote locations of the body via blood or lymph stream. The uncontrolled growth, invasion and metastasis features of malignant cells make them distinguished from benign cells. Most cancers form solid tumors while others do not. For example, malignant cells in leukemia do not form solid tumors. Cancer may occur at various sites of the body and it is often named according to its origin. Based on the origin, cancers are classified into the following categories: carcinoma (derived from epithelial cells), sarcoma (derived from connective tissues or mesenchymal cells), lymphoma/leukemia (derived from hematopoietic cells), germ cell tumor (derived from totipotent cells) and blastoma (resembling an embryonic tissue) (Peng et al., 2009). Among all the different kinds of cancers, carcinoma represents the most common cancers. Breast cancer, lung cancer and colon cancer are all different types of carcinoma. Different cancers may cause different symptoms. Generally, the symptoms of cancers include local symptoms, systemic symptoms and symptoms of metastasis. Local symptoms include abnormal swelling, pain, hemorrhage, and
ulceration. Systemic symptoms include poor appetite, weight loss, fatigue, cachexia and so on. Symptoms that indicate metastasis include enlarged lymph nodes, hepatomegaly, pain at sites other than the original tumor locus and so on.

Cancer affects people of all ages including infants and even fetuses. But in general, the risk of cancer increases with age (Meza et al., 2008). The age-specific cancer incidence curve fits a multistage model that predicts two phases: the exponentially increase phase until the age of approximately 60 and the linearly increase phase after 60 (Meza et al., 2008). Cancer, as a group of malignant diseases, is quite life-threatening. According to the American Cancer society 2009 statistics, cancer accounts for nearly one quarter of the death in the US (American Cancer Society Inc., 2009). In the year 2009, the estimated cancer incidence is 1479350 and the estimated cancer death is 562340 in the US (American Cancer Society Inc., 2009). Cancer was ranked as the second leading cause of death and has been predicted to become the first leading cause of death in 2008 (American Cancer Society Inc., 2009). Although the drastic increase of cancer incidence and cancer death has slowed down a little in recent years, the prevalence of cancer is kept at a fairly high level.

Cancer pathogenesis is traced back to the accumulation of DNA mutations that affect cell growth and metastasis. DNA replication is error-prone. Cell mutations happen spontaneously during cell division. When errors occur, cells may sense them and try to make corrections with the innate error-correcting machinery. If the correction fails, mutated cells may undergo senescence or apoptosis. Unfortunately, cell surveillance and error-correcting machinery sometimes fails in error correction and apoptosis induction. In
that case, the mutated cells survive and pass the errors to their daughter cells. After rounds of replication, mutations may accumulate to a point that cells become malignant. Malignant cells are often found to have mutations on the genes that are involved in cell growth regulation. Those genes can either be proto-oncogenes or tumor suppressor genes. Proto-oncogenes code for proteins that promote cell growth. They are often involved in mitogenic signaling transduction. The gene that codes for MAPK is an example of proto-oncogene. Proto-oncogenes can become oncogenes due to mutations or elevated expression. Mutations on one allele are usually enough to transform a proto-oncogene to oncogene. In contrast to proto-oncogenes, tumor suppressor genes code for products that control cell growth and protect cells from becoming malignant. Mutations on both alleles are often required to inactivate a tumor suppressor gene and promote cancer. Although mutations happen in DNA, most cancers are not directly inheritable. Somatic cell mutations usually do not pass to the progeny whereas germ cell mutations can be inherited.

Both internal factors and external factors are contributing to carcinogenesis. The internal factors include inherited mutations on proto-oncogenes or tumor suppressor genes, abnormal levels of growth hormones, weak or biased immune conditions, and so on. For instance, breast cancer, a common type of carcinoma, is largely associated with certain inherited mutations on \textit{BRCA1} and \textit{BRCA2} genes (Dombernowsky et al., 2009). Also, excessive estrogen receptor signaling promotes breast cancer (Deblois et al., 2009). The occurrence of cancer is under the surveillance of immune system. It has been reported that weak immune system and lack of cell-mediated immunity due to a switch
from Th1 response to Th2 response may be responsible for cancer occurrence and cancer progression (Sato et al., 1998). In addition to internal factors, external factors such as tobacco consumption (Le Marchand et al., 2008), viral or bacterial infection (DURAN-REYNALS, 1956; Parsonnet, 1995), chemical carcinogen exposure (Kuang et al., 2005), ionizing radiation (Deng et al., 2008), also play a great role in cancer promotion as most of them cause DNA mutations. As an example, prolonged exposure to ultraviolet radiation can lead to skin malignancies such as melanoma (Fink and Bates, 2005).

Cancer is very hard to cure due to its notorious malignancy, its variations and its lack of symptoms for early diagnosis. However, cancer is still treatable. Cancer can be treated by surgery, radiation therapy, chemotherapy, immunotherapy and so on. Those therapeutic methods either try to physically remove the clusters of malignant cells, or kill the malignant cells via various mechanisms including targeting the abnormal proliferative signaling, cutting off energy supply to the cells, and bursting up the immune system.

Cancer represents a class of diseases. Each cancer is unique and is only sensitive to certain medications. The choice of therapy depends on the feature, location and stage of a particular cancer. There is no single cure for the disease. And usually multiple therapies are combined when treating cancer. For instance, chemotherapy or radiation therapy is often performed in combination with surgical therapy. Even more, chemotherapy itself may involve several different medications. Early diagnoses and treatments positively correlate with good outcomes for cancer.
Colorectal Cancer

Colorectal cancer, also known as colon cancer or large bowel cancer, represents malignant growths in the colon, rectum and appendix. The symptoms of colorectal cancer may include reduction in diameter of stool, constipation, lower gastrointestinal bleeding, fatigue and so on (Bazensky et al., 2007). According to the World Health Organization cancer fact sheet information, colorectal cancer is the third most common type of cancer and the second leading cause of cancer death (World Health Organization Media Center, 2009). It accounts for about 10% of cancer incidence and cancer death, costing around 655000 lives each year worldwide (World Health Organization Media Center, 2009).

Like other cancers, colorectal cancer is due to successive accumulation of cell mutations. Colorectal cancer progression can be divided into five stages: Stage 0, I, II, III and IV (National Cancer Institute, 2009). Stage 0 is also named as carcinoma in situ. It is a stage when abnormal cells are found in the colon innermost lining (National Cancer Institute, 2009). Stage I (Dukes A colon cancer) represents a stage when abnormal cells have formed cancer and spread from the innermost lining to the middle layers of colon (National Cancer Institute, 2009). Stage II (Dukes B colon cancer) is further divided into Stage IIA and IIB. In Stage IIA, cancer has spread beyond the middle layers of colon or to the adjacent tissues (National Cancer Institute, 2009) while in Stage IIB, cancer has spread to organs near colon (National Cancer Institute, 2009). Stage III (Dukes C colon cancer) is divided into 3 substages: Stage IIIA, IIB and IIIC. Cancer has spread to the middle layers of colon and to 1-3 lymph nodes in Stage IIIA; cancer has spread beyond the middle layers of colon and to 1-3 lymph nodes in Stage IIB; cancer has spread
beyond the middle layers of colon and to at least 4 lymph nodes in Stage IIIC (National Cancer Institute, 2009). In Stage IV (Dukes D colon cancer), cancer has spread to nearby lymph nodes and to other parts of the body (National Cancer Institute, 2009). The characterization of the different colorectal cancer stages is mainly based on histopathologic assays. However, the conventional colorectal cancer staging could not always correlate well with the outcome due to the marked variability at each stage (Kahlenberg et al., 2003). As the underlying molecular mechanisms are being elucidated, colorectal cancer stages can be better characterized and colorectal cancer can be better prognosed than ever before (Kahlenberg et al., 2003). Serum carcinoembryonic antigen (CEA) is the only biomarker used currently for colorectal cancer prognosis (Kahlenberg et al., 2003). The concentration of CEA correlates well with the progression and the outcome of the malignant disease (Kahlenberg et al., 2003). Besides, an increasing body of putative biomarkers for colorectal cancer has been identified (Kahlenberg et al., 2003). Mutations in proto-oncogenes such as Ras, Erb-B2, EGFR, TGFα, TGFβ-1 have been found to be related to colorectal cancer development (Kahlenberg et al., 2003). Meanwhile, mutations in tumor suppressor genes including p53, p27, 18q LOH, 5q allelic loss, MSI and DNA methyltransferase are involved in colorectal cancer progression (el-Deiry et al., 1991; Kahlenberg et al., 2003; Lenz, 2007; Turner et al., 2007). In addition, abnormality of factors related to apoptosis such as Bcl-2, SOD and GST-π and factors involved in angiogenesis such as IL-8, VEGF, PD-ECGF, and CAMs is also found to contribute to colorectal cancer development (Kahlenberg et al., 2003; Lenz, 2007). Mutations in proto-oncogenes and tumor suppressor genes facilitate the transformation of
cells and promote the expansion of a malignancy (Kahlenberg et al., 2003). They are thereby often required for early cancer development. Malfunctions in the apoptosis machinery together with advanced angiogenesis are considered to increase cancer cell growth, invasion and metastasis, thus involved in late stage cancer progression (Kahlenberg et al., 2003).

Both genetic susceptibility and environmental factors are involved in the onset and progression of colorectal cancer. Many genes have been found to be associated with increased mutations or over-expressions in colorectal cancer cases (Ahmed, 2003). For example, polymorphisms of MTHFR C677T (Fernández-Peralta et al., 2009), A1298C (Fernández-Peralta et al., 2009), hMLH1 (Christensen et al., 2008) and hMLH2 (Christensen et al., 2008) are associated with colorectal cancer. Environmental factors such as diet also impact the risk of colorectal cancer. A great increase of colorectal cancer risk has been found to be associated with the diet rich in fat and calories and low in fibers, vegetables and fruits (Ahmed, 2003).

Balanced diet, increased activity and colon polyp screening are means to prevent colorectal cancer. Colorectal cancer is curable if caught at early stages (Ramos et al., 2007). However, it is less likely to be cured when detected at later stages (Ramos et al., 2007). Surgery is the primary treatment for colorectal cancer. Chemotherapy or radiation therapy may also be recommended under certain circumstances. 5-fluorouracil, leucovorin, and oxaliplatin are often administered in an adjuvant chemotherapy after surgery (Aranha and Benson, 2007). For treating the colorectal cancer that has metastatic situation, irinotecan (Moosmann and Heinemann, 2008), bevacizumab (Grávalos et al.,...
2007), cetuximab (Lee and Chu, 2007; Moosmann and Heinemann, 2008), and panitumumab (Lee and Chu, 2007) are options in addition to 5-fluorouracil, leucovorin, and oxaliplatin. Those medications used in chemotherapy either directly induce tumor cell death or indirectly kill the abnormal cells by starving them. The current chemotherapy medications for colorectal cancer and also for other cancers all display significant side effects due to lack of selectivity. They can hardly discriminate cancer cells from normal cells. Due to the unsatisfactory features of current chemo-drugs, continuous efforts are being made to find better therapies that have high anti-cancer potency, high therapeutic selectivity but low toxicity.

**Apoptosis**

Many anti-cancer chemotherapeutic agents and ionizing radiation therapy treat cancer by inducing programmed cell deaths (PCD) (Bold et al., 1997; Kerr et al., 1994). PCD is an activate cell death mediated by an intracellular program. It is found both in eukaryotic multicellular organisms and bacteria (Bermpohl et al., 2005; Yarmolinsky, 1995). There are two major forms of PCD: apoptosis and autophagy (Tan et al., 2009). Both are playing important roles in development, differentiation, homeostasis, immune system regulation and removal of defect or cancerous cells (Tan et al., 2009).

Apoptosis is a more common type of PCD. It is characterized by several morphological and biochemical events (Nagata, 1997). Morphological changes in apoptosis include compaction of nuclear choromatin and condensation of cytoplasm, followed by formation of apoptic bodies which are ultimately taken up by nearby cells.
without causing inflammation (Kerr et al., 1994; Kerr et al., 1972). The biochemical changes during apoptosis are characterized by fragmentation of double-stranded DNA and phosphatidylserine externalization (Tan et al., 2009). The process of apoptosis can be triggered by intracellular and extracellular insults. The intracellular insults include DNA damage (Olsson et al., 2009), ER stress (Madeo and Kroemer, 2009), and mitochondrial oxidative stress (Grishko et al., 2009). And the extracellular apoptosis inducers may be heat (Bellmann et al., 2009), radiation (Yu et al., 2009), hypoxia (Yan et al., 2009), nutrient deprivation (Akakura et al., 2001), toxins (Chaudhari et al., 2009), nitric oxide (Rakshit S et al., 2009), hormones (Lewis-Wambi and Jordan, 2009), cytokines (Abdelli et al., 2009) and even growth factors (Stewart et al., 1999a). Two signaling pathways are mediating apoptosis: the extrinsic pathway and the intrinsic pathway (Fig. 17). In the extrinsic pathway, apoptosis is mediated by the death receptors on the cell surface (Tan et al., 2009). The death receptors belong to the tumor-necrosis factor (TNF) receptor superfamily and are composed of extracellular cysteine-rich domains and intracellular death domains (Tan et al., 2009). Ligands such as TNFα, Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) bind with death receptors and recruit Fas-associated DD adapter protein (FADD) to form the death inducing signaling complex (DISC) (Tan et al., 2009). DISC recruits pro-aspartate-specific cysteineyl protease (Caspase) 8 and pro-caspase 10, which subsequently cleave themselves and activate the downstream apoptosis executioners caspase 3, caspase 6 and caspase 7 (Schulze-Osthoff et al., 1998) (Hengartner, 2000). Activation of caspase 3 or caspase 6 leads to the cleavage of DNA fragmentation factor 45 (DFF45) and the subsequent release of the
endonuclease DFF40, ultimately resulting in chromosomal degradation and cell apoptosis (Tan et al., 2009). In the intrinsic pathway, apoptosis is mediated by mitochondria (Tan et al., 2009). In response to apoptotic stimuli such as DNA damage, cytochrome c (Cyt c) (Hengartner, 2000), direct inhibitor of apoptosis binding protein (SMAC/DIABLO) (Rajalingam et al., 2007), apoptosis-inducing factor (AIF) (Lipton and Bossy-Wetzel, 2002) and endonuclease G (EndoG) (Zhang et al., 2003) are released from the mitochondria. Cyt c interacts and activates apoptotic protease activating factor-1 (Apaf-1) in the cytosol, which induces the formation of apoptosome (Tan et al., 2009). Apoptosome then recruits pro-caspase 9 which when activated induces the activation of caspase 3 and caspase 7 and leads to apoptosis (Tan et al., 2009). Although most apoptoses are mediated by caspase, caspase-independent apoptoses have also been described (Cummings et al., 2004). Many studies show that dysregulation of apoptosis contributes to the development of neoplastic transformation and tumor growth (Meng et al., 2006; Nagata, 1997). Compounds that can selectively induce tumor cell apoptosis have potentials to be used in cancer research and cancer treatment (Bunch and Eastman, 1996; Yu et al., 2002).
Apoptosis pathways. Apoptosis can be mediated by the extrinsic and intrinsic pathways. In the extrinsic pathway, when death signal molecules bind with death receptors, they together recruit FADD to form the DISC which recruits and activates caspase 8 and 10. Activation of caspase 8 and 10 further activates caspase 3 and 6, leading to DNA fragmentation by DFF40 and apoptosis. The intrinsic pathway is mediated by mitochondria. Pro-apoptotic Bcl-2 family members such as Bax and Bak form channels on mitochondrial outer membrane, causing the release of pro-apoptotic factors like Cyt c. Those released pro-apoptotic factors activates Apaf-1 and subsequently activates caspase 9. Activated caspase 9 induces cleavage and activation of caspase 3 and 7, resulting in apoptosis. This graph is adapted from (Tan et al., 2009).

**Regulation of Caspase Activation or Apoptosis by p53 and Bcl-2 family**

Both extrinsic and intrinsic signaling pathways drive cells to the final stages of apoptosis by activating caspases, especially caspase 3 (Riedl and Shi, 2004). Caspases are a family of cysteine proteases, which when activated promote PCD, inflammation and so on (Pop and Salvesen, 2009). Caspases can simply be classified into two groups:
apoptotic and pro-inflammatory (Pop and Salvesen, 2009). The apoptotic caspases include caspase 2, caspase 3, caspase 6, caspase 7, caspase 8, caspase 9, and caspase 10 while the non-apoptotic caspases contain caspase 1, caspase 4, caspase 5 and caspase 14 (Pop and Salvesen, 2009). Apoptotic caspases, once activated, undergo auto-proteolysis in a cascade manner and cleave other substrates including essential cell components, leading to apoptosis (Tan et al., 2009). And Activation of caspases is under conserved and strict regulation. The tumor suppressor protein p53 and Bcl-2 family proteins are among the key regulators of caspase activation and apoptosis induction.

p53, also known as protein 53 or tumor protein 53, is a multifunctional transcriptional factor encoded by the TP53 gene (Matlashewski et al., 1984). It belongs to the p53 family together with the family members p63 and p73 (Pietsch et al., 2008). p53 is pivotal in maintaining genomic integrity, arresting cell cycle and inducing apoptosis (Aylon and Oren, 2007; Bargonetti and Manfredi, 2002; Fridman and Lowe, 2003; Levine, 1997; Pietsch et al., 2008). Therefore, it functions as a “guardian of the genome” and tumor suppressor. In over 50% of human tumors, p53 is found to be mutated or deleted (Olivier et al., 2002).

Loss of p53 function predisposes tumor growth whereas over-expression or over-activation of p53 results in excessive cell senescence, apoptosis and aging. Therefore, the level and activity of p53 are under tight regulation (Fig. 18). Because p53 inhibits cell cycle and promotes apoptosis, it has a very short half-life and usually exists at an extremely low level in the cytosol of cells through a continuous degradation process (Pietsch et al., 2008). Mdm2 (in mouse, Hdm2 in human) is the most well-known
negative regulator of p53 (Marine and Lozano, 2009). It not only sequesters p53 in cytosol to prevent its nuclear transport and action, but also acts as a ubiquitin ligase to catalyze the ubiquitination and degradation of p53 by proteasome (Marine and Lozano, 2009). In response to stresses such as DNA damage, oxidative stress, and chronic mitogenic stimulation, p53 is temporarily stabilized and accumulated (Pietsch et al., 2008). Meanwhile, the conformation of p53 changes to make it further stable or easy to bind with DNA elements after nuclear translocation to regulate gene expression as a transcriptional factor. The accumulation and activation of p53 correlates with a decrease in Mdm2 and an increase in p53 post-translational modifications including phosphorylation, acetylation, methylation, poly(ADP-ribosyl)ation, sumoylation, neddylation, and non-proteolytic monoubiquitylation (Pietsch et al., 2008). The critical event leading to p53 activation is the phosphorylation on its N-terminal domain in response to stresses by protein kinases including the MAPK family (ERK1/2, JNK1-3 and p38 MAPK) (Bulavin et al., 1999; Cordenonsi et al., 2007; Gong et al., 2006), the protein kinases implicated in the genomic integrity checkpoint (Latham et al., 1996; Soubeyrand et al., 2004; Wang et al., 2008), PKCδ (Yoshida et al., 2006) and oncogene activated p14ARF (Buschmann et al., 2000). Phosphorylation of p53 N-terminal domain not only disrupts the binding of Mdm2 but also helps to recruit transcriptional coactivators such as p300 and PCAF (Liu et al., 1999; Sakaguchi et al., 1998). The coactivators acetylate the C-terminal end of p53, causing the conformational change of p53 and exposing the DNA binding domain to allow gene regulation by p53 (Gu and Roeder, 1997; Liu et al., 1999; Sakaguchi et al., 1998). p53, once activated, regulates the
expression of a variety of genes, through which cell cycle arrest, senescence, apoptosis and cellular damage repair are coordinated depending on the status of the cells (Aylon and Oren, 2007; Vousden and Lu, 2002). Although p53 activation is essential for guarding the integrity of genome and ensuring the discarding of defective cells, cells cannot afford unnecessary p53 activation. In addition to Mdm2, there are other factors that block p53 activation. For example, deacetylase enzymes like Sirt1 and Sirt7 can deacetylate p53, thus preventing p53 from activation (Vakhrusheva et al., 2008).

p53 is best understood as a multifunctional transcription factor, acting by regulating gene expression (Fig. 18). It binds to the consensus sequence at the promoter or intron regions of target genes and recruits other transcriptional factors or transcriptional machinery to either activate or suppress gene expression (el-Deiry et al., 1992). Hundreds of p53 target genes have been identified so far. They include Caspases, Fas, Bax, PTEN and so on (Riley et al., 2008). Several studies also characterized transcription-independent pro-apoptotic effects of p53 (Fig. 18) (Haupt et al., 1995; Moll et al., 2005), the mechanism of which mainly relies on the rapid translocation of p53 to the mitochondria (Marchenko et al., 2000). Several pieces of evidence have demonstrated that Bcl-2 family members are implicated in p53-regulated transcription-independent apoptosis at mitochondria (Moll et al., 2005).

Bcl-2 family proteins are key players in the intrinsic apoptotic pathway (Cory and Adams, 2002). They are broadly divided into two groups: the anti-apoptotic proteins and the pro-apoptotic proteins. They either prevent or promote apoptosis by controlling the release of Cyt c and other pro-apoptotic factors from mitochondria. The anti-apoptotic
Bcl-2 proteins are Bcl-2, Bcl-xl and Mcl-1 (Pietsch et al., 2008). The pro-apoptotic Bcl-2 proteins include Bax, Bak, and the BH3-only proteins Bad, Bid, Bim, Noxa and Puma (Pietsch et al., 2008). Among the pro-apoptotic Bcl-2 proteins, BH3-only proteins are the sensors of apoptotic signaling while Bax and Bak are the main pro-apoptotic effectors (Wei et al., 2001). A group of BH3-only proteins directly activate Bax and Bak by facilitating the formation of homo-oligomers of Bax and Bak which serve as pro-apoptotic factor releasing channels in the mitochondrial outer membrane (Letai et al., 2002). Another group of BH3-only proteins derepresses Bax and Bak by preventing the binding of Bax or Bak inhibitory proteins like Bcl-2 and Bcl-xl (Kuwana et al., 2005).

p53 mimics BH3-only members when inducing mitochondria-dependent apoptosis. In particular, p53 releases Bax (Chipuk et al., 2004) and Bak (Leu et al., 2004) from their inhibitory proteins and induces Bax and Bak oligomerization, consequently resulting in Cyt c release from mitochondria and apoptosis induction. In addition, p53 also interacts and inhibits Bcl-2 and Bcl-xl, which indirectly activates the pro-apoptotic Bcl-2 family members and finally leads to apoptosis (Mihara et al., 2003; Tomita et al., 2006).

In all, apoptosis is a specific type of PCD which is important in development and differentiation. Dysregulation of apoptosis leads to tumorogenesis. Apoptosis can be initiated by both extracellular and intracellular stimuli and is mediated through either the extrinsic or the intrinsic apoptotic pathway. Both extrinsic and intrinsic pathways converge at caspases to execute apoptosis. The activation of caspases and the induction of apoptosis are regulated by various factors including p53 and Bcl-2 family members. While p53 can either mediate apoptosis as a transcriptional factor in the mitochondria-
independent apoptotic pathway or induce mitochondria-dependent apoptosis, Bcl-2 family members such as Bax only regulate mitochondria-dependent apoptosis.

**Figure 18.** The p53 pathway. p53 is sequestered in cytosol, prevented from activation and maintained at a very low level by Mdm2 within unstressed cells. In response to stresses such as DNA damage and mitogenic stimulation, p53 undergoes post-translational modifications including phosphorylation by kinases. Modified p53 dissociates from Mdm2 and is temporarily stabilized and activated. Activated p53 regulates DNA damage repair, cell cycle arrest and apoptosis induction through transcription-dependent or transcription-independent pathways. And the transcription-independent pathway of p53 is mediated by Bax and Bak at mitochondria. This graph is adapted from (Marine and Lozano, 2009; Moll et al., 2005; Pietsch et al., 2008).
Previous Anti-Cancer Related Studies in Our Laboratory

α-PGG is a naturally occurring polyphenolic compound (Ren and Chen, 2007; Ren et al., 2006). Our previous work indicated that it binds to IR α-subunit at a site different from the insulin binding site and activates the IR signaling for glucose transport in adipocytes (Li et al., 2005). It is well documented that IR mediates two types of responses: a rapid metabolic response such as glucose uptake that occurs within minutes upon activation and/or a slow mitogenic (cell proliferating) response that takes hours or days and requires new gene expressions (Saltiel and Kahn, 2001; Saltiel and Pessin, 2002). It has also been reported that IR or IRSs were upregulated in some kinds of cancers including pancreatic cancer which has one of the lowest five-year survival rates and for which we have no effective treatment (Kornmann et al., 1998; Paonessa et al., 2006; Surmacz and Burgaud, 1995). The potential role of IR signaling molecule in tumor progression has been speculated. However, p53 elevation, Bax activation and apoptosis have never been reported to be induced through IR. In our preliminary studies, we observed that α-PGG, in addition to stimulating the IR-mediated glucose transport in 3T3-L1 adipocytes, also inhibited cell proliferation and differentiation in 3T3-L1 preadipocytes (unpublished data). These cell growth arresting activities led us to hypothesize that α-PGG might also inhibit tumor cell growth and even induce apoptosis in tumor cells. In this study, using various molecular and cellular assays, we investigated the potential anti-cancer effects of α-PGG and its derivative 6Cl-TGQ in human colon epithelial carcinoma cells (RKO). We also studied the potential mechanisms by which α-PGG induces these effects using RKO cells.
MATERIALS AND METHODS

Cell lines and culture conditions - Human colon epithelial carcinoma (RKO, wild-type (WT) p53), pCMV-E6 transfected human colon epithelial carcinoma (RKO-E6, low WT p53 level and activity), and human colon epithelial (FHC, WT p53) cell lines were purchased from American Type Culture Collection (ATCC; Rockville, MD). RKO and RKO-E6 cells were cultured in DMEM (Mediatech; Herndon, VA) containing 10% FBS and 1% penicillin/streptomycin at 37°C in a 5% CO₂ cell incubator. FHC cells were cultured in a specific medium according to ATCC instructions. Cells were cultured to 60% confluency before propagation or treatment unless described otherwise.

Compounds and compound treatment - α-PGG and 6Cl-TGQ were synthesized by Dr. Yulin Ren and Dr. Klaus Himmeldirk in the Department of Chemistry and Biochemistry of Ohio University. Both α-PGG and 6Cl-TGQ were dissolved in ddH₂O. Cells were treated with 25 μM of α-PGG or 6Cl-TGQ unless described otherwise.

Methyl tetrazolium (MTT) cell viability assay - Cells in 100 μL of 10% FBS and 1% penicillin/streptomycin-containing DMEM or other specific medium were seeded at a density of 1 X 10⁴ cells/well in a 96-well plate 24 hours prior to compound treatment. The media were then replaced with 100 μL of fresh media supplemented with α-PGG or 6Cl-TGQ. Mock-treated cells were used as controls and each condition was prepared in triplicate. After a 48-h treatment, the viability of the cells, which is proportional to the amount/activity of NAD(P)H-oxidoreductases present in the cells, was determined by the methyl tetrazolium (MTT) cell proliferation assay kit (Cayman Chemical Company; Ann Arbor, MI) as described previously (Van de Loosdrecht et al., 1994). Briefly, 10 μL of
5 mg/mL MTT reagent was added to each well. After a 3-hour incubation at 37°C in a CO2 incubator, media were aspirated carefully and 100 µL of crystal dissolving solution was added to each well to dissolve the formazan crystals. The absorbance was determined at 570 nm using an ELISA reader (SPECTRA max M2, Molecular Devices; Sunnyvale, CA).

**Microscopic photography** - RKO cells in 10% FBS and 1% penicillin/streptomycin-containing DMEM were seeded at a density of 1 X 10^6 cells/100 mm culture dish 24 hours prior to compound treatment. The medium was then replaced with fresh media supplemented with 25 µM of α-PGG or 25 µM of 6Cl-TGQ. Mock-treated cells were used as controls. 48 hours after treatment, microscopic cell pictures were taken using the software Motic MCCamera 1.1 with both the ocular lens and the objective lens being 10X.

**Flow cytometric analysis** - RKO cells were seeded at a density of 1 X 10^6 cells/100 mm cell culture dish and incubated for 24 hours before treatment. Cells were treated with 25 µM of α-PGG or 25 µM of 6Cl-TGQ. Mock-treated cells were used as controls. 48 hours after treatment, cells were harvested by trypsinization. Then, cells were washed with PBS and fixed with 4 mL of ice-cold 70% ethanol overnight. Finally, cells were collected by centrifugation at 1200 rpm and re-suspended in 2 mL of PBS containing 5 µg/mL RNase A (Sigma; St. Louis, MO) and 50 µg/mL propidium iodide (Sigma; St. Louis, MO) for analysis. Cell cycle analysis was performed using FACScan Flow Cytometer (Becton Dickson; Franklin Lakes, NJ) according to the manufacturer’s protocol. ModFit LT software (Verity Software House; Topsham, ME) was used to
calculate the cell cycle phase distribution from the resultant DNA histogram graph. The results were expressed as the percentage of cells in the G1, S and G2/M phases and the percentage of dying or dead cells as indicated by the subdiploid (Sub-G1) peak.

*Caspase 3 activity assay* - RKO cells were cultured in Petri dishes till 60% confluency before they were switched to DMEM containing 25 μM of α-PGG. Mock-treated cells were used as controls. Each condition was prepared in triplicate. 48 hours later, medium was removed and cells were trypsinized and washed with ice-cold PBS twice. Proteins were then extracted using the lysis buffer from the caspase 3 fluorometric kit (Invitrogen; Carlsbad, CA). Protein concentrations were measured using BCA protein assay kit (Pierce; Rockford, IL). Equal amounts of proteins with the total volume of 50 μL were added to each well of a 96-well plate. 50 μL of caspase 3 substrate DEVD-AFC was then added followed by a 45 min incubation in dark with gentle shaking. The fluorescence was determined using an ELISA reader (SPECTRA max M2, Molecular Devices; Sunnyvale, CA) with the excitation wave at 342 nm and emission wave at 441 nm.

*Western blot analysis*

*p53 induction mechanism studies* - RKO cells at 60% confluency were switched to fresh 10% FBS and 1% penicillin/streptomycin-containing DMEM supplemented with 25 μM of α-PGG and/or one of the following inhibitors: 200 μM of Insulin receptor inhibitor HNMPA-(AM)₃ (Biomol; Plymouth Meeting, PA) (Rose et al., 2007), 1 μM of Insulin-like Growth Factor-1 Receptor Inhibitor Picropodophyllin (PPP) (Calbiochem; San Diego, CA) (Vasilcanu et al., 2006), 20 μM of PI3K inhibitor
Wortmannin (Sigma-Aldrich; St. Louis, MO) (Kim et al., 2007), 10 μM of MEK inhibitor I (Calbiochem; San Diego, CA) (Hetman et al., 2002), or 50 μM of ERK inhibitor 3-(2-Aminoethyl)-5-((4-ethoxyphenyl)methylene)-2,4-thiazolidinedione (AEMT) (Calbiochem; San Diego, CA) (Hancock et al., 2005). Specifically, in the IR and IGF-1R inhibitor study, RKO cells were cultured to approximately 60% confluency and were then switched to culture medium supplemented with 25 μM of α-PGG and/or 200 μM of the IR inhibitor HNMPA-(AM)₃ / 1 μM of IGF-1R inhibitor PPP and were incubated for 48 hours. In the PI3K inhibitor study, RKO cells at 60% confluency were switched to culture medium supplemented with 25 μM of α-PGG and/or 20 μM of the PI3K inhibitor Wortmannin and were incubated for 48 hours. 1 μM of insulin (Sigma-Aldrich; St. Louis, MO) was used as a control. And in the MEK and ERK inhibitor study, RKO cells at 60% confluency were switched to culture medium supplemented with 10 μM of MEK inhibitor I / 50 μM of ERK inhibitor AEMT and were cultured for 48 hours. In the study with siRNA-transfected RKO cells, control-siRNA or IR-siRNA-transfected cells were treated with 25 μM of α-PGG. In all the studies, mock-treated cells were used as controls. Cells were lysed after treatment with a lysis buffer containing 20 mM HEPES, 350 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 20% glycerol, 1% Nonidet P-40, phosphatase inhibitor mixture, and protease inhibitor mixture. Protein concentrations were determined using BCA protein assay kit (Pierce; Rockford, IL). 30-50 μg of total protein was loaded to 7.5% Tris-glycine polyacrylamide gels (Bio-Rad; Hercules, CA) and subjected to electrophoresis. Proteins were visualized by Lumi-Light western blotting substrates (Roche; Nutley, NJ) using specific antibodies. Primary antibodies include
those that are against: phospho-Akt (Ser473) (Cell Signaling; Beverly, MA), phospho-
MEK1/2 (Ser217/221) (Cell Signaling; Beverly, MA), phospho-ERK1/2 (Thr202/Tyr204) (Cell Signaling; Beverly, MA), phospho-SAPK/JNK (Thr 183/Tyr 185) (Cell Signaling; Beverly, MA), Bp53-12 (Santa Cruz; Santa Cruz, CA), β-tubulin (Cell Signaling; Beverly, MA), and β-actin (Cell Signaling; Beverly, MA). β-tubulin and β-actin were used as protein loading controls. Secondary antibodies include: HRP-conjugated goat-anti-mouse (Santa Cruz; Santa Cruz, CA) or strepavidin-conjugated goat-anti-rabbit (Cell Signaling; Beverly, MA) depending upon the source of the primary antibodies. Each experiment was repeated at least twice.

**Bax protein analysis** - RKO cells were grown to 60% confluency and then the culture medium was switched to medium supplemented with 25 μM of α-PGG and/or 10 μM of MEK inhibitor I (Calbiochem; San Diego, CA) (Hetman et al., 2002). Mock-treated cells were used as controls. After 48 hours, cells were lysed with lysis buffer (10 mM Tris HCl pH 7.5, 1 mM EDTA, 1% triton X-100, 150 mM NaCl, 1 mM dithiothretol, 10% glycerol, 0.2 mM phenylmethysulphonyl fluoride and protease inhibitors). Protein concentrations were determined using BCA protein assay kit (Pierce; Rockford, IL). For determining Bax levels, 20 μg of total protein was loaded to 8% sodium dodecyl sulphate – polycrylamide gel electrophoresis (SDS-PAGE) gels, and for inhibitor studies, 50 μg of total protein was loaded to 10% SDS gels. The protein of interest was probed using anti-Bax (B-9) mouse IgG (Santa Cruz; Santa Cruz, CA) and the secondary antibody goat anti-mouse IgG – HRP (Santa Cruz; Santa Cruz, CA). As loading controls, β-actin or GAPDH was detected using anti-β-actin IgG (Sigma-Aldrich; St. Louis, MO) or anti-
GAPDH (Santa Cruz; Santa Cruz, CA). The proteins were visualized using ECL western blot chemiluminescence kit (GE health care; Piscataway, NJ).

**Immunofluorescence microscopy**

*p53 translocation* - RKO cells in 10% FBS and 1% penicillin/streptomycin-containing DMEM were cultured in 2-well chambers (Nunc Inc.; Naperville, IL) that were mounted on glass slides with plastic covers. At 60-70% confluency, cells were switched to fresh 10% FBS and 1% penicillin/streptomycin-containing DMEM supplemented with or without 25 μM of α-PGG. After 48 hours, cells were washed three times with cold PBS and were fixed with ice-cold 100% methanol (VWR international; West Chester, PA) at -20°C for 20 min. After washing twice with PBS, cells were incubated with 4% paraformaldehyde (Fisher Scientific; Pittsburgh, PA) in PBS to fix cell surface antigens for 30 min at room temperature. Then, cells were blocked with 5% normal donkey serum in PBS with 0.2% Triton X-100 (Sigma-Aldrich; St. Louis, MO) and 1% DMSO (Sigma-Aldrich; St. Louis, MO) at room temperature for 50 min. When the blocking solution was removed, cells were incubated with the primary antibody Bp53-12 diluted at 1:200 in 7.5% donkey serum-containing PBS overnight at 4°C. After the overnight incubation, cells were incubated with the secondary antibody (Fluorescein (FITC)-conjugated affinipure F(ad’2 fragment Donkey anti-Mouse IgG) (Jackson ImmunoResearch Laboratories; West Grove, PA) at a 1:100 dilution in 7.5% donkey serum/PBS at 37°C for 30 min. The cells were then washed three times with PBS and mounted with a drop of Vectashied with DAPI (Vector Laboratories Inc.; Burlingame, CA). Finally, the cells were visualized and photographed using an immunofluorescence
microscope (ECLIPSE E600; Nikon, Japan) with the ocular lens being 10X and the objective lens being 40X. The images were merged using the Advanced SPOT software from Diagnostic Instruments (Sterling Heights, MI).

**Bax expression** - RKO cells in 10% FBS and 1% penicillin/streptomycin-containing DMEM were cultured on glass coverslips in 30mm² chambers (Nunc Inc.; Naperville, IL). When cells reached 60% confluency, they were switched to fresh 10% FBS and 1% penicillin/streptomycin-containing DMEM supplemented with or without 25 μM of α-PGG. After being treated for 48 hours, the cells were washed three times with PBS and fixed with 95% ethanol and 5% acetic acid for 5 min at -20°C. The cells were then blocked with 2% chicken serum in PBS at room temperature for 30 min and incubated for 1 hour with the primary antibody Bax (B-9) which was diluted at 1:400 in 2% chicken serum-containing PBS. After that, cells were washed three times with PBS and incubated with Texas Red-conjugated secondary antibody (Jackson ImmunoResearch Laboratories; West Grove, PA) at a 1:500 dilution in 2% chicken serum-containing PBS for 1 hour at room temperature. The coverslips were washed three times with PBS and then incubated with DAPI for 20 min to stain the nuclei. Thereafter, the coverslips were washed three times with PBS and were mounted onto slides in anti-fade mounting medium (Sigma-Aldrich; St. Louis, MO). Cells were visualized and photographed using the same microscope and software as in the p53 immunofluorescent staining except for that the objective lens used was of 20X.

**Luciferase assay** - RKO cells were cultured till about 60% confluency. Then, using lipofectamine 2000 (Invitrogen; Carlsbad, CA), cells were transfected with pp53-
Luc (Panomics Inc; Redwood, CA), a firefly luciferase reporter plasmid constructed with a cis-acting enhancer element sequence that has a p53 binding site. pRL-TK vector (Promega; Madison, WI) was co-transfected as an internal control reporter. Six hours after transfection, the transfection medium was removed and the cells were cultured in normal culture medium for 8 hours to recover from the transfection stress. Then, cells were treated with or without 25 μM of α-PGG for 36 hours, followed by a luciferase assay. To do the luciferase assay, cells were incubated with 1X passive lysis buffer (Promega; Madison, WI) for 20 min to lyse the cells. After that, cell lysates were transferred to centrifuge tubes and were centrifuged at 14,000 rpm for 30 sec. 20 μl of supernatant for each sample was transferred to a luminometer tube. The firefly luciferase activities were then determined by the luminometer (Lumat LB9507, BERTHOLD Technologies; Oak Ridge, TN) with luciferase substrate (Promega; Madison, WI) and stop solution (Promega; Madison, WI).

Real time quantitative PCR - RKO cells were cultured in 10% FBS and 1% penicillin/streptomycin-containing DMEM to 60% confluency and then the cells were switched to new culture medium supplemented with 25 μM of α-PGG. After 48 hours, RNA was extracted using RNeasy total RNA extraction kit (Qiagen, Inc.; Valencia, CA) according to the manufacturer’s protocol. The quality of RNA was verified using 12% formaldehyde agarose gel electrophoresis and the concentration of the total RNA extracted was measured using spectrophotometer at 260 nm. 3 μg of the total extracted RNA was subjected to RT-PCR and cDNA was synthesized by the Effendorf Mastercycler gradient PCR machine (Effendorf; Westbury, NY) with the Bio-Rad
iScript™ Select cDNA Synthesis kit (Bio-Rad; Hercules, CA) containing random primers and oligo dT mix. The cDNA produced was used to specifically quantify the transcript of interest using the Bio-Rad iCycler (Bio-Rad; Hercules, CA) with the RT² PCR primer set for human bax (SuperArray; Frederick, MD), the PCR primer set for human β-actin (Biosynthesis; Lewisville, TX) and the Bio-Rad iQ™ SYBR Green Supermix kit (Bio-Rad; Hercules, CA). The SYBR green dye intercalates with the double-stranded cDNA being formed during PCR, allowing an easy quantification of cDNA to indirectly quantify the original RNA transcripts present. For determining the transcript levels, ΔCt method was used.

*Small interfering Ribonucleic acid (siRNA) transfection* - Insulin receptor siRNA (IR-siRNA) (Santa Cruz; Santa Cruz, CA) was used to knockdown the insulin receptor in RKO cells. To perform RNA interference, RKO cells were cultured till about 60% confluency. Lipofectamine 2000 (Invitrogen; Carlsbad, CA) was used to transfect the cells with IR-siRNA. Control-siRNA (Santa Cruz; Santa Cruz, CA) was transfected as an external control. Six hours after transfection, the transfection medium was removed and the cells were cultured in normal culture medium for 42 hours to recover from the transfection stress. Cells were then treated with or without 25 μM of α-PGG for 48 hours. MTT assay and western blot analysis were performed thereafter.

*Enzyme linked immuno absorbent (ELISA) apoptosis assay* - Control-siRNA or IR-siRNA-transfected RKO cells were seeded at a density of 1 × 10⁴ cells/well in a 96-well plate and incubated for 24 hours. Cells were then switched to DMEM with 10% FBS and 1% penicillin/streptomycin with 25 μM of α-PGG. Mock-treated cells were used as
controls and each condition was prepared in triplicate. After 48 hours, an ELISA assay was performed using the Cell Death Detection ELISA$^{PLUS}$ kit (Roche-Applied Science; Indianapolis, IN) that measures the amount of cleaved DNA/histone complexes in apoptotic cells. Briefly, cells were lysed with 200 μL of lysis buffer for 30 min at room temperature. The lysate was centrifuged at 200 x g for 10 min. 150 μL of supernatant was collected, of which 20 μL was incubated with anti-histone biotin and anti-DNA peroxidase at room temperature for 2 h. After washing with incubation buffer three times, 100 μL of substrate solution (2,2’azino-di(3-ethylbenzthiazolin-sulphuric acid) was added to each well and the plate was incubated for 15-20 min at room temperature. The absorbance was measured using an ELISA reader (SPECTRA max M2, Molecular Devices; Sunnyvale, CA) at 405 nm.

**Statistical analyses** - All the assays described above were repeated more than once. The quantification assays were performed in triplicate and data were calculated and presented as mean ± standard deviation (M±SD). Data were analyzed by one-way ANOVA and Turkey’s post-hoc test. Significant level was set as p<0.05. * indicated p<0.05; ** indicated p<0.01; *** indicated p<0.001.
RESULTS

*α-PGG lowered cell viability in RKO cells in a dose-dependent manner* - MTT

assay, which is a standard colorimetric assay for measuring cellular proliferation or cell growth, was applied to determine the viability of the cells. The MTT assay indicated that *α*-PGG lowered the viability of RKO cells in a dose-dependent manner after a 48-h treatment (Fig. 19A). 25 μM of *α*-PGG lowered the viability of RKO cells by approximately 50% compared to the mock-treated controls (Fig. 19A). Since the viability lowering EC₅₀ of *α*-PGG was around 25 μM, 25 μM of *α*-PGG was used for the subsequent studies. The cell killing effects of *α*-PGG in RKO cells was also demonstrated by the microscopic pictures which showed that RKO cells treated with 25 μM of *α*-PGG for 48 hours changed their morphology and lowered the cell density (Fig. 19B). In contrast to *α*-PGG, 6Cl-TGQ, the derivative of *α*-PGG, did not produce appreciable effects in lowering viability in RKO cells (Fig. 19A). And the morphology of RKO cells was observed to be affected by a 48-h treatment with 25 μM of 6Cl-TGQ (Fig. 19B).
Figure 19. α-PGG lowered cell viability in RKO cells in a dose-dependent manner while 6Cl-TGQ did not generate pronounced effects on cell viability in RKO. (A) RKO cells were treated with various concentrations of α-PGG or 6Cl-TGQ (1 μM, 2 μM, 5 μM, 10 μM, 15 μM, 25 μM, 35 μM) for 48 h. Mock-treated cells were used as controls. MTT assay was performed to detect living cells. Each data point was an average of results from three independent experiments performed in triplicate and presented as M relative to mock-treated control ± SD. (B) RKO cells were treated with 25 μM of α-PGG or 6Cl-TGQ for 48 h. Mock-treated cells were used as controls. Microscopic pictures were taken using the software Motic MCCamera 1.1 with both the ocular lens and the objective lens being 10X.

α-PGG induced cell cycle arrest and cell death in RKO cells - Inhibition of cell division and induction of cell death or apoptosis can both lower the number of viable
cells. To determine whether α-PGG decreased cell viability through cell cycle inhibition and cell death induction, a flow cytometry analysis was done. Consistent with the results from MTT, sub-G1 cells as indicated by the first green peak were shown to have a great increase in RKO cells (~41% compared to the mock-treated control of ~2.7%) when they were treated with 25 μM of α-PGG for 48 h (Fig. 20). And the sub-G1 cells were mostly apoptotic cells according to the apoptosis induction assay and Tunnel assay done by Dr. Ahmed Malki (Data not shown). Besides, α-PGG treatment resulted in G1 and G2 arrest, thus reducing RKO cell proliferation as shown by the decreased S phase cells (Fig. 20). On the other hand, 6Cl-TGQ treatment only resulted in a slight increase of G1 arrest without inducing cell death (Fig 20). And this was consistent with the results obtained from MTT assay (Fig. 19). Since 6Cl-TGQ was not as effective as α-PGG in lowering cell viability, only α-PGG was used for the following studies.

![Mock-treated vs 25 μM α-PGG](image)

Figure 20 to be continued
Figure 20. $\alpha$-PGG induced cell cycle arrest and cell death in RKO cells whereas 6Cl-TGQ did not generate pronounced effects in RKO cells. RKO cells were treated with 25 μM of $\alpha$-PGG or 6Cl-TGQ for 48 h. Mock-treated cells were used as controls. Flow cytometry was used to examine the cell cycle distribution differences between compound-treated and mock-treated cells. Three independent experiments were carried out. Samples were triplicated for each treatment condition in each experiment and were expressed as Mean percentage of cells in a particular phase ± SD.
**α-PGG lowered cell viability significantly more in RKO cells than in FHC cells** -

While RKO cells are human colon epithelial carcinoma cells, FHC cells are human normal colon epithelial cells. FHC cells were therefore used as the normal cell controls for RKO cells in our study. To determine whether α-PGG lowers cell viability more in carcinoma cells than in their normal counterparts, we compared the compound effect in carcinoma and normal cell lines using MTT assay. The MTT assay which measures the amount/activity of NAD(P)H-oxidoreductases present in the cells, indicated that a 48-h treatment with α-PGG greatly lowered viability in RKO cells at all the three treatment concentrations (15, 25 or 35 μM) compared to the mock-treated controls (Fig. 21), which is consistent with our previous results (Fig. 19 & 20). Importantly, as indicated by the cell viability relative to mock-treated controls, the viability-lowering effect of α-PGG was stronger in RKO cells (relative cell viability: 24% for 15 μM of α-PGG, 21% for 25 μM of α-PGG, 16% for 35 μM of α-PGG) than in their normal counterpart FHC cells (relative cell viability: 110% for 15 μM of α-PGG, 98% for 25 μM of α-PGG, 118% for 35 μM of α-PGG) (Fig. 21). Notably, for all the three α-PGG treatment concentrations, the post-treatment viability differences between RKO and FHC cells were significant (p<0.01 for 15 μM of α-PGG and 35 μM of α-PGG, p<0.001 for 25 μM) (Fig. 21).
Figure 21. α-PGG lowered cell viability significantly more in RKO cells than in FHC cells. RKO and FHC cells were treated with 15, 25, or 35 μM of α-PGG. Mock-treated cells were used as controls. After a 48-h treatment, the viability of the cells was assessed using the MTT assay. Each treatment condition was prepared in triplicate. The experiment was repeated three times. The results were presented as M relative to mock-treated control ± SD. One-way ANOVA was used to determine the significance. ** indicated p<0.01; *** indicated p<0.001.

**α-PGG induced caspase 3 activation in RKO cells** - Apoptosis involves a variety of mediators, among which caspases are thought to be playing a central role (Riedl and Shi, 2004; Wang et al., 2005). To determine whether caspase 3, an effector caspase which has been identified as a key regulator of apoptosis in mammalian cells (Riedl and Shi, 2004), was involved in α-PGG-induced apoptosis, a caspase 3 activity assay was performed. The results showed that a 48-h treatment with α-PGG significantly increased caspase 3 activity in RKO cells (p<0.001) (Fig. 22).
Figure 22. α-PGG induced caspase 3 activation in RKO cells. RKO cells were treated with 25 μM of α-PGG for 48 h. Mock-treated cells were used as controls. Caspase 3 activity was measured using the caspase 3 fluorometric kit. Meanwhile, protein concentration was determined using the BCA protein assay kit. Each condition was performed in triplicate and was repeated in three independent experiments. The caspase 3 activity result for each sample was normalized with correspondent protein amount and was expressed as M±SD. Data were analyzed using one-way ANOVA. The significant level was set as p<0.05. *** indicated p<0.001.

α-PGG treatment increased p53 levels, induced nuclear translocation of p53, increased p53 transcriptional activity in RKO cells, and showed stronger viability-lowering effect in RKO cells than in RKO-E6 cells - Since α-PGG induced caspase 3-mediated apoptosis, thus decreasing cell viability in RKO cells, the mechanisms for the pro-apoptotic effects of α-PGG were then investigated. Western blot analysis revealed that, in comparison with the mock-treated controls, treatment of cells with 25 μM of α-PGG increased p53 levels by approximately 2 folds in RKO cells at 48 h (Fig. 23A first panel, Fig. 23B). Meanwhile, the phosphorylation of MEK1/2 and ERK1/2 showed a
pattern similar to the elevation of p53 (Fig. 23A first panel) after a 48-h compound treatment (Fig. 23A second and third panels).

Because p53 mainly functions transcriptionally in the nucleus (Toledo and Wahl, 2006), we studied the effects of α-PGG on the subcellular localization of p53 in RKO cells. Immunofluorescence microscopy showed that in comparison with the mock-treated controls, a 48-h treatment with 25 μM of α-PGG induced the translocation of p53 (indicated in green) from the cytoplasm to the nucleus (indicated in blue) (Fig. 23C). We further studied the influence of compound treatment on the transcriptional activity of p53 using the pp53-Luc luciferase reporting system. The binding of p53 to a cis-acting enhancer element sequence of pp53-Luc drives the expression of luciferase. Our luciferase assay results indicated that p53 transcriptional activity was significantly increased 36 h after the treatment with 25 μM of α-PGG (48 h after transfection) (p<0.001) (Fig. 23D).

To determine whether p53 contributes to the lowered cell viability by α-PGG treatment in RKO cells, we measured and compared the effects of α-PGG on the viability of RKO cells and RKO-E6 cells which are derived from RKO cells but lack appreciable functional p53. The MTT assay indicated that compared to the mock-treated controls, treating cells with α-PGG for 48 h significantly lowered cell viability in RKO and RKO-E6 cells at all the three treatment concentrations (15, 25 and 35 μM) (Fig. 23E), which is again consistent with our previous observations (Fig. 19-21). Importantly, as indicated by the cell viability relative to mock-treated controls, the viability-lowering effect of α-PGG was stronger in RKO cells (relative cell viability: 38% for 15 μM of α-PGG, 22% for 25
μM of α-PGG, 23% for 35 μM of α-PGG) than in RKO-E6 cells (relative cell viability: 64% for 15 μM of α-PGG, 48% for 25 μM of α-PGG, 42% for 35 μM of α-PGG) (Fig. 23E). For all the three α-PGG treatment concentrations, the post-treatment viability differences between the two types of cells were significant (p<0.001 for 15 μM of α-PGG, p<0.01 for 25 μM and 35 μM of α-PGG) (Fig. 23E).
B

![Graph showing P53/β-tubulin levels](image)

C

**C**

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D

![Graph showing Luciferase activity](image)

Luciferase activity (arbitrary unit)
Figure 23. \(\alpha\)-PGG treatment increased p53 levels, induced nuclear translocation of p53, increased p53 transcriptional activity in RKO cells, and showed stronger viability-lowering effect in RKO cells than in RKO-E6 cells. (A) RKO cells were treated with 25 \(\mu\)M of \(\alpha\)-PGG for 48 h. Cells were then lysed and total protein was collected. Western blot analysis was used to determine the p53 protein levels in compound-treated and mock-treated control cells. \(\beta\)-tubulin was used as a loading control. This experiment was repeated at least twice and the result shown was representative and was reproducible. (B) Quantification result of the elevated p53 levels by \(\alpha\)-PGG treatment in RKO cells from multiple experiments. The result was expressed as folds of increase compared to mock-treated control \(\pm\) SD. One-way ANOVA was used to determine the significance. * indicated \(p<0.05\). (C) RKO cells were treated with 25 \(\mu\)M of \(\alpha\)-PGG for 48 h. They were then used for determination of the subcellular localization of p53 with immunofluorescence microscopy. The subcellular location of p53 was indicated by the green FITC while the position of the nucleus was shown by the blue DAPI staining. Individual pictures for either p53 or the nucleus were taken when the ocular lens was 10X and the objective lens was 40X. Pictures were merged to display the subcellular localization of p53. (D) RKO cells, which were transfected with pp53-Luc together with the control plasmid PRL-TK, were treated with 25 \(\mu\)M of \(\alpha\)-PGG for 36 h before the performance of the assay for the p53 transcriptional activity indicated by the luciferase activity. Transfected cells without being treated with \(\alpha\)-PGG were used as controls. Each condition was assayed in triplicate and was repeated in three independent experiments. The results were presented as M\(\pm\)SD and were analyzed using one-way ANOVA. \(***\) indicated \(p<0.001\). (E) RKO and RKO-E6 cells of 40% confluency were treated with 15, 25, or 35 \(\mu\)M of \(\alpha\)-PGG. Mock-treated cells were used as controls and each condition was prepared in triplicate. After a 48-h treatment, the viability of the cells was determined using the MTT cell proliferation assay. The experiment was carried out three times. The results were presented as M relative to mock-treated control \(\pm\) SD. One-way ANOVA was used to determine the significance. ** indicated \(p<0.01\); *** indicated \(p<0.001\).
α-PGG induced Bax expression in RKO cells at both the mRNA level and the protein level - Bax, a Bcl-2 family protein, induces cell death through disruption of mitochondrial permeability and subsequent release of cytochrome c (Antionani and Youle, 2006; Hardwick and Polster, 2002; Reed, 1996). Bax receives signals from both extrinsic and intrinsic pathways of apoptosis and it can be downstream of p53, relaying the apoptotic signals from p53 (Moll et al., 2005). RKO cells were treated with 25 μM of α-PGG for 48 h and the mock-treated cells were used as controls. As shown by the real-time PCR and western blot analyses, α-PGG increased Bax expression at both mRNA level (p<0.01) (Fig. 24A) and protein level (Fig. 24B) in RKO cells compared to the mock-treated controls. These results were further confirmed by the immunofluorescence microscopic study which showed that, compared to the mock-treated control group, 25 μM of α-PGG increased Bax expression in RKO cells after a 48-h treatment (Fig. 24C).
α-PGG induced Bax expression in RKO cells at both the mRNA level and the protein level. RKO cells were treated with 25 μM of α-PGG for 48 h. Mock-treated cells were used as controls. All the experiments shown in this figure were repeated at least twice. (A) mRNA was extracted and was reverse transcribed to cDNA for real-time PCR analysis of Bax mRNA level. Primers specific for Bax cDNA and iCycler iQ Real-time Detection System were applied in the analysis. β-actin levels were also measured from the same cDNA preparation to normalize Bax mRNA levels. The calculated mRNA levels of Bax were first normalized using β-actin RNA levels separately for compound-treated and mock-treated cells and then the ratio for the compound-treated to mock-treated cell RNA levels was prepared using ΔCt method. SD was calculated accordingly. Each sample was analyzed in three separate experiments. And in each experiment, cDNAs were measured in two quantitative PCR runs, yielding six determinations for each treatment condition. One-way ANOVA was used to determine the significance. ** indicated that the p value determined was <0.01. (B) Total protein was extracted and western blot analysis was applied to determine the protein levels of Bax in compound-treated and mock-treated cells. β-actin was used as a loading control. (C) Bax in RKO cells was immuno-stained using Bax (B-9) as the primary antibody and Texas Red - conjugated IgG as the secondary antibody. Individual pictures for Bax were taken using an immunofluorescence microscope with the ocular lens being 10X and the objective lens being 20X.

Figure 24. α-PGG induced Bax expression in RKO cells at both the mRNA level and the protein level. RKO cells were treated with 25 μM of α-PGG for 48 h. Mock-treated cells were used as controls. All the experiments shown in this figure were repeated at least twice. (A) mRNA was extracted and was reverse transcribed to cDNA for real-time PCR analysis of Bax mRNA level. Primers specific for Bax cDNA and iCycler iQ Real-time Detection System were applied in the analysis. β-actin levels were also measured from the same cDNA preparation to normalize Bax mRNA levels. The calculated mRNA levels of Bax were first normalized using β-actin RNA levels separately for compound-treated and mock-treated cells and then the ratio for the compound-treated to mock-treated cell RNA levels was prepared using ΔCt method. SD was calculated accordingly. Each sample was analyzed in three separate experiments. And in each experiment, cDNAs were measured in two quantitative PCR runs, yielding six determinations for each treatment condition. One-way ANOVA was used to determine the significance. ** indicated that the p value determined was <0.01. (B) Total protein was extracted and western blot analysis was applied to determine the protein levels of Bax in compound-treated and mock-treated cells. β-actin was used as a loading control. (C) Bax in RKO cells was immuno-stained using Bax (B-9) as the primary antibody and Texas Red - conjugated IgG as the secondary antibody. Individual pictures for Bax were taken using an immunofluorescence microscope with the ocular lens being 10X and the objective lens being 20X.
p53 elevation induced by α-PGG in RKO cells was abolished by IR inhibitor but not by IGF-1R inhibitor - We previously found that α-PGG binds with IR and activates the IR signaling which mainly includes the IR-PI3K-Akt and the IR-MAPK pathways (Kayali et al., 2000; Li et al., 2005). Based on these results and the observation that the phosphorylation of MEK1/2 and ERK1/2 showed a pattern similar to the elevation of p53 (Fig. 23A, first panel) after a 48-h compound treatment (Fig. 23A, second and third panels), we hypothesized that p53 elevation in RKO cells induced by α-PGG treatment might involve the action of the compound on an IR signaling cascade. To investigate the relationship between the IR signaling and the elevation of the p53 levels, an inhibitor study with the IR inhibitor HNMPA-(AM)₃ and the IGF-1R inhibitor PPP was performed. Western blot analysis showed that the inhibition of the IR signaling with the IR β-subunit tyrosine phosphorylation inhibitor HNMPA-(AM)₃ abolished the function of α-PGG in increasing p53 levels in RKO cells while inhibition of the IGF-1R signaling with the IGF-1R inhibitor PPP did not show significant effects on the elevated p53 levels induced by the compound (Fig. 25A). That the same concentration of PPP abolished IGF-1R phosphorylation in the IGF-1R overexpressing 3T3 cells indicated that PPP was functional at that particular concentration (data not shown).

Elevation of p53 in RKO cells induced by α-PGG was either abolished, slightly decreased, or not affected by inhibitors of MEK, ERK, or PI3K, respectively - After establishing that IR signaling is involved in the p53 elevation by α-PGG, we further studied the signaling molecules downstream of IR in attempt to identify the specific IR signaling pathway(s) that participate(s) in the p53 elevation. To study the relationship
between PI3K-Akt signaling and p53 elevation, the PI3K inhibitor Wortmannin was used. Western blot analysis using phospho-Akt specific and p53 specific antibodies indicated that Wortmannin, which interacts with PI3K and thus inhibits Akt phosphorylation, did not affect p53 levels (Fig. 25B). In this assay, inhibition of Akt phosphorylation was used as an indicator of Wortmannin function (Fig. 25B). Notably, insulin, which is similar to α-PGG in the activation of the IR signaling, did not induce the elevation of p53 as α-PGG did (Fig. 25B). To study the relationship between MEK-ERK activation and p53 elevation, an experiment similar to the previous inhibitor studies was carried out with MEK inhibitor I and ERK inhibitor AEMT. Addition of 10 μM of MEK inhibitor I blocked the interaction of MEK with its downstream signaling molecules including ERK, and significantly decreased the elevated p53 levels induced by 25 μM of α-PGG (Fig. 25C). In contrast, addition of 50 μM of AEMT, which interferes with the interaction of ERK with its downstream targets, generated some noticeable but not pronounced reduction in the elevated p53 levels induced by α-PGG (Fig. 25C). A significant RKO cell killing effect of AEMT was observed at the concentration of 50 μM, indicating that AEMT was functional at that particular concentration, but it functioned in a p53-independent manner (data not shown).
Figure 25. The elevation of p53 induced by α-PGG in RKO cells was mediated through the IR-MEK signaling. (A) RKO cells were cultured for 48 h in culture medium supplemented with 25 μM of α-PGG and/or 200 μM of the IR inhibitor HNMPA-(AM)_3 / 1 μM of IGF-1R inhibitor PPP. (B) RKO cells were cultured for 48 h in culture medium supplemented with 25 μM of α-PGG or 1 μM of insulin, and/or 20 μM of the PI3K inhibitor Wortmannin. (C) RKO cells were cultured for 48 h in culture medium supplemented with 25 μM of α-PGG and/or 10 μM of MEK inhibitor I / 50 μM of ERK inhibitor AEMT. Non-treated or mock-treated cells were used as controls. Total protein was extracted for western blot analyses to determine p53 protein levels under different treatment conditions. Akt phosphorylation was detected to indicate the function of Wortmannin and ERK phosphorylation was detected to indicate the function of MEK inhibitor I. Either β-tubulin or β-actin was used as a loading control. All the experiments shown in this figure were repeated at least twice. The results shown were representative and were reproducible.
Induction of Bax in RKO cells by α-PGG was abolished by MEK inhibitor - Bax activation was observed in our previous experiments (Fig. 24). We investigated the relationship between Bax activation and MEK signaling to see if like p53, compound-induced Bax activation is also regulated through MEK signaling. RKO cells were treated with 25 μM of α-PGG with or without 10 μM of MEK inhibitor I for 48 h. Bax was seen to be increased by α-PGG (Fig. 26), which is consistent with our previous observations (Fig. 24). Moreover, Bax induction by α-PGG was abolished by MEK inhibitor I (Fig. 26), which is similar to the situation of p53 (Fig. 25C).

Figure 26. The induction of Bax by α-PGG in RKO cells was mediated through the MEK signaling. RKO cells were cultured for 48 h in culture medium supplemented with 25 μM of α-PGG and/or 10 μM of MEK inhibitor I. Non-treated or mock-treated cells were used as controls. Total protein was extracted for western blot analyses to determine Bax protein levels under different treatment conditions. Structural protein GAPDH was used as a loading control. Experiment shown in this figure was repeated twice. The result shown was representative and reproducible.

Addition of insulin reversed the viability-lowering effects of α-PGG in RKO cells - α-PGG induced p53 elevation (Fig. 23 & 25) and lowered cell viability (Fig. 19-21, 27) while insulin did not (Fig. 25B). To verify the role of IR in lowering cell viability,
increasing concentrations of insulin (0 μM, 1 μM, 10 μM, 50 μM and 100 μM) were added to RKO cells together with 25 μM of α-PGG and incubated for 48 hours. MTT assay showed that insulin reversed the viability-lowering effect of α-PGG in a dose-dependent manner (Fig. 27). While 1 μM and 10 μM of insulin did not demonstrate much effect, 50 μM and 100 μM of insulin did reverse the lowered viability by 25 μM of α-PGG although not fully (Fig. 27).

Figure 27. Addition of insulin reversed the viability-lowering effects of α-PGG in RKO cells. Increasing concentrations of insulin (0 μM, 1 μM, 10 μM, 50 μM and 100 μM) were added to RKO cells together with 25 μM of α-PGG. Mock 10 μM insulin and 100 μM insulin-treated cells were used as controls. After a treatment of 48 hours, the viability of the cells was determined using the MTT cell proliferation assay. The experiment was carried out three times. And each condition was prepared in triplicate in each experiment. The results were presented as M relative to mock-treated control ± SD. One-way ANOVA was used to determine the significance. ** indicated p<0.01; *** indicated p<0.001.
Knockdown of insulin receptor with IR-siRNA attenuated the apoptosis-promoting and p53-elevating effects of α-PGG in RKO cells - IR-siRNA was applied to knockdown the insulin receptor in RKO cells. The function of IR-siRNA was verified by western blot analysis. Our results showed that compared to the control-siRNA, IR-siRNA significantly decreased the levels of insulin receptor in RKO cells 24 hours, 48 hours and 72 hours after the completion of transfection (Fig. 28A). We started the 48-hour α-PGG treatment 24 hours after siRNA transfection so that IR levels remained low in IR-siRNA-transfected cells during the entire course of compound treatment. ELISA apoptosis assay showed that compared to the mock treatment, α-PGG treatment resulted in an increase of apoptosis in both control- and IR-siRNA-transfected cells (Fig. 28B). However, the induction of apoptosis was much lower in IR-siRNA-transfected RKO cells (~3 folds) than in control-siRNA-transfected RKO cells (~6 folds) (Fig. 28B). Consistent with the apoptosis assay result, western blot indicated that the p53 elevating effect of α-PGG was diminished in IR-siRNA-transfected cells (~1 fold) in comparison with that in control-siRNA-transfected cells (~3.5 folds) (Fig. 28C & 28D).
B

Induction of apoptosis (Abs at 405 nm)

Control-siRNA  IR-siRNA

Mock/mock  α-PGG/mock

C

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p53: 53 Kd
β-actin: 43 Kd
Figure 28. The induction of apoptosis and p53 by α-PGG was attenuated in IR-siRNA-transfected RKO cells compared with control-siRNA-transfected RKO cells. RKO cells were transfected with either control-siRNA or IR-siRNA. (A) 24, 48 and 72 hours after the completion of transfection, total protein was extracted for western blot analysis of the function of RNA interference. IR protein levels were measured as an indicator of IR-siRNA function. β-tubulin served as a loading control. (B) 24 hours after transfection completion, cells were treated with 25 μM of α-PGG. 48 hours after treatment, ELISA apoptosis assay was performed to measure the level of apoptotic cells. Each condition was prepared in triplicate and was repeated in three independent experiments. Data were presented as M relative to mock-treated control ± SD. One-way ANOVA was used to determine the significance. ** indicated p<0.01; *** indicated p<0.001. (C) 24 hours after transfection completion, cells were treated with 25 μM of α-PGG. 48 hours after treatment, total protein was isolated. Western blot analysis was used to determine p53 levels under different treatment conditions. IR protein levels were measured as an indicator of IR-siRNA function. β-tubulin was used as a loading control. The results shown were representative and were reproducible. (D) Quantification result of the elevated p53 levels by α-PGG treatment in control-siRNA-transfected and IR-siRNA-transfected RKO cells. Data were presented as M relative to mock-treated control ± SD. One-way ANOVA was used to determine the significance. *** indicated p<0.001.
α-PGG induced p53 elevation and apoptosis in RKO cells through the IR-MEK pathway – Schematic diagram for the hypothetic mechanism of α-PGG-induced p53 elevation and apoptosis in RKO cells (Fig. 29).

Figure 29. Hypothetical mechanism of α-PGG-induced p53 elevation and apoptosis in RKO cells. The insulin signaling is mainly composed of the insulin receptor-MEK pathway and the insulin receptor-PI3K-Akt pathway. Based on the experimental results, α-PGG induces p53 elevation, Bax activation and the subsequent cell cycle arrest and caspase-3-mediated apoptosis in RKO cells most likely through binding to IR and activating the IR-MEK signaling pathway. ERK1/2 may also play some roles in p53 and Bax activation. In comparison, the PI3K-Akt pathway is unlikely to be involved in p53 activation.
DISCUSSION

The role of p53 in DNA damage repair, cell cycle arrest and apoptosis has been well-documented (Bargonetti and Manfredi, 2002; Fridman and Lowe, 2003; Levine, 1997; Aylon and Oren, 2007; Pietsch et al., 2008). Meanwhile, the role of IR in cell metabolism and proliferation has been well-established (Saltiel and Kahn, 2001; Saltiel and Pessin, 2002). However, p53 activation and apoptosis mediated through IR, under any condition or by any reagent, has never been described. α-PGG belongs to a large family of compounds named tannin or polyphenols (Li et al., 2005; Ren and Chen, 2007; Ren et al., 2006). Its structural isomer, β-PGG, was previously reported to have anti-cancer effects (Feldman et al., 1999; Huh et al., 2005). However, the systematic anti-cancer mechanistic studies have never been conducted for β-PGG. This study is the first to demonstrate the anti-cancer activity of α-PGG. Furthermore, to the best of our knowledge, this is also the first time that the phenomenon of p53 elevation and Bax activation through the IR-MEK signaling pathway is described.

We initiated the study with the observation that α-PGG lowered cell viability and caused cell morphology change in human colon carcinoma RKO cells (Fig. 19). Its derivative 6Cl-TGQ did not show appreciable effect in lowering cell viability or inducing morphology change in RKO cells although it is more powerful in diabetes treatment compared to α-PGG (Fig. 5-6, 19). The lowered viability in RKO cells by α-PGG could be attributed to the inhibited cell cycle progression (cell division) and induced apoptosis (Fig. 20). Due to the combinational effects of G1, G2 arrest and apoptosis induction (~40%) (Fig. 20), α-PGG lowered the viability of RKO cells by 50-80% after a 48-h
treatment (Fig. 19). Consistent with the viability assay result (Fig. 19), 6Cl-TGQ was only found to induce a slight G1 arrest without causing apoptosis in RKO cells by flow cytometry analysis (Fig. 20). Therefore, only α-PGG was carried on for the subsequent studies. A further study suggested that α-PGG is a promising anti-cancer reagent as its viability-lowering effect is stronger in carcinoma cells than in their normal counterpart cells (Fig. 21). We subsequently found that α-PGG activated caspase 3 (Fig. 22). The increased caspase 3 activity confirms α-PGG-induced apoptosis as caspase 3 is a key mediator of apoptosis (Riedl and Shi, 2004). On the other hand, considering that apoptosis can be mediated through caspase-dependent or independent pathways (Cummings et al., 2004), this result also indicated that the apoptosis induced by α-PGG is at least partially mediated through the caspase-dependent pathway. In a following study, we found in RKO cells that p53 was elevated and translocated to the nucleus to regulate transcription upon α-PGG treatment (Fig. 23). p53 is a critical cell cycle arrest and apoptosis inducer (Aylon and Oren, 2007). Although cell cycle arrest and apoptosis can go through p53-dependent (Aylon and Oren, 2007) or p53-independent pathways (Liang et al., 2007), we consider that the cell cycle arrest and apoptosis induced by α-PGG in RKO cells is largely mediated through the p53-dependent pathway. An important piece of evidence supporting this notion is that the viability of RKO-E6 cells which lack appreciable functional p53 was not affected by α-PGG as much as that of RKO cells (Fig. 23E). p53 has been found to induce apoptosis via both transcription-dependent and Bax or Bak-mediated-transcription-independent mechanisms (Moll et al., 2005). The nuclear translocation and the upregulated transcriptional activity of p53 suggested that in
α-PGG-treated RKO cells, p53 functions through transcriptional regulations. We later identified that in RKO cells, α-PGG treatment activated Bax (Fig. 24). Bax, a mitochondrial apoptosis mediator, receives pro-apoptotic signals from both the extrinsic and the intrinsic pathways (Antignani and Youle, 2006; Cory and Adams, 2002; Hardwick and Polster, 2002; Reed, 1996). Although Bax activation may not necessarily involve p53, p53 could regulate Bax expression transcriptionally (Riley et al., 2008). p53 may also activate Bax upon translocation to the mitochondria when p53 works through the transcription-independent pathway (Moll et al., 2005). Therefore, the activation of Bax by α-PGG, which contributes to the apoptosis of RKO cells, could be mediated through p53 no matter p53 works through the transcription-dependent or transcription-independent apoptotic pathways (Moll et al., 2005; Riley et al., 2008).

After we verified the elevation of p53 and Bax in RKO cells by α-PGG treatment, we concentrated on RKO cells to identify the signaling pathways responsible for these effects. Since we previously found that α-PGG binds to IR and activates the IR signaling in 3T3-L1 adipocytes (Li et al., 2005) and we observed in cancer cell lines that the IR downstream signaling factors MEK1/2 and ERK1/2 were phosphorylated in a pattern similar to the elevation of p53 after α-PGG treatment (Fig. 23A), we speculated that α-PGG induced p53 elevation in RKO cells through the IR signaling. To test this hypothesis, we used an IR inhibitor HNMPA-(AM)_3. The p53 elevation by α-PGG was abolished when HNMPA-(AM)_3 was added to RKO cells (Fig. 25A), indicating that p53 elevation was regulated through IR. The role of IR in α-PGG-induced apoptosis and p53 increase was further confirmed by the insulin supplement study (Fig. 27) and the siRNA
study (Fig. 28). In the insulin supplement study, insulin of various concentrations was
used to reverse the viability-lowering effects of α-PGG because insulin was not reported
or found to elevate p53 or induce apoptosis (Fig. 25B). The result showed that high
concentrations of insulin were able to partially reverse the viability-lowering effects of α-
PGG in RKO cells (Fig. 27). Thus, it is likely that α-PGG lowers RKO cell viability
through IR and insulin could partially compete against or counteract the viability-
lowering effects of α-PGG. In the siRNA study, knockdown of IR with IR-siRNA
attenuated the apoptosis and p53 induction effects of α-PGG in RKO cells, strongly
supporting the involvement of IR in α-PGG action (Fig. 28). In order to rule out the
possibility that IGF-1R, an IR homologous receptor, is also involved in p53 elevation,
IGF-1R inhibitor PPP was used. Western blot analysis revealed that α-PGG-elevated p53
level was not affected by PPP in RKO cells (Fig. 25A), indicating that IGF-1R was not
involved in α-PGG-induced p53 elevation. After IR was identified as the target of α-
PGG for p53 activation, we subsequently determined the downstream signaling factors
that are involved in the elevation. Two major signaling pathways are known to be
mediated through IR: the IR-PI3K-Akt pathway and the IR-MEK-ERK pathway
(Derijard et al., 1994; Kayali et al., 2000; Pollak et al., 2004; Saltiel and Kahn, 2001).
The PI3K inhibitor Wortmannin did not significantly affect p53 elevation (Fig. 25B),
suggesting that the IR-PI3K-Akt pathway is not involved in increasing p53 levels. In
contrast, when MEK inhibitor I was added to α-PGG treated RKO cells, p53 elevation
was completely abolished (Fig. 25C). This suggested that MEK is responsible for p53
elevation. On the other hand, ERK inhibitor AEMT produced some noticeable but not
pronounced effect on p53 levels (Fig. 25C), suggesting that ERK may only be playing a limited role in the p53 elevation by α-PGG in RKO cells. Together, these experiments strongly suggested that p53 elevation in RKO cells induced by α-PGG is mediated, at least in part, through the activation of the IR-MEK signaling pathway, but not through the activation of IGF-1R or the IR-PI3K-Akt pathway, as shown in the graph for the proposed hypothetical working mechanism (Fig. 29). We further studied whether this pathway is also involved in α-PGG-induced Bax activation in RKO cells. Abolishment of the α-PGG-induced Bax activation by MEK inhibitor I (Fig. 26) suggested that, like the situation of p53, the activation of Bax is regulated by the MEK pathway, in particular the IR-MEK pathway. To our best knowledge, this is the first time that the phenomenon of p53 elevation and Bax activation through the IR-MEK signaling pathway is described.

IR activation is largely associated with enhanced cell growth, thus contributing to cancer occurrence and progression. Plasma insulin level is elevated in some metabolic diseases such as type 2 diabetes. And an impressive body of epidemiologic data has been accumulated over the past decade indicating the association of the risk of cancers like colorectal cancer (Giovannucci, 2007) and breast cancer (Xue and Michels, 2007) with hyperinsulinemia in metabolic syndrome. Similarly, MEK signaling is often involved in cell proliferation. The association of MEK activation with cancer has been well-established (Legrier et al., 2007). However, recently more and more evidence showed that abnormal activation of mitogenic signals may actually lead to cell cycle arrest and even apoptosis. For instance, the outcome of increased tyrosine kinase activity including increased IGF-1R activation turned out to be apoptosis (Stewart et al., 1999a; Stewart et
Strong ERK activation was reported to target Cdc25A for degradation to induce cell cycle arrest (Isoda et al., 2009). Sustained activation of ERK may lead to cell cycle arrest in human colon cancer cells (Goulet et al., 2005). Furthermore, MEK was found to become cell cycle-inhibiting and apoptosis-inducing when it was constitutively active (Nguyen et al., 2004; Tu et al., 2007). These and our new finding both suggested that p53 elevation, Bax activation and apoptosis through MEK are possible under certain conditions.

A question arises as how \( \alpha \)-PGG is able to induce an insulin-like glucose uptake activity in adipocytes but induce p53 elevation, Bax activation, growth arrest and apoptosis in carcinoma cells, which are uncharacteristic of insulin functions. The answer to this question may lie in the existence of different types of biological responses mediated by IR and the differences between insulin and \( \alpha \)-PGG in IR binding and activation. Through IR, insulin mediates two types of cellular responses: the fast metabolic responses that take seconds to minutes to occur, and the slow mitogenic responses that require gene expression and needs days to complete (Saltiel and Kahn, 2001; Saltiel and Pessin, 2002). The differences in the ligand-receptor binding kinetics, the timing, magnitude and duration of signals as well as the subcellular localization of signaling molecules all play important roles in determining the biological consequences of IR activation (Jensen and De Meyts, 2009). Since \( \alpha \)-PGG binds and activates IR (Li et al., 2005), it is able to induce IR-mediated fast responses such as glucose transport in adipocytes (Li et al., 2005). In comparison, since \( \alpha \)-PGG binds IR at a site different from the insulin binding site with a very different binding kinetics (Li et al., 2005) (Table 1), it
is likely that the slow mitogenic signal sent out by the $\alpha$-PGG-induced IR is different in intensity and duration from the one induced by insulin, leading to growth arrest and apoptosis rather than cell proliferation in tumor cells.

Our findings may be significant for basic cancer research and clinical cancer treatment investigation. It is scientifically important to find out how the activation of IR signaling, a cell metabolism and mitogenesis advancing pathway, could upregulate p53, Bax to induce growth arrest and apoptosis under certain conditions. Some cancers including pancreatic cancer, overexpress IR or its downstream signaling molecules and exhibit over-activation of the IR signaling (Kornmann et al., 1998; Paonessa et al., 2006; Surmacz and Burgaud, 1995). It is conceivable that $\alpha$-PGG and its derivatives may offer a novel and effective way to treat these cancers.
FUTURE STUDY

Introduction and Specific Aims

Cells, as living creatures, communicate with the environment and regulate self behavior via intricate signaling networks. Cell signaling, which is mediated through various signaling molecules, is fine tuned so that cells may respond properly to intracellular and extracellular changes. The understanding of cell signaling started decades ago and the knowledge about cell signaling is still rapidly expanding. Proliferation and growth, which are important cell activities, have been found to be advanced through certain mitogenic signaling pathways. Meanwhile, some growth-inhibitory and apoptotic signaling pathways take charge of growth restriction. Traditionally, it is thought that mitogenic and growth-inhibitory signaling pathways are distinct pathways. In recent years, more and more studies revealed that mitogenic pathways are not necessarily mitogenic. Abnormal or prolonged activation of a mitogenic pathway may generate apoptotic signals. For example, MEK, which is often involved in mitogenic signalings, has been found to become apoptosis-inducing when constitutively active (Nguyen et al., 2004). It is considered that subtle differences in signaling transduction of a same pathway may elicit dramatically different biological responses (Jensen and De Meyts, 2009). Insulin receptor signaling pathway has long been accepted as a signaling pathway promoting cell proliferation and growth. However, our most recent work with human colon carcinoma cell line RKO sheds light on an unconventional function of the insulin receptor signaling. Our study indicated that the activation of the insulin receptor signaling by compound α-PGG, instead of promoting cell division and
growth, leads to p53 activation, cell cycle inhibition and even apoptosis. Specifically, we found that α-PGG inhibited cell cycle progression and induced apoptosis in RKO cells. α-PGG induced apoptosis significantly more in RKO cells than in their normal cell counterparts FHC cells. The apoptosis induced by α-PGG in RKO cells is, at least in part, p53-dependent. Importantly, we found that blocking the insulin signaling pathway, in particular the insulin receptor - MEK pathway, using chemical inhibitors or siRNAs, decreased α-PGG-induced p53 elevation. Also, addition of insulin alleviated the apoptotic effects of α-PGG. These data strongly suggested that the insulin signaling pathway plays a positive role in α-PGG-induced p53 activation and apoptosis. Since insulin, the native binding partner of insulin receptor, has not yet been found to trigger p53 activation or apoptosis in cells, we suspected that α-PGG elicits a biological response different from insulin because α-PGG binds insulin receptor with an affinity and kinetics different from that of insulin. The in vitro receptor binding experiment with the Biacore System indicated that α-PGG does bind to insulin with a quite different affinity and kinetics from insulin, supporting our hypothesis. Although our results have been relatively complete and convincing, our study is far from completed. First, we have tested the anti-cancer effects of α-PGG in cell system, but not in animal models. Second, we observed that α-PGG induced apoptosis more in tumor cells than in normal cells. However, the reasons why the difference exists remain to be investigated. Third, although we narrowed down the signaling pathway that is responsible for p53 elevation to the insulin receptor - MEK signaling pathway, we haven’t elucidated the MEK downstream signaling molecules that relay the signal from MEK to p53. Fourth, we are interested in
finding out whether α-PGG may induce apoptosis through a p53-independent pathway. Finally, pancreatic cancer is among the top malignant cancers which have a very low 5-year survival rate. Our preliminary data indicated that α-PGG induced cell death in pancreatic cancer cell lines PANC-1 and BXPC-3. PANC-1 is a human pancreatic duct epithelial cancer cell line while BXPC-3 is a human pancreatic adenocarcinoma cell line. We are planning to find out whether our compound α-PGG induces pancreatic cell apoptosis through insulin receptor-mediated signaling pathway.

Specific aim 1: evaluate the therapeutic efficacy and toxicity of compound α-PGG in mouse tumor model. Nu/J mice with tumor will be used to evaluate compound toxicity and compound effect in tumor therapy. Our goal is to learn about whether α-PGG is effective in tumor therapy in mouse model with an acceptable toxicity and to find a compound dosage with low-toxicity but high therapeutic activity.

Specific aim 2: study the mechanism(s) by which α-PGG kills tumor cells much more than the normal cell counterparts. RKO and FHC cells have dramatically different responses to α-PGG treatment. The insulin receptor amount, α-PGG-induced insulin receptor signaling activation timing and duration will be compared between RKO and FHC cells. We are aiming at elucidating why α-PGG is selectively against tumor cells so that we will have a better understanding of how to generate low toxicity anti-tumor reagents.

Specific aim 3: continue to study the insulin receptor signaling pathway to find the signaling molecules that are downstream of MEK and responsible for α-PGG-induced p53 elevation. Using the RKO cell model, intensive signaling transduction studies will be
conducted to obtain a more comprehensive view of the signaling pathway(s) that contributes to α-PGG-induced increase of p53.

Specific aim 4: investigate the p53-independent mechanisms by which α-PGG induces tumor cell apoptosis. Since we previously found that α-PGG is able to reduce glucose transport from the extracellular environment to cytosol in RKO and other tumor cells, we plan to work on whether α-PGG also induces cell apoptosis through inhibition of glucose transport.

Specific aim 5: study the mechanism of α-PGG-induced cell death in pancreatic cancer cell lines. The cell death induced in PANC-1 and BXPC-3 by α-PGG will be studied to learn whether the cell death is due to apoptosis. The action of α-PGG on the insulin receptor signaling pathway and p53 will be studied. The relationship between the insulin receptor signaling and p53 will be investigated once the involvement of the insulin signaling pathway and p53 in α-PGG action is verified.

Designs

Specific aim 1: To evaluate the therapeutic effect and toxicity of compound α-PGG in colorectal cancer treatment. 30 Nu/J mice of 5-week old will be purchased from the Jackson laboratory (Bar Harbor, MA). On day 0, RKO cells will be trypsinized and resuspended in PBS (10^6 cells/0.1 ml). Mice will be injected subcutaneously at the lumbar regions with RKO cells, 5 X 10^6 cells for each mouse. 7 days after tumor cell injection, mice will be verified for the growth of tumors. The mice with visible tumor growth will be collected and divided into 3 groups, mock treatment group, 9.4 mg/kg (10
μM) α-PGG treatment group and 18.8 mg/kg (20 μM) α-PGG treatment group. Starting from day 8, either mock (ddH₂O) or α-PGG will be injected every day intraperitoneally. Measuring will start from day 8. Food intake and body weight will be measured every 3-4 days. Blood glucose level will be monitored once or twice a week right after compound injection. Tumor size will be measured using a caliper every 2-3 days. Tumor volume will be calculated using the modified ellipsoid formula: 0.5 L x W², where L is the longer axis and W is the axis perpendicular to A (Chen et al., 2004; Shureiqi et al., 2003). One-way ANOVA will be used for statistic analysis. The significance level will be set as p<0.05. Mice will be sacrificed once tumor size reaches >5% but < 10% of the body weight. Tumor tissue and the comprisable normal tissue will be carefully isolated and immediately frozen with liquid nitrogen for future immunohistochemistry analysis. Mice will also be dissected to examine organ abnormality. The animal study will be carried out and terminated in accordance to the rules and regulations of NIH and our university IACUC.

Specific aim 2: To study the mechanism(s) by which α-PGG kills tumor cells much more than the normal cell counterparts, RKO and FHC cells will be employed as our in vitro cell models.

The insulin receptor amount or density of the two cell lines will be compared first. To determine how many IR are on the membrane of RKO and FHC cells, IR binding assay will be performed. Insulin receptor belongs to a membrane protein. So wheat germ agglutinin (WGA) will be able to capture insulin receptor. Total protein will be prepared from a given amount of RKO and FHC cells, and used to bind WGA wells of FlashPlates
pre-coated with WGA (PerkinElmer Life Sciences; Waltham, MA) at 100 μg/well in Binding Buffer (10 mM Tris, 0.12 M NaCl, 5 mM KCl, 1.2 mM MgSO₄, 15 mM sodium acetate) at 4 °C for 6 h. After removal of unbound proteins and washes, increasing concentrations of insulin labeled with ¹²⁵I (Amersham; Piscataway, NJ) will be added to the wells in the binding buffer with a final volume of 100 μL. The plates will be incubated at 4 °C overnight with gentle shaking. Following incubation and removal of unbound radioactive ligands, the wells will quickly be washed three times with 200 μL of Wash Buffer (150 mM NaCl, 20 mM Hepes, pH 7.8, 0.025% Triton X-100). The bound ligand will be detached (eluted) from the receptor with 2 x 200 μL 0.2M Glycine pH 2.8, and measured for its radioactivity using a Beckman Coulter LS 6400 Multi-Purpose Scintillation Counter (Beckman Coulter, Inc.; Fullerton, CA). WGA wells coated with BSA (100 μg/well) will be used as controls for non-specific ligand binding and proteins isolated from CHO-IR cells (0.5 mg/well) will serve as positive controls. The receptor binding results will be analyzed with the software GraphPad Prism.

The differences in timing and duration of signaling activation of RKO and FHC cells in response to α-PGG treatment will also be compared. 1 X 10⁶ cells will be cultured in a 40 mm culture dish. When cells reach 40% to 60% confluency, cells will be subjected to treatment with 25 μM α-PGG. Total protein will be isolated 0 min, 5 min, 15 min, 30 min, 60 min, 3 h, 6 h, 12 h, 24 h, 36 h, and 48 h after compound treatment. To collect total protein, lysis buffer (10 mM Tris HCl pH 7.5, 1 mM EDTA, 1% triton X-100, 150 mM NaCl, 1 mM dithiothretol, 10% glycerol, 0.2 mM phenylmethysulphonyl fluoride and protease inhibitors) will be used to scrape cells off the dish. The cell lysate
will be sonicated 3 times, 3 seconds each time followed by centrifugation for 10 min to harvest the supernatant. All protein isolation procedures should be done at 4°C. Protein concentrations will be determined using BCA protein assay kit (Pierce; Rockford, IL). 50 μg of the total protein will be loaded to 7.5% Tris-glycine polyacrylamide gels (Bio-Rad; Hercules, CA) and subjected to electrophoresis. Phospho-MEK, Phospho-ERK1/2 (phospho-MAPK), p53 will be detected using specific first antibodies and HRP-conjugated secondary antibodies; either β-actin or β-tubulin will be detected as a protein loading control. Band density will be determined using the software JImage. The patterns of MEK and ERK phosphorylation and p53 elevation will be compared between RKO cells and FHC cells.

**Specific aim 3**: Western blot analysis will be applied intensively to study the signaling molecules that are possibly involved in α-PGG-induced p53 elevation. Using RKO cells, we will search for the signaling molecules which change their activation status in response to α-PGG treatment. For instance, some MAPK family members, some extracellular stress responsive molecules and some DNA damage responsive molecules will be examined to see whether those molecules phosphorylate or translocate to the nucleus. The insulin receptor inhibitor or MEK inhibitor will then be used to block the insulin receptor-MEK signaling pathway to see whether those molecules stop responding to α-PGG treatment. If they stop responding to α-PGG treatment, they are downstream of the insulin receptor-MEK signaling pathway. Subsequently, the action of the identified MEK downstream signaling molecules will be inhibited to see whether the inhibition of the action of those molecules affect p53 level or activation. Western blot analysis will be
use to identify the levels and phosphorylation status of proteins. *In situ* immunoblot combined with fluorescence microscopy will be used to examine the localization of molecules. Luciferase assay may be used for telling the transcriptional activity of p53. For western blot, 50 μg of isolated total protein will be loaded to 7.5% or 12% Tris-glycine polyacrylamide gels (Bio-Rad; Hercules, CA) and subjected to electrophoresis. Specific antibodies will be used for detection of the proteins or phosphorylated proteins of interest. For fluorescence microscopy, RKO cells with or without treatment will be fixed with ice-cold 100% methanol (VWR international; West Chester, PA) at -20°C for 20 min. After washing twice with PBS, cells will be incubated with 4% paraformaldehyde (Fisher Scientific; Pittsburgh, PA) in PBS to fix cell surface antigens for 30 min at room temperature. Then, cells will be blocked with 5% normal donkey serum in PBS with 0.2% Triton X-100 (Sigma-Aldrich; St. Louis, MO) and 1% DMSO (Sigma-Aldrich; St. Louis, MO) at room temperature for 50 min. When the blocking solution is removed, cells will be incubated with the primary antibody diluted at 1:200 in 7.5% donkey serum/PBS overnight at 4°C. After the overnight incubation, cells will be incubated with the secondary antibody (Fluorescein (FITC)-conjugated affinipure F(ad’)2 fragment Donkey IgG) (Jackson ImmunoResearch Laboratories; West Grove, PA) at a 1:100 dilution in 7.5% donkey serum/PBS at 37°C for 30 min. The cells will then be washed three times with PBS and mounted with a drop of Vectashied with DAPI (Vector Laboratories Inc.; Burlingame, CA). Finally, the cells were visualized and photographed using an immunofluorescence microscope (ECLIPSE E600; Nikon, Japan) with the ocular lens being 10X and the objective lens being 40X. The images will be merged using
the Advanced SPOT software from Diagnostic Instruments (Sterling Heights, MI). For luciferase assay, RKO cells will be cultured till about 60% confluency. Then, using lipofectamine 2000 (Invitrogen; Carlsbad, CA), cells will be transfected with pp53-Luc (Panomics Inc; Redwood, CA), a firefly luciferase reporter plasmid constructed with a cis-acting enhancer element sequence that has a p53 binding site. pRL-TK vector (Promega; Madison, WI) will be co-transfected as an internal control reporter. Six hours after transfection, the transfection medium will be removed and the cells will be cultured in normal culture medium for 8 hours to recover from the transfection stress. Then, cells will be treated according to the designs for 36 hours, followed by an assay. To do the assay, cells will be incubated with 1X passive lysis buffer (Promega; Madison, WI) for 20 min to lyse the cells. After that, cell lysates will be transferred to centrifuge tubes and will be centrifuged at 14,000 rpm for 30 sec. 20 μl of supernatant for each sample will be transferred to a luminometer tube. The firefly luciferase activities will then be determined by the luminometer (Lumat LB9507, BERTHOLD Technologies; Oak Ridge, TN) with the luciferase substrate (Promega; Madison, WI) and the stop solution (Promega; Madison, WI).

Specific aim 4: Tumor cells have frequent mutations and p53 in many tumors has been found to be mutated. So it would be good for an anti-tumor reagent to have multiple therapeutic targets. And it would be desirable if an anti-tumor reagent may induce tumor cell apoptosis through both p53-dependent and p53-independent mechanisms. Our studies have established that α-PGG could induce apoptosis by activating p53. Meanwhile, we noticed that α-PGG could induce apoptosis in tumor cells without wild-type p53. For
instance, p53 is absent in human lung cancer cell line H1299. \( \alpha \)-PGG has been found to induce apoptosis in H1299 cells although \( \alpha \)-PGG is not as potent for H1299 cells as for RKO cells. This suggests that \( \alpha \)-PGG may induce tumor cell apoptosis through a p53-independent mechanism. We are therefore interested in finding out how \( \alpha \)-PGG manages to induce a p53-independent apoptosis. Through the studies stated in specific aim 3, we may find some molecules that are responsive to \( \alpha \)-PGG treatment but are not contributing to p53 elevation or activation. Those molecules may be involved in a p53-independent apoptosis induction by \( \alpha \)-PGG. Using western blot analysis and/or immunofluorescence microscopy as described in specific aim 3, the responses of those molecules to \( \alpha \)-PGG in H1299 cells will be verified. Besides, glucose is the major building block and energy source of tumor cells. Tumor cells rely on glucose supply much more than normal cells. Cutting glucose supply to tumors has long been proposed as a potential anti-tumor therapeutics (Du et al., 2008). We previously found that \( \alpha \)-PGG is able to greatly reduce glucose uptake in many tumor cell lines including RKO and H1299. Whether \( \alpha \)-PGG induces cell apoptosis through inhibition of glucose uptake in addition to abnormally activating the insulin receptor signaling will be studied. Using western blot analysis, the expression of GLUT1, the major glucose transporter responsible for basal glucose uptake, in RKO and H1299 cells will first be confirmed. Then pure GLUT1 proteins will be purchased. Biacore System will be applied to measure the binding affinity and kinetics of \( \alpha \)-PGG with GLUT1. If a relatively high binding affinity is observed between \( \alpha \)-PGG and GLUT1, it is very likely that \( \alpha \)-PGG interacts with GLUT1 in a cell system to inhibit glucose transport. In order to understand whether GLUT1 expression difference between
tumor and normal cells is one of the reasons why $\alpha$-PGG selectively induces tumor cell apoptosis, the extent to which $\alpha$-PGG reduces glucose transport in tumor cells (RKO) and normal cells (FHC) will be compared using glucose transport assay. To perform glucose transport assay, tumor or normal cells of 70-80% confluency will be cultured in serum-free DMEM for 2 hours. The cells will be washed 3 times with KRP buffer and will be incubated in KRP buffer for 30 min. Then cells will be treated with either mock or 25 $\mu$M of $\alpha$-PGG for 10-15 min before $^3$H-labeled radioactive glucose (Amersham; Piscataway, NJ) is added to the system for glucose transport to occur. 15 min after addition of radioactive glucose, supernatant will be removed and cells will be washed with ice-cold PBS. Cells will then be lysed for intracellular radioactive glucose analysis by Beckman Coulter LS 6400 Multi-Purpose Scintillation Counter (Beckman Coulter, Inc.; Fullerton, CA). If the potency of $\alpha$-PGG in inhibiting glucose transport in tumor cells (RKO) is different from that in normal cells (FHC), the levels of GLUT1 expression in the two types of cells will further be compared. The total proteins will be isolated from RKO and FHC cells. Using western blot analysis as previously described, the expression levels of GLUT1 in those two cells will be determined using the anti-GLUT1 specific primary antibody (Santa Cruz; Santa Cruz, CA) and the secondary antibody.

Specific aim 5: Each year, more than a quarter million people die of pancreatic cancer in the world (Sharp et al., 2009). In North America and Europe, pancreatic cancer is the top five cause of cancer death (Sharp et al., 2009). It has been found that insulin receptor and its downstream signaling molecules are involved in the progression of pancreatic cancer (Asano et al., 2005; Bergmann et al., 1996; Kolb et al., 2007;
Kornmann et al., 1998; Neid et al., 2004). It is reasonable to speculate that α-PGG might induce apoptosis in pancreatic cancer cells through the insulin receptor signaling as it does in colon cancer RKO cells. Our preliminary data showed cell killing activity of α-PGG in human pancreatic cell lines PANC-1 and BXPC-3. Our data further indicated that p53 transcriptional activity was increased by α-PGG treatment in both of those two cell lines, suggesting an involvement of p53 in α-PGG-induced cell death. Following the blueprint of RKO cell study, whether the cell death induced by α-PGG in PANC-1 and BXPC-3 is apoptosis will first be determined. MTT assay, apoptosis assay, FACS analysis and caspase 3 activity assay will be applied for the study. Western blot analysis will be used to determine the level of p53 to see whether p53 is elevated by α-PGG treatment in PANC-1 and BXPC-3. Using chemical inhibitors and the siRNAs specific for the insulin receptor signaling molecules, the relationship between the insulin receptor signaling and p53 in pancreatic cancer cell lines will be researched. All methods in specific aim 5 are the same as what have been used for the RKO cell study except for the cell lines.
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