CHAPTER 4

Discussion and Conclusion

The World Health Organization defines obesity and overweight is a condition in which excess body fat has accumulated more than optimal healthy. These condition are the risk factors which initiate the diseases that affect the health including cardiovascular disease hypertension, diabetes mellitus especially type 2 diabetes. The prevalence of these diseases will continue to escalate in worldwide population. Obesity and associated chronic inflammation can cause a state of insulin resistance. Initially inflammation a link between obesity and insulin resistance demonstrate by the expression of the pro-inflammatory cytokine TNF-α in adipose tissue of obese mice and linked to insulin resistance (80). At present, the pharmaceutical target in the treatment of insulin resistance at the molecular level, drugs that 1) interfere with TLR/IKK/NF-κ B axis, 2) target PPAR-γ, or 3) target pro-inflammatory cytokines, have demonstrated promise with respect to treating insulin resistance despite obesity.

4.1 Biological effects of rice

In previous study, there is an evidence for a protective effect of whole-grain foods with regard to the development of type 2 diabetes (81). More recently, higher whole grains consumption reduces in insulin resistance a risk factor for developing of type 2 diabetes (82). However, the cellular mechanistic and clinical studies in this field are very limited.

In this study, we used the dichloromethane and methanol extract of purple rice, Doisaket (DSK), Nan (NAN), Phayao (PYO) and unpolished white rice, Korkhor 6 (RD 6). The compounds obtained by the dichloromethane extraction are lipophilic compounds and by the methanol extraction are hydrophilic compounds. Lipophilic antioxidants compound are found in rice including vitamin E complex (tocopherols and

tocotrienols) and γ-oryzanol (81). Rice bran is a by-product from rice milling. It comprises 10% of the total rice grain, but it also has a similar nutrients and anti-oxidative compounds to rice itself, including linoleic acid, oleic acid, tocopherols, tocotrienols, phytosterols, phytic acid, phenols, γ-oryzanol and tricin (83). Among the dichloromethane rice extract, PYO and NAND had the highest content of γ-oryzanol (appendix D). The main components of active ingredient in the hydrophilic anthocyanin compounds in purple rice are cyanidin-3-glucoside, cyanidin-3-rutinoside and peonidin-3-glucoside (82). NANM showed the highest amount of total anthocyanin when compare with other methanol rice extracts (appendix D). The study in 2012 reported that anthocyanins suppress lipid accumulation in adipocytes due to broad inhibition of the transcription factors regulating lipogenesis (84).

4.2 Effect of rice extracts on 3T3-L1 adipocyte cell viability

Adipocyte is a unique model for understanding of basic cellular mechanism of obesity, diabetes and relates disorder. In present studies, we used 3T3-L1 adipocyte (mouse embryonic fibroblast - adipose like cell line) as an *in vitro* model of adipocyte. Both of the dichloromethane extracts and methanol extracts were not toxic to any of 3T3-L1 preadipocyte and mature adipocyte. However, the high dose (200 μg/mL) of the dichloromethane rice extracts slightly stimulated the cell growth. These effects may be due to various nutrients such as vitamins present in rice extract (85). Similarly, several studies reported that the dichloromethane extract of purple and unpolished rice contains high amount of lipophilic compounds, γ-oryzanol and γ-tocopherols which can stimulate the cell growth better than a hydrophilic compounds (86, 87). Moreover, the methanol extracts from eight varieties of rice from the previous study also showed no toxicity to the murine normal fibroblast NIH3T3 cells (88).

4.3 Effect of rice extracts on adipogenesis of 3T3-L1 preadipocyte and the expression of master regulators of adipocyte differentiation

Adipocyte differentiation and lipid accumulation is related to development of obesity. Hyperplastic obesity can causes the increasing of adipose tissue mass. The

decreasing of adiposity can reduce the adipocyte numbers and the lipid content of adipocytes. The effect of rice extracts on adipogenesis induced by the hormonal induction was determined. RD6D showed the highest inhibitory effect on the differentiation of preadipocyte when compare with purple rice. RD6M showed similar effect with the RD6D, it significantly reduced the preadipocyte differentiation in dose dependent manner but slightly lower effect than RD6D. DSKD and PYOD slightly decreased the differentiation of preadipocyte into the mature cells. NAND showed higher inhibitory effect on adipogenesis but the lower concentration at 50 μg/mL had a better effect than the higher concentration (100 and 200 μg/mL). This observation was occurred in DSKM, PYOM and NANM but with different concentration. DSKM, PYOM and NANM at lower concentration 50-100 μg/mL were significantly reduced the differentiation of preadipocyte but not at the higher concentration 200 μg/mL of purple rice. This observation is similar to the result in a previous study of Thai black sticky rice extract on gene expression of lipid metabolism in HepG2 cells (86). Thus, it is possible that purple rice extract contains unknown compound(s) capable of stimulating the adipogenesis effectively at high dose.

The differentiation of preadipocytes into adipocytes is regulated by a complex network of transcription factors. Nuclear receptor PPARγ and members of C/EBP family are the center of this transcription factors network. They initiate the entire adipogenic program and regulate the terminal differentiation process. We further investigated the effect of rice extracts on the mRNA expression of CEBP-αand PPAR-γ after 3 days incubation with the hormonal induction media. All of the extracts had the effect in reducing CEBP-α and PPAR-γ mRNA expression. But these inhibitory effects of transcription factor gene expression seem to be different with those effects in adipogenesis which use 7 days longer period than the gene expression. Since the adipogenesis model used longer time to study, the results of the rice extracts in this model is more reliable and beneficent than those of the shorter time.

In this study, we also determined the effect of rice extracts on the lipogenesis of preadipocyte without the hormonal induction. Both of PYOD and PYOM extract induce the lipid accumulation in preadipocyte without the hormone induction. DSKD extract

also increased the lipogenesis of these preadipocyte. However all doses of DSKM and had no effect on the preadipocyte lipogenesis. These results indicated that both of dichloromethane and methanol extracts of DSK and PYO had lower inhibitory activity in adipocyte differentiation due to DSK and PYO itself induced the lipid accumulation in these preadipocyte. These similar results were observed in NAN and RD6 rice extracts. NANM significantly decreased adipocyte differentiation but the inhibitory activity was lower than NAND, RD6M and RD6D respectively. Because of NANM also induced the adipocyte lipogenesis without the hormone induction. On the other hand, NAND, RD6M and RD6D reduced the lipogenesis of preadipocyte without the hormone induction.

The anti-obesity effect of dichloromethane rice extracts is likely due to a number of lipophilic bioactive compounds such as linoleic acid, γ-oryzanol and tocotrienol since the anti-obesity activity of these phytochemicals have been reported (88, 89, 90). The previous study has been reported the suppressive effect of tocotrienol-rich fraction from palm oil is α-tocotrienol and γ-tocotrienol which decreased the insulin induced PPAR-γ and CEBP-β mRNA expression (91). Due to rice has higher amount of linoleic and γ-oryzanol, the anti-obesity activity of rice is more likely come from these two compounds (92). In this study, NAND and PYOD showed higher amount of γ-oryzanol when compare with DSKD and RD6D. But the anti-adipogenesis activity is unrelated to the level of γ-oryzanol in rice. Moreover, RD6D which showed the highest activities in anti-obesity of adipocyte with or without hormone induction has the lowest amount of γ-oryzanol. A further study to determine the amount of linoleic acid in both of purple rice and unpolished white rice should be performed.

Hydrophilic constituents such as phytic acid, niacin, *p*-coumaric acid, thiamine, riboflavin, ferulic acid, and tricin are possibly present in the polar fraction from the grain of purple rice and unpolished rice (93) except for anthocyanin the color pigment of purple rice. Thiamine has been reported to prevent obesity and obesity-associated metabolic disorder in Otsuka Long-Evans Tokushima Fatty rats which are exhibit obesity and metabolic disorder similar to human (94). In addition, ferulic acid has been

shown the hypolipidemic actions in high fat-fed mice (95). Moreover, methanol extract of germinated unpolished rice administration reduced body weight gain and lipid accumulation in the epidermal adipocytes and liver, and improved serum lipid levels, in part, by regulating adipogenesis through a reduction in transcription factor CEBP-α and PPAR-γ (96). Even there were several studies have been reported the anti-adipogenesis activities of *p*-coumaric acid and quercitin in 3T3-L1 (97, 98). Since these two polyphenols are not presence in high amount in rice. There is no report in anti-obesity of other hydrophilic phytochemicals from unpolished rice in our model. It is interesting to investigate and identify hydrophilic compounds that could contribute to this observation in further studies.

Several studies have been reported anthocyanin activities on adipocyte differentiation. A various concentration of anthocyanin reduced the expression of C/EBPα, PPARγ, LXRα, and SREBP-1c mRNA during adipocyte differentiation in a dose-dependent manner (99). Kim *et al*. reported the anti-obesity effect of black soybean anthocyanin (100). Exposure to bilberry extracts and anthocyanidins during adipocyte differentiation inhibited 3T3-L1 differentiation (101). Furthermore, several active compounds such as polyphenol also have anti-obesity. The previous study had shown the treatment with blueberries extracts which are rich in polyphenols attenuated lipid accumulation as determined by Oil-red O staining and a triglyceride accumulation assay (101). This present study also confirmed the inhibitory effect of anthocyanin on adipocyte differentiation since NANM which found the highest amount of total anthocyanin in this study showed the highest inhibitory activity of adipocyte differentiation. Moreover, DSKM and PYOM which had the same amount of total anthocyanin also exhibited the same activity on adipocyte differentiation.

4.4 Effect of purple rice extracts on TNF-α-induced insulin resistance

The condition of insulin resistance in adipose tissue involves inflammation induced by several cytokines, especially TNF-α. The inflammatory cytokine blocks the insulin signaling pathway resulting in reduced translocation of glucose receptor to the surface of adipocyte, and increased cellular lipolysis. The previous study in obese mice

with a targeted null mutation in TNF-α and their receptor genes resulted to the significantly improved insulin sensitivity in both diet induced obesity and ob/ob model of obesity (102). 3T3-L1 adipocytes are highly response to insulin. Therefore they represent normal adipocytes instead of diabetic ones. Thus, in the present study, to better evaluate the beneficial of rice extract for the type 2 diabetes, we investigated their effects on the 2-NBDG uptake and glycerol released in mature 3T3-L1 adipocytes treated with TNF-α to induce insulin-resistance.

In this study, the methanol extracts of rice had higher anti-insulin resistant activity than the dichloromethane extracts. The study of glucose uptake assay revealed that the dichloromethane extracts of all varieties of rice reverse the effect of TNF-α induced insulin resistant in adipocyte. The treatment with dichloromethane rice extracts brought the glucose uptake of inflammation cells back to those of the non TNF-α-treated cells. The increasing order in improving of the insulin sensitivity among the dichloromethane rice extracts was PYOD, RD6D, NAND and DSKD, respectively. The methanol rice extract improved the insulin-stimulated glucose uptake of TNF-α-treated 3T3-L1 cells better than the dichloromethane rice extracts. It increased higher than those of non-inflammation control by >170%. The increasing order in anti-insulin resistant activity revealed by glucose uptake assay was PYOM, DSKM, NANM and RD6M, respectively. RD6 was more effective than other purple rice in glucose uptake of TNF-α-treated cells by >200%.

Triglyceride hydrolysis proportionally release glycerol and free fatty acid from adipocytes, and this cellular lipolytic activity was determined by measuring the amount of free glycerol released in mature 3T3-L1 adipocytes treated with TNF-α to induce insulin-resistance. This present study found that all rice extracted decreased the glycerol release in the TNF-α-treated adipocyte. The increasing order in anti-insulin resistant activity revealed by the glycerol release content was NANM, PYOD, PYOM, NAND, RD6M, RD6D, DSKD and DSKM. Among all extracts, the dichloromethane and methanol extract of DSK and RD6 highly reduced glycerol release in mature 3T3-L1 adipocytes treated with TNF-α. Our result suggested that rice varieties in the anti-

lipolytic activity were very important. DSK and RD6 is more likely contain the anti-lipolytic agents which were different with NAN and PYO. Moreover, the phytochemicals that improved glucose uptake and inhibited lipolysis should be different compound since the active fraction of rice was not the same.

This observation suggested that several lipophilic phytochemicals in our rice extracts could be a benefit for anti-insulin resistant in adipocytes. Previous reports found a hypoglycemic effect of γ-oryzanol in diabetic mice (103) and anti-inflammatory and hypoglycemic activities of γ-tocotrienol in high-fat diet-induced obesity and insulin resistance mice (104). Moreover phytic acid has been report in reducing the blood glucose levels of diabetic mice (105). The previous report showed that γ-oryzanol improved glucose metabolism (reduce blood glucose), reduce hypothalamic ER stress, and consequently, attenuate the preference for dietary fat in mice fed with high fat diet (106). According to results in this study, purple rice, PYOD, NAND and DSKD had the higher amount of γ-oryzanol than unpolished rice RD6 (Appendix D, table 3). But the effect in increasing of the insulin sensitivity of those rice varieties was not statistically significant difference. Further study will be necessary to determine the bioactive compound in the lipophilic fraction of the rice.

We also investigated the active component of rice by Thin Layer Chromatography (data not shown). RD6M and NANM, these two varieties of rice had the higher content of ferulic acid. The previous study showed that ferulic acid has protective and therapeutic effects on diabetic nephropathy by reducing oxidative stress and inflammation (96). Moreover, Tsuda et al. (2003) have been shown that cyanidin-3-glycoside from purple corn improve obesity and hyperglycemia in mice (97). Sasaki et al. (2007) have been reported the ameliorate effects of cyanidin 3-glucoside on hyperglycemia and insulin sensitivity in diabetic mice (98). Thus, it is possible that these compounds in our rice extracts are responsible for the improvement of inflammation-induced insulin resistance in adipocytes.

In conclusion, the present study demonstrated that local purple glutinous rice and unpolished glutinous white rice had the anti-obesity and anti-insulin resistant effects of

in 3T3-L1 adipocyte. In the anti-obesity activity, RD6 and NAN had the higher affect on the adipocyte than the others purple rice. Since DSK and PYO itself induced the lipid accumulation in preadipocyte without the hormonal induction, it ameliorates the inhibitory activity on adipocyte differentiation of DSK and PYO. Both of the dichloromethane and the methanol extract exhibited the anti-obesity activity. These two fractions of rice indicated that both of lipophilic and hydrophilic compounds of rice should contain the anti-obesity agent. Moreover, obesity can induce inflammation and insulin resistant. All rice varieties inhibit the insulin-resistance in adipocyte. It increased the glucose uptake and inhibited the lipolysis of the inflammation insulin-resistant adiopocyte. Thus, health benefits of the consumption of both whole grain purple and white rice would be suitable for prevention and treatment of obesity and type II diabetes.