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Insulin receptor exon $11^{+/-}$ is expressed in Zucker (*fa/fa*) rats, and chlorogenic acid modifies their plasma insulin and liver protein and DNA

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Abstract

In vivo studies confirmed that chlorogenic acid (CGA) improved glucose tolerance and mineral pool distribution in obese Zucker (*fa/fa*) rats. We found a significant decrease ($P<.05$) in postprandial blood glucose concentrations, which may have been due to an improved sensitivity to insulin. Impaired glucose tolerance and insulin resistance have been associated with differences in the hepatic mRNA expression of the spliced variants of the insulin receptor at exon 11. Spliced variants of the insulin receptor have not been studied in obese Zucker (*fa/fa*) rats, and no information exists about the effects of CGA in vivo as a possible insulin sensitizer. Thus, we studied the in vivo effect of CGA on plasma insulin concentrations during a glucose tolerance test, liver protein and DNA concentrations, the hepatic activity of glucose-6-phosphatase (G-6-PASE) and the mRNA expression of the two variants of the insulin receptor at exon 11. Zucker (*fa/fa*) rats were implanted with jugular vein catheters. Chlorogenic acid was administered (5 mg/kg body weight per day) for 3 weeks via intravenous infusion.

In the CGA-treated group, areas under the curve (AUC) for blood glucose and plasma insulin improved ($P<.005$), and the protein and DNA concentrations in the liver increased ($P<.05$). No significant differences ($P>.05$) were found between groups for the hepatic G-6-PASE activity. The insulin receptor exon 11^{+} and the exon 11^{-} variants were expressed in the liver of Zucker (*fa/fa*) rats without significant changes ($P>.05$). Chlorogenic acid improved some cellular mechanisms that are stimulated by insulin.

Keywords: Increased protein concentration; Liver DNA; Chlorogenic acid; Insulin sensitizer

1. Introduction

Elevated serum glucose, as found in individuals with non-insulin-dependent diabetes mellitus (type 2 diabetes), is associated with perturbations in insulin metabolism, mineral distribution and/or the activity of some enzymes, e.g., glucose-6-phosphatase (G-6-PASE; EC 3.1.3.9), involved in glucose metabolism [1,2]. Chlorogenic acid (CGA) is a phenolic acid formed by esterification of caffeic and quinic acids, is present as 50.3% of the total phenolics identified in potato peel extracts and exerts antioxidant and antimicrobial activities [3–5]. Additionally, CGA has been claimed to modulate the in vitro activity of G-6-PASE [6]. In previous studies, we found that intravenous infusion of CGA to insulin-resistant, obese Zucker (*fa/fa*) rats improved ($P<.05$) glucose tolerance by 22% [7]. It might be that CGA can regulate glucose metabolism by modulating G-6-PASE activity in vivo. Therefore, we studied the in vivo effect of CGA on the activity of hepatic G-6-PASE in Zucker (*fa/fa*) rats.

The insulin receptor mRNA is expressed as two variants at exon 11 [8]. Exon 11^{+} encodes for a sequence of 12 amino acids, which is not present in the exon 11^{-} isoform [8]. Tissue-specific expression of the two isoforms has been found in rats [9]. An increase in the expression of the exon 11^{-} isoform has been reported in the liver of type 2 diabetic monkeys, and this increase was associated with elevated hepatic insulin resistance [10]. In liver samples from humans with impaired glucose tolerance, the percentage of the two insulin receptor mRNA isoforms differed significantly from the ratio found in non-type 2 diabetic individuals [11]. Isoforms of the insulin receptor have not been studied in insulin-resistant, obese Zucker (*fa/fa*) rat. The obese Zucker (*fa/fa*) rat is characterized as hepatic insulin-resistant [12]; thus, it is a good model to study the in

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vivo effect of CGA as an insulin sensitizer. The purpose of this investigation was to determine the effects in Zucker (*fa/fa*) rats of intravenously infused CGA on plasma insulin concentrations, liver protein and DNA concentrations, the activity of hepatic G-6-PASE and the expression of hepatic insulin receptor mRNA.

2. Methods and materials

2.1. Animals

Approval for the care and treatment of animals was obtained from the Institutional Animal Care and Use Committee of North Dakota State University. Male Sprague–Dawley Zucker (*fa/fa)* rats (9 weeks old), obese, insulin-resistant, hyperlipidemic and type 2 diabetic, weighing between 315 and 327 g, were purchased from Harlan Sprague Dawley (Indianapolis, IN) and housed in hanging wire-bottomed stainless steel cages. The room housing the rats was temperature controlled (22°C, 40–60% relative humidity) and kept under a daily 12-h light/dark cycle. Water and AIN-93M Purified Rodent Powder Diet (Dytes, Bethlehem, PA) were supplied ad libitum. The animals ($n=12$) were allowed to acclimatize for 1 week before they were subjected to surgery.

2.2. Animal surgery

Distilled water was sterilized for 20 min at 121°C and 15 psi using a Barnstead Sterilizer (Barnstead Thermolyne, Dubuque, IA). Saline (0.9% NaCl) and heparinized saline solutions were prepared in sterile distilled water and filter sterilized using a 0.2-μm Syrfil Disposable Filter Unit. Pentobarbital and the gentamicin sulfate antibiotic Garacin solutions were filter sterilized using a 0.2-μm Syrfil Disposable Filter Unit before used.

A week after arrival, groups of up to six rats per day were anaesthetized by intraperitoneal injection of pentobarbital (60 mg/kg body weight) and implanted with indwelling jugular vein catheters [7,13]. Two weeks elapsed after surgery before the experimental period was started [7,13]. The catheter implanted and secured on a pedestal in the head facilitated intravenous treatment and the withdrawal of blood for the measurement of plasma insulin before and during glucose tolerance tests [7].

The rats (12 weeks old) were bled to determine baseline fasting blood glucose concentrations; this was immediately followed by an oral glucose tolerance test [7]. After the baseline fasting blood glucose concentrations, a total of 7 weeks elapsed before the experimental period was started. This approach was followed in an effort to understand the response to a glucose challenge at different ages (12, 19 and 22 weeks old) prior to and after CGA treatment [7].

The rats (19 weeks old) were divided randomly into two groups ($n=6$): control and treatment [7]. For a period of 3 weeks, the treatment rats were given intravenous infusion of CGA (5 mg/kg body weight per day) in a 5-mM phosphate buffer pH 7.4 (vehicle). The control rats were given intravenous infusion of the vehicle only [7]. Sixteen hours after the last CGA dose, the rats (22 weeks old) were fasted for 24 h and sacrificed [7]. Food consumed (g) and body weights (g) were recorded daily and averaged over the experimental period [7]. The liver was removed, weighed and rinsed with ice-cold isotonic saline solution. Portions of sliced liver were pulverized in liquid nitrogen, stored at −80°C and freeze-dried at 70 mtorr at −80°C using a Dura-Dry Freezer (FTS Systems, Stone Ridge, NY).

The chemicals used were purchased from Sigma (St. Louis, MO). The deionized distilled water used to prepare the reagents was 18 mΩ purity, collected from a 4-ModuleE-pure purification system (Barnstead Thermolyne, Dubuque, IA).

2.3. Blood glucose, plasma insulin and glucose tolerance test

After an overnight fast, blood was collected from the implanted catheter using heparinized syringes and transferred into heparinized tubes. Blood was kept on ice until a 32-μl aliquot was used to determine glucose concentrations using a Re-flotron System (Mannheim, Indianapolis, IN). The remaining blood was centrifuged at 4°C for 10 min at $2000\times g$ to harvest plasma. Fifty microliters of plasma was used to measure insulin concentrations using a Sensitive Rat Insulin RIA Kit (Linco Research, St. Charles, MO).

The determination of fasting blood glucose concentrations was immediately followed by stomach gavage with a glucose solution (3 g glucose/kg body weight) [7] to determine glucose tolerance. Blood glucose and plasma insulin concentrations were determined at 30 min after glucose administration and, thereafter, at 30-min intervals up to 120 min. Blood glucose concentrations (mg/dl) [7] or plasma insulin concentrations (ng/ml) were plotted vs. time (min), and the individual plots were used to determine the blood glucose or insulin peak time.

Plots of areas under the curve (AUC) for glucose and insulin were constructed using data obtained during the glucose tolerance test. The glucose–insulin index, defined as an indirect index of in vivo peripheral insulin action, was calculated as the product of the glucose and insulin AUCs [14].

2.4. Isolation of liver DNA

The isolation of DNA from freeze-dried liver (20±2.0 mg) was performed using an Easy-DNA Kit according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). The method allows for isolation of DNA from small amounts of tissue and is based on the lysis of cells, precipitation of lipids and proteins, and digestion of RNA by RNase. The isolated DNA was extracted and precipitated using chloroform and ethanol (100%). A DNA pellet obtained after centrifugation at 4°C and at $16\,000\times g$ for 20 min was reconstituted in 80% ethanol and centrifuged at 4°C at $16\,000\times g$ for 5 min. The ethanol was removed, and the pellet was allowed to air dry for 10 min. The dried

pellet was dissolved in 100 μl of Tris–EDTA buffer (10 mM Tris–HCl and 1 mM EDTA), and the samples were kept at 4°C. A sample, diluted 200 times, was used to determine DNA concentrations using an UltraSpec 2000 UV/Visible Spectrophotometer (Pharmacia Biotech, Newark, NJ) at λ=260 nm.

2.5. Isolation of hepatic microsomes and protein determination

During isolation of microsomes, all the procedures were performed at 4°C. The liver (500 mg) was thawed on ice, mixed with 4.5 ml of buffer A (0.25 M sucrose in 5 mM of HEPES, pH=7.4) and homogenized using a teflon/glass homogenizer (PRO Scientific, Monroe, CT) [15]. The homogenate obtained was centrifuged for 10 min at 10 000×g. The pellet was discarded. An aliquot of supernate, 0.2 ml, was used to determine protein concentration as described below. The remaining supernate was centrifuged for 60 min at 105 000×g. After centrifugation, a 0.2-ml aliquot was used to determine protein concentration. The pellet obtained from the second centrifugation contained the intact microsomes. The pellet was incubated for 1 h at 4°C in 0.5 ml of buffer A. Two hundred microliters (200 μl) of the resuspended microsomes was used to determine microsomal protein concentration. Half of the remaining supernatant was diluted with cold buffer A to a final concentration of 15–20 mg protein/ml, and this dilution was used to determine mannose-6-phosphatase (M-6-PASE; EC 3.1.3.-) or G-6-PASE activity (explained below). The remaining microsomal supernatant was used to prepare fully disrupted microsomes [15,16].

Aliquots of liver fractions obtained during the isolation of microsomes were treated with 0.52 M sodium dodecyl sulfate (SDS) prior to determination of protein concentrations [17]. The protein–detergent mixture was stirred gently on a stirring plate at room temperature for 1 h [17]. The mixture was centrifuged at 100 000×g for 1 h at room temperature [17]. The pellet was reconstituted in 1.0 ml of 0.85% sodium chloride solution containing SDS to a final concentration of 0.52 M [17]. The protein concentration was determined using a colorimetric microprotein kit (Sigma) according to the manufacturer's protocol. The absorbance of standards and liver fractions was read using a Beckman Du-50 Spectrophotometer (Beckman Instruments, Irvine, CA) at λ=725 nm.

2.6. Intactness of microsomal preparation

The activity of M-6-PASE toward the substrate monosodium mannose-6-phosphate (1 mM) was determined in intact microsomes or microsomes that had been disrupted by detergent action [15,16]. The latency of M-6-PASE activity represented the loss of endoplasmic reticulum integrity after detergent treatment [15,16]. The percentage of intact microsomes used was calculated as follows: 100−[100 (M-6-PASE of untreated microsomes)/(M-6-PASE of detergent-treated microsomes)] [16].

Disrupted microsomes were prepared after thawing the microsomal suspension on ice and diluting with cold buffer A to a final concentration of 1–2 mg protein/ml. Triton X-100 was added to the microsomal suspension to produce a final concentration of 0.1% [16]. Ten microliters of 1% Triton X-100 was added to a 90-μl aliquot of the diluted microsomal suspension followed by incubation at 0°C for 20 min. To test for the intactness of the microsomal preparation, M-6-PASE activity was determined.

2.7. Glucose-6-phosphatase or M-6-PASE activity

The activity of G-6-PASE toward the substrate monosodium glucose-6-phosphate (30, 10, 5 or 1 mM) using intact or fully disrupted microsomes was determined spectrophotometrically [15]. The M-6-PASE activity was determined as explained for G-6-PASE, replacing monosodium glucose-6-phosphate with 1 mM monosodium mannose-6-phosphate as substrate. The spectrophotometric method (λ=820 nm) is based on the development of a blue chromogen formed when inorganic phosphate is hydrolyzed from either substrate by the action of G-6-PASE or M-6-PASE [15].

A standard curve was plotted using appropriate dilutions of a monobasic potassium phosphate stock solution (645 nmol/ml) to prepare solutions containing 10 to 40 nmol total phosphate [18]. The absorbance of standards and liver samples was read (λ=820 nm) against a blank mixture containing deionized, distilled water instead of the phosphate solution. The activity of the enzyme was defined as units per milligram protein, where 1 U represented 10 nmol of inorganic phosphate released per minute per milliliter of enzyme [18].

2.8. Total RNA isolation and cDNA synthesis by reverse transcription

Total RNA was isolated from lyophilized liver tissue (500 mg) using an ASNAP Total RNA Isolation Kit according to the manufacturer's protocol (Invitrogen). The integrity and concentration of the isolated RNA were determined spectrophotometrically (λ=260 and 280 nm) using an UltraSpec 2000 UV/Visible Spectrophotometer (Pharmacia Biotech). The ratio of absorbances, A_{260}/A_{280} nm, for total RNA was 1.94±0.01 for the CGA-treated rats and 1.92±0.01 for the control. In solution, pure RNA typically should have an A_{260}/A_{280}=1.9–2.0.

The average concentration of RNA for the control and CGA groups was 5.1±0.3 and 5.2±0.1 μg/μl, respectively. First-strand complementary DNA was synthesized by reverse transcription of 10 μg of the isolated total RNA from liver using a cDNA Cycle Kit (Invitrogen) according to the manufacturer's protocol. The method is based on the utilization of avian myeloblastoma virus reverse transcriptase (AMV RT). The AMV RT catalyzes the 5′ to 3′ polymerization of cDNA using a template such as total RNA. The cDNA products were stored on ice until amplification by PCR.

Table 1
In vivo effect of CGA on body and liver weight, and food intake in Zucker (*fa/fa*) rats

	Control	CGA-treated rats[1]
Number of rats	6	6
Initial BW[2]	577.3±22.8[a]	538.2±21.8[a]
Final BW (g)	614.7±23.1[a]	556.7±26.0[a]
Liver (g/100 g BW)	4.72±0.47[a]	3.57±0.24[a]
Food intake (g/day)	23.39±1.6[a]	22.33±2.2[a]
Daily food intake/BW	0.038±0.003[a]	0.040±0.004[a]

Values are means±S.E.M.
Means in the same row and with different letters are significantly different ($P<.05$).
[1] CGA was injected intravenously every day for 3 weeks at 5 mg/kg body weight.
[2] BW, rat body weight.

The integrity and concentration of the synthesized cDNA were determined by simultaneously reading the absorbances of diluted cDNA samples at 260 and 280 nm using an UltraSpec 2000 UV/Visible Spectrophotometer (Pharmacia Biotech). The average absorbance ratio, A_{260}/A_{280} nm, was 1.85±0.01 for the control group and 1.87±0.02 for the CGA-treated rats. In solution, pure DNA typically should have an A_{260}/A_{280}=1.8–1.9. For the control group, the cDNA concentration was 18.33±2.74 μg/μl, and for the CGA-treated rats, it was 17.83±5.00 μg/μl.

2.9. cDNA Amplification by PCR and insulin receptor mRNA expression

The cDNA products were amplified by PCR. The method was based on the utilization of ThermalAce DNA Polymerase (2 U/μl), a thermostable enzyme with 3′ to 5′ exonuclease activity. Ten microliters of hepatic cDNA samples was amplified using a ThermalAce DNA Polymerase Kit according to the manufacturer's protocol (Invitrogen).

The two oligonucleotide primers used for the insulin receptor gene (Commonwealth Biotechnologies, Richmond, VA) spanned nucleotides 2181 to 2201 (5′-TTC ATT CAG GAA GAC CTT CGA-3′; sense primer) and 2418 to 2438 (5′-CGG CCA GAG ATG ACA AGT GAC-3′; antisense primer), flanking the exon 11 region of the rat insulin receptor [9].

To 10 μl of reverse transcription liver products, a 40-μl PCR master mix was added, containing a total of 5 μl of 10× ThermalAce Buffer, 1 μl of 100 mM deoxynucleotide-triphosphate (dNTP) mix, 0.1 μM of each oligo primer, 2 U of ThermalAce DNA polymerase, and sterile PCR water. To each sample, 70 μl of mineral oil was added before PCR.

A DNA Thermal Cycler 480 from Perkin-Elmer (PE Biosystems, Foster City, CA) was used. The PCR for the liver samples from CGA-treated rats and control rats totaled 32 cycles. The PCR conditions were set as follows: denaturation 94°C, annealing 55°C and extension at 72°C for 1 min each. A 10× Tris–borate–EDTA (TBE) stock buffer (54 g Tris-base, 27.5 g boric acid and 20 ml of a 0.5 M EDTA-di-sodium, pH=8.0) was prepared. An aliquot of 10× TBE buffer was diluted in deionized, distilled water to obtain a 1× TBE buffer.

To 20 μl of the RT-PCR amplification products, 5 μl of 10× loading buffer solution (3 ml glycerol, 2.5 ml 10× TBE and 1 ml of 0.25% bromophenol blue) was added. The mixtures and the DNA Ladder (Invitrogen) were resolved by electrophoresis on a 3.0% high-resolution SFR agarose gel containing ethidium bromide (0.5 μg/ml) in 1× TBE buffer. Electrophoresis was conducted at a constant 120 V until the bromophenol blue tracking dye migrated about three fourth the length of the gel. The gels were rinsed with deionized, distilled water and photographed using an Imaging Detecting Analyzer (Raytest, New Castle, DE). The relative abundance of the insulin receptor mRNA exon $11^{+/-}$ variants was calculated using the software SigmaGel 1.0 [19].

2.10. Statistical analysis

All results are expressed as means±S.E.M. (n=6). The data were analyzed by analysis of variance using SAS software and Duncan's multiple range model at an α=.05 [20].

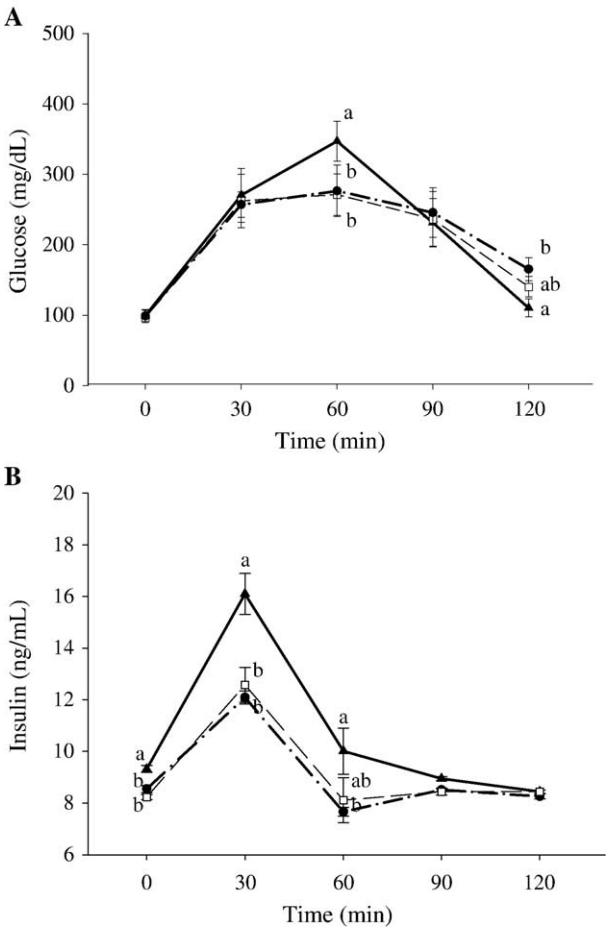

Fig. 1. Effect of CGA treatment in Zucker (*fa/fa*) rats for 3 weeks on (A) 24-h fasting concentration of blood glucose (mg/dl) [7] and (B) 24-h fasting concentration of plasma insulin (ng/ml). At 12 (•) and 19 weeks old (▲) without CGA treatment, and at 22 weeks old (□) with CGA treatment. [ab]$P<.05$. Values are means±S.E.M. (n=6).

3. Results

The results presented here are the second part of findings obtained after investigating the effect of CGA in an animal model of glucose-intolerant and insulin-resistant Zucker (*fa/fa*) rat. There was no significant difference in the body weight and mean daily food intake of CGA-treated rats when compared to controls rats (Table 1). Therefore, supplementation of CGA did not suppress food intake in the obese Zucker (*fa/fa*) rats. Liver weight was calculated per 100 g of body weight, and no significant difference ($P>.05$) was found between the CGA-treated and control rats (Table 1).

When the rats were 22 weeks old, fasting blood glucose and glucose tolerance tests were repeated for the treatment group only. Although catheters were flushed daily with 0.1 ml heparinized saline solution (5 U/ml), blood could not be drawn from the control rats as the catheters were plugged by 22 weeks.

3.1. Fasting plasma insulin concentrations and oral glucose tolerance test

Chlorogenic acid was infused daily for a period of 3 weeks to determine its insulin sensitizing effect in glucose-intolerant and insulin-resistant Zucker (*fa/fa*) rats. It has been shown that Zucker (*fa/fa*) rats develop an inability, with age, to maintain serum glucose and insulin concentrations following a glucose challenge or a meal [21,22]. We found a similar response with age as indicated by the significantly higher peak glucose response in 19-week-old rats [7]. The area under the glucose curve, calculated using data from Fig. 1A [7], for CGA-treated rats at the age of 22 weeks was decreased by 21% ($P<.05$) compared with the same group of rats at 19 weeks of age and without CGA treatment (Fig. 2A).

Insulin concentrations were estimated during the glucose tolerance test. We found that the Zucker (*fa/fa*) rats, aged 22 weeks, had a significant ($P<.05$) decrease in the peak plasma insulin concentration after 30 min when compared to the same group of rats at 19 weeks of age (Fig. 1B). In addition, the CGA-treated rats maintained the same peak plasma insulin concentration found when the rats were 12 weeks old before CGA intervention (Fig. 1B). The area under the insulin curve for CGA-treated rats at the age of 22 weeks was decreased by 20% ($P<.05$) compared with the same group of rats at 19 weeks of age and without CGA treatment (Fig. 2B).

Fasting insulin concentration was determined at the time the rats, aged 22 weeks, were sacrificed. It has been reported that the obese Zucker (*fa/fa*) rat has elevated concentrations of fasting plasma insulin starting at the fourth week of age [23]. The average for fasting plasma insulin concentrations found in the insulin-resistant and obese Zucker (*fa/fa*) rats at 12 and 24 weeks of age was 6.53 ± 1.53 and 13.33 ± 3.09 ng/ml, respectively [23]. Chlorogenic acid lowered ($P<.05$) fasting plasma insulin concentrations in the 22-week-old Zucker (*fa/fa*) rats (8.23 ± 0.09 ng/ml) vs. the same group of rats before CGA treatment at 19 weeks of age (9.31 ± 0.13 ng/ml). No significant differences ($P>.05$) were found in plasma insulin concentration between 12-week-old untreated rats (8.55 ± 0.14 ng/ml) and the 22-week-old CGA-treated rats. However, at 22 weeks of age, there was a 32% difference ($P<.05$) in plasma insulin concentration between the untreated rats (10.87 ± 0.13 ng/ml) and the 22-week-old CGA-treated rats (8.23 ± 0.09 ng/ml).

3.2. Protein and DNA concentrations in liver

Chlorogenic acid was infused daily for a period of 3 weeks to determine its effect on liver protein and DNA

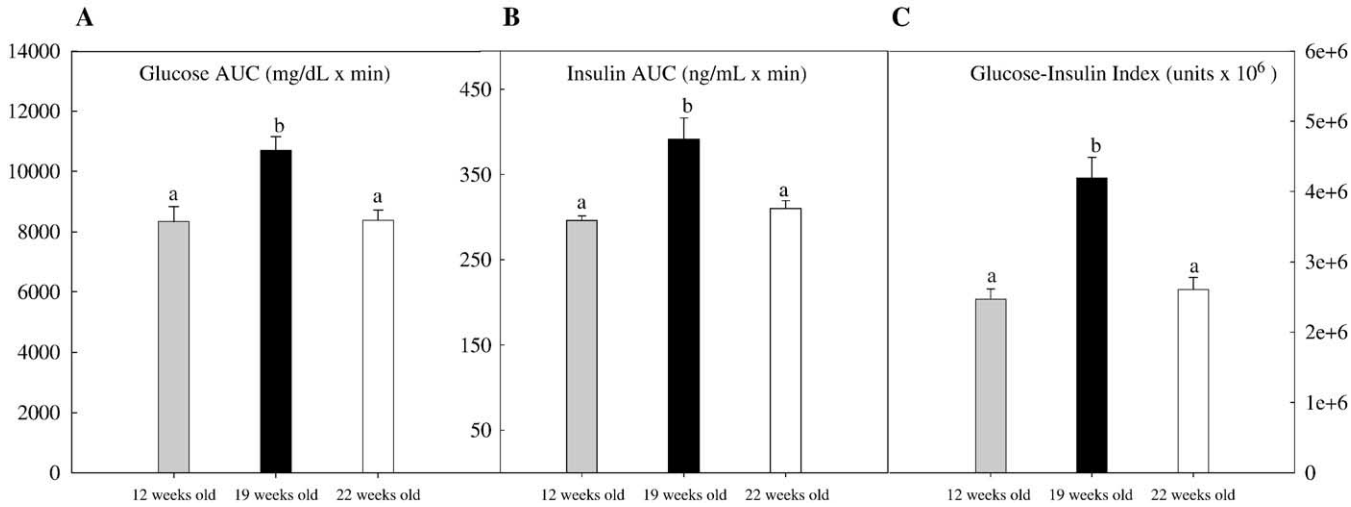

Fig. 2. Effect of CGA treatment in Zucker (*fa/fa*) rats for 3 weeks on incremental AUC during an oral glucose tolerance test at 12 and 19 weeks old without CGA treatment, and at 22 weeks old with CGA treatment. (A) AUC for glucose; (B) AUC for insulin; (C) glucose–insulin index. $^{ab}P<.05$. Values are means±S.E.M. ($n=6$).

concentration in glucose-intolerant and insulin-resistant Zucker (*fa/fa*) rats. The concentration of protein in liver fractions (I, II and III) obtained during isolation of microsomes and in total liver was estimated (Table 2). A significant ($P<.05$) increase of 15% and 29% was found in the protein concentration of CGA-treated rats in liver fractions I and III, respectively (Table 2). The concentration of total protein increased significantly ($P<.05$) by 16% in the CGA-treated rats.

Phenolic acids such as CGA are metal ion chelators [24]. Chlorogenic acid chelation of metal ions could compromise DNA synthesis and, subsequently, cellular metabolism due to perturbation of protein synthesis. Therefore, the in vivo effect of CGA on hepatic DNA concentration was investigated. In the liver of CGA-treated rats, a significant ($P<.05$) increase in the concentration of DNA was found (Table 2).

3.3. Mannose-6-phosphatase and G-6-PASE activity

The activity of M-6-PASE (U/mg protein) toward the substrate monosodium mannose-6-phosphate in intact and fully disrupted microsomes was determined and used to calculate the percentage of intact microsomes in the microsomal fraction. No significant difference ($P>.05$) was found for M-6-PASE activity between the two groups in intact or disrupted microsomes (Table 3). The percentage of intact microsomes in the microsomal fraction for the control group was 94±1.48 and for the CGA-treated group was 96±0.72.

No significant ($P>.05$) difference was found for the activity of G-6-PASE (U/mg protein) between the two groups in the intact or disrupted microsomes with glucose-6-phosphate (30, 10, 5 or 1 mM). The activity of G-6-PASE with 1 mM glucose-6-phosphate is shown (Table 3).

3.4. Hepatic insulin receptor mRNA expression by polymerase chain reaction assay

In individuals affected by insulin resistance, the cause of the abnormality appears to be heterogeneous, as, in some cases, it has been associated with defects in the insulin receptor binding, while, in other cases, it is attributed to insulin receptor numbers and/or post-receptor modifications [11,25–27]. The number of hepatic insulin receptors and/or their mRNA expression such as the exon $11^{+/-}$ variants has been found to be affected in humans and/or animal models suffering from peripheral hepatic insulin resistance [10,11,25,26].

The obese Zucker (*fa/fa*) rat is characterized as insulin-resistant, the hepatic insulin receptor numbers of which are significantly decreased starting at 20 weeks of age [26,28]. The decreased number of insulin receptors was correlated with hepatic insulin resistance [26]. However, to date, no information exists on the mRNA insulin receptor exon $11^{+/-}$ variant expression in the obese Zucker (*fa/fa*) rat. The effect of CGA as a novel insulin sensitizer on the expression of hepatic insulin receptor mRNA was investigated in obese Zucker (*fa/fa*) rats.

The liver RT-PCR products of the insulin receptor exon $11^{+/-}$ variants were resolved by electrophoresis on a 3% SFR agarose gel. An electrophoretogram of RT-PCR products of Zucker (*fa/fa*) rats treated with CGA and of controls indicates expression of both exon 11^{+} (273 bp) and exon 11^{-} (237 bp) (Fig. 3A).

In this preliminary study, the relative abundance of exon 11^{+} and exon 11^{-} isoforms was calculated as the area under the peaks obtained from bands in the electrophoretogram (Fig. 3A). The peaks for band densities were obtained using the software SigmaGel 1.0 [19]. The areas under the peaks were proportional to the density of the bands in the electrophoretogram (Fig. 3A). No significant differences in the insulin receptor mRNA expression were found for either exon 11^{+} or exon 11^{-} between the two groups. For the control group and the CGA-treated rats, the average areas (arbitrary units) for exon 11^{+} were 350.9±1.5 and 351.5±3.3, respectively. The average areas (arbitrary units) for exon 11^{-} for the control group and the

Table 2
In vivo effect of CGA after 3 weeks' intervention on liver magnesium, zinc, protein and DNA concentrations of Zucker (*fa/fa*) rats

	Controls	CGA-treated rats[1]
Number of rats	6	6
Magnesium[2] μg/g)	562.90±31.78[a]	790.36±18.26[b]
Zinc[2] (μg/g)	63.17±3.07[a]	97.66±3.60[b]
Fraction I (mg/g)	35.736±0.214[a]	42.256±0.097[b]
Fraction II (mg/g)	32.194±0.351[a]	30.944±0.215[a]
Microsomal fraction (mg/g)	34.982±1.703[a]	49.240±1.916[b]
Total protein (mg/g)	102.912±4.310[a]	122.440±5.231[b]
DNA (μg/mg)	2.720±0.468[a]	3.975±0.292[b]

Values are means±S.E.M.
Means in the same row and with different letters are significantly different ($P<.05$).
[1] CGA was injected intravenously every day for 3 weeks at 5 mg/kg body weight.
[2] Magnesium and zinc concentrations were determined previously [7].

Table 3
In vivo effect of CGA after 3 weeks' intervention on M-6-PASE and G-6-PASE activity of Zucker (*fa/fa*) rats

	Controls	CGA-treated rats[1]
Number of rats	6	6
M-6-PASE activity in intact microsomes[2]	4.34±0.09[a]	4.12±0.07[a]
M-6-PASE activity in disrupted microsomes (U/mg protein)	67.06±1.20[a]	66.82±1.55[a]
G-6-PASE activity in intact microsomes[3] (U/mg protein)	21.36±2.70[a]	18.12±3.01[a]
G-6-PASE activity in disrupted microsomes (U/mg protein)	75.87±1.80[a]	73.97±1.60[a]

Values are means±S.E.M.
Means in the same row and with different letters are significantly different ($P<.05$).
[1] CGA was injected intravenously every day for 3 weeks at 5 mg/kg body weight.
[2] M-6-PASE against 1 mM mannose-6-phosphatase as substrate.
[3] G-6-PASE against 1 mM glucose-6-phosphatase as substrate.

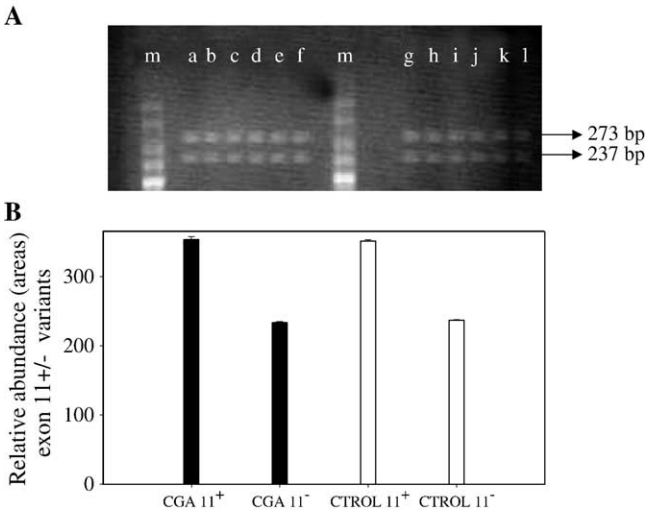

Fig. 3. (A) Representative electrophoretogram of the insulin receptor exon $11^{+/-}$ variants RT-PCR products in the liver of Zucker (*fa/fa*) rats at 22 weeks old and after 3 weeks with or without CGA treatment. Lane m, DNA ladder marker; lanes a–f, CGA-treated rats; lanes g–l, control rats. (B) Relative abundance (areas) of the alternatively spliced insulin receptor $11^{+/-}$ variants in the liver of Zucker (*fa/fa*) rats calculated using SigmaGel software. CGA $11^{+/-}$, exon $11^{+/-}$ of CGA-treated rats; CTROL $11^{+/-}$, exon $11^{+/-}$ of vehicle-treated rats ($P>.05$). Values are means±S.E.M. ($n=6$).

CGA-treated group were 296.7±1.2 and 294.3±1.5, respectively (Fig. 3B).

4. Discussion

At the time the control rats were 22 weeks old, their catheters were not viable [7]. We found a decrease (13.8%) in plasma magnesium concentration and an increased (8.4%) calcium concentration, explaining in part, why the catheters of the control group, at the age of 22 weeks, might have been plugged [7]. Low serum concentrations of magnesium, elevation of calcium concentrations in the serum and a high ratio of calcium/magnesium are found in humans and in animals with insulin resistance, diabetes mellitus and cardiovascular diseases [29,30]. The hyperlipidemic characteristic of the obese Zucker (*fa/fa*) rat, together with the disturbance in magnesium and calcium concentrations, can promote atherogenesis in these rats [29,31,32]. Thus, it is possible that the control rats used under the conditions of this experiment were undergoing atherogenesis. This suggestion is supported by observations that obese Zucker (*fa/fa*) rats develop vascular complications over time due to obesity [33].

We found that after 3 weeks of intervention with CGA, the Zucker (*fa/fa*) rats, aged 22 weeks, had a significant ($P<.05$) decrease in the peak blood glucose concentration 1 h after a glucose challenge when compared to the same group of rats at 19 weeks of age (Fig. 1A) [7]. In addition, the CGA-treated rats maintained the same peak blood glucose concentration found when the rats were 12 weeks old before CGA intervention and before they showed postprandial hyperglycemia [7]. Furthermore, we found that CGA did not lower fasting blood glucose concentrations in the 22-week-old Zucker (*fa/fa*) rats (97.94±6.33 mg/dl), when compared to the same group of rats before CGA treatment at either 12 or 19 weeks of age (98.46±9.57 and 99.25±7.33 mg/dl, respectively) (Fig. 1A) [7]. Moreover, for the control group (22 weeks old) the fasting blood glucose concentration (97.89±6.68 mg/dl) did not differ from the CGA-treated group.

The differences in plasma insulin concentrations and the improved response to a glucose challenge found under the conditions of this study indicate that CGA had an insulin-sensitivity enhancing effect in male Zucker (*fa/fa*) rats. Additionally, we found the glucose–insulin index, an indirect indicator of in vivo peripheral insulin action, decreased by 60% (Fig. 2C) in 22-week-old CGA-treated group vs. the same group of rats at 19 weeks of age without CGA treatment. These findings, plus a decreased ($P<.05$) concentration of circulating cholesterol (44%) and triacylglycerols (58%) in the 22-week-old CGA-treated rats [7], support the suggestion that CGA is a novel insulin sensitizer that potentiates insulin action similar to the known therapeutic action of metformin [34].

Protein synthesis in the liver is stimulated by insulin, a process that is altered in type 2 diabetics and/or insulin-resistant individuals, and in obese, type 2 diabetic and insulin-resistant animal models [35,36]. The Zucker (*fa/fa*) rats are a model of insulin resistance with fasting insulin concentrations reported as high as 132 μU/ml at the age of 18 weeks [31]. Bach et al. [35] reported a significant ($P<.05$) decrease of 6% in the total liver protein (mg/g of tissue) in 9-week-old Zucker (*fa/fa*) rats when compared to nonobese, non-insulin-resistant Zucker (*Fa/fa*) rats. The 16% increase (Table 2) in the concentration of total liver proteins found for the CGA-treated rats in this investigation may have been due to an increase of liver sensitivity to insulin. A decrease in liver protein concentration has been reported for obese Zucker (*fa/fa*) rats between the ages of 17 and 24 weeks when compared to lean Zucker (*Fa/fa*) rats [21]. The results reported here are in agreement with others [21]. The control rats at 22 weeks old had significantly lower total liver protein than the CGA-treated obese Zucker (*fa/fa*) rats.

Metformin, an insulin sensitizer, increases hepatic protein synthesis by improving insulin action [37]. In our study, CGA, like metformin, may have decreased hepatic peripheral, insulin resistance, leading to increased protein synthesis.

The synthesis of DNA is directly stimulated, among other things, by insulin action and by the availability of cellular metal ions such as zinc and magnesium [38]. Decreased cellular concentrations of zinc in the hepatocytes affect insulin action with a significant decrease in the catabolism of dietary carbohydrate due to decreased mRNA expression of pyruvate kinase [39]. We found that the concentrations of zinc and magnesium [7] were significantly ($P<.05$) increased (Table 2). Both of these trace minerals

are essential for the synthesis of DNA and the integrity of the DNA helix. Therefore, the decreased DNA concentration in the liver of control rats might reflect insufficient levels of the metal ions to support optimal DNA synthesis. Furthermore, the problem is exacerbated because obese Zucker (*fa/fa*) rats are insulin-resistant.

The in vivo effect of CGA on the activity of G-6-PASE found in this investigation agrees with in vitro studies reported by others [6]. Using microsomal hepatic cells, they found that only some derivatives of CGA significantly ($P<.05$) decreased the activity of G-6-PASE when compared to CGA and controls [6].

The activity of G-6-PASE is inhibited when liver microsomes are incubated with CGA [6]. In this study, the rats were sacrificed 40 h after the last CGA intervention. At this point, it is unlikely that any CGA was still present, which would explain the lack of inhibition of G-6-PASE. This was done in an effort to determine whether the 3-week treatment with CGA would induce an undesirable sustained hypoglycemia.

In this study, the insulin receptor mRNA expression of the Zucker (*fa/fa*) rats was not affected by CGA treatment possibly because in peripheral insulin-resistant receptor or post-receptor, modifications are implicated [25,26]. Thus, it is possible that the insulin resistance found in the liver of Zucker (*fa/fa*) rats is due to a post-transcriptional event such as decreased binding of insulin to its receptor and/or a decreased number of insulin receptors. This suggestion is supported by others [26], who reported a reduced number of hepatic insulin receptors in the insulin-resistant obese Zucker (*fa/fa*) rats starting at 20 weeks old. Furthermore, a decreased number of insulin receptors in the adipocytes of insulin-resistant and obese Zucker (*fa/fa*) rats have also been found [28]. In addition, it has been proposed that the two spliced variants found in the insulin receptor at exon 11 (11^+ and 11^-) are not strongly related to the affinity of insulin for binding to its receptor and/or the function of the insulin receptor once it binds insulin and internalizes in the plasma membrane [27]. This characteristic would explain why most insulin-sensitizing drugs, such as metformin and troglitazone, are referred to as post-insulin receptor modifiers [34,40]. In this preliminary study, however, the effect of CGA on ligand binding to insulin receptor or the number of insulin receptor in the liver plasma membrane was not investigated; such studies are needed to elucidate CGA properties as a novel insulin-sensitizing agent.

The results of the present study showed that CGA significantly improved the glucose–insulin index and significantly increased hepatic protein and DNA concentrations. Thus, the null hypothesis, intravenous infusion of CGA to genetically obese, insulin-resistant and hyperlipidemic Zucker (*fa/fa*) rats will have no effect on liver protein and DNA concentration, was rejected. However, CGA did not have any significant effect on the activity of G-6-PASE.

The results of this preliminary study lead us to hypothesize that the efficacy of CGA may be dependent on the presence of insulin and, thus, may act synergistically with the hormone to increase its biological action. Additional studies are needed to clarify the mechanisms involved before the benefits and/or disadvantages of using CGA as a pharmacological agent can be determined.

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