Research Article
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Can connarus rubber extract inhibit the induction of insulin resistance by hyperglycemia?

Abstract

Although it would be ideal if the induction of insulin resistance by the persistence of the hyperglycemic state could be prevented by food components that can be consumed daily, to our knowledge, it has not been reported food components that have such effect. Advanced glycation end products (AGE) is involved in the development and progression of diabetic complications. Here, we investigated whether *Connarus rubber* (CR) extract can inhibit the production of AGE and the induction of insulin resistance. Twenty-four hours prior to the cultivation under serum-free condition, cells were exposed to CR extract and the glucose concentration in the medium was measured every 24 hours. Glucose intake was promoted in the presence of insulin without but not with hyperglycemia condition, showing that insulin resistance was induced in HepG2 cells under hyperglycemia condition. At 1.25 and 2.5µg/mL CR, glucose concentration decreased time-dependently with statistical significance, showing that the induction of insulin resistance was inhibited by CR extract. Furthermore, CR at >1µg/mL suppressed glycation of human serum albumin (HSA) and oxidation of glycated HSA in a concentration-dependent manner. In conclusion, CR is shown to inhibit the chemical reaction of AGE production and the induction of insulin resistance.

Keywords: insulin resistance, glycated HAS, diabetic complications, HepG2 cells

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Introduction

Diabetes is characterized by chronic hyperglycemia and is known to be caused by impaired insulin secretion and insulin resistance. [1] Hyperglycemia destroys pancreatic beta cells, insulin is not secreted, and hyperglycemia does not improve and worsens, creating a vicious circle.[2] Furthermore, the persistence of the hyperglycemic state induces cellular insulin resistance, which results in type-2 diabetes mellitus (T2DM). T2DM is a chronic metabolic disease in modern society that will affect over 366 million people worldwide by the year 2025.[3] Insulin resistance is the main pathogenic event in T2DM and is characterized by a failure of tissues to respond to insulin, leading to a reduction in glucose uptake by peripheral tissue and increased hepatic glucose output.[4,5] Many studies have highlighted the importance of achieving optimal glucose control through strict adherence to medications, diet, and exercise to reduce the risk of serious long-term complications.[6] Because existing anti diabetic agents are often associated with side effects, there is increasing interest in the use of natural products for pharmacological purposes, to complement or replace existing therapies. To date, researchers have reported that a variety of natural products derived from medicinal herbs exhibit hypoglycemic properties, particularly triterpenes, flavonoids, and polyphenols.[7] since insulin resistance is a fundamental condition that leads to the development of various diseases including T2DM, improvement or alleviation of insulin resistance is an important health factor. It would be ideal if the induction of insulin resistance by the persistence of the hyperglycemic state could be prevented by food components that can be consumed daily. Up to date, however, it has not reported food components that have such effect. Currently, insulin resistance has been reported to occur in all insulin target organs such as skeleton, muscle, adipose tissue, and liver, [8, 9] and suppression of membrane expression of glucose transporters that cause insulin resistance. In addition, studies on insulin receptor signal down are underway.[10–13] Furthermore; the persistence of hyperglycemia due to diabetes is known to induce accumulation of advanced gyration end products (AGE). AGE, which exhibits extremely strong cytotoxicity, may be involved in the development and progression of diabetic complications. *Connarus rubber* (CR) is a plant of the *Connaraceae* that grows in the vicinity of Manaus in the middle of the Amazon. Its bark extract is consumed as an herb tea and is effective for diabetes,[14,15] and the decrease in the level of DNA damage in white blood cells been reported due to consumption of beverages containing CR.[16] Here, we investigated whether CR extract can inhibit the production of AGE and the induction of insulin resistance. As a comparison control, the extract of Chrysanthemum moratorium which is an oriental drug and has been found to include a wide variety of potentially useful chemicals was also studied.

Material and methods

Preparation of samples

Two hundred and forty g of Connarus cortex was put into 18L of distilled water and incubated at 97±2°C for 15min. The extract was separated from cortex by filtration, evaporated with a rotar evaporator and 91g of Connarus aqueous extract was obtained. Two hundred and forty eight g of flower of *Chrysanthemum morifolium* cv Abokyu was extracted with 3.5L of methanol for 72h for 3 times. The extract was separated from flowery filtration, evaporated with a rotary evaporator and 98g of Abukir methanol extract was obtained.

Anti-glycation test

Human serum albumin (HSA:SIGMA-Aldrich CO, LLC) at 2mg/mL and glucose (Wako Pure Chemical In,LTD)at 37 mM were mixed with CR extract and incubated at 37°C for 2 and 4 weeks with and without 2mg/mL glycated-HSA, respectively. After the incubation, the reaction solution was placed in an 8mm cellulose tube and dialyzed for 48 hours in cold PBS. The protein concentration was measured

using a DC protein assay kit (BIO-RAD Laboratories, Inc.). Twenty µL of Solution A and 160µL of Solution B were added to 40µL of the reaction solution, and the mixture was placed at room temperature for 15 minutes, and the absorbance at 750 nm was measured. Then the amount of AGE produced per 1µg protein was quantified by ELISA. 100µL of the reaction solution was placed in a 96-well microplate, left at room temperature for 1 hour, and washed with a washing solution (0.05% tween 20/ PBS). 200µL of blocking solution (0.5% gelatin / PBS) was added to the wells, left at room temperature for 1 hour, and washed with washing solution. 100µL of a 0.1 µg/mL primary antibody (anti-AGEs monoclonal antibody (6D12) /washing solution) was added to the wells, left at room temperature for 1 hour, and washed with the washing solution.100µL of 1µg/mL secondary antibody (Anti-IgG, Mouse, Goat / washing solution) was added to the wells, left at room temperature for 1 hour, and washed with a washing solution. 100µL of the substrate solution (2% citric acid, 5% hydrogen peroxide and OPD tablet) was added to the wells and the color was developed. Then, the absorbance at 492nm was measured with a micro plate reader.

Insulin resistance test

Human hepatoma HepG2 cells, obtained from JCRB cell bank (Osaka, Japan), were maintained in Dulbecco's modified Eagle's medium (DMEM: Nissui Pharmaceutical CO, LTD) supplemented with 10% fetal bovine serum (FBS COSMO BIO CO, LTD), 200µg/mL streptomycin at 37°C under a 5% CO_2 atmosphere. Cells were maintained in logarithmic growth. The cells were seeded in 6cm dish at a concentration of $2x10^5$ cells/mL in 5mL of the culture medium. Insulin resistance in HepG2 cells was induced according to Nakajima et al.[13] and Mohamadpour et al. Experimental schedule was shown in Figure 1. Twenty-four hours (B in Figure 1a) and 48 hours (C in Figure 1b) after the seeding, medium was changed to Ham's F12 medium (Nissui Pharmaceutical CO, LTD) not containing FBS. Then, 24 and 48 hours after the cultivation without serum, glucose was added at the final concentration of 30mM (D in Figure 1a & 1b), and insulinwas added at 1µM 6 hours after glucose addition. The glucose concentration in the medium was measured at the points A–F shown in Fig 1using LAB Gluco (Research and Innovation CO, LTD). CR dissolved in diethyl sulfoxide (Wako Pure Chemical In,LTD) was added 24 hours after(C in Fig 1a) and before (B in Figure 1b) the start of the cultivation under serum-free condition. Abukir extract dissolved in dimethyl sulfoxide was added 24 hours before (B in Figure 1b) the start of the cultivation under serum-free condition.

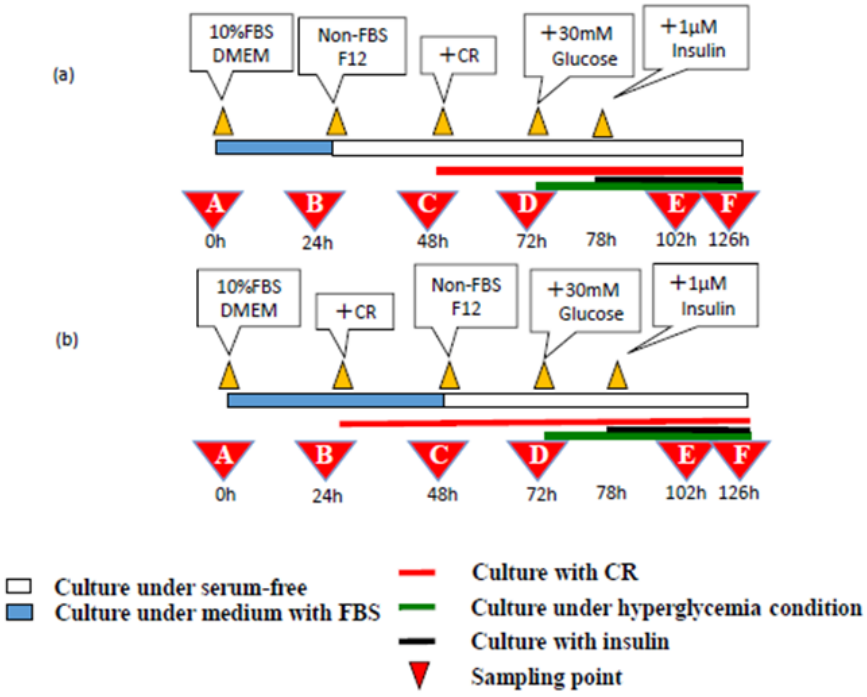

Figure 1 Experimental schedule to induce insulin resistance under serum-free and hyperglycemia condition.

Results

Anti-glycation test

CML is generated by the glycation of HSA and the oxidation of glycated-HSA. CR extract suppressed glycation of HSA (P<0.01 by Student's t-test between HSA alone and HSA glycation with glucose. Between CR treatment and CML formation, Dunnett's test is p<0.05 at 1 µg/mL and p<0.01 at 10-1000µg/mL.) (Figure 2a) and oxidation of glycated-HSA (P<0.01 by student's t-test between HSA alone and HSA glycation with glucose. Between CR treatment and CML formation, Dennett's test is p <0.05 at 1, 100, 1000 µg/mL and p<0.01 at 10µg/mL.) (Figure 2b) in a concentration-dependent manner at 1-1000µg/mL.

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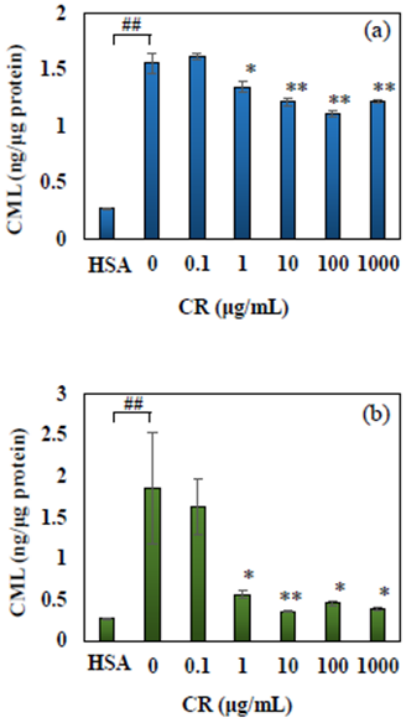

Figure 2 Inhibition of CML formation by CR HSA was incubated with or without glucose for 4 weeks.

a. and gyrated-HSA was incubated for 2 weeks.

b. The reaction solution was dialyzed for 48 hours in cold PBS, and the CML quantified by DC protein assay and ELISA. Data from three experiments were shown. Significantly higher than CML without glucose: $p<0.01$ (Student's t-test) Significant lower than CML with 0μg/mL CR $p<0.05$, $p<0.01$ (Dunnett's test).

Insulin resistance induction test

Transition of glucose concentration in the medium under serum-free condition is shown in Figure3. The experimental method was the same as the method shown in Figure 1b except that CR and Abokyuwere added. In the absence of hyperglycemia condition, any correlation between time and glucose concentration was not observed without insulin (the correlation coefficient between time and glucose concentration was 0.0789, Pearson's correlation coefficient test: $p=0.921$). With insulin, on the other hand, it decreased time-dependently, (the correlation coefficient between time and glucose concentration was -0.961, Pearson's correlation coefficient test: $p=0.0386$) with statistical significance (Figure 3). Without hyperglycemia condition, therefore, glucose intake was promoted in the presence of insulin. Under hyperglycemia with insulin, on the other hand, any time-dependent decrease in glucose concentration was not observed with and without insulin. These results shows that insulin resistance was induced in HepG2 cells under hyperglycemia condition as Nakajima et al.[13] and Mohamadpour et al. have already reported. Figure 4 shows the effects of CR and Abokyu exposure that started 24h before the initiation of serum-free condition (Figure 1b). At 0μg/mL CR, glucose concentration tended to increase time-dependently during the period between points D and F without statistical significance (the correlation coefficient between time and glucose concentration was 0.938, Pearson's correlation coefficient test: $p=0.2259$). At 1.25μg/mL CR, it decreased time-dependently with statistical significance (the correlation coefficient between time and glucose concentration was -0.999, Pearson's correlation coefficient test:$p=0.00668$). Also at 2.5μg/mL CR, it decreased time-dependently with statistical significance (the correlation coefficient between time and glucose concentration was 0.998, Pearson's correlation coefficient test: $p=0.00298$). CR extracts at 5μg/mL or higher showed cytotoxicity. On the other hand, glucose concentration increased time-dependently with Abokyu extract at 1.25-10μg/mL, glucose concentration tended to increase time-dependently without statistical significance (the correlation coefficient between time and glucose concentration was 0.991, Pearson's correlation coefficient test: $p=0.0843$ at 10μg/mL). These results shows that the induction of insulin resistance was inhibited by CR extract but not Abukir extract. In the experiment shown in (Figure 1b), cells were cultured for 24h with CR and Abokyu extracts followed by the cultivation under serum-free condition (the period between points B and C). During this culture period prior to the cultivation under serum-free condition, time-dependent decrease in glucose concentration was not observed in the presence and absence of CR and Abokyu extracts, showing that both extracts do not show insulin-like effect to promote uptake glucose. Figure 5 shows the effects of CR exposure that started 24h before the initiation of serum-free condition (Figure 1a). Glucose concentration increased time-dependently with and without CR extract, showing that the exposure to CR prior to the cultivation under serum-free condition is necessary to inhibit insulin resistance.

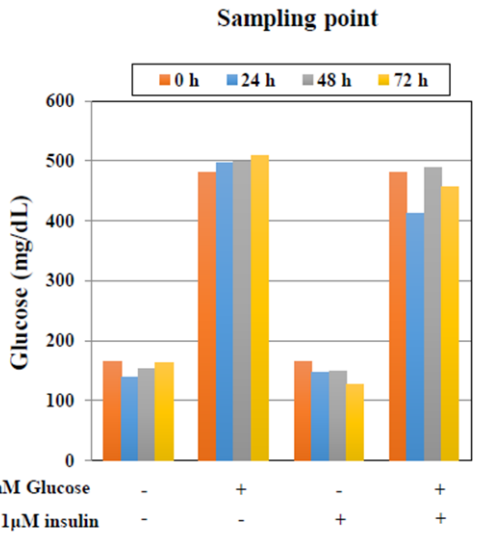

Figure 3 Transition of glucose concentration in the medium under serum-free and hyperglycemia condition.

The experimental schedule is the same as the method shown in Fig 1b except that CR and Abokyuwere added Glucose concentration in the medium was detected 0, 24, 48, and 72 h after the addition of 1μM INS.

Without 30mM glucose and 1μM INS, the correlation coefficient between time and glucose concentration was 0.0789, Pearson's correlation coefficient tes $p=0.921$. Without 30 mM glucose and with 1μM INS, the correlation coefficient between time and glucose concentration was -0.961, Pearson's correlation coefficient test $p=0.0386$.

Citation: Kawaguchi S, Kawasaki R, Murashige R, et al. Can connarus rubber extract inhibit the induction of insulin resistance by hyperglycemia? *MOJ Toxicol.* 2020;6(1):6–10. DOI: 10.15406/mojt.2020.06.00166

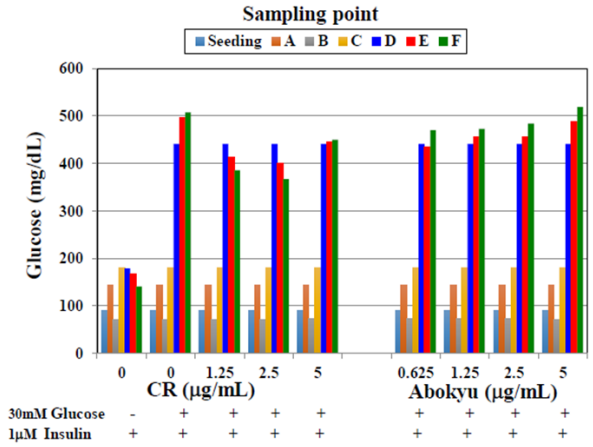

Figure 4 The effects of CR and Abokyu exposure that started 24h before the initiation of serum-free condition Experimental schedule is shown in Figure 1b Sampling points A, B, C, D, E, and F in the figure correspond to the sampling points A, B, C, D, E, and F in Figure 1b.

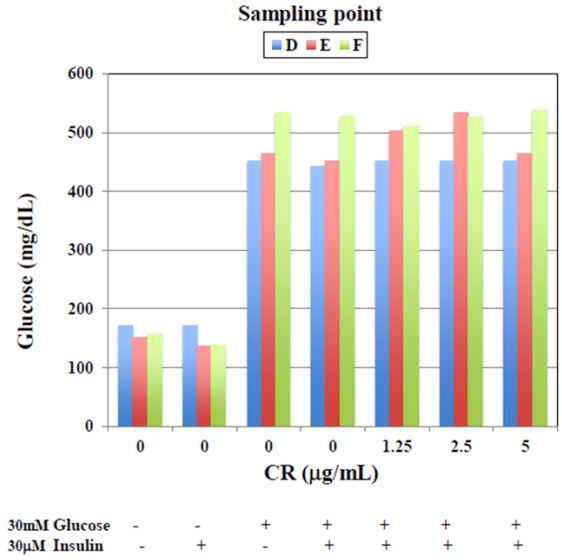

Figure 5 The effects of CR exposure that started 24 h after the initiation of serum-free condition Experimental schedule is shown in Figure 1a Sampling points D, E, and F in the figure correspond to the sampling points D, E, and F in Figure 1a.

Discussion

When insulin binds to the insulin receptor, cellular glucose reduces by consumption, and glucose is transported into cells. As a result, glucose concentration in the medium is assumed to decrease. Exposure to glucose at high concentration range (15–33mM) causes phosphorylation of serine residues of the insulin receptor substrate 1(IRS-1), reducing its electrophoretic mobility resulting in insulin receptor-down signaling.[13] In fat cells and hepatocytes, glucose transporter GLUT4 and GLUT2 acts, respectively.[9] When GLUT2-down signaling occurs in HepG2 cells, intercellular glucose is not promoted to consume even in the presence of insulin (Figure. 6), [11] which might be observed as the increase in glucose concentration in the medium. CR extract was observed to inhibit the induction of insulin resistance, when its exposure started before the cultivation without serum. Therefore, it might be possibly considered that CR affects the process of the induction of insulin resistance but not the recovery of insulin resistance. Although it is remained for further studies to reveal the mechanisms of observed inhibitory effect of insulin resistance induction and effective components included in CR extract, the important is that CR extract can inhibit the induction of insulin resistance by hyperglycemia. Nakajima et al.[13] have concluded that a high concentration of glucose causes phosphorylation of IRS-1, leading to selective attenuation of metabolic signaling of insulin. PKCε and PKCδ are involved in the down-regulation of insulin signaling, and they may lie in a pathway regulating the phosphorylation of IRS-1.[13]

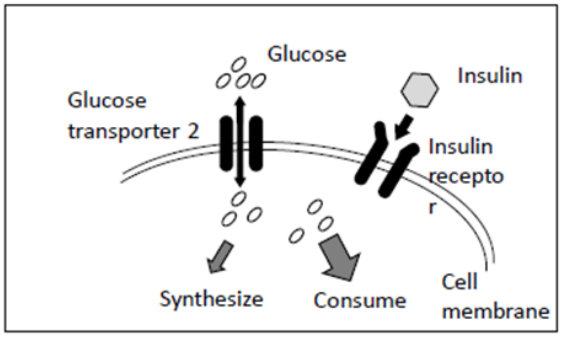

Figure 6 Effect of insulin on glucose transport in HepG2 cells. In hepatocytes, when glucose concentration decreases, glucose is released from the cell by glycogenolysis, and bidirectional GLUT2 of hepatocytes independent of insulin transports sugar to the outside of the cell.

In T2DM, excessive AGE accumulation in the vascular endothelium causes arteriosclerosis and AGE deposition in the kidney. So far, there have been reports that polyphenols and catechin derived from green tea have improved lipid and AGE accumulation, and that tea polypeptide has improved renal function in T2DMmodel mice.[17,18] T2DM is non-obese, impaired insulin secretion and insulin resistance. Although classified into those with resistance, and those with obesity and hyper insulinemia and insulin resistance. T2DM, like slowly progressive type 1 diabetes, develops insulin-independently. Thereafter, it progresses slowly, and progresses to insulin-dependent form several years later.[19] In this advanced diabetes, insulin therapy is used from the beginning as a therapeutic drug.[19] Insulin administration in this progressive diabetes may reduce the work of tired β-cells and thus inhibit progression[19] Once diabetic progresses, unlike other diseases, reduced β-cells cannot return to their pre-diabetic number and function, therefore, therapy for T2DM is considered to be delayed from the time when the subjective symptoms are already advanced, and should be started before the onset of the subjective symptoms.[19] As an early drug therapy, a drug that has the effect of stimulating the proliferation of cells by restoring β-cell fatigue is thought to be effective in preventing the progression of diabetes, and such drugs are expected to appear in the future.[19] Currently, it has been reported that Wu-Mei-Wan, a Chinese herbal formula used to treat T2DM, could alleviate palpitate-induced insulin resistance in HepG2 cells via inhibition of NLRP3 inflamma some and reduction of pro inflammatory cytokine production. [20] Low molecular weight chitosan is considered to be effective for both types of T2DM.[19] although it would be ideal if the induction of insulin resistance by the persistence

of the hyperglycemic state could be prevented by food components that can be consumed daily; they are effective to reduce insulin resistance but not to prevent the induction of insulin resistance. Up to date, little is known about food components that have the effect to prevent the induction of insulin resistance.

Conclusion

In conclusion, CR is shown to inhibit the chemical reaction of AGE production and the induction of insulin resistance. Therefore, it is considered the possibility that CR can reduce the onset and progression of diabetic complications and derivation to type II diabetes due the persistence of hyperglycemia due to diabetes.

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None.

Conflicts of interest

The authors declare that there is no conflict of interest.

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