Hypoglycemic Action of Chicken Meat Extract in Type-2 Diabetic KKAY Mice and GK Rats

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This study researched the effects of chicken meat extract on blood glucose and insulin level, membrane glucose transporter-4 (GLUT4), and tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) in type 2 diabetic KKAY mice and GK rats. Eight-week-old KKAY mice and GK rats and euglycemic control animals, C57BL/6J, mice and Wistar rats, were orally administered a liquid commercial chicken meat extract, BRAND’S Essence of Chicken (BEC), for up to 8 weeks. BEC (1.5 ml/kg) had no effect on blood insulin levels, but significantly lessened the hyperglycemia in the diabetic animals. In the BEC-treated diabetic animals, insulin induced a significant increase in plasma membrane GLUT4 and cytosolic tyrosine-phosphorylated IRS-1, indicating that it attenuates insulin resistance. The present findings are the first demonstration of the hypoglycemic action of a dietary protein, and they lend credence to the reported benefits of using chicken meat as a source of protein in the dietary management of diabetic individuals.

Key words: chicken meat; diabetes; GK rats; KKAY mice; GLUT4

Dietary epidemiological studies indicate that red meat consumption, especially the processed form, is associated with an increase risk of type-2 diabetes.1 4 However, there was an absence of correlation between poultry or chicken meat consumption and risk of type-2 diabetes.3 4 This is of particular interest, as global meat consumption for 2007 was estimated to be 278.3 million tons, of which 86.2 million tons was poultry meat.5 In two large-scale studies, poultry meat consumption has been indirectly linked to lower risk of type-2 diabetes in both men6 and women7 as high consumption of poultry meat was found to be part of a “prudent dietary pattern” that is associated with a lower risk of type-2 diabetes. Contradicting these studies is a report by the German arm of the European Prospective Investigation into Cancer (EPIC) Study, in which poultry meat consumption was part of a dietary pattern associated with a higher risk of type-2 diabetes.8 A recent study that emphasized the difference between red meat and poultry meat reported that consumption of unprocessed poultry meat was associated with a decrease in the risk of type-2 diabetes.9 Besides risks, replacement of red meat with chicken meat has been found to improve the condition as to both microalbuminuria and macroalbuminuria in type-2 diabetic patients.10 11 These studies are strong indicators of the use of chicken meat as the source of protein in lowering the risk of diabetes in high risk individuals and in the diet management of diabetic patients. To date, the cellular mechanism of chicken meat that contributes to the observed benefits in diabetes has not been studied. The present study reports that chicken meat extract attenuated hyperglycemia in two animal models of type-2 diabetes by reducing insulin resistance.

Materials and Methods

Animals. Six-to 7-week-old male C57BL/6J mice and Wistar rats were purchased from the National University of Singapore Animal Centre. Seven-to-8-week-old type-2 diabetic KKAY mice and 6-to-7-week-old GK rats were purchased from CLEA (Tokyo). The animals were fed standard rat chow and water at libitum. The temperature of the animal house was set at 25 ± 1°C with lighting from 0700–1900h daily. The animals were allowed 1 week of acclimatization before experimentation. All the experimental protocols were approved by the National University of Singapore Institutional Animal Care and Use Committee and were carried out following its guidelines.

Antibodies and chemicals. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) USA. An enzymatic glucose reagent kit was from Thermo Electron (Victoria, Australia). An insulin ELISA kit was from Crystal Chem (Downers Grove, IL). A colorimetric protein assay kit was from Bio-Rad Laboratories (Hercules, CA). Protein A-agarose was from Invitrogen (Carlsbad, CA). All other chemicals used in the preparation of the buffers were from Sigma-Aldrich (St. Louis, MO).

Chicken meat extract. For the following two logistic reasons, chicken meat extract instead of chicken meat was studied: (i) compared to solid food, liquid food is easier to administer to animals, especially for chronic oral administration; (ii) the dose in relation to protein content and the consistency of other nutrients could be standardized. The chicken meat extract used in the study was supplied by Cerebos Pacific Limited, Singapore. This manufacturer has been in the business of producing a liquid chicken meat extract known as BRAND’S Essence of Chicken (BEC) for the last 170 years. The current annual sale of this product was more than 145 million bottles in South-East Asia (data supplied by the manufacturer). The composition of BEC is given in Table 1.

Administration of BEC and casein. The C57BL/6J mice served as control animals for the diabetic KKAY mice, and Wistar rats as control animals for the diabetic GK rats. Animals were randomly assigned to

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groups of 7–8 and administered orally (by gavage) either diluted BEC or water. BEC was diluted with sterilized water so that 0.1 ml contained a dose of either 1 or 1.5 ml of the undiluted BEC/kg of animal weight. These two doses were selected based on an earlier study showing that 0.4 to 3.2 ml/kg BEC was effective in attenuating cardiac hypertrophy in rats.12) Casein solution, which served as a non-chicken-meat protein control, was similarly administered to the diabetic animals. The protein content of the casein solution was equivalent to that of the BEC. BEC, water, and the casein were administered daily for up to 8 weeks.

Oral glucose tolerance test (OGTT) and serum insulin determination. OGTT was performed as described previously.13) Briefly, after every 2 weeks of water or BEC treatment, the animals were fasted overnight (16h), and the weight of each animal was recorded. Each animal was then administered orally 2 g/kg glucose solution. Blood was obtained from the orbital sinus of each animal using a glass capillary at 0 min (just before glucose administration), and at 30, 60, and 120 min after glucose administration. The glucose concentration in sera of the clotted blood samples was determined using a commercial kit from Thermo Electron (Noble Park, Vic.). In preliminary experiments performed to determine the onset of the hypoglycemic action of BEC, only one blood sampling, at 30 min, was taken. The insulin in the serum of the 8-week treated animals obtained at 30 min of the OGTT was determined using a commercial insulin kit from Crystal Chem (Downers Grove, IL).

Correlation of serum glucose and insulin with insulin resistance. Whole-body insulin sensitivity was determined using an equation defined as an index of whole-body insulin sensitivity [10,000 square root of (fasting glucose × fasting insulin) × (mean glucose × mean insulin during OGTT)]. This index has been found to correlate with the rate of whole-body glucose disposal during the euglycemic insulin clamping.14)

Skeletal muscle preparation. After an overnight fast, the vehicle- and BEC-treated animals were intraperitoneally administered 0.5 U/kg of insulin or an equivalent volume of PBS. The animals were sacrificed by cervical dislocation at 30 min post insulin or PBS administration, and hind limb skeletal muscles were excised and frozen in liquid nitrogen. The muscles were stored at −80 °C until they were used in a GLUT4 translocation study by immunocytochemistry and Western blotting. Skeletal muscles for the IRS-1 tyrosine phosphorylation study were similarly prepared, except that the fasted animals were administered 40 U/kg insulin, and killed 5 min after insulin administration.

Fluorescence immunohistochemistry of GLUT4. Cryostat sections (10 μm) of frozen skeletal muscle were prepared and probed with anti-GLUT antibody, followed by FITC-conjugated secondary antibody. Briefly, the frozen skeletal muscle samples were mounted onto a specimen block with Tissue-Tek Optimal Cutting Temperature Compound (Sakura, Northbrook, IL). The mounted samples were allowed to cool in a cryostat (Leica CM1850, Houston, TX) for 10 min at −30 °C, and then were cut into 10-μm-thick sections and picked up onto gelatin-coated slides. The slides were warmed at room temperature for 30 min and fixed in cold acetone for 2 min. After fixation, the slides were washed with PBS and allowed to air-dry at room temperature for 1 h. Each sample was incubated with anti-GLUT4 antibody (1:100 dilution), followed by FITC-conjugated secondary antibody (1:800 dilution). Fluorescence images were captured using a fluorescence microscope, and the intensity was quantified using Imaging software (Image Processor, New York University, New York, NY).

Membrane preparation for GLUT4 study. Skeletal muscle plasma membrane was prepared by the method of Dombrowski et al.15) Briefly, 3 g of frozen skeletal muscle was allowed to thaw over an ice pack, and was minced with a pair of fine scissors, and was homogenized at a low-speed setting with Polytron in ice-cold lysis buffer containing 10 mm sodium bicarbonate, 0.25 m sucrose, 5 mm sodium azide, and 100 μmole PMSF at a ratio of 1 g of muscle/15 ml of buffer. The homogenate was spun at 1,200 g for 10 min, and the supernatant was re-spun at 9,000 g for 10 min. The second-time supernatant was spun at 190,000 g for 60 min to obtain a pellet, which contained the crude membranes. A sample of the crude membranes was reserved for Western blot analysis. The remain of the pellet was resuspended in 2 ml buffer and subjected to discontinuous sucrose gradient (25, 32, and 35% w/w) centrifugation for 16 h at 150,000 g. Fractions were collected and resuspended in sucrose-free buffer, and subjected to 190,000 g for 1 h. The pelleted membrane fractions were resuspended in 200 μl of buffer and were used in protein assay, alkaline phosphodiesterase-1 activity assay, and Western blot assay for GLUT4 protein.

Protein, alkaline phosphodiesterase-1 and Western blot assay. Sample protein concentrations were determined by Bio-Rad DC Protein Assay with bovine serum albumin as the standard. Alkaline phosphodiesterase-1 is a plasma membrane-bound enzyme. Its activity was used to determine the plasma membrane concentration in the sucrose gradient pelleted membrane fractions. Its activity was measured by the release of p-nitrophenol from a buffered thymidine 5'-monophosphate p-nitrophenol ester solution.16) In Western blot assay, plasma membrane fractions were normalized to alkaline phosphodiesterase-1 activity or to soluble protein. Antibody for GLUT4 was diluted of 800 times in 5% non-fat milk solution prior to use. Alkaline phosphodiesterase-1 enriched plasma membranes were found in the 25% sucrose gradient fraction, which was previously found to contain the highest level of GLUT4 among the sucrose gradient fractions.13) This fraction was used in GLUT4 determinations. The plasma membrane recovery in terms of total alkaline phosphodiesterase-1 activity was 9–13% for all groups, similar in range to that reported by Dombrowski et al.15)

Immunoprecipitation of IRS-1. Frozen muscles were minced and homogenized in 12 volumes w/v of lysis buffer containing 50 mm HEPES/NaOH pH 7.5, 150 mm NaCl, 2 mm EDTA, 1% glycerol, 10 mm NaF, 2 mm NaVO4, 1 mm PMSF, and 10 mm Na2PO4 using a Polytron at high speed as described previously.13) The homogenate was centrifuged at 10,000 g for 10 min, and the supernatant was stored at −20 °C until use in for IRS-1 and protein determination.

Protein A-agarose beads in suspension (Invitrogen) were washed with PBS and incubated with monoclonal IRS-1 antibody at a ratio of 6:1 v/v at room temperature for 3 h with end-over-end rotational mixing. Antibody-conjugated protein A-agarose was washed free of untagged antibody and incubated with muscle supernatant with similar rotational mixing. The beads were then washed in RIPA buffer, 0.5 m LiCl, and Tris–HCl to remove non-specific binding proteins. They

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### Table 1. Composition of BRAND’S Essence of Chicken

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
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<tbody>
<tr>
<td>Protein and peptide</td>
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<tr>
<td>Amino acid (free; mg/ml)</td>
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<tr>
<td>Hexose (mg/ml)</td>
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<tr>
<td>Lipid (mg/ml)</td>
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<tr>
<td>Mineral (mg/ml)</td>
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<tr>
<td>Ca</td>
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<tr>
<td>Mg</td>
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<tr>
<td>Zn</td>
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<td>Mn</td>
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<tr>
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<td>Niacin</td>
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<tr>
<td>Vitamin C</td>
<td>15</td>
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</table>

*Data provided by Cerebos Pacific, Ltd., the manufacturer of BEC.*
were denatured at 95 °C in Laemmli sample buffer for 5 min, and the immunoprecipitated products were assayed for IRS-1 and its phosphotyrosine residue by Western blotting. Mouse monoclonal antibody against p-Tyr and IRS-1 were used at dilutions of 400 and 800 respectively.

Statistical analysis. All statistical analyses were performed using the SPSS 11.9 statistical program. Statistical significance of differences between the mean serum glucose level for the control and treatment groups was analyzed by One Way Analysis Of Variance (ANOVA). The Tukey test was used for post hoc comparison. The unpaired Student’s t-test (two-tailed) for independent samples was used to detect statistical differences between insulin- and non-insulin treated animals. *Significantly different from the control value (p < 0.05, ANOVA followed by post hoc Tukey test).

Results

OGTT and insulin level

Figure 1 shows, BEC, administered orally, exerted significant hypoglycemic action in KKAy mice after 6 weeks of treatment. The effect was sustained at 8 weeks of treatment at which the lower dose (1 ml/kg) also had significant hypoglycemic action-similar trend of hypoglycemic action was seen in the GK rats after 8 weeks of BEC treatment (Fig. 2). The C57BL/6J mice, which significantly increased whole-body sensitivity in both diabetic and non-diabetic animals. Similar data have been reported between GK and Wistar rats (i) insulin stimulated translocation of GLUT-4 from the cytoplasmic vesicles to the plasma membranes in the skeletal muscles of the Wistar rats (ii) stimulation was impaired in the GK rats, and (iii) BEC attenuated the impairment (Fig. 3). Quantitation of this observation is given in the Western blot data (Fig. 4). Similar quantitative data were also obtained for the skeletal muscles of KKAy mice (Fig. 5). There was no significant difference in total GLUT4 protein between the diabetic and non-diabetic animals. Similar data have been reported between GK and Wistar rats (i) and between the KKAy and C57BL/6J mice. BEC enhanced the tyrosine phosphorylation of IRS-1 in the skeletal muscles of GK rats and KKAy mice (Figs. 6 and 7).

Serum insulin and insulin sensitivity index

Table 2 shows, BEC had no significant effect on insulin levels in blood samples taken at 30 min of OGTT after 8 weeks of treatment. The data show that BEC significantly increased whole-body sensitivity in both the diabetic KKAy mice and the GK rats.

Discussion

Nutritional dieting is an important component of diabetic therapy, but information on meat consumption is sparse and, is pared down to percentages of protein in terms of energy requirements. The variety of meat sources is great, and circumstantial evidence from recent studies favors chicken meat as a source of protein in diet management for diabetic patients. The present study presents evidence, for the first time, that chicken meat extract has significant hypoglycemic action in type-2 diabetic GK rats and KKAy mice. The GK rat is an animal model of spontaneous-onset, non-obese type-2 diabetes, featuring postprandial hyper-
glycemia, insulin resistance, impaired insulin secretion, progressive reduction of β-cell mass, and the development of long-term diabetic complications.\textsuperscript{16,20–23} The KK-Ay mouse is a cross between the glucose-intolerant black KK female mouse and the male yellow obese Ay mouse and is known to serve as an excellent model of type-2 diabetes.\textsuperscript{24} Diabetes is a multifactorial disease, and the use of two different animal models of diabetes in the present study lessened the chances of obtaining idiosyncratic positive responses to BEC. In terms of glycemic profile, BEC showed similar trends in the two animal species. The magnitude of hypoglycemic responses to BEC corresponded to reported values obtained with other hypoglycemic agents in the KKAy mouse\textsuperscript{25} and the GK rat.\textsuperscript{26} Although the hypoglycemic action of BEC in the C57BL/6J mice was unexpected, these animals have been found to be sensitive to metabolic manipulation\textsuperscript{27–29} and to respond to the

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**Fig. 3.** Effects of BEC on the Distribution of GLUT-4 Protein in Skeletal Muscles of Insulin-Challenged and Non-Challenged Wistar and GK Rats.

Ten-µm cryostat sections were cut from frozen skeletal muscles and probed with anti-GLUT4 antibody, followed by FITC-conjugated secondary antibody. Upper panel, 20X magnified sections of stained skeletal muscle. Sections A and B were from Wistar rats that were not treated and treated with insulin respectively. Sections C and D were from GK rats that were not treated and treated with insulin. Sections E and F were from BEC (1.5 ml/kg)-treated GK rats that were not treated and treated with insulin. Lower panel, Quantitation of GLUT4 fluorescence. The vertical bars represent the SEM of samples obtained from four individual animals. *Significantly different from the corresponding content in non insulin treated animals (p < 0.05, two-tail t-test).

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**Fig. 4.** Effects of BEC on Insulin-Induced GLUT4 Translocation in the Skeletal Muscle of GK Rats.

Upper panel, Representative Western blots of GLUT4 protein from plasma membrane (PM, 25% sucrose gradient fraction) and crude membrane (CM) of non insulin- and insulin-treated animals. Bottom panel, GLUT4 content (relative to content in non insulin-treated animals). The vertical bars represent the SEM of samples obtained from four individual animals. *Significantly different from the corresponding content in non insulin-treated animals (p < 0.05, two-tail t-test). The total membrane GLUT4 content in insulin-treated animals was not significantly different from that of the non insulin-treated animals (data not shown).

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**Fig. 5.** Effects of BEC on Insulin-Induced GLUT4 Translocation in the Skeletal Muscle of KKAy Mice.

Upper panel, Representative Western blots of GLUT4 protein from plasma membrane (PM, 25% sucrose gradient fraction) and crude membrane (CM) of non insulin- and insulin-treated animals. Bottom panel, GLUT4 content (relative to content in non insulin-treated animals). The vertical bars represent the SEM of samples obtained from four individual animals. *Significantly different from the corresponding content in non insulin treated animals (p < 0.05, two-tail t-test). The total membrane GLUT4 content in insulin-treated animals was not significantly different from that of the non insulin-treated animals (data not shown).
hypoglycemic action of des-aspartate-angiotensin I. 13) These animals are normo-glycemic, and it is possible that physiological homeostatic responses set in after 6 weeks of treatment, resulting in the waning of the BEC effect.

BEC enhanced the insulin-stimulated translocation of GLUT4 from the cytoplasmic vesicles to the plasma membranes in the skeletal muscles of both type-2 diabetic animals. In the upstream pathways of GLUT4 translocation, BEC enhanced the tyrosine phosphorylation of IRS-1 in the skeletal muscles of both type-2 diabetic animals. These responses signify that the hypoglycemic actions of BEC are specific and are brought about by the attenuation of defective cellular events that underlie the hyperglycemia of type-2 diabetes. Substantial decreases in insulin-stimulated receptor kinase activity and a defect in receptor-mediated IRS-1 phosphorylation have been found in muscle and fat tissue in type-2 diabetic patients and a rodent model of the disease. 30, 31) Thus, BEC attenuates insulin resistance in the skeletal muscle of the diabetic animals by enhancing insulin-induced tyrosine phosphorylation of IRS-1, which led to a corresponding increase in the translocation of cytoplasmic GLUT4 to the plasma membrane. GLUT4 then took membrane up postprandial glucose and attenuated the hyperglycemia of the diabetic animals. Insulin resistance is a hallmark of type-2 diabetes, and the ability of BEC to increase whole-body insulin sensitivity indicates that its use as a source of protein in the diet credence to the reported beneficial effects of using chicken meat extract in KKAy mice.

Table 2. Effects of BEC on Whole-Body Insulin Sensitivity Index (ISWb) in KKAy Mice and GK Rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>KKAy mice</th>
<th>GK rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n = 8)</td>
<td>BEC (n = 8)</td>
</tr>
<tr>
<td>Plasma glucose (0 min) mmol/l</td>
<td>10.8 ± 0.7</td>
<td>10.2 ± 0.8</td>
</tr>
<tr>
<td>Plasma insulin (0 min) ng/ml</td>
<td>1.1 ± 0.1</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Plasma glucose (30 min) mmol/l</td>
<td>30.9 ± 1.7</td>
<td>25.2 ± 1.2*</td>
</tr>
<tr>
<td>Plasma insulin (30 min) ng/ml</td>
<td>1.9 ± 0.2</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>ISWb – 10000/1((glucose)0 × (insulin)b) × (G × 1)/2</td>
<td>560.7 ± 35.7</td>
<td>701.3 ± 41.6*</td>
</tr>
</tbody>
</table>

Data represent means ± SEM; I = mean serum insulin level during OGTT; G = mean serum glucose level during OGTT.

*Significantly different from the corresponding control (p < 0.05, two-tail t-test)
BEC action is parallel to the similar long onset of action of des-aspartate-angiotensin I. Des-aspartate-angiotensin I was effective at a dose of 600 nmole/kg, which equates to about 0.71 mg/kg (the molecular weight of the peptide is 1,181 Daltons). At a dose of 1.5 ml/kg, BEC contained 124.5 mg protein/peptides, which is over two orders of magnitude in weight as compared to the dose of the angiotensin peptide.

In summary, the present findings on the hypoglycemic action of chicken meat extract are important information for diabetic individuals and caregivers. They provide a rationale for the use of chicken meat as a source of protein for diabetes. Taken as a food, it is potentially free of adverse effects. The effects of BEC on other protein kinases of the insulin receptor-mediated phosphorylation cascade and the survival of the insulin-protein kinases of the insulin receptor-mediated phosphorylation cascade and the survival of the insulin-producing beta cells in diabetic animals and human remain to be investigated.

Acknowledgments

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References
