Contribution to glucose tolerance of insulin-independent vs. insulin-dependent mechanisms in mice

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Pacini, Giovanni, Karl Thomaseth, and Bo Ahren. Contribution to glucose tolerance of insulin-independent vs. insulin-dependent mechanisms in mice. Am J Physiol Endocrinol Metab 281: E693–E703, 2001.—To study the contributions of insulin-dependent vs. insulin-independent mechanisms to intravenous glucose tolerance (KGl), 475 experiments in mice were performed. An intravenous glucose bolus was given either alone or with exogenous insulin or with substances modulating insulin secretion and sensitivity. Seven samples were taken over 50 min. Insulin [suprabasal area under the curve (ΔAUCins)] ranged from 0 to 100 mU·ml⁻¹·50 min. After validation against the euglycemic hyperinsulinemic clamp, the minimal model of net glucose disappearance was exploited to analyze glucose and insulin concentrations to measure the action of glucose per se independent of dynamic insulin (KGl), and the combined effect of insulin sensitivity (SI) and secretion. Sensitivity analysis showed that insulin [through disposition index (DI)] contributed to glucose tolerance by 29 ± 4% in normal conditions. In conditions of elevated hyperinsulinemia, contribution by insulin increased on average to 69%. KGl correlated with DI but was saturated for ΔAUCins above 15 mU·ml⁻¹·50 min. Insulin sensitivity related to ΔAUCins in a hyperbolic manner, whereas SI did not correlate with the insulin peak in the physiological range. Thus glucose tolerance in vivo is largely mediated by mechanisms unrelated to dynamic insulin and saturates with high insulin.

Insulin sensitivity; insulin secretion; glucose tolerance; glucose effectiveness; mathematical modeling; intravenous glucose tolerance test

GLUCOSE DISAPPEARANCE is mainly due to two processes, one mediated by insulin and the other independent of the hormone. In the former, the two main components are insulin secretion and insulin action. Although the importance of insulin must not be neglected, it has been envisioned that insulin-independent mechanisms are also among the major determinants of glucose tolerance (10, 19). These mechanisms include glucose’s own disappearance by mass action and the combined actions of glucose to inhibit hepatic glucose production and accelerate uptake into the liver and peripheral tissues (1, 10).

The minimal-model analysis of glucose and insulin data during an intravenous glucose tolerance test (IVGTT) provides well-established indexes of insulin-dependent [insulin sensitivity (SI)] and insulin-independent [glucose effectiveness, (SG)] actions on glucose tolerance (2, 8, 10). This test also yields a quantitative description of β-cell secretion from peripheral hyperinsulinemia induced by the glucose injection (18). The importance of SG in the understanding of the etiology of glucose intolerance has been widely demonstrated (1, 10, 19), and it has been proposed that SG significantly contributes to glucose tolerance under conditions of reduced insulin action being thus an independent risk factor for type 2 diabetes (10). SG has been defined as the action of glucose per se, without any change in insulin concentration, to normalize glucose concentration by reducing liver glucose production and increasing glucose uptake (2).

Insulin-dependent and -independent net glucose disappearance was obtained in mice by exploiting the minimal model of net glucose disappearance. The model was applied to the seven-sample IVGTT performed in the mouse. It has already been successfully used to evaluate the effect of insulin-stimulating agents on glucose tolerance, SG, SI, and insulin secretion in mice (6, 13). However, the parameters emanating from it have never been validated against a reference method. Thus a primary aim of this study was the validation of the SI index obtained with the minimal model in the mouse against the same variable measured with the gold standard euglycemic hyperinsulinemic clamp. Once the validation has been carried out successfully, the exploitation of the minimal-model analysis of IVGTT data allows the evaluation of the relative contribution to glucose tolerance of insulin-dependent vs. insulin-independent mechanisms in several different conditions characterized by a very wide range of insulin response, from no response to elevated hyperinsulinemia, and by variable SI. The use of mice allowed a very large set of data, several different metabolic conditions, and a homogeneous group of animals.

Finally, because some studies have raised the issue on a possible dependency of SG on the immediate insulin release (first phase) (14, 29), we aimed also to verify...
whether prevailing insulin influences the estimation of $S_0$.

METHODS

Experimental Procedures

General design. A total of 475 experiments was performed in nonfasted female NMRI mice (Bomholdgaard Breeding and Research Center, Ry, Denmark) weighing 25–30 g and kept on a 12:12-h light-dark schedule (lights on at 0600). Experiments were performed in late morning without food from the cages being removed. The experimental procedures were approved by the Ethics Committee of Lund University. The animals, fed with a standard pellet diet (fat 11.4%, carbohydrate 62.8%, protein 25.8%, total energy 12.6 kJ/g) and tap water ad libitum, were anesthetized with an intraperitoneal injection of midazolam (Dormicum, 0.4 mg/mouse; Hoffman-La Roche, Basel, Switzerland) and a combination of fluanison (0.9 mg/mouse) and fentanyl (Hypnorm; 0.02 mg/mouse; Janssen, Beerse, Belgium). The anesthesia persists for >1 h. In the clamp experiments, which lasted longer, administration of anesthetics was repeated every 60 min. During the whole procedure, animals were kept on a heating pad. The animals under high-fat (HF) diet consumed 58% fat, 25.6 carbohydrate, and 6.4% protein (total energy 23.4 kJ/g; Research Diets, New Brunswick, NJ) on energy basis from their 4th wk of age.

The experiments can be divided into two major groups: IVGTT with glucose alone [control experiments (CNT), $n = 202$] and IVGTT with glucose plus various substances to obtain different amounts of systemic insulin concentration. In particular, glucose was administered with different doses of substances stimulating insulin secretion (ENDO group, $n = 201$), of insulin alone (EXO group, $n = 48$), or of substances blocking insulin secretion (BAS group, $n = 24$).

IVGTT: A blood sample was taken from the retrobulbar intraorbital capillary plexus into a 100-μl pipette that had been preinjected in heparin solution (100 U/ml in 0.9% NaCl; Lövens, Ballerud, Denmark). Thereafter, β-glucose (10 g/dl; British Drug Houses, Poole, UK) was injected intravenously over 3 s at a dose of 1 g/kg in a tail vein without flushing of the 27-gauge needle after injection. Every ENDO, EXO, and BAS experiment actually comprised two randomly performed tests: i.e., the same mouse went through a control IVGTT and a test with administration of glucose plus another agent; the second test was always performed with >1-mo interval from the first one. During this period, all of the effects of the endogenous substances disappeared, as observed by comparing the basal levels. Because the mice were inbred and therefore genetically the same, the variation among animals was the same as within animals studied 1 mo apart. Furthermore, all individual experiment series included a control group given glucose alone. In RESULTS, when comparisons within a group are reported, we refer to “relative controls,” meaning the control tests done in the very same mice that underwent the experiments proper of that group. The eight mice that also underwent the clamp experiment (see Euglycemic hyperinsulinenic clamp) were part of the CNT group. In all experiments, the volume load was 10 μl/g body wt. We used quite a high dose of glucose (1 g/kg) because of the rapid metabolism in mice. In fact, we observed a rise in insulin only at 1 min, and rarely at 5 min, when giving lower doses of glucose such as 0.3 or 0.25 g/kg (not shown), whereas in this study, we wanted a higher and prolonged insulin response to better examine its influence under dynamic conditions in vivo. At 1, 5, 10, 20, 30, and 50 min after injection, blood samples (75 μl each) were collected. The first sample was at 1 min, because by that time, the mixing phase of the glucose bolus could be considered terminated; the last sample was at 50 min to avoid possible influence on the measurements of awakening from anesthesia.

ENDO group. To stimulate insulin secretion, we used synthetic gut hormone glucagon-like peptide-1 (GLP-1), synthetic ovine pituitary adenylate cyclase-activating polypeptide (PACAP) (both from Peninsula Laboratories Europe, Merseyside, UK), and synthetic COOH-terminal octapeptide of cholecystokinin (CCK, Sigma Chemical, St. Louis, MO). All types of experiments, number of animals, and doses are summarized in Table 1. These peptides are potent insulinotropic agents in mice, being gut hormones and islet neuropeptides (4, 6, 13, 15, 20), and induce elevated endogenous insulin levels. GLP-1 and PACAP augment glucose-stimulated insulin secretion mainly by raising the cellular content of cAMP (3, 4), whereas CCK stimulates insulin secretion through phospholipase activity in the β-cells, where both phospholipase C and phospholipase A2 are involved (20, 32).

PACAP consists of two forms, with 38 and 27 amino acid residues, respectively, the latter being equivalent to the NH2-terminal PACAP-38 (1–27) (3). Both of these forms, PACAP-38 and PACAP-27, were used in this study. They are equipotent in stimulating insulin secretion in mice, where they also modulate $S_1$ independently on the elevated insulin secretion (13). Some of the experiments of this group have been already published in previous reports, where the effects of GLP-1 (6) and PACAP (13) on insulin secretion and sensitivity were described.

EXO group. In this series, human insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark) was administered together with glucose at various doses (Table 1) to exogenously induce hyperinsulinemic patterns different from those induced endogenously. In particular, we aimed at obtaining a first-phase peak insulin more elevated than that in the ENDO group, maintaining, at the same time, amounts of systemic insulin on average as close as possible to corresponding values of the ENDO group.

BAS group. To block endogenous insulin secretion and maintain insulin concentration at a level as close as possible

Table 1. Doses of administered substances together with 1 g/kg of glucose and ΔAUC<sub>ins</sub>

<table>
<thead>
<tr>
<th>Substance</th>
<th>$n$</th>
<th>Dose Range</th>
<th>ΔAUC&lt;sub&gt;ins&lt;/sub&gt;, mU·ml&lt;sup&gt;-1&lt;/sup&gt;·50 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diazoxide</td>
<td>24</td>
<td>25</td>
<td>0.13 ± 0.08</td>
</tr>
<tr>
<td>GLP-1</td>
<td>12</td>
<td>0.03</td>
<td>5.58 ± 1.82</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.1–0.3</td>
<td>6.75 ± 0.87</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>1.0–3.0</td>
<td>12.39 ± 1.75</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>10–30</td>
<td>15.69 ± 1.51</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>100</td>
<td>29.08 ± 4.11</td>
</tr>
<tr>
<td>PACAP-38</td>
<td>4</td>
<td>0.0005</td>
<td>1.35 ± 0.65</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.005–0.150</td>
<td>3.63 ± 0.51</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>0.5–1.5</td>
<td>6.57 ± 0.86</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>5.0</td>
<td>10.75 ± 2.46</td>
</tr>
<tr>
<td>PACAP-27</td>
<td>20</td>
<td>0.04–0.40</td>
<td>8.60 ± 1.66</td>
</tr>
<tr>
<td>CCK</td>
<td>12</td>
<td>6.0</td>
<td>5.12 ± 0.72</td>
</tr>
<tr>
<td>Insulin</td>
<td>34</td>
<td>1.0–5.0</td>
<td>4.73 ± 0.45</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>10.0</td>
<td>14.99 ± 1.09</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>2.5 + GLP-1*</td>
<td>23.34 ± 2.28</td>
</tr>
<tr>
<td>Control experiments</td>
<td>202</td>
<td>2.67 ± 0.19</td>
<td></td>
</tr>
</tbody>
</table>

ΔAUC<sub>ins</sub>, relative total incremental area under the insulin concentration curve; $n = no.$ of experiments/group. Units: mg/kg for diazoxide; nmol/kg for glucagon-like peptide-1 (GLP-1), pituitary adenylate cyclase-activating peptide (PACAP), and cholecystokinin (CCK); U/kg for insulin; *dose of GLP-1, 10 nmol/kg.
to the fasting value, diazoxide (Sigma Chemical) was injected at a dose of 25 mg/kg together with glucose. This dose was chosen because it was the dose eliciting the absence of dynamic insulin response in a preliminary dose-response study (not shown), where increasing doses of diazoxide were administered. The inhibitory effect of diazoxide on insulin secretion was observed throughout the experiment. Diazoxide is a potent inhibitor of insulin secretion by hyperpolarizing the β-cells through opening the ATP-regulated K⁺ channels (17). In a different group of animals, 0.25 μmol/kg of synthetic somatostatin-14 (Sigma Chemical) was injected instead of diazoxide to abolish insulin secretion, but dynamic insulin secretion was not completely abolished in these experiments (not shown), which, therefore, were excluded from this study.

Euglycemic hyperinsulinemic clamp. To validate SI obtained with the IVGTT, a euglycemic hyperinsulinemic clamp was performed in eight mice fed a normal diet (32 ± 1 g body wt) and five animals that were given a HF diet for 6–12 wk (38 ± 1 g, P < 0.003). The clamp and IVGTT were randomly performed in the very same mice with a 1-mo interval elapsing between the two experiments. The clamp was carried out according to previous work of Deems et al. (12) and Niswender et al. (25). Mice were anesthetized as described in General design. The right jugular vein and the left carotid artery were catheterized. The venous catheter was used for infusion of glucose and insulin, and the arterial catheter was used for sampling. Thirty minutes after introduction of the catheters, synthetic human insulin (Actrapid) was infused at a rate of 60 mU·kg⁻¹·min⁻¹ for 1 min, followed by a continuous and constant infusion of 30 mU·kg⁻¹·min⁻¹. The volume load was 4 μl for the 1st min, followed by 2 μl/min thereafter. Blood glucose levels were determined at 5-min intervals for 120 min by the glucose dehydrogenase technique with the use of a Hemocue (Hemocue, Angelholm, Sweden). A variable rate of glucose (solution of 40 g/dl) was infused to maintain blood glucose levels at 100–120 mg/dl. A blood sample was taken at 60, 90, and 120 min for determination of plasma insulin. Insulin sensitivity was calculated as the glucose infusion rate during the second h (M) divided by the mean insulin concentration at 60, 90, and 120 min (I), and the clamp glucose concentration per unit of insulin was calculated as M/I divided by the clamped glucose concentration.

In another series of experiments (6 control mice), diazoxide was administered at minute 60 during the clamp (same dose of 25 mg/kg used in the IVGTT) to assess whether this substance alters glucose clearance and insulin sensitivity during clamp. Results were compared with those of seven different mice in which, at time 60, saline was given instead.

Assays

Collected sample blood was kept in heparinized tubes; then, after immediate centrifugation, plasma was separated and stored at −20°C until analysis. Plasma insulin was determined in single samples radioimmunochemically with the use of a guinea pig anti-rat insulin antibody, 125I-labeled human insulin as tracer, and rat insulin as standard (Linco Research, St. Charles, MO). The rat insulin standard resulted in a perfect parallel curve with a mouse and human insulin standard (data not shown). Free and bound radioactivity were separated by use of an anti-IgG (goat anti-guinea pig) antibody (Linco). The sensitivity of the assay is 2 pU/ml, and the coefficient of variation (CV) is <3% within assays and <5% between assays. Plasma glucose was determined with the glucose oxidase method (CV ± 1%).

Data Analysis

Insulin and glucose data from the seven-sample IVGTT were analyzed with the minimal-model technique as already reported (6, 13). The model assumes first-order, nonlinear, insulin-controlled kinetics and accounts for the effect of insulin and glucose alone on net glucose disappearance after exogenous glucose injection. The single-compartment model assumed for glucose kinetics is a reasonable approximation, because the changes of glucose concentration after the first peak are relatively gradual. In addition, given the low number of data points, it would be unlikely to obtain reliable parameter estimates with multicompartamental structures. This modeling analysis provides the parameter Sᵥ (insulin sensitivity index), which is defined as the ability of insulin to enhance net glucose disappearance and inhibit glucose production (8), and the parameter Sᵥ, which is the glucose effectiveness, representing net glucose disappearance per se from plasma without any change in dynamic insulin (2, 10).

The area under the curve (AUC) of insulin concentration (ΔAUCins) was calculated using the trapezoidal rule. We also determined a unitless index called global disposition index (DI) by multiplying Sᵥ times suprabasal or dynamic ΔAUCins. This is an extension of the concept proposed in humans by Kahn et al. (18) and describes insulin effect by including both insulin action and insulin secretion related by the hyperbolic equation Sᵥ = k × ΔAUCins. The glucose distribution volume was calculated as the ratio of the glucose dose to the difference between the extrapolated zero-intercept (a model parameter) and glucose basal level (16).

The net glucose elimination rate after the glucose injection [kg, the glucose tolerance index (7)] was calculated as the slope for the interval 1–20 min after glucose injection of the logarithmic transformation of the individual plasma glucose values (13). Saturation relationships of kg and DI vs. ΔAUCins were tested with the Michaelis-Menten equation, the parameters of which, as well as k and h, were estimated by using the nonlinear least-squares technique.

In the experimental series with diazoxide (BAS group), the virtual absence of dynamic insulin made it impossible to estimate Sᵥ with the minimal model. Because we demonstrated that diazoxide per se does not change Sᵥ (see RESULTS), Sᵥ values for the diazoxide-treated animals have been considered those obtained in the same animals during the control experiment. In the absence of dynamic insulin, net glucose disappearance during IVGTT is described by a monoequational function (2); thus Sᵥ can be calculated as the linear regression of the logarithm of glucose, because the insulin-dependent component of the minimal model would be zero.

Data and results are reported as means ± SE unless otherwise designated. Statistical comparisons between any group or subgroup with the relative controls were performed with paired t-test, being the same animals before and after some intervention. When two data vectors of different animals were compared, the unpaired t-test was used. Finally, ANOVA was exploited for multiple comparisons. Accuracy of the minimal model estimates was evaluated from the CV as fractional SD, calculated from the variance values in the main diagonal of the inverse of the Fisher’s information matrix (33).

Assessment of Contributions to Glucose Tolerance

The dynamic insulin-independent action on net glucose disappearance, i.e., at basal insulin, is described by parameter Sᵥ, whereas the effect of insulin is expressed by the combined parameter DI. Glucose tolerance is a combination of insulin-independent and insulin-dependent processes, and
a linear relationship appears to be a suitable representation. It has, in fact, been demonstrated that the IVGTT-derived $K_G$ can be linearly related to $S_G$ and $DI$ (34). We can thus write the model

$$K_G = \alpha S_G + \beta DI$$  

(1)

Parameters $\alpha$ and $\beta$ are estimated with a multiple regression. To evaluate the relative contribution of one of the factors, $S_G$ or $DI$, to the variable $K_G$, the definition of sensitivity must be considered. The sensitivity of changes in $K_G$ with respect to changes in $S_G$ is given by

$$S(K_G|S_G) = (dK_G/dS_G) \times (S_G/K_G)$$

(2)

With respect to changes in $DI$, the sensitivity is

$$S(K_G|DI) = (dK_G/dDI) \times (DI/K_G)$$

(3)

Considering that, in our model, $S_G$ and $DI$ are the only contributors to $K_G$, the sum of their sensitivities must give 1 (or 100% if we want it in percentage). Therefore

$$S(K_G|S_G) + S(K_G|DI) = (dK_G/dS_G) \times (S_G/K_G) + (dK_G/dDI) \times (DI/K_G) = 1$$

(4)

From Eq. 1, it follows that the derivatives $dK_G/dS_G$ and $dK_G/dDI$ are equal to $\alpha$ and $\beta$. Thus it is possible to calculate in every group the value (in percentage) of the two contributions $S(K_G|S_G)$ and $S(K_G|DI)$, given the estimates of $\alpha$ and $\beta$ and nominal values for $S_G$ and $DI$. For these, we chose the mean value ($S_G$ and $DI$) in every group. The corresponding $K_G$ is $\alpha S_G + \beta DI$, such that

$$S(K_G|S_G) = \alpha S_G/\alpha S_G + \beta DI$$

(5)

$$S(K_G|DI) = \beta DI/\alpha S_G + \beta DI$$

(6)

These sensitivities are point estimates for each group, and their standard deviations can be obtained by conventional statistical calculations of variance.

RESULTS

Validation of $S_I$ Index with Glucose Clamp

The euglycemic hyperinsulinemic clamp experiments are shown schematically in Fig. 1 and the resulting values in Table 2. Figure 2 shows that glucose levels were reduced by the insulin infusion in both groups of mice and that the glycemia was stable through the 2nd h of experiment. The mean glucose levels during the 2nd h of experiments were 104.4 ± 1.8 mg/dl in controls vs. 100.8 ± 1.8 mg/dl in HF mice. M/I was 7.32 ± 1.46 (mg·kg⁻¹·min⁻¹)/(μU/ml) in control animals and 1.63 ± 0.37 (mg·kg⁻¹·min⁻¹)/(μU/ml) in HF mice ($P < 0.013$). These M/I values strongly correlated ($r = 0.964, P = 0.0001$) with $S_I$ from the IVGTT (Fig. 3), which was 7.79 ± 2.06 10⁻⁶ min⁻¹/(μU/ml) in control and 1.34 ± 0.23 10⁻⁶ min⁻¹/(μU/ml) in HF mice ($P < 0.035$). The maintenance of steady state was demonstrated by correlating the values of M/I in the interval 60–90 min with those in 90–120 min. They correlated highly ($r = 0.98, P = 0.0001$), and the regression line was virtually coincident with the unit line (slope 1.07 ± 0.07, $P = 0.0001$). All animals were grouped together, because for the purpose of the comparison it is not important to segregate control from HF mice. Because $S_I$ represents a fractional glucose clearance per insulin concentration unit, a more comparable clamp parameter is the clamp glucose clearance that was 0.67 ± 0.13 (ml·kg⁻¹·min⁻¹)/(μU/ml) in control and 0.16 ± 0.03 (ml·kg⁻¹·min⁻¹)/(μU/ml) in HF mice ($P = 0.01$). An even stronger correlation with $S_I$ was observed with the use of this parameter ($r = 0.974, P = 0.0001, Fig. 3$). From inspection of Fig. 3, one point of elevated sensitivity can be seen that could be an outlier. However, even without that point, the correlation still remains very strong ($r = 0.909, P = 0.0001$), as well as the discriminative power between controls and HF (e.g., $P = 0.002$ for $S_I = 5.88 ± 0.90$). The glucose distribution volume ($V_d$) from IVGTT was
8.2 ± 0.3 ml in control and 8.1 ± 0.5 ml in HF animals, corresponding to ~23% body weight. The IVGTT glucose clearance obtained as $S_I = \frac{V_d}{3} \times \text{average insulin}$ was 0.14 ± 0.03 and 0.09 ± 0.002 ml/min, respectively.

Assessment of Diazoxide Effect on $S_I$

In the six mice given diazoxide at 60 min during the clamp, $M/I$ was 12.6 ± 3.7 (mg·kg⁻¹·min⁻¹)/mU/ml from 60 to 90 min and 13.3 ± 5.5 (mg·kg⁻¹·min⁻¹)/mU/ml from 90 to 120 min. These values were not different ($P > 0.95$) from 12.5 ± 5.6 and 13.0 ± 5.2 (mg·kg⁻¹·min⁻¹)/mU/ml, respectively, calculated in the seven mice where only saline was administered, showing that diazoxide does not exert any effect on insulin sensitivity.

IVGTT

Basal glucose and insulin in all animals were 180 ± 2 mg/dl and 68 ± 3 mU/ml, respectively. Calculated values of $\Delta AUC_{ins}$ for the various types of experiments are reported in Table 1 and show the increasing effect of the insulinotropic agents with the increasing dose. The IVGTT data are shown in Fig. 4. We pooled different situations in only four patterns for simplicity, being well aware that, for instance, the ENDO pattern was grouping together several different types of time courses of glucose and insulin. The calculations were performed in every single subgroup according, for instance, to a particular range of any of the parameters. Insulin responses were markedly higher than those of the relative controls, with a peak in ENDO of 831 ± 35 vs. 316 ± 18 mU/ml of CNT ($P < 0.0001$). Insulin returned to values not different from preinjection ones at 20 min. In the EXO group, the peak was 1,389 ± 126 mU/ml ($P < 0.0001$ vs. ENDO), and insulin concentration was back to basal at 10 min.
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Fig. 4. Means ± SE of glucose and insulin concentration curves after intravenous injection of glucose alone (CNT group, ○, n = 202); glucose plus insulinotropic substances (ENDO, ●, n = 201); glucose plus insulin (EXO, □, n = 48); and glucose plus substances blocking endogenous insulin response (BAS, ■, n = 24). Percent coefficient of variation of the data was ranging from 1.2 to 5.9% (mean 2.6%) for glucose and from 4.2 to 33.1% (mean 11.6%) for insulin. In all experimental sets, glucose dose was 1 g/kg. For details on the other substances and their doses see METHODS and Table 1.

together, total AUC_{ins} was 13.4 ± 0.9 mU·ml^{-1}·50 min in ENDO and 8.2 ± 0.7 mU·ml^{-1}·50 min in EXO vs. 5.5 ± 0.3 mU·ml^{-1}·50 min of CNT (both P < 0.0001).

In the experiments where insulin secretion was prevented (BAS), a very low dynamic insulin (0.12 ± 0.08 mU·ml^{-1}·min) was evident compared with that of the relative control mice (1.33 ± 0.15 mU·ml^{-1}·min, P < 0.0001). The inhibition occurred immediately after the glucose bolus, and insulin concentration at any data point remained not different from the basal preinjection value (P > 0.1). Consequently, a slow decay of glucose concentration was observed: value at 50 min 334 ± 11 vs. 159 ± 5 mg/dl at preinjection, P = 0.0001; KG: 0.52 ± 0.06 vs. 1.44 ± 0.14%·min^{-1} of relative controls, P < 0.0001.

Metabolic and Model Parameters

In Table 3, the minimal-model parameters, the tolerance index, and the dynamic insulin are shown for the pooled groups. In general, despite the low number of data points, the estimated minimal-model parameters were quite accurately and precisely estimated, although there were cases with elevated CV values (15% of CNT experiments and 20% of ENDO + EXO exhibited a CV >80% for either one of the estimated parameters SI and SG). The average CV value was 45 ± 2% for SG and 25 ± 2% for SI, without appreciable differences among groups (see for instance, the CVs of the already published PACAP experiments along with those of the relative control in Ref. 13).

KG was different among groups (P < 0.003). SI of ENDO and EXO were different from CNT and from each other (P < 0.001), whereas SG of EXO was different from both CNT and ENDO (P < 0.0001) but not between CNT and ENDO (P = 0.344). Differences in SI and ΔAUC_{ins} were reflected by differences in the DI, which was 14.8 ± 0.6 in ENDO, 18.1 ± 1.4 in EXO and 18.7 ± 1.2 in CNT (P < 0.005 ENDO vs. CNT; P = 0.872 EXO vs. CNT; P < 0.015 ENDO vs. EXO). In the BAS group, the DI (4.6 ± 0.9) was lower than that of the whole CNT group and that of the relative controls (15.4 ± 1.5), P < 0.0001 in both cases. Also, SG of BAS was lower than that of the relative controls (0.050 ± 0.006 min^{-1}, P < 0.0001).

Relationships Among Parameters

No correlation was found between SG and SI in any single group or when they were taken together (ranges

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>CNT n = 202</th>
<th>ENDO n = 201</th>
<th>EXO n = 48</th>
<th>BAS n = 24</th>
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<tbody>
<tr>
<td>ΔAUC_{ins}</td>
<td>mU·ml^{-1}·50 min</td>
<td>2.67 ± 0.19</td>
<td>10.62 ± 0.81</td>
<td>6.98 ± 0.72</td>
<td>0.12 ± 0.08</td>
</tr>
<tr>
<td>KG</td>
<td>% min^{-1}</td>
<td>2.57 ± 0.07</td>
<td>3.84 ± 0.11</td>
<td>4.61 ± 0.23</td>
<td>0.52 ± 0.06</td>
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<tr>
<td>SG</td>
<td>min^{-1}</td>
<td>0.050 ± 0.002</td>
<td>0.048 ± 0.002</td>
<td>0.075 ± 0.004</td>
<td>0.014 ± 0.002</td>
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<tr>
<td>SI</td>
<td>10^{-6} min^{-1}·(mU/l)</td>
<td>4.10 ± 0.20</td>
<td>1.69 ± 0.10</td>
<td>2.87 ± 0.30</td>
<td>6.40 ± 0.95^4</td>
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Percent contribution to glucose disappearance of processes (∆ coefficient of variation, as SD)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>CNT n = 202</th>
<th>ENDO n = 201</th>
<th>EXO n = 48</th>
<th>BAS n = 24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin independent</td>
<td>(SG)</td>
<td>71 ± 4</td>
<td>37 ± 3</td>
<td>26 ± 9</td>
<td>99 ± 5</td>
</tr>
<tr>
<td>Insulin dependent</td>
<td>(DI)</td>
<td>29 ± 4</td>
<td>63 ± 3</td>
<td>74 ± 9</td>
<td>1 ± 5</td>
</tr>
</tbody>
</table>

Experimental groups: CNT, control; ENDO, glucose plus insulin stimulation; EXO, insulin alone; BAS, with insulin blocking. KG, glucose tolerance index; SG, glucose effectiveness; SI, insulin sensitivity index; DI, global disposition index. ^4 The insulin sensitivity index cannot be estimated in the absence of dynamic insulin; because diazoxide does not affect insulin sensitivity (see RESULTS), SI obtained in the same animals with a control intravenous glucose tolerance test has been used for the calculation of the contribution to glucose disappearance.

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for $r$ value $0.03–0.05$, for $P$ value $0.3–0.7$). $S_I$ and $\Delta AUC_{\text{ins}}$ exhibited the classic hyperbolic relationship (Fig. 5); that is, $S_I = k \times \Delta AUC_{\text{ins}}^{-1}$. From our data (excluding the BAS group, where $S_I$ was not estimated), it resulted in $S_I = 11.1 \times \Delta AUC_{\text{ins}}^{-0.88}$, with $r = 0.75$ and $P < 0.0001$; 95% confidence intervals were $9.5–12.9$ for $k$ and $0.80–0.95$ for $h$. When the total of 475 cases was considered, a significant, direct relationship was found between parameter $S_G$ and insulin peak ($r = 0.24$, $P = 0.0001$); this relationship holds in the two hyperinsulinemic groups ($n = 249$, $r = 0.30$, $P = 0.0001$, Fig. 6) but not in the CNT alone ($n = 202$, $r = 0.08$, $P = 0.25$) and when only insulin peak values $<1,700 \mu U/ml$ are considered ($n = 449$, $r = 0.075$, $P = 0.116$). The correlation begins to be statistically significant for peaks $>1,900 \mu U/ml$.

Relative Contribution of Insulin-Dependent vs. Independent Processes

Table 3, bottom, shows the percent relative contribution to net glucose disappearance of insulin-dependent vs. -independent mechanisms for the various groups. In the control animals, only 29% of intravenous net glucose disappearance was due to dynamic insulin. The contribution of insulin-independent processes was reduced ($P < 0.001$), with increased $K_G$ possibly due to the increasing prevailing insulin. In fact, $K_G$ increased with $\Delta AUC_{\text{ins}}$, and this relationship exhibited a saturation above $\sim15 \text{ mU} \cdot \text{ml}^{-1} \cdot 50 \text{ min}$ (Fig. 7, top). Michaelis-Menten parameters were $2.02 \pm 0.35 \text{ mU} \cdot \text{ml}^{-1} \cdot 50 \text{ min}$ for the abscissa of the half-maximum level and $5.25 \pm 0.23 \text{min}^{-1}$ for the saturation. In Fig. 7, all of the data were clustered in only eight categories for better readability. A similar saturation pattern was also observed for the processes involving insulin as shown by DI (Fig. 7, middle; Michaelis-Menten parameters were $3.08 \pm 0.45 \text{ mU} \cdot \text{ml}^{-1} \cdot 50 \text{ min}$ for the abscissa of the half-maximum level and $17.65 \pm 0.75$ for the saturation). On the contrary, $S_G$ did not exhibit significant changes with $\Delta AUC_{\text{ins}}$ (Fig. 7, bottom), and the lowest value ($0.038 \pm 0.006 \text{ min}^{-1}$) observed for very high $\Delta AUC_{\text{ins}}$ (range $30–96 \text{ mU} \cdot \text{ml}^{-1} \cdot 50 \text{ min}$, $n = 8$) was not statistically different ($P > 0.05$) even from $0.059 \pm 0.004$ calculated for the $\Delta AUC_{\text{ins}}$ range of $15–28 \text{ mU} \cdot \text{ml}^{-1} \cdot 50 \text{ min}$, $n = 46$.

For the BAS group, in the virtual absence of dynamic insulin, net glucose disappearance resulted in being entirely due to insulin-independent mechanisms, despite the lowest $S_G$ ($P < 0.0001$ vs. CNT).

DISCUSSION

This study quantifies the relative contribution of insulin-dependent vs. -independent mechanisms to intravenous glucose tolerance in animals characterized by a wide spectrum of insulin sensitivity and insulin response to glucose challenge. In a normal condition for the IVGTT, i.e., when insulin release is stimulated only by the glucose load, the contribution of insulin-independent mechanisms was found to be predominant, accounting for 71% of net glucose disappearance. This may, at first sight, be an unexpected finding; however, it has long been recognized that insulin-independent glucose uptake, mainly by the central nervous system, contributes by a major degree to net glucose disappearance in the postabsorptive state (28). The importance of this issue has been stressed in several previous publications describing studies that exploited the minimal-model approach in humans (19) and dogs (2) or used tracer injection in rats (23). All of these investigations showed that $S_G$ was a major factor in intrav-
AUCins: 0, 1, 2, 3, 5, 8, 15, 30, and 100 mU

8 groups according to the intervals between the following values of

ments (71, 78, 63, 72, 73, and 63, respectively), except the last 2
in the interval. Every group includes a similar number of experi-

glucose to normalize itself, accounting, in fact, for 72% of the values of SG and DI. Nonetheless, it may be speculated that the main contributor to the variation in glucose tolerance observed in ENDO and EXO is due to the increasing Rd. Figure 7 shows that the dependence of glucose tolerance on the amount of insulin resembles that of DI, confirming what has been found in humans of different ethnic origins (19, 27). Also renal excretion of glucose, which is an insulin-independent process, might have contributed to SG, since the peak glucose levels were above the kidney glucose threshold. However, it is known that the renal glucose threshold for mice is ~400 mg/dl (26), and because such high values were observed for only a short period of time after the glucose challenge, it is likely that the contribution of this process to SG is minimal.

A further observation from our study arises from the possibility of reaching very high insulin levels in the mice. This allowed the evaluation of the quantitative behavior of metabolic parameters in a very wide spectrum of hyperinsulinemia, and the results showed a saturation for insulin action. This can occur at two sites: during transport from plasma to the interstitial space or at the level of binding to cell surface receptors (30). The present study cannot elucidate whether one or both of these are involved, but it is possible to state that insulin action does not change above approximately six times the average incremental insulin observed in a normal situation. On the other hand, a negligible effect has been shown, in rats, of small changes of insulin concentration from fasting (23). Thus both of these pieces of evidence must be taken into account when planning metabolic studies involving insulin modulation, such as, for instance, hyperinsulinemic glucose clamps, experiments with somatostatin, and insulin-modified intravenous glucose tests for minimal-model analysis.

It is worth noting that not only the amount of prevailing insulin, given by AUCins, but also the shape of the insulin concentration curve seem to govern glucose tolerance. If the two groups with elevated insulin are compared, we notice that, although the incremental insulin area is slightly reduced from ENDO to EXO, all of the parameters related to net glucose disappearance (K_G, S_I, S_G) significantly increase with the increase of the peak level of plasma insulin. The direct relationship of the peak insulin with the aforesaid metabolic parameters confirms its importance as a marker of impaired glucose tolerance (5, 22). Related to this issue is the possible existence of a direct relationship between S_G and the insulin secretory function that has been reported as a model artifact (14). In the BAS experiments, insulin remained at basal levels; therefore, by definition, the minimal model should provide the “true” S_G (2). Comparing this value with that calculated in the same animals in normal conditions, we observe that the latter is significantly higher,
meaning that either S_G is overestimated by the minimal model or it is actually insulin dependent during conditions of very low or very high dynamic insulin. In fact, in the wide span of insulin concentrations of this study, a significant correlation was found between S_G and the peak insulin (Fig. 6), whereas no correlation was observed with ΔAUC_{ins} (Fig. 7). These results show that the correlation holds only when insulin peak reaches very elevated levels; in fact, for insulin peaks <1,700 μU/ml, which included all of the CNT and the majority of the ENDO, no significant correlation was observed. Therefore, we can add some more information to the controversial issue of the dependency of S_G on peak insulin by noticing that, despite a possible overestimation, S_G did not correlate with first-phase insulin in physiological conditions and for values of peak insulin concentration not extremely elevated.

The importance of S_G on glucose tolerance was already proposed by Kahn et al. (19) from analyzing simple linear regression between K_G and S_G. In the present study, in addition to the simple regression, we used sensitivity analysis, which provides estimates of changes of a dependent variable (K_G) for a unit change of independent variables and accurately describes in quantitative terms the relationships among those variables. Some requirements, however, had to be fulfilled for a correct use of this method. First, we had to demonstrate that the two independent variables of Eq. 4 (S_G and DI) are independent. Estimated model parameters S_G and S_I did not correlate, as was also independently assessed in humans (24) and in other circumstances by simulation studies (D. T. Finegood, Simon Fraser University, Burnaby, BC, Canada, personal communication). Because ΔAUC_{ins} is a measured variable, DI is also uncorrelated with S_G, and Eq. 4 can be used. The lack of correlation between S_G and the other parameters indicates that, even in the presence of an overestimation of S_G, the assessment of S_I is not affected. Accuracy of the method can be also evaluated by checking that it reflects known facts. For this reason, we considered the results of the BAS group as evidence that the method was correct, since we noted that the total contribution to net glucose disappearance was ascribed to S_G, given the almost total lack of dynamic insulin. Finally, to increase the accuracy and precision of the results, it would have been better if the data points could have spanned the widest range possible. Insulin secretagogues and exogenously administered hormone allowed a very wide spectrum of insulin concentration, and insulin sensitivity was allowed to vary independently.

About this latter aspect, we point out that we purposely chose different protocols to have a wide range of ΔAUC_{ins} and at the same time also different S_I for similar ΔAUC_{ins}. Our goal was, in fact, to evaluate the relative contribution of the various parameters in different situations characterized by common and independent features such as different insulin profiles. For this reason, PACAP as well as GLP-1 contributed to generating different conditions that allowed S_I, S_G, and ΔAUC_{ins} to vary either together or independently. For instance, we employed PACAP, which is known to modulate S_I (13), in the ENDO group experiments, where a reduced S_I acted to compensate for the elevated hormone levels. The possible actions of these secretagogues on S_G or other parameters were not important in this study, because we were interested only in the relative relationships among K_G, S_G, and DI, regardless of the nature of the causes that might have produced changes in the parameter itself. We thus obtained a uniform distribution of DI values within the ΔAUC_{ins} range.

The choice of DI instead of using parameter S_I directly deserves some comment. The existence of a relation between insulin sensitivity and secretion was first proposed by Bergman et al. (9) in humans and then elegantly extended by Kahn et al. (18), who demonstrated that the interaction between S_I and insulin secretion is a better predictor of glucose tolerance than its components taken alone. This concept has been stressed further by the introduction of the “adaptation index,” which more appropriately measures insulin resistance by correcting S_I with β-cell function expressed by glucose-stimulated prompt C-peptide release (5). Thus the DI as originally proposed (18) relates S_I to a compensation in the posthepatic insulin levels, which is of great importance for understanding the physiological adaptation in insulinemia to the ambient insulin sensitivity. However, this adaptation requires an adaptive alteration in insulin secretion, which is executed at the level of the β-cell. To quantify this adaptation, the so-called adaptation index was introduced (5), which therefore more accurately determines the mechanistic basis of the adaptation than the DI. Calculation of the adaptation index also requires C-peptide measurements, however, due to the high degree of hepatic extraction of insulin. Therefore, it is not possible to calculate the adaptation index in the present study in mice where it was not possible to analyze C-peptide.

Bergman et al. (9) and Kahn et al. (18) showed that the relationship between S_I and the insulin response can be described by a hyperbolic function. We recently confirmed this finding in humans (5, 21), and the present study clearly shows the hyperbolic function between insulin sensitivity and secretion in mice (Fig. 5). This demonstrates that the same concepts regarding the relationship between insulin sensitivity and secretion apply in humans and in mice, making us confident that the methodological approach we have used, i.e., the minimal model of net glucose disappearance to analyze IVGTT data, was correct.

Regarding the IVGTT protocol, it is necessary to point out some features related to the sampling schedule. The minimal model has four parameters to be estimated; therefore, in theory, only four sampling points should be sufficient to obtain a unique value for every single parameter; however, more data points are necessary for reconstructing the insulin pattern. Number, timing, and location of the samples depend on several constraints. One is the length of the experiment; in fact, the mouse was anesthetized, and the
experiment time had to end before the mouse awoke. Nonetheless, given the know-how on minimal modeling in humans and dogs, the dynamics observed during 180 min in humans can be associated with that in 50–60 min in the mouse. The timing was chosen arbitrarily, but the frequency was increased at the beginning of the test to better observe the early insulin response. The first sample was constrained by the mixing phase of the glucose bolus. Given the size of the mouse and its fast metabolism, this phase can be considered to be terminated within 1 min. In fact, the 1-min glucose value falls on the straight line (after logarithmic transformation) that includes the 5-, 10-, and 20-min values. Had it still been a mixing phase, the 1-min value would have fallen off that curve. The amount of blood that could be collected in these small animals limited the number of samples. A compromise of seven samples was considered acceptable in terms of blood volume and sample size. Each sample consisted of 75 µl, and the removal of such an amount of blood in 50 min has previously been shown not to alter baseline glucose, insulin, and epinephrine levels in mice (13). In general, more samples would have been preferable; but we want to point out that all of the IVGTT experiments, including those whose results were compared with the clamp, adopted the same schedule, thus avoiding possible errors due to different protocols.

The outcome of the minimal-model analysis (namely $S_2$) was compared with the similar parameter obtained with the gold standard glucose clamp. This procedure was adapted in mice to successfully maintain glucose concentration constant at the desired euglycemia with a constant glucose infusion for a steady-state period of 1 h. It can be argued that both basal glucose and stimulated insulin levels during clamp and IVGTT were quite different, and this could invalidate the comparison of the results. The higher glucose levels in HF-fed mice are due to insulin resistance in combination with islet dysfunction. Thus the difference between the two groups in circulating glucose persists also after prolonged fasting (data not shown). Regarding the insulin discrepancy, we would like to point out that we were the first to measure insulin during the insulin infusion in a clamp, for which we used established insulin infusion rates (12, 25). In these previous clamp experiments in mice, only the amount of glucose infused (the M-value) was used as a measure of insulin sensitivity, without any insulin measurement. Indeed, the two indexes of insulin sensitivity are related by the glucose $V_d$, which yielded a glucose clearance of 0.12 ± 0.02 ml/min in all 13 animals. This value was calculated at physiological levels of insulin such as those reached during the IVGTT. If we consider the insulin levels of the clamp, this clearance would result in 8.8 ± 2.5 ml/min, a clearly implausible value. Therefore, we conclude that the sensitivities from the two tests are related, but a direct comparison with the same units is not possible because of the different insulin levels. Nevertheless, the very good regression between the two indexes of insulin sensitivity and the equivalent capacity of discriminating between control and insulin-resistant animals demonstrate that the minimal-model analysis of IVGTT data in the mouse with the adopted sampling schedule is able to provide a reliable index of insulin sensitivity.

In conclusion, this study demonstrates, in a very large number of animals, the importance of the mechanisms not mediated by dynamic insulin for glucose tolerance in vivo. Glucose effectiveness ($S_G$) was quantified in several different situations characterized by various degrees of insulin secretion and insulin sensitivity. The processes described by parameter $S_G$, which was estimated with the minimal model, contributed in normal conditions to approximately three-fourths of net glucose disappearance. This figure is quite large and is not greatly affected, even if we consider a possible overestimation of the parameter $S_G$ by the minimal model. Conversely, increasing hyperinsulinemia switches the process toward a prevalence of insulin-mediated mechanisms, but the global effect of the processes involving insulin (i.e., DI) saturates for insulin values that are not particularly high. If these results are extrapolated to humans, they stress the need for further studies to deeply determine the factors involved in glucose effectiveness, for possible pharmacological interventions and for detecting additional candidate genes of impaired glucose tolerance and diabetes.

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REFERENCES

CONTRIBUTIONS TO GLUCOSE TOLERANCE