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ORIGINAL ARTICLE

Effects of age on the glucoregulatory response following acute glucoprivation induced by 2-deoxyglucose (2DG) in the adrenal medulla of Sprague Dawley rats

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Abstract

OBJECTIVES: Impairment in glucose homeostasis is one of the factors that may alter the feeding drive, hunger and satiety signals, which essential to maintain a sufficient level of energy for daily activities especially among the elderly. Adrenal medulla is one of the important organs that involves in glucose homeostasis through secretion of catecholamines. The catecholamines biosynthesis pathway utilizes various enzymes and protein kinases. The aims of this study are to investigate the effects of age on the biosynthetic pathway of catecholamines in adrenal medulla by determining the level of blood glucose and blood catecholamines, the gene and protein expression of biosynthetic catecholamine enzymes (TH, DBH and PNMT) as well as protein kinase substrates that involved in the phosphorylation of TH in 2DG-induced rats.

METHODS: Adrenal medulla from male Sprague Dawley rats at the age of 3-months (n=12) and 24-months (n=12) were further divided into two groups: 1) treatment group with 2DG to create glucoprivation condition and 2) the vehicle group which received normal saline as control.

RESULTS: The results showed that the level of glucose, adrenaline and noradrenaline were increased in response to acute glucoprivation conditions in both young and old rats. No age-related differences were found in the basal gene expression of the enzymes that involved in the catecholamines biosynthesis pathway. Interestingly the expressions of TH and DBH protein as well as the level of TH phosphorylation at Ser40, PKA, PKC and ERK1/2 substrates were higher in basal condition of the aged rats. However, contradicted findings were obtained in glucoprivic condition, which the protein expressions of DBH, pERK1/2 and substrates for pPKC were increased in young rats. Only substrate for pCDK was highly expressed in the old rats in the glucoprivic condition, while pPKC and pERK1/2 were decreased significantly. The results demonstrate that adrenal medulla of young and old rats are responsive to glucose deficit and capable to restore the blood glucose level by increasing the levels of blood catecholamines.

CONCLUSION: The present findings also suggest that, at least in rats, aging alters the protein expression of the biosynthetic catecholamine enzymes as well as protein kinase substrates that may attenuate the response to glucoprivation.

INTRODUCTION

Glucose is a metabolic energy provider, which is very important in all tissues, particularly the brain and it is essential to maintain a sufficient level of energy for daily activities especially among the elderly. Therefore, the blood glucose level must strictly maintain at a normal level. The fall of blood glucose level or known as hypoglycemia will stimulate glucose sensor system located in the central nervous system, which are the hypothalamus and brain stem and also in the peripheral system mainly in the liver (Marty *et al.* 2007; Roberts & Rosenberg 2006; Verberne *et al.* 2014). Part of the hypothalamus namely ventromedial hypothalamus (VMH) comprising of the ventromedial nucleus (VMN) and arcuate nucleus (ARC) initiate and coordinate the counterregulatory response against hypoglycemia through the secretion of glucocorticoid hormones from the pituitary gland, glucagon from the pancreas gland and catecholamines released by the adrenal medulla (Watts & Donovan 2010; Roberts & Rosenberg 2006; Cryer 2004). These counterregulatory hormones act to increase blood glucose by stimulating the production of glucose in the liver through the process of gluconeogenesis and glycogenolysis as well as reducing the use of glucose by the peripheral tissues (Marty *et al.* 2007).

In addition, among normal young adults, the condition of hypoglycemia or lack of glucose sparked hunger and increased food intake behavior (Melanson *et al.* 1998) to increase back the blood glucose to normal level. Impairment in glucose homeostasis is one of the factors may alter the feeding drive as well as hunger (Tank & Lee Wong 2015) and satiety signals. A reduction in food intake is associated with aging. The reduction in food intake is considered as a normal response and a physiological adaptation process for the reduction of energy consumption for healthy aging (Hauser & Neumann 2005). However, the chronic decline in appetite and food intake can cause the occurrence of anorexia of aging and increasing the cases of malnutrition among elderly (Morley 2001). Anorexia of aging is defined as the decline in appetite and food intake that leads to the decline of body weight and cause a dangerous risk to health. It may contribute to various adverse consequences, such as loss of skeletal muscle mass, frailty (Martone *et al.* 2013), increased hospital admissions and treatment costs (Hickson 2006). Therefore, specific treatment and prevention of anorexia in the elderly are an important challenge for the health care systems. However, a precise understanding of the mechanisms that underlie anorexia of aging in elderly is necessary to develop an effective treatment or prevention.

Several studies were carried out to study the factors that cause the decline in appetite and food intake. It involves the central and peripheral regulatory that controls hunger and the feeling of fullness and then induced the response towards food intake. Interference in the hypothalamus include arcuate nucleus (ARC), paraventricular nucleus (PVN), ventromedial, dorsomedial and lateral hypothalamus may interfere the regulatory of the dietary intake behavior (Kmiec 2006; Sousa-Ferreira *et al.* 2014). In addition, disruption of peripheral regulatory included the secretion of insulin, leptin (Varela & Horvath 2012; Woods & Seeley 2000) and gastrointestinal peptides such as cholecystokinin (CCK) can also cause reduction in appetite and food intake (Sousa-Ferreira *et al.* 2014). The increases in the level of leptin and insulin have decreased the function of the arcuate nucleus (ARC) in producing of the neuropeptide Y (NPY) and Agouti-related peptide (AgRP) thus inhibits the desire to take food and stimulate energy consumption (Horwitz *et al.* 2002). The level of cholecystokinin (CCK) also found high in the elderly as compared to young subjetcs (MacIntosh *et al.* 2001; Sturm *et al.* 2003; Martinez *et al.* 1993). The high level of CCK inhibits the production of NPY and AgRP by ARC and reduced hunger in the elderly (MacIntosh *et al.* 2001; Morley 2001).

However, the involvement of catecholamines in the regulation of food intake and appetite, especially among the elderly is still unknown. Catecholamines such as adrenaline and noradrenaline, which secreted by adrenal medulla are known in increasing the blood glucose concentration (Ritter *et al.* 2011) through activation of α and β adrenergic receptors and indirectly increase the release of glucagon by the pancreatic cells and hinder the secretion of insulin into the blood (Karam *et al.* 1966; Malaisse *et al.* 1967). Catecholamines also act on adipose and muscle to reduce the intake of glucose by blocking the glucose transporter GLUT4 through inhibition the merger of insulin to its receptor (Verberne *et al.* 2014). Adrenaline also activates lipase to encourage the lipolysis mainly in adipose tissues to increase the blood glucose level and provide energy to the cells (Verberne *et al.* 2014; Lafontan *et al.* 1997; Lonnqvist *et al.* 1990; Ritter *et al.* 2011).

The biosynthesis of these catecholamines depend on the activity of the rate limiting enzyme, *tyrosine hydroxylase* (TH) as well as *dopamine β-hydroxylase* (DBH) and *phenylethanolamine N-methyltransferase* (PNMT). The activity of TH is controlled by both short term and long term regulatory mechanisms. Short term or acute regulatory of TH involve feedback inhibition (Daubner *et al.* 2011; Dunkley *et al.* 2004), phosphorylation and allosteric regulatory (Kleppe *et al.* 2001; Kumer & Vrana 1996; Lloyd & Kaufman 1974). This regulatory mechanism involves the modification of the existing TH molecule and replenishment of the catecholamines

(Bobrovskaya *et al.* 2010). While in the long-term or chronic regulation, it involves enzymatic stability of TH, the regulation of transcription, the stability of ribonucleic acid (RNA), consolidation of the alternative RNA and translation activities (Kumer & Vrana 1996). The chronic regulatory mechanism involves the expression of TH gene and the production of a new TH molecule (Dunkley *et al.* 2004).

In the acute regulation, phosphorylation of the serine residues (Ser8, Ser19, Ser31 and Ser40) at the N-terminal regulatory domain of the TH enzyme (Dunkley *et al.* 2004) was shown previously in bovine adrenal chromaffin cells (Haycock *et al.* 1992) and PC12 cells (Haycock 1990). However, only Ser19, Ser31 and Ser40 found to be regulated in vivo (Haycock & Haycock 1991). Ser40, Ser31 and Ser19 was phosphorylated by a number of protein kinases in the brain tissue and adrenal medulla in vivo when exposed to several stimulus including angiotensin II (Bobrovskaya *et al.* 2001; Bobrovskaya *et al.* 1998), nicotine (Bobrovskaya *et al.* 2007), glucoprivation (Bobrovskaya *et al.* 2010; Damanhuri *et al.* 2012) and foot shock (Ong *et al.* 2011; Ong *et al.* 2014). For example, Ser19 is phosphorylated by calcium calmodulin-dependent protein kinase II (CaMKII), Ser31 by extracellular signal-regulated kinase 1/2 (ERK1/ERK2) and indirectly by PKC, while Ser40 by protein kinase A (PKA), protein kinase G, mitogen and stress-activated protein kinase (MAPK), protein kinase C (PKC), CaMKII and *mitogen-activated protein kinase-activated kinases* (MAPKAP) 1 and 2 (Dunkley *et al.* 2004; Haycock 1990; Haycock & Haycock 1991). Ser40 has been a major site in mediating the activation of TH molecules. Only PKA is can directly phosphorylate and responsible for increasing the phosphorylation of TH at Ser40 (Salvatore *et al.* 2001). Phosphorylation of TH determines the enzymatic activity and the amount of catecholamine synthesis.

Based on the previous studies, the glucoregulatory mechanism in the brain and in the periphery can induce food intake behavior. Mechanism of glucoregulatory in the brain and adrenal medulla following glucoprivation was explored in young Sprague Dawley rats (Bobrovskaya *et al.* 2010; Damanhuri *et al.* 2012). However, up to now, studies on the effects of age on glucoregulatory response, particularly in adrenal medulla following glucoprivation is still unclear. Therefore, this study was conducted to determine the effect of age on the glucoregulatory mechanism following acute glucoprivation in the adrenal medulla of young and old rats. We used 2-deoxy-D-glucose (2DG) to evoke glucoprivation in conscious Sprague-Dawley rats. 2DG is a glucose analogue that does not undergo glycolysis providing an environment of low effective concentrations of glucose (Bobrovskaya *et al.* 2010).

Tab. 1. List of primers.

Gene	Primer sequence (5′ – 3′)	Amplicon size (bp)	No. accession
TH	Forward: TTCCGCGTGTTTCAATGCAC Reverse: TGCAAGTCCAATGTCCTGGG	150	NM_012740.3
DBH	Forward: ACGGGGACAGGACCTACTTT Reverse: AGGCTGTTTGACACCCTCTG	112	NM_013158.2
PNMT	Forward: GAAGCGAGTCTTGCCCATTG Reverse: ATGATACAAAGCCTGCCGGA	148	NM_031526.1
β-actin	Forward: CAACCTTCTTGCAGCTCCTC Reverse: TCTGACCCATACCCACCATC	200	NM_031144.3

EXPERIMENTAL DESIGN

Materials

2-deoxy-D-glucose (2DG), ethylene glycol tetraacetic acid (EGTA), ethylene diamine tetraacetic acid (EDTA), dithiothreitol (DTT), sodium 1-octanesulfonate (Nacalai Tesque, Kyoto, Japan), sodium dodecyl sulfate (SDS) and aluminium oxide (Sigma). Clear conical bottom verex insert, diameter 5 mm, 100 µL (4520552190, Phenomenex, USA) and Ultrafree-MC DURA 0.22 µm filter tube unit (UFC30GV00, Millipore) were used in the HPLC analysis. The following antibodies were used in western blot analysis: primary monoclonal rat anti-tyrosine hydroxylase (T1299, Sigma), polyclonal rabbit anti-dopamine β-hydroxylase (AB1585, Millipore), polyclonal rabbit anti-phenylethanolamine N-methyltransferase (PNMT) (ab69579, Abcam), polyclonal rabbit anti-tyrosine hydroxylase phosphoSer40 (AB5935, Millipore), polyclonal rabbit anti-phospho-(Ser) PKC substrate (#2261, Cell Signaling), monoclonal rabbit IgG anti-phospho-PKA substrate (#9624, Cell Signaling), monoclonal rabbit anti-p44/42 MAPK (Erk1/2)(137F5) (#4695, Cell Signaling), monoclonal rabbit anti-phospho-p44/42 MAPK (Erk1/2)(Thr202/Tyr204) (4370, Cell Signaling), polyclonal rabbit anti-CaM Kinase II (#07-1496, Millipore), polyclonal rabbit anti-phospho-CaM Kinase II α/β (T286/287) (#06-881, Upstate), monoclonal rabbit anti-phospho-MAPK/CDK substrate (#2325, Cell Signaling) and monoclonal rat anti-β-actin (Sigma). Secondary polyclonal goat anti-rabbit IgG H&L (HRP) (Abcam) and anti-mouse IgG HRP (#H2814, SantaCruz Biotechnology) were used. Standards that were used in HPLC analysis: noradrenaline bitartrate salt (3414-63-9, Sigma), adrenaline hydrochloride (329-63-5, Sigma) and 3,4-dihydroxybenzylamine (DHBA) hydrobromide (16290-26-9, Sigma). DHBA was used as internal standard. The primers that were used in RT-PCR analysis are shown in Table 1. β-actin was used as the housekeeping gene.

Animal treatment, sample collection and preparation

Experiments were conducted on adult male Sprague Dawley rats aged 10–13 weeks for the young group and 23–24 months for the old group weighing between 300–500 grams. All experiments were approved by the Animal Ethics Committee, National University of

Malaysia. Rats were purchased from Animal Laboratory Resource Centre, National University of Malaysia. In conscious animals (n=6), 2DG (400 mg/kg) or saline, were administered intraperitoneally (0.4 mL) following the removal of food and water. Animals were sacrificed 30 min following treatment by administration of Zoletil 50 (Virbac, France) intramuscularly. When rats no longer responded to painful stimuli, they were decapitated by guillotine and blood and tissue samples were collected. Whole trunk blood (5 mL) was collected into tubes containing 95 mg/mL EGTA and 30 mg/mL reduced glutathione and kept cold (4 °C). Blood glucose level was measured immediately using Accu-check performa glucometer (Roche, Mannheim, Germany). Blood samples were centrifuged for 10 min at 1800 rpm at 4 °C. Resulting plasma was then spun for 10 min at 2700 rpm at at 4 °C. Plasma samples were kept at −80 °C until further analysis. Whole adrenal glands were removed rapidly then the adrenal medulla was dissected free from the adrenal cortex prior to freezing in dry ice and kept at −80 °C until further analysis.

High Performance Liquid Chromatography (HPLC) for measurement of catecholamines

Plasma catecholamines were extracted using the alumina extraction methods (Wang *et al.* 1999). Adrenaline and noradrenaline were analyzed by the Prominence HPLC system (Shimadzu Corporation, Kyoto, Japan). 10 µL samples were injected into Synergi 4U Fusion-RP C18 column (Phenomenex, USA) that connected to Coulochem III electrochemical detector (ESA Inc., Chelmsford, MA, USA). The analytical cell was set at +450 mV and kept a constant flow of 1.0 mL/min of mobile phase [(0.1 M phosphate buffer (pH 5.7), 50 mg/L of disodium EDTA, 700 mg/L of sodium 1-octanesulfonate (Nacalai Tesque, Kyoto, Japan), 12% methanol)]. The specific retention time for each compound was determined by using adrenaline and noradrenaline standards. The concentration of catecholamines from each sample was calculated from the peak area ratio by using LC Solution software (Shimadzu, Kyoto, Japan).

Western blot for protein expression analysis

Frozen tissue samples were weighed and homogenized, in 40 volumes of homogenization buffer (2% SDS, 2 mM EDTA, 50 mM Tris (pH 6.8)), by sonication, on ice (3 times, 30 Sec at 10,000 A), then boiled for 10 min and centrifuged for 20 min. Supernatants were carefully removed. Aliquots (5 µL) of the homogenate were used for the protein content determination. 20 µL of supernatant was mixed with 1 µL of 10% dithiothreitol (DTT), 7 µL of sample buffer (40% glycerol, 50 mM Tris) and minimal bromophenol blue (pH 6.8). Equal amounts of protein (50 µg), from each sample were loaded onto 10% polyacrylamide gels, and the proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidine difluoride membranes (PALL, Life Sciences, Mexico). The membrane was incubated with primary antibody: monoclonal rat anti-tyrosine hydroxylase (1:5000), polyclonal rabbit anti-dopamine β-hydroxylase (1:1000), polyclonal rabbit anti-PNMT (1:1000), polyclonal rabbit anti-tyrosine hydroxylase phosphoSer40 (1:2000), polyclonal rabbit anti-phospho-PKC substrate (1:1000), monoclonal rabbit IgG anti-phospho-PKA substrate (1:1000), monoclonal rabbit anti-p44/42 MAPK (Erk1/2)(1:1000), monoclonal rabbit anti-phospho-p44/42 MAPK (ERK1/2) (1:1000), polyclonal rabbit anti-CaM Kinase II (1:1000), polyclonal rabbit anti-phospho-CaM Kinase II α/β (1:1000), monoclonal rabbit anti-phospho-MAPK/CDK substrate (1:1000) or monoclonal rat anti-β-actin (1:2000) for overnight at 4 °C. The membrane was further incubated with secondary antibody polyclonal goat anti-rabbit IgG HRP (1:1000) or anti-mouse IgG HRP (1:10000 for TH antibody or 1:4000 for other proteins) for 1 hour at room temperature. The protein on the membrane was detected by chemiluminescent detection kit. Intensity quantification of the bands was done by using ImageMaster^{TM} 2D Platinum 6.0 (GE Healthcare) software.

Real Time-Polymerase Chain Reaction (RT-PCR) for gene expressions analysis

Adrenal medulla (n=5 for each group) was lysed by QIAzol reagent (54804664, QIAGEN, USA) and total mRNA was extracted according to RNeasy Lipid Tissue (74804, QIAGEN, USA) instruction kit. Two step RT-PCR was used to analyze the gene expression by using QuantiNova Reverse Transcription kit (205411, QIAGEN, USA) and QuantiNova SYBR Green PCR kit (208054, QIAGEN, USA). The protocol was based on the recommended protocol from the manufacturer. Primers for TH, DBH, PNMT and β-actin were synthesized by Integrated DNA Technologies. The β-actin was used as an internal control. Real time RT-PCR was performed on iQ5 Real-Time PCR Detection System (BioRad). In the first step of RT-PCR, the reaction mixture for cDNA synthesis contained: 2 µg total RNA and QuantiNova Reverse Transcription master mix buffer. The reaction condition was set as followed: 45 °C for 2 min for gDNA elimination reaction, 25 °C for 3 min for annealing step, and 45 °C for 10 min for reverse transcription step. For the second step, the reaction mixture contained: 500 ng cDNA, QuantiNova SYBR Green PCR master mix and 0.7 µM forward and reverse primers. PCR was carried out at 95 °C for 2 min for one cycle, followed by 40 cycles of 95 °C for 5 sec and 60 °C for 10 sec. Data from the RT-PCR was reported as a C_T number (cycle threshold number). The expression mRNA level of each gene was normalized to β-actin and calculated using $2^{-\Delta\Delta CT}$ method. Each sample was tested in duplicate.

Statistical evaluation of the data

Data of western blot, HPLC and RT-PCR for gene expression analysis were expressed as mean ± SEM.

Analysis was performed using GraphPad Prism (version 7). Comparisons of baseline data between young and older rats, comparisons between baseline and 2DG injection in both group young and old rats were performed by using the Students's T-test for independent samples. Differences having a value of $p<0.05$ were considered to be statistically significant.

RESULTS

Effects of age and glucoprivation on blood glucose and plasma catecholamines level

The basal circulating level of glucose (Figure 1A) and adrenaline (Figure 1B) in old group were not significantly different compared to the young group. In contrast, the basal level of noradrenaline was significantly increased in the control of old group compared to young control ($^{\#}p<0.05$, Figure 1C). The level of blood glucose, adrenaline and noradrenaline were relatively increased by 2DG administration in all groups. The blood glucose levels were significantly increased by 2DG administration in young group ($^{***}p<0.001$, 1.8 fold) and old group ($^{***}p<0.001$, 1.6 fold) relative to the control group (Figure 1A). The significant increased were also observed in the adrenaline level in 2DG administered young group ($^{***}p<0.001$, 25 fold) and 2DG administered old group ($^{***}p<0.001$, 55 fold) as shown in Figure 1B as well as noradrenaline level in 2DG administered young group ($^{**}p<0.01$, 13 fold) and 2DG administered old group ($^{*}p<0.05$, 3 fold) relative to the control group (Figure 1C).

Effects of age on the TH, DBH, PNMT gene expression in the adrenal medulla

In basal condition, there were no significant differences in expression of TH (Figure 2A), DBH (Figure 2B) and PNMT (Figure 2C) genes in both groups of rats.

Effects of age and glucoprivation on the protein expression of DBH, PNMT, total TH and phosphorylation of TH at Ser40 (pSer40) in the adrenal medulla

In the basal level, the expression of DBH (Figure 3D), TH (Figure 3E) and pSer40 (Figure 3F) protein were significantly increased ($^{\#}p<0.05$), but not for the PNMT

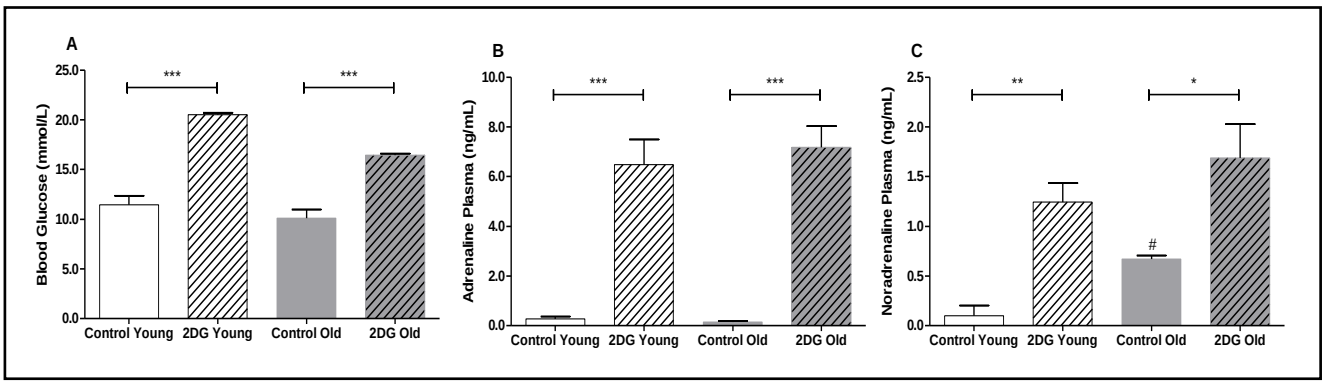

Fig. 1. Effects of age and 2DG or saline on blood glucose (A), plasma adrenaline (B) and noradrenaline levels (C). Each bar and associated error bar represent the mean value of analyte concentrations ± SEM from one experiment (n=6). $^{\#}(p<0.05)$ represents a significant difference between control group of young and old rats and $^{***}(p<0.001)$, $^{**}(p<0.01)$ and $^{*}(p<0.05)$ represent a significant difference between 2DG and control group in the same age group of rats.

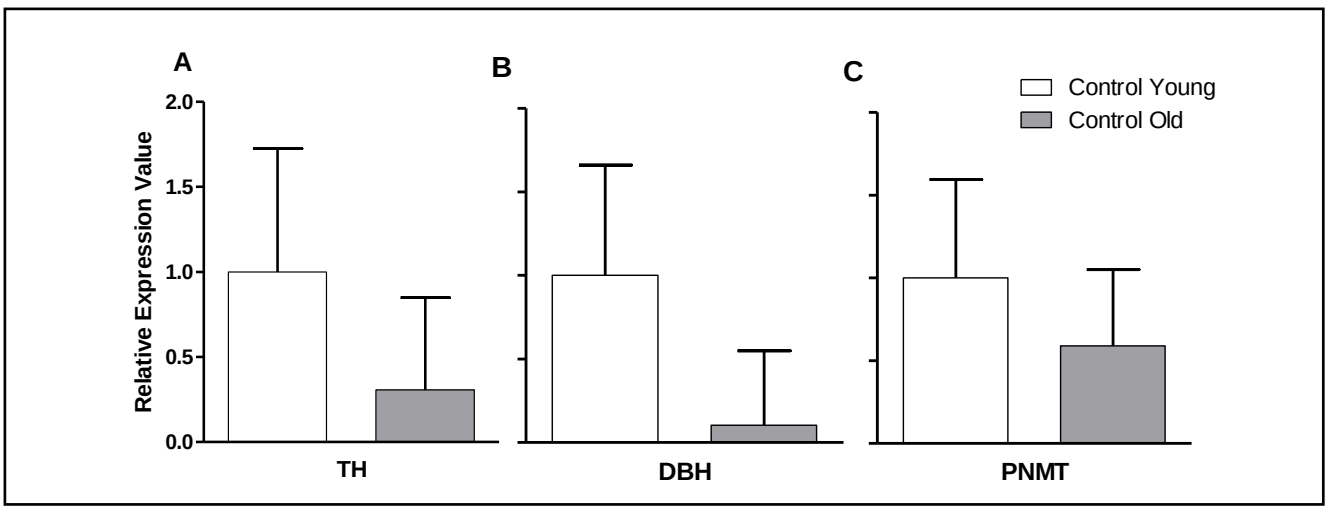

Fig. 2. Effects of age on the gene expression of TH (A), DBH (B) and PNMT (C). Each bar and associated error bar represent the mean of relative expression values ($2^{-\Delta\Delta CT}$) ± SEM from one experiment (n=5).

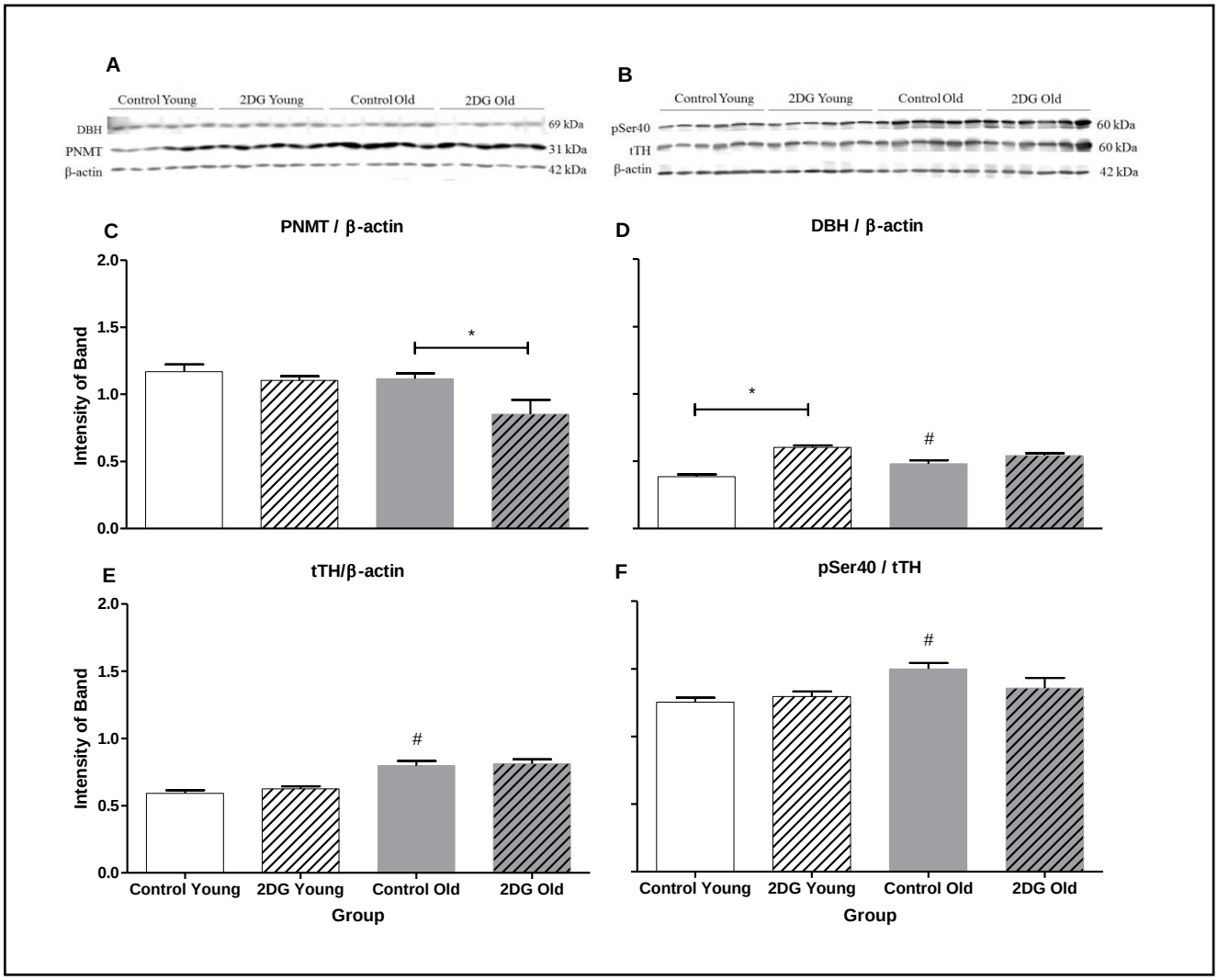

Fig. 3. Effects of age and glucoprivation condition on the level of PNMT (C), DBH (D) and total TH (tTH) protein (E) with respect to β-actin as well as phosphorylation of TH at Ser40 residue (F) with respect to tTH in the adrenal medulla. Each bar and associated error bar represent the mean value of intensity bands ± SEM from one experiment (n = 6 for each group). Representative Western blots (A, B) show the effects of age and the administration of 2DG or saline and each lane represents a single animal. # represents significant difference between control group of young and old rats ($p<0.05$) and * represents a significant difference between 2DG and control group in the same age group of rats ($p<0.05$).

protein (Figure 3C) when compared between the old control and the young control. In the young group, only DBH protein was significantly increased (*$p<0.05$) in response to glucoprivation condition (Figure 3C) while others were shown not significantly different. In the old group, the expression of PNMT protein was significantly decreased in response to glucoprivation (*$p<0.05$, Figure 3C). The expression of TH protein in the glucoprivic condition of old group was significantly higher than in the 2DG administered young group (*$p<0.05$, Figure 3E), while expression of PNMT (Figure 3C) and DBH (Figure 3D) proteins were significantly decreased (*$p<0.05$). The phosphorylation of TH at Ser40 did not show any difference in response to glucoprivation condition.

Effects of age and glucoprivation on the protein kinases activation in the adrenal medulla

The phosphorylation of PKA (Figure 4E), PKC (Figure 4F) and ERK1/2 (Figure 4H) protein in basal condition were significantly increased (#$p<0.05$) in old group compared to the younger group. In young group, the magnitude of phosphorylated PKC (Figure 4F) and ERk1/2 (Figure 4G) protein following 2DG administration were significantly higher than the basal group (*$p<0.05$). However, a significant decrease was observed in the expression of total ERK1/2 proteins (*$p<0.05$, Figure 4H) and no significant differences in the expression of phosphorylated PKA (Figure 4E) and CDK protein (Figure 4I). In contrast, in the old group treated with 2DG, the expression of phosphorylated

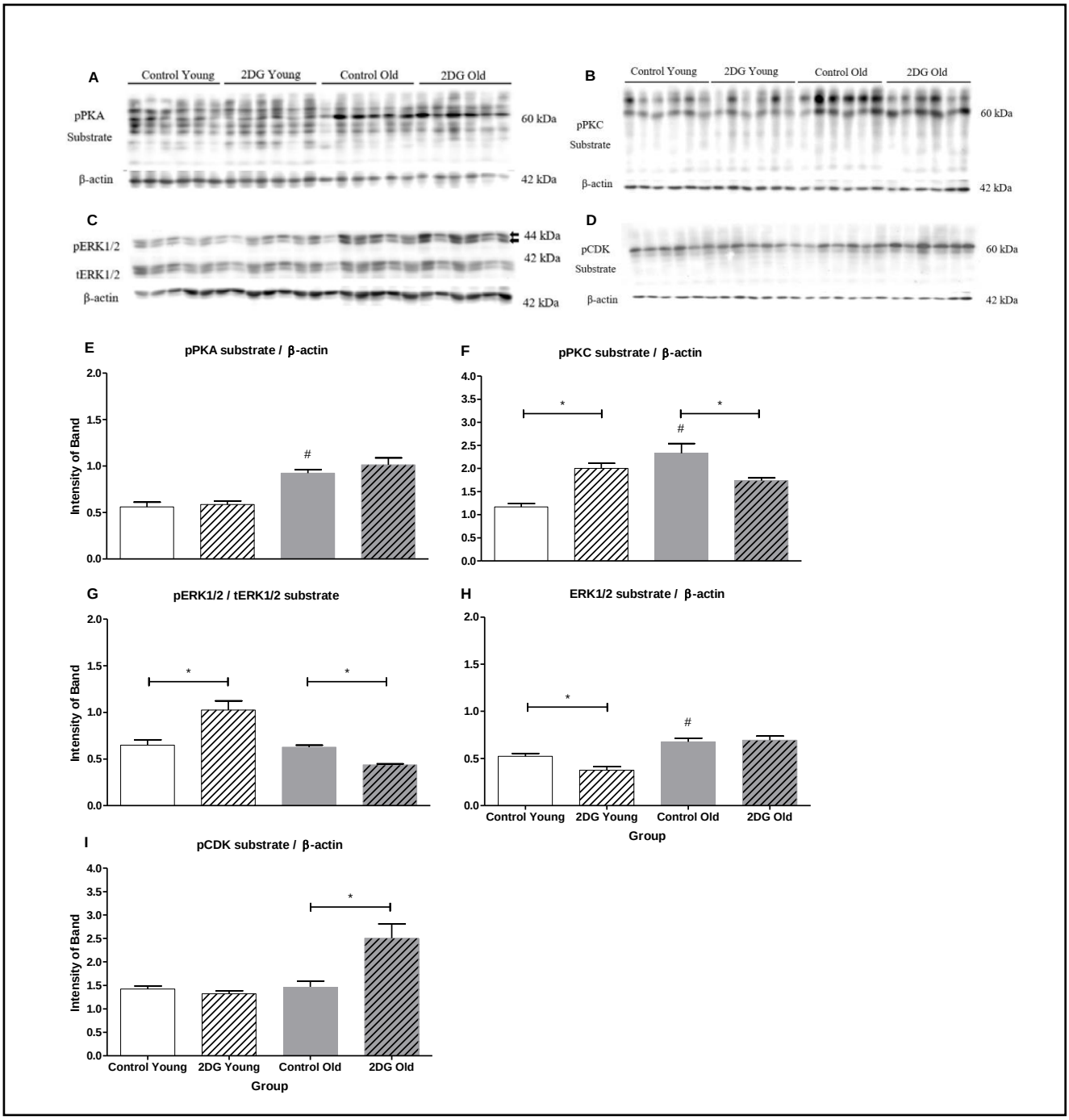

Fig. 4. Effects of age and glucoprivation condition on the level of protein kinases pPKA (E), pPKC (F) ERK1/2 (H) and pCDK (I) with respect to β-actin and pERK1/2 (G) with respect to ERK1/2 in regulation of phosphorylation of TH in the adrenal gland. Each bar and associated error bar represent the mean value of intensity band ± SEM from one experiment (n = 6 for each group). Representative Western blots (A, B, C and D) show the effect of age and 2DG or saline and each lane represents a single animal. # represents significant difference between control group of young and old rats ($p<0.05$) and * represents a significant difference between 2DG and control group in the same age group of rats ($p<0.05$).

CDK protein was significantly increased (*$p<0.05$, Figure 4I) when compared to the control group. Whilst, the expression of phosphorylated PKC (Figure 4F) and ERK1/2 proteins (Figure 4G) were significantly decreased (*$p<0.05$) and no differences were found in the expression of phosphorylated PKA (Figure 4E) and total ERK1/2 protein (Figure 4H).

DISCUSSION

Aging is associated with several changes in functions and physiological regulation includes glucoregulatory mechanisms by catecholamines, which released by the adrenal medulla. The glucoregulatory mechanisms are one of the important factors in the regulation of

food intake and control the signal of hunger and fullness. One of the objectives of this study is to examine the effect of age on the glucoregulatory mechanism by focusing on the catecholamines biosynthesis in the adrenal medulla. Almost all the adrenaline in blood circulation is released by chromaffin cells of the adrenal medulla, while most of the noradrenaline release from sympathetic nerve (Kvetnansky *et al.* 2009).

Our data demonstrated that the basal level of noradrenaline in aged rats was increased, while the level of glucose and adrenaline did not vary with age. Similarly found in previous studies which secretion of adrenaline from the adrenal medulla appeared to be either normal or low in the elderly compared to the young group (Franco-Morselli *et al.* 1977; Seals & Esler 2000). The concentration of noradrenaline in serum is found higher among older people than young people (Heffner 2011; Zjačić-Rotkvić *et al.* 2010). Other than the adrenal medulla, noradrenaline also secreted by sympathetic nerve terminals in some organs, such as muscle, liver, kidney, heart, skin and lung (Iwase *et al.* 1991). This happens probably resulting from the compensatory process due to the loss of responsive some tissue against noradrenaline (Zjačić-Rotkvić *et al.* 2010; Schwartz *et al.* 1987). A previous study also suggested that there may be interference on the sensitivity of adrenergic receptors that causes reduced response of tissues against noradrenaline (Cryer *et al.* 1980). Sympathetic nervous system activity is controlled by the central nervous system. The decline in the tissue response to noradrenaline may trigger the central nervous system to increase sympathetic nervous system activity to free up more noradrenaline. The level of noradrenaline increased with age is probably due to either increase in the release of noradrenaline into plasma by the sympathetic nervous system or decreased the clearance rate of plasma noradrenaline or a combination of both factors. The rate of noradrenaline released in healthy elderly participants was 32% higher and the rate of the noradrenaline clearance was 19% lower than the young participants (Veith *et al.* 1986; Quang T 2016).

We did further investigation on the expression of genes and proteins of tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DBH) and phenylethanolamine N-methyltransferase (PNMT) in adrenal medulla to determine the effects of age on the pathway of catecholamines biosynthesis. The results showed that the expression of TH, PNMT and DBH genes in adrenal medulla were not influenced by aged. Interestingly, the expression of TH and DBH proteins were increased in the older rats compared to the young rats, but no differences occur in the expression of PNMT protein. The expression of TH gene was not increased but the level of TH protein increased in the old rats as compared to the young rats. This possibly explained by the fact that in basal condition, TH protein is found in the sympathetic nerve or in the chromaffin cells of adrenal medulla is readily to be activated upon stimulation. DBH and PNMT enzymes are involved in the synthesis of noradrenaline and adrenaline. DBH enzyme is a catalyst for the conversion of dopamine to noradrenaline. While the enzyme PNMT is the catalyst for the production of adrenaline from noradrenaline. In this study, the increase in the level of DBH protein was associated with an increase of noradrenaline level in the blood circulation of the old rats and this in agreement with the previous study (Tümer *et al.* 1999). This study also showed that age difference does not affect the expression of PNMT gene nor at PNMT protein and this can be linked with the discovery from our finding that there are no significant differences in the level of adrenaline between young and old rats.

In basal condition, we also investigate the association between signaling pathways of protein kinases with the phosphorylation of TH. TH activity is regulated hierarchically by phosphorylation of Ser19, Ser31 and Ser40. Several studies suggested that Ser40 is the main site involved in the direct activation of TH (Haycock *et al.* 1998; Toska *et al.* 2002; Bobrovskaya *et al.* 2004). Phosphorylation of Ser19 does not directly regulate TH activity (Sutherland *et al.* 1993; Haycock *et al.* 1998; Toska *et al.* 2002). While, phosphorylation of Ser31 may direct or indirectly regulates TH activation. Phosphorylation of Ser31 alone only induces a small increase in TH activity (Haycock *et al.* 1992; Sutherland *et al.* 1993; Bobrovskaya *et al.* 2010). Phosphorylation of Ser19 and Ser31 are able to increase the rate of phosphorylation of Ser40 by approximately 3-fold and 9-fold, respectively (Bevilaqua *et al.* 2001; Lehmann *et al.* 2006). Phosphorylation of these serine residues is involving multiple protein kinases as signaling substrates. The pSer40 is phosphorylated by PKA, PKG, PKC and CaMKII. Phosphorylation of Ser31 is mediated by ERK1/2 and indirectly by PKC. Cyclin-dependent kinase (CDK) protein also found associated with phosphorylation of TH at Ser31 (Moy & Tsai 2004; Lehmann *et al.* 2006). While, Ser19 is phosphorylated by CaMKII (Haycock 1990; Haycock & Haycock 1991; Haycock *et al.* 1998; Dunkley *et al.* 2004; Bobrovskaya *et al.* 2010; Aita *et al.* 2012). Similar pattern was found in our study, in which the phosphorylation of Ser40 was increased in basal condition of old rats. Phosphorylated level of PKA and PKC as well as the level of ERK1/2 were also increased in the older group. Therefore in a basal condition, the phosphorylation of TH molecule is greater in the aged rats, more closely related to the phosphorylation at the site Ser40 which is activated by the PKA, PKC and ERK1/2 substrates.

Next, we investigated the effects of glucoprivation by 2DG injection of young and old rats. Glucoprivation is a metabolic condition that elicits multiple glucoregulatory responses, including hyperphagia, adrenal medullary secretion of epinephrine, secretion of glucocorticoids, and stimulation of feeding (Sanders & Ritter 2000; Li *et al.* 2006; Andrew *et al.* 2007). 2DG is a glucose analogue and act as a competitive inhibi-

tor of intracellular glucose. It competitively inhibits the production of glucose-6-phosphate from glucose that catalyzed by glucose hexokinase, thus inhibits the glycolysis and gluconeogenesis processes. This action provides a hypoglycemic environment (Bobrovskaya *et al.* 2010). We then compared the blood glucose and plasma catecholamines between glucoprivation and the basal condition in both groups of rats. Our results found that glucoprivation evoked the release of adrenaline and noradrenaline from the adrenal medulla into the plasma which in turn raised blood glucose. Similar results were found in previous studies where significant increased were observed in plasma adrenaline (Korim *et al.* 2016; Senthilkumaran *et al.* 2016) and noradrenaline level after 20 minutes of 2DG injection when compared to saline in young rats (Bobrovskaya *et al.* 2010). The findings suggested that catecholamines were secreted rapidly following administration of 2DG to prepare the body to meet the emergency of physical stress (Kim *et al.* 2009).

We further analyzed the expression of TH, DBH and PNMT protein, phosphorylation of Ser40 and the signaling pathway of protein kinases of catecholamine biosynthesis in association with glucoprivation condition. In young group, we found that glucoprivation led the increasedof DBH protein, while the expression of PNMT and TH protein were not elicited by glucoprivation. For the young group, the expression of TH protein did not show any differences even in the glucoprivation state. There are several possibilities that can be associated with these findings. First, the regulation of the acute glucoprivation might have been modified the phosphorylation of existing TH molecule in the chromaffin cell (Kumer & Vrana 1996) and increase the release of stored adrenaline from vesicle. Second, if the activation of existing TH molecules is sufficient to offset the loss of catecholamines due to its release upon glucoprivation stimulation, then the new TH protein is not needed to be produced. In this regard, the translation process of TH mRNA to TH protein will be reduced (Xu *et al.* 2007). Third, the high level of catecholamines found in this study due to the glucoprivation condition may inhibited TH activity and TH mRNA through feedback inhibition and this is supported by other studies (Daubner *et al.* 2011; Kvetnansky *et al.* 2008). The reduction of the expression of PNMT protein occurs as a result of the release of the adrenaline into blood circulation. In this study, the level of DBH protein was increased. This finding suggests that the possibility of glucoprivation condition in young rats has increased the activity of preganglionic sympathetic nerves and chromaffin cell for the production and release of the noradrenaline as well as to produce new noradrenaline to replenish the level of noradrenaline in storage.

Studies show that within the young rats, glucoprivation condition has increased the phosphorylation of PKC and ERK1/2. While the level of total ERK1/2 shows a significant decrease and the phosphorylation of Ser40 (Senthilkumaran *et al.* 2016), PKA and CDK did not show any change even in a glucoprivation state. However, there were contradicting results obtained between our results and previous study by Bobrovskaya et al. (2010)According to their findings, the phosphorylation of TH at Ser40 and PKA substrates were significantly increased and PKC and pERK1/2 did not show significant changes in response to short term glucoprivation (Bobrovskaya *et al.* 2010). The causes of the contradicted results happen are still unclear. These contradict results probably due to the mechanism that involved in the secretion of catecholamines. Two mechanisms are involved in the secretion of catecholamines either via the ionotropic acetylcholine nicotinic receptor (nAChR) or/and the metabotropic muscarinic receptor (mAChR). Catecholamines are stored in vesicles until a stress-induced increase in splanchnic nerve activity. Upon excitation of splanchnic nerves, acetylcholines are released from the splanchnic nerve of adrenal terminals, and then activates nAChR and/or mAChR of the chromaffin cells, causing secretion of catecholamines by exocytosis (Wakade 1981). Acetylcholine induces sodium and calcium ion influx via nAChR causes depolarization. Depolarization activates voltage gated calcium ion channels and results in a transient rise in intracellular free calcium ion concentration, and then trigger the catecholamine exocytosis (Kilpatrick *et al.* 1982; Wada *et al.* 1985). This accumulation of intracellular free calcium ion leads to activation of CaMKlI and cAMP leading to PKA activation (Kim *et al.* 1994). Calcium ion influx also associates with increased inositol triphosphate (IP3) and activates diacylglycerol (DAG). Accumulations of DAG and calcium ion activate PKC which phosphorylates ERK1/2 followed by phosphorylation of TH on Ser31. The mAChR stimulation activates both adenylyl cyclase and phospholipase C to activate PKA and PKC for Ser40 and Ser31 phosphorylation, respectively (Haycock *et al.* 1992; Sutherland *et al.* 1993). However, how these types of ACh receptors on chromaffin cells will be induced is still not clear.

This study has shown that glucoprivation increase the phosphorylation of PKC and ERK1/2 in young rats. However, the stimulus does not increase the level TH protein and the phosphorylation of TH at Ser40. Phosphorylation of TH at Ser40 can be increased through hierarchical mechanisms, namely through the phosphorylation at Ser31 and Ser19 (Lehmann *et al.* 2006). Previous studies found that, ERK1/2 can increase the phosphorylation of TH at Ser31 (Aita *et al.* 2012; Bobrovskaya *et al.* 2001; Floras 1992; Haycock 1990; Haycock *et al.* 1998). ERK1/2 also indirectly phosphorylated by PKC and then phosphorylates TH molecule at Ser31. However, the phosphorylation of Ser31 only drives a marginal increase in the TH activity compared at Ser40 (Haycock *et al.* 1992; Almela *et al.* 2008; Bobrovskaya *et al.* 2010; Sutherland *et al.* 1993). Besides that, this phenomenon may occur due to increased level of catecholamines found in this study

that probably inhibits the expression of TH proteins through the feedback inhibition mechanisms (Daubner *et al.* 2011; Kvetnansky *et al.* 2008). Catecholamines can form a complex with the recombinant TH enzyme and inhibit the binding of substrates to the active site of the enzymes that cause the inhibition of enzymatic activities (Daubner *et al.* 1992). The phosphorylation of PKA will reduce the enzymes binding affinity with catecholamines and free up TH enzyme (Ramsey & Fitzpatrick 1998; Bevilaqua *et al.* 2001). The unchanged level of pPKA found in this study even in the glucoprivation state may causes the decreased of free TH enzyme and thus inhibit the binding of the PKC and ERK1/2 substrates to phosphorylate TH molecule, even though, the level of pPKC and pERK1/2 were significantly elevated.

We also analyzed the expression of TH, DBH and PNMT protein, phosphorylation of Ser40 and the signaling pathway of protein kinases of catecholamine biosynthesis in association with glucoprivation condition in aged rats. The results show increased of age does not affect the expression of TH protein in the glucoprivation state. These findings similar to the data obtained in young rats and the likelihood of such event may also be similar to those described in the young rats following glucoprivation conditions, which: (1) activation of existing molecule TH was sufficient to offset the loss of catecholamines (Xu *et al.* 2007), and (2) the high level of catecholamines released into the blood circulation upon acute glucoprivation activates the feedback inhibition and inhibit the expression of the TH protein (Xu *et al.* 2007; Daubner *et al.* 2011; Kvetnansky *et al.* 2008). While, the decline in the level of PNMT and DBH protein may be caused by these PNMT and DBH enzymes were activated and used for the production and release of adrenaline and noradrenaline in response to the stimulus of acute glucoprivation.

This study found that the increase in aged rats does not affect the phosphorylation of TH molecule at Ser40 as well as the protein kinase PKA, PKC, ERK1/2 and CaMKII in the acute glucoprivation except the expression of pCDK substrates which showed a significant increased. The reasoning for this condition remains unclear. These findings may have been influenced by aging factors. Aging is characterized as a staged decline on all functions of tissues and organs and lead to loss of the ability to maintain and restore homeostasis in stress condition. Aging also includes the changes in the number and sensitivity of receptors, which can change the response of target tissue to the hormone and neurotransmitter (Cai *et al.* 2012; Jonas *et al.* 2015; Quang T 2016). Aging is also associated with a reduction in transmission in neurons, contraction of synaptic neurons and the decline in the number of dendrites (Dickstein *et al.* 2007). This condition may delay signals from the brain and sympathetic nerves towards chromaffin cells and affects the phosphorylation of TH molecule mediated by the protein kinases. Among other effects caused by aging is the increase of oxidative stress (Dröge & Schipper 2007; Lee 1999; Murphy & Partridge 2008) that causes damage to DNA (Quang T 2016) and decreases the production of proteins which is essential for the production of receptors, protein kinases, neurotransmitters, hormones, cause the changes in gene expressions (Han *et al.* 2006) as well as the increasing number of senescent cells (Lee 1999; Quang T 2016). These circumstances may affect the ability of cell to function, especially cells that involved in the mechanism of glucose regulation. This phenomenon is likely to influence the physiological system in the mechanism of food intake, such as the center of hunger and fullness as well as mechanisms of the central and peripheral glucoregulatory that become irregular or uneven.

In conclusion, a normal mechanism for glucoregulatory response shows that glucoprivation induce the release of catecholamines into the blood and then increase the blood glucose concentrations. This mechanism is not affected by the age difference. This study found that in acute glucoprivation, the adrenal medulla may not produce new catecholamines, but instead free up the stored catecholamines that ready to use for flight and fight response. Acute glucoprivation in the young rats activates the signaling pathway of PKC and ERK1/2 to enhance the production of catecholamines and replenish the level of catecholamines in the adrenal medulla. However, the explanation why the level of phosphorylated protein kinases showed no significant difference between the basal condition and following stimulation by acute glucoprivation in the older rats remains unclear. This situation may have influenced by aging, which is a complex phenomenon and is still not completely understood. A natural process of aging involves the deterioration of many physiological functions may have been one of the factors the causes of decline in feeding response among the elderly, consequently increasing the case of anorexia of aging.

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