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Regulation of Adipocyte Differentiation and Metabolism: Rab5-Guanine Nucleotide Exchange Factors and Methylglyoxal

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REGULATION OF ADIPOCYTE DIFFERENTIATION AND METABOLISM: RAB5-GUANINE NUCLEOTIDE EXCHANGE FACTORS AND METHYLGLYOXAL

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Praew Chantarasinlapin

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To: Dean Tomás R. Guilarte
R.Stempel College of Public Health and Social Work

This dissertation, written by Praew Chantarasinlapin, and entitled Regulation of Adipocyte Differentiation and Metabolism: Rab5-Guanine Nucleotide Exchange Factors and Methylglyoxal, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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DEDICATION

I dedicate this dissertation to my family, especially my parents, Krisada and Sumonmarn, and my sister Manunya. I am forever grateful for your unconditional love, wholehearted support and unwavering belief in me. I could not imagine myself go through this long academic journey without you. You are my strength and power to keep going and never give up. I am blessed to have you by my side.

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ABSTRACT OF THE DISSERTATION

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Internalization and trafficking of ligand-receptor complex rely on a particular set of proteins, e.g. small GTPase protein Rab5 and its activators called guanine nucleotide exchange factors. Rab5-activating protein 6 (RAP6), a Vps9-containing protein, may participate in Rab5-mediated insulin signaling and receptor trafficking. A dicarbonyl compound methylglyoxal was found to alter insulin signaling in preadipocytes. This dissertation aimed to investigate the association of RAP6 activity on 3T3-L1 preadipocyte differentiation and those driven by methylglyoxal. Overexpression of RAP6 inhibited preadipocyte differentiation, Ser473-phosphorylation of Akt1, and expression of adipogenic marker PPARγ, but not C/EBPα. Methylglyoxal (10 μM) increased preadipocyte differentiation, proliferation and expression of PPARγ, C/EBPα and p-Akt1-Ser473, but appeared to be neutralized by RAP6 overexpression. The findings suggest that RAP6 may be a key modulator in regulating the stimulatory effect of methylglyoxal on preadipocyte differentiation.

The associations of predominant methylglyoxal-derived adduct, methylglyoxal hydroimidazolone 1 (MGH1), with selected risk factors of chronic diseases in Black participants with and without type 2 diabetes (n=234 controls and n=254 cases) were also investigated. Only in individuals with diabetes, MGH1 levels were positively associated with fasting plasma glucose (B=0.240, p=0.037), homocysteine (B=0.355, p=0.014) and triglyceride (B=0.190, p=0.049). Being African Americans with type 2 diabetes was associated with lower MGH1 levels as compared to being Haitian American with diabetes (B=-0.334, p=0.016). The findings suggest that methylglyoxal may be linked to hyperglycemia and metabolic changes in type 2 diabetes, and may differently impact the development of diabetes across Black subgroups.

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ABBREVIATIONS AND ACRONYMS

Advanced glycation end products	AGEs
Alternate healthy eating index	AHEI
Adenosine-5’-triphosphate	ATP
Coefficient	B
Body mass index	BMI
Cyclic adenosine monophosphate	cAMP
Complementary DNAs	cDNAs
CCAAT enhancer-binding protein	C/EBP
Confidence interval	CI
Copper ion	Cu^{2+}
Dihydroxyacetone phosphate	DHAP
Enzyme-linked immunosorbent assay	ELISA
Extracellular signal–regulated kinase	ERK
Forkhead box protein O1	FOXO1
Fasting plasma glucose	FPG
glyceraldehyde 3-phosphate	G3P
GTPase-activating proteins	GAPs
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH
Guanine-nucleotide dissociation inhibitor	GDI
Guanosine diphosphate	GDP
Guanine nucleotide exchange factors	GEFs
Green fluorescent protein	GFP

Glucose transporter	GLUT
Glycogen synthase kinase-3	GSK-3
Guanosine triphosphate	GTP
Homocysteine	Hcy
High-density lipoprotein cholesterol	HDLC
Healthy eating index 2005	HEI-2005
High sensitivity C-reactive protein	HsCRP
Insulin-like growth factor I	IGF-I
Insulin-like growth factor I receptor	IGF-IR
Interquartile range	IQR
Insulin receptor	IR
Insulin receptor substrate	IRS
Low-density lipoprotein cholesterol	LDLC
Natural logarithm	ln
Mitotic clonal expansion	MCE
3-Isobutyl-methylxanthine, dexamethasone, and insulin	MDI
Mitogen-activated protein kinase kinase	MEK
Methylglyoxal	MG
Methylglyoxal-bovine serum albumin	MG-BSA
N_{δ}-(5-Hydro-5-methyl-4-imidazolon-2-yl)-L-ornithine	MGH1
Messenger RNA	mRNA
3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide	MTT
Oil red O	ORO

Pancreatic duodenal homeobox-1	PDX1
Phosphodiesterase 3b	PDE3b
Phosphoinositide-dependent kinase 1	PDK1
Proline-rich domain	PH
Phosphoinositide 3-kinases	PI3Ks
Phosphatidylinositol 3-phosphate	PI3P
Protein kinase C	PKC
Peroxisome proliferator-activated receptor γ	PPARγ
Receptor for advanced glycation end products	RAGE
Rab5-activating protein 6	RAP6
Rat sarcoma	Ras
Ras GTPase-activating protein binding domain	Ras-GAP
Ras interfering 1	Rin1
Reactive oxygen species	ROS
Receptor tyrosine kinases	RTKs
Standard deviation	SD
Sodium dodecyl sulfate polyacrylamide gel electrophoresis	SDS-PAGE
Standard error	SE
Small interfering RNA	siRNA
Solid fats, alcohol, and added sugars	SoFAAS
Sarcoma	Src
Sterol regulatory element–binding protein 1c	SREBP1c
Semicarbazidesensitive amine oxidase	SSAO

Total cholesterol	TC
Triglyceride	TG
Vacuolar protein sorting-associated protein 9	Vps9
Wild type	WT

CHAPTER I

INTRODUCTION

Cell signaling is a complex communicating network which governs basic cellular activities. Multiple signaling pathways in this biological system are regulated by extracellular ligands or stimuli within the cells (Jordan, Landau, & Iyengar, 2000). Receptor tyrosine kinases (RTKs) have been found to play an essential role in proper cell growth, cell development, cell metabolism, tissue repair and homeostasis (Li & Hristova, 2006; Volinsky & Kholodenko, 2013) as well as oncogenesis (Pawson, 2002). Upon binding, a certain ligand activates its responsive receptor(s) which is associated with the plasma membrane. This increases receptor kinase activity and enables the receptor to eventually activate different intracellular downstream effectors, leading to distinct cell signaling events. Ligands induce signaling events not only at the plasma membrane, but they are also internalized together with their receptor as a complex, and enhance signal transduction intracellularly (Ceresa, Kao, Santeler, & Pessin, 1998; Romanelli et al., 2007). The internalization and trafficking of ligand-receptor complex rely on a particular set of proteins such as a small GTPase protein Rab5 and its activators (Barbieri, 2004; Hunker, Kruk, et al., 2006). Error of cell signaling likely contributes to disease development e.g. diabetes (Fröjdö, Vidal, & Pirola, 2009), obesity (Gustafson, Hedjazifar, Gogg, Hammarstedt, & Smith, 2015) and cancer (Inoue, Goi, Hirono, Katayama, & Yamaguchi, 2011). The cell signaling can be altered by numerous stimuli such as hormones, growth factors, and a dicarbonyl compound methyglyoxal. Methylglyoxal can be ubiquitously produced in the cells (Phillips & Thornalley, 1993) as well as obtained from foods (Marceau & Yaylayan, 2009; Poulsen et al., 2013). Previous

studies indicated that methylglyoxal blunted insulin signaling (Afridi et al., 2016; Jia & Wu, 2007; Nigro et al., 2014; Riboulet-Chavey, 2006), caused beta-cell dysfunction (Chang et al., 2016; Fiory et al., 2011; Gao, Liao, et al., 2016), interfered with adipocyte growth and differentiation (Jia et al., 2012; Yang et al., 2013) and aggravated vascular complications (Brouwers et al., 2010; Hadas, Randriamboavonjy, Elgheznawy, Mann, & Fleming, 2013). Its glycation ability also links methylglyoxal to formation of advanced glycation end products (AGEs) which is commonly associated with chronic illnesses.

Basic information about signaling pathways and receptor-mediated endocytosis, particularly in relation to insulin-responsive preadipocytes are presented here. A growing evidence suggests that methylglyoxal possibly causes perturbation of cellular signaling and development of chronic diseases. Therefore, metabolism and harmful effects of methylglyoxal are also reviewed in the following section.

The Insulin Receptors

Insulin receptor family comprises insulin receptor (IR), insulin-like growth factor I receptor (IGF-IR), insulin-like growth factor II receptor, and insulin receptor-related receptor (Werner, Weinstein, & Bentov, 2008). These transmembrane receptors belong to a receptor tyrosine kinase (RTK) superfamily, which is activated through phosphorylation by specific ligands. Ligand activation causes conformational change and following autophosphorylation, which enhances receptor kinase activity (Werner et al., 2008). This renders the receptor to phosphorylate multiple intracellular kinase proteins that involve in ligand-dependent signal transduction (Werner et al., 2008). Particular interest of this project is IR and IGF-IR.

Unlike other classes of RTKs, IR and IGF-IR exist as heterotetramers of two α subunits and two β subunits linked by covalent disulfide bonds (Werner et al., 2008). Heterodimer between IR and IGF-IR, which appears one half of each, can be found in cells expressing both receptors (Federici et al., 1997; Soos, Field, & Siddle, 1993). The α subunits of IR and IGF-IR reside extracellularly and contain cysteine-rich domain which is required for ligand binding (Werner et al., 2008). The β subunits feature several domains: hydrophobic transmembrane region; intracellular juxtamembrane domain which is speculated to participate in receptor internalization (Werner et al., 2008) and docking of intracellular effectors; and tyrosine kinase domain that plays a role in autophosphorylation and downstream cascade activation (De Meyts, 1994).

Even though there is high resemblance in gene structures encoding IR and IGF-IR (Werner et al., 2008), existing evidences suggest different biological functions of the receptors. The difference is possibly due to several factors such as their affinity for a certain ligand, their distribution among cell types, and their signaling events. Insulin receptor and IGF-IR appeared to have higher affinity for their cognate ligand (Werner et al., 2008). It was reported that IGF-I had at least 100-fold lower affinity to IR than insulin (Werner et al., 2008). Expression of IR and IGF-IR was also found to be different among cell types. For example, IR expressed in liver and adipocytes, while IGF-IR was nearly absent in liver and low in adipocytes (Werner et al., 2008). A previous study demonstrated that proportion of total IR fraction assembled as hybrid receptors was low in adipocytes as compared with placenta and hepatoma (Federici et al., 1997). Hybrid receptors between IR and IGF-IR had high affinity for IGF-I, but not insulin, under physiological condition (Federici et al., 1997). Given that, high distribution of IR, but low

distribution of hybrid receptors in adipocytes may possibly favor insulin-dependent pathways related to growth and differentiation rather than those that are IGF-I-dependent. Through their preferential intracellular effectors, insulin is speculated to mainly participate in cell metabolism, e.g. glucose transport through Akt activity (Manna & Jain, 2013), while IGF-I predominantly involves cell growth and survival, e.g. cell proliferation through activity of extracellular signal–regulated kinase 1/2 (ERK1/2) (Bost, Aouadi, Caron, & Binétruy, 2005). Later, additional data indicate that these downstream effectors are not exclusively activated by specific receptors. For example, IGF-IR involved in sustained activation of Akt in glial progenitor cells (Romanelli et al., 2007), whereas insulin appeared to strongly activate ERK1/2 in cultured adipocytes (Kayali, Austin, & Webster, 2000). These observations show that multiple ligands work together by participating in the complex signaling network that, in turn, precisely causes distinct cellular events.

Insulin Action and Signaling Pathways in Adipocytes

Adipocytes play an important role in energy storage in a form of triacylglycerols when excess energy is consumed in the diet. Triacylglycerols are used as fuel during post-absorptive state (Ali, Hochfeld, Myburgh, & Pepper, 2013). Adipogenesis is the differentiation of premature preadipocyte to form fat-laden adipose tissues (Attie & Scherer, 2009), which is tightly regulated by ligands such as insulin and IGF-I. An increase in adipogenesis possibly contributes to obesity which is characterized by an excessive fat accumulation in the body (Attie & Scherer, 2009).

Established preadipocyte cell lines have been used to study adipocyte signaling pathways (Boney, Smith, & Gruppuso, 1998; Cao, Umek, & McKnight, 1991; Tang &

Lane, 2012; Tang, Otto, & Lane, 2003). Cultured preadipocytes, such as 3T3-L1 cells, grow to growth arrest e.g. at G_1 phase in the cell cycle (Tang & Lane, 2012). Differentiation is induced by stimulant cocktail, including high concentration of insulin, dexamethasone (a synthetic glucocorticoid agonist), and 3-isobutyl-1-methylxanthine (a cAMP phosphodiesterase inhibitor) (Cornelius, MacDougald, & Lane, 1994). The stimulants activate signaling pathways dependent on IGF-I, glucocorticoid, and cAMP level, respectively (Tang & Lane, 2012), initiating differentiation. Several hours after induction, preadipocytes re-enter the cell cycle and go through about two rounds of mitosis in which the process is called mitotic clonal expansion (MCE) (Tang & Lane, 2012). The cells then lose their fibroblast-like appearance, assume rounded shape, and become adipocytes by accumulating cytoplasmic triglycerides (Cornelius et al., 1994). Preadipocyte cell lines successfully commit to adipocytes, therefore; they have been extensively utilized to examine adipocyte signaling events, such as those caused by insulin.

Insulin plays a key role in adipogenesis by promoting glucose uptake (Charron, Brosius, Alper, & Lodish, 1989; Watson, Kanzaki, & Pessin, 2004) and stimulating downstream cascades that initiate adipocyte differentiation (Siersbaek & Mandrup, 2011) (Figure 1). Receptor tyrosine kinases, e.g. IR and IGF-IR, have been widely accepted as key players for insulin signal transduction. Upon insulin activation, the receptors serve as a docking platform for multiple signaling cascades. One of the well-characterized pathways include a cascade constituting insulin receptor substrate 1 (IRS1), phosphoinositide 3-kinases (PI3Ks) and Akt, which acts as a central pathway harnessing adipogenesis and cell survival (Stenkula et al., 2004). Once activated, PI3K produces

phosphatidylinositol (3,4,5)-trisphosphate in the plasma membrane creating anchoring sites for downstream effectors, e.g. Akt (McCurdy & Klemm, 2013) and storage vesicles of glucose transporter 4 (GLUT4) (Bai et al., 2007; Calera et al., 1998). Along with other proteins, activated Akt subsequently induces GLUT4 translocation to the plasma membrane where glucose uptake takes place (Cong et al., 1997; McCurdy & Klemm, 2013). Simultaneously, insulin-dependent PI3K activity suppresses lipolysis through Akt activation and following phosphorylation of phosphodiesterase 3b (PDE3b) (Choi et al., 2006).

Even a weaker affinity than IGF-I, insulin can stimulate IGF-IR whose downstream pathway involves in the early phase of adipogenesis (Boney et al., 1998; Smith, Wise, Berkowitz, Wan, & Rubin, 1988). Major IGF-IR-mediated signaling cascades consist of ERK1/2 and PI3K pathways (Siersbaek & Mandrup, 2011). Kinases ERK1/2 play a role in the early phase of adipogenesis by activating cell cycle regulatory proteins (Yohannes & Yohannes, 2009) and inducing MCE (Tang et al., 2003). This leads to expression of CCAAT enhancer-binding protein β (C/EBPβ) which is required for initiating MCE (Tang et al., 2005; Tang, Otto, & Lane, 2003). Then, C/EBPβ and δ cause co-expression of transcription factors, i.e. C/EBPα and peroxisome proliferator-activated receptor γ (PPARγ), that subsequently induces adipogenic activity during the late phase of adipogenesis (Lefterova & Lazar, 2009; Siersbaek & Mandrup, 2011). Insulin signaling also regulates other pro-adipogenesis transcription factors such as sterol regulatory element–binding protein 1c (SREBP1c), which involves in lipogenesis (Petersen et al., 2008), and foxhead box protein O1 (FOXO1), which plays a role in early stage of terminal adipocyte differentiation (Munekata and Sakamoto, 2009). Therefore, a

series of insulin signaling pathways is essential for proper and complete proliferation and differentiation of adipose tissues.

Receptor-mediated Endocytosis and Receptor Trafficking

Endocytosis is a process that molecules are internalized from the plasma membrane to intracellular compartments. Available data indicate an association of endocytosis with cell signaling. Apart from transmitting signal at the plasma membrane, receptors and their ligands can be endocytosed and amplified the signal intracellularly. Clathrin-mediated endocytosis is a well-characterized mechanism which plays an important role in internalization of multiple receptors, e.g. IR (Ceresa et al., 1998), IGF-IR (Romanelli et al., 2007) and low-density lipoprotein receptor (Goldstein & Brown, 2009) as well as nutrients and other molecules (Le Roy & Wrana, 2005). When ligands bind to receptors, clathrin is recruited to the plasma membrane at which the cluster of receptors is located to form clathrin-coated pits (Le Roy & Wrana, 2005). The coated pits then bud and pinch off from the cell membrane to form clathrin-coated vesicles. Subsequently, the vesicles are uncoated and fuse with early endosomes (Le Roy & Wrana, 2005). The early endosomes containing ligand-receptor complex are further directed to specific intracellular compartments for either receptor degradation and signal termination, or receptor recycling to the plasma membrane and maintaining signal transduction (Irannejad, Tsvetanova, Lobingier, & von Zastrow, 2015). A small GTPase Rab5 has been found to facilitate receptor-mediated clathrin-dependent endocytosis (Kajiho et al., 2003; Nielsen, Severin, Backer, Hyman, & Zerial, 1999; Su, Lodhi, Saltiel, & Stahl, 2006) as well as early endosome biogenesis, sorting and fusion (Bucci et al.,

1992; Rybin et al., 1996; Zeigerer et al., 2012). Rab5 is considered a key component that links clathrin-mediated endocytosis to ligand-activated cell signaling.

Rab5 is a member of Ras GTPase superfamily that mainly participates in membrane trafficking. Rab5 switches between an inactive form bound to guanosine diphosphate (GDP), and an active form bound to guanosine triphosphate (GTP; Figure 2) (Mizuno-Yamasaki, Rivera-Molina, & Novick, 2012). The interchange between the two Rab5 conformations is very slow by itself. The switching process is thus accelerated by two sets of proteins: guanine nucleotide exchange factors (GEFs) activate Rab5 by promoting the exchange of GDP to GTP; and GTPase-activating proteins (GAPs) deactivate Rab5 by stimulating hydrolysis of GTP to GDP (Mizuno-Yamasaki et al., 2012). Typically, Rab5 is localized at the plasma membrane and early endosomal membrane (Chavrier, Parton, Hauri, Simons, & Zerial, 1990). In cytosol, GDP-bound Rab5 is formed as a complex to Rab guanine-nucleotide dissociation inhibitor (GDI) which is speculated to translocate Rab5 to or from the functional target sites (Ullrich, Horiuchi, Bucci, & Zerial, 1994).

Rab5-specific GEFs contain a highly-conserved Vps9 domain which is required for Rab5 activation (Burd, Mustol, Schu, & Emr, 1996; Delprato & Lambright, 2007; Tsukamoto et al., 2015). Among Rab5-GEFs, Ras interference 1 (Rin1) (Balaji et al., 2012; Kavitha Balaji & Colicelli, 2013; Ding, Wang, & Chen, 2009; Galvis, Balmaceda, et al., 2009; Galvis, Giambini, Villasana, & Barbieri, 2009; Hunker, Giambini, et al., 2006; Tall, Barbieri, Stahl, & Horazdovsky, 2001), and Rabex-5 (Aikawa, 2012; Delprato, Merithew, & Lambright, 2004; Kalesnikoff et al., 2007; Stenmark, Vitale, Ullrich, & Zerial, 1995; Zhang et al., 2014) have been widely studied to examine their

involvement in Rab5 activity. However, available data remains scarce for Rab5-activating protein 6 (RAP6), also known as GAPex-5 (Lodhi et al., 2007) and hRME-6 (Sato et al., 2005), which is newly discovered to participate in Rab5 activation (Hunker, Galvis, et al., 2006).

Rab5 was reported to mediate clathrin-dependent endocytosis in response to insulin (Cormont, Van Obberghen, Zerial, & Le Marchand-Brustel, 1996), suggesting the link of Rab5 activity to insulin-stimulated signaling events. Similar mechanism also applied to glucose uptake through GLUT4 function (Watson et al., 2004). These evidences indicate that Rab5 and Rab5-GEFs, such as RAP6, possibly take part in modulating insulin-dependent homeostasis of energy storage in adipocytes. Supportively, overexpression of wild-type RAP6 markedly inhibited GLUT4 translocation, whereas RAP6 mutant lacking Vps9 domain caused less inhibition (Lodhi et al., 2007). These findings suggest that there may be an interaction between Vps9 and other domains, e.g. proline-rich domain and Ras-GAP domain, in RAP6 to fully regulate Rab5-mediated glucose uptake and adipogenesis.

In addition, decline in Rab5 and RAP6 expression may perturb insulin-dependent signaling cascades that involve in endogenous synthesis of triacylglycerol from glucose, and delivery of glucose and triacylglycerol between cellular compartments. In response to insulin activation, RAP6 was required for Rab5-mediated formation of phosphatidylinositol 3-phosphate (PI3P) in the plasma membrane of 3T3-L1 preadipocytes, thereby regulating GLUT4 translocation (Lodhi et al., 2008) (Figure 3). In the same study, knockdown of RAP6 inhibited GLUT4 translocation and insulin-stimulated glucose uptake (Lodhi et al., 2008), suggesting an association of RAP6 with

insulin signaling. In insulin-responsive NIH3T3/hIR cells, knockdown of all Rab5 isoforms (Rab5a, Rab5b and Rab5c) or RAP6 markedly reduced insulin-dependent activation of Akt (Su et al., 2006). This was speculated to be due to interference with interaction between IRS1 and PI3K-p85 subunit (Su et al., 2006). Interestingly, expression of Rab5 isoforms was significantly suppressed in obese Zucker rats as compared to lean rats (Su et al., 2006), suggesting the involvement of Rab5 in obesity. Collectively, Rab5 and RAP6 activity possibly have a potential role in insulin-dependent signaling and energy metabolism in adipocytes.

Methylglyoxal: Dicarbonyl Compound

Methylglyoxal is a dicarbonyl compound that can be produced endogenously from the breakdown of macronutrients (Phillips & Thornalley, 1993). Methylglyoxal is also found in foods, such as coffee, milk, bakery products (Poulsen et al., 2013) and honey (Marceau & Yaylayan, 2009), as well as biological samples, e.g. blood, urine, (Nemet, Turk, Duvnjak, Car, & Varga-Defterdarović, 2005; Nemet, Varga-Defterdarović, & Turk, 2006; Turk, Vrdoljak, Misur, Trescec, & Benko, 2009), and tissues (Ahmed, Brinkmann Frye, Degenhardt, Thorpe, & Baynes, 1997; Ahmed et al., 2003). As a highly reactive glucose derivative, methylglyoxal can bind to free amino acid residues and subsequently form advanced glycation end products (AGEs), suggesting its association with chronic diseases. It is hypothesized that intrinsic methylglyoxal transports to plasma by passive diffusion through interstitial fluid in its unhydrated form (Rabbani & Thornalley, 2015). Methylglyoxal concentrations have been estimated in human plasma at 100-120 nM, while cellular concentrations range from 1-5 μM (Thornalley, 2008). Greater than 95% of methylglyoxal is reversibly bound to plasma proteins with a half-life

of about 10 minutes, whereas irreversible binding is approximately 3.6 hours (Rabbani & Thornalley, 2015). Amino group of arginine and lysine is a target site of methylglyoxal-mediated glycation (Lo, Westwood, McLellan, Selwood, & Thornalley, 1994). Methylglyoxal reacts with arginine (Klöpfer, Spanneberg, & Glomb, 2011; Oya et al., 1999) and lysine forming glycosylamine adducts (Lo et al., 1994). Chemical structure of methylglyoxal, amino acid residues, and derived adducts are illustrated in Figure 4. Among AGEs in blood and tissues, a quantitatively prominent adduct is N_{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-L-ornithine (MGH1), which results from the reaction between methylglyoxal and arginine (Thornalley, 2005). Less stable isomers of methylglyoxal-derived hydroimidazolones consist of 2-amino-5-(2-amino-5-hydro-5-methyl-4-imidazolon-1-yl)pentanoic acid (MGH2) (Ahmed et al., 2003) and 2-amino-5-(2-amino-4-hydro-4-methyl-5-imidazolon-1-yl)pentanoic acid (MGH3) (Ahmed, Argirov, Minhas, Cordeiro, & Thornalley, 2002; Klöpfer et al., 2011). It is proposed that MGH3 is an intermediate in methylglyoxal-mediated glycation mechanisms, leading to more stable compounds N^7-(1-carboxyethyl)-arginine (CEA) (Alt & Schieberle, 2005) and MGH1 (Klöpfer et al., 2011).

Other arginine-derived methylglyoxal crosslinks are also identified *in vitro*, including N_{δ}-(5-hydroxy-4,6-dimethylpyrimidine-2-yl)-L-ornithine (argpyrimidine) and N_{δ}-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyrimidine-2-yl)-L-ornithine (tetrahydropyrimidine; THP) (Ahmed et al., 2002). The adducts derived from the interaction between methylglyoxal and lysine residues include N_{ε}-(1-carboxyethyl)-lysine (CEL) (Ahmed et al., 1997) and methylglyoxal-lysine dimer 1,3-di(N_{ε}-lysino)-4-methyl-imidazolium (MOLD) (Brinkmann, Wells-Knecht, Thorpe, & Baynes, 1995).

With arginine and lysine residues, methylglyoxal also forms 2-ammonio-6-({2-{4-ammonio-5-oxido-5-oxopentyl)amino]-4-methyl-4,5-dihydro-1*H*-imidazol-5-ylidene}amino)hexanoate (MODIC) (Lederer & Klaiber, 1999).

Methylglyoxal Metabolism

Production

Glycolysis is postulated to be a central pathway of methylglyoxal production from carbohydrates (Phillips & Thornalley, 1993; Figure 5). A majority of methylglyoxal is converted from glycolytic triosephosphates, including glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) (Kalapos, 1999; Phillips & Thornalley, 1993). Methylglyoxal synthase is mainly involved in enzymatic conversion of methylglyoxal from G3P and DHAP (Kalapos, 1999). Methylglyoxal can also be non-enzymatically generated by phosphate removal of triosephosphates (Phillips & Thornalley, 1993), glucose autoxidation, and glycation of amino group-containing biomolecules (Rabbani & Thornalley, 2012; Semchyshyn & Lushchak, 2012). Glycation is a complex series of sequential reactions between reactive carbonyl groups of reducing sugars, such as glucose, and nucleophilic amino groups of proteins, lipids or nucleic acids (Semchyshyn & Lushchak, 2012). An early step of glycation is where glucose reacts with amino groups, reversibly forming an unstable Schiff's base (Rabbani & Thornalley, 2012). Schiff's base can be subjected to a reversible Amadori rearrangement resulting in more stable Amadori products or ketosamines (Semchyshyn & Lushchak, 2012). Amadori products can further undergo various chemical modifications, that subsequently lead to formation of carbonyl compounds, including methylglyoxal (Rabbani & Thornalley, 2012). In parallel to the sequential reactions, methylglyoxal can also be generated from

fragmentation of glucose and Schiff's base (Thornalley, Langborg, & Minhas, 1999), which is likely accelerated by oxidation reaction or reactive oxygen species (ROS) (Sakai, Oimomi, & Kasuga, 2002; Semchyshyn, 2013). In addition, fructose is another carbohydrate substrate for methylglyoxal formation (Thornalley, 1996). Fructose, such as from food intake or endogenously generated from glucose and sorbitol via polyol pathway, can be converted to G3P and DHAP. Therefore, high-fructose diet (Jia & Wu, 2007; Masterjohn et al., 2013) and fructose generating system using inulin and inulinase (Wei, Wang, Moran, Estrada, & Pagliassotti, 2013) were used in animal models to induce methylglyoxal production. Like glucose, fructose can also lead to methylglyoxal formation through non-enzymatic glycation.

Less significant amount of methylglyoxal is possibly produced from malondialdehyde, ketone bodies and threonine. Malondialdehyde is one of reactive aldehyde products from lipid peroxidation. By using rat liver fractions, a glycolytic enzyme phosphoglucose isomerase was hypothesized to convert malondialdehyde to methylglyoxal (Agadjanyan, Dugin, & Dmitriev, 2006). Ketone bodies are synthesized from fatty acids when fats become main fuel, such as during nutritional deprivation. Ketone bodies include acetoacetate and its byproducts acetone and acetol, which can be further catalyzed into methylglyoxal (Beisswenger, Howell, Nelson, Mauer, & Szwergold, 2003; Nemet et al., 2006). A few human studies suggest that ketone bodies become significant precursors of methylglyoxal under certain conditions, such as low-carbohydrate diet and diabetes (Beisswenger et al., 2003; Turk, Nemet, Varga-Defteardarović, & Car, 2006).

In addition, threonine is speculated to be a substrate of methylglyoxal (Dhar, Desai, Kazachmov, Yu, & Wu, 2008). Threonine is typically catalyzed into glycine and acetyl-CoA. When the ratio of acetyl-CoA to CoA increases, which commonly occurs during starvation and diabetes, threonine can be non-enzymatically converted into aminoacetone instead (Nemet et al., 2006). Aminoacetone is subsequently catalyzed into methylglyoxal by copper/iron-dependent semicarbazidesensitive amine oxidase (SSAO) (Lyles & Chalmers, 1992, 1995) or ferricytochrome c (Sartori et al., 2013). Supportively, the increase in SSAO activity (Boomsma et al., 1999) and Cu^{2+} levels (Abou-Seif & Youssef, 2004) were reported in participants with type 1 and type 2 diabetes. The data suggest that non-carbohydrate substrates may become other significant sources for methylglyoxal formation, under abnormal metabolic conditions such as diabetes.

Degradation

Methylglyoxal is mainly degraded by glyoxalase system (Thornalley, 2003). Glyoxalase system is a ubiquitous set of metalloenzymes which consists of glyoxalase-1, glyoxalase-2, and cofactors, including reduced glutathione and reduced zinc ion (Thornalley, 2003; Vander Jagt & Hunsaker, 2003). The binding between methylglyoxal and glutathione forms a non-toxic intermediate hemithioacetal that is subsequently detoxified to S-lactoylglutathione by glyoxalase-1 (Thornalley, 2003). Glyoxalase-2 converts S-lactoylglutathione to D-lactate, simultaneously recycling back the reduced glutathione (Thornalley, 2003). Apart from D-lactate, the conversion of methylglyoxal eventually to pyruvate and other metabolites was speculated to be very small comparing to the total metabolites of methylglyoxal (Best & Thornalley, 1999). Lesser amount of methylglyoxal can be degraded by other enzymes such as aldose reductase, betaine

aldehyde dehydrogenase, and 2-oxoaldehyde dehydrogenases (Rabbani & Thornalley, 2015; Vander Jagt & Hunsaker, 2003).

Methylglyoxal and Pathological Consequences

Insulin Resistance

Insulin resistance is generally characterized by defective response of the cells to insulin stimulation (Højlund, 2014). Methylglyoxal treatment impaired systemic insulin sensitivity in mice as compared to the controls (Dhar, Dhar, Jiang, Desai, & Wu, 2011; Nigro et al., 2014). Supportively, an elevation of methylglyoxal levels in blood (Nemet et al., 2005) and urine (Turk, Čavlović-Naglić, & Turk, 2011; Turk et al., 2009) was shown in participants with diabetes or impaired glucose metabolism (Maessen et al., 2015). Augmented plasma methylglyoxal was associated with diabetic nephropathy progression (Nakayama et al., 2008) and self-report pain in individuals with diabetic neuropathy (Bierhaus et al., 2012). These observations are convincible that methylglyoxal may involve in insulin resistance and type 2 diabetes.

Existing data support that methylglyoxal can cause insulin resistance through perturbation of insulin signaling. Methylglyoxal decreased glucose uptake by abolishing insulin-stimulated phosphorylation of IRS1 (Afridi et al., 2016) and PI3K activity in adipocytes (Jia & Wu, 2007), muscle cells (Riboulet-Chavey, 2006), and endothelial cells (Nigro et al., 2014). In response to insulin stimulation, insulin receptor phosphorylates IRS1 at tyrosine residues to further activate downstream effectors such as PI3K and Akt. The tyrosine phosphorylation of IRS1 was reportedly attenuated by IRS1 serine phosphorylation, which was proposed to interfere with an activity of insulin receptor to interact or phosphorylate IRS1 (Tanti, Grémeaux, van Obberghen, & Le Marchand-

Brustel, 1994). In mouse preadipocytes and human hepatocytes, methylglyoxal appeared to impaired insulin signaling by decreasing IRS1 tyrosine phosphorylation and simultaneously increasing serine phosphorylation of IRS1 (Afridi et al., 2016). Phosphorylation of insulin receptor and downstream effector Akt was reduced in methylglyoxal-treated HepG2 cells, corresponding to the reduction of glucose uptake and GLUT2 expression (Cheng, Cheng, Chiou, & Chang, 2012). In contrast, unaltered IRS1 expression and elevated glucose uptake were observed in cultured L6 myoblasts treated with methylglyoxal (Engelbrecht et al., 2014). The study showed that methylglyoxal increased glucose uptake by reducing GLUT4 endocytosis, in turn, prolonged GLUT4 expression in the plasma membrane (Engelbrecht et al., 2014). Similar findings were also found in L6 myoblasts that underwent methylglyoxal accumulation provoked by siRNA-mediated knockdown of glyoxalase-1 (Engelbrecht, Stratmann, Hess, Tschoepe, & Gawlowski, 2013). The inconsistent findings may be due to differences in methylglyoxal concentrations, glucose uptake assays used among studies, and efficiency of glyoxalase systems among cell types.

A few studies demonstrate that methylglyoxal modulates cellular activities through ligand receptors, including IGF-IR. Pretreatment of methylglyoxal, 1 mM to HEK293 cells and 0.25 mM to NIH3T3 cells, increased the IGF-I-induced activation of MEK/ERK that subsequently elevated expression of a cyclin-dependent kinase inhibitor p21 (Du et al., 2003). By using thymidine uptake assay, the increased p21 expression resulted in decreased cell proliferation in both cell lines. In hepatic HEK293 cell, co-stimulation of IGF-I and methylglyoxal did not show significant effect on activation of PI3K or protein kinase C (PKC). The effect of methylglyoxal on IGF-I-dependent

pathways, however, was not investigated on fibroblast NIH3T3 cells. Co-stimulation of insulin and 0.5-mM-methylglyoxal increased PKC phosphorylation which, in turn, decreased glucose uptake in mouse 3T3-L1 preadipocyte cells (Afridi et al., 2016). The findings suggest that methylglyoxal may differently modulate effector proteins among cell types.

Methylglyoxal possibly aggravate insulin resistance by causing functional decline in pancreatic beta-cells, which play a pivotal role in regulation of glucose-induced insulin synthesis and secretion. A recent study demonstrated that methylglyoxal downregulated activity of IRS1/PI3K pathway and subsequent downstream effectors, Akt and glycogen synthase kinase-3 (GSK3) (Fiory et al., 2011). Mutual activity of Akt and GSK3 was reported to activate pancreatic duodenal homeobox-1 (PDX1), a key transcription factor that regulates beta-cell maturation and function such as modulation of genes encoding insulin and glucokinase (Ashizawa, Brunicardi, & Wang, 2004; Kaneto et al., 2007). The methylglyoxal-induced downregulation of Akt and GSK3 significantly decreased mRNA expression of genes *Pdx1*, *Ins1*, and *Gck,* simultaneously reducing insulin secretion stimulated by glucose (Fiory et al., 2011). A transcription factor FOXO1, which is phosphorylated and inactivated by Akt, was proposed to suppress beta-cell maturation through inhibition of *Pdx1* transcription (Kitamura et al., 2002). Accordingly, methylglyoxal increased activation and nuclear translocation of FOXO1 through Akt downregulation, contributing to a marked decrease in glucose-induced insulin secretion of beta-cells (Gao, Liao, et al., 2016). In addition, pancreatic mitochondrial function was deteriorated by methylglyoxal *in vitro*, concomitant with the reduction of mitochondrial transmembrane potential (Gao, Liao, et al., 2016), oxygen consumption, and ATP

production (Chang et al., 2016; Gao, Liu, et al., 2016). Taken together, methylglyoxal appears to diminish a responsiveness of target cells, and even beta-cells, to insulin stimulation through blunted multiple insulin-dependent pathways. Disturbance in glucose-driven insulin secretion from beta-cells possibly occurs due to methylglyoxal-induced beta-cell dysfunction and increased apoptosis. Therefore, the development of insulin resistance and type 2 diabetes may be evoked by methylglyoxal.

Obesity

It is widely accepted that obesity is positively associated with the risk of type 2 diabetes and cardiovascular diseases. Greater accumulation of methylglyoxal in adipocytes was exhibited in obese Zucker rats compared to lean rats (Jia, Chang, Wilson, & Wu, 2012). Recent studies showed that methylglyoxal was inversely associated with activity of glyoxalase-2 in extracted adipocytes from fructose-fed rats (Masterjohn et al., 2013), and expression of glyoxalase-mRNA in obese participants with at least one risk factor of metabolic syndrome (Uribarri et al., 2015). These observations implicate the association of methylglyoxal and obesity.

Recent studies indicated methylglyoxal induced adipogenesis. An increase in cell differentiation was observed in methylglyoxal-treated 3T3-L1 cells (Jia et al., 2012). Methylglyoxal upregulated Akt1, in turn, stimulated cell cycle progression and increased adipocyte-specific markers, e.g. leptin, adiponectin, PPARγ, and C/EBPα (Jia et al., 2012). Receptor for AGEs (RAGE) is pro-inflammatory and presents in multiple tissues including adipose tissues (Brett et al., 1993; Dozio et al., 2016; Neeper et al., 1992; Schmidt et al., 1992). An interaction of AGEs and RAGE promoted 3T3-L1 cell differentiation through transactivation of IGF-I-dependent pathway, and increased ROS

production. Activation of tyrosine kinase Src by ROS led to phosphorylation of caveolae structure element caveolin1 and IGF-IR, stimulating downstream signaling proteins e.g. IRS1, phosphoinositide-dependent kinase 1 (PDK1), and Akt. (Yang et al., 2013). This, in turn, increased adipogenesis and expression of adipogenic markers, PPARγ and C/EBPα (Yang et al., 2013). In this process, caveolae was required as docking sites where signaling proteins were recruited and activated. The study synthesized AGEs by incubating bovine serum albumin and glyceraldehyde, which unnecessarily yielded methylglyoxal-derived adducts. AGE/RAGE axis is possibly one of intracellular pathways mediated by methylglyoxal-derived AGEs. The assumption is supported by Chen et al. (2012) who reported the effect of methylglyoxal-AGEs on quiescent adipocyte maturation. Through an axis of AGEs and RAGE, adipogenic function was restored in senescent adipocytes that featured the same phenotypes as adipose tissues extracted from obese animal and human cells (Chen et al., 2012). It was hypothesized that methylglyoxal suppressed expression of tumor suppressor p53 and p21 in senescent preadipocytes by direct binding between methylglyoxal and p53 (Chen et al., 2012). Collectively, individuals with preexisting metabolic disease have either increased methylglyoxal production and/or ineffective methylglyoxal elimination that, in turn, may contribute to the risk of obesity development.

Vascular Conditions

Vascular endothelial cell plays a pivotal role in maintenance of blood vessel and circulatory function, such as regulating vasomotor tone and blood flow, controlling the balance of thrombosis and thrombolysis, and modulating generation of new blood vessels (Sumpio, Riley, & Dardik, 2002). As a barrier, the functions of endothelial cells are

dynamic in response to stimuli such as inflammation (Sumpio et al., 2002). However, overstimulation of stimuli may disturb endothelial integrity and function contributing to multiple pathological processes, such as hypertension and atherosclerosis (Sumpio et al., 2002). It is widely accepted that inflammation takes part in vascular complication, with which methylglyoxal may involve. Levels of MGH1 were positively associated with levels of C-reactive protein in children and adolescents with type 1 diabetes of short duration (Heier et al., 2015), who were previously reported to have an increasing trend of carotid artery intima-media thickness (Margeirsdottir, Stensaeth, Larsen, Brunborg, & Dahl-Jorgensen, 2010). The findings suggest that methylglyoxal-derived AGEs may link to inflammation, which is a leading factor for atherosclerosis since early stages of the disease.

Methylglyoxal-treated aortic cells showed a decrease in levels of nitric oxide, which is an important endothelium-dependent relaxing agent (Brouwers et al., 2010; Sena et al., 2012; Turkseven, Ertuna, Yetik-Anacak, & Yasa, 2014). This was, at least in part, due to interference with nitric oxide production (Turkseven et al., 2014) and breakdown (Brouwers et al., 2010) as well as increased oxidative stress (Brouwers et al., 2010; Sena et al., 2012, 2012). Interestingly, prolonged exposure of methylglyoxal, but not methylglyoxal-derived AGEs, showed the harmful effect on the blood vessel dilation (Brouwers et al., 2010). Supportively, overexpression of glyoxalase-1 abolished such effect and MGH1 formation evoked by methylglyoxal in mice (Brouwers et al., 2010). The findings suggest that detrimental actions of methylglyoxal on vascular relaxation likely emerge from extracellular methylglyoxal and intrinsic methylglyoxal-mediated AGEs. Collectively, methylglyoxal may trigger the release of pro-inflammatory

cytokines and the generation of ROS, leading to compromised endothelium-dependent vasorelaxation. Moreover, methylglyoxal appeared to provoke platelet aggregation, decrease thrombus stability (Hadas, Randriamboavonjy, Elgheznawy, Mann, & Fleming, 2013), increase macromolecule endocytosis by endothelial cells (Shinohara et al., 1998) and inhibit hypoxia-induced neovascularization (Ceradini et al., 2008). These evidences suggest a possible link of methylglyoxal to the development of macro- and microvascular conditions.

Dietary Intervention Targeting Methylglyoxal

Like other AGEs, methylglyoxal adducts can be produced in food. This is due to methylglyoxal can be generated from the non-enzymatic Maillard reaction between sugar and protein in food (Nemet et al., 2006; Poulsen et al., 2013; Sharma, Kaur, Thind, Singh, & Raina, 2015). Then, further chemical reactions contribute to the production of dietary methylglyoxal-derived AGEs (Nemet et al., 2006; Poulsen et al., 2013; Sharma et al., 2015). The formation of methylglyoxal depends on various factors, such as alkaline condition, low water activity, and high contents of sugar (Nemet et al., 2006), protein and fat (Sharma et al., 2015). The Maillard reaction play a role in a generation of diverse aroma, color and flavor compounds used in food products (Poulsen et al., 2013; Wang & Ho, 2012). High heat cooking methods (e.g. browning and roasting) and processing procedures to extend shelf-life (e.g. curing and canning) appear to enhance AGEs formation through the reaction (Poulsen et al., 2013; Sharma et al., 2015).

Daily consumption of dietary AGEs was positively associated with serum methylglyoxal adducts in obese adults (Uribarri et al., 2015). The finding suggests that blood methylglyoxal may be modulated by food intake. A recent randomized control trial

reported that high AGE diet increased overall postprandrial response of glucose, ghrelin, vascular cell adhesion protein 1, and urinary oxidative marker F2-isoprostrane in healthy overweight participants as compared to low AGE group (Poulsen et al., 2014). Self-reported appetite and insulin sensitivity remained unchanged, which may be due to the study period of 24 hours (Poulsen et al., 2014). Another double-blind trial found a significant improvement in insulin sensitivity in healthy overweight individuals who had low AGE diet for 2 weeks, as compared to high AGE group (de Courten et al., 2016). Despite inconclusive findings, it suggests that endogenous methylglyoxal may be controlled by restricted exogenous intake.

Taken together, endocytosis and endosome trafficking are required for insulin signal transduction. Rab5-guanine nucleotide exchange factors, such as Rab5-activating protein 6 (RAP6), appear to facilitate insulin signaling through Rab5 activation. Due to the complexity of insulin signaling network, further evidence is still needed to reveal the novel function of RAP6 and additional protein effectors. Also, the association of methylglyoxal with insulin resistance, obesity, atherogenesis, and dietary intake deserves further investigation. Therefore, the current study was conducted to explore cellular mechanism(s) by which RAP6 regulates *in vitro* preadipocyte differentiation driven by methylglyoxal. Furthermore, the association of methylglyoxal, and various risk factors of chronic disease was also examined in ethnic participants, including African Americans and Haitian Americans.

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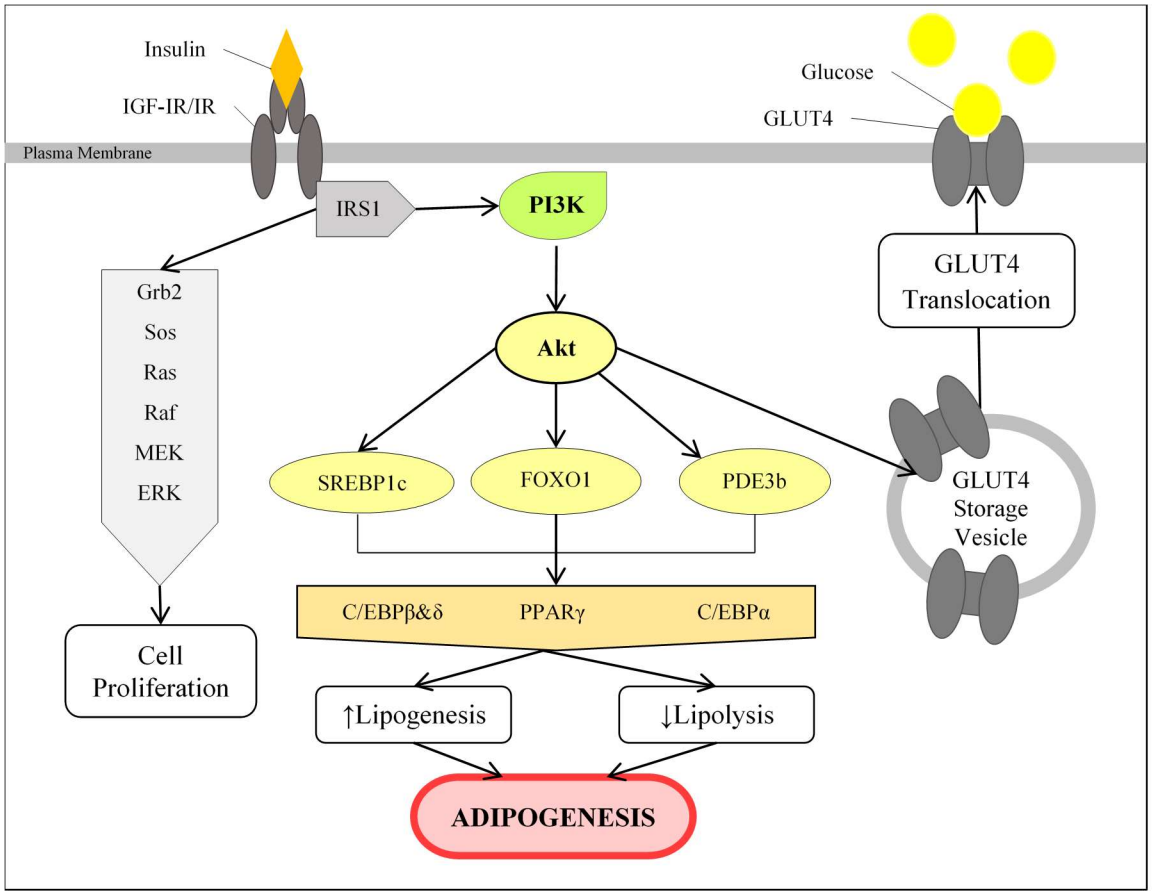

Figure 1. Insulin signaling pathway in adipocytes. C/EBP= CCAAT enhancer-binding protein; ERK=extracellular signal-regulated kinase; FOXO1=forkhead box protein O1; GLUT4=glucose transporter 4; IGF-IR=insulin-like growth factor I receptor; IR=insulin receptor; IRS1=insulin receptor substrate 1; MEK=mitogen-activated protein kinase kinase; PDE3b=phosphodiesterase 3b; PI3K=phosphoinositide 3-kinase; PI3P=phosphatidylinositol 3-phosphate; PPARγ=peroxisome proliferator-activated receptor γ; Ras=rat sarcoma; SREBP1c=sterol regulatory element–binding protein 1c.

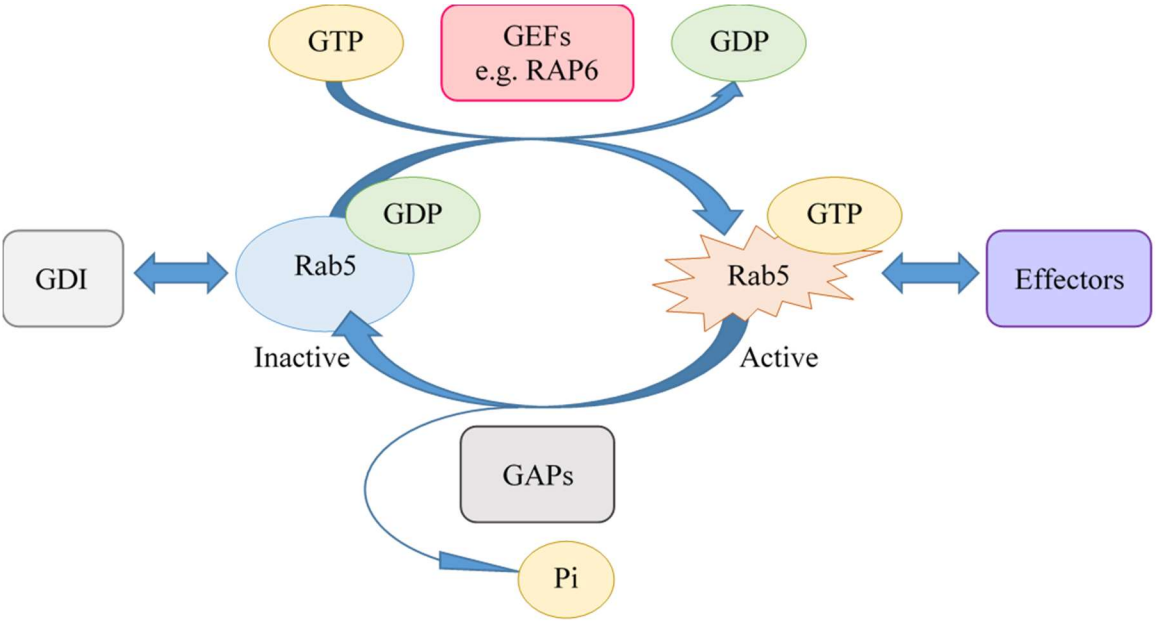

Figure 2. Regulation of small GTPase Rab5. GAPs=GTPase-activating proteins; GDI=Rab guanine-nucleotide dissociation inhibitor; GDP=guanosine diphosphate; GEFs=guanine nucleotide exchange factors; GTP=guanosine triphosphate; Pi=phosphate; RAP6=Rab5-activating protein 6.

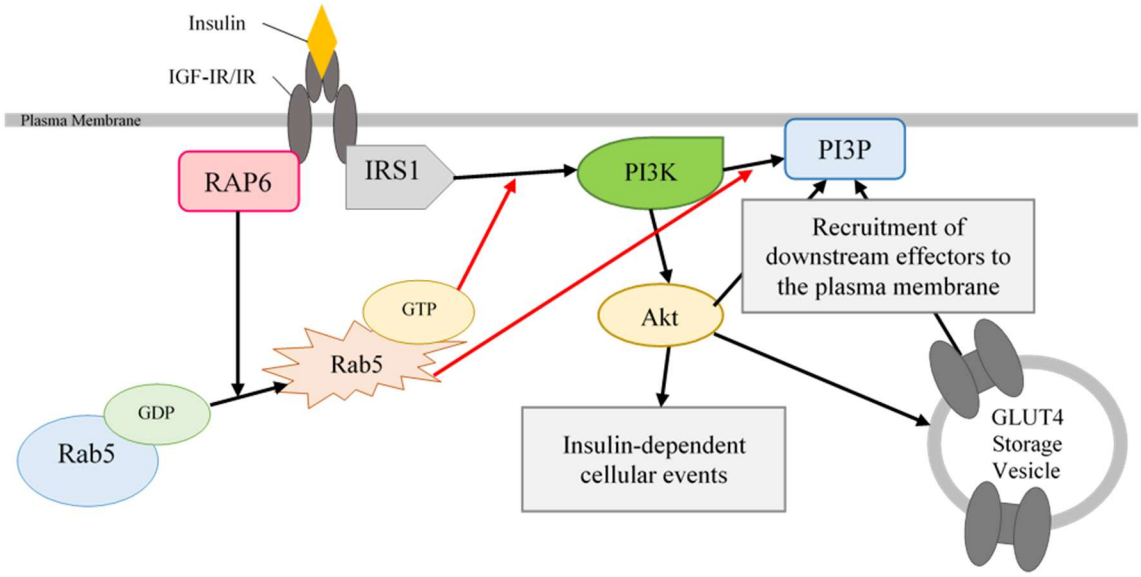

Figure 3. Rab5 and RAP6 activities in insulin signaling according to existing data. Akt=protein kinase B; GDP=guanosine diphosphate; GLUT4=glucose transporter 4; GTP=guanosine triphosphate; IGF-IR=insulin-like growth factor I receptor; IR=insulin receptor; IRS1=insulin receptor substrate 1; PI3K=phosphoinositide 3-kinase; PI3P=phosphatidylinositol 3-phosphate; RAP6=Rab5-activating protein 6.

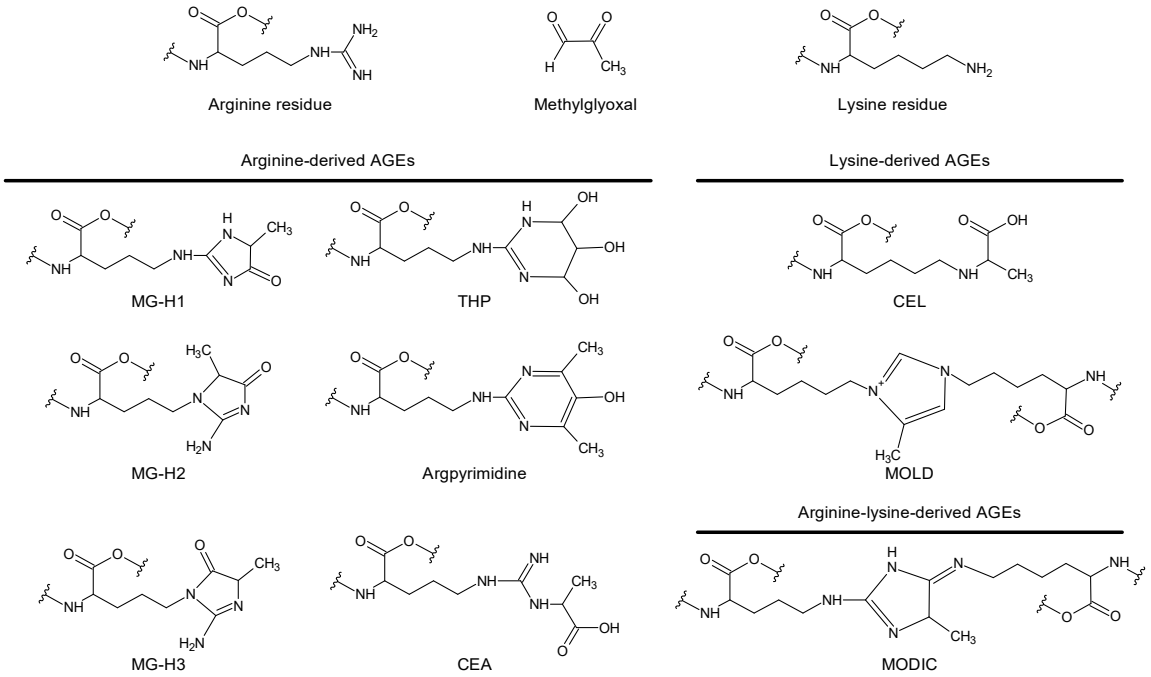

Figure 4. Chemical structures of methylglyoxal, amino acid residues, and advanced glycation end products. Argpyrimidine=N_{δ}-(5-hydroxy-4,6-dimethylpyrimidine-2-yl)-L-ornithine; CEA=N^{7}-(1-carboxyethyl)-arginine; CEL=N_{ε}-(1-carboxyethyl)-lysine; MG-H1=N_{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-L-ornithine; MG-H2=2-amino-5-(2-amino-5-hydro-5-methyl-4-imidazolon-1-yl)pentanoic acid; MG-H3=2-amino-5-(2-amino-4-hydro-4-methyl-5-imidazolon-1-yl)pentanoic acid; MODIC=2-ammonio-6-({2-{4-ammonio-5-oxido-5-oxopentyl)amino]-4-methyl-4,5-dihydro-1*H*-imidazol-5-ylidene}amino)hexanoate; MOLD=1,3-di(N_{ε} -lysino)-4-methyl-imidazolium; THP=N_{δ}-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyrimidine-2-yl)-L-ornithine

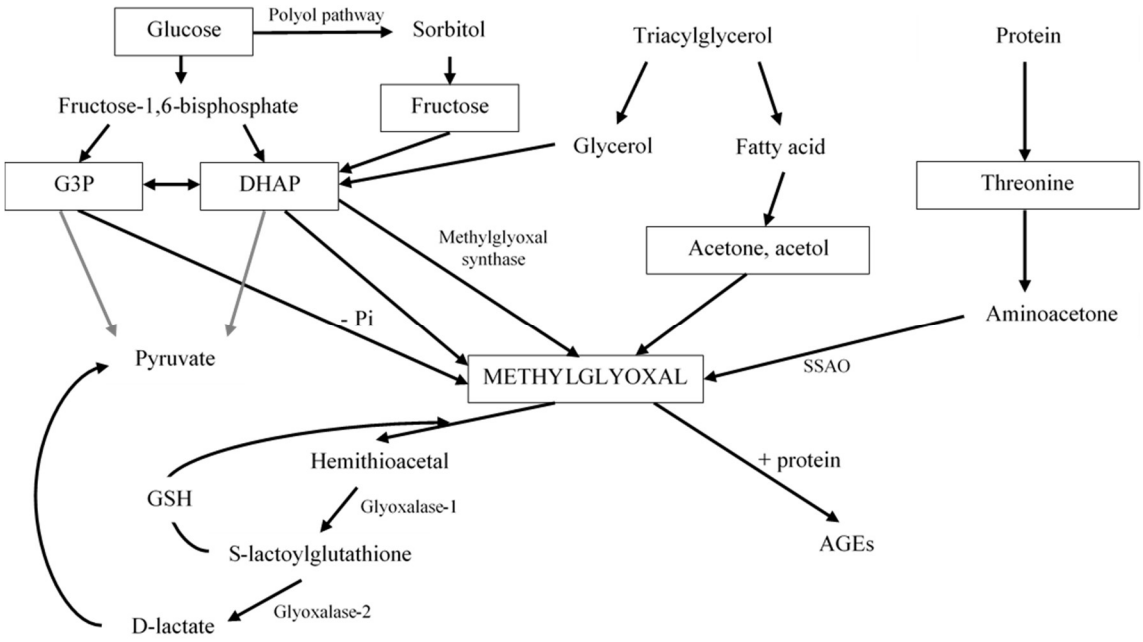

Figure 5. Metabolism of methylglyoxal. Adapted from Chaplen (1998), Desai et al. (2010) and Rabbani & Thornalley (2015). AGEs=advanced glycation end products; DHAP=dihydroxyacetone phosphate; G3P=glyceraldehyde 3-phosphate; GSH=reduced glutathione; Pi=phosphate; SSAO=semicarbazide sensitive amino oxidase.

CHAPTER II

SPECIFIC AIMS AND HYPOTHESES

Available data has been shown that Rab5-activating protein 6 (RAP6) plays a role in receptor-mediated endocytosis and insulin signal transduction. As the internalization of receptors facilitates either sustaining or terminating insulin signals, RAP6 activity may be able to modulate stimuli-induced cellular signaling. Methylglyoxal has been found to alter insulin-dependent signaling pathways, including those related to preadipocyte differentiation. These evidences suggest that RAP6 activity is possibly associated with methylglyoxal-driven cellular events in preadipocytes. Particularly, expression of RAP6 protein has been found in both insulin-responsive preadipocytes and adipocytes, which gives rise to an opportunity for further investigation. However, the association between RAP6 and methylglyoxal is still lacking and remains to be studied. Therefore, the first part of the dissertation was aimed to examine the effect of RAP6 overexpression on *in vitro* preadipocyte differentiation in relation to the potent metabolite methylglyoxal.

Attention to methylglyoxal is growing due to its association with chronic conditions, especially insulin resistance and diabetes. One factor that contributes to methylglyoxal accumulation is high glucose condition. However, available data indicates a diversity of health between different racial/ethnic groups which may lead to inconsistent findings regarding the relationship between methylglyoxal and other risk factors. Due to existing knowledge gap, the latter part of the dissertation was conducted to examine the association of a prominent methylglyoxal-derived adducts, namely methylglyoxal hydroimidazolone 1 (MGH1), with risk factors of chronic diseases in Haitian American and African American participants with and without type 2 diabetes.

By studying ethnic participants, the current study aimed to establish additional understanding in the relationship among potential risk factors of chronic diseases in high risk populations.

Specific aims and hypotheses are listed as follows.

***Specific Aim 1*:** To determine the activity of Rab5-activating protein 6 (RAP6), Rab5-guanine nucleotide exchange factor, on *in vitro* preadipocyte differentiation.

Hypothesis 1a: Through Rab5 activation, RAP6 overexpression will modulate cell proliferation and differentiation of mouse 3T3-L1 preadipocyte cells.

Hypothesis 1b: Through Rab5 activation, RAP6 overexpression will alter selective insulin downstream effector(s) in mouse 3T3-L1 preadipocyte cells (Figure 1).

- Hypothesis 1a and 1b were examined in chapter III.

Specific Aim 2: To elucidate the effect of a dicarbonyl compound methylglyoxal on *in vitro* preadipocyte differentiation mediated by RAP6.

Hypothesis 2a: Methylglyoxal will increase proliferation and differentiation of mouse 3T3-L1 preadipocyte cells.

Hypothesis 2b: RAP6 overexpression will alter an effect of methylglyoxal through specific insulin downstream effector(s) in mouse 3T3-L1 preadipocyte cells (Figure 1).

- Hypothesis 2a and 2b were tested in chapter III.

Specific Aim 3: To examine relationships between serum levels of methyglyoxal-derived adduct, methylglyoxal hydroimidazolone 1 (MGH1) and risk factors of chronic disease in African American and Haitian American participants with and without type 2 diabetes.

Hypothesis 3a: Participants with type 2 diabetes will have serum MGH1 level greater than those without type 2 diabetes.

- Hypothesis 3a was examined in chapter IV, V, and VI.

Hypothesis 3b: Serum MGH1 levels will be positively associated with levels of fasting plasma glucose and hemoglobin A1c.

- Hypothesis 3b was examined in chapter IV.

Hypothesis 3c: Serum MGH1 levels will be associated with levels of selected cardiovascular risk factors.

- Hypothesis 3c was examined in chapter V. The selected cardiovascular risk factors were homocysteine, high sensitivity C-reactive protein, triglycerides, total cholesterol, low-density lipoproteins and high-density lipoproteins.

Hypothesis 3d: Serum MGH1 level will be negatively associated with healthy eating indices, healthy eating index 2005 and alternate healthy eating index.

- Hypothesis 3d was tested in chapter VI.

SIGNIFICANCE

An integration of findings from *in vitro* and *in vivo* studies substantially contributed to a better understanding of the development of obesity as well as the relationship between risk factors of chronic diseases. This, in turn, may lead to prevention strategies. Biochemical, cellular, and molecular assays were utilized in this dissertation to provided new information at several levels: (1) They determined whether RAP6 represented a novel regulatory protein of preadipocyte differentiation, presumably through Rab5 activation; (2) They identified key cellular mechanism of RAP6 that was

required in methylglyoxal-mediated preadipocyte differentiation; (3) They examined whether there were the associations between levels of methylglyoxal and other risk factors of chronic diseases. Finally, these observations may have implications for Rab5 regulation and function in adipogenesis, presenting RAP6 as the center of a cellular mechanism for controlling specific Rab5 function. Therefore, the observations established by this study provided a strong foundation to explore the roles of RAP6 through Rab5 function in receptor internalization and signaling in normal and pathological conditions. Additionally, the current study suggested a significant association of methylglyoxal with biomarkers and modifiable risk factors of diabetes and cardiovascular disease among Black subgroups, which deserves further investigation.

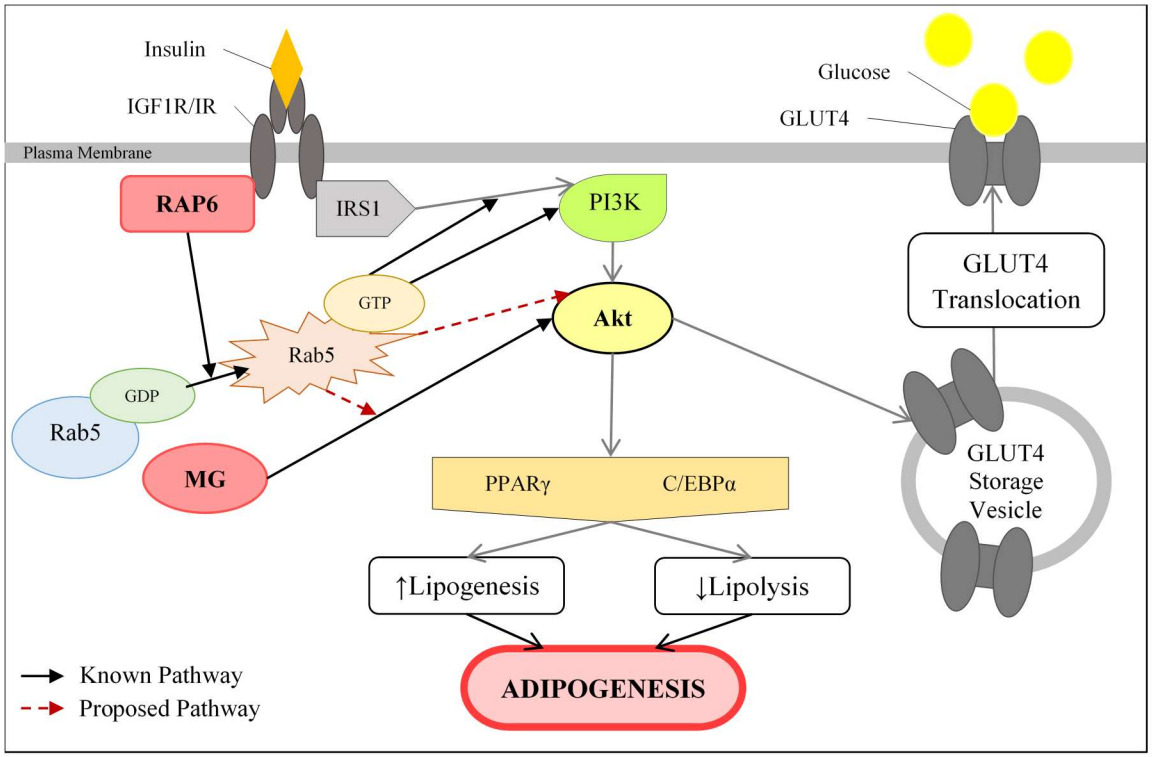

Figure 1. The working model for the purposed *in vitro* study. The illustration showed the pathways speculated to be modulated by RAP6 activity (Specific Aim 1) and methylglyoxal (Specific Aim 2). C/EBP= CCAAT enhancer-binding protein; GDP=guanosine diphosphate; GLUT4=glucose transporter 4; GTP=guanosine triphosphate; IGF-IR=insulin-like growth factor I receptor; IR=insulin receptor; IRS1=insulin receptor substrate 1; MG=methylglyoxal; PI3K=phosphoinositide 3-kinase; PPARγ=peroxisome proliferator-activated receptor γ; RAP6=Rab5-activating protein 6.

CHAPTER III

Rab5-Activating Protein 6 and Methylglyoxal: A Role in Adipocyte Differentiation

ABSTRACT

INTRODUCTION: Rab5-activating protein 6 (RAP6), a Vps9-containg protein, has recently discovered to facilitate insulin signaling and insulin-receptor trafficking through Rab5 activation. Methylglyoxal is a dicarbonyl compound that has been reported to alter insulin signaling and subsequent cellular events. This suggests an association of methylglyoxal with RAP6-mediated insulin signal transduction, which remains unknown.

PURPOSE: The study was aimed to investigate the impact of RAP6 overexpression on mouse 3T3-L1 preadipocyte differentiation in a presence of methylglyoxal.

METHOD: Mouse 3T3-L1 preadipocytes overexpressing GFP (control) and RAP6 were generated. The 3T3-L1 differentiation was measured by oil red o staining. Expression of selected proteins was detected by Western blot analysis. Cell proliferation was determined by MTT assay, while cell cycle progression was examined by flow cytometry. Levels of methylglyoxal-derived hydroimidazolone 1 (MGH1) in 3T3-L1 cells were measured by competitive ELISA.

RESULTS: Methylglyoxal treatment (10 μM) increased 3T3-L1 differentiation as evidenced by a significant increase in expression of adipogenic markers, PPARγ and C/EBPα. This was corresponded to a marked elevation in Akt phosphorylation at Ser473. Methylglyoxal also promoted 3T3-L1 proliferation and accelerated cell cycle progression. However, RAP6 overexpression inhibited 3T3-L1 differentiation, concomitant with suppression of PPARγ. These suppressions were unaltered by methylglyoxal. Phosphorylation of Akt1 at Ser473 was abolished by combination of

RAP6 overexpression and methylglyoxal treatment, even more than that by RAP6 overexpression alone. In addition, differentiated 3T3-L1 cells had elevated levels of MGH1, which was enhanced by high glucose condition.

CONCLUSION: Methylglyoxal at low concentration stimulated 3T3-L1 differentiation and proliferation which appeared to be neutralized by RAP6 overexpression. Therefore, RAP6 may be a key regulator that modulates methylglyoxal-stimulated preadipocyte differentiation.

INTRODUCTION

Adipocytes play an important role in energy storage in a form of triacylglycerols when excess energy is consumed in the diet. Adipogenesis is the differentiation of premature preadipocyte to form fat-laden adipose tissues (Ali, Hochfeld, Myburgh, & Pepper, 2013). An increase in adipogenesis can lead to an excess fat accumulation in the body which is a key feature of obesity (Attie & Scherer, 2009). Therefore, adipogenesis needs to be tightly regulated so as to function properly.

Insulin plays a key role in adipogenesis by promoting glucose uptake (Charron, Brosius, Alper, & Lodish, 1989; Watson, Kanzaki, & Pessin, 2004) and stimulating downstream cascades that initiate adipocyte differentiation (Siersbaek & Mandrup, 2011). Receptor tyrosine kinases, e.g. insulin receptor (IR) and insulin-like growth factor I receptor (IGF-IR), have been recognized as key components for insulin signal transduction. The receptors, which are associated with the plasma membrane, serve as a docking platform for multiple signaling cascades in response to insulin stimulation. One of the well-characterized pathways include a cascade constituting insulin receptor substrates (IRS 1-6), phosphoinositide 3-kinases (PI3Ks) and Akt, which acts as a central

pathway harnessing adipogenesis and cell survival (Stenkula et al., 2004). Once activated, PI3K produces phosphatidylinositol (3,4,5)-trisphosphate in the plasma membrane creating anchoring sites for downstream effectors, e.g. Akt (McCurdy & Klemm, 2013) and storage vesicles of glucose transporter 4 (GLUT4) (Bai et al., 2007; Calera et al., 1998). Along with other proteins, activated Akt subsequently induces GLUT4 translocation to the plasma membrane where glucose uptake takes place (Cong et al., 1997; McCurdy & Klemm, 2013).

Insulin can also activate extracellular signal–regulated kinase 1/2 (ERK1/2) (Porras & Santos, 1996) which play a role in activation of cell cycle regulatory proteins (Yohannes & Yohannes, 2009) and induction of mitotic clonal expansion (MCE) during the early phase of adipogenesis (Tang, Otto, & Lane, 2003). This lead to expression of CCAAT enhancer-binding protein β (C/EBPβ) which is required for initiating MCE (Tang et al., 2005, 2003). Then, C/EBPβ and δ cause co-expression of transcription factors, i.e. C/EBPα and peroxisome proliferator-activated receptor γ (PPARγ), that subsequently induces complete maturation of adipocytes (Lefterova & Lazar, 2009; Siersbaek & Mandrup, 2011).

Apart from transmitting signal at the plasma membrane, receptors and their ligands can amplify or degrade the signal through endocytosis. Clathrin-mediated endocytosis is a well-characterized mechanism which play an important role in internalization of multiple receptors, including IR (Ceresa, Kao, Santeler, & Pessin, 1998) and IGF-IR (Romanelli et al., 2007). Upon ligand activation, ligand-bound receptors are internalized from the plasma membrane to early endosomes (Le Roy & Wrana, 2005). The early endosomes containing ligand-receptor complex are further

directed to target intracellular compartments for either receptor degradation and signal termination, or receptor recycling to the plasma membrane and maintaining signal transduction (Irannejad, Tsvetanova, Lobingier, & von Zastrow, 2015). A small GTPase Rab5 has been found to facilitate receptor-mediated clathrin-dependent endocytosis (Kajiho et al., 2003; Nielsen, Severin, Backer, Hyman, & Zerial, 1999; Su, Lodhi, Saltiel, & Stahl, 2006) as well as early endosome biogenesis, sorting and fusion (Bucci et al., 1992; Rybin et al., 1996; Zeigerer et al., 2012). Therefore, Rab5 is a key component that links clathrin-mediated endocytosis to ligand-activated cell signaling.

Rab5 is a member of Ras GTPase superfamily that mainly participates in membrane trafficking. Rab5 switches between an inactive form bound to guanosine diphosphate (GDP), and an active form bound to guanosine triphosphate (GTP) (Mizuno-Yamasaki, Rivera-Molina, & Novick, 2012). The switching process is accelerated by two sets of proteins: guanine nucleotide exchange factors (GEFs) activate Rab5 by promoting the exchange of GDP to GTP; and GTPase-activating proteins (GAPs) deactivate Rab5 by stimulating hydrolysis of GTP to GDP (Mizuno-Yamasaki et al., 2012). Typically, Rab5 is localized at the plasma membrane and early endosomal membrane (Chavrier, Parton, Hauri, Simons, & Zerial, 1990). In cytosol, GDP-bound Rab5 is formed as a complex to Rab guanine-nucleotide dissociation inhibitor (GDI) which is speculated to translocate Rab5 to or from the functional target sites (Ullrich, Horiuchi, Bucci, & Zerial, 1994).

Rab5-specific GEFs contain a highly-conserved Vps9 domain which is required for Rab5 activation (Burd, Mustol, Schu, & Emr, 1996; Delprato & Lambright, 2007; Tsukamoto et al., 2015). Among Rab5-GEFs, Ras interference 1 (Rin1) (Balaji et al.,

2012; Kavitha Balaji & Colicelli, 2013; Ding, Wang, & Chen, 2009; Galvis, Balmaceda, et al., 2009; Galvis, Giambini, Villasana, & Barbieri, 2009; Hunker, Giambini, et al., 2006; Tall, Barbieri, Stahl, & Horazdovsky, 2001), and Rabex-5 (Aikawa, 2012; Delprato, Merithew, & Lambright, 2004; Kalesnikoff et al., 2007; Harald Stenmark, Vitale, Ullrich, & Zerial, 1995; Zhang et al., 2014) have been widely studied to examine their involvement in Rab5 activity. However, available data remains scarce for Rab5-activating protein 6 (RAP6), also known as GAPex-5 (Lodhi et al., 2007) and hRME-6 (Sato et al., 2005), which is newly discovered to participate in Rab5 activation (Hunker, Galvis, et al., 2006).

Activities of RAP6 and Rab5 were reported to mediate insulin-stimulated cellular events. In insulin-responsive NIH3T3/hIR cells, knockdown of all Rab5 isoforms (Rab5a, Rab5b and Rab5c) or RAP6 markedly reduced insulin-dependent activation of Akt (Su et al., 2006). Interestingly, expression of Rab5 isoforms was significantly suppressed in obese Zucker rats as compared to lean rats (Su et al., 2006), suggesting the involvement of Rab5 in obesity. Overexpression of wild-type RAP6 inhibited GLUT4 translocation, whereas RAP6 mutant lacking Vps9 domain caused less inhibition (Lodhi et al., 2007). These findings suggest that there may be an interaction between Vps9 and other domains, e.g. proline-rich domain and Ras-GAP domain, in RAP6 to fully regulate Rab5-mediated glucose uptake and adipogenesis. In addition, RAP6 and Rab5 activities participated in GLUT4 translocation by facilitating insulin-induced formation of phosphatidylinositol 3-phosphate in the plasma membrane of 3T3-L1 preadipocytes (Lodhi et al., 2008). Collectively, Rab5 and RAP6 activity possibly have a potential role in insulin-dependent signaling and metabolism in preadipocytes.

Methylglyoxal is a dicarbonyl compound that can be produced endogenously from the breakdown of macronutrients (Phillips & Thornalley, 1993) as well as obtained from foods (Poulsen et al., 2013). Recent studies indicated that methylglyoxal induced adipogenesis. An increased proliferation was shown in methylglyoxal-treated 3T3-L1 cells (Jia, Chang, Wilson, & Wu, 2012). Methylglyoxal upregulated Akt1 that, in turn, stimulated cell cycle progression and elevated adipocyte-specific markers, e.g. leptin, adiponectin, PPARγ, and C/EBPα. (Jia et al., 2012). As a highly reactive glucose derivative, methylglyoxal can bind to amino acid residues forming advanced glycation end products (AGEs). Receptor for AGEs (RAGE) is pro-inflammatory and presents in multiple tissues including adipose tissues (Brett et al., 1993; Dozio et al., 2016; Neeper et al., 1992; Schmidt et al., 1992). Through the interaction of methylglyoxal-AGEs and RAGE, adipogenic function was restored in senescent adipocytes that featured the same phenotypes as adipose tissues extracted from obese animal and human cells (Chen, Abell, Moon, & Kim, 2012). It was hypothesized that methylglyoxal directly bound tumor suppressor p53 and thereby reduced expression of tumor suppressor p53 and p21 in senescent preadipocytes (Chen et al., 2012). In contrast, methylglyoxal was also reported to inhibit insulin signaling. A recent study demonstrated that methylglyoxal treatment diminished tyrosine phosphorylation of IRS1, Akt activation and subsequent glucose uptake in 3T3-L1 preadipocytes (Afridi et al., 2016). Regardless, methylglyoxal possibly alter ligand signaling and thereby interfere with preadipocyte proliferation and differentiation.

Given that receptor trafficking plays a role in ligand signal transduction, a change in RAP6 activity may impact ligand-activated cellular events elicit by methylglyoxal. As

previously reported, protein RAP6 expresses in predipocyte and differentiated adipocyte cells (Lodhi et al., 2007), which provides an opportunity to observe RAP6 activity in preadipocyte model. Therefore, the current study was aimed to explore the impact of RAP6 overexpression on *in vitro* preadipocyte differentiation driven by methylglyoxal.

MATERIAL AND METHOD

Materials

Mouse 3T3-L1 preadipocyte cells, Platinum A (Plat-A) packaging cells, human HEK293T kidney cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Anti-GFP antibodies were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-RAP6 antibodies were prepared and obtained as described elsewhere (Hunker, Galvis, et al., 2006). Other primary antibodies (e.g. antibodies against Akt1, p-Akt1 (Ser473), PPARγ, C/EBPα, p-ERK1/2, and GAPDH) and secondary antibodies were obtained from Cell Signaling Technology (Boston, MA, USA). Methylglyoxal was purchased in a concentration of 40% w/v (6.5 M) in water (Sigma Aldrich, St. Louis, MO, USA). All other reagents and supplies were purchased from Fisher Scientific (Pittsburgh, PA, USA) except as specifically described.

Generation of Stable Cell Lines

Complementary DNAs (cDNAs) of GFP were subcloned into pMX-puro vector, while cDNAs of RAP6:wild type (WT) were cloned into pBABE-puro vector as described elsewhere (Barbieri, Fernandez-Pol, Hunker, Horazdovsky, & Stahl, 2004). Production of retroviruses containing GFP constructs was perform on Plat-A packaging cells, whereas human HEK293T cells were used to generate retroviruses containing RAP6:WT constructs. Plat-A cells were grown in Dulbecco's minimum essential medium

(DMEM) containing 10% fetal bovine serum (FBS), 100 unit/ml penicillin G sodium and 100 μg/ml streptomycin sulfate, 1 ug/ml puromycin, and 10 μg/mL blasticidin. HEK293T were cultured in DMEM containing 10% FBS, penicillin and streptomycin.

By using Lipofectamine® 2000 kit (Invitrogen, Grand Island, NY, USA), the 80%-confluent Plat-A or HEK293T cells were transfected with GFP or RAP6:WT construct, respectively. After 48-hour incubation, retroviral supernatant was harvested and further used along with 5ug/ml polybrene to transfect 3T3-L1 cells, generating GFP- and RAP6-overexpressing cell lines. After 72 hours, transfected 3T3-L1 cells were selected by using regular growth media containing 4 ug/ml puromycin for at least 9 days. The successfully transfected cells then were maintained in growth media constituting 0.5 μg/ml puromycin.

Cell Culture and 3T3-L1 Differentiation Assay

Mouse embryonic fibroblasts, especially 3T3-L1 cell line, have been widely used as *in vitro* preadipocyte models to investigate novel signaling pathways in adipose tissues (Jia et al., 2012; Lodhi et al., 2008; Siersbaek & Mandrup, 2011). Mouse 3T3-L1 preadipocytes were cultured to confluence in DMEM containing 10% FBS, 100 unit/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. An *in vitro* differentiation procedure of 3T3-L1 preadipocyte cells was performed (Green & Meuth, 1974), with a slight modification in differentiation-inducing stimulant mix based on Jia et al. (2012). Briefly, 3T3-L1 cell differentiation was induced by a cocktail mix constituting 0.5 μM 3-isobutyl-methylxanthine, 0.25 μM dexamethasone, and 0.43 μM insulin (MDI). On the fifth day after differentiation induction, the cells were then maintained in DMEM containing 10% FBS, penicillin, streptomycin, and 2.5 ug/ml insulin until fully

differentiation (about 10-14 days). The media was changed every other day. To determine effect of methylglyoxal on 3T3-L1 differentiation, working methylglyoxal solution was freshly prepared before use. The cells were treated with or without methylglyoxal, which was added as same time as when media was changed. Similar protocols of differentiation assay and methylglyoxal treatment were applied to GFP- and RAP6-overexpressing cells.

Quantification of Lipid Accumulation

Intracellular triacylglycerols were quantified using oil red O (ORO) staining protocol (Halvorsen et al., 2001), with a slight modification. Briefly, differentiated 3T3-L1 cells were fixed with 10% formaldehyde and stained with working ORO solution for 15 minutes at room temperature and washed three times with distilled water. The remained dye was eluted with 100% isopropanol for 10 minutes at 37 ºC. Optical density of the solution was measured using a spectrophotometer at a wavelength of 540 nm. Normal 3T3-L1 cells without methylglyoxal treatment served as a control to compare with cells treated with methylglyoxal, whereas GFP-overexpressing cells were used to compare with those overexpressing RAP6.

Measurement of Cell Size

The cells were induced to differentiate in an absence or presence of methylglyoxal and subsequently underwent ORO staining. All photographs of dyed cells were captured using QCapture software (Surrey, BC, Canada), which were randomly selected and used for cell size measurement by ImageJ64 software (Bethesda, MD, USA). Perimeter of differentiated 3T3-L1 cells was measured in the unit of micrometer (μm). To ensure a circular shape, only data from differentiated cells that had roundness between 0.35 and 1.00, was included in the analysis.

Western Blot Analysis

Western blot analysis described by Kohn et al. (1996) was utilized to measure expression of selected proteins, including GFP, RAP6, Akt1, p-Akt1 (Ser473), PPARγ, C/EBPα, p-ERK1/2, and GAPDH. Briefly, differentiated 3T3-L1 cells were lysed in ice-cold lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 50 mM NaF, 0.2 mM Na_3VO_4, and 1 mM PMSF). The cell lysates were separated through SDS-PAGE and transferred onto a nitrocellulose membrane (Millipore, Billerica, MA, USA). After blocking, the membranes were incubated in primary antibodies overnight at 4 ^oC. The membranes were washed thrice with TBST, incubated in appropriate secondary antibodies for an hour, and underwent three washes of TBST. Protein expression images were acquired by using darkroom development technique for enhanced chemiluminescence (Thermo Fisher Scientific, MA, USA). Protein expression was quantified by densitometry analysis using ImageJ64 software (Bethesda, MD, USA). GAPDH was used as a protein loading control.

Cell Proliferation Assay

Cell proliferation was measured by a 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay which was described elsewhere (Kang, Nam, Kim, Huh, & Lee, 2013), with a slight modification. 3T3-L1 cells were seeded in a 96-well plate at 1 x 10^4 cell/ml. After incubation in serum-free medium overnight, the cells were treated with or without methylglyoxal in regular growth medium for 24 hours. After methylglyoxal treatment, the cells were incubated in MTT-containing growth medium for 2 hours in the dark until purple precipitate became visible. Then, detergent reagent was added to dissolve purple formazan crystals. Optical density at 570 nm of the solutions

were determined using a microplate reader (BioTek®, Winooski, VT, USA). Normal 3T3-L1 cells without methylglyoxal treatment served as a control which was used to compare with methylglyoxal-treated cells.

Cell Cycle Analysis

Cell cycle progression was measured by flow cytometry. Once GFP- and RAP6-overexpressing cells reached 50% confluence, the cells were treated with or without methylglyoxal for 24 hours. After incubation, the cells were harvested, washed, and re-suspended in 0.5 ml of PBS, and fixed in 4.5 ml of ice-cold 100% ethanol for 30 minutes. The cells were centrifuged, re-suspended in 0.5 ml of 0.1% Triton-X solution containing 5 µl of RNase (20 µg/ml), and incubated in the dark at 37 ºC for 20 minutes. The cell suspension was added with 5 µl of propidium iodide (50 µg/ml) and incubated in the dark at room temperature for 30 minutes. The flow cytometry then was performed on a BD Accuri™ C6 flow cytometer (BD Biosciences, San Jose, CA, USA). The data for cell cycle analysis were collected and analyzed from 20,000 to 30,000 gated events by BD Accuri™ software (BD Biosciences, San Jose, CA, USA).

Methylglyoxal Measurement

A prominent methylglyoxal adduct, hydroimidazolone 1 (MGH1), in sample lysates of differentiated 3T3-L1 cells were measured by OxiSelect™ MG competitive ELISA kit (Cell Biolabs, Inc., San Diego, CA, USA). The quantity of MGH1 was compared to a standard curve of predetermined MG-BSA.

Statistical Analyses

A Shapiro-Wilk's test and visual inspection of their histograms, normal Q-Q plots, and box plots were used to examine whether variables were normally distributed.

Normal distributed data were expressed in mean±standard deviation (SD), percentage (%). Skewed distributed variable were shown in median and interquartile range (IQR). The difference between groups were determined by Student's t-test for normal distributed variables, while Mann-Whitney U test was used for skewed distributed variables. Tests were considered statistically significant if *p*-value was less than 0.05. All data were analyzed by using SPSS version 23.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Sample Characteristics

According to a Shapiro-Wilk's test ($p>0.05$; Razali & Wah, 2011) and a visual inspection of histograms, normal Q-Q plots and box plots, the data for cellular protein expression, cell cycle phase distribution, and cellular MGH1 concentration were approximately normally distributed for all experiment groups. However, the data distribution for cell differentiation in oil red O assay, cell perimeter, and cell proliferation in MTT assay were skewed for some subgroups as evidenced by *p*-value less than 0.05 in Shapiro-Wilk's test and/or apparent skewness of data in visual plots.

Methylglyoxal impacted 3T3-L1 differentiation

To determine whether methylglyoxal impacted 3T3-L1 differentiation, the 3T3-L1 differentiation assay with or without methylglyoxal treatment (0-200 μM) was performed. Methylglyoxal treatment at 10 μM significantly increased 3T3-L1 differentiation (median: 106.34%; IQR: 105.04%-110.96%; $p=0.016$ vs control) as compared to untreated cells (median: 100.56%; IQR: 96.06%-103.10%; Figure 1). The differentiation was gradually decreased when methylglyoxal concentrations increased. The significant reduction of differentiation emerged at 50 μM (median: 89.77%; IQR:

86.80%-96.83%; p=0.016 vs control), 150 μM (median: 85.28%; IQR: 80.88%-92.00%; p=0.010 vs control) and 200 μM of methylglyoxal (median: 79.17%; IQR: 78.17%-86.15%; p=0.004 vs control).

Methylglyoxal enhanced 3T3-L1 preadipocyte enlargement and increased expression of adipogenic markers.

Measurement of cell size was then performed to investigate whether methylglyoxal increased differentiation through adipocyte enlargement, one of the key features in adipogenesis. 3T3-L1 cells were induced to differentiate with or without 10 μM of methylglyoxal. The methylglyoxal concentration of 10 μM was chosen due to the highest effect on 3T3-L1 differentiation. Subsequently, the cells underwent ORO staining, in which photos of the stained cells were captured and used for measurement of cell perimeter (Figure 2A). Methylglyoxal-treated cells significantly had a greater cell perimeter than untreated cells (control (n=310), median: 60.13 μm, IQR: 55.28-69.73 μm vs methylglyoxal (n=354), median: 65.64 μm, IQR: 59.95-78.77 μm, p<0.001; Figure 2B).

Western blot analysis was also performed to determine expression of adipogenic markers, PPARγ and C/EBPα, in an absence or presence of methylglyoxal (10 μM). Marginally, methylglyoxal treatment resulted in elevated PPARγ expression as compared to the control (1.18±0.10 folds relative to the control, p=0.091 vs control, Figure 3A and 3B). Methylglyoxal treatment caused a significant increase in expression of C/EBP-p42 (1.16±0.03 folds relative to the control, p=0.002 vs control, Figure 3A and 3C) and C/EBP-p30 isoforms (1.23±0.10 folds relative to the control, p=0.016 vs control, Figure 3A and 3D).

Methylglyoxal upregulated ERK1/2 in the early phase of 3T3-L1 differentiation.

As previously reported, ERK1/2 was required for cell cycle progression and mitotic clonal explansion (MCE) (Tang et al., 2003). To determine whether methylglyoxal altered ERK1/2 activation, differentiation of 3T3-L1 cells was induced for 5 minutes in an absence or presence of methylglyoxal (10 μM). Western blot results showed that methylglyoxal treatment markedly increased phosphorylation of ERK1/2 as compared to untreated cells (Figure 4).

Methylglyoxal increased Akt1 phosphorylation at Ser473 in 3T3-L1 cells.

Methylglyoxal was reported to alter Akt phosphorylation (Afridi et al., 2016; Jia et al., 2012), which plays an important role in adipocyte differentiation (Kim et al., 2010; Maiuri, Ho, & Stambolic, 2010; Xu & Liao, 2004). Therefore, Western blot analysis was conducted to determine Akt1 activation in fully differentiated 3T3-L1 cells. The results showed that phosphorylation of Akt1 at Ser473 was significantly increased in methylglyoxal-treated cells as compared to untreated cells (1.16±0.03 folds relative to the control, p=0.002 vs control, Figure 5A and 5B).

RAP6 overexpression caused inhibition of 3T3-L1 differentiation which was unaltered by methylglyoxal treatment.

RAP6 has been speculated to activate Rab5 (Hunker, Galvis, et al., 2006), suggesting its association with receptor-mediated endocytosis and signal transduction. To determine the effect of RAP6 activity on 3T3-L1 differentiation, RAP6-overexpressing cell lines were generated along with GFP-expressing cells which served as the control. Amino acid sequences of RAP6 and its constituting domains were illustrated in Figure 6A. The expression of GFP and RAP6 was confirmed by Western blot analysis (Figure

6B). The GFP- and RAP6-overexpressing cells underwent differentiation assay with or without methylglyoxal. The methylglyoxal concentration of 10 μM was chosen due to the highest effect observed in non-transfected 3T3-L1 cells. Similar to non-transfected cells, methylglyoxal treatment significantly increased differentiation of the GFP-overexpressing cells (median: 120.40%, IQR: 113.64%-127.33%, p=0.006 vs non-treated GFP control) as compared to non-treated cells (median: 99.68%, IQR: 95.66%-104.02%; Figure 6C). Overexpression of RAP6 markedly diminished 3T3-L1 differentiation as compared to the untreated cells expressing GFP (median: 58.87%, IQR: 48.55%-72.85%, p=0.004 vs non-treated GFP control). The decreased differentiation by RAP6 overexpression was unaltered by methylglyoxal treatment as the differentiation remained markedly lower than untreated GFP-overexpressing cells (median: 55.74%, IQR: 50.09%-60.15%, p=0.004 vs non-treated GFP control), but not different from untreated RAP6-overexpresing cells (p=0.631 vs non-treated RAP6).

Expression of adipogenic markers was decreased by RAP6 overexpression and remained suppressed in a presence of methylglyoxal.

To determine whether RAP6 overexpression could affect expression of PPARγ and C/EBPα, Western blot analyses were performed after differentiation assay with or without 10 μM of methylglyoxal. Overexpression of RAP6 resulted in PPARγ suppression in 3T3-L1 cells (p=0.018 vs untreated GFP; Figure 7A and 7B). A slight decrease in expression of C/EBPα-p42 and -p30 isoforms in RAP6-overexpressing cells was shown, yet not statistically significant (p=0.125 and p=0.191 vs untreated GFP, respectively; Figure 7A, 7C and 7D). In GFP-overexpressing cells, methylglyoxal treatment moderately increased expression of PPARγ in GFP-overexpressing cells

(p=0.077 vs untreated GFP) as well as caused a significant increase in expressions of C/EBPα-p42 (p=0.014 vs untreated GFP) and C/EBPα-p30 (p=0.041 vs untreated GFP). Interestingly, methylglyoxal treatment caused a marginal suppression of C/EBPα-p30 (p=0.073 vs untreated GFP), but not expression of PPARγ (p=0.483 vs untreated GFP) and C/EBPα-p42 (p=0.111 vs untreated GFP) in a presence of RAP6 overexpression. There was no significant difference in expression of PPARγ (p=0.483 vs untreated RAP6), C/EBPα-p42 (p=0.454 vs untreated RAP6), and C/EBPα-p30 (p=0.262 vs untreated RAP6) between RAP6-overexpressing cells with and without methylglyoxal treatment.

RAP6 overexpression significantly inhibited Akt1 phosphorylation. Methylglyoxal appeared to enhance Akt1 downregulation by RAP6 overexpression.

It was speculated that RAP6 modulated an activity of insulin-dependent downstream protein such as PI3K (Lodhi et al., 2008) as well as GLUT4 translocation (Lodhi et al., 2007). Given that Akt has been recognized as a major downstream effector of PI3K, RAP6 may also impact Akt activity. Following differentiation assay with or without methylglyoxal (10 μM), Western blot analyses were performed to determine Akt1 activation. Phosphorylation of Akt1 at Ser473 was significantly inhibited by RAP6 overexpression (p=0.019 vs untreated GFP; Figure 8A and 8B). Interestingly, this inhibition was markedly enhanced by methylglyoxal treatment as compared to untreated GFP control (p<0.001vs untreated GFP) and untreated RAP-overexpressing cells (p=0.007 vs untreated RAP6). In contrast, methylglyoxal significantly increased Akt1 phosphorylation at Ser473 in GFP-overexpressing cells (p=0.010 vs untreated GFP).

Methylglyoxal increased 3T3-L1 proliferation

It was previously reported that methylglyoxal increased proliferation in 3T3-L1 cells (Jia et al., 2012), cell proliferation with or without methylglyoxal treatment (0-200 μM) was determined. Methylglyoxal markedly increased median 3T3-L1 proliferation at 10, 30, 50 and 200 μM to 130.50% (IQR: 125.75%-149.75%; p=0.004), 136.50% (IQR: 132.50%-185.25%; p=0.004), 147.50% (IQR: 140.50%-175.00%; p=0.004), and 136.00% (IQR: 123.50%-151.5%; p=0.004) of untreated cells, respectively (Figure 9).

Methylglyoxal treatment moderately led to a faster cell cycle progression, but not in a presence of RAP6 overexpression

As methylglyoxal was found to increase 3T3-L1 proliferation in MTT assay, analysis of cell cycle phase distribution was performed to determine the effect of methylglyoxal in an absence or presence of RAP6 overexpression. At 24 hours of proliferation, only S phase distribution in RAP6-overexpressing cells was significantly higher than that in the control (GFP: 9.13±0.34 vs RAP6: 9.87±0.23, p=0.034; Figure 10). There was no difference in distribution of G1 phase (p=0.354 vs untreated GFP) or G2/M phase (p=0.861 vs untreated GFP) between the control and RAP6-overexpressing cells. Methylglyoxal treatment (10 μM) appeared to accelerate cell cycle progression in GFP-overexpressing cells, as evidenced by a lower G1 phase distribution (GFP: 57.87±1.42 vs GFP+MG: 50.23±4.28, p=0.042) and a higher distribution of S phase (GFP: 9.13±0.34 vs GFP+MG: 10.56±0.43, p=0.011) and G2/M phase (GFP: 33.00±1.36 vs GFP+MG: 39.22±4.51, p=0.081) as compared to untreated cells. However, the distribution of all phases in RAP6-overexpressing cells unaltered in a presence of methylglyoxal (G1: p=0.555, S: p=0.457, G2: p=0.609 vs untreated GFP).

Levels of methylglyoxal adduct in 3T3-L1 cells were elevated in a presence of differentiation stimulants and high glucose condition.

As compared to the control, insulin treatment appeared to cause methylglyoxal accumulation in 3T3-L1 cells (Liu, Desai, Wang, & Wu, 2013). In addition, high glucose-induced methylglyoxal accumulation was found in cell culture (Liu et al., 2013), animal model (Schlotterer et al., 2009) and human studies (Nemet, Turk, Duvnjak, Car, & Varga-Defterdarović, 2005; Turk, Čavlović-Naglić, & Turk, 2011). These evidences suggest that insulin-stimulated glucose uptake may increase production of methylglyoxal and subsequent methylglyoxal-derived adducts as compared to non-stimulation. Inducers of 3T3-L1 differentiation include insulin which stimulates glucose uptake (Leney & Tavare, 2009). Therefore, the prominent methylglyoxal adduct, methylglyoxal hydroimidazolone 1 (MGH1), in 3T3-L1 cells was measured in undifferentiated and differentiated 3T3-L1 cells. Levels of MGH1 in 3T3-L1 cells were increased about 1.56 folds in a presence of MDI as compared to non-induced cells ($p<0.001$; Figure 11).

It was reported that high glucose condition gave rise to methylglyoxal accumulation in cultured cells (Liu et al., 2013; Shinohara et al., 1998; Thornalley, 1988) and *C. elegan* model (Schlotterer et al., 2009). Methylglyoxal accumulation may also lead to an increased production of methylglyoxal-derived adducts. To determine the effect of glucose on cellular MGH1 formation, 3T3-L1 cells were induced to differentiate by MDI in media containing different concentrations of glucose (5, 12, and 25 mM). As compared to 5 mM-glucose group, the cells differentiated in 12 mM and 25 mM glucose containing media had a 1.35-fold and 1.38-fold increase in MGH1 levels, respectively (12 mM-glucose: $p<0.001$ and 25 mM-glucose: $p<0.001$ vs 5 mM-glucose).

DISCUSSION

The present study showed that methylglyoxal altered 3T3-L1 differentiation as evidenced by increased expression of selected adipogenic markers, PPARγ and C/EBPα. This was corresponded to an increase in phosphorylation of ERK1/2 in the early phase of differentiation and Akt1 phosphorylation at Ser473 in the late phase. Methylglyoxal also increased 3T3-L1 proliferation and enhanced cell cycle progression. However, RAP6 overexpression suppressed 3T3-L1 differentiation, concomitant with significant suppression of PPARγ. These suppressions were unaltered by methylglyoxal. Surprisingly, Akt1 phosphorylation at Ser473 was abolished by combination of RAP6 overexpression and methylglyoxal treatment, even more than that by RAP6 overexpression alone. In addition, differentiated 3T3-L1 cells had elevated levels of methylglyoxal-derived adduct, MGH1, which was enhanced by high glucose condition.

Methylglyoxal increased 3T3-L1 differentiation in the current study. The highest increase was shown in 10-μM methylglyoxal treatment. Adipocyte enlargement was also significantly enhanced in methylglyoxal-treated cells. It was speculated that ERK1/2 activity was required for mitotic clonal expansion (MCE) and expression of transcription factors during the early phase of adipogenesis (Tang et al., 2005). This sequential induction gives rise to expression of late phase transcription factors, e.g. PPARγ and C/EBPα, and thereby terminal adipocyte differentiation (Lefterova & Lazar, 2009; Siersbaek & Mandrup, 2011). The current findings showed that methylglyoxal increased phosphorylation of ERK1/2 after induction of differentiation for 5 minutes as compared to the control. This suggests the stimulatory effect of methylglyoxal during the early phase of adipocyte differentiation. Methylglyoxal caused a marginal elevation of PPARγ

expressions as well as significant increased expression of C/EBPα-p42 and -p30 subunits in the current study. The findings indicate that methylglyoxal may be associated with stimulation of differentiation during the late phase. Protein kinase Akt which is a key component in preadipocyte differentiation (Calera et al., 1998; Kim et al., 2010; Maiuri et al., 2010; Xu & Liao, 2004), was found to be upregulated through phosphorylation at Ser473 in methylglyoxal-treated cells. In contrast, a recent study reported that methylglyoxal (500 μM) suppressed Akt activation and subsequent glucose uptake in 3T3-L1 cells (Afridi et al., 2016). As compared to the present study (10 μM), the methylglyoxal concentration was much higher which possibly gave rise to different effect on Akt activation. In addition, methylglyoxal significantly increased 3T3-L1 proliferation and accelerated cell cycle progression in the current study, consistently with the previous report by Jia and colleagues (Jia et al., 2012). Interestingly, a significant decrease in 3T3-L1 differentiation was observed in methylglyoxal treatment at 50, 150 and 200 μM. Even though gradually declined, methylglyoxal concentrations of 50 and 200 μM still appeared to stimulate 3T3-L1 proliferation. These findings suggest that methylglyoxal concentration may govern its effect of cell signaling events. Collectively, methylglyoxal at low concentration appear to promote both 3T3-L1 proliferation and differentiation.

RAP6 is a newly discovered guanine exchange factor specific to Rab5 (Hunker, Galvis, et al., 2006). Given that Rab5 activity has been reported to mediate insulin signaling (Hunker, Kruk, et al., 2006; Jozic, Blanco, & Barbieri, 2011), Rab5 activators such as RAP6 may be associated with signal transduction in insulin-responsive cells, including preadipocytes. In the current study, RAP6 overexpression inhibited 3T3-L1 differentiation, concomitant with a significant decrease in PPARγ expression.

Surprisingly, expression of C/EBPα-p42 and -p30 subunits was unaltered by RAP6 overexpression. These findings suggest that RAP6 may selectively mediate expression of adipogenic transcription factors. Phosphorylation of Akt1 at Ser473 was also inhibited by RAP6 overexpression in the current study. However, knockdown of RAP6 was previously reported to abrogate Akt activation in insulin-responsive NIH3T3 cells (Su et al., 2006). Possibly, RAP6 function may depend on intensity of its activity as well as cell types.

As RAP6 activity was found to mediate Akt activation, downstream signaling events may be altered. A possible event included glucose uptake by GLUT4. An *in vitro* study demonstrated that GLUT4 translocation to the plasma membrane was abolished by RAP6 overexpression in 3T3-L1 preadipocytes (Lodhi et al., 2007). It was proposed that RAP6 participated in regulation of GLUT4 trafficking between GLUT4 storage compartment and endosomes under basal condition (Lodhi et al., 2007). When RAP6 was overexpressed, this recycling of GLUT4 was augmented and became futile that, in turn, retained GLUT4 in the intracellular vesicles and impaired insulin-stimulated GLUT4 translocation (Lodhi et al., 2007). Furthermore, RAP6 overexpression possibly inhibits 3T3-L1 differentiation via Ras-GAP domain. Through GAP activity for GTPase protein Ras, RAP6 was able to deactivate Ras (Hunker, Galvis, et al., 2006) which is an upstream activator of Raf/MEK/ERK pathway (Marshall, 1995; Murholm, Dixen, & Hansen, 2010). Downregulation of the pathway may impede the initiation of differentiation during the early phase by ERK1/2.

Methylglyoxal significantly increased 3T3-L1 differentiation, expression of PPARγ, C/EBPα, and cell cycle progression in GFP-expressing control cells. However,

these stimulatory effects of methylglyoxal did not show in RAP6-overexpressing cells. The combination of RAP6 overexpression and methylglyoxal treatment abolished Akt1 activation more than that in a presence of RAP6 overexpression alone. These findings implicate that RAP6 activity may involve in methylglyoxal-mediated alteration of 3T3-L1 differentiation, especially in Akt1 phosphorylation. Previous studies suggest that methylglyoxal altered various signal pathways, including insulin-dependent cascades (Afridi et al., 2016; Chen et al., 2012; Jia et al., 2012; Yang et al., 2013). At least in part, methylglyoxal may rely on receptor trafficking which is modulated by RAP6 activity.

As previously reported, insulin-dependent production of phosphatidylinositol 3-phosphate (PI3P) by PI3K required RAP6 and Rab5 activities that, in turn, facilitated a recruitment of downstream effectors at the plasma membrane (Lodhi et al., 2008). Simultaneously, phosphorylated insulin receptors were internalized (Inoue, 1998; Wang, Balba, & Knutson, 1996) and thereby recruited intracellular effectors such as IRS1 and PI3K (Di Guglielmo et al., 1998; Kublaoui, Lee, & Pilch, 1995). These processes were speculated to be mediated by RAP6 and Rab5 (Hunker, Galvis, et al., 2006; Hunker, Kruk, et al., 2006; Su et al., 2006). As mentioned, intensity of RAP6 activity may dictate its function and subsequent cellular signaling events. Apart from the plasma membrane, Rab5 also led to PI3P production on early endosome membranes where more Rab5 and Rab5-effectors were recruited to maintain its function in endosome trafficking (Christoforidis et al., 1999). Generally, Rab GTPases are localized in a membrane-specific fashion to govern membrane trafficking at distinct sites. For example, Rab5 resides at the plasma membrane and early endosomes which enables its function in endocytosis and early endosome trafficking (Chavrier et al., 1990). Disassembling of

Rab5 is required to allow the next Rab protein to assemble with endosomal membranes and continue the trafficking sequence such as endosome recycling to the plasma membrane or endosome degradation. Overactivation of Rab5 by RAP6 overexpression may inhibit Rab5 dissociation from endosomal membranes that, in turn, interfere with sequential membrane trafficking. It was demonstrated that a constitutively active Rab5 increased early endosome enlargement, but simultaneously abolished receptor recycling back to the plasma membrane (Stenmark et al., 1994; Sun et al., 2012). Inhibition of receptor recycling possibly prevents insulin from sustaining its signal. In addition, RAP6-mediated Rab5 hyperactivation possibly increases the rate of endocytosis, which may promote futile internalization of inactivated receptors. According to previous studies, methylglyoxal affected cell proliferation and differentiation through insulin-dependent downstream proteins (Chen et al., 2012; Jia et al., 2012; Liu et al., 2013; Yang et al., 2013). Alteration of RAP6 activity possibly modify insulin signal transduction as well as that driven by methylglyoxal. It was demonstrated that RAP6 facilitated IRS and PI3K interaction which was required for Akt activation. Taken together, RAP6 overexpression possibly suppressed methylglyoxal-driven Akt activation by altering receptor trafficking and impairing activities of Akt upstream effectors.

Elevated MGH1 levels in differentiated 3T3-L1 cells were observed in the present study. This may be due to glucose uptake elicit by insulin in differentiation stimulant mix. Methylglyoxal can be endogenously produced through multiple pathways such as glycolysis. Glycolytic intermediates, e.g. glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, are speculated to be major substrates of methylglyoxal (Thornalley, 2005). Given that glucose predominantly enters glycolysis, insulin-

stimulated glucose uptake may lead to glycolytic flux and following methylglyoxal generation in differentiated 3T3-L1 cells. Elevated glucose can also lead to formation of methylglyoxal and advanced glycation end products through non-enzymatic glycation (Rabbani & Thornalley, 2012; Semchyshyn & Lushchak, 2012). The current findings showed that high glucose conditions (12 and 25 mM) significantly rose MGH1 levels in 3T3-L1 cells as compared to low glucose condition (5 mM). Supportively, previous studies reported methylglyoxal accumulation resulted from high glucose condition in cell cultures (Liu et al., 2013; Shinohara et al., 1998; Thornalley, 1988) and animal model (Schlotterer et al., 2009). Elevated methylglyoxal was also observed in participants with diabetes (Kong et al., 2014; McLellan, Thornalley, Benn, & Sonksen, 1994; Nemet et al., 2005; Turk et al., 2011; Turk, Vrdoljak, Misur, Trescec, & Benko, 2009). Collectively, intrinsic methylglyoxal production may be harnessed by abundance of substrates which possibly manipulate its effects on cellular events.

To summarize, low concentration of methylglyoxal increased 3T3-L1 differentiation, presumably through Akt activation and expression of transcription factors PPARγ and C/EBPα (Figure 12). Overexpression of RAP6 inhibited 3T3-L1 differentiation, even in a presence of methylglyoxal. The findings suggest that RAP6 activity, at least in part, may be able to modulate adipogenic stimulatory effect of methylglyoxal. Therefore, mechanisms by which RAP6 is required for methylglyoxal-driven preadipocyte differentiation deserve further investigation.

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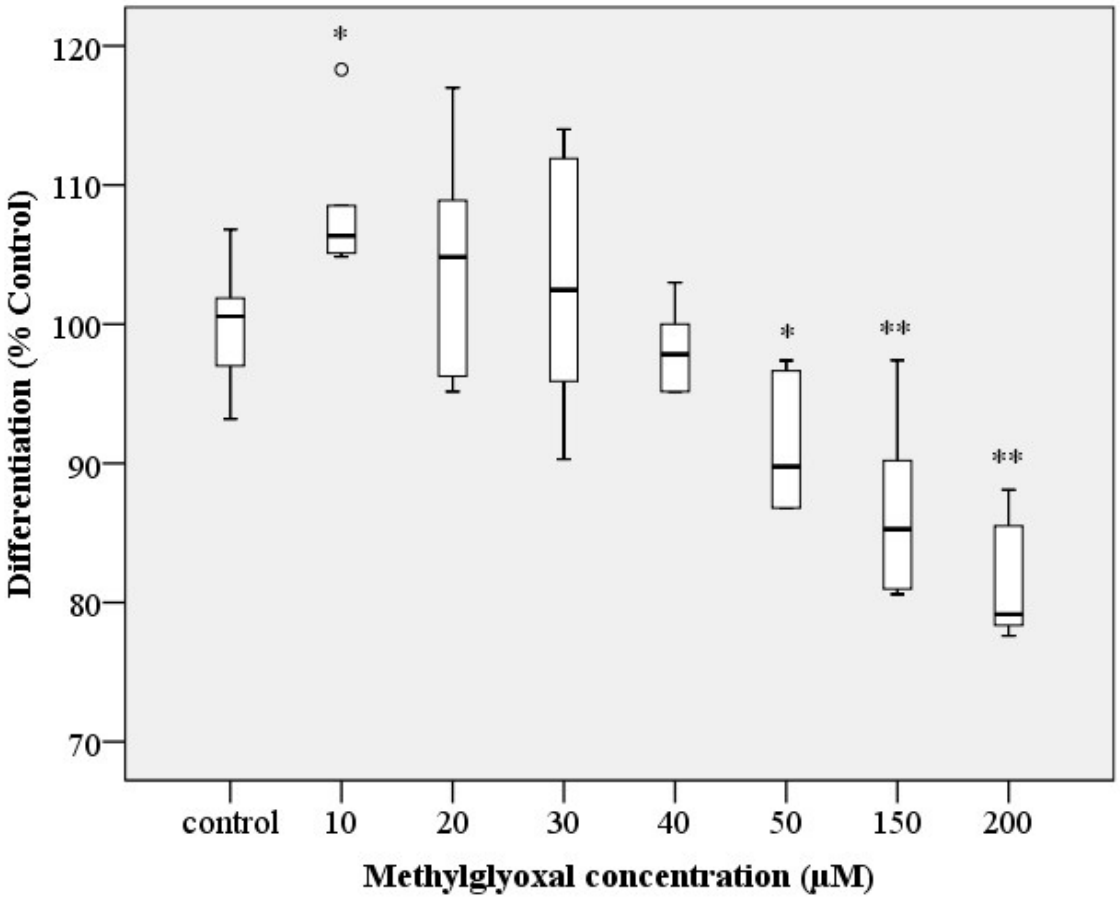

Figure 1. Effect of methylglyoxal on 3T3-L1 differentiation. Once confluence, 3T3-L1 cells were induced to differentiation with or without methylglyoxal (0-200 µM). The differentiated 3T3-L1 cells were then underwent oil red O staining. Data was presented in percentages (%) of arbitrary absorbance on 540 nm relative to that of the untreated control cells from two independent experiments (n=6 for each group). *p<0.05, **p<0.01 vs control by Mann-Whitney U test.

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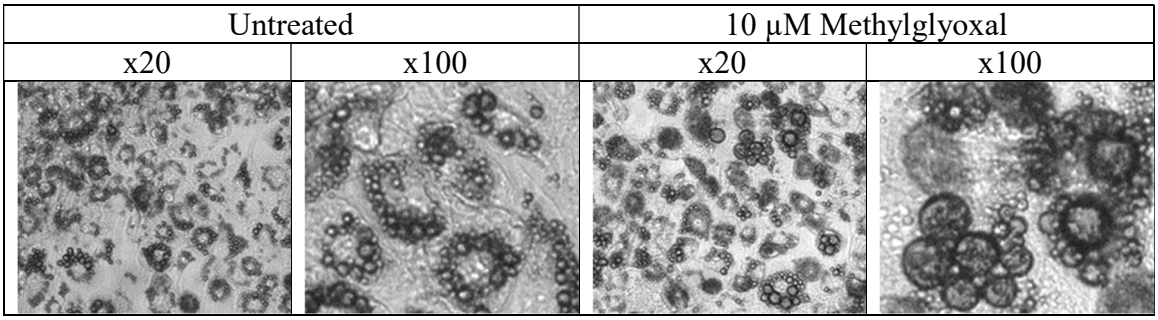

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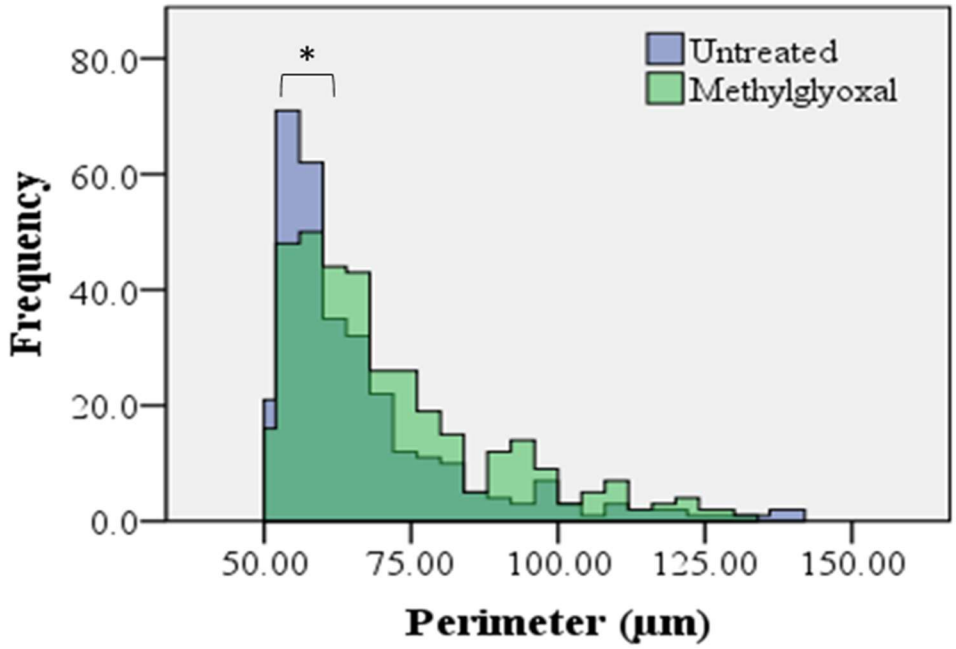


Figure 2. Effect of methylglyoxal on cell size of differentiated 3T3-L1 cells. 3T3-L1 cells were induced to differentiate with or without methylglyoxal (10 μM) and underwent oil red O staining. Then, the photos of stained cells were used for measurement of cell perimeter. (A) Photos of untreated and methylglyoxal-treated cells from oil red O staining was illustrated at magnification of x20 and x100. (B) The chart represented cell count for perimeter of untreated 3T3-L1 cells (blue area, n=310) and methylglyoxal-treated cells (green area, n=354). *p<0.05 vs untreated cells by Mann-Whitney U test.

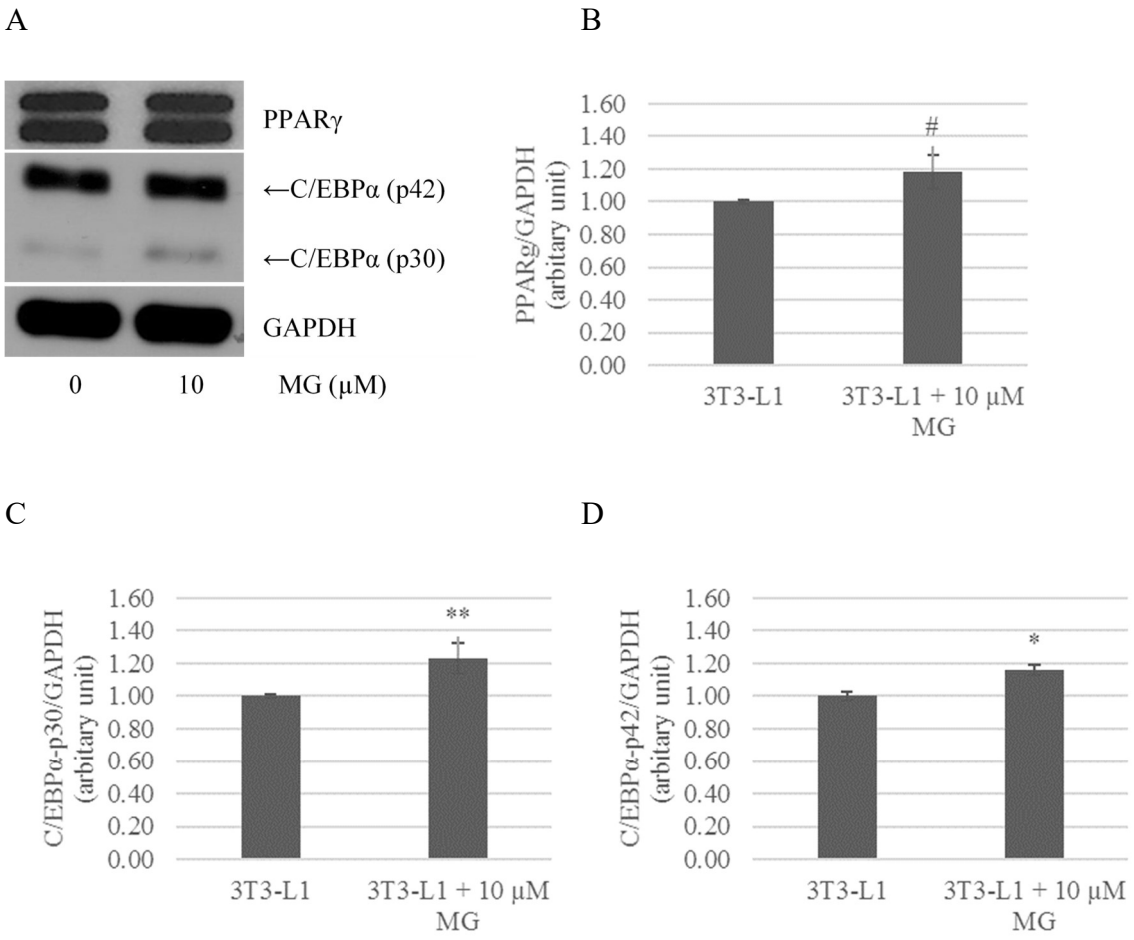


Figure 3. Effect of methylglyoxal on expression of adipogenic markers in 3T3-L1 cells. The 3T3-L1 cells were induced to fully differentiate with or without methylglyoxal (10 μM). Then the cells were harvested, lysed and subject to Western blot analysis. (A) Western blot of PPARγ, C/EBPα-p42/p30 was illustrated. A quantification of (B) PPARγ, (C) C/EBPα-p42, (D) C/EBPα-p30 expressions was shown corresponding to the Western blot results. Data represented arbitrary unit of protein expression as compared to the untreated cells from three independent experiments (n=3 for each group). ** $p<0.01$, * $p<0.05$, # $p<0.1$ vs control by Student's t-test. MG=methylglyoxal.

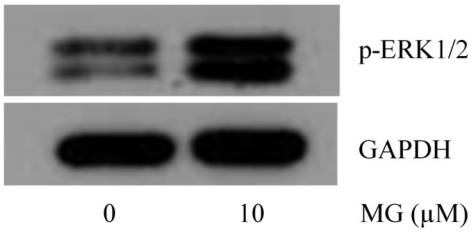
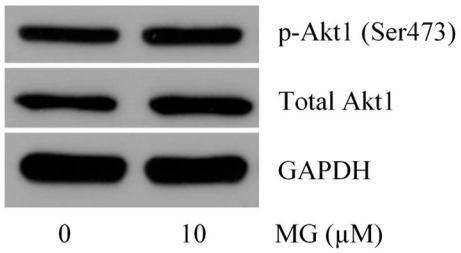


Figure 4. Effect of methylglyoxal on ERK1/2 phosphorylation in the early phase of 3T3-L1 differentiation. The differentiation of 3T3-L1 cells were induced for 5 minutes with or without 10 μM of methylglyoxal. Then the cells were lysed and subjected to Western blot analysis. Western blot of p-ERK1/2 was shown in an absence or presence of methylglyoxal. MG=methylglyoxal.

A



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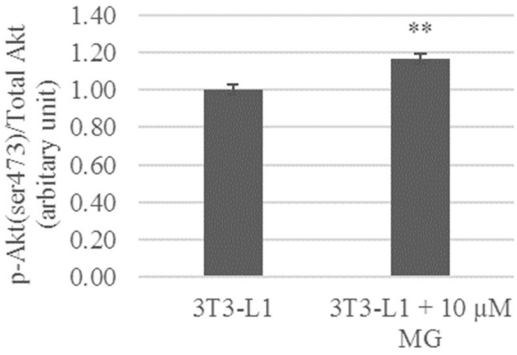


Figure 5. Effect of methylglyoxal on Akt1 phosphorylation in 3T3-L1 cells. The 3T3-L1 cells were induced to fully differentiate with or without methylglyoxal (10 μM). Then the cells were harvested, lysed and subject to Western blot analysis. (A) Western blot of p-Akt1 (Ser473) and total Akt1 was illustrated. (B) A quantification of p-Akt1-Ser473 expression was shown corresponding to the Western blot results. Data represented arbitrary unit of protein expression as compared to the untreated cells from three independent experiments (n=3 for each group). ** $p<0.01$ vs control by Student's t-test. MG=methylglyoxal.

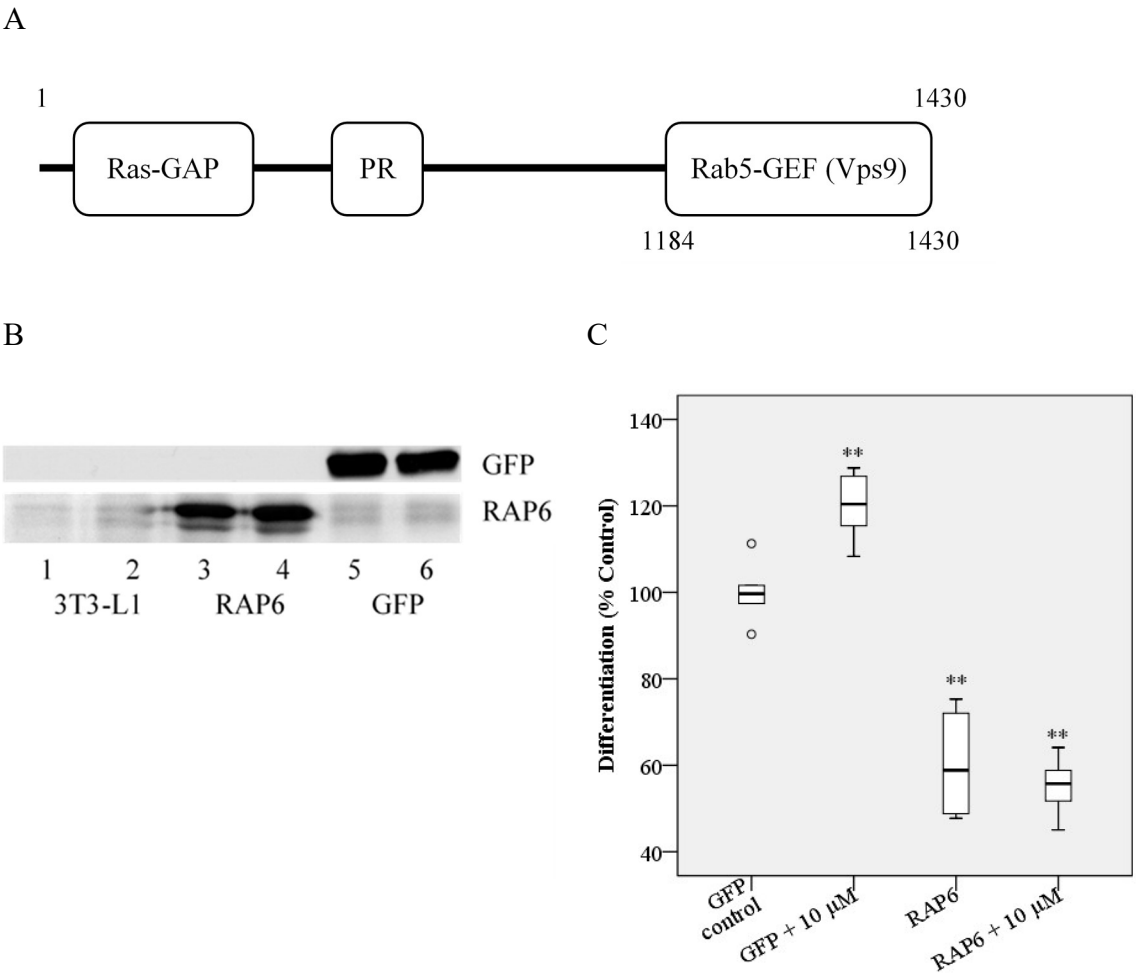


Figure 6. Effect of methylglyoxal and RAP6 overexpression on 3T3-L1 differentiation. (A) Amino acid sequence and constituting domains of RAP6 were illustrated. (B) Western blot of GFP and RAP6 in 3T3-L1 cells was shown in duplicate for each type of cells. Also, differentiation assay with or without 10 μM methylglyoxal was performed in GFP- and RAP-overexpressing cells, followed by oil red O staining. (C) Data represented percentages (%) of arbitrary absorbance on 540 nm as compared to that of the GFP control cells from two independent experiments (n=6 for each group). ** $p<0.01$ vs control by Mann-Whitney U test. MG=methylglyoxal, PR=proline-rich domain, Rab5-GEF=Rab5-specific guanine nucleotide exchange factor binding (Vps9) domain, Ras-GAP=Ras GTPase-activating protein binding domain.

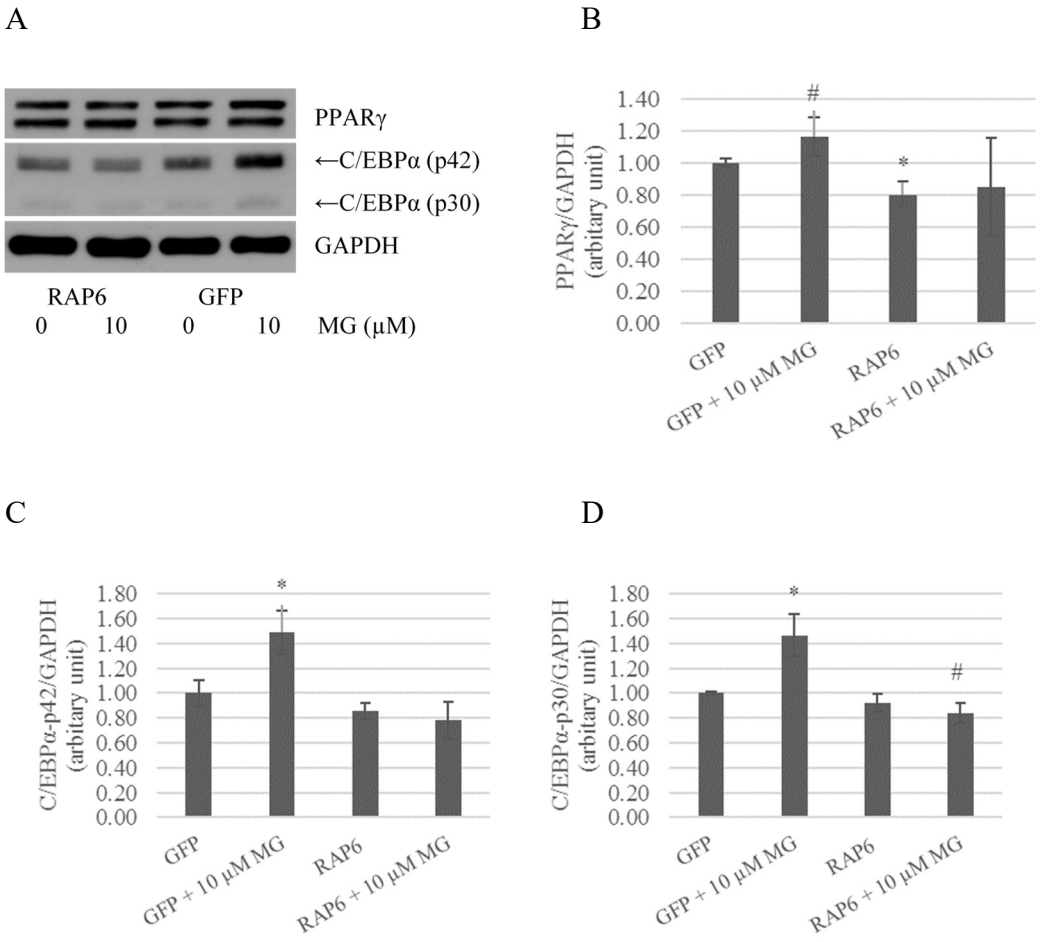


Figure 7. Effect of methylglyoxal and RAP6 overexpression on expression of adipogenic markers in 3T3-L1 differentiation. GFP- and RAP6-overexpressing cells were induced to differentiate with or without 10 μM of methylglyoxal. Then the cells were lysed and subjected to Western blot analyses. (A) Western blot of PPARγ, C/EBPα-p42, and C/EBPα-p30 in GFP- and RAP6-overexpressing cells, with or without 10 μM of methylglyoxal. A quantification of (B) PPARγ, (C) C/EBPα-p42 and (D) C/EBPα-p30 expressions was shown corresponding to the Western blot results. Data represented arbitrary unit of protein expression as compared to the untreated GFP-overexpressing cells from three independent experiments (n=3 for each group). *$p<0.05$, #$p<0.1$ vs untreated GFP-overexpressing cells by Student's t-test. MG=methylglyoxal.

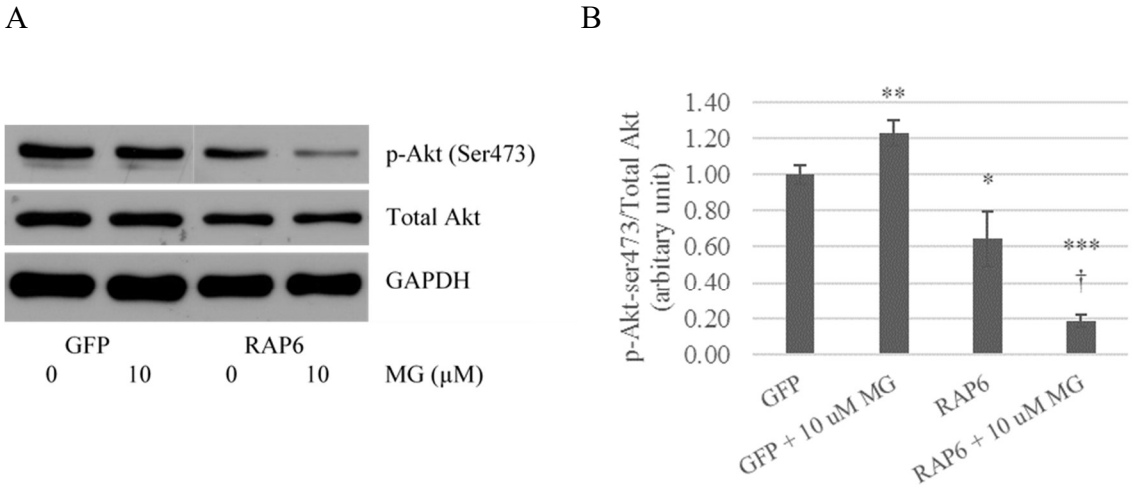


Figure 8. Effect of methylglyoxal and RAP6 overexpression on Akt1 phosphorylation (Ser473) in 3T3-L1 differentiation. GFP- and RAP6-overexpressing cells were induced to differentiate with or without 10 μM of methylglyoxal. Then the cells were lysed and subjected to Western blot analyses. (A) Western blot of p-Akt1 (Ser473) and total Akt1 in GFP- and RAP6-overexpressing cells, with or without 10 μM of methylglyoxal. (B) A quantification of p-Akt1 (Ser473) over total Akt1 was shown corresponding to the Western blot results. Data represented arbitrary unit of protein expression as compared to the untreated GFP-overexpressing cells from three independent experiments (n=3 for each group). *p<0.05, **p<0.01, ***p<0.001 vs untreated GFP control and †p<0.01 vs untreated RAP6-overexpressing cells by Student's t-test. MG=methylglyoxal.

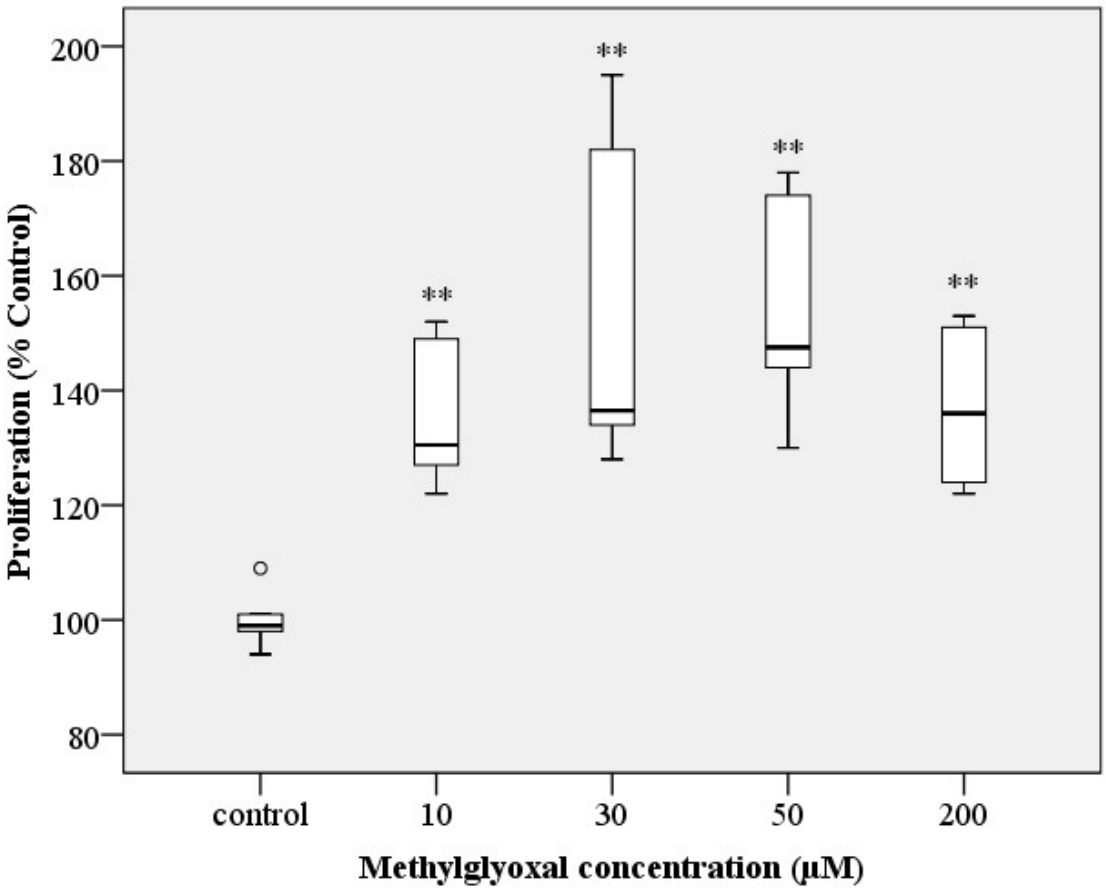


Figure 9. Effect of methylglyoxal on 3T3-L1 proliferation. The MTT assay with or without 10, 30, 50 and 200 μM methylglyoxal was performed. The cell proliferation of each group was presented in percentages (%) of absorbance on OD570 nm relative to that of the untreated control cells from two independent experiments (n=6 for each group). **p<0.01 vs control by Mann-Whitney U test.

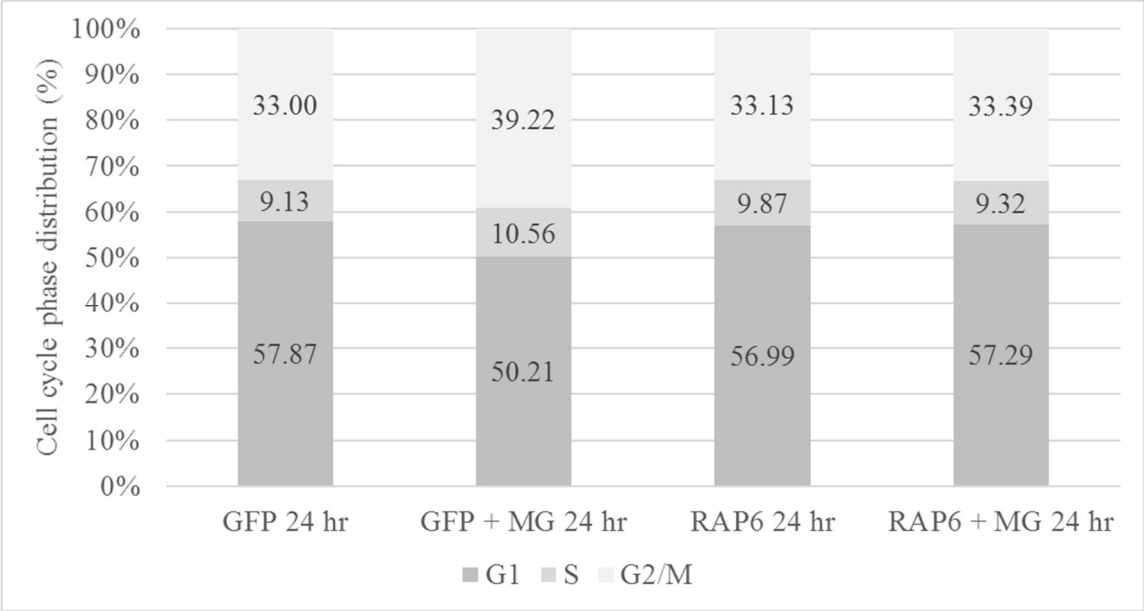
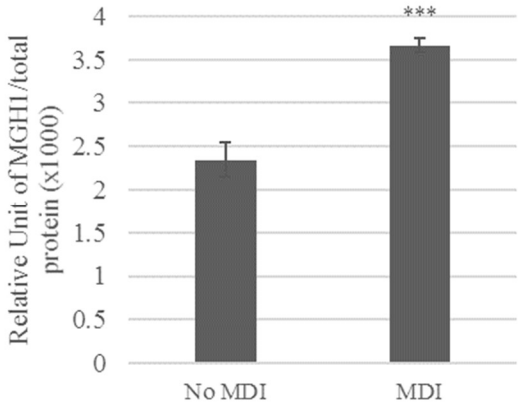


Figure 10. Effect of methylglyoxal and RAP6 overexpression on cell cycle phase distribution. Once 50% confluence, GFP- and RAP6-overexpressing cells were incubated in growth media with or without 10 μM of methylglyoxal for 24 hours. The cell samples were collected and thereby used for cell cycle analysis by flow cytometry. Data was expressed in percentage distribution (%) of cell cycle phases (G1, S, and G2/M) from three independent experiments (n=3 for each group). MG=methylglyoxal.

A



B

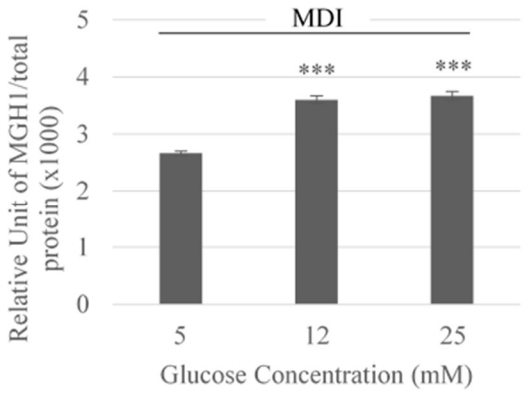


Figure 11. Effect of differentiation stimulant mix and glucose on cellular MGH1 formation in 3T3-L1 cells. (A) MGH1 levels of 3T3-L1 cells in an absence or presence of stimulants as described in METHOD. ***p<0.001 vs non-stimulated cells by Student's t-test. (B) MGH1 levels of 3T3-L1 cells induced to differentiate in media containing 5, 12 or 25 mM glucose. ***p<0.001 vs 5 mM glucose-treated cells by Student's t-test. Data was presented in arbitrary unit of MGH1 levels relative to total cellular protein concentrations (n=3 for each group). MDI=3-isobutyl-1-methylxanthine, dexamethasone and insulin, MGH1=methylglyoxal hydroimidazolone 1.

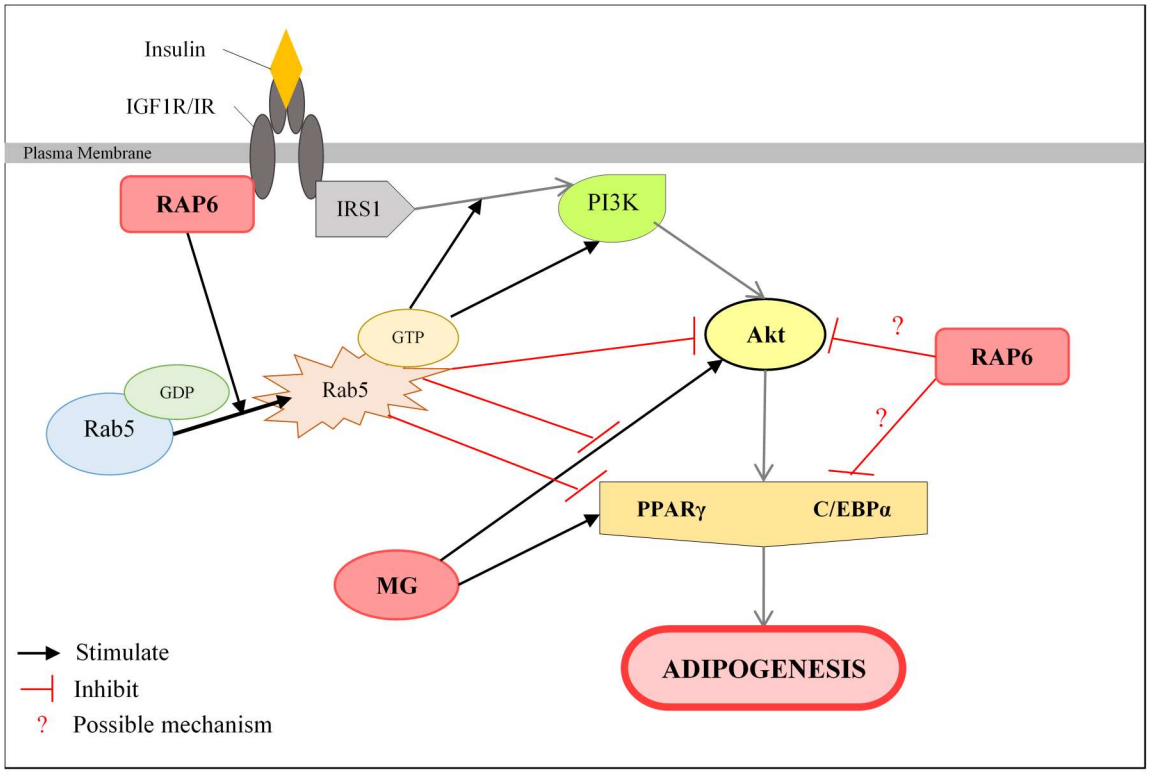


Figure 12. A possible model of insulin-dependent adipogenesis modulated by RAP6 and methylglyoxal. RAP6 possibly modulates Akt activity, expression of PPARγ and C/EBPα, and subsequent adipogenesis through Rab5 activation and/or direct interaction with the effectors. The findings suggest that methylglyoxal-induced adipogenesis may be regulated by RAP6 activity. Akt=protein kinase B; C/EBPα= CCAAT enhancer-binding protein α; GDP=guanosine diphosphate; GTP=guanosine triphosphate; IGF-IR=insulin-like growth factor I receptor; IR=insulin receptor; IRS1=insulin receptor substrate 1; MG=methylglyoxal; PI3K=phosphoinositide 3-kinase; PPARγ=peroxisome proliferator-activated receptor γ; RAP6=Rab5-activating protein 6.

CHAPTER IV

Relationship between Methylglyoxal Adduct and Fasting Plasma Glucose in Blacks with and without Type 2 Diabetes

ABSTRACT

INTRODUCTION: Methylglyoxal is a dicarbonyl compound reported to be associated with insulin resistance, diabetes, and other chronic illnesses. However, such observation among Black populations, including African Americans and Haitian Americans, remains scarce.

PURPOSE: The current study aimed to investigate the relationship between levels of serum methylglyoxal adduct and biomarkers of diabetes in African American and Haitian American participants, with and without type 2 diabetes.

METHODS: Participants were recruited by community outreach in Broward and Miami-Dade counties, Florida. The study included a total of 488 eligible participants (n = 234 non-diabetics and n = 254 diabetics) over 30 years of age who had complete data of serum methylglyoxal and fasting plasma glucose. Serum levels of prominent methylglyoxal adduct, methylglyoxal hydroimidazolone 1 (MGH1) were measured by using a commercially available competitive ELISA kit. Fasting plasma glucose levels were determined by hexokinase enzymatic methods, whereas whole blood A1c was measured by DCA2000+ system. Multiple regression analysis was used to examine the association of MGH1 with fasting plasma glucose and A1c.

RESULTS: In participants with diabetes, there was an increase in MGH1 levels of 0.24% for every 1% increase in fasting plasma glucose levels, after adjusting for pertinent variables (95% CI [0.02%, 0.46%], p=0.037). However, such association was not found

in controls (p=0.977). For every year increase in age, the geometric mean of MGH1 levels rose about 1.34% (95% CI [0.44%, 2.25%], p=0.004). Interestingly, the geometric mean of MGH1 levels for African Americans with type 2 diabetes was about 28.40% lower than that for Haitian Americans with type 2 diabetes, holding other variables constant (95% CI [6.01%, 45.39%], p=0.016). No significant association between MGH1 and A1c levels was shown in participants with (p=0.188) and without diabetes (p=0.808). CONCLUSION: The findings suggest that methylglyoxal may be linked to hyperglycemia and metabolic changes in type 2 diabetes, and may differently impact the development of the disease across age and Black subgroups.

INTRODUCTION

Methylglyoxal is a dicarbonyl compound that can be produced endogenously from the breakdown of macronutrients (Phillips & Thornalley, 1993). Methylglyoxal is mainly converted from glycolytic intermediates, including glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (Kalapos, 1999; Phillips & Thornalley, 1993). In lesser amount, methylglyoxal can be generated through lipid peroxidation (Agadjanyan, Dugin, & Dmitriev, 2006) and metabolism of threonine (Dhar, Desai, Kazachmov, Yu, & Wu, 2008) and ketone bodies (Beisswenger, Howell, Nelson, Mauer, & Szwergold, 2003; Nemet, Varga-Defterdarović, & Turk, 2006). It is hypothesized that intracellular methylglyoxal transports to plasma by passive diffusion (Rabbani & Thornalley, 2015). Greater than 95% of methylglyoxal is reversibly bound to plasma proteins, which can lead to formation of advanced glycation end products (AGEs) (Rabbani & Thornalley, 2015). Free amino groups of arginine and lysine are main target sites to which methylglyoxal interacts (Kalapos, 2008). For example, the reaction between

methylglyoxal and arginine produces N_{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-L-ornithine (methylglyoxal hydroimidazolone 1; MGH1), which is one of quantitatively prominent adducts from dicarbonyl compounds in blood and tissues (Thornalley, 2005).

Typically, methylglyoxal is degraded by glyoxalase system which is a main sequential pathway responsible for degradation of dicarbonyl compounds (Thornalley, 2003). The glyoxalase system is a ubiquitous set of metalloenzymes which comprises glyoxalase-1, glyoxalase-2, and cofactors including reduced glutathione and reduced zinc ion (Thornalley, 2003; Vander Jagt & Hunsaker, 2003). Binding between methylglyoxal and reduced glutathione forms a non-toxic intermediate hemithioacetal that is subsequently detoxified to S-lactoylglutathione by glyoxalase-1 (Thornalley, 2003). Glyoxalase-2 converts S-lactoylglutathione to D-lactate, simultaneously recycling back the reduced glutathione (Thornalley, 2003). Apart from D-lactate, the conversion of methylglyoxal eventually to pyruvate and other metabolites was speculated to be very small as compared to the metabolites of methylglyoxal (Best & Thornalley, 1999).

Methylglyoxal has been widely studied due to pathological relevance, such as in relation to insulin resistance and diabetes. Elevated methylglyoxal levels in blood (Kong et al., 2014; McLellan, Thornalley, Benn, & Sonksen, 1994; Nemet, Turk, Duvnjak, Car, & Varga-Defterdarović, 2005) and urine (Turk, Čavlović-Naglić, & Turk, 2011; Turk, Vrdoljak, Misur, Trescec, & Benko, 2009) were shown in participants with diabetes or impaired glucose metabolism (Maessen et al., 2015). Also, changes in activity (McLellan et al., 1994) and mRNA expression of glyoxalase enzymes (Uribarri et al., 2015) were reported under pathological condition. The evidence suggests hyperglycemic condition possibly increases production and suppresses degradation of methylglyoxal.

Methylglyoxal-derived AGEs increased in individuals with diabetes, which may be due to hyperglycemia-induced methylglyoxal accumulation (Ahmed, Babaei-Jadidi, Howell, Thornalley, & Beisswenger, 2005). Additionally, methylglyoxal appeared to impair insulin signaling (Dhar, Dhar, Jiang, Desai, & Wu, 2011; Fiory et al., 2011), decrease function, and cause apoptosis in pancreatic beta-cells *in vitro* (Bo et al., 2016; Gao, Liu, et al., 2016). Such effects lead to decrease in cellular response to insulin stimulation, and reduce insulin secretion by beta-cells (Best, Miley, Brown, & Cook, 1999; Cook et al., 1998; Dhar et al., 2011; Gao, Liao, et al., 2016). Taken together, the findings suggest the association of methylglyoxal with insulin resistance and beta cell dysfunction which are main hallmarks of diabetes.

Diabetes remains one of major causes of death in the United States (National Center for Health Statistics, 2016). Available evidence indicate that Blacks are at high risk for aberrant cardiometabolic illnesses including diabetes (National Center for Health Statistics, 2016). Age-adjusted rates of death caused by diabetes for Black population were about 1.9 times higher than that for White population (Kochanek, Murphy, Xu, & Tejada-Vera, 2016). In 2015, age-adjusted prevalence of diabetes among adults aged 18 and over was 13.1% in non-Hispanic Blacks or African Americans only (Blackwell & Villarroel, 2016). Correspondingly, Black population tended to have 4 or more chronic conditions than White and other populations (National Center for Health Statistics, 2016). Obesity and cigarette smoking are widely accepted as important contributing factors for the development of diabetes. The percent of overweight or obese in non-Hispanic Blacks is increasing over the years, at a greater rate than Whites (National Center for Health Statistics, 2016). The prevalence appeared to be more pronounced in Black females than

Black males (National Center for Health Statistics, 2016). Black males had higher rates for cigarette smoking than White males (National Center for Health Statistics, 2016). These statistics indicate the need for further scientific studies and public health programs to tackle chronic illnesses in all subpopulations, including Blacks.

Due to African descent, Haitian Americans and African Americans are typically considered as non-Hispanic Blacks and not categorized separately. In fact, some modifiable factors and genetic variations of Haitian Americans and African Americans are heterogeneous. A meta-analysis revealed that higher A1c levels were observed across studies for African American as compared to Whites (Kirk et al., 2006). Specifically, Haitians had mean A1c levels higher than African Americans and non-Hispanic Whites (Vimalananda, Rosenzweig, Cabral, David, & Lasser, 2011) and tended to have impaired fasting glucose (Rosen, Sharp, Abad, Doddard, & Rosen, 2007). The prevalence of diabetes was 33% among Haitian Americans (Rosen et al., 2007), which was even greater than the overall age-adjusted percentage for non-Hispanic Blacks (Blackwell & Villarroel, 2016). In addition, previous studies suggest that subgroups of non-Hispanic Blacks have high genetic diversity across individuals and different groups across parts of the United States. Populations in Haiti and African Americans shared substantial proportions of African and European ancestry (Bryc, Durand, Macpherson, Reich, & Mountain, 2015; Simms et al., 2012). Low proportion of Native American ancestry was detected in self-identified African Americans resided in the United States (Bryc et al., 2015), yet hardly determined in Haitian populations (Simms et al., 2012). These observations suggest that the risk for chronic diseases in Black subgroups is possibly different and should be examined separately. However, there is a scarcity of evidence in

the relationship between methylglyoxal and risk factors of chronic diseases in Black population. Therefore, the current study aimed to examine the association of methylglyoxal and biomarkers for diabetes in Haitian African and African American participants with and without type 2 diabetes.

METHOD

Participants

Data and de-identified blood samples were retrieved from the cross-sectional study conducted on Haitian American and African American participants by Huffman et al. (2013). Haitian American participants were recruited by community-based approaches. The recruitment took place in Miami-Dade and Broward counties, Florida. Invitational letters explaining the study were mailed to African American participants by using a mailing list (Knowledge Base Marketing, Inc., Richardson, TX, USA). Health professionals and diabetes educators receiving the flyer were requested their assistance to enroll individuals with type 2 diabetes. Faculty, staff and students at Florida International University (FIU) received the flyers explaining the protocol and were asked for their cooperation. Advertisements were published in local newspapers and high-trafficking areas. Radio advertisements were announced on local Creole stations.

Inclusion criteria of the parent study consisted of self-identified Haitian American and African American males and females; age 30 years or older; absence or presence of type 2 diabetes; free of thyroid disorders, coronary heart disease, chemo- or radiation therapy, major psychiatric disorders, and HIV/AIDS; not pregnant or lactating. By using an initial phone interview, potential participants were informed about the study purpose and determined the age and gender. To ascertain type 2 diabetes status, the participants

were asked for the duration of diagnosis and initial treatment regimens. Eligible individuals were invited to the Human Nutrition Laboratory at FIU to participate in the study. Participants were instructed to refrain from smoking, consuming any food or beverages except water, and any unusual exercise for at least eight hours prior to collection of blood samples. All participants gave written informed consents in either English or Creole. The study was approved by the Institutional Review Board at FIU prior to measurement of blood samples.

Sample Size

Sample size was calculated by using G*Power software (Faul, Erdfelder, Buchner, & Lang, 2009). By using a medium conventional effect size to produce an equal number of controls and cases, the total sample size was estimated at 128 individuals based on independent t-test, whereas multiple linear regression yielded a total of 55 participants. A total of 507 target participants (Haitian American=258: non-diabetics=120, diabetics=138; African American=249: non-diabetics=120, diabetics=129) from the parent study was adequate for both calculated sample sizes to meet the predetermined 80% statistical power at significance level of 0.05.

Biochemical Analysis

Approximately 300 microliters of de-identified serum from the parent study were used for methylglyoxal measurement. A prominent methylglyoxal adduct, MGH1, in blood samples was measured by OxiSelect™ MG competitive ELISA kit (Cell Biolabs, Inc., San Diego, CA, USA). The quantity of MGH1 was compared to a standard curve of predetermined MG-BSA. All blood samples were stored at -80 C^{o}. Existing data of other biomarkers was retrieved from the parent study. Briefly, hexokinase enzymatic method

was used to measure fasting plasma glucose (FPG). Whole blood A1c was measured by DCA2000+ system (Bayer HealthCare, Whippany, NJ, USA). This method is reliable as evidenced by 99% correlation with high-standard HPLC method.

Anthropometric data

Participant's height was measured when the participant stood upright without shoes. Weight measurement was taken with the participant wearing light clothes by using a SECA clinical scale (SECA Corp, Columbia, MD, USA). Body mass index (BMI) was calculated as weight (kg)/height (m^2).

Socio-demographic data

Socio-demographic data regarding age, gender, ethnicity were collected by using a standardized questionnaire.

Statistical Analysis

Values were presented in mean ± standard deviation or percentages. Continuous variables were analyzed by independent t-test, whereas Chi-square test was employed for categorical variables. Multiple regression analysis was used to further examine the relationship between serum MGH1 levels and other biomarkers, including FPG and A1c. Age, gender, ethnicity, BMI, and diabetes status were potential variables that were adjusted as appropriate during data analysis. Due to skewed distribution, levels of MGH1, FPG, A1c, and BMI underwent logarithm transformation. The log-transformed data were used in all analyses. Tests were considered statistically significant if *p*-value was less than 0.05. All data were analyzed by using SPSS version 23.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

In the current study, eligible participants were individuals who had complete data of serum MGH1, FPG 500 mmol/l or less, and BMI between 18.5 kg/m^2 and 60 kg/m^2. A total of 488 participants (n = 234 controls and n = 254 cases) were included in the data analyses, which was adequate to achieve 80% power.

Characteristics of study participants

The participants with type 2 diabetes tended to be older (p<0.001) and had higher lnBMI (p<0.001), lnFPG (p<0.001), and lnA1c (p<0.001) than those without diabetes (Table 1). The levels of lnMGH1 were marginally higher in the participants with diabetes as compared to those without diabetes (p=0.057).

Relationship of MGH1 with FPG and A1c in study participants

In unadjusted model, MGH1 levels were positively associated with levels of FPG (B=0.273, 95% CI [0.064, 0.481], p=0.011) and A1c (B=0.337, 95% CI [0.023, 0.651], p=0.036). However, the relationships of MGH1 with FPG (p=0.118) and A1c (p=0.419) lost insignificance after controlling for age, gender, ethnicity, diabetes status, lnBMI, smoking status, and two-way interactions among gender, ethnicity, and diabetes status.

Relationship between MGH1 and FPG stratified by diabetes status

To determine whether relationships of MGH1 with diabetes parameter were existed in subgroups of participants, stratification by diabetes status was performed prior to further multiple regression analyses. There was no significant relationship of MGH1 with FPG in control participants, either in unadjusted (p=0.977) or adjusted model (p=0.977; Table 2). In participants with type 2 diabetes, the significantly positive relationship between MGH1 and FPG was observed in unadjusted model (p=0.027) and

such association remained after controlling for age, gender, ethnicity, interaction of gender by ethnicity, lnBMI, and smoking status. In adjusted model, the increase in MGH1 levels in participants with diabetes was about 0.24% when fasting plasma glucose levels increased by 1% (95% CI [0.02%, 0.46%], p=0.037). For every year increase in age, the geometric mean of MGH1 levels rose about 1.34% (95% CI [0.44%, 2.25%], p=0.004). The geometric mean of MGH1 levels for African Americans was about 28.4% lower than that for Haitian Americans, holding other variables constant (95% CI [6.01%, 45.39%], p=0.016). There was no significant interaction effect between gender and ethnicity in the model (p=0.148).

The adjusted multiple regression analysis showed insignificant relationship between MGH1 and A1c levels in participants without diabetes, controlling for age, gender, ethnicity, interaction of gender by ethnicity, lnBMI, and smoking status (p=0.808; Table 3). Similarly, there was no relationship between MGH1 and A1c levels in participants with type 2 diabetes after adjusting for age, gender, ethnicity, interaction of gender by ethnicity, lnBMI, and smoking status (p=0.188). In individuals with diabetes, the geometric mean of MGH1 levels was positively associated with age (B=0.014, 95% CI [0.005, 0.023], p=0.003). African American participants with diabetes were more likely to have lower MGH1 levels as compared to Haitian American counterparts, holding other variables constant (B=-0.323, 95% CI [-0.597, -0.048], p=0.021).

DISCUSSION

Serum concentrations of dominant methylglyoxal adduct, MGH1, was marginally higher in participants with types 2 diabetes than controls in this study. A positive

relationship between levels of MGH1 and FPG was seen only in individuals with type 2 diabetes. Also, increasing years of age and being Haitian American positively influenced serum concentrations of MGH1 in participants with type 2 diabetes. However, there was no significant relationship between levels of MGH1 and A1c.

An interest in methylglyoxal has been increasing due to its pathological significance, especially in relation to insulin resistance and diabetes. Consistent with previous observations (Ahmed et al., 2005; Kilhovd et al., 2003), the current study found partially increased levels of MGH1 adduct in participants with type 2 diabetes as compared to the controls. Methylglyoxal levels were markedly elevated in blood (Kong et al., 2014; McLellan et al., 1994; Nemet et al., 2005) and urine of participants with diabetes or impaired glucose metabolism in other studies (Maessen et al., 2015; Turk et al., 2011, 2009).

High glucose-induced methylglyoxal augmentation was also shown in previous cell culture studies (Liu, Desai, Wang, & Wu, 2013; Shinohara et al., 1998; Thornalley, 1988) and animal studies (Schlotterer et al., 2009). The findings suggest that the increased availability of substrates, such as glucose, likely contributes to elevated methylglyoxal levels. However, the association between MGH1 and fasting plasma glucose was inconsistent in the literature (Ahmed et al., 2005; Kilhovd et al., 2003; Kong et al., 2014). The current study found significantly positive relationship of MGH1 and FPG levels in Black diabetic participants. The significant findings may be partially due to participants' characteristics of the study. As compared to Whites, Non-Hispanic Blacks had higher prevalence of prediabetes and diabetes (Bullard et al., 2013; Menke, Casagrande, Geiss, & Cowie, 2015; Selvin, Parrinello, Sacks, & Coresh, 2014). Less

glycemic control and adherence to treatment also observed in non-Hispanic Blacks than Whites (Selvin et al., 2014). These characteristics contribute to persistent hyperglycemia, which likely promotes methylglyoxal accumulation.

The increase in methylglyoxal levels may results from triosephosphate flux, converted from glucose via glycolysis and from fructose and sorbitol through polyol pathway. Elevated glucose and fructose can also lead to formation of methylglyoxal and advanced glycation end products (AGEs) through non-enzymatic glycation (Rabbani & Thornalley, 2012; Semchyshyn & Lushchak, 2012), which is likely accelerated in aberrant cardiometabolic condition like diabetes. Additionally, the marginal increase in MGH1 levels in the present study may reflect disproportion between methylglyoxal production and degradation in diabetes. A recent human study demonstrated that methylglyoxal accumulation was correlated with suppression of glyoxalase-mRNA expression in obese participants with at least one metabolic syndrome risk factor as compared to those with none (Uribarri et al., 2015). In contrast, the increased activity of glyoxalase-1 and glyoxalase-2 was found in isolated erythrocytes of individuals with type 2 diabetes, corresponding to elevated blood concentrations of methylglyoxal (McLellan et al., 1994). The increase in glyoxalase system activity may be due to compensation for the decline in gene expression, and chronic exposure of high circulating methylglyoxal under hyperglycemic condition. In addition, the two selected studies conducted on different groups of individuals, which may contribute to differential findings in activity of glyoxalase system.

Similar to other studies, null relationship between levels of MGH1 and A1c was observed in the present study. However, Kong et al. (2014) found a strong correlation

between levels of methylglyoxal and A1c in newly diagnosed with type 2 diabetes. The difference in duration of diabetes may contribute to such inconsistency. Among the studies with insignificant relationship, the participants were diagnosed with diabetes ranged from 4 to 26 years, in which glyoxalase system may increase its activity to adjust for prolonged exposure to high glucose (McLellan et al., 1994). The relationship between methylglyoxal and A1c levels may be stronger in recently diagnosed individuals than those with chronic condition. In addition, Kong and colleagues utilized the HPLC-MS/MS method to determine total methylglyoxal levels (Kong et al., 2014). However, the current study used an immunoassay for measurement of major adduct MGH1, which may not be comparable. Dietary factors may also impact methylglyoxal levels. As previously reported, methylglyoxal was positively associated with postprandial blood glucose excursion, but not with A1c (Beisswenger et al., 2001; Maessen et al., 2015).

The current study found a positive relationship between MGH1 levels and ages of the participants with type 2 diabetes, after adjusting for demographic and diabetes-related parameters. Supportively, elevation of methylglyoxal adducts in human lens was significantly correlated with participant's age (Ahmed, Brinkmann Frye, Degenhardt, Thorpe, & Baynes, 1997). This may be described, in part, by glyoxalase-1 activity. A trend of glyoxalase-1 activity in human aortic tissues appeared to decrease with ages (Kirk, 1960). In individuals aged 50 to 79 years, significantly lower activity of glyoxalase-1 was found in arteriosclerotic tissues than normal tissue section (Kirk, 1960). In this study, the positive association between serum MGH1 levels and age in participants with diabetes may reflect the decreased activity of glyoxalase-1, exacerbated by diabetes.

In diabetic participants, African Americans were more likely to have lower MGH1 levels than Haitian Americans. Such dissimilarity may be partially due to difference in glycemic control and variation in glyoxalase-1 polymorphism among ethnicities. Black population features diversity of ancestry and language, which contribute to unique characteristics and genetics across Black subgroups. Non-Hispanic Blacks were more likely to have multiple chronic conditions and diseases than Whites (National Center for Health Statistics, 2016), leading them to become a high risk population. As compared to African American and non-Hispanic White counterparts, Haitian Americans with diabetes had poor glycemic control as evidenced by significantly higher levels of FPG and A1c (Vimalananda et al., 2011). This may strengthen the association between levels of MGH1 and FPG in Haitian American participants as compared with African American participants in the current study. In addition, genetic variation may be another factor to describe the diverged findings. The significant difference in erythrocyte GLO allele frequencies between White and Black Americans was reported by Weitkamp (1976). Unfortunately, the study did not have further analysis to examine GLO frequency among subgroups of Black Americans. Another study reported similarity of erythrocyte GLO allele frequency in Black Americans and Bantu-speaking Black habitants in South Africa (Bender, Frank, & Hitzeroth, 1977). The study mentioned significant differences in the GLO allele frequency among some Black subpopulations (Bender et al., 1977). Additionally, populations from Haiti and African Americans had variation in proportions of African and European ancestry (Bryc et al., 2015; Simms et al., 2012). A recent genome-wide study revealed that a small proportion of Native American ancestry was detected in African Americans resided in the United

States (Bryc et al., 2015), whereas it was hardly found in population from Haiti (Simms et al., 2012). Taken together, the observation suggest GLO allele frequency in African Americans may be distinct from those in Haitian Americans which, in turn, lead to difference in glyoxalase-1 activity and following methylglyoxal degradation.

This study had some limitations. Due to the observational study design, changes in clinical and behavioral factors were not observed overtime. Therefore, it lacked to generate causality and provide a complete phenomenon. Also, the participants were only recruited from two counties in South Florida, which likely contributed to low generalizability. However, strengths of the study are worth mentioning. Apart from patient report, diabetes status was ascertained using standard criteria established by American Diabetes Association. This is the first study that investigated the relationship between methylglyoxal and biomarkers of diabetes particularly in Haitian Americans and African Americans. The evidence contributed to existing knowledge concerning Blacks, which provides the better understanding in health disparities among subpopulations.

In conclusion, serum levels of prominent adduct MGH1 was marginally higher in Haitian American and African participants with type 2 diabetes than those without diabetes. Levels of MGH1 was positively associated with fasting plasma glucose in diabetic participants, suggesting the link of methylglyoxal to hyperglycemia. The relationship between MGH1 and biomarkers of diabetes appeared to be distinct across age and ethnicities. This may imply the needs of invention studies and public health programs for specific subpopulations. Future studies are required to warrant and elucidate the true nature of such relationship.

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Table 1. Characteristics of the participants

Variable	without diabetes (n=234)	with diabetes (n=254)	*p*-value
Age (years)	52.59 ± 10.00	56.00 ± 10.14	<0.001*
Gender, n (%)			0.381
Female	116 (49.6)	136 (53.5)	
Male	118 (50.4)	118 (46.5)	
Ethnicity, n (%)			0.868
Haitian Americans	118 (50.4)	130 (51.2)	
African Americans	116 (49.6)	124 (48.8)	
BMI (kg/m^2)	29.91 ± 6.06	32.46 ± 7.17	<0.001*
lnBMI	3.38 ± 0.19	3.46 ± 0.21	<0.001*
Smoke (%)			0.555
Yes	54 (23.1)	53 (20.9)	
No	180 (76.9)	201 (79.1)	
FPG (mmol/l)	97.28 ± 15.08	151.71 ± 64.88	<0.001*
lnFPG	4.57 ± 0.15	4.94 ± 0.39	<0.001*
A1c (%)	5.92 ± 0.45	8.03 ± 2.27	<0.001*
lnA1c	1.78 ± 0.08	2.05 ± 0.26	<0.001*
MGH1 (μg/ml)	2.29 ± 2.50	2.36 ± 2.44	0.764
lnMGH1	0.42 ± 0.93	0.56 ± 0.73	0.057

Continuous variables were expressed as mean ± standard deviation (SD), while categorical variables were expressed as n (%). ln=natural log-transformed; A1c=hemoglobin A1c; BMI=body mass index; FPG=fasting plasma glucose; MGH1=methylglyoxal hydroimidazolone 1; *p<0.05 is considered statistically significant.

Table 2. Multiple regression analysis for relationship of lnMGH1 with lnFPG stratified by diabetes status

Participants without diabetes (n=234)				
Variables	B	SE	95% CI	*p*-value
Unadjusted model				
lnFPG	-0.012	0.416	-0.831, 0.807	0.977
Adjusted model[a]				
Age	-0.006	0.007	-0.019, 0.006	0.328
Gender (male)	0.033	0.396	-0.748, 0.813	0.934
Ethnicity (AA)	-0.063	0.190	-0.437, 0.312	0.741
Gender*ethnicity (male*AA)	0.001	0.252	-0.495, 0.498	0.996
lnBMI	0.041	0.343	-0.635, 0.718	0.904
Smoking status (yes)	0.100	0.169	-0.233, 0.433	0.555
lnFPG	0.013	0.431	-0.836, 0.861	0.977
Participants with diabetes (n=254)				
Variables	B	SE	95% CI	*p*-value
Unadjusted model				
lnFPG	0.259	0.116	0.030, 0.487	0.027*
Adjusted model[b*]				
Age	0.013	0.005	0.004, 0.023	0.004*
Gender (male)	-0.238	0.285	-0.799, 0.323	0.404
Ethnicity (AA)	-0.334	0.138	-0.605, -0.062	0.016*
Gender*ethnicity (male*AA)	0.264	0.182	-0.094, 0.623	0.148
lnBMI	0.423	0.256	-0.081, 0.927	0.100
Smoking status (yes)	-0.046	0.121	-0.285, 0.193	0.707
lnFPG	0.240	0.114	0.015, 0.466	0.037*

AA=African American; B=coefficient, SE=standard error, CI=confidence interval, ln=natural log; BMI=body mass index; FPG=fasting plasma glucose; MGH1=methylglyoxal hydroimidazolone 1, *$p<0.05$ is considered statistically significant.

[a]Model Summary: Adjusted R^2=-0.023, $F(7,226)=0.256$, $p=0.970$.

[b]Model Summary: Adjusted R^2=0.057, $F(7,246)=3.169$, $p=0.003$.

[a,b]The model included age, gender, ethnicity, interaction of gender by ethnicity, lnBMI, smoking status, and lnFPG.

Table 3. Multiple regression analysis for relationship of lnMGH1 with lnA1c stratified by diabetes status

Participants without diabetes (n=234)				
Variables	B	SE	95% CI	*p*-value
Unadjusted model				
lnA1c	0.013	0.800	-0.831, 0.807	0.987
Adjusted model[a]				
Age	-0.007	0.007	-0.020, 0.006	0.307
Gender (male)	0.035	0.395	-0.745, 0.814	0.930
Ethnicity (AA)	-0.061	0.188	-0.433, 0.310	0.745
Gender*ethnicity (male*AA)	0.016	0.250	-0.477, 0.509	0.950
lnBMI	0.007	0.355	-0.693, 0.708	0.983
Smoking status (yes)	0.107	0.170	-0.228, 0.443	0.529
lnA1c	0.285	0.884	-1.457, 2.028	0.747
Participants with diabetes (n=254)				
Variables	B	SE	95% CI	*p*-value
Unadjusted model				
lnA1c	0.257	0.178	-0.094, 0.608	0.150
Adjusted model[b*]				
Age	0.014	0.005	0.005, 0.023	0.003*
Gender (male)	-0.255	0.288	-0.823, 0.313	0.377
Ethnicity (AA)	-0.323	0.139	-0.597, -0.048	0.021*
Gender*ethnicity (male*AA)	0.271	0.184	-0.091, 0.633	0.141
lnBMI	0.386	0.258	-0.123, 0.895	0.136
Smoking status (yes)	-0.063	0.122	-0.303, 0.178	0.608
lnA1c	0.243	0.180	-0.113, 0.598	0.180

AA=African American; B=coefficient, SE=standard error, CI=confidence interval, ln=natural log; BMI=body mass index; A1c=hemoglobin A1c; MGH1=methylglyoxal hydroimidazolone 1, *$p<0.05$ is considered statistically significant.

[a]Model Summary: Adjusted R^2=-0.022, $F(7,226)=0.270$, $p=0.965$.

[b]Model Summary: Adjusted R^2=0.047, $F(7,246)=2.772$, $p=0.009$.

[a,b]The model included age, gender, ethnicity, interaction of gender by ethnicity, lnBMI, smoking status, and lnA1c.

CHAPTER V

Relationship of Methylglyoxal-derived Adduct with Homocysteine and Triglycerides in Blacks with and without Type 2 Diabetes

ABSTRACT

INTRODUCTION: A diacarbonyl intermediate methylglyoxal gained an increasing interest due to pathogenesis relevant to atherosclerosis, diabetes, and other chronic illnesses. However, there is still lack of observations particularly in Black populations.

PURPOSE: The study investigated the association of major methylglyoxal adduct, methylglyoxal hydroimidazolone 1 (MGH1) with selected markers for cardiovascular risk in African American and Haitian American participants with and without type 2 diabetes.

METHODS: The study included a total of 488 eligible participants (n = 234 non-diabetics and n = 254 diabetics) over 30 years of age who had complete data of serum methylglyoxal and risk factors for cardiovascular disease. Community outreach approach was used to recruit participants resided in Broward and Miami-Dade counties, Florida.

RESULTS: In adjusted multiple regression analyses, there was an increase in MGH1 levels of 0.35% for every 1% increase in homocysteine levels in participants with diabetes (95% CI [0.07%, 0.64%], p=0.014). Being African American was associated with approximately 30% lower MGH1 levels than being Haitian American (95% CI [7.41%, 46.10%], p=0.012). Levels of MGH1 in participants with diabetes rose about 0.19% when triglyceride levels increased by 1% (95% CI [0.00%, 0.38%], p=0.049). However, there was no significant association in control participants.

CONCLUSION: Methylglyoxal was associated with increased risk of cardiovascular disease in participants with type 2 diabetes. The relationship between MGH1 and biomarkers of cardiovascular disease appeared differential between Haitian Americans and African Americans. Further investigation should be conducted separately across all Black subgroups.

INTRODUCTION

Glycation is a complex series of sequential reactions between reactive carbonyl groups of reducing sugars, such as glucose, and nucleophilic amino groups of proteins, lipids or nucleic acids (Semchyshyn & Lushchak, 2012). Formation of advanced glycation end products (AGEs) is typically considered a potential contributing process for the development of diabetes. The glycation process is likely to accelerate under chronic conditions, placing individuals with diabetes at risk. Hyperglycemia was speculated to give rise to dicarbonyl compounds (Schlotterer et al., 2009) which appeared to be a highly reactive AGE intermediate even more than glucose (Thornalley, 2005). Methylglyoxal is proposed to be one of the key precursors in protein glycation (Kalapos, 2008; Suravajjala, Cohenford, Frost, Pampati, & Dain, 2013; Zdenka Turk, Čavlović-Naglić, & Turk, 2011).

Methylglyoxal can be enzymatically converted from glycolytic intermediates (Kalapos, 1999; Phillips & Thornalley, 1993) and from non-enzymatic glycation of glucose (Thornalley, Langborg, & Minhas, 1999). Lesser amount of methylglyoxal can be generated through lipid peroxidation (Agadjanyan, Dugin, & Dmitriev, 2006), and metabolism of threonine (Dhar, Desai, Kazachmov, Yu, & Wu, 2008) and ketone bodies (Beisswenger, Howell, Nelson, Mauer, & Szwergold, 2003; Nemet, Varga-Defterdarović,

& Turk, 2006). Methylglyoxal possibly transports to plasma by passive diffusion, over 95% of which reversibly binds to plasma proteins (Rabbani & Thornalley, 2015). A major methylglyoxal-derived adduct is methylglyoxal hydroimidazolone 1 (N_{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-L-ornithine; MGH1), generated from reaction between methylglyoxal and free arginine residue (Thornalley, 2005). Methylglyoxal is mainly degraded by glyoxalase system which is responsible for degradation of dicarbonyl compounds (Thornalley, 2003; Vander Jagt & Hunsaker, 2003). However, persistent exposure of high glucose appears to alter methylglyoxal degradation and cause methylglyoxal accumulation (Schlotterer et al., 2009). This contributes to increased formation of AGEs in diabetes (Ahmed, Babaei-Jadidi, Howell, Thornalley, & Beisswenger, 2005; Kilhovd et al., 2003).

Apart from through glycation, available evidence indicated involvement of methylglyoxal in pathophysiology of cardiovascular disease. Methylglyoxal-induced nitric oxide reduction corresponded to increased generation of reactive oxygen species (Sena et al., 2012) and blood vessel stiffness *in vitro* (Brouwers et al., 2010). Other potential biomarkers for cardiovascular condition include homocysteine (Sreckovic et al., 2016) and C-reactive protein (Cardoso, Leite, & Salles, 2016). Homocysteine was speculated to rise under glycemic and oxidative stress (Karamshetty, Acharya, Ghaskadbi, & Goel, 2016), which likely increased in diabetes (Kulkarni, Acharya, Ghaskadbi, & Goel, 2014). C-reactive protein is an acute-phase biomolecule which is mainly released from hepatocytes in response to inflammatory cytokines (Gilstrap & Wang, 2012). The measurement of C-reactive protein may be useful to evaluate cardiovascular risk in some adult groups (Greenland et al., 2010). In addition, lipid

profile is suggested in assessment of chronic inflammatory disease (Ungurianu et al., 2016). Positive relationships of cardiovascular disease with hypercholesterolemia and diabetes was found in a previous cohort study (Sardarinia et al., 2016). As mentioned, methylglyoxal is generated from lipid peroxidation and related to oxidative stress. Therefore, methylglyoxal may be associated with these factors which, in turn, links diabetes to the development of vascular complication.

Heart disease and diabetes remain major causes of death in the United States (National Center for Health Statistics, 2016). Available evidence indicate that Blacks are at high risk for cardiometabolic illnesses (National Center for Health Statistics, 2016). The ratio of age-adjusted death rates for Blacks to Whites were about 1.2 for heart disease and 1.9 for diabetes (Kochanek, Murphy, Xu, & Tejada-Vera, 2016). In 2015, age-adjusted prevalence of diabetes among adults aged 18 and over was 13.1% in non-Hispanic Blacks or African Americans only (Blackwell & Villarroel, 2016). Correspondingly, Black population tended to have 4 or more chronic conditions than White and other populations (National Center for Health Statistics, 2016). It is widely accepted that the increased risk of morbidity and mortality among populations are related to obesity (Calle, Thun, Petrelli, Rodriguez, & Heath, 1999) and tobacco use (U.S. Department of Health and Human Services, 2014). The prevalence of overweight or obese in Blacks continued to be greater than Whites, and more pronounced in Black females than Black males (National Center for Health Statistics, 2016). Black males had higher rates for cigarette smoking than White males (National Center for Health Statistics, 2016). Further scientific studies and public health programs are demanded to tackle preventable chronic diseases, especially in ethnic minorities.

Haitian Americans and African Americans are typically characterized as non-Hispanic Blacks due to similarity of ancestry. As previously reported, subgroups of non-Hispanic Blacks had diversity in genetic variants and modifiable factors across subgroups. Haitians had poorer glycemic control than Whites and African Americans (Vimalananda, Rosenzweig, Cabral, David, & Lasser, 2011). The prevalence of diabetes in Haitians was observed at 33% (Rosen, Sharp, Abad, Doddard, & Rosen, 2007), which was greater than the overall age-adjusted percentage for non-Hispanic Blacks (Blackwell & Villarroel, 2016). Apart from African and European ancestry, small proportion of Native American ancestry was also detected in African Americans (Bryc, Durand, Macpherson, Reich, & Mountain, 2015), but hardly determined in Haitian populations (Simms et al., 2012). Possibly, the risk for chronic diseases is different in Black subgroups and should be investigated. However, existing evidence in the relationship between methylglyoxal and risk factors of chronic diseases in Black population is still lacking. Therefore, the current study aimed to examine the association of methylglyoxal and selected risk factors of cardiovascular disease in Haitian African and African American participants with and without type 2 diabetes.

METHOD

Participants

Data and de-identified blood samples were retrieved from the cross-sectional study conducted on Haitian American and African American participants by Huffman et al. (2013). Haitian American participants were recruited by community-based approaches. The recruitment was taken place in Miami-Dade and Broward counties, Florida. Invitational letters reviewing the study were mailed to African American

participants by using a mailing list (Knowledge Base Marketing, Inc., Richardson, TX, USA). Health professionals and diabetes educators receiving the flyer were requested for their assistance to enroll individuals with type 2 diabetes. Faculty, staff and students at Florida International University (FIU) received the flyers explaining the protocol and were asked for their cooperation. Advertisements were published in local newspapers and high-trafficking areas. Radio advertisements were announced on local Creole stations.

Inclusion criteria of the parent study included self-identified Haitian American and African American males and females; age 30 years or older; absence or presence of type 2 diabetes; free of thyroid disorders, coronary heart disease, chemo- or radiation therapy, major psychiatric disorders, and HIV/AIDS; not pregnant or lactating. By using an initial phone interview, potential participants were informed about the study purpose and determined the age and gender. To ascertain type 2 diabetes status, the participants were asked for the duration of diagnosis and initial treatment regimens. Eligible individuals were invited to the Human Nutrition Laboratory at FIU to participate in the study. Participants were instructed to refrain from smoking, consuming any food or beverages except water, and any unusual exercise for at least eight hours prior to collection of blood samples. All participants gave written informed consents in either English or Creole. The study was approved by the Institutional Review Board at FIU prior to measurement of blood samples.

Sample Size

Sample size was calculated by using G*Power software (Faul, Erdfelder, Buchner, & Lang, 2009). By using a medium conventional effect size to produce an equal number of controls and cases, the total sample size was estimated at 128 individuals

based on independent t-test, whereas multiple linear regression yielded a total of 55 participants. A total of 507 target participants (Haitian American=258: non-diabetics=120, diabetics=138; African American=249: non-diabetics=120, diabetics=129) from the parent study was adequate for both calculated sample sizes that design to meet the predetermined 80% statistical power at significance level of 0.05.

Biochemical Analysis

Approximately 300 microliters of de-identified serum from the parent study were used for methylglyoxal measurement. A prominent methylglyoxal adduct, MGH1, in blood samples was measured by OxiSelect™ MG competitive ELISA kit (Cell Biolabs, Inc., San Diego, CA, USA). The quantity of MGH1 was compared to a standard curve of predetermined MG-BSA. All blood samples were stored at -80 C^{o}. Existing data of selected parameters for cardiovascular risk was retrieved from the parent study. Briefly, hexokinase enzymatic method was used to measure fasting plasma glucose (FPG). Whole blood A1c was measured by DCA2000+ system (Bayer HealthCare, Whippany, NJ, USA). This method is reliable as evidenced by 99% correlation with high-standard HPLC method. Based on fluorescence polarization immunoassay method, the IMx System was utilized to determine total plasma homocysteine (Hcy; Abbott Laboratories, Abbott Park, IL, USA). High sensitivity C-reactive protein (HsCRP) was analyzed by Immulite (Diagnostic Products Corporation, Los Angeles, CA). Serum total cholesterol (TC), triglyceride (TG), and HDL cholesterol (HDLC) were measured using enzymatic spectrophotometric methods by Laboratory Corporation of America (LabCorp, Miami, FL, USA). The Friedewald equation was used to estimate LDL cholesterol (LDLC) levels as described elsewhere (Friedewald, Levy, & Fredrickson, 1972).

Anthropometric data

Participant's height was measured when the participant stood upright without shoes. Weight measurement was taken with the participant wearing light clothes by using a SECA clinical scale (SECA Corp, Columbia, MD, USA). Body mass index (BMI) was calculated as weight (kg)/height (m^2).

Socio-demographic data

Socio-demographic data regarding age, gender, ethnicity, and smoking status were collected by using a standardized questionnaire.

Statistical Analysis

Values were presented as mean ± standard deviation (mean ± SD) or percentage (%). Continuous variables were analyzed by independent t-test, whereas Chi-square test was employed for categorical variables. Multiple regression analysis was used to further examine the relationship between serum MGH1 levels and other biomarkers, including homocysteine total cholesterol, triglycerides, LDL cholesterol and HDL cholesterol. Age, gender, ethnicity, BMI, diabetes status and smoking status were potential variables that were adjusted as appropriate during data analysis. Due to skewed distribution, levels of MGH1, homocysteine, triglycerides, and BMI underwent logarithm transformation. The log-transformed data were used in all analyses. Tests were considered statistically significant if *p*-value was less than 0.05. All data were analyzed by using SPSS version 23.0 (SPSS Inc., Chicago, IL, USA).

RESULT

In the current study, eligible participants were individuals who had complete data for serum MGH1 and other selected variables, and BMI between 18.5 kg/m^2 and 60

kg/m^2. A total of 488 participants (n = 234 controls and n = 254 cases) were included in the data analyses, which was adequate to achieve 80% power.

Characteristics of study participants

The participants with type 2 diabetes tended to be older (p<0.001) and had greater lnBMI (p<0.001), lnFPG (p<0.001), lnA1c (p<0.001), and lnTG (p=0.010) than those without diabetes (Table 1). The levels of lnMGH1 were partially higher in the participants with diabetes as compared to those without diabetes (p=0.057).

Association of MGH1 with selected biomarkers in study participants

Multiple regression analyses were used to examine the association of MGH1 with selected biomarkers in participants with and without type 2 diabetes. A positive relationship between MGH1 and homocysteine levels was found (B=0.346, 95% CI [0.135, 0.557], p=0.001; Table 2), and persisted after adjusting for age, gender, ethnicity, diabetes status, lnBMI, smoking status, and two-way interactions among gender, ethnicity, and diabetes status (B=0.338, 95% CI [0.111, 0.566], p=0.004).

There was no significant association of MGH1 levels with TC (p=0.426), TG (p=0.664), LDLC (p=0.515), HDLC (p=0.850), and HsCRP (p=0.457). After adjusting for age, gender, ethnicity, diabetes status, lnBMI, smoking status, and two-way interactions among gender, ethnicity, and diabetes status, null association of MGH1 with TC (p=0.488), TG (p=0.515), LDLC (p=0.591), HDLC (p=0.746), and HsCRP (p=0.250) was remained.

Relationship between MGH1 and homocysteine stratified by diabetes status

Due to the fact that diabetes may contribute to increased cardiovascular risk, data were stratified by diabetes status prior to further multiple regression analyses. Null

association between MGH1 and homocysteine was shown in participants without diabetes (p=0.152; Table 3). The marginal relationship between MGH1 and homocysteine emerged in control participants after adjusting for age, gender, ethnicity, interaction of gender by ethnicity, lnBMI, and smoking status (p=0.085). However, the adjusted model failed to meet the significance level of 0.05 (p=0.684).

There was a significant association between MGH1 and homocysteine in participants with diabetes (p=0.001), and remained after adjustment. Levels of MGH1 in participants with diabetes increased about 0.35% when homocysteine levels increased by 1% (95% CI [0.07%, 0.64%], p=0.014). Being African American was associated with approximately 30% lower MGH1 levels than being Haitian American, holding other variables constant (95% CI [7.41%, 46.10%], p=0.012). Levels of MGH1 were also marginally associated with age (p=0.057) and BMI (p=0.068). There was no significant association of MGH1 with gender (p=0.405) and smoking status (p=0.594) as well as interaction effect between gender and ethnicity (p=0.405).

Association of MGH1 with selected cardiovascular risk factors stratified by diabetes status

In participants without diabetes, MGH1 levels were associated with TG levels (B=-0.288, 95% CI [-0.547, -0.028], p=0.030; Table 4), and persisted after adjusting for age, gender, ethnicity, interaction of gender by ethnicity, lnBMI, and smoking status (B=-0.345, 95% CI [-0.624, -0.066], p=0.016). However, the adjusted model did not reach significance level at 0.05. In unadjusted models, there was no significant association of MGH1 with HDLC (p=0.169), LDLC (p=0.832), TC (p=0.924), and HsCRP (p=0.260). Null associations of MGH1 levels with HDLC (p=0.075; Table 5), LDLC (p=0.752), TC

(p=0.959), and HsCRP (p=0.179) remained in control participants after adjusted for age, ethnicity, gender, lnBMI, smoking status, and interaction of gender by ethnicity.

In participant with diabetes, the association between MGH1 and TG levels did not show in unadjusted model (p=0.133), but emerged only in adjusted model (Table 4). After adjusted for age, ethnicity, gender, lnBMI, smoking status, and interaction of gender by ethnicity, MGH1 levels in participants with diabetes rose about 0.19% when TG levels increased by 1% (95% CI [0.00%, 0.38%], p=0.049). Elevated MGH1 levels were associated with an increasing age (B=0.014, 95% CI [0.005, 0.023], p=0.003) and being Haitian American (B=-0.341, 95% CI [-0.613, -0.070], p=0.014). A marginal relationship between MGH1 and HDLC was observed in participants with diabetes holding other variables constant (p=0.063; Table 5). Similarly, MGH1 levels was significantly associated with age (B=0.015, 95% CI [0.006, 0.024], p=0.002) and ethnicity (B=-0.349, 95% CI [-0.621, -0.077], p=0.012) in the adjusted model. In unadjusted model, there was no significant association of MGH1 with LDLC (p=0.281), TC (p=0.313), HsCRP (p=0.626) in participants with diabetes. After adjusted for age, ethnicity, gender, lnBMI, smoking status, and interaction of gender by ethnicity, null association of MGH1 with LDLC (p=0.292), TC (p=0.331), HsCRP (p=0.788) remained.

DISCUSSION

The present study found that serum concentrations of dominant methylglyoxal adduct, MGH1, were higher in participants with types 2 diabetes than controls. The elevated MGH1 levels were significantly associated with an increase in homocysteine and triglyceride levels in participants with diabetes. Also, being Haitian American

appeared to positively influence serum concentrations of MGH1 in participants with type 2 diabetes.

Aberrant metabolic condition appeared to increase methylglyoxal levels. Elevated methylglyoxal levels were observed in individuals who had impaired glucose metabolism (Maessen et al., 2015), diabetes (Kong et al., 2014; Nemet, Turk, Duvnjak, Car, & Varga-Defterdarović, 2005; Turk et al., 2011; Turk, Vrdoljak, Misur, Trescec, & Benko, 2009) or metabolic syndrome (Uribarri et al., 2015). As previously reported (Ahmed et al., 2005; Kilhovd et al., 2003), the current study found that serum levels of prominent adduct, MGH1, was marginally higher in participants with diabetes than the controls. This may be due to differential capacity of methylglyoxal degradation among individuals. Even though an increased activity of erythrocyte glyoxalase enzymes was found in diabetes (McLellan, Thornalley, Benn, & Sonksen, 1994), hyperglycemia-induced methylglyoxal overproduction may disproportionate to degradation (Schlotterer et al., 2009). This possibly leads to methylglyoxal accumulation. Existing evidence suggests that methylglyoxal is related to alteration of glucose metabolism which, in turn, increase risk of chronic illnesses overtime.

Homocysteine is identified as an important biomarker for atherosclerosis and vascular disease (Homocysteine Studies Collaboration, 2002; Sreckovic et al., 2016). Levels of MGH1 were positively associated with homocysteine levels in pooled analyses. However, the significant relationship between MGH1 and homocysteine persisted only in participants with diabetes after stratified by diabetes status and adjusted for confounders, suggesting such association likely presented under chronic condition. The current study firstly reported the relationship between MGH1 and homocysteine in diabetes. The

possible candidate to describe the findings was reduced glutathione. High glucose condition was speculated to cause overproduction of methylglyoxal (Ahmed et al., 2005; Schlotterer et al., 2009; Thornalley, 2005), which likely stressed the glyoxalase system. Reduced glutathione is required in glyoxalase-1 activity and recycled back after D-lactate production catalyzed by glyoxalase-2 (Thornalley, 2003). The study in isolated erythrocytes from individuals with diabetes found that reduced glutathione concentrations were negatively correlated with diabetes-related microvascular complications, and D-lactate concentrations (Thornalley, McLellan, Lo, Benn, & Sönksen, 1996). Induced by methylglyoxal accumulation, an increased glyoxalase-1 activity coupled with a decreased glyoxalase-2 function may reduce abundance of glutathione by trapping it in the system (Chakraborty, Karmakar, & Chakravortty, 2014). As glutathione is essential for oxidative stress defense (Aoyama & Nakaki, 2013; Franco & Cidlowski, 2012; Matafome, Sena, & Seiça, 2013), systemic anti-oxidative property may decline. Methylglyoxal also caused the formation of reactive oxygen species (Brouwers et al., 2010; Sena et al., 2012) which, in turn, aggravated preexisting oxidative stress. In addition, almost half of hepatocyte glutathione was obtained from homocysteine through transsulfuration pathway (Mosharov et al., 2000). The positive relationship between MGH1 and homocysteine conceivably reflected physiological response against methylglyoxal accumulation and increased oxidative stress in diabetes.

In participants with diabetes, high MGH1 levels were significantly associated with elevated triglyceride levels, whereas marginally negative relationship was found with HDL cholesterol. However, Turk and colleagues (2011) reported the significant correlation between blood levels of MG-adduct and LDL cholesterol as well as that

between urinary MG-adduct and triglyceride levels. About 30% of the study participants had a history of macrovascular disease, and about 12% had type 1 diabetes (Turk et al., 2011). The difference in participant characteristics may create different findings from the current study.

The positive association between MGH1 and triglycerides supported the possibility of methylglyoxal production through lipid peroxidation. By using rat liver fractions, a glycolytic enzyme phosphoglucose isomerase was hypothesized to convert malondialdehyde to methylglyoxal (Agadjanyan et al., 2006). Ketone bodies are synthesized from fatty acids when fats become main fuel, such as during nutritional deprivation. Ketone bodies include acetoacetate and its byproducts acetone and acetol, which can be further catalyzed into methylglyoxal (Beisswenger et al., 2003; Nemet et al., 2006). A few human studies indicated that elevated ketone bodies were found corresponding to increased methylglyoxal in diabetes or individuals who chronically consumed low-carbohydrate diet (Beisswenger et al., 2003; Turk, Nemet, Varga-Defteardarović, & Car, 2006). Taken together, non-carbohydrate substrates may be other significant sources for methylglyoxal formation under abnormal metabolic conditions such as diabetes.

The association of MGH1 positively with triglycerides and negatively with HDL cholesterol suggested that methylglyoxal may involve in pathological manifestation of vascular complication. As endothelium serves as a semipermeable barrier, it plays a critical role in selective transfer of small and large molecules, and stability of blood vessel (Sumpio, Riley, & Dardik, 2002). High glucose-induced methylglyoxal accumulation in bovine endothelial cells appeared to increase endocytosis of

macromolecules (Shinohara et al., 1998). The increased internalization of macromolecules, such as lipids from circulating lipoprotein, possibly increased endothelial permeability linking to fatty deposit at vascular lesion (Cester et al., 1996).

Additionally, HDL cholesterol was susceptible for methylglyoxal glycation (Bacchetti, Masciangelo, Armeni, Bicchiega, & Ferretti, 2014). As compared to healthy individuals, MGH1 contents of high density lipoprotein (HDL) were significantly greater in individuals with type 2 diabetes (Godfrey, Yamada-Fowler, Smith, Thornalley, & Rabbani, 2014). High density lipoprotein participates in reverse cholesterol transport where HDL is proposed to have a protective effect against atherogenesis (Vergeer, Holleboom, Kastelein, & Kuivenhoven, 2010). In part, HDL interacts with cholesteryl ester transfer protein (CETP), which mediates triglyceride transfer from triglyceride-rich lipoprotein to HDL in exchange for cholesteryl ester (Oliveira & de Faria, 2011). As a result, HDL enriched in triglyceride can undergo multiple fates such as taken up and catabolized by the liver. In animal model, the modification of HDL by methylglyoxal reduced particle stability and cholesteryl ester transfer through the interaction between HDL and CETP (Godfrey et al., 2014). In addition, methylglyoxal may enhance cardiovascular risk through interference with antioxidant enzyme activity. Paraoxonase-1 features anti-oxidative and anti-inflammatory properties, including detoxification of homocysteine-thiolactone (Perła-Kaján & Jakubowski, 2012). Homocysteine converts to homocysteine-thiolactone which subsequently leads to the formation of *N*-homocysteinylated protein (*N*-Hcy-protein; (Gurda, Handschuh, Kotkowiak, & Jakubowski, 2015). *N*-Hcy-protein was proposed to play a role in thrombosis and cell damage (Gurda et al., 2015; Jakubowski, 2001). Activity of paraoxonase-1 appeared to

protect against accumulation of *N*-Hcy-protein (Perła-Kaján & Jakubowski, 2012), which possibly delayed atherogenesis. Methylglyoxal-mediated glycation found to reduce activity of paraoxonase-1 enzyme on HDL particle surface (Bacchetti et al., 2014), at least in part, connecting methylglyoxal to impaired oxidative defense by HDL cholesterol. At least 33% decrease in plasma half-life of methylglyoxal-modified HDL was also observed (Godfrey et al., 2014), implicating the involvement of methylglyoxal in blood HDL cholesterol levels which commonly manifest as low in individuals with coronary heart disease or type 2 diabetes. The marginally negative relationship between MGH1 and HDL cholesterol supported this hypothesis.

The current study did not find the significant association between MGH1 and HsCRP levels among participants. Levels of MGH1 were positively associated with levels of CRP in children and adolescents with type 1 diabetes of short duration (Heier et al., 2015), who were previously reported to have an increasing trend of carotid artery intima-media thickness (Margeirsdottir, Stensaeth, Larsen, Brunborg, & Dahl-Jorgensen, 2010). Short duration of diabetes, preexisting vascular event, and participant's age may contribute to different findings from the current study. Possibly, the relationship of methylglyoxal with CRP may emerge during early stages of the disease, but not late stages.

In participants with diabetes, African Americans were more likely to have lower MGH1 levels than Haitian Americans. Such dissimilarity may be partially due to diversity of modifiable risk factors and ancestry among Black populations. Haitians with diabetes were more likely to have poor glycemic control as compared to White and African American counterparts (Vimalananda et al., 2011). Prolonged exposure of high

glucose appeared to cause methylglyoxal overproduction (Schlotterer et al., 2009), subsequently aggravating methylglyoxal detoxification system. Reduced glutathione concentrations in erythrocytes from individuals with type 2 diabetes were negatively correlated with plasma glucose levels (McLellan et al., 1994). Low glutathione concentration may cause methylglyoxal accumulation and simultaneously increase homocysteine for precursor supply. In addition, *Glo1* gene was considered one of hotspots for genetic variation (Cahan, Li, Izumi, & Graubert, 2009). The similarity of erythrocyte GLO allele frequency in Black Americans and Bantu-speaking Black habitants in South Africa (Bender, Frank, & Hitzeroth, 1977). Yet, the study also mentioned significant differences in the GLO allele frequency among some Black subpopulations (Bender et al., 1977). The diversity of ancestry among Black populations, including Haitians (Simms et al., 2012) and African Americans (Bryc et al., 2015) was recently reported. Collectively, glyoxalase-1 expression and activity may be different in Black subgroups, impacting methylglyoxal detoxification.

The nature of observational study limited the researchers to examine causality of the findings. Low generalizability was assumed due to participant recruitment from the same pool of population and area. However, the study also features some strengths. Apart from patient report, diabetes status was ascertained using standard criteria established by American Diabetes Association. By far, this was the first study investigated the relationship between methylglyoxal and biomarkers for cardiovascular risk particularly in Haitian Americans and African Americans. The evidence, at least in part, provided the better understanding in biomarkers that possibly predisposed Black populations to cardiovascular condition.

To sum up, elevated serum MGH1 levels were shown in Haitian American and African American participants with type 2 diabetes. A positive association of MGH1 with homocysteine and triglyceride placed methylglyoxal a possible candidate that linked diabetes to cardiovascular risk. The relationship between MGH1 and biomarkers for cardiovascular disease appeared differential between Haitian Americans and African Americans. Disparity in susceptibility for chronic illnesses among subpopulations deserve further investigation. Also, replication studies are demanded to warrant and elucidate the true nature of such relationship.

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Table 1. Characteristics of the participants

Variable	without diabetes (n=234)	with diabetes (n=254)	*p*-value
Age (years)	52.59 ± 10.00	56.00 ± 10.14	<0.001*
Gender, n (%)			0.381
Female	116 (49.6)	136 (53.5)	
Male	118 (50.4)	118 (46.5)	
Ethnicity, n (%)			0.868
Haitian Americans	118 (50.4)	130 (51.2)	
African Americans	116 (49.6)	124 (48.8)	
BMI (kg/m^2)	29.91 ± 6.06	32.46 ± 7.17	<0.001*
lnBMI	3.38 ± 0.19	3.46 ± 0.21	<0.001*
FPG (mmol/l)	97.28 ± 15.08	151.71 ± 64.88	<0.001*
lnFPG	4.57 ± 0.15	4.94 ± 0.39	<0.001*
A1c (%)	5.92 ± 0.45	8.03 ± 2.27	<0.001*
lnA1c	1.78 ± 0.08	2.05 ± 0.26	<0.001*
Homocysteine (mg/l)	10.07 ± 4.80	10.33 ± 4.09	0.534
lnHcy	2.24 ± 0.36	2.27 ± 0.34	0.273
HsCRP (mg/l)	4.75 ± 7.20	5.23 ± 7.28	0.468
lnHsCRP	0.76 ± 1.28	0.96 ± 1.22	0.076
Total cholesterol (mg/dl)	194.27 ± 38.90	191.53 ± 42.31	0.458
TG (mg/dl)	110.01 ± 59.90	123.78 ± 69.43	0.020*
lnTG	4.59 ± 0.46	4.69 ± 0.49	0.014*
HDL cholesterol (mg/dl)	51.68 ± 13.73	51.43 ± 14.36	0.847
LDL cholesterol (mg/dl)	120.59 ± 36.61	115.34 ± 36.47	0.114
Smoke (%)			0.555
Yes	54 (23.1)	53 (20.9)	
No	180 (76.9)	201 (79.1)	
MGH1 (μg/ml)	2.29 ± 2.50	2.36 ± 2.44	0.764
lnMGH1	0.42 ± 0.93	0.56 ± 0.73	0.057

Continuous variables were expressed as mean ± standard deviation (SD), while categorical variables were expressed as n (%). ln=natural log-transformed; A1c=hemoglobin A1c; BMI=body mass index; FPG=fasting plasma glucose; HsCRP=high sensitive C-reactive protein; Hcy=homocysteine; MGH1=methylglyoxal hydroimidazolone 1; TG=triglycerides; *p<0.05 is considered statistically significant.

Table 2. Multiple regression analysis for relationship of lnMGH1 with lnHcy in the study participants (n=488)

Unadjusted model				
Variables	B	SE	95% CI	*p*-value
lnHcy	0.346	0.107	0.135, 0.557	0.001*
Adjusted model*				
Variables	B	SE	95% CI	*p*-value
Age	9.030E-5	0.004	-0.004, 0.011	0.982
Gender (male)	-0.026	0.250	-0.525, 0.462	0.917
Ethnicity (AA)	-0.038	0.138	-0.314, 0.231	0.784
Diabetes status (yes)	0.460	0.248	-0.149, 0.852	0.063
lnBMI	0.259	0.211	-0.212, 0.620	0.220
Smoking status (yes)	0.021	0.102	-0.176, 0.229	0.837
Ethnicity*diabetes status (AA*yes)	-0.243	0.152	-0.522, 0.080	0.111
Gender*ethnicity (male*AA)	0.040	0.151	-0.231, 0.366	0.791
Diabetes status*gender (yes*male)	0.033	0.151	-0.249, 0.350	0.829
lnHcy	0.338	0.116	0.111, 0.566	0.004*

AA=African American, B=coefficient, SE=standard error, CI=confidence interval, ln=natural log; Hcy=homocysteine, MGH1=methylglyoxal hydroimidazolone 1, *$p<0.05$ is considered statistically significant.

Model Summary: Adjusted $R^2=0.019$, $F(10,477)=1.927$, $p=0.040$.

Table 3. Multiple regression analysis for relationship of lnMGH1 with lnHcy stratified by diabetes status

Participants without diabetes (n=234)				
Variables	B	SE	95% CI	*p*-value
Unadjusted model				
lnHcy	0.246	0.171	-0.091, 0.584	0.152
Adjusted model[a]				
Age	-0.009	0.007	-0.022, 0.004	0.178
Gender (male)	0.050	0.393	-0.725, 0.824	0.900
Ethnicity (AA)	-0.051	0.187	-0.420, 0.319	0.788
Gender*ethnicity (male*AA)	-0.032	0.251	-0.526, 0.462	0.898
lnBMI	0.077	0.336	-0.586, 0.739	0.819
Smoking status (yes)	0.104	0.168	-0.227, 0.435	0.535
lnHcy	0.314	0.181	-0.044, 0.671	0.085
Participants with diabetes (n=254)				
Variables	B	SE	95% CI	*p*-value
Unadjusted model				
lnHcy	0.425	0.131	0.167, 0.683	0.001*
Adjusted model[b*]				
Age	0.009	0.005	0.000, 0.019	0.057
Gender (male)	-0.236	0.284	-0.795, 0.322	0.405
Ethnicity (AA)	-0.348	0.137	-0.618, -0.077	0.012*
Gender*ethnicity (male*AA)	0.227	0.182	-0.131, 0.585	0.405
lnBMI	0.469	0.256	-0.035, 0.974	0.068
Smoking status (yes)	-0.065	0.121	-0.303, 0.174	0.594
lnHcy	0.355	0.144	0.071, 0.639	0.014*

AA=African American, B=coefficient, SE=standard error, CI=confidence interval, ln=natural log; Hcy=homocysteine; MGH1=methylglyoxal hydroimidazolone 1; *$p<0.05$ is considered statistically significant.

[a]Model Summary: Adjusted R^2=0.021, $F(7,226)$=0.686, p=0.684.

[b]Model Summary: Adjusted R^2=0.089, $F(7,246)$=3.425, p=0.002.

Table 4. Multiple regression analysis for relationship of lnMGH1 with lnTG stratified by diabetes status

Participants without diabetes (n=234)				
Variables	B	SE	95% CI	*p*-value
Unadjusted model				
lnTG	-0.288	0.132	-0.547, -0.028	0.030*
Adjusted model[a]				
Age	-0.005	0.006	-0.017, 0.008	0.468
Gender (male)	0.206	0.397	-0.576, 0.988	0.604
Ethnicity (AA)	-0.009	0.187	-0.378, 0.360	0.960
Gender*ethnicity (male*AA)	-0.074	0.250	-0.567, 0.420	0.769
lnBMI	0.246	0.344	-0.432, 0.923	0.476
Smoking status (yes)	0.154	0.168	-0.178, 0.486	0.361
lnTG	-0.345	0.142	-0.624, -0.066	0.016*
Participants with diabetes (n=254)				
Variables	B	SE	95% CI	*p*-value
Unadjusted model				
lnTG	0.142	0.094	-0.043, 0.328	0.133
Adjusted model[b]*				
Age	0.014	0.005	0.005, 0.023	0.003*
Gender (male)	-0.173	0.284	-0.734, 0.388	0.544
Ethnicity (AA)	-0.341	0.137	-0.613, -0.070	0.014*
Gender*ethnicity (male*AA)	0.213	0.182	-0.148, 0.573	0.246
lnBMI	0.329	0.256	-0.183, 0.841	0.207
Smoking status (yes)	-0.068	0.121	-0.308, 0.171	0.574
lnTG	0.190	0.096	0.001, 0.380	0.049*

AA=African American, B=coefficient, SE=standard error, CI=confidence interval, ln=natural log; MGH1=methylglyoxal hydroimidazolone 1, TG=triglycerides; *$p<0.05$ is considered statistically significant.

[a]Model Summary: Adjusted R^2=0.033, $F(7,226)$=1.109, p=0.359.

[b]Model Summary: Adjusted R^2=0.081, $F(7,246)$=3.092, p=0.004.

Table 5. Multiple regression analysis for relationship of lnMGH1 with HDLC stratified by diabetes status

Participants without diabetes (n=234)				
Variables	B	SE	95% CI	*p*-value
Unadjusted model				
HDL cholesterol	0.006	0.004	-0.003, 0.015	0.169
Adjusted model[a]				
Age	-0.007	0.006	-0.020, 0.005	0.259
Gender (male)	0.140	0.397	-0.643, 0.923	0.724
Ethnicity (AA)	-0.049	0.187	-0.418, 0.320	0.793
Gender*ethnicity (male*AA)	-0.024	0.250	-0.517, 0.469	0.923
lnBMI	0.148	0.341	-0.523, 0.819	0.664
Smoking status (yes)	0.121	0.168	-0.210, 0.453	0.473
HDL cholesterol	0.009	0.005	-0.001, 0.018	0.075
Participants with diabetes (n=254)				
Variables	B	SE	95% CI	*p*-value
Unadjusted model				
HDL cholesterol	-0.004	0.003	-0.010, 0.002	0.197
Adjusted model[b]*				
Age	0.015	0.005	0.006, 0.024	0.002*
Gender (male)	-0.283	0.288	-0.850, 0.284	0.327
Ethnicity (AA)	-0.349	0.138	-0.621, -0.077	0.012*
Gender*ethnicity (male*AA)	0.258	0.182	-0.101, 0.618	0.158
lnBMI	0.310	0.263	-0.208, 0.828	0.240
Smoking status (yes)	-0.052	0.121	-0.291, 0.187	0.668
HDL cholesterol	-0.006	0.003	-0.013, 0.000	0.063

AA=African American, B=coefficient, SE=standard error, CI=confidence interval, ln=natural log; MGH1=methylglyoxal hydroimidazolone 1; *$p<0.05$ is considered statistically significant.

[a]Model Summary: Adjusted R^2=0.022, $F(7,226)$=0.716, p=0.658.

[b]Model Summary: Adjusted R^2=0.079, $F(7,246)$=3.028, p=0.005.

CHAPTER VI

Relationship between Methylglyoxal Adduct and Eating Indices in Blacks with and without Type 2 Diabetes

ABSTRACT

INTRODUCTION: A dicarbonyl compound methylglyoxal found in food leads to formation of dietary advanced glycation end products which are generally known for association with chronic diseases. Methylglyoxal and its adducts tend to form in food high in sugars, proteins, and fats which are recommended to be consumed in moderation.

PURPOSE: The current study aimed to investigate the relationship between levels of serum methylglyoxal adduct and diet quality using the 2005 healthy eating index (HEI-2005) and alternate healthy eating index (AHEI) in African American and Haitian American participants, with and without type 2 diabetes.

METHODS: Participants were recruited by community outreach in Broward and Miami-Dade counties, Florida. The study included a total of 452 eligible participants (n = 212 non-diabetics and n = 240 diabetics) over 30 years of age who had complete data of serum methylglyoxal and scores for the HEI-2005 and the AHEI. Serum levels of prominent methylglyoxal adduct, methylglyoxal hydroimidazolone 1 (MGH1) were measured by a commercially available competitive ELISA kit. The eating indices were calculated from completed food frequency questionnaire. Multiple regression analysis was used to determine the association of MGH1 with the eating indices.

RESULTS: The significantly positive relationship between MGH1 levels and HEI-2005 scores were observed after adjusted for age, gender, ethnicity, diabetes status, interaction of diabetes status by ethnicity, and physical activity (B=0.009, 95% CI [0.002, 0.014],

p=0.012). Such relationship appeared to be more pronounced in Haitian American participants (B=0.012, 95% CI [0.002, 0.022], *p*=0.014). Among Haitian Americans, elevated MGH1 levels were associated with having diabetes (B=0.853, 95% CI [0.316, 1.391], *p*=0.002), higher education (B=0.248, 95% CI [0.027, 0.469], *p*=0.028), lower physical activity (B=-0.080, 95% CI [-0.161, 0.000], *p*=0.051), and being older (B=0.010, 95% CI [0.000, 0.022], *p*=0.054). No significant association between MGH1 levels and the AHEI scores was shown in unadjusted comparison (*p*=0.897) or adjusted model (B=-0.001, *p*=0.813).

CONCLUSION: The relationship between diet quality and methylglyoxal may be different due to ethnicities, disease state, and modifiable factors, which should be assessed.

INTRODUCTION

Diabetes remains one of the major causes of death in the United States (National Center for Health Statistics, 2016). Available evidence indicate that Blacks are at high risk for aberrant cardiometabolic illnesses including diabetes (National Center for Health Statistics, 2016). Age-adjusted rates of death caused by diabetes for Black population were about 1.9 times higher than that for White population (Kochanek, Murphy, Xu, & Tejada-Vera, 2016). In 2015, age-adjusted prevalence of diabetes among adults aged 18 and over was 13.1% in non-Hispanic Blacks or African Americans only (Blackwell & Villarroel, 2016). Correspondingly, Black population tended to have 4 or more chronic conditions than White and other populations (National Center for Health Statistics, 2016). Obesity and cigarette smoking are widely recognized as important contributing factors for the development of diabetes. The percent of overweight or obese non-Hispanic Blacks

has increased over the years, which was greater than Whites (National Center for Health Statistics, 2016). The prevalence of obesity appeared to be more pronounced in Black females than Black males (National Center for Health Statistics, 2016). These statistics indicate the need for further scientific studies and public health programs to tackle chronic illnesses in all subpopulations, including Black minorities.

Eating well is an important feature for managing diabetes (Gallivan, Greenberg, & Brown, 2008). The dietary guidelines for Americans has been widely used as a foundation of dietary recommendation for chronic diseases (Evert et al., 2013; Kushi et al., 2012). However, the national data between 2003-2004 indicated that most Americans still had low adherence to the dietary guidelines (Ervin, 2011). Of those, the poorer compliance was found in non-Hispanic Blacks as compared with other populations (Ervin, 2011), suggesting the need of racial/ethnic specific dietary intervention. To assess overall diet quality among populations, healthy eating index (HEI) was developed by the United States Department of Agriculture in 1995 (Bowman, Lino, Gerrior, & Basiotis, 1998; Kennedy, Ohls, Carlson, & Fleming, 1995) and has been revised to reflect the current dietary guidelines. The HEI-2005 is a measure of diet quality that features twelve diet-related components of the 2005 dietary guidelines for Americans by using scoring system (Guenther, Reedy, & Krebs-Smith, 2008). Alternate healthy eating index (AHEI) is another scoring system created to determine diet quality (McCullough et al., 2002). Some diet-related attributes in the original HEI still included in the AHEI scoring. However, AHEI scores also take into account for proportion of white and red meat, fat quality, cereal fibers, duration of multivitamin supplement, and moderate alcohol consumption, which are chosen due to association with reduced risk for chronic illnesses

(McCullough et al., 2002). Both HEI-2005 and AHEI has been used to determine overall diet quality in diverse populations (Akbaraly et al., 2011; Chiuve et al., 2012; Guenther, Reedy, Krebs-Smith, & Reeve, 2008; Lin, Gao, & Lee, 2013; McCullough & Willett, 2006; Savoca et al., 2009). The high scores of the indices were associated with less all-cause mortality and those caused by type 2 diabetes, cardiovascular disease, and cancer (Schwingshackl & Hoffmann, 2015), indicating the importance of diet quality in delaying mortality among populations.

Advanced glycation end products (AGEs) have been widely mentioned due to association with chronic diseases. Apart from endogenous production, AGEs are also formed in food through non-enzymatic Maillard reaction (Poulsen et al., 2013; Sharma, Kaur, Thind, Singh, & Raina, 2015). The Maillard reaction is a chemical process in which protein reacts with reducing sugar (Nemet, Varga-Defterdarović, & Turk, 2006). This reaction can generate dicarbonyl compounds, such as methylglyoxal, that can further lead to AGE formation (Nemet et al., 2006; Poulsen et al., 2013; Sharma et al., 2015). Generation of AGEs driven by methylglyoxal suggested its relevance to pathogenesis of diabetes (Yamagishi, Matsui, & Nakamura, 2008) and cardiovascular disease (Yamagishi & Matsui, 2016). Increased dietary methylglyoxal was significantly associated with serum methylglyoxal adduct levels in overweight or obese participants (Uribarri et al., 2015), suggesting that blood methylglyoxal may be possibly altered by food intake. In a recent double-blind cross-over randomized trial, healthy overweight individuals in low AGE diet group had a significantly greater insulin sensitivity than those having high AGE diet (de Courten et al., 2016). As compared to high AGE group, low AGE group also had an increased excretion of methylglyoxal hydroimidazolone 1 (MGH1) (de

Courten et al., 2016), which is a prominent adducted derived from methylglyoxal (Thornalley, 2005). The observations indicate that dietary methylglyoxal may be manipulated by food intake, and possibly impact physiological response. Methylglyoxal and AGE formation in food depends on high contents of sugar (Nemet et al., 2006), protein and fat (Sharma et al., 2015), which can be found in some diet components of HEI-2005 and AHEI. This suggest a possible link of eating pattern to dietary and subsequent circulating methylglyoxal-derived AGEs. However, diet quality assessment in relation to methylglyoxal remains scarce, especially in Black populations. Therefore, the current study aimed to examine the association between serum methylglyoxal adduct (MGH1) and eating indices (HEI-2005 and AHEI) in Haitian Americans and African Americans with and without type 2 diabetes.

METHOD

Participants

Data and de-identified blood samples were retrieved from the cross-sectional study conducted on Haitian American and African American participants by Huffman et al. (2013). Haitian American participants were recruited by community-based approaches. The recruitment was taken place in Miami-Dade and Broward counties, Florida. Invitational letters reviewing the study were mailed to African American participants by using a mailing list (Knowledge Base Marketing, Inc., Richardson, TX, USA). Health professionals and diabetes educators receiving the flyer were requested their assistance to enroll individuals with type 2 diabetes. Faculty, staff and students at Florida International University (FIU) received the flyers explaining the protocol and

were asked for their cooperation. Advertisements were published in local newspapers and high-trafficking areas. Radio advertisements were announced on local Creole stations.

Inclusion criteria of the parent study comprised self-identified Haitian American and African American males and females; age 30 years or older; absence or presence of type 2 diabetes; free of thyroid disorders, coronary heart disease, chemo- or radiation therapy, major psychiatric disorders, and HIV/AIDS; not pregnant or lactating. By using an initial phone interview, potential participants were informed about the study purpose and determined the age and gender. To ascertain type 2 diabetes status, the participants were asked for the duration of diagnosis and initial treatment regimens. Eligible individuals were invited to the Human Nutrition Laboratory at FIU to participate in the study. Participants were instructed to refrain from smoking, consuming any food or beverages except water, and any unusual exercise for at least eight hours prior to collection of blood samples. All participants gave written informed consents in either English or Creole. The study was approved by the Institutional Review Board at FIU prior to collection of blood samples and other study related data.

Sample Size

Sample size was calculated by using G*Power software (Faul, Erdfelder, Buchner, & Lang, 2009). By using a medium conventional effect size to produce an equal number of controls and cases, the total sample size was estimated at 128 individuals based on independent t-test, whereas multiple linear regression yielded a total of 55 participants. A total of 507 target participants (Haitian American=258: non-diabetics=120, diabetics=138; African American=249: non-diabetics=120,

diabetics=129) from the parent study was adequate for both calculated sample sizes to meet the predetermined 80% statistical power at significance level of 0.05.

Biochemical Analysis

Approximately 300 microliters of de-identified serum from the parent study were used for methylglyoxal measurement. A prominent methylglyoxal adduct, MGH1, in blood samples was measured by OxiSelect™ MG competitive ELISA kit (Cell Biolabs, Inc., San Diego, CA, USA). The quantity of MGH1 was compared to a standard curve of predetermined MG-BSA. All blood samples were stored at -80 C^{o}. Existing data of other biomarkers was retrieved from the parent study. Briefly, hexokinase enzymatic method was used to measure fasting plasma glucose (FPG). Whole blood A1c was measured by DCA2000+ system (Bayer HealthCare, Whippany, NJ, USA). This method is reliable as evidenced by 99% correlation with high-standard HPLC method.

Dietary intake and eating indices

Dietary intake was assessed by using a semi-quantitative food frequency questionnaire (Willett et al., 1985). Participants were asked to report food and vitamin consumption over the past year. Then, macro- and micronutrient intake was estimated accordingly. The instrument has been validated and standardized in diverse populations (Hernández-Avila et al., 1998; Holmes et al., 2007; Nath & Huffman, 2005).

The HEI-2005 score was calculated from each completed food frequency questionnaire. The HEI-2005 included a total of twelve diet-related items corresponding to the food guidance system MyPyramid which was described extensively elsewhere (Guenther, Reedy, & Krebs-Smith, 2008). Briefly, diets that met the dietary requirement obtained the maximum scores for nine adequacy attributes: total fruit (5 points), whole

fruit (5 points), total vegetables (5 points), dark green and orange vegetables and legumes (5 points), total grains (5 points), whole grains (5 points), milk (10 points), meat and beans (10 points), and oils (10 points). The minimum scores (0 point) of these attributes represented no intake of the food. In contrast, three restricted food items received maximum score representing moderate intake, whereas minimum standard score reflected overconsumption of the food: saturated fat (10 points), sodium (10 points), and energy from solid fats, alcohol, and added sugars (SoFAAS; 20 points). The scores were expressed in either per 1,000 calories or percentage of calories. All attribute scores were summed to a total score which ranged from 0 to 100. Higher scores reflected more compliance to the dietary guidelines and better diet quality (Guenther, Reedy, & Krebs-Smith, 2008).

The AHEI score was calculated from each completed food frequency questionnaire. The AHEI included some food components corresponding to the dietary guidelines and food items that appeared to be associated with lower risk for chronic illnesses in epidemiology and clinical studies (McCullough et al., 2002). Eight of the 9 diet components contributed 0 to 10 score points to a total score: vegetables (servings/day), fruit (servings/day), nuts and soy protein (servings/day), ratio of white to red meat, cereal fiber (grams/day), trans fat (percentage of energy), ratio of polyunsaturated to saturated fatty acids, and alcohol (servings/day). A score of 0 indicated the least desirable dietary behavior, while a score of 10 represented the dietary recommendations were fully met. Scores between 0 and 10 indicated intermediate intakes which were determined proportionately. Scores for duration of multivitamin use were dichotomous, which contributed either 2.5 points for use of less than 5 years or 7.5 points

for use of 5 years or over. A total AHEI score was obtained from sum of all attribute scores. The total score ranged from 2.5 to 87.5, which indicated least and most desirable dietary patterns and eating behaviors, respectively.

Anthropometric data

Participant's height was measured when the participant stood upright without shoes. Weight measurement was taken with the participant wearing light clothes by using a SECA clinical scale (SECA Corp, Columbia, MD, USA). Body mass index (BMI) was calculated as weight (kg)/height (m^2). Physical activity was assessed by using a modifiable activity questionnaire (Pereira et al., 1997). The instrument determined physical activity by using estimated metabolic equivalent (MET; 1 kcal/kg/hour) which represented a resting metabolic rate of a typical person. The number of METs for each activity was calculated using the average metabolic cost for that activity. The duration and frequency of physical activity that individuals participated in the past year were collected. Each activity was calculated by duration of the activity (hours), monthly frequency, and the numbers of months that individuals engaged in the activity. The total duration of each performed activity was divided by 52 weeks per year, yielding hours per week. The corresponding MET of each activity was multiplied by the total duration to obtain MET-hour per week. Then, all MET-hour per week of leisure and occupational activity were summed to estimate the participant's physical activity levels.

Socio-demographic data

Socio-demographic data regarding age, gender, ethnicity and education level were collected by using a standardized questionnaire.

Statistical Analysis

Values were presented in mean ± standard deviation or percentage. Continuous variables were analyzed by independent t-test, whereas Chi-square test was employed for categorical variables. Multiple regression analysis was used to further examine the relationship between serum MGH1 levels and eating indices, including HEI-2005 and AHEI. Age, gender, ethnicity, BMI, diabetes status, smoking status, and education level were potential variables that were adjusted as appropriate during data analysis. Due to skewed distribution, levels of MGH1 and physical activity underwent logarithm transformation. The log-transformed data were used in all analyses. By using general linear model, pairwise comparisons were performed to determine interaction effects as appropriate. Tests were considered statistically significant if *p*-value was less than 0.05. All data were analyzed by using SPSS version 23.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

In the current study, eligible participants were individuals who had complete data of serum MGH1, scores for the HEI-2005 and the AHEI, and BMI between 18.5 kg/m^2 and 60 kg/m^2. A total of 452 participants (n = 212 controls and n = 240 cases) were included in the data analyses, which was adequate to achieve 80% power.

Characteristics of study participants

The participants with type 2 diabetes tended to be older ($p<0.001$) and had higher BMI ($p<0.001$), FPG ($p<0.001$), A1c ($p<0.001$), HEI-2005 score ($p=0.002$), and lnMGH1 ($p=0.026$) than those without diabetes (Table 1). Calorie intake ($p=0.026$) and physical activity ($p=0.014$) appeared to be higher in control participants as compared to

participants with diabetes. There was a significant difference in education level between participants with and without diabetes (p=0.032).

Relationship of MGH1 with HEI-2005 and AHEI in study participants

Surprisingly, unadjusted comparison showed that there was an increase in MGH1 levels of 0.08% for every unit increase in HEI-2005 scores (95% CI [0.20%, 1.41%], p=0.005; Table 2). The significantly positive relationship between MGH1 levels and HEI-2005 scores persisted after adjusted for age, gender, ethnicity, diabetes status, interaction of diabetes status by ethnicity, and physical activity (B=0.009, 95% CI [0.002, 0.014], p=0.012). Marginally, individuals with diabetes were associated with higher MGH1 levels as compared to those without diabetes (B=0.471, 95% CI [-0.010, 0.952], p=0.055). The adjusted model showed that an increase in physical activity was partially associated with a decrease in MGH1 levels (B=-0.051, 95% CI [-0.107, 0.005], p=0.074). Males tended to have MGH1 levels higher than females after holding other variables constant, but only marginally (B=-0.051, 95% CI [-0.107, 0.005], p=0.090).

There was no significant association between MGH1 and AHEI in unadjusted comparison (p=0.897; Table 2). Null relationship of MGH1 with AHEI remained after adjusted for age, gender, ethnicity, diabetes status, interaction of diabetes status by ethnicity, and physical activity (B=-0.001, p=0.813). Even though insignificant, the negative relationship of MGH1 levels with AHEI scores was shown, which was opposite to those with HEI-2005. Having diabetes was significantly associated with elevated MGH1 levels as compared to not having diabetes (B=0.511, 95% CI [0.027, 0.994], p=0.038). Noted that the adjusted model was partially significant.

Relationship between MGH1 and HEI-2005 stratified by ethnicity

To determine whether relationships of MGH1 with diabetes parameter were existed in subgroups of participants, stratification by ethnicity was performed prior to further multiple regression analyses (Table 3). In Haitian American participants, the elevated MGH1 level was significantly associated with increased HEI-2005 scores in unadjusted comparison (B=0.013, 95% CI [0.004, 0.022], p=0.006). Such relationship persisted in the final adjusted model which controlled for age, gender, diabetes status, education, interaction of diabetes status by education, and physical activity (B=0.012, 95% CI [0.002, 0.022], p=0.014). Being Haitian American with diabetes was significantly associated with higher MGH1 levels as compared to that without diabetes (B=0.853, 95% CI [0.316, 1.391], p=0.002). Higher education was significantly associated with increased MGH1 levels (B=0.248, 95% CI [0.027, 0.469], p=0.028). There was an interaction between disease state and education (B=-0.389, 95% CI [-0.680, -0.097], p=0.009), therefore; pairwise comparisons by general linear model were performed. Among Haitian American participants with education less than high school graduate, having type 2 diabetes was significantly associated with elevated MGH1 levels as compared to not having the disease when controlling for other variables (F(7,228)=14.104, p<0.001). Among Haitian Americans without diabetes, having high school or some college degree was significantly associated with higher MGH1 levels as compared to having less than high school graduate, holding other variables constant (F(7,228)=9.687, p=0.006). Being older (B=0.010, 95% CI [0.000, 0.022], p=0.054) and having lower physical activity (B=-0.080, 95% CI [-0.161, 0.000], p=0.051) were

partially associated with elevated levels of MGH1 in Haitian American participants after controlling for other variables.

In African American participants, null relationship between MGH1 and HEI-2005 scores showed in the unadjusted comparison (p=0.493) as well as in the final adjusted model after controlling for age, gender, disease state, education, and interaction of disease state by education (p=0.161). Noted that the adjusted model did not reach a significance level of 0.05.

Relationship between MGH1 and AHEI stratified by ethnicity

Stratification analyses by ethnicity were also performed to determine the relationship between MGH1 levels and AHEI scores (Table 4). In Haitian American individuals, a negative relationship between MGH1 and AHEI scores was observed in the unadjusted comparison, however; it did not reach a significance level of 0.05 (p=0.309). Such insignificant findings remained after controlling for age, gender, disease state, education, and interaction of disease state by education (p=0.263). A significant relationship of MGH1 levels with age (B=0.011, 95% CI [0.000, 0.022], p=0.043), diabetes status (B=0.940, 95% CI [0.403 ± 1.477], p=0.001), education (p=0.309), and interaction of diabetes status by education (B=0.273, 95% CI [0.053 ± 0.494], p=0.008) were similar to the association between MGH1 levels and HEI-2005.

In African American participants, a positive relationship between MGH1 and AHEI scores was shown in the unadjusted comparison, yet not statistically significant (p=0.910). The final adjusted model did not show a significant association of MGH1 with AHEI after controlling for age, gender, disease state, education, and interaction of disease

state by education (p=0.426). Noted that the adjusted model did not reach a predetermined significance level.

DISCUSSION

The current study found that individuals with type 2 diabetes had significantly higher serum MGH1 levels and HEI-2005 scores than those without diabetes. Interestingly, there was a positive relationship between MGH1 levels and HEI-2005 scores, which appeared to be more pronounced in Haitian American participants. Having diabetes was associated with higher MGH1 levels. Particularly in Haitian Americans, age, education, diabetes status and physical activity also uniquely impacted levels of MGH1. However, there was no significant relationship between MGH1 levels and AHEI scores in study participants.

Consistent with previous studies, elevated levels of MGH1 adduct was shown in participants with type 2 diabetes as compared to control participants (Ahmed, Babaei-Jadidi, Howell, Thornalley, & Beisswenger, 2005; Kilhovd et al., 2003). Additionally, methylglyoxal levels were markedly elevated in blood (Kong et al., 2014; McLellan, Thornalley, Benn, & Sonksen, 1994; Nemet, Turk, Duvnjak, Car, & Varga-Defterdarović, 2005) and urine of participants with diabetes or impaired glucose metabolism (Maessen et al., 2015; Z. Turk, Čavlović-Naglić, & Turk, 2011; Turk, Vrdoljak, Misur, Trescec, & Benko, 2009). These evidences suggest that methylglyoxal is possibly associated with hyperglycemia and insulin resistance.

By using HEI-2005, individuals with diabetes appeared to have healthier eating pattern than those without diabetes. However, AHEI scores tended to be lower in diabetic participants than controls. This may be partially due to difference in calorie intakes as

well as calculation method and food components of indices. Calorie intake was used in adjustment for HEI-2005 scores where AHEI did not. Individuals with diabetes had significantly lower energy consumption as compared to those without diabetes. The HEI-2005 incorporated food components regrading amounts of oil, saturated fat, sodium, and SoFFAS, while AHEI featured duration of multivitamin use, ratio of consumed fatty acids and types of meat. Solid fats, alcohol and added sugar were ranked with highest weighing scores in HEI-2005 (Guenther, Reedy, & Krebs-Smith, 2008), which may be more relevant to dietary recommendation for diabetes. However, the higher scores of HEI-2005 may possibly reflect nutrition education bias among participants with diabetes (Huffman et al., 2011; Yu et al., 2015).

Interestingly, the positive association between MGH1 levels and HEI-2005 scores emerged in the current study. This may be, at least in part, described by diet-related components in the HEI-2005. The scoring standard of the HEI-2005 was set at the lowest level of MyPyramid recommendation for assessing diverse populations, including sedentary individuals who were more likely to consume less healthful diet and energy-dense foods (Hobbs, Pearson, Foster, & Biddle, 2015). Given that the HEI-2005 was adjusted for energy consumption, the least restrictive of the standard may inflate the scores. High contents of sugar (Nemet et al., 2006), protein and fat (Sharma et al., 2015) appeared to increase formation of methylglyoxal and AGEs through the non-enzymatic Maillard reaction. Sugar, protein, fat, and oils were taken into account in the HEI-2005, however; it did not directly reflect excess consumption of these AGE-containing food groups (Guenther, Reedy, & Krebs-Smith, 2008). High heat cooking methods (e.g. browning and roasting) and processing procedures to extend shelf-life (e.g. curing and

canning) appear to enhance AGEs formation (Poulsen et al., 2013; Sharma et al., 2015). Therefore, methylglyoxal-derived and other AGEs can be formed in some foods during processing such as bake goods, milk, grilled/broiled meat, and processed food (Negrean et al., 2007; Poulsen et al., 2013; Sharma et al., 2015; Stirban et al., 2008). The HEI-2005 incorporated only grain portions of foods and adequate consumption of meat and beans, which did not necessarily cover foods with high content of methylglyoxal and its adducts. Even though Haitian American participants and individuals with diabetes reportedly had higher HEI-2005 scores as compared to their counterparts, some food components in the indices were still lower in the study participants as compared to those from the national data (Huffman et al., 2011). Taken together, dietary pattern of the study participants should not be straightforwardly assumed as healthy and needs to be interpreted carefully in relation to methylglyoxal.

Disease state, environmental factors, and heterogeneity of participants may differently influence the relationship between MGH1 levels and HEI-2005. There was a significant effect of interaction between diabetes status and education. Non-diabetic individuals with high school and some college degree appeared to have greater MGH1 levels than those with less than high school graduate. At education level of less than high school graduate, having diabetes was associated with increased MGH1 levels. These findings suggest that healthy eating pattern may not be prompted in individuals with diabetes and education levels less than high school, or in participants without diabetes with high school and some college degree.

In stratified analyses, the association between MGH1 levels and HEI-2005 scores appeared to be more pronounced in Haitian American participants than African American

participants. This may be due to differences in modifiable behaviors, dietary patterns, and genetic variation among Black subgroups. Less glycemic control and adherence to treatment were observed in non-Hispanic Blacks than Whites (Selvin, Parrinello, Sacks, & Coresh, 2014). Haitian Americans with diabetes had poor glycemic control as evidenced by significantly higher levels of fasting plasma glucose and A1c, comparing to African American and non-Hispanic White counterparts (Vimalananda, Rosenzweig, Cabral, David, & Lasser, 2011). Persistent hyperglycemia possibly leads to subsequent methylglyoxal accumulation, particularly in Haitian Americans. According to the national nutrition survey, non-Hispanic blacks born in the United States had lower intake of fruits and grains, but higher calorie intake, than those born outside the United States (Lancaster, Watts, & Dixon, 2006). An observational study reported that African-American subgroups resided in Florida had unique pattern of food consumption which was substantially varied among groups (James, 2009). Therefore, the difference in the relationship of MGH1 levels with HEI-2005 between ethnicities may be resulted from heterogeneity of eating pattern among ethnic subgroups. Additionally, a growing evidence supported that inherited ancestry among Blacks was substantially diverse across parts of the United States and subgroups (Bryc, Durand, Macpherson, Reich, & Mountain, 2015; Simms et al., 2012). *Glo1* gene encoding methylglyoxal detoxifying enzyme, glyoxalase-1, was found to be one of genetic variation hotspots in an animal study (Cahan, Li, Izumi, & Graubert, 2009). Difference in allele frequency of GLO was reported in some Black subpopulations (Bender, Frank, & Hitzeroth, 1977), suggesting dissimilar activity of glyoxalase-1 and subsequent methylglyoxal degradation among Blacks. Collectively, poor glycemic control, diverse eating patterns, and, genetic

variation may differentially impact the findings. That, in turn, uniquely contributes to the positive relationship between MGH1 and the HEI-2005 in study participants. So far, there was no available data regarding the association of methylglyoxal with eating indices in Black populations for comparison with the current observation.

The adjusted model showed that an increase in physical activity levels was marginally associated with lower levels of MGH1 in adjusted model. In a small randomized control trial in overweight men, combination of low AGE diet and aerobic exercise for 12 weeks appeared to decrease methylglyoxal and other AGE levels (Macías-Cervantes et al., 2015). Muscle contraction during exercise was proposed to activate antioxidative pathways and subsequent glutathione biosynthesis (Dieter & Vella, 2013). Glutathione plays an important role in systemic oxidative stress defense (Aoyama & Nakaki, 2013; Franco & Cidlowski, 2012; Matafome, Sena, & Seiça, 2013), including glyoxalase system which is responsible for methylglyoxal detoxification (Thornalley, 2003). An increase in physical activity may, in part, enhance the activity of methylglyoxal degradation system that subsequently decreases methylglyoxal accumulation.

The positive relationship between MGH1 levels and age was observed after adjusted for demographic and diabetes-related parameters. Augmented methylglyoxal adducts in human lens was significantly correlated with increasing participant's age (Ahmed, Brinkmann Frye, Degenhardt, Thorpe, & Baynes, 1997). This may be described, in part, by glyoxalase-1 activity. In the glyoxalase system, glyoxalase-1 and co-factor reduced glutathione converted methylglyoxal to a non-toxic intermediate (Thornalley, 2003). A trend of glyoxalase-1 activity in human aortic tissues appeared to decrease with

ages (Kirk, 1960). In individuals aged 50 to 79 years, significantly lower activity of glyoxalase-1 in arteriosclerotic tissues than normal tissue section was found (Kirk, 1960). The positive association between serum MGH1 levels and age found in participants with diabetes may reflect the decreased activity of glyoxalase-1, exacerbated by diabetes.

Even though insignificant, decreased MGH1 levels appeared to be associated with high AHEI scores. This was opposite to the positive relationship of MGH1 levels with the HEI-2005 scores. Unlike the HEI-2005, the AHEI also featured nuts and soy protein, and duration of multivitamin use. These components possibly contain food items that showed the effect in reducing methylglyoxal levels. A study in diabetic rats found the beneficial effect of soybean isoflavones in lowering methylglyoxal levels (Lu et al., 2008). Isoflavone genistein showed the effect in trapping methylglyoxal and preventing subsequent AGE formation *in vitro* (Lv, Shao, Chen, Ho, & Sang, 2011) and in mice (Wang, Chen, & Sang, 2016). In animal studies, vitamin B6 derivative, pyridoxamine, appeared to decrease methylglyoxal-induced glycation moderately in adipose tissues (Rodrigues, Matafome, Santos-Silva, Sena, & Seiça, 2013) and significantly in heart (Almeida et al., 2013). In a small intervention study, a 5-day vitamin E supplementation enriched in γ-tocopherol markedly diminished postprandial plasma methylglyoxal in healthy men (Masterjohn, Mah, Guo, Koo, & Bruno, 2012). Collectively, these evidence would partially describe the negative relationship between MGH1 levels and AHEI scores.

The current study had several limitations. Due to nature of the observational study, changes in clinical and behavioral factors were not observed overtime. Therefore, it lacked to generate causality and provide a complete phenomenon. The participants

were only recruited from two counties in South Florida, which likely contributed to low generalizability. Food frequency questionnaire heavily depended on long-term memory. However, the instrument has extensively been validated and standardized in diverse populations (Hernández-Avila et al., 1998; Holmes et al., 2007; Nath & Huffman, 2005). Also, dietary assessment over the past year may captured seasonal food intake which better reflected on overall food consumption. Despite these aspects, strengths of the study were worth mentioned. Apart from patient report, diabetes status was ascertained using standard criteria established by American Diabetes Association. This is the first study investigated the relationship between methylglyoxal and eating pattern particularly in Haitian Americans and African Americans. The evidence contributed to existing knowledge concerning Blacks, which provides a better understanding in health disparities among subpopulations.

In conclusion, serum levels of prominent adduct MGH1 and HEI-2005 scores were significantly higher in Haitian American and African participants with type 2 diabetes than those without diabetes. Levels of MGH1 were positively associated with the HEI-2005 scores. Such relationship may reflect the low sensitivity of the HEI-2005 to capture diet high in methylglyoxal and its adducts. Also, disease state and modifiable behaviors possibly confound the findings through aggravating methylglyoxal accumulation even with relatively healthy eating pattern. The interpretation of diet quality should be performed discreetly, especially in relation to methylglyoxal and AGEs. The positive association between MGH1 levels and the HEI-2005 score was more pronounced in Haitian American participants, indicating the need for culturally sensitive

nutrition education for diabetes management. Future studies are required to warrant and elucidate the true nature of such relationship.

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Table 1. Characteristics of the participants

Variable	without diabetes (n=212)	with diabetes (n=240)	*p*-value
Age (years)	52.47 ± 9.88	56.15 ± 10.30	<0.001*
Gender, n (%)			0.486
Female	107 (50.5)	129 (53.8)	
Male	105 (49.5)	111 (46.3)	
Ethnicity, n (%)			0.805
Haitian Americans	112 (52.8)	124 (51.7)	
African Americans	100 (47.2)	116 (48.3)	
BMI (kg/m^2)	30.00 ± 5.96	32.50 ± 7.23	<0.001*
FPG (mmol/l)	97.21 ± 15.09	152.80 ± 65.73	<0.001*
A1c (%)	5.93 ± 0.45	8.07 ± 2.31	<0.001*
Education			
< High school graduate	57 (26.9)	87 (36.3)	0.032*
High school/some college	111 (52.4)	121 (50.4)	
College degree and beyond	44 (20.8)	32 (13.3)	
Kcal	2,057.30 ± 1,025.56	1,851.25 ± 929.65	0.026*
Physical activity (MET-hr/wk)	28.82 ± 61.39	17.57 ± 27.00	0.014*
HEI-2005	59.49 ± 13.40	63.29 ± 12.66	0.002*
AHEI	48.31 ± 13.85	46.47 ± 12.80	0.142
MGH1 (µg/ml)	2.26 ± 2.55	2.40 ± 2.49	0.555
lnMGH1	0.40 ± 0.93	0.58 ± 0.74	0.026*

Continuous variables were expressed as mean ± standard deviation (SD), while categorical variables were expressed as n (%). ln=natural log-transformed; A1c=hemoglobin A1c; AHEI=alternate healthy eating index; BMI=body mass index; FPG=fasting plasma glucose; HEI-2005=healthy eating index 2005; MGH1=methylglyoxal hydroimidazolone 1; *p<0.05 is considered statistically significant.

Table 2. Multiple regression analysis for relationship of lnMGH1 with HEI-2005 and AHEI in study participants

HEI-2005 scores				
Variables	B	SE	95% CI	*p*-value
Unadjusted model				
HEI-2005	0.008	0.003	0.002, 0.014	0.005*
Adjusted model[a]*				
Age	0.001	0.004	-0.006, 0.009	0.745
Gender (male)	0.141	0.083	-0.022, 0.303	0.090
Diabetes status (yes)	0.471	0.245	-0.010, 0.952	0.055
Ethnicity (AA)	0.102	0.119	-0.132, 0.337	0.391
Diabetes status*ethnicity (yes*AA)	-0.229	0.156	-0.536, 0.078	0.143
Physical activity[c]	-0.051	0.028	-0.107, 0.005	0.074
HEI-2005	0.009	0.003	0.002, 0.014	0.012*
AHEI scores				
Variables	B	SE	95% CI	*p*-value
Unadjusted model				
AHEI	0.000	0.003	-0.006, 0.005	0.897
Adjusted model[b]				
Age	0.002	0.004	-0.006, 0.010	0.598
Gender (male)	0.080	0.080	-0.078, 0.238	0.224
Diabetes status (yes)	0.511	0.246	0.027, 0.994	0.038*
Ethnicity (AA)	0.012	0.118	-0.219, 0.244	0.917
Diabetes status*ethnicity (yes*AA)	-0.235	0.157	-0.545, 0.074	0.136
Physical activity[c]	-0.039	0.029	-0.097, 0.018	0.175
AHEI	-0.001	0.003	-0.007, 0.006	0.813

AA=African American, B=coefficient, SE=standard error, CI=confidence interval, ln=natural log; AHEI=alternate healthy eating index, HEI-2005=healthy eating index 2005, MGH1=methylglyoxal hydroimidazolone 1; *$p<0.05$ is considered statistically significant.

[a]Model Summary: Adjusted R^2=0.027, $F(7,444)$=2.759, p=0.008.

[b]Model Summary: Adjusted R^2=0.013, $F(7,444)$=1.839, p=0.078.

[c]Leisure-time physical activity was transformed to ln(1+ physical activity).

Table 3. Multiple regression analysis for relationship of lnMGH1 with HEI-2005 stratified by ethnicity

Haitian American (n=236)				
Variables	B	SE	95% CI	*p*-value
Unadjusted model				
HEI-2005	0.013	0.005	0.004, 0.022	0.006*
Adjusted model[a]*				
Age	0.010	0.005	0.000, 0.022	0.054
Gender (male)	0.122	0.113	-0.101, 0.345	0.283
Diabetes status (yes)	0.853	0.273	0.316, 1.391	0.002*
Education	0.248	0.112	0.027, 0.469	0.028*
Diabetes status*education	-0.389	0.148	-0.680, -0.097	0.009*
Physical activity[c]	-0.080	0.041	-0.161, 0.000	0.051
HEI-2005	0.012	0.005	0.002, 0.022	0.014*
African American (n=216)				
Variables	B	SE	95% CI	*p*-value
Unadjusted model				
HEI-2005	0.003	0.005	-0.006, 0.012	0.493
Adjusted model[b]				
Age	-0.007	0.006	-0.019, 0.005	0.277
Gender (male)	0.143	0.117	-0.088, 0.374	0.224
Diabetes status (yes)	-0.805	0.419	-1.632, 0.022	0.056
Education	-0.341	0.146	-0.628, -0.054	0.020*
Diabetes status*education	0.419	0.199	0.027, 0.810	0.036*
HEI-2005	0.007	0.005	-0.003, 0.016	0.161

B=coefficient, SE=standard error, CI=confidence interval, ln=natural log; HEI-2005=healthy eating index 2005; MGH1=methylglyoxal hydroimidazolone 1, *$p<0.05$ is considered statistically significant.

[a]Model Summary: Adjusted R^2=0.080, $F(7,228)=3.938$, $p<0.001$.

[b]Model Summary: Adjusted R^2=0.011, $F(6,209)=1.388$, $p=0.221$.

[c]Leisure-time physical activity was transformed to ln(1+ physical activity).

Table 4. Multiple regression analysis for relationship of lnMGH1 with AHEI stratified by ethnicity

Haitian American (n=236)				
Variables	B	SE	95% CI	*p*-value
Unadjusted model				
AHEI	-0.004	0.004	-0.013, 0.004	0.309
Adjusted model[a]*				
Age	0.011	0.005	0.000, 0.022	0.043*
Gender (male)	-0.017	0.109	-0.232, 0.197	0.874
Diabetes status (yes)	0.940	0.273	0.403, 1.477	0.001*
Education	0.273	0.112	0.053, 0.494	0.015*
Diabetes status*education	-0.400	0.149	-0.693, -0.107	0.008*
AHEI	-0.005	0.004	-0.013, 0.004	0.263
African American (n=216)				
Variables	B	SE	95% CI	*p*-value
Unadjusted model				
AHEI	0.001	0.005	-0.009, 0.010	0.910
Adjusted model[b]				
Age	-0.006	0.006	-0.018, 0.006	0.331
Gender (male)	0.107	0.115	-0.120, 0.334	0.354
Diabetes status (yes)	-0.783	0.424	-1.618, 0.053	0.066
Education	-0.319	0.146	-0.607, -0.032	0.030*
Diabetes status*education	0.425	0.204	0.024, 0.827	0.038*
AHEI	0.004	0.005	-0.006, 0.013	0.426

B=coefficient, SE=standard error, CI=confidence interval, ln=natural log; AHEI=alternate healthy eating index 2005; MGH1=methylglyoxal hydroimidazolone 1, *$p<0.05$ is considered statistically significant.

[a]Model Summary: Adjusted R^2=0.052, $F(6,229)$=3.156, p=0.005.

[b]Model Summary: Adjusted R^2=0.004, $F(6,209)$=1.157, p=0.330.

CHAPTER VII

CONCLUSION AND FUTURE WORK

The first part of the dissertation aimed to investigate the association of RAP6 activity on preadipocyte differentiation as well as those driven by methylglyoxal *in vitro*. Overexpression of RAP6 inhibited preadipocyte differentiation as well as expression of key adipogenic marker PPARγ, but not C/EBPα. Activation of Akt1 was decreased by RAP6 overexpression, suggesting that RAP6 may regulate preadipocyte differentiation through Akt1 activity. Another part of the *in vitro* study showed that low concentration of methylglyoxal increased preadipocyte differentiation, proliferation and expression of PPARγ, C/EBPα and p-Akt1-Ser473. However, the stimulatory effect of methylglyoxal appeared to be neutralized by RAP6 overexpression. Methylglyoxal-induced Akt1 phosphorylation was suppressed even greater than that in a presence of RAP6 overexpression alone. The findings suggest that RAP6 may be a key modulator in regulating the effect of methylglyoxal on stimulating preadipocyte differentiation. Presumably, RAP6 modulated preadipocyte differentiation and stimulatory effect of methylglyoxal through Rab5 activation. However, a direct interaction between RAP6 and related proteins should not be ruled out.

The latter part of the dissertation focused on determining levels of a predominant methylglyoxal-derived adduct, MGH1, in two ethnic participants with and without type 2 diabetes. Elevated MGH1 levels in participants with diabetes reported earlier (Ahmed, Babaei-Jadidi, Howell, Thornalley, & Beisswenger, 2005; Kilhovd et al., 2003) were supported by the current study. Additionally, the association between levels of MGH1 and fasting plasma glucose emerged in participants with type 2 diabetes, but not in

healthy individuals. The findings indicated that methylglyoxal was more likely to be elevated by hyperglycemia under pathological condition.

The relationship of MGH1 with other risk factors of chronic disease were also investigated. Levels of MGH1 were positively associated with levels of homocysteine and triglycerides. These observations suggested the association of methylglyoxal with macro- and microvascular disease conditions. It was previously reported that methylglyoxal accumulation was shown in participants with diabetic vascular complications, e.g. neuropathy (Bierhaus et al., 2012), nephropathy (Beisswenger et al., 2013; Beisswenger et al., 2014), and retinopathy (Haik, Lo, & Thornalley, 1994). Suggestively, methylglyoxal may be associated with pathogenesis of diabetic vascular complications.

The positive relationship between MGH1 levels and the 2005-healthy eating index (HEI-2005) was also found in ethnic participants with type 2 diabetes, which was surprising. It was reported a significant association between high dietary advanced glycation end products (AGEs) and serum methylglyoxal levels in overweight and obese participants (Uribarri et al., 2015). Possibly, methylglyoxal may be more related to quantity of AGEs-containing food than overall food quality. Also, dietary pattern in relation to methylglyoxal should be interpreted cautiously due to diverse food pattern across Black subgroups as previously reported (James, 2009).

The current findings showed that some factors also influenced circulating MGH1 levels. Augmented MGH1 levels were associated with increasing age of the study participants, which was consistent with that reported earlier (Ahmed, Brinkmann Frye, Degenhardt, Thorpe, & Baynes, 1997). In participants with type 2 diabetes, the increase

in MGH1 levels were more pronounced in Haitian American participants than African American participants. This may be due to differences in genetic variation and behavioral factors. According to previous studies, Black subpopulations had variation in proportion of African and European ancestry (Bryc, Durand, Macpherson, Reich, & Mountain, 2015; Simms et al., 2012). This possibly contributed to different frequencies of some alleles such as GLO1 encoding enzyme glyoxalase 1 which was required for methylglyoxal detoxification. Supportively, the difference in GLO1 frequency was observed in Black subgroups residing in South Africa (Bender, Frank, & Hitzeroth, 1977). Haitian Americans reportedly had poor glycemic control as compared to Whites and African Americans (Vimalananda, Rosenzweig, Cabral, David, & Lasser, 2011). Given that, persistent hyperglycemia possibly caused the elevation of MGH1 levels in Haitian American participants even more than in African American participants. Culturally sensitive health counseling and intervention may be better applied to ethnic minorities who have diversity of genetics and health-related lifestyles.

In this dissertation, there are some possible connections between the *in vitro* and human studies. High glucose condition appeared to elevate formation of MGH1 in cultured preadipocytes as well as in participants with type 2 diabetes. Methylglyoxal also increased preadiopocyte differentiation and proliferation *in vitro*, at least in part, through alteration of insulin signaling. These observations suggest that methylglyoxal may be a link between diabetes and adipogenesis. Based on the current findings, RAP6 activity possibly modulated the effect of methylglyoxal on preadipocyte differentiation. However, there still have discrepancies that remain to be elucidated. Mechanism(s) by which RAP6 regulates Akt1 activation, e.g. through upstream effectors and/or direct interaction,

deserves further investigation. Moreover, it is convincible to examine the direct interaction between RAP6, receptors, and other effectors as well as the need of Rab5 activation in RAP6-mediated preadicocyte differentiation. The future studies should be conducted to also explore whether the activity of RAP6 and the effect of methylglyoxal alter cell differentiation in a cell-type specific fashion. These observations may contribute to existing knowledge in regulation of adipogenesis as well as other pathological conditions resulted from dysregulation of receptor signaling and trafficking.

The current human study particularly examined the association between methylglyoxal and modifiable risk factors in Black subgroups residing in two counties of Florida, which was unique. However, this may contribute to low generalization. Replicate studies in a larger sample size are required to warrant the findings of the current study. Not only variation of GLO1 allele frequency was reported in Black subgroups residing in South African (Bender, Frank, & Hitzeroth, 1977) but also between American Whites and Blacks (Weitkamp, 1976). Inclusion of other races may give rise to a whole spectrum of understanding in relationships between methylglyoxal and other risk factors of chronic diseases among populations.

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