NEW PHYTOCHEMICAL BASED COCKTAIL FOR IN VITRO ADIPOCYTES DIFFERENTIATION

F.A. ABDUL MAJID[1*], M. TAHER[1] AND M.R. SARMIDI[2]

[1]*Department of Bioprocess,* [2]*Chemical Engineering Pilot Plant, Faculty of Chemical and Natural Resources Engineering, Universiti Teknologi Malaysia, 81310 Skudai, Johor.*

Keywords: adipocytes, cell differentiation, insulin, cinnamtannin B1

*Corresponding Author:

Department of Bioprocess, Faculty of Chemical and Natural Resources Engineering,

Universiti Teknologi Malaysia, 81310 Skudai, Johor

Tel: 07-5535533

Fax: 07-5581463

Email: adibah@fkkksa.utm.my

NEW PHYTOCHEMICAL BASED COCKTAIL FOR IN VITRO ADIPOCYTES DIFFERENTIATION

F.A. ABDUL MAJID[1*], M. TAHER[1] AND M.R. SARMIDI[2]

[1]Department of Bioprocess, [2]Chemical Engineering Pilot Plant, Faculty of Chemical and Natural Resources Engineering, Universiti Teknologi Malaysia, 81310 Skudai, Johor.

ABSTRACT

Adipocyte (fat tissue) is commonly used in in vitro study of diabetes and many other areas in endocrinology. Prior to testing, preadipocytes (fat cells) culture will be differentiated to adipocytes using a mixture of three compounds, which are insulin, dexamethasone and isobutylmethylxanthine. The cocktail of these components is expensive. Furthermore, dexamethasone has been recently categorized as a restricted item in Malaysia. Therefore, in this work, an alternative component to replace these compounds is necessary. The first scope of the study is to replace insulin with suitable phytochemical based compound using Malaysian rich biodiversity. Based on the lead compound screening of phytochemicals for diabetes, cinnamon water extract has shown to have the ability to increase glucose uptake in adipocytes culture tested. The active component in the extract has been identified as Cinnamtannin B1 and the results indicates that this compound react similar to insulin in promoting preadipocytes differentiation to adipocytes tissue. First scope of the project has been achieved where Cinnamtannin B1 is found to be a potential candidate to replace insulin in currently used adipocytes differentiation cocktail. This finding is hoped to offer the possibility of using a non biological alternative to insulin in adipocytes differentiation.

Keywords: adipocytes, cell differentiation, insulin, cinnamtannin B1

INTRODUCTION

Adipocytes are highly specialized cells that play a major role in energy homeostasis in the organism. Their primary function is to synthesize and store triglycerides at times of caloric excess and to mobilize these stores when caloric intake is low (Wu et al., 1998).

Confluent 3T3-L1 preadipocytes can be differentiated synchronously by a defined adipogenic cocktail. This adipogenic cocktail, consist of 1-methyl-3-isobutyl xanthine (IBMX), dexamethasone, and insulin, is commonly abbreviated as MDI. Conversion of 3T3-L1 preadipocytes to fully developed adipocytes in culture under the influence of dexamethasone, 1-methyl-3-isobutyl xanthine (IBMX), and insulin offers a unique system to investigate differentiation-related changes in lipid metabolism. IBMX, a cAMP phosphodiestrease inhibitor, increase intracellular cAMP. Dexamethasone a synthetic glucocorticoid, activates the glucocorticoid receptor pathway. Insulin is known to act through the insulin-like growth factor 1 (IGF-1) receptor. IGF-1 can be substituted for insulin in the adipogenic cocktail (Smith et al., 1988).

The tropical plant *Cinnamomum zeylanicum* (Cinnamon) (*kayu manis* in the Malay) has been known to have antidiabetic properties (Khan et al., 2003). In this study, the ability of cinnamtannin B1 (Fig.1) isolated from *C. zeylanicum* to differentiate adipocytes differentiation was examined and compared to insulin. In addition the effect of cinnamtannin B1 on glucose uptake was also investigated.

MATERIALS AND METHODS

General

3T3-L1 was purchased from European Collection of Cell Cultures (ECACC, UK). All cell culture grade chemicals were purchased from Sigma-Aldrich and Gibco BRL and analytical grade chemical were purchased from Merck-Schuchardt (Mallinckrodt). 2-deoxy-D-[1-^{3}H] glucose were obtained from

Amersham Biosciences (Little Chalfont, Buckinghamsire, UK). Molecular weight markers for proteins (broad range) were purchased from Promega (Madison, USA).

Cell Culture

The 3T3-L1 cells were cultured and maintained as previously described by Student et al. (1980). Cell culture were propagated at 37°C in a humidified atmosphere of 5% CO_2 in DMEM containing 10% foetal bovine serum, 1 % penicillin (10.000 U/mL) and 1 % streptomycin (10.000 μg/mL). Induction of differentiation was done one to two days post confluence (Fig. 3A). Cells were maintained in differentiation medium DMEM, 10% foetal bovine serum, 1% penicillin and streptomycin, and inducer cocktail (0.25 mM dexamethasone, 0.5 mM 1-isobutyl-3-methylxanthine/IBMX, and 1 μg/mL insulin) for four days, medium was changed every two days. At day 4, the dexamethasone and IBMX were removed with insulin remaining on the cells for an additional two days. Differentiation was allowed to continue in DMEM supplemented with 10% fetal bovine serum. The sample was tested at day 9-14 post-induction (Fig.3B). Prior to the cellular assays, cells were serum starved in DMEM for three hours, washed two times with KRPH buffer (5 mM Na_2HPO_4, 20 mM HEPES, pH 7.4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 136 mM NaCl, 4.7 mM KCl) and equilibrated 15 minutes in KRPH buffer.

Glucose Uptake

Glucose uptake assays were performed based upon previously described methods (Modan-Moses et al., 1998; Frost and Lane, 1985). The 3T3-L1 adipocytes were differentiated with 10 ml medium in 25 cm^2 T-flask. Following pretreatment, after serum starving of differentiated 3T3-L1 adipocytes for 3 hours, adipocytes cells were washed twice and equilibrated 30 minutes in 10 ml KRPH buffer. Tested samples were added 30 minutes prior to addition of 2-deoxy-D-[1, 2-^{3}H]-glucose. Glucose uptake proceeded in radio labeled sugar (2-deoxy-D-[1, 2-^{3}H]-glucose, 0.025 μCi/mL) for the times indicated in the results.

Following indicated time, the reaction was terminated by washing the cells triplicate in cold phosphate-buffered saline (pH 7.4). Cells were washed and lysed with 0.5 N NaOH/0.1% SDS. Samples from each lysate were counted using liquid scintillation Counter (Packard Tricarb 2700 TR/SL liquid scintillation analyzer, Packard Instrument Co.). Uptake was routinely measured in triplicate with each experiment repeated a minimum of twice.

Adipocytes differentiation (Adipogenesis) and Oil Red O Staining

3T3-L1 cells were plated in 24-well plates at 60 000 cells/well. The cells were incubated in DMEM containing fetal bovine serum alone (no inducers), with IBMX, dexamethasone (+ inducers). Cells were then incubated in the same media without IBMX and dexamethasone. The insulin, cinnamtannin B1 or water extract is maintained for 2 days. The medium was routinely replaced every 2 days. At day-11 after induction, the cells were washed three times with PBS, fixed by 10% formalin in PBS for 1 h at room temperature, washed once again with PBS, then stained with 60% filtered oil red O stock solution (0.5 g of oil red O in 100 ml isopropanol) for 3 hours, washed four times with water, and analyzed under a microscope (Miki et al., 2001). The stained cells was then extracted 1 ml with 4% Igepal CA-630 in isopropanol (Bennet et al., 2002). Extracted dye was transferred 1 ml to a 96-well plate and absorbance of extracted oil red O was measured with ultraviolet spectrophotometer (Hitachi U-1100) at 520 nm.

RESULTS AND DISCUSSION

Effect of Cinnamtannin B1 on Glucose Uptake

The use of 3T3-Ll cells to investigate the regulation of the glucose transport system offers several advantages. Fully differentiated 3T3-Ll adipocytes are particularly responsive to insulin, *e.g.* sugar uptake can be acutely activated 15-20-fold by insulin. In contrast to isolated adipocyte suspensions,

which lose cell viability rapidly, 3T3-Ll adipocytes can be studied in stable cell monolayers which maintain cell viability and hormonal responsiveness for extended periods of time (Frost and Lane, 1985).

In muscle and adipose tissue, insulin accelerates the uptake of glucose by facilitating the translocation of the glucose transporter-4 (GLUT-4) to the cell membrane. Deoxyglucose uptake rate by mature 3T3-Ll adipocytes is achieved within 3-4 min after activation by insulin and then proceeds at a constant rate for at least 10 min. In the saturating insulin level, deoxyglucose uptake was activated by insulin 17-fold (Frost and Lane, 1985).

These compounds increase glucose transport in skeletal muscle when the recognized pathway of insulin stimulated glucose transport via insulin receptor substrates-1 and -2 (IRS1/2), phosphatidylinositol 3-kinase (PI 3-kinase) and protein kinase B [PKB (also known as Akt)] has been blocked. Accordingly, vanadium compounds have been reported to act via an alternative signaling pathway to increase the translocation or activity of glucose transporters (Bailey, 2000).Glucose transport across the cell plasma membrane is the first step of glucose metabolism in the target tissue. To determine that effect, the activity of the cinnamtannin B1 on glucose uptake was investigated.

After incubation of the cells with 100 μg/mL cinnamtannin B1, the radioactive labeled glucose uptake into cells was analyzed. As shown in Fig.2, 100 nM insulin maximally stimulated glucose uptake in adipocytes. Cinnamtannin B1 alone exhibited signicantly stimulated glucose uptake at 100 μg/mL. However, a combination of cinnamtannin B1 with the maximal effective concentration of insulin (100 nM) exhibited no significant effect in increasing stimulation of glucose uptake compared to cinnamtannin B1 or insulin alone.

Cinnamtannin B1 stimulated glucose uptake in 3T3-L1 adipocytes significantly increased by the treatment time. At 15 minutes post treatment, activity of the compound was not significant. After 15 minutes, its activity was increasing gradually. After 60 minutes, there was a significant increase in the uptake by a one and half of basal uptake. As expected, insulin stimulated glucose uptake was similar to

that of the cinnamtannin B1 activity. The combination of the compound with insulin result in similar effect compared to uptake by insulin or the compound alone. It was suggested that cinnamtannin B1 exerted insulin-mimetic activities in adipocytes.

Effect of Cinnamtannin B1 on Adipogenesis

In this experiment, cinnamtannin B1 was evaluated for its activity in adipogenesis. It was used to induce differentiation of preadipocytes into adipocytes. Two days post confluent (day-0), preadipocytes were induced with the cocktail of dexamethasone (0.25 mM) and 3-isobutyl-1-methylxanthine (0.5 mM) containing cinnamtannin B1 (100 μg/mL) and/or insulin (1 μg/mL). On the day-two post confluent, preadipocytes were treated with different inducers as summarized in Table 1. On the day-11, preadipocytes differentiation was terminated and stained with oil red O. Fat droplets in these cells were visualized and photographed.

Fig.3 shows that replacing insulin with cinnamtannin B1 exhibited high density of lipid droplets. This result indicated that cinnamtannin B1 or water extract of cinnamon induce adipocyte differentiation of 3T3-L1 cells. The figure shows the formation lipid droplets in all induced cells. The stained cells was then extracted 1 ml with 4% Igepal CA-630 in isopropanol and measured with ultraviolet spectrophotometer to quantify the concentration of oil red O. Fig. 4 shows an increment of lipid droplets in the samples treated with inducers as compared to negative control

Before induction, 3T3-L1 cells free of any fat droplets in cytoplasm. On the day-three after induction, fat droplets were observed in the cytoplasm of 3T3-L1 cells and cells having two nuclei were detected. On the day-eight after induction by hormones and the cinnamtannin B1, about 90% cells differentiated, and there were fat droplets in the cells, which were bigger and circular. It was observed that some adipocytes, which had two nuclei, were dividing into two cells, and also there were significant fat droplets in both dividing cells. Therefore, it can be concluded that cinnamtannin B1 promoted adipogenesis similar to that of insulin.

ACKNOWLEDGEMENTS

The authors wish to thank Intensified Research in Priority Area (IRPA) Ministry of Science, Environment and Technology Malaysia for the grant of 72139 and 74054 for financial supports. Authors also would like express gratitude to Dr. Norimah Yusof and Dr. Shaharuddin Mohamad at Malaysian Institute of Nuclear Technology Research (MINT) for their kindness in providing facilities of Liquid Scintillation Counter.

REFERENCES

Bailey, C.J. 2000. Potential new treatments for type 2 diabetes. Trends in Pharmacological Sciences, 21(7): 259-265.

Bennett, C.N., Ross, S.E., Longo, K.A., Bajnok, L., Hemati, N., Johnson, K.W., Harrison, S.D., and MacDaugald, O.A. 2002. Regulation of Wnt signaling during adipogenesis. Journal of Biological Chemistry, 277(34): 30998-31003.

Frost, S.C., and Lane, M.D. 1985. Evidence for the involment of vicinal sulfhydryl groups in insulin-activated hexose transport by 3T3-L1 adipocytes. Journal of Biological Chemistry, 260(5): 2646-2652.

Khan, A., Safdar, M., Ali Khan, M.M., and Anderson, R.A. 2003. Cinnamon improves glucose and lipids of people with type 2 diabetes. Diabetes Care, 26(12):3215-3218.

Miki, H., Yamauchi, T., Suzuki, R., Komeda, K., Tsuchida, A., Kubota, N., Terauchi, Y., Kamon, J., Kaburagi, Y., Matsui, J., Akanuma, Y., Nagai, R., Kimura, S., Tobe, K., and Kadowaki, T. 2001. Essential role of insulin receptor substrate 1 (IRS-1) and IRS-2 in adipocyte differentiation. Molecular and Cellular Biology., 21(7): 2521-2532.

Modan-Moses, D., Janicot, M., McLenithan, J.C., Lane, M.D., and Casella, S.J. 1998. Expression and function of insulin/insulin-like growth factor 1 hybrid receptors during differentiation of 3T3-L1 preadipocytes. *Biochemical Journal*, 333: 825–831.

Smith, P.J., Wise, L.S. Berkowitz, R., Wan, C., and Rubin, C.S. 1988. Insulin-like growth factor-I is an essential regulator of the differentiation of 3T3-L1 adipocytes. Journal of Biological Chemistry, 263(19): 9402-9408.

Student, A.K., Hsu, R.Y., and Lane, M.D. 1980. Induction of fatty acid synthase synthesis in differentiating 3T3-L1 preadipocytes. Journal of Biological Chemistry, 255 (10): 4745-4750.

Wu, Z., Xie, Y., Morrison, R.F., Bucher, N.L., and Farmer, S.R. 1998. PPARγ induces the insulin-dependent glucose transporter GLUT4 in the absence of C/EBPα during the conversion of 3T3 fibroblasts into adipocytes. Journal of Clinical Investigation, 101(1): 22-32.

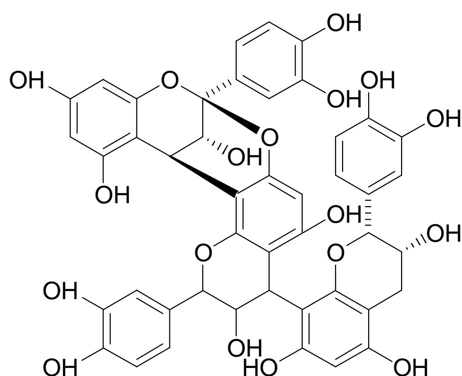

Fig. 1. Chemical structure of cinnamtannin B1

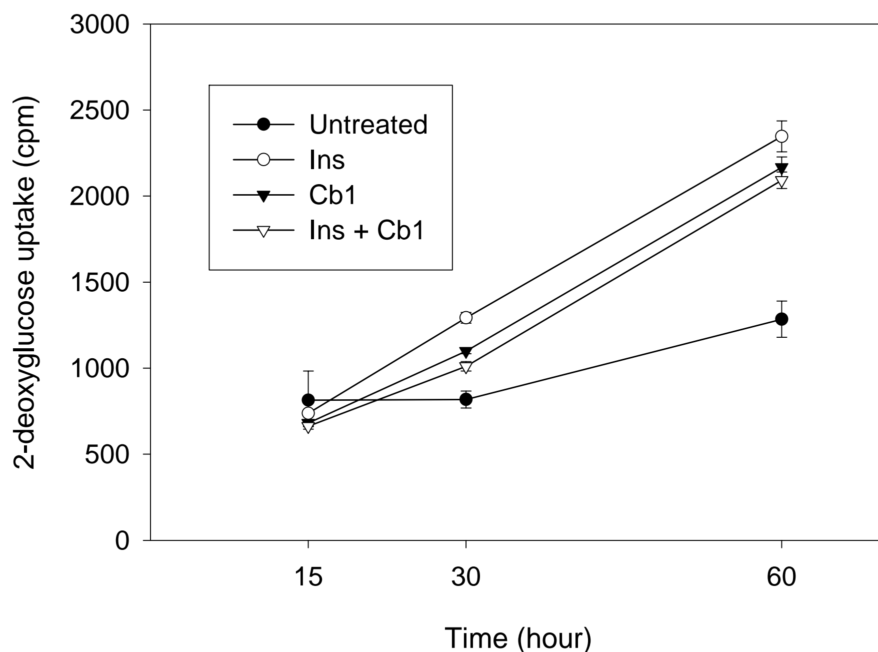

Fig. 2. Effect of cinnamtannin B1 in stimulating glucose uptake. 3T3-L1 adipocytes in 25 cm^2-T flask were incubated with 100 nM insulin, 100 μg/mL cinnamtannin B1 and 100 nM insulin + 100 μg/mL cinnamtannin B1. 2-deoxy-D-[^{3}H] glucose uptakes were assayed using liquid scintillation counter. Results are means ± SE of three experiments.

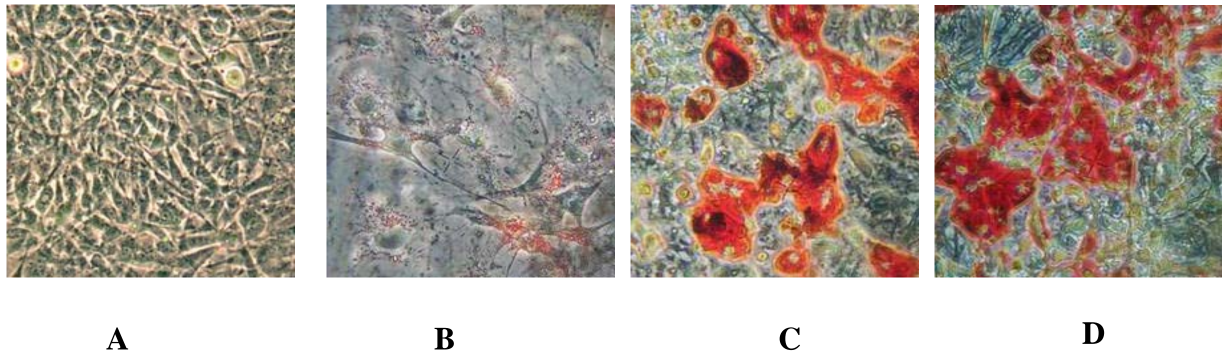

Fig. 3. Effects of cinnamtannin B1 on adipogenesis of 3T3-L1 preadipocytes. Differentiation is induced by addition inducers as indicated in Table 4.3. After day-11, preadipocytes differentiation was terminated and stained with oil red O. The figure shows the lipid accumulation can be seen in all the induced samples. **A**= the confluent of undifferentiated preadipocytes (unstained), **B**= non-induced, **C=** control**,** containing (insulin + dexamethasone + isobutyl methylxanthine), **D=** treatment, containing (cinnamtannin B1+ dexamethasone+ isobutyl methylxanthine). Red areas show lipid droplets.

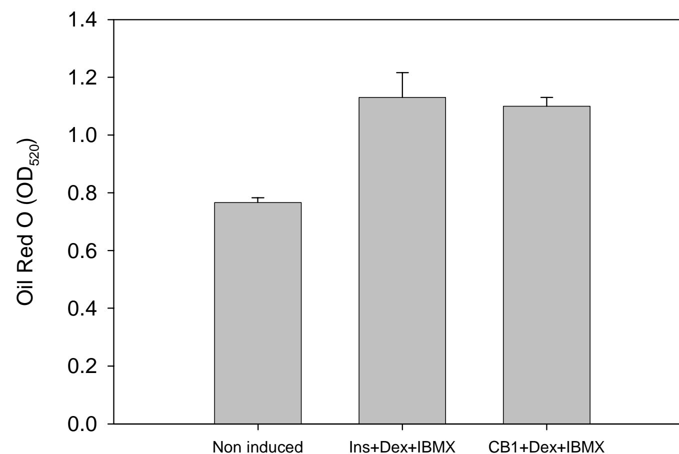

Figure 4 Adipogenesis assay. After day-11, preadipocytes differentiation was terminated and stained with oil red O and extracted with isopropanol and measured with UV spectrophotometer. Results are means ± SE of three experiments.

Table 1: Composition of inducer agent to convert 3T3-L1 preadipocytes to 3T3-L1 adipocytes

Day	Negative control	Positive control	Treatment
Inducers added at day 0	No inducer	Dex. 0.25 mM IBMX 0.5 mM Insulin 1 µg /mL	Dex. 0.25 mM IBMX 0.5 mM Cinnamtannin B1 100 µg/mL
Inducers added at day 2	No inducer	Insulin 1 µg /mL	Cinnamtannin B1 100 µg/mL