While the incidence of diabetes increases with age, a decrease in β-cell function independent of age-related insulin resistance has not been conclusively determined. We studied insulin secretion (by hyperglycemic clamp) in 3-, 9-, and 20-month-old chronically catheterized, awake, Sprague Dawley (SD) rats (n = 78). Insulin action was modulated in a group of old rats by caloric restriction (CR) or by surgical removal of visceral fat (VF–). During the first 2 h of the clamp (11 mmol/l glucose), insulin secretion and insulin resistance (S_Hyper clamp) demonstrated the characteristic hyperbolic relationship. However, after hyperglycemia for an additional 2 h, the ability to maintain insulin secretion, commensurate with the degree of insulin resistance, was decreased in all aging rats (P < 0.05). Increasing plasma glucose levels to 18 mmol/l glucose, after clamp at 11 mmol/l, increased insulin secretion by approximately threefold in young rats, but failed to induce similar magnitude of response in the aging rats (~50%). However, elevation of plasma free fatty acid (FFA) levels by twofold (by intralipid infusion during 11 mmol/l glucose clamp) resulted in a robust, approximate twofold response in both young and old rats. Thus, prolonged stimulation by hyperglycemia unveiled a functional defect in insulin secretion with aging. This age-related defect is independent of insulin action and is specific to glucose and not FFAs. We suggest that prolonged hyperglycemic stimulation can be a tool to identify functional defects in insulin secretion, particularly in the context of the hyperbolic relationship with insulin action, in elderly subjects or those at risk for type 2 diabetes. Diabetes 53:441–446, 2004

Age is a significant risk for development of type 2 diabetes (1). This is evident in the significantly higher prevalence of impaired glucose tolerance test (GTT) and type 2 diabetes in Americans older than 65 years (2). As in the younger age-group, decreased physical activity and increased adiposity impair insulin sensitivity in the elderly. This is initially compensated with an increase in insulin secretion (1); however, the appearance of impaired GTT and type 2 diabetes suggests that adequate insulin secretion cannot be maintained. Thus, relative insulin secretory defects in the presence of insulin resistance may contribute to the increased incidence of age-related glucose intolerance and diabetes.

Many studies have utilized oral GTTs (OGTTs) to assess insulin response in aging; however, as the relative role of insulin resistance, β-cell function, and gastrointestinal and neural factors cannot be deduced from these studies, they do not provide clear evidence that aging per se decreases insulin secretion (3,4). Age-related decrease in insulin response has been demonstrated with intravenous GTT, suggesting impaired β-cell function (5,6). Recently, a clinical study utilizing a mixed meal and an intravenous injection of glucose demonstrated that age-related diminution in insulin secretion contributes to the glucose intolerance in the elderly (7). However, only few studies have utilized the hyperglycemic clamp, the gold standard technique, to assess glucose-mediated insulin secretion, and the results have not been consistent. While some studies have shown an impairment in β-cell function with aging (8), few have shown no differences in the insulin secretion between the young and old subjects (9,10). Thus far, while these studies have shown some age-related impairment in insulin secretion in human subjects, they have failed to account for the great variability in the metabolic profile and the role of insulin resistance on β-cell function in elderly subjects. As a result, a conclusive statement about the decline in β-cell mass or function with aging has yet to be made (3).

To overcome some of these difficulties and to suggest a novel approach to assess functional decline in insulin secretion, we studied the effects of aging on insulin secretion in a rodent model utilizing the hyperglycemic clamp technique. Rodent models are genetically homogeneous and can be subjected to similar controlled environment and levels of activity. Furthermore, anthropometric and metabolic parameters can be manipulated as they age. In addition, Sprague Dawley (SD) rats offer a unique advantage because they do not develop diabetes with aging. We used two additional models to specifically delineate the effect of aging on glucose-stimulated insulin secretion. First, we employed caloric restriction (CR) to allow us to study effects of age without the confounder of age-related obesity and insulin resistance (11,12). Second, we reversed insulin resistance, without limiting nutrients, by surgically removing visceral fat (VF) (13,14). We also continued the clamp for 4 h to study the relationship...
between insulin secretion and insulin action across ages. In addition to glucose, we used intralipid infusions to study the effects of free fatty acids (FFAs) on insulin secretion in young and old rats.

**RESEARCH DESIGN AND METHODS**

**Animals.** Male SD (n = 78) (Charles River Laboratories, Wilmington, MA) were used for this study. Rats were housed in individual restrictive cages and were subjected to a standard light (6:00 a.m. to 6:00 p.m.)–dark (6:00 p.m. to 6:00 a.m.) cycle. Five groups of rats—3-month-old (human equivalent of young adult), 9-month-old (human equivalent of mature adult), and 20-month-old rats (human equivalent of ~70 years); 20-month-old calcific restricted (CR) rats; and 20-month-old rats that underwent surgical removal of VF at 5 months of age (VF−)—were studied (8–11). All rats, except 20-month-old CR rats, were fed ad libitum using regular rat chow that consisted of 64% carbohydrate, 30% protein, and 6% fat with a physiologic fuel value of 3.3 kcal/g chow. The fed ad libitum using regular rat chow that consisted of 64% carbohydrate, 30% protein, and 6% fat with a physiologic fuel value of 3.3 kcal/g chow. The fed ad libitum using regular rat chow that consisted of 64% carbohydrate, 30% protein, and 6% fat with a physiologic fuel value of 3.3 kcal/g chow. The fed ad libitum using regular rat chow that consisted of 64% carbohydrate, 30% protein, and 6% fat with a physiologic fuel value of 3.3 kcal/g chow. The fed ad libitum using regular rat chow that consisted of 64% carbohydrate, 30% protein, and 6% fat with a physiologic fuel value of 3.3 kcal/g chow. The fed ad libitum using regular rat chow that consisted of 64% carbohydrate, 30% protein, and 6% fat with a physiologic fuel value of 3.3 kcal/g chow. The fed ad libitum using regular rat chow that consisted of 64% carbohydrate, 30% protein, and 6% fat with a physiologic fuel value of 3.3 kcal/g chow. The fed ad libitum using regular rat chow that consisted of 64% carbohydrate, 30% protein, and 6% fat with a physiologic fuel value of 3.3 kcal/g chow. The fed ad libitum using regular rat chow that consisted of 64% carbohydrate, 30% protein, and 6% fat with a physiologic fuel value of 3.3 kcal/g chow. The fed ad libitum using regular rat chow that consisted of 64% carbohydrate, 30% protein, and 6% fat with a physiologic fuel value of 3.3 kcal/g chow. The fed ad libitum using regular rat chow that consisted of 64% carbohydrate, 30% protein, and 6% fat with a physiologic fuel value of 3.3 kcal/g chow. The fed ad libitum using regular rat chow that consisted of 64% carbohydrate, 30% protein, and 6% fat with a physiologic fuel value of 3.3 kcal/g chow. The fed ad libitum using regular rat chow that consisted of 64% carbohydrate, 30% protein, and 6% fat with a physiologic fuel value of 3.3 kcal/g chow. The fed ad libitum using regular rat chow that consisted of 64% carbohydrate, 30% protein, and 6% fat with a physiologic fuel value of 3.3 kcal/g chow. The fed ad libitum using regular rat chow that consisted of 64% carbohydrate, 30% protein, and 6% fat with a physiologic fuel value of 3.3 kcal/g chow. The fed ad libitum using regular rat chow that consisted of 64% carbohydrate, 30% protein, and 6% fat with a physiologic fuel value of 3.3 kcal/g chow. The fed ad libitum using regular rat chow that consisted of 64% carbohydrate, 30% protein, and 6% fat with a physiologic fuel value of 3.3 kcal/g chow. The fed ad libitum using regular rat chow that consisted of 64% carbohydrate, 30% protein, and 6% fat with a physiologic fuel value of 3.3 kcal/g chow. The fed ad libitum using regular rat chow that consisted of 64% carbohydrate, 30% protein, and 6% fat with a physiologic fuel value of 3.3 kcal/g chow. The fed ad libitum using regular rat chow that consisted of 64% carbohydrate, 30% protein, and 6% fat with a physiologic fuel value of 3.3 kcal/g chow. The fed ad libitum using regular rat chow that consisted of 64% carbohydrate, 30% protein, and 6% fat with a physiologic fuel value of 3.3 kcal/g chow. The fed ad libitum using regular rat chow that consisted of 64% carbohydrate, 30% protein, and 6% fat with a physiologic fuel value of 3.3 kcal/g c

**TABLE 1**

<table>
<thead>
<tr>
<th></th>
<th>3-month-old</th>
<th>9-month-old</th>
<th>20-month-old</th>
<th>20-month-old VF−</th>
<th>20-month-old CR</th>
</tr>
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<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td>9</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>322 ± 14.3*</td>
<td>583 ± 17.3</td>
<td>558 ± 28</td>
<td>572 ± 22</td>
<td>374 ± 5.2*</td>
</tr>
<tr>
<td>Fat mass (g)</td>
<td>26.6 ± 2.9*</td>
<td>170.1 ± 7.1</td>
<td>179.8 ± 9.8</td>
<td>166.8 ± 23.4</td>
<td>29.8 ± 6.2*</td>
</tr>
<tr>
<td>Total VF (g)</td>
<td>4.9 ± 0.3*</td>
<td>322.5 ± 5.3</td>
<td>30.9 ± 2.3</td>
<td>8.0 ± 1.0‡</td>
<td>5.3 ± 0.6‡</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>1.5 ± 0.3‡</td>
<td>3.1 ± 0.6</td>
<td>3.0 ± 0.5</td>
<td>1.4 ± 0.4‡</td>
<td>1.4 ± 0.2‡</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>8.1 ± 0.2</td>
<td>8.1 ± 0.2</td>
<td>8.0 ± 0.3</td>
<td>7.2 ± 0.3</td>
<td>7.2 ± 0.1</td>
</tr>
<tr>
<td>FFAs (nmol/l)</td>
<td>0.7 ± 0.1*</td>
<td>1.3 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>1.2 ± 0.2</td>
<td>0.7 ± 0.1*</td>
</tr>
</tbody>
</table>

Data are means ± SE. *P < 0.05 vs. 9-month-old, 20-month-old, and 9-month-old and 20-month-old rats.

**RESULTS**

**Preclamp basal metabolic characteristics.** The preclamp basic metabolic characteristics of the rats from the various groups are summarized in Table 1. There was a progressive increase in the weight of the rat and fat mass with age (P < 0.0001), which was prevented by CR. CR led to a decrease in total fat mass and VF (P < 0.0001). Though the total fat mass in VF− rats was similar to age-matched controls, VF was only one-third of the fed rats (P < 0.0001). While preclamp basal glucose level was similar across all ages, insulin levels were higher in the 9-month-old and 20-month-old compared with young 3-month-old, 20-month-old CR, or 20-month-old VF− rats (P < 0.05).

**Relationship between glucose-stimulated insulin secretion and insulin action during 2-h hyperglycemic clamp.** Glucose was clamped at 11 mmol/l in all the groups. During first 2 h of hyperglycemic clamp, there was a progressive increase in insulin levels in all the groups, with highest insulin levels in 20-month-old rats (Fig. 1A). Insulin levels were lower in the 20-month-old VF− and CR groups compared with age-matched controls. C-peptide levels paralleled the changes in insulin with highest levels in the 20-month-old rats (3,457 ± 195, 3,488 ± 206, 4,708 ± 245, 4,093 ± 354, and 2,634 ± 429 pmol/l in 3-month-old, 9-month-old, 20-month-old VF−, and 20-month-old CR rats, respectively, P < 0.05). First-phase insulin response (FPIR), as assessed by the area under the...
curve for insulin (AUC$_I$), was also highest in the 20-month-old rats (11.79 ± 2.1, 16.94 ± 3, 21 ± 2.1, 15.31 ± 2, and 12.79 ± 1.3 in 3-month-old, 9-month-old, 20-month-old, 20-month-old VF−, and 20-month-old CR rats, respectively, $P < 0.05$). FFAs and insulin sensitivity, as assessed by $S_I$ hyper clamp, demonstrate a hyperbolic relationship, with a decrease in insulin sensitivity accompanied by higher FFPIR (Fig. 1B). A hyperbolic relationship can also be demonstrated across all ages between the insulin response during the clamp and insulin sensitivity ($S_{I_{hyper clamp}}$) (Fig. 1C). As expected, the $S_{I_{hyper clamp}}$ was highest in the 3-month-old and lowest in the 20-month-old rats. VF− and CR improved the insulin sensitivity by ~30% and twofold, respectively, compared with the 20-month-old rats ($P < 0.05$). The FFA levels were significantly suppressed with the clamp in all groups ($P < 0.05$) (Table 2).

### Relationship between glucose-stimulated insulin secretion and insulin action during 4-h hyperglycemic clamp

To test if the relationship between insulin resistance and insulin secretion is altered with prolonged stimulation, we subjected the rats to an additional 2 h of hyperglycemic clamp at 11 mmol/l. As previously shown, with continued clamp at 11 mmol/l, insulin secretion continued to increase in all the groups (19,20). After an additional 2 h of hyperglycemic clamp, highest insulin levels were reached in 9-month-old rats, although 20-month-old rats started the clamp with highest insulin levels at 2 h (Fig. 2A and B). Similar trends were seen in the C-peptide levels among the various groups (6,215 ± 617, 6,553 ± 636, 6,710 ± 590, 5,226 ± 660, and 2,964 ± 317 pmol/l in 3-month-old, 9-month-old, 20-month-old, 20-month-old VF−, and 20-month-old CR rats, respectively, $P < 0.05$). The $S_{I_{hyper clamp}}$ was highest in 3-month-old, approximately twofold higher than 9- and 20-month-old rats ($P < 0.05$). Interestingly, the highest $S_{I_{hyper clamp}}$ and lowest insulin levels were seen in the 20-month-old CR group. When insulin levels were plotted against insulin sensitivity, the hyperbolic relationship was not maintained and the old rats “left” the hyperbola (Fig. 2C); the insulin levels were lower compared with the degree of insulin resistance in the 20-month-old ($P = 0.03$), VF− ($P < 0.005$), and CR ($P < 0.0001$) groups (Table 1).

### Stimulated insulin secretion with 18 mmol/l glucose and FFAs

When 3-month-old, 20-month-old, and 20-month-old CR rats were subjected to an 18 mmol/l glucose clamp for 90–180 min, insulin secretion was similar in 3-month-old and 20-month-old rats. However, considering the higher insulin levels achieved during the first 90 min of the clamp by 20-month-old rats, the further increment with the 18 mmol/l clamp was significantly lower (from 13.7 ± 2.0 to 16.11 ± 1.9 in the 20-month-old rats and from 11.6 ± 2.4 to 13.5 ± 2.1 in 20-month-old CR rats compared with 12.6 ± 2.1 to 19.02 ± 2.2 in the 3-month-old rats). The increase in insulin with the 18 mmol/l clamp was much less in old rats, both fed and CR rats (50, 25, and 20% increase in 3-month-old, 20-month-old, and 20-month-old CR rats, respectively, $P < 0.05$). C-peptide levels showed a similar trend: 45, 26, and 28% increase (from 2,398 ± 11 to 3,848 ± 178, 3,252 ± 399 to 5,946 ± 287, and 2,245 ± 237 to 3,023 ± 71 pmol/l, $P < 0.05$) in 3-month-old, 20-month-old, and 20-month-old CR rats, respectively (Fig. 3).

Intralipid infusion in addition to the 11 mmol/l clamp increased FFA levels by 3-fold, 2.8-fold, and 2-fold in 20-month-old, 20-month-old CR, and 3-month-old rats, respectively (0.47 ± 0.19 to 1.21 ± 0.28, $P < 0.001$). Insulin secretion increased significantly in both young and old rats with the addition of intralipid: 12.6 ± 2.1 to 20.7 ± 3.3 in 3-month-old, 13.7 ± 2.0 to 27.2 ± 2.3 in 20-month-old, and 11.6 ± 2.4 to 22.4 ± 2.9 in 20-month-old CR rats (75, 90, and 100% in 3-month-old, 20-month-old, and 20-month-old CR rats, respectively, $P < 0.001$). The changes in C-peptide levels were parallel to changes in insulin (from 2,355 ± 126 to 4,946 ± 375, 3,148 ± 413 to 10,960 ± 1,352, and 2,552 ± 329 to 7,183 ± 937 pmol/l, a 2-fold, 3.5-fold, and 2.8-fold increase in 3-month-old, 20-month-old, and 20-month-old CR rats, respectively, $P < 0.001$). Stimulated insulin secretion with glucose and intralipid infusion was similar in the young, whereas there was a marked difference in the insulin levels between the two stimuli in the old rats ($P < 0.05$).
FIG. 1. Plasma insulin levels during a hyperglycemic clamp (0–120 min). A: Three-month-old (3M; n = 6), 9-month-old (9M; n = 6), 20-month-old (20M; n = 9), 20-month-old VF− (20M VF−; n = 6), and 20-month-old CR rats (20M CR; n = 6) were studied under 11-mmol/l hyperglycemic clamp conditions. The highest insulin levels were demonstrated in the 20-month-old rats (P < 0.05 vs. 3-month-old and 20-month-old CR rats). B: Hyperbolic curve was obtained with 95% CI was obtained by plotting the insulin levels during the first phase against insulin sensitivity in 27 individual rats of the 3-month-old and 9-month-old groups (r = 0.5; p < 0.002). C: Similarly, a hyperbolic curve was obtained by plotting insulin sensitivity against insulin levels during the second hour of the clamp (r = 0.77; P < 0.0001). Mean values of data from 3-month-old, 9-month-old, 20-month-old, 20-month-old VF−, and 20-month-old CR rats were then plotted on the hyperbolic curve (B and C).

FIG. 2. Plasma insulin levels during prolonged hyperglycemic clamp (120–240 min). Plasma glucose was maintained at 11 mmol/l for an additional 2 h of the hyperglycemic clamp (120–240 min) in all the groups. A: Insulin secretion is significantly different in the 9-month-old (*P < 0.05) and 20-month-old CR rats (#P < 0.05) groups compared with 3-month-old, 20-month-old, and 20-month-old VF− rats. B: Changes in insulin levels during the last hour of the clamp over the level at 120 min. The older rats (20-month-old, 20-month-old VF, and 20-month-old CR rats) demonstrate significantly lower insulin when compared with 3-month-old and 9-month-old rats (P < 0.05). C: Hyperbolic curve was obtained initially by plotting the insulin levels during the last hour of the clamp against insulin sensitivity in 27 individual rats of the 3-month-old and 9-month-old groups (r = 0.7 and P < 0.001). Mean values of data from all the groups were then plotted on the curve. The 20-month-old (*P < 0.05), 20-month-old VF− (**) P < 0.005), and 20-month-old CR rats (** P < 0.0001) had significantly lower insulin secretion commensurate to the degree of insulin resistance compared with the 3-month-old and 9-month-old groups. 9M, 9-month-old; 3M, 3-month-old; 20M, 20-month-old; 20M VF−, 20-month-old VF−; 20M CR, 20-month-old CR rats.
DISCUSSION

In this study we show that glucose-mediated insulin secretion is impaired independent of insulin resistance and nutritional intake in the old rats. While insulin action could predict insulin secretion during a short physiologic stimulation, a more prolonged stimulation in aging animals led to a decrease in insulin secretion. This defect seems to be specific for glucose and not to stimuli such as FFAs.

With increasing age, changes in anthropometric characteristics, such as an increase in fat mass and visceral fat, may lead to insulin resistance in both rodents and humans (19,20). Sedentary lifestyle, genetic predisposition, and presence of other diseases contribute further to the diminished insulin sensitivity in humans. The relationship between increasing age and the decline in insulin action has been demonstrated under euglycemic-hyperinsulinemic clamp conditions (21). This decrease in insulin sensitivity is associated with a compensatory increase in insulin secretion (3) under basal and certain stimulated conditions. The recently coined term “glucose allostasis” describes the slightly higher glucose levels, albeit within the normal range, in insulin-resistant states that continue to drive the β-cells to produce higher levels of insulin (22). During the first 2 h of hyperglycemic clamp, insulin secretion and insulin action demonstrated a hyperbolic relationship across all ages—a decrease in insulin sensitivity associated with higher insulin levels.

Although a hyperbolic relationship between insulin action and insulin secretion has been demonstrated in many studies in humans through the use of a variety of techniques (23), a causative role of insulin action in determining insulin secretion has not been established. Prevention of insulin resistance with CR and improvement in insulin sensitivity with removal of VF is associated with lower insulin levels and a marked improvement in insulin sensitivity. However, the model of VF removal is unique as these animals are similar in body weight to the ad libitum–fed animals and only differs significantly in the amount of VF (Table 1). Similarly, the food intake of the VF– rats was comparable to that of ad libitum–fed animals, while the food intake of CR animals was restricted. Through the demonstration of hyperbolic relationship between insulin action and insulin secretion in these two unique models and in ad libitum–fed 3-month-old, 9-month-old, and 20-month-old models, we demonstrate that insulin action, independent of nutrients, age, and fat mass, determines acute insulin secretion.

Prospective studies in Pima Indians have shown that subjects with increasing insulin resistance, when compensated, move on the hyperbolic curve, increasing insulin secretion capacity with time (24). Studies have also demonstrated that subjects who eventually developed type 2 diabetes with time had declines in insulin secretion and moved “off the curve” (24). In this study we demonstrate that in aging animals, a decline in insulin secretion can be demonstrated not in years but in just a few hours of stimulation. Stimulation, either by increasing the duration of the clamp to 4 h or by increasing the glucose stimuli to 18 mmol/l, unmasked the differences between the old and the young rats. With continued clamp for an additional 2 h, although all rats continued to increase the insulin secretion throughout the period of study as described earlier (17,25,26), the magnitude of increase in insulin levels was much lower in the old rats. It is rather striking that a relatively short period of hyperglycemia distinguished the old rats, with old rats demonstrating lower insulin secretion for the degree of insulin resistance compared with 3- and 9-month-old rats. Glucose-stimulated insulin secretion as assessed by an 18-mmol/l clamp is also decreased in old age. Because insulin clearance may be decreased in aging (27,28), the lower insulin levels demonstrated here may underestimate the degree of the defect in insulin secretion. In addition, parallel changes in C-peptide suggest a defect in insulin secretion. Inherent defects in insulin secretion with aging, when superimposed on increased insulin requirements, may contribute toward the higher risk for abnormal glucose tolerance in old age. This is corroborated by a study in elderly humans, using mixed-meal challenge and intravenous glucose infusion, that showed a decrease in both insulin secretion and insulin action with aging, suggesting that the effect on insulin secretion is independent of the effects on insulin action (7).

In light of a functional decrease in insulin secretion with aging, it is rather interesting that islet number, islet size, and secretory granules are actually increased in old SD rats compared with young animals (29,30). Despite the increased size of the islets, the release of insulin in response to glucose is decreased in old age in vitro (31–34). This functional defect is demonstrable in both sexes and across species of rats and is independent of body weight, although the pancreas of old males has higher islet tissue coupled with more impaired secretion (31). An impaired stimulation-secretion coupling has been demonstrated in the β-cells of aging rats in response to glucose and arginine (35).

Interestingly, the defect in insulin secretion in old age...
appears to be specific to glucose. Additional stimulation with FFAs seems to be necessary to elicit a higher insulin response. FFAs are potent insulin secretagogues and augment insulin release only in the presence of glucose (36–38), and this effect may be mediated through an increase in cytoplasmic long-chain acyl CoA (39,40).

In conclusion, we demonstrate that there is a functional impairment of glucose-stimulated insulin secretion with old age. This defect in insulin secretion in aging appears to be specific to glucose. β-cells in old age seem particularly vulnerable and a few hours of hyperglycemia during the clamp appears sufficient to unmask significant defects in glucose-stimulated insulin secretion. Prolonged hyperglycemic stimulation may be a tool in identifying defects in insulin secretion in relation to insulin action in elderly subjects or those at risk for type 2 diabetes.

REFERENCES


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