Is Insulin Resistance in the Spontaneously Hypertensive Rat Related to Changes in Protein Kinase C in Skeletal Muscle?

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The mechanism of insulin resistance in the spontaneously hypertensive rat (SHR) has not been clearly identified, but protein kinase C (PKC) has been implicated as a mechanism of insulin resistance in obesity and diabetes mellitus and in a diet-induced (fructose-fed) model of insulin resistance and hypertension. This study compared PKC enzyme activity (cytosol and particulate fractions) and expression of the muscle-specific isoform, PKC-\(\theta\) (Western blotting), in red (soleus) and white (tensor fascia latae) hindlimb muscles from SHR (\(n = 12\)) and WKY (\(n = 12\)) rats. SHRs were hypertensive and insulin resistant, as shown by higher insulin (188 ± 34 \(\mu\)mol/L), triglycerides (1.65 ± 0.07 mmol/L), and nonesterified fatty acids (0.99 ± 0.05 mmol/L) concentrations. PKC activity was significantly greater in the membrane fraction, compared with the cytosol, but there were no significant differences either in PKC activity or subcellular distribution, or expression of PKC-\(\theta\), between the two strains. Thus, insulin resistance in the SHR (in contrast to the fructose-fed dietary model of insulin resistance and hypertension) is not related to changes in PKC signaling or expression of PKC-\(\theta\) in skeletal muscle. Am J Hypertens 1997;10:1053–1057 © 1997 American Journal of Hypertension, Ltd.

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specific protein kinase that plays an important regulatory role in a variety of cellular responses. Activation and translocation of PKC occurs in response to a transient increase in diacylglycerol and exposure to exogenous tumor promoting agents such as phorbol esters. Changes in PKC signaling in muscle and adipose tissues have been associated with physiological responses to insulin and the development of insulin resistance in models of aging, obesity, hypertension, and muscle denervation. In particular, there is evidence that PKC phosphorylates and down-regulates one or more intracellular substrates involved in glucose transport and metabolism, eg, the insulin receptor and glycogen synthase.

The ability of insulin to stimulate glucose uptake by skeletal muscle varies as a function of muscle type, with muscles containing primarily oxidative fibers (red) having two- to threefold greater insulin sensitivity and responsiveness compared with muscles composed mainly of glycolytic fibers (white). We have previously shown that expression of PKC-θ (the major isoenzyme of PKC in skeletal muscle) varies between hindlimb muscles of different fiber-type composition and insulin sensitivity, with more PKC-θ expressed in a pure white muscle (tensor fascia latae; TFL) as compared with a red muscle (soleus). Furthermore, tissue and isof orm-selective changes in PKC signaling were observed in a fructose-fed model of insulin resistance and hypertension. The purpose of this study was to see whether insulin resistance in a genetic model of hypertension is also associated with differences in PKC activity or distribution (ie, cytosol versus membrane fraction), or expression of PKC-θ.

METHODS

General A detailed protocol was approved by the Animal Care Ethics Committee of the University of Sydney. Male SHR and WKY rats were obtained at approximately 8 weeks of age and maintained on a 12 h light/dark cycle (6 am to 6 pm) with free access to water and standard laboratory chow. Blood samples were drawn from the tail veins of unanesthetized rats for measurement of fasting serum glucose, insulin, and triglyceride concentrations, as described previously. Blood pressure was measured using tail cuff without actual recordings. The tail cuff method, without fluid N2, and stored at −70°C until assayed.

Preparation of Tissue Samples Frozen muscle samples were ground with a pestle and mortar, and then homogenized in a Polytron (Ultra-Turrax T8, IKA Labortechnik, Staufen, Germany) in 100 mg/ml buffer A [20 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, 0.25 mmol/L EGTA, 0.25 mmol/L sucrose, 2 μg/ml leupeptin, and 4 μg/ml each of apro tin, calpain-I, and calpain-II]. For subfractionation of the crude homogenate into cytosol and particulate fractions, samples were first spun at 400 g for 15 min; the supernatant was then ultracentrifuged at 105,000 g for 60 min and removed. The sediment (particulate fraction) was resuspended in 0.5 ml buffer A. An aliquot of each fraction was saved for protein quantification (Bradford method), and cytosol and particulate samples were then used for Western blotting and assay of PKC activity.

PKC Activity Following ultracentrifugation of total muscle homogenate, the supernatant was removed as the cytosol fraction; the residue (particulate fraction) was resuspended in 0.5 ml Buffer A, then mixed with 0.5 ml of Buffer B (Buffer A without sucrose) containing 2% Triton X-100 and solubilized for 30 sec in a sonic dismembrator at 4°C. The supernatant and particulate fractions were then applied to DEAE-Sephacryl columns. After washing with 5 ml Buffer B, the enzyme fractions were eluted in 3 ml fractions from the column using Buffer B containing 0.15 mol/L NaCl. Enzyme activity was assayed immediately after completion of the chromatography step by following the incorporation of [32P] from [γ-32P]-ATP into acceptor GS peptide (H-Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys-NH2), a synthetic dodecapeptide of glycogen synthase as a PKC substrate; Auspep, Melbourne, Australia), as described previously. The standard incubation mixture consisted of the following components in a final volume of 100 μL: 20 μL aliquots of chromatography fractions, 25 mmol/L PIPES-NaOH (pH 6.8), 10 mmol/L magnesium acetate, 20 μmol/L GS peptide, 5 mmol/L 2-mercapto ethanol, 0.1 mmol/L [γ-32P]-ATP (200 to 300 cpm/ pmol), 1.0 mmol/L CaCl2, 150 μg/ml phosphatidyliner e, and 10 μg/ml diolein. Phosphatidyliner serine and diolein were dissolved in chloroform, dried in N2 and the residues resuspended in 10 mmol/L PIPES, pH 6.8, before addition to the assay. Basal activity was determined in the presence of 0.5 mmol/L EGTA (instead of Ca2+, phosphatidyliner serine and diolein). The reaction was initiated by the addition of [γ-32P]-ATP at 30°C. After incubation for 15 min, the reaction was terminated by spotting 50 μL of the mixture onto 2 × 2 cm phosphocellulose strips, which were dropped immediately into 75 mmol/L phosphoric acid. The
strips were then washed four times, including one overnight wash, in 75 mmol/L phosphoric acid. The dried strips were counted in a liquid scintillation counter. PKC activity was calculated by subtracting the enzyme activity observed in the presence of 0.5 mmol/L EGTA from that measured in the presence of phosphatidylserine, diolein, and calcium. One unit of PKC activity is defined as that amount catalyzing the transfer of 1 pmol of [\( ^{32} \text{P} \)]-phosphate from [\( ^{\gamma} \text{P} \)]-ATP to GS peptide per min at 30°C.

**Western Blotting** Samples were mixed with equal volumes of 2X sample-loading buffer (4.6% [w/v] SDS, 10% [v/v] \( \beta \)-mercaptoethanol, 16% [w/v] sucrose and 0.1 M Tris-HCl, pH 6.8), heated at 95°C for 5 min and cooled to room temperature. The mixture was then centrifuged at 5,000 rpm for 5 min, and the supernatant subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (7% [w/v] polyacrylamide) followed by electrotransfer to a Polyvinylidene fluoride (PVDF) transfer membrane (DuPont, Boston, MA). For each sample, 50 μg of total protein was loaded onto the gel, with equal numbers of SHR and WKY samples on each gel. Protein standards were also loaded onto each gel (myosin 200 kDa, \( \beta \)-galactosidase 116 kDa, phosphorylase b 97 kDa, albumin 66 kDa, and ovalbumin 45 kDa; Bio-Rad Laboratories, Richmond, CA). The membrane was then incubated for 12 h at 4°C in phosphate buffered saline-Tween (PBS-T: 10 mmol/L sodium phosphate, 0.15 mol/L NaCl, 0.5 g/L MgCl\(_2\) and 0.2% Tween-20) containing 5% (w/v) nonfat dried milk. After the blocking step, membranes were washed (4 × 10 min) in rinsing solution (PBS-T with 1% dried milk, pH 7.4), and then incubated for 2 h with immune serum (anti-PKC-\( \theta \)-isozyme, dilution 1:5000). After further washings in rinsing solution (4 × 15 min), membranes were incubated with horseradish peroxidase conjugated IgG fraction of goat antirabbit IgG, diluted 1:20,000 in PBS-T. An enhanced chemiluminescence (ECL) detection kit was used for the revealing step and development onto Kodak XOMAT film (Rochester, NY). Each film was exposed for 90 sec. The bands obtained from immunoblotting were scanned by one-dimensional laser densitometry (Molecular Dynamics, Santa Cruz, CA), with automated software analysis of the areas under the peaks.

**Statistical Analysis** Data were analyzed by two-way analysis of variance and the results are expressed as mean ± SEM.

**RESULTS** Compared with WKY rats (n = 12), SHRs (n = 12) were hypertensive (175 ± 5 mm Hg v 143 ± 3) and insulin resistant, as shown by higher fasting insulin (188 ± 34 v 169 ± 22 pmol/L), triglyceride (1.65 ± 0.07 v 1.38 ± 0.06 mmol/L), and nonesterified fatty acid (NEFA, 0.99 ± 0.05 v 0.78 ± 0.04 mmol/L) concentrations.

PKC activity in TFL (white) and soleus (red) muscles was significantly greater in the membrane fraction, as compared with the cytosol, but there were no significant differences in enzyme activity or PKC distribution between SHR (n = 12) and WKY (n = 12) rats (Figure 1). PKC-\( \theta \) (the major isoenzyme of PKC in muscle) was identified as a 79 kDa band in cytosol and particulate fractions. The Western blots showed no difference in expression or subcellular distribution of PKC-\( \theta \) between the two rat strains, either in red or white muscle (Figure 1).

**DISCUSSION**

The mechanisms of insulin resistance both in human and rodent hypertension have not been clearly established, but detailed metabolic studies have suggested that there is a tissue- and pathway-specific defect in glucose utilization involving skeletal muscle at or beyond the level of the insulin receptor.\(^{1-6,14}\) There is increasing evidence that PKC plays an important role in insulin signal transduction,\(^5\) as well as blood pressure regulation,\(^{15}\) and that PKC activation phosphorylates (and down-regulates) one or more steps involved in glucose transport and metabolism.\(^{7,8}\)

In light of our previous data\(^{11,12}\) showing that a diet-induced model of hypertension and insulin resistance is associated with increased PKC activity and inducible expression of the muscle-specific isoform, PKC-\( \theta \), it seemed reasonable to ask whether insulin resistance in a primary genetic model of hypertension is also associated with changes in PKC activity or subcellular distribution in red and white hindlimb muscles of different fiber-type composition. Although most of the available evidence suggests that the SHR is resistant to insulin-mediated glucose disposal,\(^{1-5}\) there is some inconsistency in the observations and even the suggestion that insulin sensitivity is increased, not decreased, in the SHR.\(^{16}\) In the present study, there was evidence that the SHRs were insulin resistant, relative to WKY rats, by virtue of higher fasting insulin, TG and NEFA concentrations, as shown previously by several groups.\(^{1-5}\) but measurements of PKC activity and immunoblots of PKC-\( \theta \) in cytosol and particulate fractions were similar in the two strains. It is worthy of note that the animals in this study were fasted prior to collection of tissue samples, and that feeding may have an impact on the results. For example, although there was no difference in PKC activity under fasting conditions, we cannot exclude the possibility that glucose-induced PKC activation in the fed state is greater in SHR compared with WKY rats, which could perhaps explain why the metabolic differences between the two strains are more pronounced 2 h after an oral glucose challenge.\(^1\)
At least two studies have shown that the hyperinsulinemia in SHRs is due, at least in part, to a reduction in insulin clearance,\(^2,4\) which reflects slower internalization of the insulin-receptor complex in specific tissues, including muscle.\(^4\) Although PKC has the capacity to modify insulin receptor function at the level of the plasma membrane,\(^7,8\) the present study has shown quite clearly that membrane-associated PKC activity is not increased in the SHR. Another factor that might affect insulin clearance in the SHR is dietary Na\(^+\) intake.\(^5\)

Although other isoforms of PKC are expressed in skeletal muscle, PKC-\(\theta\) is the major isoenzymic form\(^10\) and (in contrast to other isoforms that were unchanged) the only isoenzyme that was up-regulated by fructose feeding.\(^11\) Thus, PKC-\(\theta\) is by far the biggest contributor to PKC enzyme activity measured in skeletal muscle, but unfortunately isoform-selective substrates are not yet available for use in the PKC assay. It seems unlikely, however, that differences in expression of other PKC isoforms could explain the peripheral defect in glucose utilization in the SHR.

Thus, although changes in PKC signaling have been implicated in diet-induced and obesity-associated models of insulin resistance and hypertension, there was no evidence of PKC involvement (especially PKC-\(\theta\)) in the abnormality of glucose utilization identified in the SHR.

REFERENCES


