

***Cd36* and Molecular Mechanisms of Insulin Resistance in the Stroke-Prone Spontaneously Hypertensive Rat**

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Insulin resistance is of pathogenic importance in several common human disorders including type 2 diabetes, hypertension, obesity and hyperlipidemia, but the underlying mechanisms are unknown. The spontaneously hypertensive rat (SHR) is a model of these human insulin resistance syndromes. Quantitative trait loci (QTLs) for SHR defects in glucose and fatty acid metabolism, hypertriglyceridemia, and hypertension map to a single region on rat chromosome 4. Genetic analysis of an SHR derived from a National Institutes of Health colony led to the identification of a causative mutation in the SHR *Cd36*. We have investigated glucose and fatty acid metabolism in the stroke-prone SHR (SHRSP). We demonstrate defects in insulin action on 2-deoxy-D-glucose transport (SHRSP 3.3 ± 1.5 vs. 21.0 ± 7.4 pmol \cdot min⁻¹ \cdot [20 μ l packed cells]⁻¹, SHRSP vs. WKY, respectively, $P = 0.01$) and inhibition of catecholamine-stimulated lipolysis ($P < 0.05$ at all concentrations of insulin) in adipocytes isolated from SHRSP. In contrast, basal levels of catecholamine-stimulated nonesterified free fatty acid (NEFA) release and plasma levels of NEFA are similar in SHRSP and WKY. These results are in agreement with the data on the SHR.4 congenic strain, which suggested that the QTL containing *Cd36* mutations accounted for the entire defect in basal catecholamine action but only for ~40% of the SHR defect in insulin action. In the SHR, both abnormalities appear consequent of defective *Cd36* expression. Because *Cd36* sequence and expression are apparently normal in SHRSP, it is likely that the molecular mechanism for defective insulin action in this strain is caused by a gene(s) different than *Cd36*. *Diabetes* 49:2222–2226, 2000

The spontaneously hypertensive rat (SHR) is a model of human insulin resistance syndromes and is characterized by reduced insulin-mediated glucose disposal and defective fatty acid metabolism in isolated adipocytes (1,2). Three recent studies identified that quantitative trait loci (QTLs) for SHR defects in glucose and fatty acid metabolism, hypertriglyceridemia, and hypertension map to a single locus on rat chromosome 4 (2–4). Recently, Aitman et al. (4) identified a chromosomal deletion mutation in the SHR *Cd36* at the peak of linkage of these QTLs and concluded that *Cd36* deficiency gives rise to insulin resistance, defective fatty acid metabolism, and hypertriglyceridemia in this strain. In support of these conclusions, mice with a null mutation in *Cd36* demonstrate a significant increase in plasma nonesterified free fatty acids (NEFAs) and triglycerides (5); transgenic mice overexpressing *Cd36* show the opposite changes (6).

Recent data from Gotoda et al. (7) confirmed the presence of the SHR *Cd36* deletion mutation in three SHR sublines originating from the National Institutes of Health (NIH), but found that the mutation was absent in SHR/Izm and SHRSP/Izm, SHR-derived strains that have been maintained in Japan since 1966 and 1969, respectively. These findings led to the conclusion that the SHR *Cd36* mutation arose de novo in the NIH colony (7). One subline of SHR/Izm studied in the Gotoda et al. experiment reportedly showed insulin and catecholamine resistance in isolated adipocytes, despite the absence of the *Cd36* deletion mutation. However, no genetic linkage data on the origin of these phenotypes has yet to be presented.

Despite extensive studies on multiple cardiovascular phenotypes, insulin action has not been studied in the SHRSP rat strain, and the SHRSP strain originating from the NIH was not examined by Gotoda et al. (7). The current study was designed to detect putative abnormalities in glucose and fatty acid metabolism in the SHRSP originating from the colonies maintained at the University of Glasgow (NIH-derived) and also to establish whether the deletion variant of *Cd36* is associated with these metabolic abnormalities.

At 3 months of age, male SHRSP had increased blood pressure as well as markedly reduced insulin-stimulated glucose uptake and markedly diminished ability of insulin to inhibit

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KRH, Krebs-Ringer-HEPES; NEFA, nonesterified free fatty acid; NIH, National Institutes of Health; QTL, quantitative trait locus; RT-PCR, reverse transcriptase-polymerase chain reaction.

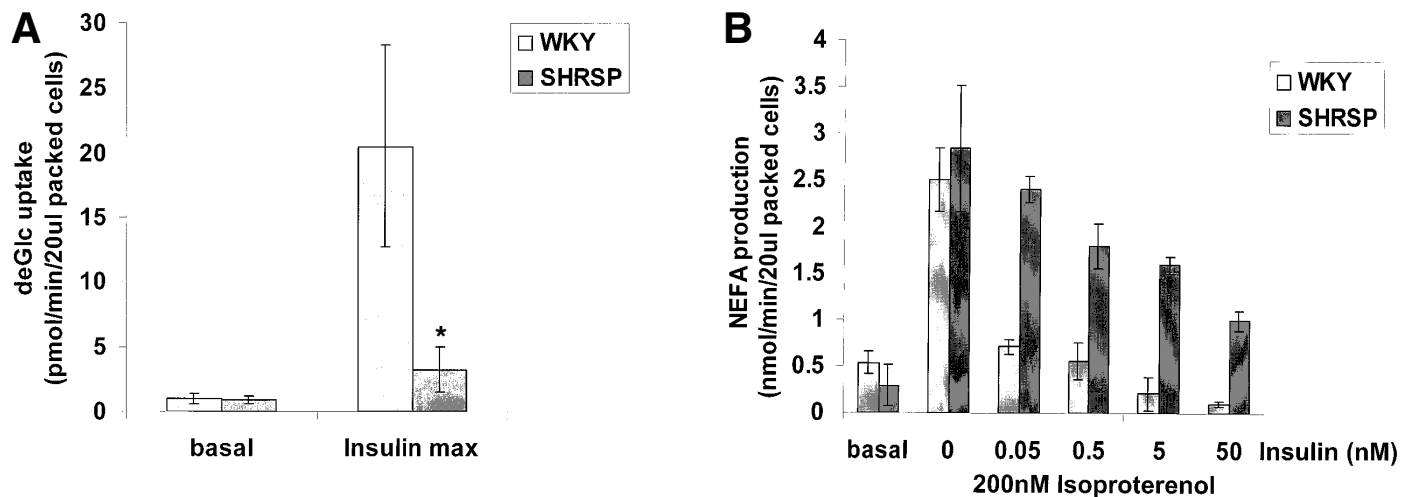


FIG. 1. A: Insulin-stimulated glucose transport in epididymal adipocytes. The uptake of 2-deoxy-D-glucose transport was measured in epididymal adipocytes from WKY or SHRSP animals under basal (nonstimulated) or insulin-stimulated (50 nmol/l for 10 min) conditions. Shown are the data from three independent experiments of this type. All transport rates were corrected for nonspecific association of deGlc with the cells by performing parallel assays in the presence of 10 μ mol/l cytochalasin B, a potent inhibitor of facilitative glucose transporters. This value typically amounted to <35% of the basal rate, and <5% of the insulin-stimulated rate. In experiments not shown here, we observed half-maximal stimulation of deoxyglucose uptake at an insulin concentration of \sim 0.1 nmol/l in WKY animals and at \sim 0.5 nmol/l in SHRSP animals. *Indicates a significant difference ($P = 0.01$) in the maximal insulin-stimulated rates. **B:** Insulin-mediated inhibition of isoproterenol-stimulated NEFA release. Adipocytes were incubated with 200 nmol/l isoproterenol in the presence or absence of the indicated concentrations of insulin for 30 min, and NEFA release was quantified. Within each experiment, triplicate determinations of NEFA release were made at each condition. Shown are the values (means \pm SD) from three independent assays. The difference between WKY and SHRSP is statistically significant at each concentration of insulin ($P < 0.05$ at all concentrations of insulin). The differences between WKY and SHRSP were not significant in either basal or isoproterenol-stimulated conditions in the absence of insulin.

catecholamine-mediated lipolysis in isolated adipocytes compared with male WKY rats (Fig. 1 and Table 1). However, in contrast to SHR, neither basal nor catecholamine-stimulated rates of NEFA release were different between SHRSP and WKY (Fig. 1), and plasma levels of NEFA were similar between the two strains. Nevertheless, at the same age, both male and female SHRSP had significantly elevated serum triglyceride levels (Table 1).

The entire *Cd36* coding sequence of SHRSP, SHR, WKY, and BN rats was determined by direct sequencing of reverse transcriptase-polymerase chain reaction (RT-PCR) products. *Cd36* cDNA from SHR contained multiple variants as previously reported (4). However, the SHRSP cDNA was found to be identical to the WKY cDNA, except at amino acid position 262, where a conservative substitution of arginine for glutamine was present (Table 2). Genotype analysis showed that the *Cd36* deletion mutation in SHR (4) was not found in SHRSP, WKY, BN, or any of an additional 27 rat strains examined (data not shown).

To determine the expression level and transcript size of SHRSP cDNA, Northern blot analysis of *Cd36* expression

was carried out in adipose tissue from SHRSP, SHR, WKY, and BN rats (Fig. 2A, top panel). Using a probe from the 5' end of the *Cd36* cDNA, the overall level of expression was found to be similar in all four strains when compared with the amount of RNA loaded as seen on the ethidium bromide stain (Fig. 2A, bottom panel). The transcript size in SHRSP was identical to the major 2.8-kb transcript seen in WKY and BN. The two major SHR transcripts (3.8 and 5.4 kb) were not observed in SHRSP or the other controls. Likewise, immunoblot analysis demonstrated similar levels of Cd36 protein in adipocyte membranes from both SHRSP and WKY (Fig. 2B). Cellular levels of the insulin-responsive glucose transporter, Glut 4, were modestly elevated in the SHRSP compared with WKY (Fig. 2B).

The current study demonstrates profound abnormalities of insulin action on both glucose transport and inhibition of catecholamine-stimulated lipolysis in adipocytes isolated from the SHRSP. In contrast, basal levels of catecholamine-stimulated NEFA release and plasma levels of NEFA are similar in the SHRSP and WKY. These results are in keeping with the conclusion that defects in the SHR *Cd36* account for the

TABLE 1
Blood pressure and serum levels of triglycerides, glucose, and insulin

	Systolic blood pressure tail-cuff (mmHg)	Systolic blood pressure telemetry (mmHg)	Diastolic blood pressure telemetry (mmHg)	Triglycerides (mmol/l)	Glucose (mmol/l)	NEFA (mmol/l)
SHRSP	162 \pm 11*	180 \pm 14*	128 \pm 10*	1.8 \pm 0.3†	8.3 \pm 0.5	0.30 \pm 0.06
WKY	116 \pm 10	131 \pm 12	94 \pm 10	0.8 \pm 0.1	7.9 \pm 0.4	0.39 \pm 0.07

Data are means \pm SE and are from 6–10 rats from each strain. * $P < 0.001$; † $P < 0.05$.

TABLE 2
Comparison of codon 262 in four rat strains

Rat strain	Codon 262	
	Nucleotide sequence	Amino acid
SHRSP	CGA	Arg
SHR/NCr1Br	CAA	Gln
WKY	CAA	Gln
BN	CGA	Arg

reported SHR defect in fatty acid metabolism (4). However, the demonstrated SHRSP defect in insulin action on both carbohydrate and lipid metabolism and increased serum triglyceride levels are likely due to genes other than *Cd36*, because we found that *Cd36* cDNA sequence and expression are apparently normal in this rat strain.

Although the SHRSP defect in insulin action shown here is similar to that reported in SHR adipocytes (1,2), the current study measured deoxyglucose rather than glucose transport. Because there is a good correlation between these two types

of assays (1), it is unlikely that assay differences account for contrasts between the current study and those carried out previously in SHR (1,2,4,7). The chromosome 4 QTL accounts for 20–40% of the genetic defect in insulin action in SHR (2,4), and it is possible that some insulin resistance genes at other chromosomal locations may be common to both the SHR and SHRSP strains.

The significance of the glutamine to arginine substitution at position 262 is presently uncertain. However, because the WKY strain studied here carries a glutamine, the BN strain carries an arginine, and both strains have normal insulin action and fatty acid metabolism, the substitution is unlikely to explain functional differences between SHR lines, SHRSP, and control strains. Northern blot analysis revealed that, unlike the SHR (4), the size of the major transcript in the SHRSP is identical to that seen in the WKY and BN strains. This finding is in keeping with the data of Pravenec et al. (8), who demonstrated using Southern blot analysis of *Cd36* that the deletion variant was present only in the SHR but not in the SHRSP or BN strains. Finally, immunoblot analysis showed that the *Cd36* protein is equally expressed in the adipocyte mem-

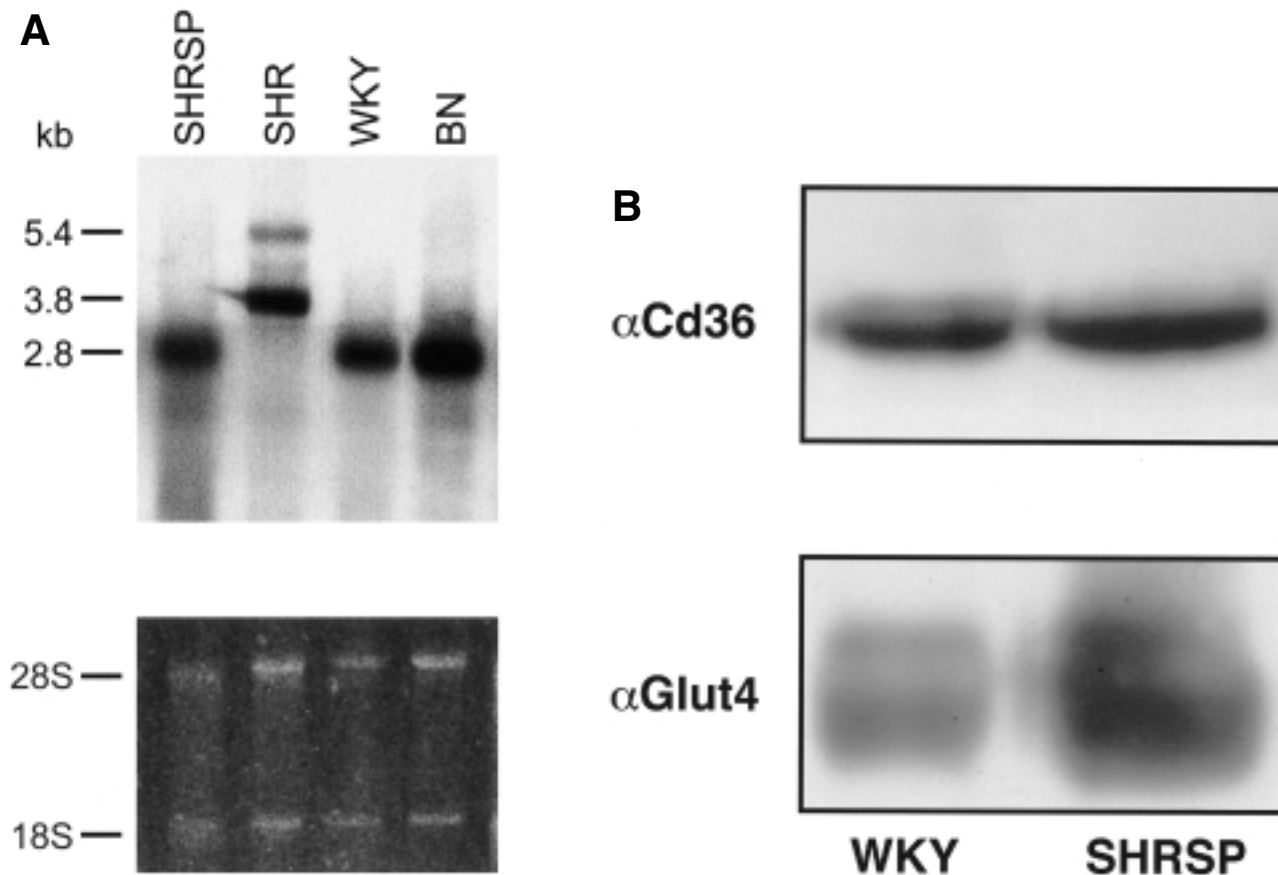


FIG. 2. *A*: The top panel shows a Northern blot of total RNA from SHRSP, SHR, WKY, and BN rats. The filter was hybridized with a PCR-amplified cDNA probe amplified from the 5' end of the WKY cDNA, extending from nucleotides 462–1,052 of the published sequence (15). Probe identity was confirmed by direct sequencing. The bottom panel shows an ethidium bromide stain of the Northern blot indicating the amount of RNA loaded in each lane. *B*: Immunoblot of total membranes from WKY and SHRSP adipocytes. The top panel shows an immunoblot using anti-*Cd36* (a gift from Narendra Tandon, Otsuka America Pharmaceutical), and the lower panel shows an immunoblot using anti-Glut4 antibodies. To quantify the relative levels of expression, increasing loads (typically 5, 10, 20, and 40 μ g) of protein were loaded into adjacent lanes, and the linearity of the immunoblot signal was determined by densitometric analysis of blots developed using enhanced chemiluminescence. Multiple exposures of X-ray film were performed to ensure linearity of response of film to signal, and all immunoblot signals were quantified from linear regions of the protein titration curve. Levels of *Cd36* were not significantly different between SHRSP and WKY rats. Levels of Glut4 were slightly higher in the SHRSP ($145 \pm 12\%$, $P = 0.047$, from three separate experiments) compared with WKY rats. Note that the adipocytes from these two strains were similar in size (data not shown).

branes of the SHRSP and WKY, in contrast with the previous findings in the SHR in which the protein was undetectable (4). Together, these findings indicate that Cd36 expression is normal in SHRSP.

The current data and previous data from our group (9–11) suggest that blood pressure levels are higher in the SHRSP than in the SHR at the same age. We have now demonstrated that this strain is also characterized by significant insulin resistance, which is dissociated from the *in vitro* and *in vivo* basal NEFA abnormalities and is not causally related to a defect in *Cd36*. Consistent with *Cd36* as the likely cause of defective fatty acid metabolism in SHR (2,4), we did not observe differences in either the basal or the maximum rate of catecholamine-induced lipolysis (in the absence of insulin) between SHRSP and WKY. It is probable, therefore, that the molecular causation of abnormal insulin action on glucose and fatty acid metabolism is not identical in SHR and SHRSP. Further genetic analysis of insulin action in SHRSP is likely to identify alternative genes, additional to *Cd36*, which can underlie development of these clinically important phenotypes.

METHODS

Rat strains. Rat strains used in this study were inbred SHRSP and WKY rats originating from the NIH and maintained in the Department of Medicine and Therapeutics, University of Glasgow, as previously described (9–11). In addition, for sequencing experiments and mRNA expression studies, we also used SHR/NCr1BR (Charles River) and BN rats (Harlan). SHRSP and WKY rats were housed under controlled temperature (21°C) and light conditions (12-h light/dark cycle, 7 A.M. to 7 P.M.) and maintained on standard rat diet (Rat and Mouse No. 1 Maintenance Diet; Special Diet Services) and water ad libitum. The pups were weaned and sexed after 3 weeks and were housed according to sibling group and sex thereafter. At 12 weeks of age, systolic blood pressure and heart rate were measured by tail-cuff plethysmography in conscious restrained rats, as previously described (11). To verify physiological measurements obtained with plethysmography, littermates of SHRSP and WKY underwent direct blood pressure and heart rate recordings with a radio-telemetry system (Data Sciences), as previously described (9,11). Hemodynamic data were sampled every 5 min for 10 s. To allow for a full stabilization of blood pressure postoperatively, observations were collected from day 7 to 42 after surgery with a total of 10,080 measurements made on each rat. Glucose was measured by the glucose oxidase technique, and triglycerides were measured using standard enzymatic techniques (8).

Preparation of adipocytes. Epididymal adipocytes were prepared from WKY and SHRSP rats using the collagenase digestion method (12) and suspended at 10% cytocrit in Krebs-Ringer-HEPES (KRH) buffer (118 mmol/l NaCl, 5 mmol/l NaHCO₃, 4.7 mmol/l KCl, 1.2 mmol/l KH₂PO₄, 1.2 mmol/l MgSO₄, 25 mmol/l HEPES, and 2.5 mmol/l CaCl₂) supplemented with 1% bovine serum albumin and 200 nmol/l adenosine at 37°C. 2-Deoxy-D-glucose uptake was then measured using a modification of the method of Cerialdi (13). Briefly, cells were incubated in the absence or presence of varying concentrations of insulin for 10 min. Glucose uptake was initiated by the addition of 10 μmol/l 2-deoxy-D-glucose (2.5 μCi per assay) for 3 min. Thereafter, cells were separated by centrifugation through oil and the cell-associated radioactivity determined by scintillation counting. Nonspecific association of 2-deoxy-D-glucose was determined by performing parallel incubations in the presence of 10 μmol/l cytochalasin B, and this value was subtracted from the total cell-associated radioactivity to obtain a measurement of glucose transport activity at each condition.

Measurement of NEFA production. Isolated adipocytes were incubated in the absence or presence of 200 nmol/l isoprenaline and varying concentrations of insulin for 30 min at 37°C. After this time, 50 μl of the infranatant was removed and NEFA release measured using the Wako NEFA-C kit (Alpha Laboratories) according to the manufacturer's protocol. The same kit was used to measure plasma levels of NEFA.

Preparation of adipocyte membranes and immunoblotting. Adipocytes prepared exactly as described above were washed three times in KRH at 37°C and homogenized using a Dounce homogenizer in 20 mmol/l HEPES, 1 mmol/l EDTA, and 225 mmol/l sucrose, pH 7.4 at 4°C, supplemented with protease inhibitor tablets (Boehringer Mannheim). Total cell membranes were prepared by sedimentation of the homogenate at 100,000g for 60 min at 4°C. SDS-PAGE and immunoblotting were performed as previously described (14).

Anti-CD36 antibody was provided by Narendra N. Tandon (Thrombosis Research Laboratory, Otsuka America Pharmaceutical), and anti-Glut4 was obtained as previously described (14).

RT-PCR and direct sequencing. PCR amplification of cDNA with AmpliTaq Gold (Perkin Elmer) was carried out with Dnase-treated total RNA extracted from epididymal adipose tissue with the DNA/RNA extraction kit (Qiagen). cDNA was prepared with the 1st Strand Synthesis Kit (Boehringer) and purified through a G50 spin column (Pharmacia). PCR products were purified with Centricon 100 columns (Millipore) and directly sequenced with the BigDye Sequencing Kit (PerkinElmer) on an ABI Prism 377 DNA sequencer.

Northern blot. Total RNA, prepared from epididymal fat by acid phenol extraction, was separated by electrophoresis on formaldehyde gels. The RNA was transferred onto Hybond-N (Amersham) and hybridized with ³²P-labeled probes. The probes were labeled using the RediPrime random prime labeling system (Amersham) and purified through a G50 Nick Spin Column (Pharmacia). The filters were prehybridized for 20 min with QuikHyb solution (Stratagene), followed by hybridization to the ³²P-labeled probes for 2 h, at 65°C. A PCR-amplified cDNA probe was amplified from the 5' end of the WKY cDNA, extending from nucleotides 462 to 1052 of the published sequence (15). Filters were washed at high stringency and exposed to Bio-Max film (Kodak).

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