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RESEARCH PAPER

Diarylheotanoid from *Alnus hirsuta* Improves Glucose Metabolism *via* Insulin Signal Transduction in Human Hepatocarcinoma (HepG2) Cells

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Abstract *Alnus hirsuta* is a deciduous, broad-leaved tree that thrives in damp locations, and the bark of the *Alnus* species has been used in traditional oriental medicine as a remedy for hemorrhage, fever, diarrhea, and alcoholism. Two diarylheptanoids, 1,7-bis-(3,4-dihydroxyphenyl)-heptane-3-one-5-*O*-β-D-xylopyranoside (Compound I) and 1,7-bis-(3,4-dihydroxyphenyl)-heptane-5-*O*-β-D-xylopyranoside (Compound II), were isolated from the stem bark of *A. hirsuta*. In the present study, the investigations of two diarylheptanoids on insulin signaling were performed in insulin-responsive human hepatocarcinoma (HepG2) cells co-treated with high glucose. As the results, the insulin metabolic effect of glucose uptake was inhibited by high glucose. However, treatment of Compound I enhanced the glucose uptake ability in HepG2 cells, whereas, Compound II did not affect glucose incorporation noticeably. In addition, we demonstrated that treatment of HepG2 cells with Compound I improved the mRNA expression level of insulin receptor (IR) and insulin receptor substrate-1 (IRS-1).

Keywords: antidiabetic, *Alnus hirsuta*, diarylheptanoid, insulin receptor

1. Introduction

Diabetes mellitus is a metabolic disorder and is likely to become more prevalent in coming decades. The number of diabetic people is expected to rise to 221 million by the year 2010 and to 300 million by 2025 [1]. Despite genetic factors may play an important role, stressful lifestyle as well as improper dietary habits which can be a factor also contributing to the increase of this disease [2]. Diabetes mellitus is classified into two types: type 1 diabetes (insulin-dependent diabetes mellitus, or IDDM), and type 2 diabetes (non-insulin dependent diabetes mellitus, or NIDDM). In particular, type 2 diabetes is the most common, accounting for approximately 90% of cases [3]. Although the two types of diabetes have disparate pathogenic mechanisms, hyperglycemia and various life-threatening complications are the same and are major causes of morbidity and death. Hence, effective control of blood glucose levels is a key step in preventing or reversing diabetic complications and improving the quality of life in both types 1 and 2 diabetic patients [4]. Many oral hypoglycemic agents are currently used in clinical practice, but these synthetic agents are unsatisfactory in terms of efficacy and adverse side-effects [5]. Recently, the search for novel anti-diabetic medicines has received increasing attention on the plant kingdom because of their efficacy in human clinical trials and the minimal side effects [6].

Mammalian cells are constantly exposed to reactive oxygen species (ROS) as a result of normal metabolic processes occurring during aerobic respiration. However, excessively high levels of free radicals or ROS create oxidative stress, which leads to detrimental effects, including lipid peroxidation of cellular membranes, alteration of lipid-protein interaction, enzyme inactivation, and DNA breakage [7]. The oxidative stress in diabetes mellitus is characterized by increased production of ROS, with a sharp reduction in antioxidant defense and altered cellular redox status. Hyperglycemia may lead to increase generation of ROS from protein glycation and glucose autoxi-

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dation [8]. Clinical research has confirmed that supplementation with some natural antioxidants are effective in modulating the oxidative stress associated with diabetes mellitus [9,10].

Alnus hirsuta is a deciduous, broad-leaved tree that thrives in damp locations, and the bark of the *Alnus* species has been used in traditional oriental medicine as a remedy for hemorrhage, fever, diarrhea, and alcoholism [11]. Two diarylheptanoids, which were isolated from the stem bark of *A. hirsuta*, revealed significant free radical scavenging ability in our pervious study. Since oxidative stress has been implicated in the etiology of diabetic complication, two diarylheptanoids may have a therapeutic role in diabetic mellitus. Therefore, the present research aimed to characterize the effect of two diarylheptanoids on glucose uptake in human hepatocarcinoma (HepG2) cells and to investigate whether the action of two diarylheptanoids are involved in insulin signaling under high glucose condition.

2. Materials and Methods

2.1. Chemicals

1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT), and 2-NBDG 2-[N-(7-nitrobenz-2-oxa-l,3-diazol-4-yl) amino]-2-deoxy-D-glucose purchased from Sigma (St, Louis, MO, USA). The kit for RNA isolation and the first strand cDNA synthesis kit were obtained from Invitrogen (Carlsbad, CA). Dulbecco's modied Eagle's medium (DMEM), trypsin-EDTA, and fetal bovine serum (FBS) were acquired from Gibco BRL (Grand Island, NY, USA). The culture supplies were obtained from SPL Brand Products (SPL, Suwon, Korea). All other chemicals were of analytical grade.

2.2. Extraction and isolation

The stem bark of *A. hirsuta* was purchased from a market in Gangwon-Do (Republic of Korea) and indentified and authenticated by Professor M. H. Wang (College of Biomedical Science, Kangwon National University). The stem bark (2 kg) of *A. hirsuta* was refluxed with methanol (MeOH) for 24 h. The total filtrate was concentrated and dried in vacuum to render the MeOH extract (232.6 g). The extract was then suspended in distilled water and sequentially partitioned with hexane, dichloromethane, ethyl acetate. The solvent of hexane fraction, dichloromethane fraction, ethyl acetate fraction was then evaporated using a vacuum rotary evaporator. The final yields of hexane fraction, dichloromethane fraction, ethyl acetate fraction, and water fraction were 5.07, 10.40, 26.61 and 40.71%, respectively. The ethyl acetate fraction (20 g) was column chromatographed on a silica gel column using dichloromethane: ethyl acetate: methanol (10:5:1, isocratic) to afford ten fractions (Ex1-Ex10). Ex6 was further column chromatographed on a MCI gel column using water/methanol (gradient) to afford four fractions (Me1-Me4). Me3 was purified using Sephadex LH-20 column with methanol: water (1:3, v/v) to yielded Compound I (421 mg). The water fraction (20 g) was column chromatographed on a silica gel column using ethyl acetate: methanol: water (8:4:0.5, isocratic) to afford five fraction (Wa1-Wa5). Wa3 was further column chromatographed on a RP 18 gel column using 50% methanol to afford three fractions (Mw1-Mw3). Mw2 fraction was purified using Sephadex LH-20 column with methanol: water (1:4, v/v) to yielded Compound II (1,103 mg).

Mass spectra data were recorded on an Autospec. M363 series (Micromass, Euroscience, Manchester, UK) mass spectrometer. ^{1}H and ^{13}C NMR spectra were measured using a Bruker DPX 400 (400 MHz for ^{1}H, 100 MHz for ^{13}C) spectrometer.

1,7-bis-(3,4-dihydroxyphenyl)-heptane-3-one-5-*O*-β-D-xylopyranoside (I): light-brown amorphous powder

Negative FAB MS: *m/z* 477$[M-H]^{-}$

^{1}H-NMR (400 MHz, CD_3OD) δ: 1.71 (2H, m, H-6), 2.45-2.77 (8H, m, H-1, 2, 4 and 7), 3.13 (1H, dd, *J* = 8.9, 7.5 Hz, xyl-2), 3.18 (1H, m, xyl-5ax), 3.32 (1H, m, xyl-3), 3.52 (1H, m, xyl-4), 3.86 (1H, dd, *J* = 11.4, 5.3 Hz, xyl-5eq), 4.07 (1H, m, H-5), 4.21 (1H, d, *J* = 7.6 Hz, xyl-1), 6.47 (2H, dd, *J* = 8.0, 2.3 Hz, H-6' and 6"), 6.62 (2H, d, *J* = 2.3 Hz, H-2' and 2"), 6.68 (2H, d, *J* = 8.0 Hz, H-5' and 5")

^{13}C-NMR (100 MHz, CD_3OD) δ: 212.8 (C-3), 146.5 (C-3"), 146.4 (C-3'), 144.8 (C-4"), 144.5 (C-4'), 135.7 (C-1"), 134.6 (C-1'), 121.4 (C-6"), 121.3 (C-6'), 117.2 (C-5"), 117.2 (C-5'), 117.0 (C-2"), 116.8 (C-2'), 104.7 (xyl-1), 78.3 (xyl-3), 76.9 (C-5), 75.6 (xyl-2), 71.7 (xyl-4), 67.4 (xyl-5), 49.0 (C-4), 46.8 (C-2), 38.9 (C-6), 32.2 (C-7), 30.5 (C-1).

1,7-bis-(3,4-dihydroxyphenyl)-heptane-5-*O*-β-D-xylopyranoside (II): dark-brown amorphous powder

ESI-MS: *m/z* 464$[M]^{+}$, 332[M-xylose]$^{+}$

^{1}H-NMR (400 MHz, CD_3OD) δ: 1.67-1.79 (4H, m, H-2, 3), 2.41-2.78 (8H, m, H-1, 6, 4 and 7), 3.16 (2H, m, xyl-5ax and xyl-2), 3.35 (1H, m, xyl-3), 3.49 (1H, m, xyl-4), 3.85 (1H, dd, *J* = 11.4, 5.3 Hz, xyl-5eq), 4.08 (1H, m H-5), 4.21 (1H, d, *J* = 7.8 Hz, xyl-1), 6.47 (2H, dd, *J* = 7.1, 2.0 Hz, H-6' and 6"), 6.61 (2H, d, *J* = 2.0 Hz, H-2' and 2"), 6.65 (2H, d, *J* = 7.1 Hz, H-5' and 5").

^{13}C-NMR (100 MHz, CD_3OD) δ: 144.7 (C-3"), 146.6 (C-3'), 143.0 (C-4"), 142.7 (C-4'), 138.1 (C-1"), 132.7 (C-1'), 115.3 (C-5"), 115.2 (C-5'), 119.4 (C-6"), 119.3 (C-6'), 115.1 (C-2"), 114.9 (C-2'), 102.9 (xyl-1), 76.5 (xyl-3), 75.0 (C-5), 73.7 (xyl-2), 69.9 (xyl-4), 65.6 (xyl-5), 48.1 (C-4), 45.0 (C-2), 37.2 (C-6), 30.4 (C-7), 28.7 (C-1), 24.2 (C-3).

2.3. Cell line and cell culture

HepG2 cell line was purchased from American Type Culture Collection (Rockville, MD, USA). HepG2 cells were grown in DMEM supplemented with 10% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

2.4. Cytotoxicity of Compound I and II on HepG2 cells

Cytotoxic effects of the treatments of Compound I and/or II on HepG2 were determined by the MTT assay. A total of 1×10^4 cells were plated per well in 96-well plates with 100 μL culture medium for 24 h and exposed to 1, 5, 10, 20, and 100 μM of Compound I or II for 24 h. After removing supernatant of each well, 10 μL of MTT (2 mg/mL in phosphate-buffered saline (PBS) were added to each well at the time of incubation. After 4 h of incubation, the supernatant was discarded and 100 μL of DMSO was added to each well to terminate the reaction. The absorbance was measured at 550 nm using an enzyme-linked immunosorbent assay multiplate spectrophotometer (ELx800TM, BioTek, USA). The mean value of optical density (OD) of three wells was used for calculating the viability (% of control).

2.5. Glucose uptake assay

A new fluorescent derivative of 2-NBDG was used to determine glucose uptake. A total of 1×10^4 cells were plated per well in 96-well black plates with 100 μL culture medium for overnight. When experiments were conducted, the normal culture medium was replaced by serum-free DMEM. The various concentration of D-glucose were added and then incubated for 24 h to determine the optimum conditions for the glucose uptake assays. At the end point of treatment, cells were chased by 100 nM insulin and 80 μM 2-NBDG for 10 min and then washed twice with 50 mM phosphate buffer (pH 7.2). The fluorescence intensity of the precipitated cells was measured with a fluorescence spectrophotometer (Victor 3, PerkinFlmer, Newyork, USA). The excitation and emission wavelengths were 475 and 550 nm, respectively.

Followed treatment, various concentration of Compound I or II were added in presence of 30 mM D-glucose for 24 h in order to evaluate the influence on glucose uptake. The fluorescence intensity of the precipitated cells was assessed by previous protocol.

2.6. Confocal microscopic studies

For confocal microscopy studies, HepG2 cells were cultured on 20 mm glass cover-slips in (12-well plates) with 2 mL medium for 18 h and exposure to various concentration of Compound I in presence of 5.5 or 30 mM D-glucose for 24 h. At the end point of treatment, cells were incubated with 80 μM 2-NBDG along with or without 100 nM insulin for 20 min and then washed twice with 50 mM phosphate buffer (pH 7.2). The glass cover-slips were transferred to confocal laser-scanning microscopy techniques (Zeiss Axiovert 135 microscope, laser excitation 488 nm, emission long-pass LP-515 filter set). Images were analyzed using Fluoview 2.0 Software (Olympus).

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was used to analyze gene expression level in the HepG2 cells. HepG2 cells (1×10^5) were grown in 6-well plates for 24 h and treated with various concentrations of Compound I in presence of 5 or 30 mM D-glucose for 24 h. At the end point of treatment, cells were incubated with 80 μM 2-NBDG in presence or absence of 100 nM insulin for 20 min and then washed twice with 50 mM phosphate buffer (pH 7.2). Total RNA was isolated from the cells using a Trizol RNA isolation kit (Invitrogen, Carlsbad, CA, USA). Subsequently, 1 μg of the RNA was reverse-transcribed into cDNA by first strand cDNA synthesis kit (Invitrogen, Carlsbad, CA) and used as the template for PCR amplification. The primer sequence for IR, IRS-1, and β-actin were as follows: IR sense 5'-AGTTTGAGGA-CATGGAGAATGTG-3' antisense 5'-ATAGGAACGATC-TCTGAACTCCAC-3'; IRS-1 sense 5'-GCTGCTCTCCT-GACATTGGA-3' antisense 5'-GTCCTCAGGGCCGTAG-TAGC-3'; β-actin sense 5'-ACGTTGCTATCCAGGCTGT-G-3', antisense 5'-GCGACGTAGCACAGCTTCTC-3'. The conditions for IR, IRS-1 and β-actin were 94°C for 5 min followed by 25 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec, with a final extension at 72°C for 7 min. The products were separated by 1% agarose gel electrophoresis. The gels were stained with ethidium bromide and photographed. Densitometric analysis was done using image analysis software (Gel Quant, DNR Bio-Imaging Systems, USA).

2.8. Data analysis

All tests were carried out independently in triplicate (n = 3). The data are expressed as the mean ± standard derivation (SD). All analyses were performed using SPSS 16 (SPSS Institute, Cary, NC, USA); data were analyzed using one-way of variance (ANOVA) and group means were compared with Duncan's multiple range test using p-value was less than 0.01 as the level of significance.

3. Results and Discussion

3.1. Identification of Compounds (I and II)

Compound I, light-brown amorphous powder, has a molecular formula of $C_{24}H_{30}O_{10}$ by FAB-MS ([M-H]$^-$ at *m/z* = 477). The ^{1}H-NMR (CD_3OD, 400 MHz) spectrum of Compound I showed multiples at δ 1.71-2.71 corresponding to 10H that were attributed to five methylene groups and two pairs of 1,3,4-trisubstituted aromatic rings: 6.47 (2H, dd, *J* = 8, 2.3 Hz, H-6' and 6"), 6.62 (2H, d, *J* = 2.3 Hz, H-2' and 2"), 6.68 (2H, d, *J* = 8 Hz, H-5' and 5"). The ^{1}H-NMR spectrum also showed an anomeric proton signal at δ 4.21 (1H, d, *J* = 7.6 Hz). These spectral data indicated that Compound I was a bis-(3,4-dihydroxyphenyl) heptane glycoside. The signals at δ 3.13 (1H, m, *J* = 8.9, 7.5 Hz, xyl-2), 3.18 (1H, m, *J* = 10.9 Hz, xyl-5ax), 3.32 (1H, m, xyl-3), 3.52 (1H, m, xyl-4), 3.86 (1H, dd, *J* = 11.4, 5.3 Hz, xyl-5eq), and 4.21 (1H, d, *J* = 7.6 Hz, xyl-1) indicated the presence of xylopyranosyl groups. The assignment of the sugar as a xylopyranosyl was supported by the signals for oxygenated carbons at δ 104.7 (xyl-1), 78.3 (xyl-3), 76.9 (C-5), 75.6 (xyl-2), 71.7 (xyl-4) and 67.4 (xyl-5). Also, the structure of Compound I was elucidated to be (5*S*)-1,7-bis(3,4-dihydroxyphenyl)-heptane-3-one-5-*O*-β-D-xylopyranoside (oregonin), by comparing its spectroscopic data with previously reported data [12,13].

Compound II, dark-brown amorphous powder, has a molecular formula of $C_{24}H_{32}O_9$ by ESI-MS ([M$^+$]) at *m/z* = 464). The spectroscopic data of Compound II is very similar with Compound I. Comparing the ^{1}H-NMR spectrum of Compound II with Compound I, Compound II has one more methylene at δ 1.67-1.79 than Compound I. ^{13}C-NMR showed the presence of a methylene at δ 24.2 (C-3) instead of 212.8 (C-3) in Compound I. Thus, the structure of Compound II was identified as (5*R*)-1,7-bis-(3,4-dihydroxyphenyl)-heptane-5-*O*-β-D-xylopyranoside by comparison to previously reported data [14].

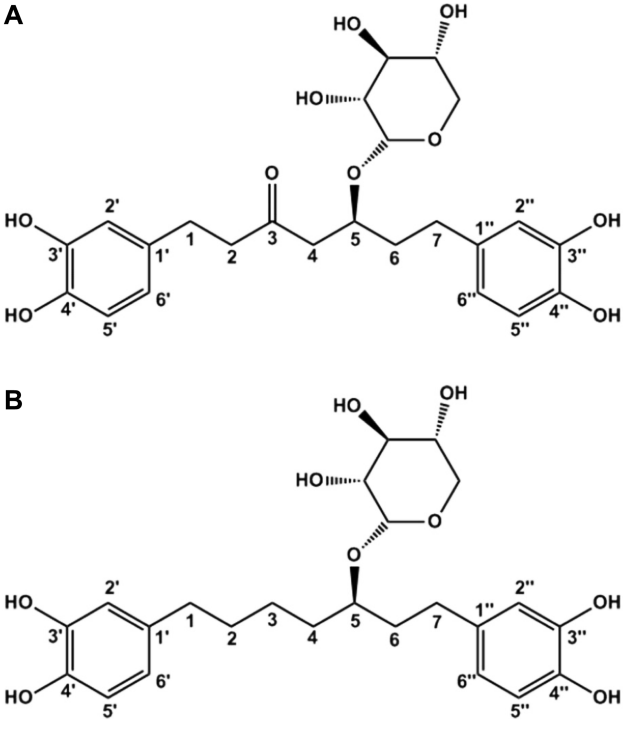

Fig. 1. Structure of two isolated compounds.
A: Compound I:
1,7-bis-(3,4-dihydroxyphenyl)-heptane-3-one-5-*O*-β-D-xylopyranoside
Compound II:
1,7-bis-(3,4-dihydroxyphenyl)-heptane-5-*O*-β-D-xylopyranoside.

3.2. Cytotoxicity of Compound I and II on HepG2 cells

It has been recently suggested that diabetes is accompanied by increased oxidative stress including superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$) or reduction of antioxidant defense system through metabolism of excessive glucose and free fatty acids. Therefore, reduction of the oxidative stress in diabetic patients by use of antioxidants may alleviate the severity of the disease [15].

The liver muscle and adipose tissue are target organs of insulin. One of the causes of hyperglycemia or high levels of glucose in type 2 diabetes is the inability of hepatic control on glucose homeostasis [16]. The liver plays a pivotal part in modulating metabolism and metabolism is known to be regulated by a wide range of cytokines and hormones, such as epidermal growth factor, and insulin [17]. It has been reported that human hepatoma cells are a suitable cell model in insulin signaling investigation due to their common physiological function to lipid, glucose, and RNA metabolism with normal hepatic cells [18]. Thus, HepG2 was selected as a model to describe the molecular mechanism underlying the actions of diarylheptanoids on insulin signaling under high glucose condition.

After 24 h treatments with various concentrations of Compound I or II, the MTT assay was performed to determine cytotoxicity on HepG2 cells. As shown in Fig. 2A, Compound I and II at the concentrations up to 100 μM did not influence the viability of HepG2 cells. Thus we employed the test compounds at less than 100 μM in subsequent experiments.

3.3. Glucose uptake assay

2-NBDG, a new fluorescent derivative of D-glucose can be incorporated into cells through the glucose transport system. It has been previously developed for the analysis of glucose uptake activity by living cells. D-glucose is transported into the cell by the glucose transporter. Previous studies showed that 2-NBDG uptaken by several cell types was significantly inhibited in the presence of D-glucose, suggesting that 2-NBDG and D-glucose are competing for the glucose transporter to enter the cell [19,20].

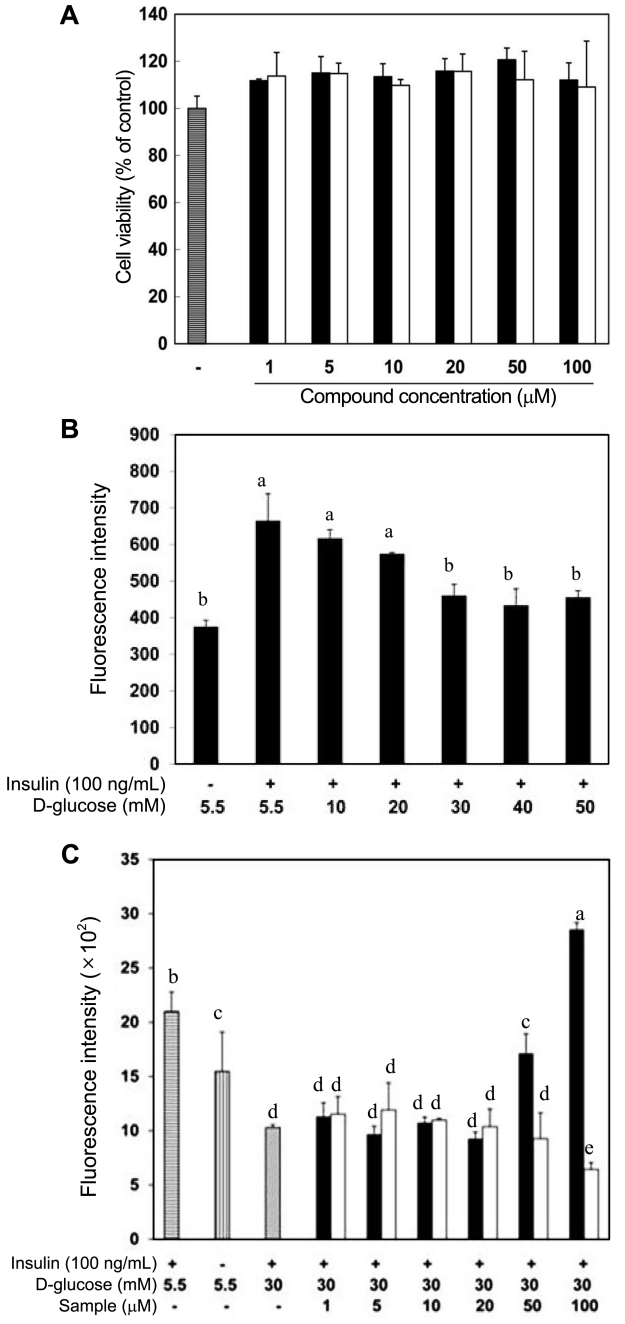

Fig. 2. Cytotoxicity and glucose uptake ability of two isolation Compounds in HepG2 cells. (A) Cytotoxicity of isolated Compounds on HepG2 cells. (▤: Control, ■: Compound I, □: Compound II) (B) Glucose uptake capability of HepG2 cells under different concentration of glucose. (C) The effects of Compound I and II on glucose uptake in HepG2 cells. (▤: control, ▥: no insulin, ▨: 30 mM D-glucose, ■: Compound I, □: compound II). Each value is expressed as the mean ± SD (n = 3). Values with the same superscript letters are not significantly different from each other at $p < 0.01$.

In order to optimise high glucose conditions for the assay, the HepG2 cells incubated with 10, 20, 30, 40, or 50 mM of D-glucose for 24 h. As shown in Fig. 2B, addition of D-glucose to the media reduced the initial rate of 2-NBDG uptake (Fig. 2B). 2-NBDG uptake was stable when the concentration of D-glucose up to 30 mM approaching the minimum value of fluorescence obtained. Thus, 30 mM D-glucose was used in all further studies of glucose uptake.

Previous findings had indicated that insulin treatment could directly promote HepG2 cells glucose uptake in evaluating hepatic insulin sensitivity [21]. In response to insulin, glucose uptake increased over basal levels (Fig. 2C). In contrast to the insulin-stimulated control group, the high glucose treatment (30 mM) in HepG2 cells inhibited insulin-stimulated glucose uptake by 50.9%. To establish whether two diarylheptanoids stimulate glucose uptake in HepG2 cells, we evaluated their effect on the 2-NBDG uptake. Compounds I displayed a remarkable ability to stimulate glucose uptake in HepG2 cells after administration: Compound I enhanced 2-NBDG incorporation by 66.23 and 177.1% at 50 and 100 μM, respectively. In our study, however, Compound II did not affect glucose incorporation noticeably.

3.4. Confocal microscopic studies

To order to confirm the enhancement of glucose uptake capability of Compound I, HepG2 cells were treated with different concentration of Compound I in addition of high D-glucose and were monitored using 2-NBDG. Observation with confocal microscopy reveled that glucose incorporation was inhibited markedly by high concentration of D-glucose (Fig. 3). In contrast, the glucose uptake capability was enhanced in presence of Compound I, which consist with data derived from fluorescence spectrophotometer.

3.5. RT-PCR analysis

The movement of glucose into or out of hepatocytes and the activity of many key enzymes are regulated by the insulin signal system and there are many factors that affect the amount of insulin signal proteins, such as insulin receptor (IR) and insulin receptor substrates (IRS) [22]. The IR is a heterotetrameric membrane protein consisting of two identical α and β subunits. Insulin binds to the α-subunits of the IR, thereby activating the intrinsic kinase activity in the β subunit [23]. In order to explore the molecular mechanisms responsible for the effect of Compound I on insulin signal transduction, levels of insulin signaling molecules were examined. As shown in Fig. 4, incubation with Compound I (10, 20, 50, and 100 μM) resulted in enhancing of levels of IR and IRS-1 mRNA compared with control, whereas IRS-2 was not affected (data not shown).

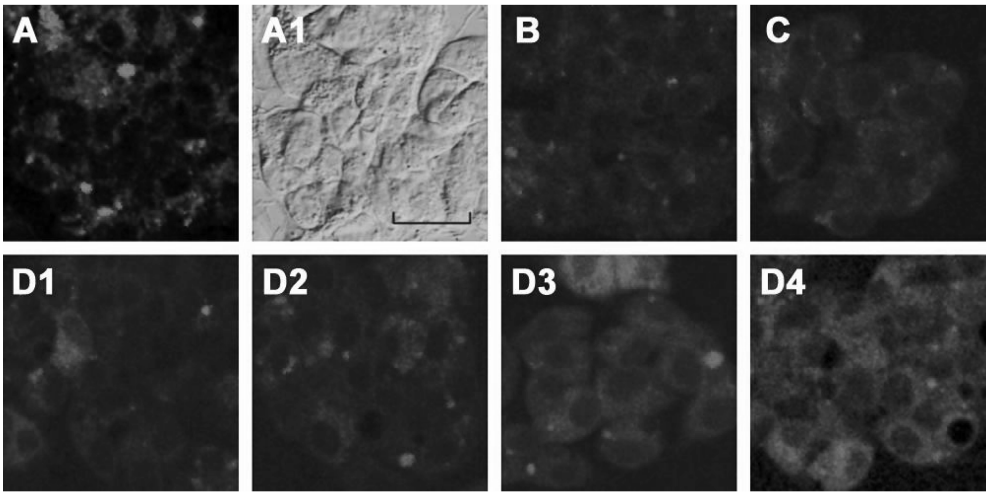

Fig. 3. Confocal laser microscopic analysis of glucose uptake in HepG2 cells. A, A1: 5.5 mM glucose + 100 ng/mL insulin; B: 5.5 mM glucose; C: 30 mM D-glucose; D1: + 10 μM Compound I; D2: + 20 μM Compound I; D3: + 50 μM Compound I; D4 : + 100 μM Compound I. ※ D1, D2, D3, and D4 were treated 30 mM D-glucose equally.

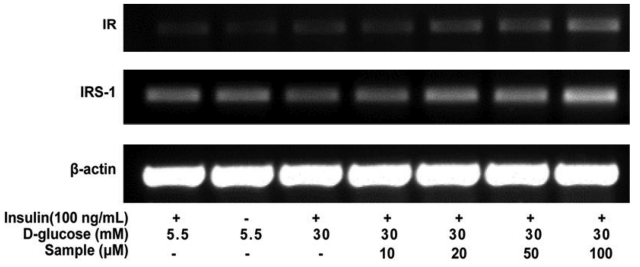

Fig. 4. RT-PCR analysis IR and IRS-1 mRNA expression level after treatment with different concentration of Compound I under high glucose condition from HepG2 cells. β-actin was co-amplified as internal control.

4. Conclusion

Theses results suggest that Compound I improves glucose metabolism and insulin signal transduction of HepG2 cells partly by enhancing the expression level of IR and IRS-1. Further experiments are currently in progress to perform a more detailed characterization.

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