Loss of Postprandial Insulin Sensitization During Aging

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With aging, there is a decrease in parasympathetic nervous activity. Since the hepatic parasympathetic nerves (HPNs) are essential to the disposal of nutrients, through the hepatic insulin sensitizing substance (HISS), we tested the hypothesis that aging leads to a lowering of post-prandial glucose disposal by a decrease of the HISS-dependent component of insulin action. Insulin sensitivity was quantified in fed or fasted, male and female Wistar rats (from 6 to 52 weeks), using a euglycemic clamp. The HISS-dependent component was quantified by administration of the muscarinic antagonist atropine. Total insulin action decreased gradually up to 52 weeks of age: The HISS-independent component of insulin action decreased until 9 weeks of age and remained unchanged thereafter; the HISS-dependent component decreased from 9 weeks of age throughout aging. The continuous decrease of HISS action, uncovered by blocking the HPN, is the key phenomenon for the gradual decrease of insulin sensitivity with aging.

Key Words: Insulin resistance—Hepatic parasympathetic nerves—Hepatic insulin sensitizing substance—Type 2 diabetes mellitus.

The hepatic parasympathetic nerves (HPNs) play a decisive role in the increment of insulin sensitivity, which allows the body to handle the nutrient load of a meal, both in rats (1,2) and in humans (3). Indeed, in the immediate postprandial state, the activation of the HPNs leads to a near doubling of insulin-stimulated whole-body glucose uptake, through a mechanism associated with the action of the putative hepatic insulin-sensitizing substance (HISS), which is released from the liver to act on skeletal muscle (4). This activation is gradually lost with subsequent fasting time (1).

The HISS hypothesis arose from several studies that indicate the existence of a blood-borne factor, released from the liver, that determines the optimal regulation of glucose uptake in the skeletal muscle in the immediate postprandial state (1,5,6). Furthermore, it was observed that the integrity of the HPNs is critical in this process (7). Thus, after ingestion of a meal, concomitantly with the release of insulin by the pancreas, the HPNs release acetylcholine that reacts with M1 cholinergic muscarinic receptors in the liver (8). This leads to the production of hepatic nitric oxide, which is a mediator, in conjunction with hepatic glutathione, of the release of HISS (9–11) into the bloodstream. HISS subsequently acts to increase insulin sensitivity selectively on the skeletal muscle.

In the present study, we were able to evaluate the HISS mechanism through the administration of the muscarinic antagonist atropine, which has been shown to produce a similar degree of decrease of the HISS-dependent insulin sensitivity as the selective surgical ablation of the anterior hepatic nerve plexus (12), thus enabling us to separate the two components: HISS-dependent insulin action, which is eliminated by blockade of the HPN signal, and another, independent of the HPNs, representing the direct action of insulin on target tissues.

Dysfunction of the HISS mechanism has been related to the development of insulin resistance in pathologies derived from both environmental (13) and genetic factors (14,15), ultimately contributing to the rise of type 2 diabetes (4). Likewise, it has been proposed that many of the pathologies that develop with aging spring from the gradual loss of parasympathetic function (16).

Glucose intolerance develops with age mainly due to decreased skeletal muscle insulin sensitivity and responsiveness in humans (17) and in several rat strains (18–22). Moreover, gender-related differences in the way insulin sensitivity changes with age have been reported (23).

In this study, the Wistar rat strain was chosen since it has been considered the best suited for aging-associated studies, having less confounding factors (24) and with alterations in body composition similar to humans (25).

The objectives of this study were to evaluate if there is an impairment of the meal-induced increment of insulin sensitivity with age in Wistar rats, if that development of insulin resistance is due to an impairment of the HISS pathway and if it occurs similarly in male and female animals.

Methods

Animals

Male and female Wistar rats, obtained from Charles River (Barcelona, Spain), were maintained on a 12-hour light–dark cycle (8:00 AM–8:00 PM), under temperature control, with free access to standard chow (Panlab A04, Charles
POSTPRANDIAL INSULIN ACTION AND AGING

River, Spain) and water until the day before the experiment (further feeding protocol below). Animals were used and cared for according to the European Union Directive for Protection of Vertebrates Used for Experimental and other Scientific Ends (86/609/CEE) and the Guidelines of the National Research Council. The recorded mortality rate for Wistar rats from Charles River is, by 52 weeks of age, 7.2% for males and 0.0% for females. In our animals, we have seen no mortality until 52 weeks of age in either males or females.

Male animals were randomly divided into four groups, each group tested at a different age. Respectively, at 6, 9, 16, or 52 weeks of age, these male Wistar rats were fasted overnight and allowed access to food for 1 hour (8:00 AM–9:00 AM). For gender comparison, this protocol was also done with female Wistar rats at the ages of 9 and 52 weeks. Additionally, for prandial status comparison, two other groups of male Wistar rats were tested still in the fasted state, at 9 and 52 weeks of age. All rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (65 mg/kg) and were placed on a heating pad (Harvard Apparatus, Holliston, MA, U.S.A.) to maintain body temperature at 37.0° ± 0.5°C, monitored with a rectal probe thermometer.

Surgical Protocol

The trachea was cannulated (polyethylene tubing, PE 240, Becton Dickinson, Franklin Lakes, NJ, U.S.A.) to allow spontaneous respiration. The femoral artery and the jugular vein were cannulated (polyethylene tubing, PE 50, Becton Dickinson) to insert an external arteriovenous shunt, primed with a saline-heparin solution (200 IU/ml). Anesthesia was maintained by continuous infusion of sodium pentobarbital solution (10 mg/kg/h) through the venous side of the shunt. All other infusions, prior and during insulin sensitivity testing, were also administered intravenously (IV) through this shunt.

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 using a glucose analyzer (1500 Sidekick, Yellow Springs Instruments, Yellow Springs, OH, U.S.A.) until three successive stable glucose concentrations were obtained. The mean of these three values is referred to as the baseline glucose level.

Rapid Insulin Sensitivity Test (RIST)

The minute 0 was set at the start of a 5-minute insulin bolus (50 mU/kg, 0.1 ml/min, IV) with an infusion pump (Perfusor fm, B. Braun Medical, Barcarena, Portugal). 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/min to avoid hypoglycemia. Arterial blood glucose concentrations were measured at 2-minute intervals, and the rate of the glucose infusion rate was adjusted to maintain euglycemia with the baseline glucose level. When no further glucose was required, the RIST was concluded. The amount of glucose infused quantifies insulin sensitivity and is referred to as the RIST index (mg glucose/kg bw).

Experimental Protocol

After the control RIST was performed, atropine (3 mg/kg, IV), a cholinergic muscarinic antagonist, was infused into the arteriovenous shunt over 5 minutes. The animal was allowed to stabilize for at least 30 minutes, after which a new glycemic baseline was determined and a second RIST performed.

Data Analysis

The RIST index obtained after atropine administration represents the HISS-independent component of insulin action. By subtracting the postatropine RIST index from the control RIST, one obtains the HISS-dependent component of insulin action.

Data were expressed as mean ± standard error (SE) and were analyzed by one-way analysis of variance (ANOVA) (age comparison), followed by the Tukey multiple comparison test or two-way ANOVA (age/gender and age/prandial state comparisons), followed by the Bonferroni posttest. Differences were accepted as statistically significant at p < .05.

Drugs

Atropine and D-Glucose were purchased from Sigma-Aldrich Chemical Co (Sintra, Portugal). The human insulin (Humulin regular) was obtained from Lilly Farma (Algés, Portugal). All chemicals were dissolved in saline (B. Braun).

RESULTS

Total Insulin Sensitivity in Fasted Versus Fed Male Wistar Rats at 9 and 52 Weeks of Age

At 9 weeks of age, we observed a higher insulin sensitization after a meal than in the fasting state (fasted: 101.6 ± 7.3, n = 8, vs fed: 260.0 ± 21.1 mg glucose/kg bw, n = 6, p < .001), while at 52 weeks of age this meal effect had been lost, as the fasting and postprandial total insulin responses were similar between them (fasted: 107.8 ± 10.3, n = 7, vs fed: 118.0 ± 19.1 mg glucose/kg bw, n = 9), and also similar to the fasting response at 9 weeks of age (Figure 1).

HISS-Dependent and HISS-Independent Components of Insulin Sensitivity in Fed Male Wistar Rats at 6, 9, 16, and 52 Weeks of Age

Total insulin sensitivity, quantified by the control RIST index, decreased progressively with age (351.8 ± 27.3 mg glucose/kg bw at 6 weeks, n = 6, 297.4 ± 18.5 mg glucose/kg bw at 9 weeks, n = 11, 221.7 ± 24.3 mg glucose/kg bw at 16 weeks, n = 6, and 135.6 ± 12.8 mg glucose/kg bw at 52 weeks, n = 13; p < .01 between 6 and 16 weeks of age, p < .05 between 16 and 52 weeks of age) (Figure 2).

The two components that constitute total insulin action had different contributions to the observed decrease of total insulin sensitivity (Figure 2). While the HISS-independent component (the postatropine RIST) decreased from 6 to...
9 weeks (201.6 ± 21.6 to 139.6 ± 14.8 mg glucose/kg bw, p < .05), and remained constant thereafter (127.1 ± 17.2 at 16 weeks and 111.0 ± 9.4 mg glucose/kg bw at 52 weeks), the HISS-dependent component remained constant from 6 to 9 weeks of age (from 150.2 ± 19.2 to 157.8 ± 17.7 mg glucose/kg bw), it was decreased at 16 weeks (94.6 ± 15.3 mg glucose/kg bw, p < .05), and further by 52 weeks (24.8 ± 6.8 mg glucose/kg bw, p < 0.05).

The highest rate of body weight gain occurred between 9 and 16 weeks of age. The body weight increased from 234.0 ± 8.9 g at 6 weeks, to 314.5 ± 10.8 g at 9 weeks, and to 566.0 ± 12.9 g at 16 weeks. It further increased to 826.5 ± 25.4 g at 52 weeks.

Basal postprandial glycemia, measured as the baseline for the control RIST, was not altered with age (137.3 ± 12.2 at 6 weeks, 125.1 ± 7.7 at 9 weeks, 132.5 ± 3.3 at 16 weeks and 113.7 ± 5.2 mg/dl at 52 weeks). Administration of atropine also did not alter the baseline glycemia (data not shown).

**Gender Comparison of Insulin Sensitivity in Fed Wistar Rats at 9 and 52 Weeks of Age**

Total insulin action showed a similar decrease in male and female fed Wistar rats, from 9 weeks (male: 299.5 ± 20.8, n = 8, and female: 265.7 ± 29.8 mg glucose/kg bw, n = 7) to 52 weeks of age (male: 166.4 ± 13.1, n = 12, and female: 175.5 ± 20.1 mg glucose/kg bw, n = 12). After atropine administration, the HISS-independent component was similar for all age and gender groups (male: from 132.1 ± 19.5 at 9 weeks to 133.8 ± 9.8 mg glucose/kg bw at 52 weeks of age; female: from 127.2 ± 10.5 at 9 weeks to 129.7 ± 12.3 mg glucose/kg bw at 52 weeks of age), with the decrease in total insulin sensitivity with age being solely due, in both genders, to a decrease of the HISS-dependent component of insulin action (male: from 167.3 ± 21.0 at 9 weeks to 32.7 ± 5.7 mg glucose/kg bw at 52 weeks of age, p < .001; female: from 138.6 ± 29.0 at 9 weeks to 45.8 ± 13.0 mg glucose/kg bw at 52 weeks of age, p < .001) (Figure 3).

**DISCUSSION**

Our results suggest that both male and female Wistar rats lose the capability of incrementing insulin sensitivity after a meal by 52 weeks of age. Furthermore, we observed that the decrease of postprandial total insulin action is gradual with aging, with the HISS-independent component of insulin action decreasing until 9 weeks of age and remaining unchanged thereafter, and the HISS-dependent component decreasing radically from 9 weeks to 52 weeks of age.

This evaluation was done by blocking the effect of the HPNs, an essential step of the HISS pathway. The RIST, a euglycemic clamp, was used since it enables the quantification of insulin sensitivity both in the fasted and fed state, as well as before and after HISS pathway blockade.

We propose that a continuous decrease of HISS action, as assessed here through the HPN-dependent component of postprandial insulin action, is the key phenomenon responsible for the gradual decrease of insulin sensitivity with aging.

**Postprandial Insulin Resistance With Aging and Gender**

At 52 weeks of age, the male Wistar rats showed a total loss of the meal-induced increment of insulin sensitization seen at 9 weeks of age. Furthermore, we observed that this loss is related solely to a decrease of the HISS-dependent component of total insulin action. The HISS-independent component remained similar between 9 and 52 weeks of age. Similar results were observed in fed female Wistar rats.

It has long been described that insulin resistance develops with age during maturation, with a slight increase or no increase afterwards (17–22). In apparent contrast, we observed that total postprandial insulin sensitivity decreased gradually and steadily from 6 to 52 weeks of age, due to a decrease of the HISS-independent component only from 6 to 9 weeks of age, and, thereafter, from 9 up to 52 weeks of age, solely due to a decrease of the HISS-dependent component.

Considering that the previous studies found in the literature relate to animals fasted for 4 to 14 hours (18–22), the differences between those studies and our present results are explained by a partial or even total biological shutdown of the HISS-dependent component with fasting. Indeed, activation of the parasympathetic nervous system occurs at the onset and during food ingestion (26). Accordingly, the HISS-dependent component of insulin action is highly modulated by the prandial state, and increased fasting periods have been shown to produce a time-dependent inhibition of the HISS-dependent component, promptly reversed by refeeding (1,2,5). Thus, the previous studies of the effect of age on insulin sensitivity in whole-body glucose uptake can only be compared with our data for the HISS-independent component of insulin action, as quantified by the postatropine RIST index.

In this respect, our observation that the HISS-independent component decreases from 6 to 9 weeks and remains stable thereafter is consistent with other studies in fasted Wistar
rats, which conclude that insulin-stimulated glucose uptake decreases somewhere between 4 and 12 weeks (22) or 8 and 16 weeks (20), with no decrease or only a slight decrease with further age.

This period also coincides with the greatest rate of weight gain, which is indicated as a possible determinant or confounding factor for the decrease of insulin sensitivity (27). Although a difference has not been found when analyzing the data according to weight or fat-free mass (20), previous studies done by our group have found a relation between obesity and insulin resistance (15). However, as much as our data may suggest that body weight changes may be involved

Figure 2. Rapid insulin sensitivity test (RIST) indexes for total insulin action and its two components for male Wistar rats at 6, 9, 16, and 52 weeks of age. Total insulin action decreased gradually with age. The hepatic insulin sensitizing substance (HISS)-independent component decreased from 6 to 9 weeks and remained unchanged thereafter, whereas the HISS-dependent component decreased radically from 9 to 52 weeks of age (*p < .05; **p < .001; ***p < .0001).

Figure 3. Rapid insulin sensitivity test (RIST) indexes for male and female Wistar rats at 9 and 52 weeks of age. The decrease of the hepatic insulin sensitizing substance (HISS)-dependent component that determines the development of insulin resistance with age occurred similarly in both genders. The HISS-independent component was unchanged either with age or gender.
in the early decrease of insulin sensitivity, the HISS-dependent component kept on decreasing in a phase of nearly stabilized weight. The quantification of this component of insulin sensitivity has been shown to remain relatively constant until 9 weeks, and then decrease until 1 year of age, which may thus argue against weight gain as a confounding factor in the loss of the HISS-dependent component with aging.

**Insulin Resistance and the Parasympathetic Nervous System in Aging**

Although the structure of the rat vagus nerve does not seem to deteriorate, not even in old age (28), there is an age-related functional reduction in parasympathetic activity (29,30). We have observed that the effect of this impairment on glucose uptake starts after 9 weeks of age, and, at 1 year of age, has already developed dramatically. The fact that humans also show a decrease of parasympathetic tonus with age (31) leads to the hypothesis that the HISS-component is also important in the gradual glucose intolerance seen in elderly humans (17,32).

The occurrence of autonomic neuropathy has been widely related to the development of insulin resistance. Although type 2 diabetes is usually described as leading to autonomic neuropathy, the observation of mainly parasympathetic autonomic impairment in prediabetics seems to hint at a role of a HISS component impairment in the etiology of diabetes (33,34). Another observation that supports this is that parasympathetic neuropathy precedes both sympathetic and peripheral neuropathies (35). It is also interesting that the autonomic system seems as compromised in nondiabetic elderly persons as in younger diabetic patients (36), leading to the notion that, in prediabetes, there is an acceleration of the degeneration as seen in biological aging.

The HPN impairment may play an important role in other pathological states also related both to insulin resistance and aging. Parasympathetic tonus decrease seems to be involved in such clinical conditions as chronic liver diseases (37), obesity (38), essential hypertension (39), and type 2 diabetes (40), which also have been related to dysfunctions of the HISS pathway (4). This leads tentatively to a connection between the dysfunction of the HPNs and its influence in the HISS mechanism, the gradual increase in insulin resistance observed in humans starting in the third or fourth decade of age (41), and the higher prevalence of impaired glucose tolerance, type 2 diabetes, and hypertension in aged persons (42).

**Future Studies**

Age also may be associated with an anomalous expression of other mediators of the HISS pathway, such as hepatic nitric oxide (NO) or glutathione (11). Indeed, strategies to prevent the age-dependent lowering of glutathione content in the rat liver, also seen as indispensable for HISS release (11), have partially prevented aging-induced insulin resistance (43), while it has been described that the decreased activity of hepatic NO synthase in aging seems to contribute to insulin resistance (44).

The impact of age on postprandial insulin sensitivity may thus be the result of the impairment of the HPNs, further impaired by several steps downstream, connected to the HISS mechanism. This opens a field of future studies in such a multifactor condition as aging, with the aim of preventing or ameliorating the deleterious effects of the cluster of pathologies related to dysfunctions of the parasympathetic nervous system and the HISS pathway.

**Conclusion**

We have shown that there is a similar loss of the postprandial sensitization of insulin sensitivity with age in both genders. Furthermore, meal-induced insulin sensitivity decreased gradually with age in Wistar rats; however, while the HISS-independent component decreased until approximately 9 weeks of age, the HISS-dependent component remained stable until 9 weeks of age and decreased steadily with further aging.

Our present results stressed the importance of the evaluation of insulin sensitivity on the postprandial state, in opposition to the traditional evaluation on the fasting state, as the determination before and after atropine enabled us to uncover changes in both components of total insulin action.

These findings suggest that the impairment of the HISS-dependent insulin pathway is associated with the development of insulin resistance and parasympathetic dysfunction known to occur with aging, and possibly to several related pathologies also known to be associated with parasympathetic dysfunction.

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