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Hepatic parasympathetic role in insulin resistance on an animal model of hypertension

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Abstract

The hepatic insulin sensitizing substance (HISS) pathway, which includes the hepatic parasympathetic nerves and hepatic nitric oxide (HNO), has been shown to be crucial to the action of insulin on glucose metabolism. Insulin resistance in essential hypertension has been related to parasympathetic dysfunction; thus, we tested the hypothesis that the HISS pathway is impaired in spontaneously hypertensive rats (SHR) when compared with their normotensive controls, Wistar (WIS) and Wistar Kyoto (WKY) rats. A modified euglycemic clamp quantified insulin sensitivity. Differentiation of the HISS-dependent and HISS-independent components of insulin action was achieved by administration of a muscarinic receptor antagonist (atropine, 3 mg/kg) or of a nitric oxide synthase inhibitor (N^g-methyl-arginine, 0.73 mg/kg). Both SHR and WKY had lower postprandial total insulin action when compared with WIS (209.1 ± 13.6 for WKY and 217.8 ± 19.8 for SHR vs 296.1 ± 16.9 mg glucose/kg body weight for WIS, $P < .05$). Furthermore, we observed that this is due to a decrease of the HISS-dependent component of insulin action (154.8 ± 16.4 for WIS vs 87.1 ± 14.5 for WKY and 55.9 ± 15.6 mg glucose/kg body weight for SHR; $P < .05$ and $P < .001$, respectively; data concerning the atropine protocol). Blockade of HISS action by inhibition of hepatic nitric oxide synthase with N^g-methyl-arginine showed similar results to those obtained with atropine, suggesting that they indeed act through the same pathway. In conclusion, our results support our hypothesis that impairment of the HISS pathway is responsible for the development of insulin resistance between WIS and SHR.

1. Introduction

A pathway that includes both the hepatic parasympathetic nerves (HPNs) and hepatic nitric oxide (HNO) has been shown to be crucial to the action of insulin on glucose metabolism. The activation of the HPNs during the immediate postprandial state leads to the release, by the liver, of a putative hormone referred to as hepatic insulin sensitizing substance (HISS), in a mechanism mediated by acetylcholine, hepatic glutathione, and HNO [1,2]. The HPN-HNO pathway determines the ability of insulin to cause the release of HISS. Hepatic insulin sensitizing substance then travels through the bloodstream to enhance skeletal muscle insulin-stimulated glucose uptake, consequently accounting for 50% to 60% of whole-body glucose uptake [2].

Hepatic insulin sensitizing substance action is highest in the immediate postprandial state and decreases with the duration of fasting [3]. By blockade of HISS release through hepatic parasympathetic denervation or pharmacologic manipulation, by muscarinic receptor blockade or nitric oxide (NO) synthase inhibitors, it is possible to distinguish the HISS-dependent and HISS-independent components of insulin action [1,4].

Impairment of the HISS pathway as the primary cause for the development of insulin resistance has been recently reported for several pathologies usually related to the metabolic syndrome [2,5], in which hypertension is included. Furthermore, insulin resistance in essential hypertension seems to be related to parasympathetic [6] and NO [7] dysfunction.

This prompted us to study the spontaneously hypertensive rat (SHR), a genetic pathologic model widely used for

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the study of the relationship between essential hypertension and insulin resistance, because of the similarity of symptoms to the corresponding human pathology [8]. However, contradicting results put in question both the condition of insulin resistance and which of its common normotensive controls should be used—Wistar (WIS) or Wistar Kyoto (WKY). Whereas several studies show a decreased insulin-stimulated glucose disposal in SHR [9,10], others report a similarity [11] or even an increase [12,13] in comparison to WKY or WIS.

Considering this, besides testing our hypothesis that an impairment of the HISS-dependent component of insulin action is involved in the development of hypertension-related insulin resistance, we aimed to clarify this controversy in the present study by quantifying and characterizing both the HISS-dependent and HISS-independent components of insulin action in SHR, WIS, and WKY rats. Using a modified euglycemic clamp [14], we assessed the 2 components at 2 separate steps of the HISS pathway: either by administration of the nonselective muscarinic cholinergic antagonist atropine, which blocked the HPNs, or by intraportal (IPV) administration of the NO synthase inhibitor N^g-methyl-arginine (L-NMMA), to prevent the production of HNO.

2. Methods

2.1. Animals

Male WIS, WKY, and SHR were obtained from Charles River, Spain. After arriving, at 5 weeks of age, they were maintained on a 12-hour light-dark cycle (8:00 AM-8:00 PM), housed 1 per cage under temperature control, with free access to drinking water and standard chow (Panlab A04, Charles River, Barcelona, Spain) until the day before the experiment.

Animals were cared for according to the European Union Directive for Protection of Vertebrates Used for Experimental and other Scientific Ends (86/609/CEE) and the US National Research Council Guide for the Care and Use of Laboratory Animals.

2.2. Presurgical protocol

At 9 weeks of age, rats (250-300 g) were fasted overnight and allowed access to food for 1 hour (8:00-9:00 AM). They were anesthetized with an intraperitoneal injection of sodium pentobarbital (65 mg/kg) and were placed on a heating pad (Homeothermic Blanket Control Unit 50-7061, Harvard Apparatus, Holliston, MA) to maintain body temperature at 37.5°C ± 0.5°C, monitored with a rectal probe thermometer.

2.3. Surgical protocol

The trachea was cannulated (polyethylene tubing, PE 240, Becton Dickinson, Franklin Lakes, NJ) to allow spontaneous respiration. The stomach was checked to confirm that the animals had indeed eaten. The femoral artery and the jugular vein were cannulated (polyethylene tubing, PE 50, Becton Dickinson) to establish an external arterial-venous shunt, primed with a saline-heparin solution (200 IU/mL). This setup also enabled the continuous monitoring of mean blood pressures (Powerlab 8/s, ADInstruments, Colorado Springs, CO; recorded by Maclab software, Colorado Springs, CO). Animals of each strain were randomly divided in 2 groups. A catheter was also inserted on the portal vein (Abbocath 24G Optiva IV 19 mm, Johnson & Johnson, Barcarena, Portugal), connected to a syringe by PE50 tubing, in the animals to be subjected to HNO synthase inhibition. Anesthesia was maintained throughout the experiment by continuous infusion of sodium pentobarbital solution (1.0 mg/mL saline given at 1.0 mL/100 g body weight). All drug and saline solutions were administered either intravenously (IV) through this shunt or IPV through the portal vein catheter.

The rats were allowed to stabilize after surgery for at least 30 minutes before any tests were carried out. After that time, arterial blood samples (25 μL) were taken from the arterial branch of the shunt every 5 minutes, and the glucose concentration was immediately determined by the oxidase method with a glucose analyzer (1500 Sidekick, Yellow Springs Instruments, Yellow Springs, OH) until 3 successive stable glucose concentrations were obtained. The mean of these 3 values is referred to as the basal glucose level.

2.4. Rapid insulin sensitivity test

A modified euglycemic clamp was used, as previously described [5,14]. Briefly, the minute 0 was set at the start of a 5-minute IV insulin perfusion (Perfusor fm, B Braun, Melsungen, Germany). At minute 1, the arterial blood glucose concentration was measured and a glucose infusion (D-glucose/saline, 100 mg/mL, IV) was started at a rate of 5 mg kg^{-1} min^{-1} to avoid hypoglycemia. From then on, arterial blood glucose concentrations were measured at 2-minute intervals, and the rate of the infusion was adjusted whenever necessary to maintain the glycemia as near as possible to the basal glucose level. When no further glucose was required, the rapid insulin sensitivity test (RIST) was concluded. The amount of glucose infused quantifies insulin sensitivity and is referred to as the RIST index (mg glucose/kg body weight).

2.5. Experimental protocol for HPN blockade

After the control RIST was performed, atropine (3 mg/kg) was infused IV over 5 minutes. Glucose levels were allowed to stabilize for at least 30 minutes, after which another RIST was performed.

2.6. Experimental protocol for HNO inhibition

After the control RIST was performed, L-NMMA (0.73 mg/kg) was infused IPV. Like in the previous protocol, the second RIST was only started when a stable basal glycemia was achieved.

2.7. Calculations

The RIST index obtained after atropine or L-NMMA administration represents the HISS-independent component

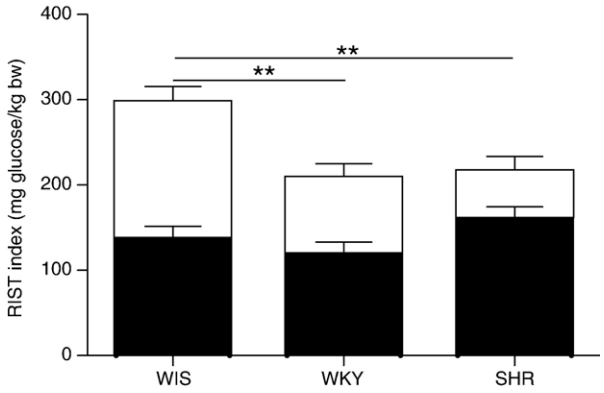

Fig. 1. Rapid insulin tolerance test indexes for the HPN-independent (filled) and HPN-dependent (blank) components of total insulin action. The sum of both components represents the control RIST index for each strain. Insulin sensitivity was significantly higher for WIS than for WKY (**P < .01) or SHR (**P < .01). The HPN-dependent component accounted for these differences (P < .05 for WKY and P < .001 for SHR, not shown on the graph).

of insulin action. By subtracting the RIST index obtained after pharmacologic intervention from the control RIST, quantification of the HISS-dependent component of insulin action was achieved [14].

To obtain the curves representing the time course of each RIST we plotted the mean values of glucose perfusion rate, represented at 0.1-minute intervals. The HISS-dependent component action curve was obtained by subtracting the posttreatment curve values from the corresponding control curve [14].

2.8. Data analysis

Data were expressed as mean ± SE and were analyzed with unpaired Student t test or 1-way analysis of variance followed by Tukey test. Differences were accepted as statistically significant at P < .05 on all analyses.

2.9. Drugs

Atropine, L-NMMA, and D-glucose were purchased from Sigma-Aldrich Chemical, Madrid, Spain. Sodium pentobarbital (Eutasil) was obtained from Ceva, Algés, Portugal. Heparin was purchased from B Braun Medical, Barcarena, Portugal. Human insulin (Humulin) was obtained from Lilly, Algés, Portugal. All chemicals were prepared in saline (B Braun). All perfusions were made with the help of Perfusor pumps (B Braun).

3. Results

3.1. Blood pressure

The SHR had higher systolic blood pressure than WIS or WKY (159.2 ± 3.7 mm Hg for SHR [n = 29] vs 118.1 ± 3.7 mm Hg for WIS [n = 18] and 106.7 ± 2.5 mm Hg for WKY [n = 20], P < .001, pooled control data from both protocols), confirming the hypertensive condition in those animals. These values were not significantly altered with the

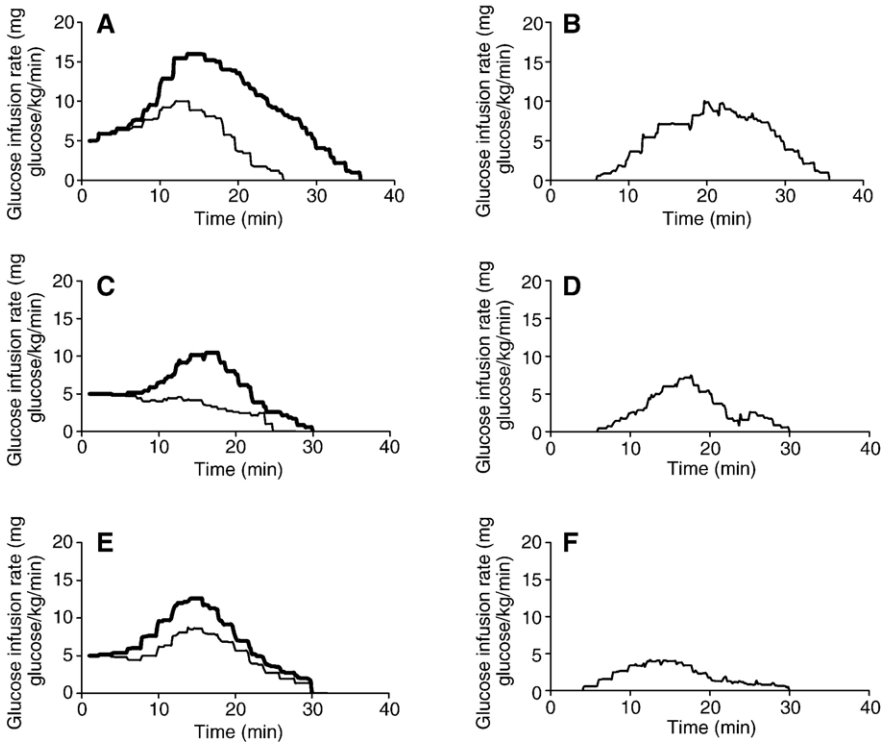

Fig. 2. A, C, and E, Dynamic profiles of the control (bold line) and post-atropine (simple line) RIST of WIS, WKY, and SHR, respectively. B, D, and F, Dynamic profile of the HPN-dependent component of insulin action—obtained by subtracting the post-atropine curve from the control curve—of WIS, WKY, and SHR, respectively.

administration of either IV atropine or IPV L-NMMA at the given doses.

3.2. Hepatic parasympathetic nerve blockade by atropine

The 2 normotensive control strains for SHR showed very different results. Indeed, whereas SHR showed to be insulin-resistant when compared with WIS (296.1 ± 16.9 mg glucose/kg body weight for WIS [n = 12] vs 217.8 ± 19.8 for SHR [n = 13], $P < .01$), WKY showed similar total insulin action to SHR (209.1 ± 13.6 for WKY [n = 12]), thus also being insulin-resistant when compared with WIS ($P < .01$).

The RIST indexes obtained after atropine administration—representing the quantification of the HPN-independent component of insulin action—did not show any significant difference between the 3 strains (141.3 ± 13.6 for WIS, 121.8 ± 12.8 for WKY, and 161.8 ± 19.7 mg glucose/kg body weight for SHR) (Fig. 1).

The SHR presented a lower HPN-dependent component of insulin action than WIS (55.9 ± 16.0 for SHR vs 154.8 ± 16.4 mg glucose/kg body weight for WIS, $P < .001$), and with WKY showing an intermediate value, also statistically different from WIS (87.1 ± 14.6 mg glucose/kg body weight, $P < .05$).

The characteristics of the dynamic profiles of the RISTs obtained before (control RIST) and after atropine administration (HPN-independent component) as well as the HPN-dependent component (Fig. 2) are described in Table 1.

3.3. Hepatic NO inhibition by L-NMMA

Again, both WKY and SHR showed lower total insulin sensitivity indexes than WIS (225.3 ± 23.8 for WKY [n = 8] and 238.2 ± 10.6 for SHR [n = 16] vs 298.9 ± 8.6 mg glucose/kg body weight for WIS [n = 6], $P < .05$).

The HNO-independent component, quantified by the RIST after L-NMMA IPV administration, showed a significant increase in the SHR when compared with the WKY (151.2 ± 6.8 for WIS; 123.3 ± 19.1 for WKY vs 170.0 ± 10.9 mg glucose/kg body weight for SHR, $P < .05$) (Fig. 3).

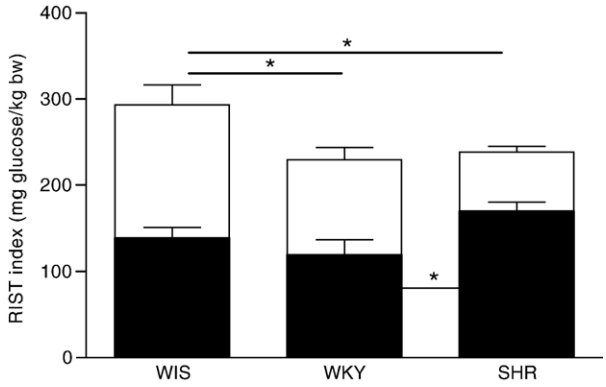

Fig. 3. Rapid insulin sensitivity test indexes for the HNO-independent (filled) and HNO-dependent (blank) components of insulin action. The sum of both components represents the control RIST index for each strain. As before, total insulin sensitivity was significantly higher for WIS than for WKY or the SHR (*$P < .05$). The HPN-dependent component accounted for these differences ($P < .05$ for WKY and $P < .001$ for SHR, not shown on the graph). Furthermore, the HNO-independent component is augmented in SHR in relation to WKY (*$P < .05$).

As in the previous protocol, the HNO-dependent component was lower in SHR than in WIS (68.2 ± 7.1 mg for SHR vs 147.2 ± 13.6 glucose/kg body weight for WIS, $P < .001$), and with WKY showing an intermediate value, also statistically different from WIS (102.0 ± 13.5 glucose/kg body weight, $P < .05$).

The data for the dynamic profiles for this protocol, similar to the ones obtained in the previous protocol, are not shown.

4. Discussion

Our study aimed to characterize the HISS-dependent and HISS-independent components of insulin-stimulated

Table 1
Dynamic profile characteristics for the control RIST (total insulin sensitivity), post-atropine RIST (HPN-independent component), and the HPN-dependent component

		WIS	WKY	SHR
Total insulin sensitivity	Peak (mg glucose kg^{-1} min^{-1})	16.2 ± 0.7*	11.7 ± 1.7*	13.3 ± 1.2
	Peak time (min)	11.9 ± 0.5	13.4 ± 1.6	12.7 ± 0.6
	Offset (min)	34.9 ± 1.3	29.1 ± 1.7	30.4 ± 1.7
	Duration (min)	34.9 ± 1.3	29.1 ± 1.7	30.4 ± 1.7
HPN-independent component	Peak (mg glucose kg^{-1} min^{-1})	11.3 ± 1.3*	6.3 ± 0.7*	9.6 ± 1.1
	Peak time (min)	10.7 ± 0.8	12.5 ± 2.7	12.1 ± 0.9
	Offset (min)	23.4 ± 2.0	22.1 ± 2.8	27.3 ± 1.9
	Duration (min)	23.4 ± 2.0	22.1 ± 2.8	27.3 ± 1.9
HPN-dependent component	Onset (min)	7.7 ± 1.2	7.1 ± 0.6	6.4 ± 1.0
	Peak (mg glucose kg^{-1} min^{-1})	10.9 ± 1.1†	8.4 ± 1.2	6.5 ± 1.0†
	Peak time (min)	16.2 ± 1.3†	15.5 ± 1.0	12.1 ± 1.1†
	Offset (min)	34.4 ± 1.4†,‡	25.5 ± 1.5‡	26.3 ± 1.8†
	Duration (min)	26.7 ± 1.7*,†	18.4 ± 1.8*	19.9 ± 2.0†

* $P < .05$ between WIS and WKY.
† $P < .05$ between WIS and SHR.
‡ $P < .01$ between WIS and WKY.

glucose uptake in an animal model of essential hypertension, the SHR, and its normotensive controls, WIS and WKY.

We observed that the insulin resistance found when comparing SHR and WIS is solely due to a dysfunction of the HISS-dependent component. However, the WKY has shown similar insulin action to SHR, thus either arguing for a secondary role of hypertension on insulin resistance development or to a specific defect on WKY that independently also impairs the HISS-dependent component.

4.1. Methodological considerations

We used the modified euglycemic clamp, RIST [14], to quantify whole-body insulin sensitivity. This choice was made on the basis that the RIST avoids the counterregulatory effects of hypoglycemia, it is reproducible up to 4 consecutive tests on the same animal, and it is comparable with the insulin tolerance test [15]. Furthermore, the current "gold standard" test to evaluate insulin sensitivity, the hyperinsulinemic euglycemic clamp, has been shown to be less sensitive in detecting the HISS-dependent component than the RIST [15]. This is consistent with the observation by others [16] that the hyperinsulinemic euglycemic clamp promotes vagal impairment, thus leading to the blockade of the HISS-dependent component of insulin action.

Although our experiments were performed when the animals were under anesthesia, studies with the RIST have shown no difference in glucose uptake between anesthetized and conscious rats [17]. A possible explanation is that maintaining euthermia, as we did with the help of a heating pad and a rectal probe, prevents the anesthesia-induced alterations in glucose metabolism [18].

Another major factor that influences the HISS-dependent component of insulin action is the prandial state. Hepatic insulin sensitizing substance–dependent insulin sensitivity is maximized in the immediate postprandial state and decreases gradually with fasting [3,17], whereas the HISS-independent component remains constant [3]. The decrease of insulin sensitivity obtained by fasting is similar in magnitude to the effect of atropine and is reversed by refeeding [17]. This led us to submit the animals to an overnight fast, followed by 1 hour of free access to food to assure that they were studied in the immediate postprandial state.

The administration of the cholinergic antagonist (atropine) or the NO synthase inhibitor (L-NMMA) were also chosen, at the given doses, because they do not alter the hepatic glucose output inhibition by insulin and do not alter glycemias or insulinemias, as previously reported [4,19]. The insulinemias of the 3 strains have been described as being similar [13,20,21].

4.2. Insulin resistance and hypertension

According to our findings, SHR and WKY show similar total insulin sensitivity as measured by the control RIST index. Furthermore, both strains show decreased total insulin sensitivity when compared with WIS rats. This is due to a decrease of the HISS-dependent component of insulin action. Surprisingly, SHR show the highest values of HISS-independent component, possibly hinting to a compensatory mechanism.

The results obtained with atropine or L-NMMA are similar both in magnitude and in the dynamic profiles, which indicate that we are indeed affecting the same pathway, as previously described [2].

Intravenous administration of the muscarinic cholinergic antagonist atropine has been reported to induce insulin resistance in a magnitude similar to specific hepatic parasympathetic denervation [4] or fasting [3]. This is consistent with the observation that food intake enhances parasympathetic tone [22]; therefore, special care has to be taken when comparing the available literature with our results. Almost all reported studies were performed in fasted animals; thus, they are strictly comparable with our HISS-independent component data. In this respect, the quantification of the HISS-independent component, given by the RIST index obtained after atropine or L-NMMA, that is, the direct metabolic effect of insulin on its target tissues, showed a tendency to be augmented in SHR when compared with WKY. This is consistent with results obtained by other authors with animals either in the fasted state or where the prandial state was not monitored [11,12,20]. A possible explanation for the greater HISS-independent insulin sensitivity in SHR is an excess insulin-stimulated glucose disposal accounted for by the nonoxidative glucose metabolism pathway, representing an increase of glycogen accumulation in the skeletal muscle [11,20], with increased activity of skeletal muscle glycogen synthase [20].

The HISS-dependent component, obtained by subtracting the RIST index after inhibition of the HISS pathway from the control RIST index, showed a decrease from WIS to WKY, and even more dramatically to SHR. That is consistent with the results of other studies where insulin sensitivity was measured before and after feeding [21,23]. There, skeletal muscle glycogen content, identical between WKY and SHR in the fasted state, increased from fasting to refeeding in WKY, whereas in SHR it remained similar to fasting levels [21]. In addition, feeding increased muscle glycogen content much more in WIS than in SHR [23]. However, in both studies, liver glycogen accumulation increased similarly in both strains and liver glycogen total content was similar [21,23], which indicates that the difference in postprandial insulin sensitivity between the strains is specific to the skeletal muscle. This supports our concept that, in the postprandial state, the skeletal muscle is further sensitized to glucose uptake and glycogen synthesis by a mechanism that is unable to properly function in the SHR.

As Swislocki et al [21], we propose the existence of a circulating factor that increases skeletal muscle insulin-stimulated glucose uptake and glycogen accumulation.

Furthermore, this factor, suggested by us as coincident with the HISS mechanism, is dependent on HPNs and on HNO.

Insulin resistance in essential hypertension seems to be related to parasympathetic dysfunction [6]. It has been also proposed that hypertension is rather related to an increased sympathetic activity resulting in sympathetic/parasympathetic imbalance [24], but, even if that is the case, it seems to be preceded by an impairment of the parasympathetic nervous system [25].

Considering that WKY already show some decrease of HISS-dependent component, there seems to be a common genetic factor between WKY and SHR that, at the administered insulin dose, impairs HISS action in both strains. In fact, a common genetic factor in SHR and WKY has already been proposed by others. Interestingly, it has been related to diminished sensitivity to vagal activity [26], which argues in favor of the secondary role of hypertension in the development of insulin resistance in this animal model. However, the lack of definition in genetics [27] between SHR, WKY, and WIS, from which both the previous 2 strains were derived, further disturbs the analysis of this animal model.

Because hypertensive humans show marked insulin resistance when compared with normotensive subjects [28], the similar total insulin sensitivity of WKY and SHR, as given by the control RIST indexes, suggests the use of the WIS rat as the appropriate normotensive control for SHR in insulin sensitivity studies.

We can further consider that SHR show other metabolic problems that may contribute to the impairment of HISS action, such as a defective NO synthesis/action. Several authors reported higher levels of plasma NO in SHR (20% higher than in WKY), but that this NO is ineffective [7,29].

Others [30] link nitric oxide inhibition with a decrease in insulin sensitivity by way of a decrease of blood flow and nutrient availability to the skeletal muscle cells. Although we have seen no alteration in blood pressure in any of the strains, after the administration of the given dose of L-NMMA, a case could be presented based on the hypothesis of changes in the microvasculature feeding the myocytes. However, the fact that we obtained the same results with atropine as with L-NMMA indicates that hemodynamic changes were not responsible for altering glucose uptake. Furthermore, in healthy animals, the administration of the nitric oxide donor SIN-1 was able to revert the insulin resistance produced with L-NMMA, but only when it was given IPV and not IV [1], despite having a clear vasodilator effect in both cases, clearly showing that the organ that is in control of insulin sensitivity dependent on HISS action is the liver.

The aim of this study was to characterize the HISS-dependent and HISS-independent components of whole-body insulin action in an animal model of hypertension. The present study leads us to the conclusion that the development of hypertension may not cause, by itself, impairment of this component. In contrast, the HISS-dependent component, herein assessed in this animal model of hypertension for the first time, shows a decrease in both WKY and SHR that may be related to the genetic makeup of both strains, which worsens with the presence of hypertension and is solely responsible for the insulin resistance observed.

Furthermore, we have observed that the SHR, when compared with WKY, showed a tendency to compensate for the impairment of the HISS-dependent component by an increase in the HISS-independent component. Alterations of this ability of the SHR can be a possible contributor to the debate found in the literature concerning the observation or not of insulin resistance, and can be of scientific relevance to the understanding of how this strain is able to compensate for the insulin resistance that is HISS-dependent, a feature not yet seen in any other animal model of HISS-related insulin resistance [2,5].

In summary, our data show that, albeit hypertension seems to have a secondary role to the impairment of insulin sensitivity, the insulin resistance observed in the SHR animal model is strictly due to an impairment of the HISS-dependent component.

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