Bistable insulin response: The win-win solution for glycemic control

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Bistable insulin response: The win-win solution for glycemic control

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SUMMARY
To satisfy both the safety and rapidity of glycemic control, muscles’ insulin response must be bistable, as theoretically predicted. Here, we test the bistability hypothesis by combining cellular experiments (to measure the threshold values in vitro) with mathematical modeling (to test the relevance of bistability in vivo). We examine bistability in C2C12 myotubes by both single-cell analysis (Förster resonance energy transfer) and cultured cells analysis (immunoblot). These technologies demonstrate bistable insulin response, with typical switch-on and switch-off thresholds of approximately 300 and 100 pM, respectively. Our mathematical model demonstrates the indispensability of bistability in interpreting experimental data, reveals fine details of plasma glucose-insulin dynamics, and explains unclear phenomena. These results suggest that the body’s ability to simultaneously avoid both hypoglycemia and hyperglycemia is mediated by bistability. The switch-on threshold is a promising biomarker for metabolic complications due to its deep quantitative connection with body composition, which is easy to measure.

INTRODUCTION
The related epidemics of obesity and diabetes demand a deeper understanding of the body’s insulin response, particularly the concept of insulin resistance.1 In the fasting state, our plasma glucose and insulin maintain their basal concentrations G0 (4-6 mM) and I0 (tens of pM), respectively. Following meal ingestion, both concentrations first rise and then decline. The decline is due to the removal of glucose from the blood by body tissues (primarily the muscles) in response to insulin stimulus. The muscles then face a dilemma2-5: a strong response is desired for fast glucose removal to avoid hyperglycemia; however, such a response may cause hypoglycemia and endanger the brain, which needs to consume as much as 60% of the total glucose supply.1 This large glucose requirement appears to be an insurmountable task for the brain because its size is too small to compete with the other tissues. Nature may have solved this problem by imposing an insulin-dependent condition upon peripheral tissues. Specifically, while the neurons’ glucose intake is largely independent of insulin,6 the muscles depend on insulin for massive glucose transport. From the perspective of control theory, a meal ingestion disturbs glucose-insulin homeostasis (inducing the excursion of G and I), and the muscle acts as a controller to restore homeostasis (bringing G and I back to G0 and I0). Understanding v(I), a myocyte’s insulin response curve, with v denoting its rate of glucose uptake, is important for fighting obesity and diabetes.

To avoid both hyperglycemia and hypoglycemia, v(I) should be a bistable response characterized by a switch-on threshold I0, a switch-off threshold I0, and a maximal rate νmax.2 See Figure 1. On the one hand, the value of I0 should be far larger than the fasting insulin level I0 (tens of pM), which minimizes the risk of hypoglycemia (Figure 1A). Only after an ample meal so that the peak insulin level exceeds I0, can the muscles switch on glucose uptake (Figure 1C). On the other hand, bistability also minimizes the risk of hyperglycemia. By using Pontryagin’s maximum principle, an optimal control theory, we proved that v(I) must be bistable to achieve the fastest restoration of glucose homeostasis.2,7 Taken together, bistability reconciles two seemingly opposing requirements: safety (avoiding hypoglycemia) and rapidity (avoiding hyperglycemia). This discovery extends the many merits of bistable regulation.7-10

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Continued
In addition to the organismal-level study, we performed molecular-level mathematical modeling to determine whether a cell’s insulin signaling pathway (Figure 2A) can provide a bistable response. Since Akt is the hub of insulin signaling, AktActivity(I) was used to quantify a cell’s insulin response. The shape of AktActivity(I) is determined by the values of the many parameters of the pathway. In the steady state model (which is in the form of coupled algebraic equations), several parameters lump together to form a composite parameter, which results in three composite parameters in total: b, a, K. The parameter b corresponds to the PI3K activity. The parameter a is the strength of the positive feedback from Akt to insulin receptor substrate (IRS), illustrated by the cyan arrow in Figure 2A. The parameter K = K_m/[total Akt], where K_m is the Michaelis constant of a much simplified model of the enzyme reactions of Akt activation. Mathematical analysis revealed that there are three types of response curves: the graded response, the toggle switch, and the irreversible switch, which can be best illustrated on the parameter plane spanned by a and K (Figure 2B). The plane is divided into three regions by the light yellow curve (defined by Eq. (A.12) in 11) and the deep yellow curve (defined by Eq. (A.10) in 11). Each region corresponds to a type of AktActivity(I): the graded response (the light yellow region), the toggle switch (the white region), and the irreversible switch (the deep yellow region). By fixing a K value, the change in the shape of AktActivity(I) can be visualized along the a-axis (Figures 2C–2H). When a < a_0, AktActivity(I) is graded (Figures 2C and 2D). As a increases, AktActivity(I) turns into a toggle switch (Figure 2F). When a > a_1, AktActivity(I) becomes negative, turning the toggle switch into an irreversible switch (Figure 2H): once Akt is activated, the deactivation of Akt becomes impossible even if I = 0. Note that a_0 and a_1 correspond to the two critical shapes in Figures 2 E and 2G, which qualitatively divide system dynamics. In summary, the insulin signaling pathway can provide three types of responses according to its parameter values, and different cells may adopt different types. In the following experiments, we found that AktActivity(I) of myocytes and HepG2 cancer cells are a toggle switch (Figure 2 F) and an irreversible switch (Figure 2 H), respectively.

In addition to the organismal-level study, we performed molecular-level mathematical modeling to determine whether a cell’s insulin signaling pathway (Figure 2A) can provide a bistable response. Since Akt is the hub of insulin signaling, AktActivity(I) was used to quantify a cell’s insulin response. The shape of AktActivity(I) is determined by the values of the many parameters of the pathway. In the steady state model (which is in the form of coupled algebraic equations), several parameters lump together to form a composite parameter, which results in three composite parameters in total: b, a, K. The parameter b corresponds to the PI3K activity. The parameter a is the strength of the positive feedback from Akt to insulin receptor substrate (IRS), illustrated by the cyan arrow in Figure 2A. The parameter K = K_m/[total Akt], where K_m is the Michaelis constant of a much simplified model of the enzyme reactions of Akt activation. Mathematical analysis revealed that there are three types of response curves: the graded response, the toggle switch, and the irreversible switch, which can be best illustrated on the parameter plane spanned by a and K (Figure 2B). The plane is divided into three regions by the light yellow curve (defined by Eq. (A.12) in 11) and the deep yellow curve (defined by Eq. (A.10) in 11). Each region corresponds to a type of AktActivity(I): the graded response (the light yellow region), the toggle switch (the white region), and the irreversible switch (the deep yellow region). By fixing a K value, the change in the shape of AktActivity(I) can be visualized along the a-axis (Figures 2C–2H). When a < a_0, AktActivity(I) is graded (Figures 2C and 2D). As a increases, AktActivity(I) turns into a toggle switch (Figure 2F). When a > a_1, AktActivity(I) becomes negative, turning the toggle switch into an irreversible switch (Figure 2H): once Akt is activated, the deactivation of Akt becomes impossible even if I = 0. Note that a_0 and a_1 correspond to the two critical shapes in Figures 2 E and 2G, which qualitatively divide system dynamics. In summary, the insulin signaling pathway can provide three types of responses according to its parameter values, and different cells may adopt different types. In the following experiments, we found that AktActivity(I) of myocytes and HepG2 cancer cells are a toggle switch (Figure 2 F) and an irreversible switch (Figure 2 H), respectively.

In this article, we used Förster resonance energy transfer (FRET) technique to examine the insulin response in single mouse myotubes derived from C2C12 cells and used western blotting to examine the myotube population. We found that the myotubes did respond to insulin in a bistable manner, with typical I_on and I_off values of approximately 300 pM and 100 pM, respectively (Figures 3 and S4). We then evaluated the feasibility of using bistability to explain related physiologic processes. For glucose dynamics, we found

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that $v(I)$ has to be bistable to fit with blood sample data of the oral glucose tolerance test (OGTT)\textsuperscript{23} (Figure 4) and that $I_{\text{on}}$ and $I_{\text{off}}$ values can be inferred (Figure 5A). For fat dynamics, we found that $I_{\text{on}}$ is the primary determinant of body fatness\textsuperscript{24} and that its value can be inferred from body composition (Figure 5B). Although entirely different, the two studies led to fairly close estimations of $I_{\text{on}}$ and thus corroborated each other. Furthermore, the bistable model has provided great insights into glycemic control, including novel explanations of elusive reactive hypoglycemia\textsuperscript{25} and the sigmoid shape of the whole-body insulin dose response.\textsuperscript{26} These results will play important roles in precision medicine applications such as biomarker discovery and precise insulin administration (timing and dosage).
RESULTS

\textbf{Förster resonance energy transfer live-cell imaging}

We used a genetically encoded FRET biosensor to investigate Akt activity in skeletal muscle cells. Overnight-starved C2C12-derived myotubes stably expressing the transfected Eevee-iAkt probe\textsuperscript{27} were placed on a microscope heating stage and analyzed under a laser scanning confocal microscope (Figure S1). For each experiment, at least five cells that were highly transfected and differentiated were selected for live-cell FRET imaging. To determine \( I_{\text{on}} \) and \( I_{\text{off}} \), we increased the medium insulin concentration in a stepwise manner to see at which point the switch occurs. Since 500 pM insulin almost always achieved the highest FRET efficiency, we considered 500 pM as the saturation dosage and thus used six insulin concentrations (0, 100, 200, \ldots, 500 pM) to stimulate the myotubes. The cells were initially placed in a live-cell imaging chamber, incubated for 2-3 h, and then exposed to laser scanning without insulin for 30 min. Then, the insulin-free medium was replaced with 100 pM insulin medium to stimulate the myotubes for 10 min, after which the 100 pM medium was replaced with 200 pM medium. Under this procedure, the myotubes were stimulated for 10 min at each concentration of a stepwise increase in insulin concentration (100, 200, \ldots, 500 pM). Then, the myotubes were stimulated for 10 min at each concentration of a stepwise decrease in insulin concentration (400, 300, 200, 100, and 0 pM). During the process, time-lapse images of the cells were taken at one frame every 10 s using a 40× oil immersion lens.

Figure 3C shows the change in FRET efficiency of a myotube in the incremental and then decremental stages of insulin stimulation. Clearly, the insulin response switched on at \( I_{\text{on}} \approx 300 \text{ pM} \) and remained fully active as \( I \) increased to 400 and 500 and then decreased to 400, 300, and 200 pM. The fact that Akt remained fully active at the decremental 200 pM insulin demonstrated bistability because the incremental 200 pM insulin did not activate Akt. As \( I \) decreased to 100 pM, the FRET efficiency declined sharply and remained steady in the washout stage (0 pM insulin), implying \( I_{\text{off}} \approx 100 \text{ pM} \). The fact \( I_{\text{on}} < I_{\text{off}} \) indicates hysteresis and conforms with our theoretical predictions.\textsuperscript{23,31} Figures 3A and 3B show the myotube FRET image at the incremental 300 and decremental 100 pM insulin, respectively. Figure S2 shows the FRET change of another myotube.

Thus, we tested 50 myotubes, which all exhibited bistability. The histogram in Figure 3D illustrates the cell counts of all the \((I_{\text{on}}, I_{\text{off}})\) combinations. The highest bar with 23 cells having \((I_{\text{on}}, I_{\text{off}}) = (300, 100)\) subsequently refer to as canonical cells. Figures 3D1, 3D2, and 3D3 show the bistable response of a canonical cell, a (500, 100) cell, and a (400, 200) cell, respectively. Figure S3 gives more detailed information, particularly the timeline of the FRET data. The above results were obtained with an incubation time of 10 min for every \( I \). Incubation times ranging from 15 to 20 min were also tested, but no significant differences were found. Therefore, 10 min was sufficient for the stabilization of Akt activation.

To determine the threshold values more precisely, we used 20 pM interval insulin concentrations to examine 46 new myotubes. The rising and falling insulin concentrations were (0, 220, 260, 280, and 300 pM) and (180, 160, 140, 120, 100, 80, and 0 pM), respectively. We found that 16 cells were canonical (the highest bar in Figure S4B), among which two are presented, one with the rising and falling insulin concentrations placed side by side (Figure S4A) and the other without discriminating them (Figure S4B1). Among the 30 noncanonical cells, five had (280, 100) (Figure S4B2) and four had (300, 80) (Figure S4B3). The marginal distributions of \( I_{\text{on}} \) and \( I_{\text{off}} \) values are shown in Figures S4C and S4D, respectively.

In addition to myotubes, we also analyzed a HepG2 cancer cell and found that the cell responded to insulin as an irreversible switch (Figures 2H’ or SSA), manifesting constitutive Akt activation. The response was fairly flat.

\textit{Figure 3. Experimental measurement of C2C12 myotube threshold values}

(A) A terminally differentiated C2C12 myotube shows the highest FRET efficiency when stimulated with 300 pM insulin.

(B) The myotube shows suddenly diminished FRET efficiency when \( I \) changes from 500 pM to 100 pM.

(C) FRET efficiency signal of C2C12 myotubes as a function of \( I \), demonstrating that \( I_{\text{on}} = 300 \) and \( I_{\text{off}} = 100 \). Since \( I_{\text{on}} < I_{\text{off}} \), hysteresis is demonstrated.

(D) The three-dimensional histogram counting the \((I_{\text{on}}, I_{\text{off}})\) values. The highest bar counts the 23 cells with (300, 100), from which one is selected to show its bistable FRET signal (D1). Examples (500, 100) and (400, 200) are shown in D2 and D3, respectively.

(E) The two-dimensional histogram counting \( I_{\text{on}} \).

(F) The two-dimensional histogram counting \( I_{\text{off}} \).

(G) Immunoblot of pAkt\textsuperscript{S473} and total Akt in C2C12 myotubes. \( \beta \)-Actin was used as a loading control. The right five bands correspond to the cells first primed with 500 pM insulin and then stimulated with the indicated insulin concentration. The six bands on the left correspond to the directly stimulated cells (not primed).

(H) The western blot density was quantified in terms of pAkt\textsuperscript{S473}/Akt, with the mean and standard deviation (error bar) shown.

(I) The p-value of the mean pAkt\textsuperscript{S473}/Akt.
\[ \text{Zone of glycosuria} \]

\[ \Delta I = 354 \text{ (mmol)} \]

\[ \Delta I = 351 \text{ (mmol)} \]

\[ \Delta I = 259 \text{ (mmol)} \]

\[ \Delta I = 454 \text{ (mmol)} \]

\[ \text{V}_{\text{peak}} \]

\[ \text{V}_{\text{peak}} \]

\[ \text{V}_{\text{peak}} \]

\[ \text{V}_{\text{peak}} \]

\[ \text{EC50} \]

\[ \text{V}_{\text{peak}} = 0.10 \]

\[ \text{F(r)} \]

\[ \text{physiologic F(r)} \]

\[ \text{prototypical F(r)} \]

\[ \text{I} \]

\[ \text{I} \]

\[ \text{I} \]

\[ \text{I} \]

\[ \text{I} \]
Figure 4. Testing the bistable insulin response by mathematical modeling of the plasma glucose dynamics induced by OGTT

Columns 1-4 correspond to groups 1-4 subjects in. The dots with error bars represent plasma data (Mean and SD), sampled at specific times.

(A) The rates of oral and endogenous glucose appearing in the blood.
(B) The plasma insulin concentration.
(C) The plasma glucose concentration; the curves are the simulation runs.
(D) The glucose disappearance rate.
(E) The bistable response curve \( v(t) \) of those cells whose switch-on threshold is the estimated \( I_{\text{on}} \) and whose hysteresis is the estimated \( I_{\text{off}} \). The numbers with (without) parentheses correspond to the choice of the normal (uniform) distribution for \( r(I_{\text{on}}) \). The same applies below.
(F) The whole-body response curve \( V(t) \).
(G) The estimated \( V_0 \) values.
(H) The estimated \( V_{\text{max}} \) values.
(I) The \( I_{\text{on}} \) values estimated by the glucose-based method (solid bars) and the fat-based method (hollow bars).
(J) The kinetics of glycogenolysis \( k(G) \).
(K1) A schematic insulin pulse following a meal.
(K2) The normal probability distribution \( p \) of the cells over \( I_{\text{on}} \).
(K3) The gray curve shows the prototypical \( V(t) \); the blue curves constitute the double-branched physiologic \( V(t) \).
(L) The rate of glucose uptake \( V_{\text{gl}} \). The blue and gray curves correspond to the physiologic and prototypical \( V(t) \), respectively.

as \( I \) increased to 300 pM, after which it became much stronger, as \( I \) decreased, the FRET signal persisted even with \( I = 0 \). This irreversible switch agrees with our theoretical prediction (Figure 2H). Thus, we analyzed three HepG2 cells. Their averaged FRET signal is presented as a function of the elapsed time (Figure S5B). Constitute Akt activation may drive uncontrolled cell proliferation and thus carcinogenesis. 28

Western blot results

Single-cell analysis, although best at characterizing the thresholds, entails the transfection of exogenous biosensors that might perturb endogenous Akt kinetics. We, therefore, performed western blotting on terminally differentiated C2C12 cells stimulated with different concentrations of insulin (Figure 3G). We found that 500 pM insulin achieved the highest Akt phosphorylation at serine 473 (lane 6), which is consistent with FRET analysis. Although lanes 1-5 and 11-7 correspond to the same range of insulin concentrations (0-400 pM), the cells of the latter lanes had been primed by 500 pM insulin for 30 min before restimulation with the respective \( I \), mimicking the insulin-decremental stage of FRET analysis. The total Akt levels were similarly obtained from new myotubes, which remained constant under all conditions (Figure 3G, the second row). The densiometric analysis showed that pAkt \( k(G) \)/Akt changed gradually with \( I \) (Figure 3H). The graded response could be attributable to the cellular heterogeneity of \( I_{\text{on}} \), as will be discussed in Figure 4K2. Nevertheless, the data still reflect key features of bistability. First, the right five bars are higher than the left five bars, which is an indication of hysteresis. Second, the mean pAkt \( k(G) \)/Akt values were significant (p < 0.05) only at insulin concentrations above \( I_{\text{on}} \) or below \( I_{\text{off}} \) (Figure 3L, red text). More specifically, 100 or 200 pM insulin caused statistically significant Akt phosphorylation (some cells were phosphorylated, but many others were not); Akt phosphorylation was statistically significant only with 300 pM insulin (p = 0.0097, 0.0075, 0.0072), implying \( I_{\text{on}} = 300 \) pM. For those cells first primed with 500 pM insulin, restimulation with 400-200 pM insulin caused statistically insignificant dephosphorylation of pAkt \( k(G) \). Only when the restimulation \( I \) was 100 pM, did the dephosphorylation of pAkt \( k(G) \) become nearly complete (p = 0.0201), implying that \( I_{\text{on}} = 100 \) pM.

Glucose-based method to test bistability in vivo

Single-cell insulin response

First, \( N = 1000 \) in silico cells, numbered by \( i = 1, 2, \ldots, N \), were created. The \( i \)-th cell exhibits a bistable response to insulin:

\[
\nu_i(t) = \begin{cases} 
0 & 0 < t < (l_{\text{off}}), \\
\nu_i((l(t)^-) - (l_{\text{on}})) & (l_{\text{off}}) \leq t < (l_{\text{on}}), \\
\nu_{\text{max}} & t > (l_{\text{on}}), 
\end{cases}
\]  

(Equation 1)

where \( t^- \) is the time infinitesimally behind of \( t \). When \( I \) is below \( I_{\text{on}} \), \( v \) is definitely 0; when \( I \) is above \( I_{\text{on}} \), \( v \) is definitely \( \nu_{\text{max}} \); when \( I \) is between \( I_{\text{on}} \) and \( I_{\text{off}} \), \( v \) just keeps its original value (which is either 0 or \( \nu_{\text{max}} \)). The value of \( \nu_{\text{max}} \) can be deduced as follows. When the body is stimulated by supraphysiologic insulin concentrations such as those used in an euglycemic-hyperinsulinemic clamp experiment, the whole-body’s rate of
Insulin-mediated glucose disposal saturates at $V_N$, which corresponds to the activation of all the insulin-responsive cells in the body. That is, $v_{\text{max}}N = V_N$. In, $V_N$ was estimated to be approximately $0.1 \text{ min}^{-1}$ based on the experimental data presented in. Therefore, $v_{\text{max}} = V_N / N = 10^{-2} \text{ min}^{-1}$.

At a given time $t$, a cell either abstains from glucose or absorbs glucose completely, and the decision is made by Equation 1 according to the values of its $I_{\text{on}}$, its $I_{\text{off}}$, and the insulin concentration $I(t)$. We let the cells have the same value of hysteresis $\Delta I = I_{\text{on}} - I_{\text{off}}$ (to be determined), but different values of $I_{\text{on}}$. We let the $I_{\text{on}}$ values follow a normal probability distribution $\rho(I_{\text{on}})$ with mean $\bar{I}_{\text{on}}$ (to be determined) and standard deviation (SD) $\sigma = 100 \text{ pM}$. In this way, the $N$ cells are parametrized by only two unknown parameters $\bar{I}_{\text{on}}$ and $\Delta I$. Once a set of $\bar{I}_{\text{on}}$ and $\Delta I$ values are given, the $N$ cells’ bistable responses are constructed as follows. For every $i$, the value of $I_{\text{on}}(i)$ is randomly drawn from the normal probability distribution with mean $\bar{I}_{\text{on}}$ and SD $\sigma$; the value of $I_{\text{off}}(i)$ is subsequently determined to be $I_{\text{on}}(i) - \Delta I$. In this way, the $i$-th bistable response is constructed (Equation 1), and it acts in the mathematical model through Equation 2.

**Whole-body insulin response**

$$V(I(t)) = \sum_{i=1}^{N} v_i(I(t))$$  \hspace{1cm} \text{(Equation 2)}

Figure 5. Two methods were used to infer bistable insulin response in vivo

(A) The glucose-based method.
(B) The body-composition-based method.
(C) $I_{\text{on}}$ is estimated by the two methods independently, and the results are compared.
should be a graded, sigmoid curve even though each \( v_i(t) \) is bistable. The sigmoidity can be explained by the normal distribution \( \rho(\lambda_v) \) (Figure 4K2) of all of the insulin-responsive cells in the body, primarily the myocytes. As \( I \) increases, the number of activated cells gradually increases, producing a graded response at the population level. At a given \( I \), the percentage of activated cells is

\[
\eta(I) = \int_{-\infty}^{\infty} \rho(\lambda_v) d\lambda_v,
\]

which is a cumulative Gaussian function and is well known for its sigmoidal shape. The whole-body response is thus \( V(I) = \eta(I) \cdot V_{\text{m}} \), where \( V_{\text{m}} = N \cdot \nu_{\text{max}} = 0.1 \text{ min}^{-1} \). As the increasing \( I \) becomes very large, \( \eta(I) \) approaches 1, and \( V(I) \) saturates at \( V_{\text{m}} \). This full range insulin response, illustrated by the gray curve in Figure 4K3, is called the prototypical \( V(I) \) because its shape conforms well with the measurements made by euglycemic hyperinsulinemic clamp experiments, which use supraphysiologic insulin concentrations to activate virtually all of the insulin-responsive cells in the body (see e.g.\textsuperscript{25}). Note that we have provided a novel explanation of the sigmoidity of \( V(I) \) based on the heterogeneity of \( \lambda_v \), which is different from the traditional explanation based on cooperative binding between insulin and its receptor. Importantly, Figures 4K2 and 4K3 together reveal that

\[
\overline{I}_\text{on} = EC50 \quad \text{(Equation 3)}
\]

where \( EC50 \) is the half-maximal effective insulin concentration of the prototypical \( V(I) \). Indeed, when \( I = \overline{I}_\text{on} \), precisely half of the cells are activated, and \( V = V_{\text{m}}/2 \).

The physiologic \( V(I) \) refers to the actual insulin response curve, which differs from the prototypical \( V(I) \) in the following aspects. After a meal, the peak insulin concentration \( I_{\text{peak}} \) is actually very small when compared with the supraphysiologic insulin concentrations used in an insulin clamp experiment. Therefore, as \( I \) increases from \( I_{\text{peak}} \) to \( I_{\text{on}} \) (Figure 4K1), only a small percentage of the cells are activated (the blue balls in Figure 4K2). This percentage is denoted by \( \eta_{\text{peak}} \) and the corresponding \( V \) is denoted by \( V_{\text{peak}} \). As \( I \) decreases from \( I_{\text{peak}} \) to \( I_{\text{on}} \), \( V \) is sustained at \( V_{\text{peak}} \) for quite some time due to cellular hysteresis before the subsequent decrease. In summary, the physiologic \( V(I) \) should be a double-branched graph (the blue curves in Figure 4K3) with its forward branch coinciding with a short segment of prototypical \( V(I) \).

**Mathematical model of the oral glucose tolerance test**

Mitrakou et al. performed OGGT on four groups of human subjects,\textsuperscript{26} denoted by IGT\textsuperscript{−}OB\textsuperscript{−} (group 1), IGT\textsuperscript{+}OB\textsuperscript{−} (group 2), IGT\textsuperscript{+}OB\textsuperscript{+} (group 3), and IGT\textsuperscript{+}OB\textsuperscript{+} (group 4), where IGT represents impaired glucose tolerance and OB represents obesity. Since IGT\textsuperscript{−} is better than IGT\textsuperscript{+} and OB\textsuperscript{−} is better than OB\textsuperscript{+}, the smaller the group number, the healthier the subjects were. The subjects ingested glucose at time 0, and their blood was then sampled at 30-min intervals for 300 min to determine the rate of oral glucose appearing in the blood \( S_{\text{oral}}(mr) \) (for \( t = 30 \text{ min} \) and \( m = 0, 1, \ldots, M \), where \( M = 10 \) represents the number of data points), the rate of endogenous glucose appearing in the blood \( S_{\text{endo}}(mr) \), the plasma insulin concentration \( I(mr) \), the plasma glucose concentration \( G(mr) \), and the rate of glucose disappearance \( D(mr) \) (the dots in Figure 4D) from the blood. These data are illustrated by the dots in Figures 4A–4D.

We developed a mathematical model to simulate the dynamics of plasma glucose concentration \( G(t) \) induced by the OGTT:

\[
\frac{dG(t)}{dt} = S_{\text{oral}}(t) + S_{\text{endo}}(t) - V_0 \cdot G(t) - V(I(t)) \cdot G(t) - f(G(t)), \quad \text{(Equation 4)}
\]

where \( t \) is the time starting from the ingestion of oral glucose. The plasma glucose concentration \( G \) is the only state variable of the model. \( I(t) \), \( S_{\text{oral}}(t) \), and \( S_{\text{endo}}(t) \) are continuous time functions obtained by pointwise connecting the discrete dots \( \bar{I}(mr) \) (Figure 4B), \( S_{\text{oral}}(mr) \) (Figure 4A), and \( S_{\text{endo}}(mr) \) (Figure 4A), respectively, with straight lines; they are thus part of known parameters for the numerical solution of \( G(t) \). The unit of \( S_{\text{oral}} \) and \( S_{\text{endo}} \) is mmol/min/(kg body weight).\textsuperscript{26} \( \Omega = 0.075 \text{ L/kg} \) is the blood volume per kg body weight.\textsuperscript{26} Thus, \( (S_{\text{oral}} + S_{\text{endo}})/\Omega \), with the unit being mmol/min, is the rate of total glucose appearing in the blood. \( V_0 \) is the rate of basal (non-insulin dependent) glucose utilization per unit glucose concentration. \( V(I(t)) \), the whole-body’s rate of insulin-mediated glucose disposal per unit glucose concentration, has been defined by Equation 2. \( f(G) \) is the rate of glucose leaking into the urine (glycosuria) due to hyperglycemia. It is commonly modeled by the following function\textsuperscript{26}.
where RTG is the renal threshold for glucose, the plasma glucose concentration above which renal tubules become overwhelmed and begin to excrete glucose into the urine. See Figure 4J for the graph of f(G). For nondiabetes, the commonly accepted value of RTG is approximately 180 mg/dL (i.e., 10 mM) \(^3\). we, therefore, take RTG = 10 mM in this article. V\(_{\text{ur}}\) is the rate of glucose leakage when G exceeds RTG. For groups 1 and 2, f(G(t)) = 0 because glycosuria never occurred, as reflected by the fact that G(t) does not encroach the “zone of glycosuria” in Figures 4C1 and 4C2.

**Parameter estimation and model validation**

The value of V\(_{0}\) is determined simply from the blood sample data at t = 0, which was the end of the fasting state (a steady state), in which Equation 4 is reduced to 0 = (S\(_{\text{oral}}\)(0) + S\(_{\text{endo}}\)(0))/V\(_{0}\) - G(0). Therefore,

\[
V_{0} = (S_{\text{oral}}(0) + S_{\text{endo}}(0))/G(0)/U
\]

(Taking group 1 as an example. One reads from Figures 4A1 and 4C1 to learn that S\(_{\text{oral}}\)(0) = 0, S\(_{\text{endo}}\)(0) = 0.011, and G(0) = 5.5, which renders V\(_{0}\) = 0.0265 min\(^{-1}\). For groups 2, 3, and 4, V\(_{0}\) = 0.0251, 0.0262, and 0.0242 min\(^{-1}\), respectively (Figure 4G). These values are all close to 0.025 min\(^{-1}\), a previous estimation based on a different dataset\(^4\) and a different method.\(^5\) The consistent results demonstrate that our basal rate of glucose utilization is uniform.

Then, the model had at most three unknown parameters \(I_{\text{on}}\), A\(_{1}\), and V\(_{\text{ur}}\) which are collectively denoted by a vector p. Note that V\(_{\text{ur}}\) is not applicable to the subjects in groups 1 and 2; thus, p = (\(I_{\text{on}}\), A\(_{1}\)) for the two groups. The aim is then to find a specific p that allows G(t), namely, the solution to Equation 4, to fit best with \(G_{\text{original}}\) (the dots in Figure 4C). We used random sampling to explore the search space. An arbitrary value of \(p\) is first selected. Based on the selected \(I_{\text{on}}\) and A\(_{1}\) values, N = 1000 bistable cells are created. These cells have the same \(V_{\text{max}} = 10^{-4}\) min\(^{-1}\), the same A\(_{1}\) (which was just selected), but different \(I_{\text{on}}\) values. That is, every cell’s \(I_{\text{on}}\) value is randomly drawn from \(p(\text{random})\) with mean \(I_{\text{on}}\) (which was just selected) and \(\sigma = 100\) pM. At this point, the parameters of Equation 4 are completely specified. Equation 4 is then integrated by fourth-order Runge-Kutta method to obtain G(t), which is then compared to the experimental data \(G(\text{mr})\) to obtain the fitting error

\[
J = \sum_{m=1}^{M} (G(\text{mr}) - \hat{G}(\text{mr}))^2, \quad \text{(Equation 7)}
\]

where \(\hat{G}(\text{mr})\) corresponds to the m-th dot in Figure 4C and M = 10. The aim is then to reduce J by iteratively changing the value of \(p\). Each update of \(p\) entails the reconstruction of the N cells according to the new \(I_{\text{on}}\) and A\(_{1}\) values. This is followed by a new round of solving Equation 4 for a new G(t) and then the new J. If the new J is smaller (larger) than the old one, then the new J is kept (rejected). In this way, J decreases consistently. When J cannot significantly decrease any more, it is considered as a local minimum. To obtain the global minimum, the algorithm is implemented in parallel on 1200 computer nodes so that the smallest of the local minima is determined. We found that most of the local minima are identical to the global minimum; thus, the global minimum should be the genuine one. The easy determination of the global minimum thus, the global minimum is not strange because the model has only three unknown parameters.

The best-fit G(t) is shown as the blue curves in Figure 4C panels. The estimated V\(_{0}\) values are presented as black texts in Figure 4H. The estimated \(I_{\text{on}}\) values are presented as the solid bars in Figure 4I. The estimated A\(_{1}\) values are presented together with their corresponding \(I_{\text{on}}\) values to form bistable responses (Figure 4E). These bistable responses belong to the cells satisfying \(I_{\text{on}} = I_{\text{on}}\), which are represented by the green balls in Figure 4K2. However, they were not activated by the OGTT because \(t_{\text{peak}}\) was smaller than \(I_{\text{on}}\). Taking group 1 as an example, one finds from Figure 4B1 that \(t_{\text{peak}} = 459\) pM, which is smaller than \(I_{\text{on}} = 551\) pM. As a consequence, only a small fraction of the cells (\(n_{\text{peak}} = 17.5\%\)) were activated. We then computed V(t) by Equation 2 along with the numerical solution of G(t), which are indeed physiologic (Figure 4F) as predicted in Figure 4K3.

For the data \(\hat{D}(\text{mr})\), our mathematical model does not have the corresponding term D(t). Based on the physiologic meaning of \(\hat{D}(\text{mr})\), as well as its unit (mmol/kg/min), we deduced that D(t) = (V\(_{0}\)G(t) + V(t) G(t) + f(G(t)))\(\Omega\). If under the estimated \(p\), D(t) happens to fit with \(\hat{D}(\text{mr})\), then the validity of the mathematical
obtained. Thus, an individual

The two coefficients $g_1$ and $g_2$ were obtained. By substituting the obtained $FM$ and $LM$ values into Equation 8, the

methods, although entirely different, obtained similar and parallel results. The two coefficients $g_0$ and $g_1$ can be estimated by regression analysis of a number of experimentally measured $(\text{EC}50, \text{FM}, \text{LM})$. Although there are abundant data for $\text{FM}$ and $\text{LM}$, $\text{EC}50$ was a recent definition and was never measured. However, Equation 3 demonstrates that $\text{EC}50$ equals the EC50 of the prototypical $\text{V}(j)$, which was frequently measured in insulin clamp experiments. We performed a literature survey to collect the experimental data of (EC50, FM, LM) in units of pM, kg, kg. To be included, the following conditions must be satisfied. First, EC50 and (FM, LM) must be obtained from the same subject. A lot of data were excluded because either EC50 or (FM, LM) had been obtained but not both. Second, the experimental subjects must be adults in their natural state; otherwise, their body composition may not faithfully reflect their degree of insulin resistance. For example, in, the subjects’ data were obtained both before and after bicycle training. While they became significantly thinner after the training, their EC50 values had almost no change and even increased slightly. Therefore, the thinner body resulting from training was temporary (i.e., not stabilized) and thus unnatural. We, therefore, excluded the posttraining data and only kept the pretraining data. Finally, we excluded subjects with severe health problems such as cancers. In total, we collected 28 sets of data from 23 articles. These articles all have body composition data, but most of them are in the form of BMI and BW (body weight) instead of $\text{FM}$ and $\text{LM}$. In this case, we first used the prediction equation of

$$\text{FM}\% = 0.01294 \times \text{BMI} + 0.002 \times \text{Age} - 0.114 \times \text{Sex} - 0.08$$

Fat-based method to infer the insulin response thresholds

In, we studied insulin response thresholds from an entirely different perspective, namely, body fat dynamics, and found that $\text{EC}50$ is primarily determined by body composition:

$$\text{lg}\text{EC}50 = g_0 + g_1 \frac{\text{lg}\text{FM}}{\text{LM}}$$

where $\text{FM}$ and $\text{LM}$ are the fat mass and lean mass of a human subject, respectively, in units of kilograms; $g_0$ and $g_1$ are two coefficients. By Equation 8, a person’s $\text{EC}50$ value can be obtained from his/her $\text{FM}$ and $\text{LM}$ values, provided that the values of $g_0$ and $g_1$ are known.

The two coefficients $g_0$ and $g_1$ can be estimated by regression analysis of a number of experimentally measured $(\text{EC}50, \text{FM}, \text{LM})$. Although there are abundant data for $\text{FM}$ and $\text{LM}$, $\text{EC}50$ was a recent definition and was never measured. However, Equation 3 demonstrates that $\text{EC}50$ equals the EC50 of the prototypical $\text{V}(j)$, which was frequently measured in insulin clamp experiments. We performed a literature survey to collect the experimental data of (EC50, FM, LM) in units of pM, kg, kg. To be included, the following conditions must be satisfied. First, EC50 and (FM, LM) must be obtained from the same subject. A lot of data were excluded because either EC50 or (FM, LM) had been obtained but not both. Second, the experimental subjects must be adults in their natural state; otherwise, their body composition may not faithfully reflect their degree of insulin resistance. For example, in, the subjects’ data were obtained both before and after bicycle training. While they became significantly thinner after the training, their EC50 values had almost no change and even increased slightly. Therefore, the thinner body resulting from training was temporary (i.e., not stabilized) and thus unnatural. We, therefore, excluded the posttraining data and only kept the pretraining data. Finally, we excluded subjects with severe health problems such as cancers. In total, we collected 28 sets of data from 23 articles. These articles all have body composition data, but most of them are in the form of BMI and BW (body weight) instead of $\text{FM}$ and $\text{LM}$. In this case, we first used the prediction equation of

$$\text{EC}50 = 454 + 597 \times \text{BW}$$

$$\text{LM} = \text{BW} - \text{FM}$$

and thus unnatural. We, therefore, excluded the posttraining data and only kept the pretraining data. Finally, we excluded subjects with severe health problems such as cancers. In total, we collected 28 sets of data from 23 articles. These articles all have body composition data, but most of them are in the form of BMI and BW (body weight) instead of $\text{FM}$ and $\text{LM}$. In this case, we first used the prediction equation of

The percent differences were only 2%, −5.3%, 2.3%, and −5.1%. Moreover, both methods found the same order:

$$\text{EC}50\ (> \text{group 3} < \text{EC}50\ (> \text{group 1} < \text{EC}50\ (> \text{group 2} < \text{EC}50\ (> \text{group 4})$$

In the following, expression (10) will be abbreviated as $\text{EC}50\ (> (3 < 1 < 2 < 4)$. It is remarkable that the two methods, although entirely different, obtained similar and parallel results.
DISCUSSION

Insulin response thresholds: From theoretical prediction to experimental quantification

The evolution of multicellularity necessitates systemic regulation to coordinate growth and metabolism between tissues, and it is well-known that insulin is an important regulator of these processes. In a multicellular organism, some cell types are more important than others; thus, glucose acquisition must be prioritized. The priority level is achievable if the tissue cells have differential glucose sensitivity. Unfortunately, glucose is only a small molecule and is thus not suitable for signaling. It appears that nature has solved this problem by developing insulin, which might serve as an information carrier for glucose because its concentration changes in parallel with the glucose concentration. As a protein, insulin has a complex three-dimensional structure and is thus suitable for molecular recognition and signal transduction. On the other hand, a switch-on threshold is developed in cells. Through the regulation of the insulin signaling pathway, different cells may have different insulin values, thereby achieving differential glucose acquisition. This regulated energy distribution is crucial for the human brain, which is larger and more sophisticated than the brains of other species. To spare glucose for the brain, the insulin values of the peripheral tissues must be sufficiently large, and this might be the origin of peripheral insulin resistance (PIR). To most individuals, insulin resistance is understood only as the pathologic condition underlying type 2 diabetes, although Neel already proposed in 1962 that PIR evolved as a fine mechanism to preserve glucose for use by the brain during starvation. In addition to this immediate, glucose-related protection, PIR also offers long-term protection by securing fat storage. These theories, however, are inherently speculative and have not been proven.

Since skeletal muscle is a major site of postprandial glucose disposal in the body, we have studied muscle insulin responses in recent years. In addition to the discovery of insulin values, we also found a switch-off threshold, which, together with the larger insulin, renders a bistable response to insulin. We found that a myocyte’s optimal insulin response should be bistable, and more importantly, that a myocyte’s insulin signaling pathway can indeed provide bistability, and bistable model is also supported by the fact that two entirely different methods obtained consistent estimations of the bistable insulin response (Figure 4I). In vivo validation of the bistable insulin response

The cellular bistable model is fully compatible with the organismal level glucose dynamics, as demonstrated by our mathematical modeling of the OGTT in the Mitrakou et al. study. The fitting of \( \hat{G} \) and \( \hat{D} \) was both successful, as manifested not only by the small fitting errors but also by the fact that the fitting was achieved by tuning at most three parameters. Moreover, the data \( \hat{D} \) were not used in the parameter estimation, yet the resultant \( \hat{D} \) can still fit \( \hat{D} \) well. In addition to the excellent data-fitting, the bistable model is also supported by the fact that two entirely different methods obtained consistent estimations of the bistable insulin response (Figure 4I). In the following, group 1 is used as a control because its members were the healthiest. The estimation \( T_{on} \approx 551 \text{ pM} \), although larger than our myotube-based measurement \( T_{on} \approx 300 \text{ pM} \), is actually reasonable. According to the glucose-based model, \( T_{on} \) is in terms of the insulin concentration of blood and should be greater than the insulin concentration of the interstitial fluid bathing the muscles. Indeed, following glucose ingestion, insulin concentrates much more slowly in the interstitial fluid than in the blood due to the delay caused by rate-limiting capillary delivery. By the time the interstitial insulin concentration reaches 300 pM and activates the majority of myocytes, the plasma insulin concentration must have reached a much higher value, namely, 551 pM. In the myotube-based measurement, \( T_{on} \) is in terms of the insulin concentration of the culture medium, which bathes the myotubes and thus mimics the interstitial fluid bathing the muscles well. Therefore, it is quite reasonable that the blood level estimation of \( T_{on} \) is greater than the cellular measurement of \( T_{on} \).

To further demonstrate the reality of the bistable insulin response in vivo, we tested other models of \( V(t) \) to determine whether they can obtain reasonable data fitting. These models are called prototypical, tunable, and polynomial. Together with the physiologic \( V(t) \), there are in total four models under comparison.

- **Physiologic \( V(t) \).** It has been described in detail. The best-fitting \( G(t) \) is shown as the blue curves in Figure 4C.
• **Prototypical** \( V(t) \). It can be modeled by two different ways. The first way is simply to let it be the gray curve in Figure S8A. The second way is to use our \( N = 1000 \) in silico cells setting with \( \Delta I = 0 \) enforced for all the cells; the \( \bar{I}_{on} \) values are still 551, 514, 657, and 740 pM for groups 1, 2, 3, and 4, respectively. In either case, the mathematical model (Equation 4) has only one tunable parameter \( V_{sw} \). By tuning \( V_{sw} \) the best-fitting \( G(t) \) are obtained and shown as the gray curves in Figure S8B. One sees that the best-fittings do not fit at all. The main problem is hyperglycemia, i.e., \( G(t) \) is still very high even at \( t = 300 \). This implies that hysteresis (\( \Delta I > 0 \)) is important for glycemic control.

• **Tunable prototypical** \( V(t) \). It is the same as the prototypical \( V(t) \) except that \( \bar{I}_{on} \), \( \sigma \) are now allowed to be tuned, with an upper bound of 2000 pM for both parameters. The tunable parameters are now \( p = (\bar{I}_{on}, \sigma, V_{sw}) \). The best-fittings are shown as the green curves in Figure S8B. The fittings are generally not good, although better than the gray curves. Moreover, the corresponding \( V(t) \) (the green curves in Figure S8A) are not realistic because both \( \bar{I}_{on} \) and \( \sigma \) are too large.

• **Polynomial** \( V(t) \). It is a polynomial function

\[
V(l(t)) = k_1(l(t)) + k_2(l(t))^2 + k_3(l(t))
\]

(Equation 11)

to be directly used in Equation 4 in lieu of the \( N = 1000 \) cell setting. In total, the model has four tunable parameters \( p = (k_1, k_2, k_3, V_{sw}) \). For the fitting of the OGTT data, the parameter estimation scheme is the same as the above except that the constraint \( V \leq V_{sw} \) is explicitly imposed (this constraint is unnecessary for the prototypical \( V(t) \) because it is naturally satisfied by using \( N = 1000 \) and \( v_{max} = 10^{-5} \)). The best fittings are obtained and are shown as the yellow curves in Figure S8B. Although the fittings are generally acceptable, the corresponding \( V(t) \) (the yellow curves in Figure S8A) are all unrealistic because they are not even monotonically increasing. The rate \( V \) finally decreases as \( l \) increases and even becomes negative when the \( l \) value is still physiologic.

Figure S8 demonstrates that only the physiologic model can fit well with the OGTT data under reasonable parameter values. To make the above intuitive evaluations more precise, we used the Akaike information criterion (AIC)\(^{22} \) to estimate the suitability of each model. In terms of the present application, AIC has the following form

\[
\text{AIC} = M \ln \frac{\nu}{M} + 2|p|
\]

(Equation 12)

where \( M = 10 \) is the number of data points; \( \nu \) is defined by Equation 7; and \( |p| \) is the dimensionality of \( p \), namely, the number of tunable parameters (Table S1). Using Equation 12, we calculated the AIC value of each of the four models combined with each of the four groups; in total 16 AIC values were obtained (Table S2). Clearly, the physiologic \( V(t) \) is far better than the other models because the smaller AIC is, the better the model.

**Peripheral insulin resistance is a good indicator of body fatness**

Equation 8 reveals that \( \bar{I}_{on} \) (which quantifies PIR) positively correlates with body fatness. Therefore, our finding \( \bar{I}_{on} (3 < 1 < 2 < 4) \) implies that group 3 subjects were the thinnest, followed by the subjects of groups 1, 2, and 4. To verify this, we checked Table 1 of\(^{22} \) and found that the body mass index (BMI) of groups 3, 1, 2, and 4 were 24.1 ± 1.7, 24.3 ± 2.1, 29.6 ± 2.6, and 31.2 ± 3.4, respectively, which is precisely in the order BMI(3 < 1 < 2 < 4). The exact parallelism suggests that \( \bar{I}_{on} \) is a potential good biomarker for body fatness. Since BMI has been widely used to measure body fat, a new biomarker seems unnecessary. However, BMI is not always reliable.\(^{24,63} \) For example, a muscular man with little fat may have the same BMI value as a fat man with little muscle, but the former is much healthier than the latter. In this regard, the \( \bar{I}_{on} \) value is more advantageous because according to Equation 8, \( LM \) is taken into account and counteracts \( FM \); it essentially measures “relative fatness,” which better reflects health condition. The ingenuity of Equation 8 is not strange because its development entails many efforts, including the discoveries in,\(^{24} \) the \( \bar{I}_{on} = EC50 \) insight obtained in this article, the laborious experiments behind the data used to estimate \( \gamma_0 \) and \( \gamma_1 \), and so forth.

An ideal biomarker should be simple, just as body temperature can be easily monitored by a thermometer. Obtaining \( \bar{I}_{on} \) through Equation 8 is indeed simple because it only requires \( FM \) and \( LM \), which can be easily measured by dual-energy X-ray absorptiometry or bioelectrical impedance analysis (BIA), which is
inexpensive and can be used as a household appliance. A future BIA may be fitted with a calculator to determine the \( I_{on} \) value immediately, which informs the body’s relative fatness anytime anywhere, making fat reduction easier and healthier.

\( I_{on} \) can also be estimated by the glucose-based method, which is however not suitable for direct biomarker implementation due to the complexity of several hours’ OGTT, blood sampling, and the subsequent mathematical analysis. Nevertheless, the glucose-based method can be used to fine-tune the parameters of Equation 8 during extensive preclinical studies that perform both fat and glucose-based experiments and collect both types of data. Thereafter, Equation 8 will become more sophisticated and more accurate. Equation 8 can be further elaborated by taking into account new factors such as gender and race.

**Peripheral insulin resistance is not a good indicator of glucose tolerance**

Our finding \( I_{on} \) (3 < 1 < 2 < 4) implies that \( I_{on} \) is not a good indicator of glucose tolerance. Group 3 subjects had the smallest \( I_{on} \) corresponding to the best body composition, yet their glucose tolerance was impaired (recall that the larger the group number was, the worse their health was). Therefore, an ideal PIR does not guarantee normal glucose tolerance. Reciprocally, an exacerbated PIR does not necessarily lead to IGT because the muscles eventually uptake normal or even supranormal amounts of glucose due to compensation by hyperglycemia.\(^{23,64}\) This mechanism corresponds to the term \( V(I)G \) in Equation 4, and an overly large \( G \) can actually accelerate glucose uptake. On the other hand, mechanisms such as dynamic compensation (DC)\(^{65}\) would make the pancreas secrete more insulin to maintain the percentage of activated myocytes (peak) during a bout of meal ingestion. It is now widely agreed that hepatic insulin resistance is more responsible for inducing hyperglycemia, and PIR may not play an appreciable role. These viewpoints agree with our philosophy that PIR is first a fine and indispensable mechanism,\(^{2,3,57}\) and it is even healthful to have a PIR that slowly increases during aging.\(^{24}\)

Our quantitative analysis has provided great insights into the health status of the subjects of the Mitrakou et al. study, particularly group 3. Before the analysis, it was unclear that the blood sample data contained information about the PIR. By the glucose-based analysis, we found that group 3 had the smallest \( I_{on} \), but the reason was still unclear. Indeed, the hyperglycemia of group 3 would suggest a large \( I_{on} \) for this group. Based on the fat-based analysis, we again found that group 3 had the smallest \( I_{on} \), and it could be explained by the fact that the perfect body composition of group 3 subjects made their \( I_{on} \) the smallest. The best body composition notwithstanding, group 3 subjects unfortunately developed pancreatic problems (insufficient insulin secretion and thus overly small \( I_{on} \)) and hepatic insulin resistance (overly large \( S_{endo}(t) \)), which caused impaired glucose tolerance.

Finally, it should be emphasized that aberrant (extremely large or small) values of \( I_{on} \) or \( \Delta I \) will of course cause health problems. When \( I_{on} \) becomes so large that the maximal capacity of the pancreas has been reached, the value of \( \eta_{peak} \) will dwindle as \( I_{on} \) further increases. In other words, an overly large \( I_{on} \) will eventually reduce the number of tissue cells participating in insulin-mediated glucose uptake, causing hyperglycemia and contributing to type 2 diabetes. As such, an overly large \( \Delta I \) may lead to severe reactive hypoglycemia and is dangerous. The vanish of hysteresis (\( \Delta I \leq 0 \)), or too small a positive \( \Delta I \), may lead to hyperglycemia, as demonstrated by the gray curves in Figure S8B.

**Insights into the “transitional low blood-glucose state”**

The “transitional low blood-glucose state” refers to the frequent occurrence of low plasma glucose concentration in a 5-hr glucose-tolerance test, during which approximately 50% of the tested normal subjects demonstrate blood-glucose nadirs that are below 50 mg/100 mL (i.e., 2.78 mM).\(^{66-68}\) This phenomenon, if associated with disorders characterized by their postprandial onset, adrenergic mediated symptoms, and other relatively benign causes, is called “reactive hypoglycemia.”\(^{69}\) Reactive hypoglycemia is discovered in mice after the intraperitoneal injection of insulin.\(^{70}\) Although ubiquitous, the transitional low blood-glucose state is poorly understood.

Based on our mathematical proof that bistability is necessary for the fastest restoration of glucose homeostasis,\(^{2}\) we hypothesize that the low glucose state is due to the great efficiency of muscle glucose uptake conferred by the bistable insulin response, of which both \( I_{on} \) and \( \Delta I \) contribute.
A sufficiently large positive $I_{on}$ are to confer a delayed switch-on of muscles’ glucose uptake. This sounds somewhat puzzling: how could “delay” instead accelerate the process? An intuitive explanation is similar to why it is difficult for PIR to cause IGT. The rate of cellular glucose uptake $V/I$G implies that in addition to a large $v$, a large $G$ (high glucose peak) can also increase the speed. To generate a high glucose peak, the muscles should abstain from glucose for a long time while letting it build up in the blood so that the subsequent glucose transportation is the most efficient, which can be achieved by building a sufficiently large $I_{on}$ into each myocyte. Remarkably, a sufficiently large $I_{on}$ is also required to avoid fasting hypoglycemia (Figure 1A). Taken together, bistability is a win-win solution for, rather than a commonplace trade-off between, the two seemingly opposing requirements of insulin action: safety (avoiding hypoglycemia) and rapidity (avoiding hyperglycemia).

A sufficiently large positive $\Delta I$ is to confer a delayed switch-off of muscles’ glucose uptake so that it can be sufficiently sustained. A feature of the glucose-insulin system is that the peak insulin concentration is generally close to the cells’ $I_{on}$ values, which is demonstrated by the comparison between $I_{peak}(=459, 551, 388, 608, \text{as shown in Figure 4B})$ and $I_{on}(=551, 657, 514, 740, \text{as shown in Figure 4E})$. Note that $I_{on} < I_{peak}$ for all the activated cells, which implies that $I_{peak}$ and $I_{on}$ are even closer. When a typical myocyte is activated by the rising insulin, the insulin concentration is precisely equal to the cell’s $I_{on}$ and is thus only slightly smaller than $I_{peak}$; this implies that the rising insulin will soon reverse its direction. In the absence of hysteresis ($\Delta I = 0$, i.e., $I_{off} = I_{on}$), the myocyte will be switched off soon after it is switched on because the falling insulin concentration will soon drop below $I_{off}$. To delay the switch-off, $I_{off}$ must be sufficiently smaller than $I_{on}$, that is, $\Delta I$ must be sufficiently large. To demonstrate the importance of hysteresis, we solved Equation 4 in the absence of hysteresis (i.e., let $\Delta I = 0$). The obtained $G(t)$ is shown as the gray curves in Figure 4B, which do not fit the data $G$ (the black dots). They are characterized by prolonged hyperglycemia. In the presence of hysteresis, the glucose concentrations decrease so rapidly that they even drop below the baseline $G_0$ (see the undershoot of $G(t)$ in Figure 4C). Figure 4L presents $V/I$G as a function of time for both $\Delta I = 0$ (the gray curves) and $\Delta I = 354, 351, 259,$ and 454 (the blue curves). For the former, $V/I$G decreases rapidly soon after it reaches the peak, which explains the hyperglycemia as demonstrated by the gray curves in Figure 4B.

**Limitations of the study**

The present study focused on the accurate quantification of insulin response thresholds, including the reliable estimation of $I_{on}$ and $\Delta I$ based on the fitting of the OGTT data with the glucose-based mathematical model, for which the model should be as simple as possible and the number of unknown parameters should be as small as possible. To this end, we chose not to model the insulin dynamics by an additional ODE to be coupled with Equation 4 because that will introduce a good number of new mechanisms and unknown parameters that are not directly related to the muscles’ insulin response. Although not modeled, the insulin dynamics have been faithfully considered in Equation 4 because the term $I(t)$ was constructed from blood sample insulin concentrations.

Our choice inevitably leads to a limitation: the present mathematical model is specialized and not suitable for a complete study of glucose-insulin homeostasis. The insulin dynamics are shaped by important and complex physiologic processes such as various causes of insulin degradation and the pancreatic insulin secretion in delicate response to glucose stimulation. The latter is particularly interesting given that a new mechanism “dynamic compensation (DC)” (namely, the regulated glucose dynamics reciprocally controls the pancreatic cell mass) was discovered to explain the precise and robust glucose dynamics in the face of the wide variation in the physiological parameters. These interesting mechanisms were not modeled in the present article because they will introduce a good number of unknown parameters.

To overcome the limitation, we will develop a relatively complete mathematical model for glucose-insulin homeostasis in future, which will be based on the present model (bistable insulin response) and embody the aforementioned physiologic mechanisms. For example, in the new model, we will use DC to effect the increase in the pancreatic $\beta$ cell mass and in the insulin secretion. $I_{on}$ slowly increases during aging or weight gain; thus, the peak insulin concentration has to increase accordingly to maintain a certain percentage ($\eta_{peak}$) of the activated cells. Thanks to DC, which accordingly increase the pancreatic $\beta$ cell mass, the increase in $I_{peak}$ is a natural consequence of the increased insulin secretion. The new mathematical model will be beneficial to future in-depth studies of intermediary metabolism and the related physiologic processes.
For the experimental component, the present study focused on individual insulin signaling proteins and did not examine the dynamics of insulin signaling pathway at other biological levels such as transcriptome, which represents another limitation. Fortunately, recently there emerged several excellent trans-omics analyses on insulin signaling and the related pathways and tissues.\textsuperscript{70–77} These studies have provided a holistic perspective, which led to important discoveries. They even shed light on the present study, such as the phenomenon of reactive hypoglycemia.\textsuperscript{70}

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
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  - Western blotting
- **QUANTIFICATION AND STATISTICAL ANALYSIS**

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105561.

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**AUTHOR CONTRIBUTIONS**

Conceptualization: GW, Data curation: JA, GW, Formal Analysis: JA, GW, Funding acquisition: GW, LC, RY, GL, Investigation: JA, YH, SH, WL, CC, RG, IAB, QJ, YY, GC, YZ, RY, GL, LC, GW, Methodology: JA, GW, Project administration: RY, GL, LC, GW, Resources: RY, GW, Supervision: GW, LC, Validation: JA, GW, Visualization: JA, GW, Writing: JA, LC, GW.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

**INCLUSION AND DIVERSITY**

We support inclusive, diverse, and equitable conduct of research.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb | Cell Signaling Technology | Cat#4060, RRID:AB_2315049 |
| Akt (pan) (11E7) Rabbit mAb | Cell Signaling Technology | Cat#4685, RRID:AB_2225340 |
| β-Actin Rabbit mAb (High Dilution) | ABclonal | Cat#AC026, RRID:AB_2768234 |
| Goat Anti-Rabbit IgG H&L (HRP) | Abcam | Cat#ab6721, RRID:AB_955447 |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| DMEM, high glucose | Gibco | Cat#11965092 |
| GlutaMAX | Gibco | Cat#35050061 |
| Insulin-Transferrin-Selenium (ITS) | Gibco | Cat#41400045 |
| Acrodisc® Syringe Filters with Supor® Membrane, Sterile - 0.45 μm, 25 mm | Pall Corporation | Cat#4614 |
| Plasmocin® prophylactic | Invivogen | Cat#ant-mpp |
| UltraPure™ DNase/RNase-Free Distilled Water | Invitrogen | Cat#10977015 |
| Penicillin-Streptomycin | Gibco | Cat#15070063 |
| **Critical commercial assays** |        |            |
| Insulin | Sigma-Aldrich | Cat#I5500-50MG |
| Dimethyl sulfoxide | Sigma-Aldrich | Cat#D2650 |
| Pierce™ BCA Protein Assay Kit | Thermo Scientific | Cat#23227 |
| M-PER™ Mammalian Protein Extraction Reagent | Thermo Scientific | Cat#78501 |
| Halt™ Protease and Phosphatase Inhibitor Cocktail, EDTA-free (100X) | Thermo Scientific | Cat#78441 |
| Immobilon®-P PVDF Membrane | EMD Millipore | Cat#PVH00010 |
| Can Get Signal® Immunoreaction Enhancer Solution | TOYOBO | Cat#NK101/NYPBR |
| Clarity™ Western ECL Substrate | Bio-Rad | Cat#1705060 |
| Tris Buffered Saline with Tween®20 | Takara Bio | Cat#T9142 |
| Bovine serum albumin (BSA) | VWR Life Science | Cat#N208-10g |
| Gibco™ Fetal Bovine Serum, qualified, Australia | Gibco | Cat#10099141 |
| Gibco™ Horse Serum | Gibco | Cat#26050088 |
| Costar® 6-well Clear TC-treated Multiple Well Plates | Corning | Cat#3516 |
| Corning® Cryogenic Vials | Corning | Cat#430659 |
| Corning® 15 mL centrifuge tubes | Corning | Cat#430790 |
| Corning® 96-well Clear Flat Bottom Polystyrene TC-treated Microplates | Corning | Cat#3599 |
| Corning® 100 mm TC-treated Culture Dish | Corning | Cat#430167 |
| FluoroDish™ | WPI | Cat#FD35-100 |
| **Deposited data** |        |            |
| pEevee-iAkt-NES (7033 bp) | Miura, H., Matsuda, M. & Aoki, K. Development of a FRET biosensor with high specificity for Akt. Cell Struct. Funct. 39, 9–20, (2014). | https://doi.org/10.1247/csf.13018 |
| pLVX-IRES-Neo | Takara Bio | Cat# 632181; https://benchling.com/s/seq-q46zzYCF0swL4un0t28/edit |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Guanyu Wang (wangguanyu@cuhk.edu.cn).

Materials availability
This study did not generate new unique reagents.

Data and code availability
- Raw FRET data and original western blot images have been deposited at Mendeley and are publicly available as of the date of publication. The DOI is listed in the key resources table.
- All original code has been deposited at GitHub and is publicly available as of the date of publication. DOIs are listed in the key resources table.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines
C2C12, HEK293, and HepaG2 cell lines were purchased from the National Collection of Authenticated Cell Cultures and were cultured in an incubator at 37°C and 5% CO₂. The cells were routinely cultured and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine
serum (FBS) from Gibco (Invitrogen Co., USA), GlutaMAX (1:100 dilution), 50 I.U./mL penicillin, and 50 μg/mL streptomycin (Gibco-Invitrogen Co., USA) and 2.5 μg/mL Plasmocin prophylactic (InvivoGen). C2C12 myoblasts were routinely maintained in growth medium at 60% confluence before subculture, and the number of passages was kept low (up to 15 passages) to maximize differentiation potential. Cells were cryopreserved in Corning® Cryogenic Vials (Corning Inc, Corning, NY, USA) containing 10% (v/v) Dimethyl sulfoxide (DMSO) (Sigma-Aldrich), 70% (v/v) FBS, and 10% (v/v) DMEM. For short-term storage of up to 30 days, cells were stored in a low-temperature freezer at −80°C. For long-term storage, cells were placed in liquid nitrogen (LN2).

Antibodies
The antibodies used in this study are as follow: phospho-Akt (Ser473) (D9E) XP® Rabbit mAb (1:2000, Cell Signaling Technology, MA, USA, 2139S); Akt (pan) (11E7) Rabbit mAb (1:1000, Cell Signaling Technology, MA, USA, 2139S); β-Actin Rabbit mAb (High Dilution) (1:100000, Abclonal Technology Co., Ltd., China); Goat Anti-Rabbit IgG H&L (HRP) (1:500, Abcam, Cambridge, MA, USA).

FRET biosensors
We tested the insulin (Sigma-Aldrich, USA) response of 50 + 46 = 96 myotubes by using live-cell FRET imaging with the Akt biosensor (pEevee-iAkt-NES), which contains an optimized Akt substrate sequence derived from human glycogen synthase kinase 3β (GSK3β) (SGRPRTTTFADSCKP), whose phosphorylation is detected by a highly efficient FRET pair: the enhanced cyan fluorescent protein (ECFP; the donor) and the brightest variant of yellow fluorescent protein (YPet; the acceptor).78 pEevee-iAkt-NES is an improved version of Eevee-Akt shown to be highly specific for and sensitive to the detection of Akt phosphorylation in HeLa and Cos7 cells.27 The plasmid was kindly provided by Prof. Kazuhiro Aoki, National Institute for Basic Biology, National Institutes of Natural Science, Okazaki Institute for Integrative Bioscience SOKENDAI (The Graduate University for Advanced Studies). A lentiviral expression vector pLVX-IRES-Neo (Takara Bio USA) (Figure S6) based on HIV-1 was used to replace the backbone of pEevee-iAkt-NES for stable expression of FRET biosensor in C2C12 derived myotubes.

METHOD DETAILS

Transfections
Fourth-generation lentiviral packaging systems (Lenti-X packaging single shots) (Takara Bio USA) were used for the packaging and production of the lentivirus in HEK293T cells (Figure S7). One day prior to transfection, HEK293T cells were seeded at a density of 4–5×10⁶ in 8 mL of culture medium in Corning® 100 mm tissue culture (TC) treated culture dish (Corning Inc, Corning, NY, USA) and incubated at 37°C and 5% CO₂ overnight. When the cells reached 80–90% confluence, 7 μg of lentiviral vector plasmid DNA was diluted in 600 μL of sterile water and mixed thoroughly by vortexing. Then, diluted DNA was added to a Lenti-X packaging single-shot tube. The nanoparticle complexes were vortexed at high speed for 20 s and incubated at room temperature for 10 min. After incubation, 600 μL of nanoparticle complex solution was dropwise applied to the 8 mL cell culture. The plates were gently rocked back and forth to mix well, and they were then placed in the cell culture incubator at 37°C supplied with 5% CO₂. After 4 h, 6 mL of fresh complete medium was added, and the plates were incubated for an additional 24–48 h.

Forty-eight hours after transfection, the lentivirus-containing supernatant was collected in 15 mL conical tubes and stored at 4°C. The supernatant was centrifuged briefly (500×g for 10 min) and then filtered through a 0.45 μm Acrodisc® Syringe Filters (Pall Corporation) to remove cell debris. Three volumes of the clarified supernatant and one volume of Lenti-X concentrator (Takara Bio USA, Inc.) were mixed by gentle inversion, and the mixture was incubated at 4°C for 30 min overnight. After incubation, the sample was centrifuged at 1500×g for 45 min at 4°C, and an off-white pellet was formed. The supernatant was carefully removed, and the pellet was titrated and stored at −80°C in single-use aliquots for later experiments. Lenti-X GoStix Plus (Takara Bio USA, Inc.) was used to determine the viral titer. A 20 μL aliquot of supernatant was added to a GoStix cassette. After 10 min of incubation, the test and control bands appeared, indicating the presence of lentiviral p24. Lenti-X GoStix Plus App for smartphones (Takara Bio USA, Inc.) was used to capture the bands with proper alignment and focal length by using the outline of the cassette in the scanning window; the GoStix value (ng/mL p24) was then displayed. The actual infectious units per milliliter (IFU/mL) were calculated from the reference value and the GoStix value of an unknown stock. The above procedure was repeated 72 h posttransfection to harvest more viruses.
C2C12 myoblasts seeded in Corning® 96-well Clear Flat Bottom Polystyrene TC-treated Microplates (Corning Inc, Corning, NY, USA) were transduced with the optimum viral titer. The transduction efficiency was confirmed by observation under a confocal microscope.

**C2C12 myotube differentiation and starvation**

Mouse-derived C2C12 myoblasts were plated in 35-mm dishes with cover glass bottom (FluoroDish, Glass Diameter ø: 23.5 mm) from WPI. To induce myotube fusion, cells were allowed to reach 80% confluence before being moved to differentiation media (DM) (DMEM high glucose, supplemented with 2% (v/v) horse serum (HS) and 1% (v/v) insulin-transferrin-selenium (ITS-G) (Gibco-Invitrogen Co., USA). DM was refreshed every other day for five days. Myotube differentiation was observed under Phase-contrast microscopy (Nikon, Tokyo, Japan). Differentiated myotubes were overnight starved in serum-free DMEM (high glucose), containing 0.1% (w/v) bovine serum albumin (BSA) (VWR Life Science, USA).

**FRET data quantification and analysis**

FRET imaging and analysis were essentially performed as previously reported. A1 Laser Scanning Confocal Microscope (A1, Nikon, Tokyo, Japan) with a live-cell system (Stage Top Incubator from TOKAI HIT) and spectral detector (DUS; Nikon) was used to image a myotube expressing both ECFP (the donor) and YPet (the acceptor), with the sensitized emission (SE) technique applied. Then, the background correction was performed by measuring the average intensity of a cell-free region of interest (ROI) and subtracting this intensity from each pixel gray value within the image. The background-corrected image can be inspected through three filter sets: $I_{DD}$ (donor excitation-donor emission) (Figure S1 A), $I_{AA}$ (acceptor excitation-acceptor emission) (Figure S1 B), and $I_{DA}$ (donor excitation-acceptor emission). Note that $I_{DA}$ was also called $FRET_{raw}$.22 The corrected FRET image (Figure S1 C) was determined by

$$FRET_{corrected} = FRET_{raw} - d \cdot I_{DD} - a \cdot I_{AA}$$  \hspace{1cm} (Equation 13)

where $d$ and $a$ were the donor and acceptor bleedthrough coefficients in the $I_{DA}$ filter set, respectively; they had been determined by the software package NIS-Elements-AR (Nikon, Tokyo, Japan) with myotubes expressing either the donor (for $d$) or the acceptor (for $a$). To enhance the contrast, the FRET efficiency image (Figure S1 D), defined by

$$FRET_{efficiency} = \frac{FRET_{corrected}}{I_{DD}}$$  \hspace{1cm} (Equation 14)

was used. The vertical axis of an insulin-FRET response curve (e.g., Figure 3 D1) is quantified by the total $FRET_{efficiency}$ of a single myotube.

**Western blotting**

C2C12 myoblasts were seeded in Costar®6-well Clear TC-treated Multiple Well Plates (Corning Inc, Corning, NY, USA) and cultured with growth medium for 2–3 days. When myoblasts reached confluence, the growth medium was replaced with differentiation medium (2% horse serum) (Gibco, Invitrogen Co., USA). The cells were routinely observed under a microscope for morphological changes. Myotubes were starved for four hours after being rinsed with cold GibcoTM phosphate buffered saline (PBS) (Gibco, Invitrogen Co., USA). The cells were then stimulated with different concentrations of insulin. We divided the myotubes grown in 6-well plates into two groups. In the first group, the starved cells were directly stimulated with different concentrations of insulin for 20 min. In the second group, the cells were treated with 500 pM insulin for 20 min to fully activate Akt and then re-stimulated with different concentrations of insulin for 20 min.

To detect Akt phosphorylation in cell population, myotubes were rinsed with cold PBS. Approximately 400 µL of M-PER® Reagent (Mammalian Protein Extraction Reagent, Thermo Scientific) supplemented with 10 µL/mL HaltTM Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific) was added in each 6-well plate and was gently shaken for 5 min. The homogenized samples were incubated on ice for 5 min then spun at ~ 14, 000×g for 10 min to pellet the cell debris. The clear supernatant was collected and the protein concentrations in the cell lysates were measured using a PierceTM BCA Protein Assay (Thermo Fisher Scientific, Inc, MA, USA). For immunoblotting, the extracted protein samples were denatured at 100°C for 10 min. Twenty micrograms (20 µg) of each protein sample was loaded into a gel and...
separated by high-performance precast mini polyacrylamide gels 4–20% (ExpressPlus PAGE Gel) (GenScript USA Inc.). Protein transfer was carried out using Immobilon®-P PVDF membrane (polyvinylidene fluoride Membrane, Pore size, 0.45 µm) (EMD Millipore Co., Darmstadt, Germany). The membranes were first blocked in Can Get Signal® Immunoreaction Enhancer Solution (Toyobo Co., Ltd., Japan) for two hours at room temperature before being incubated with primary antibody overnight at 4°C. All primary antibodies were diluted in Can Get Signal® Solution 1 (Toyobo Co., Ltd., Japan). Membranes were washed with Tris-Bufered Saline with 0.1% Tween® 20 (TBS-T) (Takara Bio USA) three times for 10 min. Secondary antibodies were diluted in Can Get Signal® Solution 2 (Toyobo Co., Ltd., Japan) and incubated for 1 h at room temperature. Finally, the immunoblots were washed three times with 1xTBS-T for 30 min before developed with Clarity Western ECL Substrate (Takara Bio USA).

Quantification of band intensities of pAkt, total Akt, and β-Actin was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The latter two were used as protein-loading controls to normalize the protein concentration variation across different samples.

QUANTIFICATION AND STATISTICAL ANALYSIS
Statistical analysis was performed by using GraphPad Prism 9 (GraphPad Software, La Jolla, CA). The data were presented as mean ± SD. Two-tailed unpaired Student’s test was used for comparison of n = 2 groups. p < 0.05 was considered statistically significant.