Seminarios de diabetes

Euglycemic hyperinsulinemic clamp

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Abstract

Insulin is the principal hormone of glucose metabolic regulation. Reduced glucose responses to insulin constitute an underlying feature of type 2 diabetes. In addition, insulin resistance is a common condition related with the metabolic syndrome and strongly associated with an increased risk of cardiovascular disease. The importance of the insulin-resistant phenotype for the assessment of cardiovascular risk and response to intervention is increasingly being recognized. Therefore, there is a need for an accurate and reproducible method for measuring insulin resistance in vivo. The euglycemic hyperinsulinemic clamp (EHC) is currently the gold standard method available for the determination of whole glucose uptake in response to insulin, from which several derived indices of insulin sensitivity are obtained. The clamp technique is both expensive and complex to undertake and has prompted the use of surrogate methods, notably the insulin tolerance test and frequently sampled intravenous glucose tolerance test. Indices may be derived from these methods and correlate well with those derived from clamp studies. However, important limitations of these procedures are that not only does insulin sensitivity change in pathological situations, but also in normal physiology. Variations also occur in time–depending on the physiological state of the individual or following diurnal rhythms. In conclusion, the quantitative assessment of insulin sensitivity with EHC is not used for routine clinical purposes, but the emerging importance of insulin resistance has led to its wider application to research studies that have examined its pathogenesis, etiology and consequences.

Keywords: insulin action, insulin resistance, clamp technique.

Introduction

Insulin resistance is an independent risk factor for cardiovascular disease and Type 2 diabetes. Hypertension, dyslipidemia and obesity are often found in association with insulin resistance. Insulin resistance is widely regarded as an important component of Type 2 diabetes. Some authors have used the term "insulin sensitivity", which is the reciprocal of insulin resistance, because the term "normal insulin sensitivity" is more consequential than "normal insulin resistance". Glucose tolerance is an expression of the efficiency with which homeostatic mechanisms restore glycemia to basal levels after a perturbation. Clinically, the most common assessment is following an oral glucose load, a surrogate for a more physiological meal. The homeostatic response includes an increase in the insulin levels followed by insulin-dependent processes that lower glycemia.

Theoretically, the oral glucose tolerance test should yield an estimate of insulin sensitivity, if insulin concentrations are measured. However, after oral glucose or meals, the increments in insulin do not depend entirely on glucose, but also on such factors as gut hormones and neural stimulation, the insulin response deviates from the purely glucose-dependent pattern.[1] Different tissues may have very distinct sensitivities to the actions of insulin, and such measures may not correlate well. A variety of methods are available for assessing insulin sensitivity. The choice of test depends on the purposes of the investigator (clinical or research), available resources (research staff expertise, equipment, funds) and the information required (insulin sensitivity alone or information on β-cell function). In this review we have focused on glucose metabolism and the biological effect of insulin assessed the glucose clearance in the periphery and insulin suppression of hepatic glucose output by the euglucemic hyperinsulinemic clamp (EHC).

Assessment of insulin sensitivity

Insulin resistance cannot be measured separately. Terminology describing its occurrence must specify the cir-

cumstances under which it was assessed as well as the method. For example, basal insulin resistance following an overnight fast is not the same as stimulated insulin resistance.[2] Insulin response *in vivo* will augment over short periods of time because receptor occupancy is a function of exposure as well as the prevailing insulin. But it may also decrease as a function of time because of the processes of down-regulation. In addition, if insulin resistance is assessed by examining metabolic clearance of glucose, then it should be aware that glucose clearance is a function of glucose concentration due to glucose-mediated glucose uptake. Finally, after exercise muscle glycogen stores are depleted and the capacity to clear glucose from the circulation increases (table 1). In consequence, subjects should undergo meticulous preparation in order to minimize acute insulin perturbations and to obtain meaningful results.

Smoking and alcohol consumption should be avoided for at least 24 h prior to the test. The former can lower insulin sensitivity and the latter impairs gluconeogenesis. Any activity that may elevate catecholamine concentrations should be avoided (i.e. caffeine consumption on the morning of the test and stress, discomfort and excessive noise during testing). Physical activity increases peripheral glucose disposal independently of insulin action and should be kept to a minimum on the morning of the test and during the test itself.

Dynamic function tests
Euglycemic-hyperinsulinemic clamp
There is general agreement that the glucose clamp technique, particularly the euglycemic clamp, is the best method available for the measurement of insulin action. The EHC has been referred to as the "gold standard" method for measuring insulin resistance under a wide variety of circumstances. It allows researchers to quantify β cell sensitivity to glucose and the sensitivity of body tissues (muscle, fat, and liver) to insulin. The clamp test was developed by Andres, Swerdloff, Pozefsky and Coleman in 1965, in response to perceived limitations of the prevailing tests, the oral glucose tolerance test and the rapid intravenous glucose tolerance test. By analogy with the voltage clamp method used in the neurosciences, this technique was further developed and widely studied by DeFronzo et al.[3] The EHC is rarely performed in clinical care, but its use is increasing mainly for medical research purposes, for example to assess the efficacy of new medications.

Procedures
The EHC is performed in the morning following a 12-hr fasting period. Technically, there are three major requirements for successfully clamping plasma glucose level. First, the procedure for the clamp begins with the insertion of two intravenous lines. One is placed in an antecubital vein for glucose and insulin infusion; the other for frequent blood samples. The latter, if arterial catheterization is not feasible, must drain arterialized blood. This situation is commonly accomplished by retrograde cannulation of a wrist or hand vein while heating the hand at 60-70 °C. The reason for that is due the stimulated tissue glucose uptake by insulin and thereby the important increases in the arterio-venous difference in plasma glucose level, typically from 0.1-0.2 to 1.0-2.0 mmol/l. Thus, if one used venous glucose readings to adjust the exogenous glucose infusion rate, the corresponding arte-

Table 1. Interpretation of quantitative estimates of insulin resistance		
Descriptor	**Mechanism by which insulin resistance is affected**	**Effect on insulin resistance**
Fasting or stimulated	Following stimulation, insulin response augments with time. Basal and stimulated insulin resistance are not the same	Estimates of stimulated insulin resistance may be lower than estimates of basal insulin resistance
Hyperglycemia	Increased glucose-mediated glucose uptake. Increased β-cell stimulation	Estimates of insulin resistance derived from a hyperglycemic clamp may be lower than estimates obtained at normoglycemia
Exercise exposure	Increased glucose-mediated glucose disposal. Increase insulin-mediated glucose uptake	Insulin resistance is decreased following exercise
Time of day	Increased cortisol and FFA levels at 8-9 a.m. leading to increased HGO	Insulin resistance is increased in the morning
Stress	Increased adrenaline and cortisol levels leading to increased HGO	Insulin resistance is increased by stress
FFA: free fatty acids; HGO: hepatic glucose output.		

rial glucose levels would rise as insulinization proceeds. Exogenous glucose would be overinfused, the clamp would be variably hyperglycemic rather than euglycemic and insulin sensitivity would consequently be overestimated by an unpredictable amount.

Second, well calibrated pumps must be used for the infusion of glucose and insulin in order to achieve the desired hyperinsulinemic plateau and to calculate glucose infusion rates accurately. The goal of the insulin infusion is to raise the plasma insulin concentration to a plateau approximately of 100 µU/ml of insulin over basal insulin levels to approximate usual postprandial levels and to maintain it at that plateau for a period of 2 to 4 hr. For purposes of comparison, clamp data must be standardized. First, the insulin infusion should be administered per unit of body surface area to avoid over insulinization of obese individuals (BMI >30 kg/m^{-2}). Thus, 6-12 pmol $\times$ min $\times$ kg^{-1} (~1-2 mU $\times$ min^{-1} $\times$ kg^{-1}) insulin (i.e. the most common clamp dose), should rather be calculated as 0.24 nmol $\times$ min^{-1} $\times$ m^{-2} (40-80 mU $\times$ min^{-1} $\times$ m^{-2}) for general application.[4] The insulin infusate is prepared with regular insulin in isotonic saline, to which 2 ml of the subject's blood or 5 cc of 25% albumin is added to prevent the absorption of the insulin by the plastic IV bag. DeFronzo, Tobin, and Andres[3] (1979) noted that "the priming infusion (of insulin) can be adjusted upward or downward on a proportional basis to achieve any desired level of plasma insulin concentration". The objective is to raise insulin to a level that will suppress hepatic glucose production. By doing this, insulin action is measured *in vivo* under comparable conditions of stimulus (the plasma insulin concentration) and substrate (the plasma glucose concentration).

Third, concentrations of glucose in the arterialized blood samples must be measured on line (i.e. within 2-3 min) in order to make the necessary adjustments to the exogenous glucose infusion rate. For this, to maintain this plateau and to keep the individual euglycemic, variable amounts of 20% dextrose must be infused.[3] Insulin dosage is at a fixed level and the amount of dextrose infused will depend on the subject's insulin sensitivity. On the other hand, maintaining plasma glucose concentrations at approximately 95 $\pm$ 2 mg/dl (euglycemia) requires frequent blood sampling (every 3-5 min) for plasma glucose concentrations, which are measured with a precise glucose analyzer. The glucose analyser device must be frequently calibrated, both before and during the clamp

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Table 2. Formula for glucose infusion for the euglycemic clamp

Initially, begun at 4 min after the initiation of the insulin infusion at 2,0 mg/(kg min), then:

$$Si = \frac{(Gd\text{-}Gi) \times 10 \times (0.19 \times body\ wt\ in\ kg)}{Ginf \times 15} \times PF + [(SMi\text{-}2) \times Gb/Gi) \times FMi\text{-}1)]$$

where Si = the glucose concentration at any specified point of time i

Gd = the desired glucose concentration

Gi = the glucose concentration at any specified point of time i

Ginf = the glucose infusate concentration in mg per ml

PF = an infusion pump factor

SMi = the setting for the metabolic component of the infusion rate

Gb = the basal glucose concentration

(measured) plasma glucose level from the goal glucose level

Note: The Ginf $\times$ 15

FMi = a correction factor that compensates for the deviation of the actual

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FMi-1 for the initial (10 min) plasma glucose concentration is assumed to be 1,00; after that time the computed FMi-1 is used.[3]

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study, against a well-preserved standard. When a steady state is attained, the exogenous glucose infusion rate equals the amount of glucose disposed of by all the tissues in the body and thus provides a quantitation of overall insulin sensitivity. The desired plasma glucose level should be attained within 60-90 min. There are a number of ways to control the glucose infusion rate during a clamp study. The most popular and simplest way is manual infusion adjustments based on operator experience. Generally, the rate and amount of glucose infused is determined by trial and error by the principal investigator. Alternatively, a mathematical algorithm can be used. To use this method, after the first 20 min of the test, glucose measurements are obtained at 5-min intervals; the rate of glucose infusion is adjusted according to the formula noted in table 2.

Irrespective of the method used to determine rates of glucose infusion, the results of the test are then analyzed by computing *M* values for each 20-min interval of the test. A value of *M* reflects the amount of glucose metabolized[3] and is reported in values of kg $\times$ min. *M*, is calculated using the following formula:

$$M = INF - UC - SC,$$

where INF is the glucose infusion rate, UC is equal to the amount of glucose lost in the urine, and SC is a glu-

cose distribution space correction. To determine insulin sensitivity, an M/I ratio is calculated. I is the plasma insulin response, and an M/I ratio is a "measure of the quantity of glucose metabolized per unit of insulin concentration and is thus a reasonable index of tissue sensitivity to insulin".

Therefore, insulin sensitivity will be measured as an insulin sensitivity index (ISi), calculated by dividing the average glucose infusion rate (referred to whole body or to lean body mass and expressed in nanomoles per minute) by the average plasma insulin concentration (picomoles per liter) at 140, 160, and 180 min of the EHC. The kinetics of peripheral insulin metabolism could be evaluated by measurement of metabolic clearance rate (MCR) of exogenously administered insulin during the EHC

MCR (ml $\times$ m^{-2} $\times$ min^{-1}) = IIR (μUI $\times$ m^{-2} $\times$ min^{-1})/[ICs - ICe (μU/ml)], where IIR= insulin infusion rate during the EHC; ICs = steady state of mean insulin concentration during the EHC (140, 160, and 180 min); ICe= endogenous steady-state insulin concentration assessed from the mean of two fasting insulin concentration per (steady-state C-peptide levels during the EHC/mean of two fasting C-peptide levels).[5]

Even though in strict terms the glucose infusion rate never reaches a steady state. Its average value during the final 40 min of a 3 h study is a satisfactory insulin-sensitivity index for ordinary purposes. It must be noted that the exogenous glucose infusion rate equals whole-body glucose disposal only when endogenous glucose output is nil. Otherwise, total glucose disposal is the sum of endogenous and exogenous glucose entry. After insulin administration, endogenous glucose release is effectively suppressed (figure 1), so that, after 30-50 min, essentially all the glucose metabolized is of exogenous origin. Inhibition of endogenous glucose output is insulin-dependent. Finally, blood samples for glucose, insulin, C-peptide, plasma lactate, and serum free fatty acid (FFA) will be collected at fasting (–5) and 120, 140, 160, and 180 min. The average value for the period from 140 to 180 will be used to calculate the rates of whole-body glucose uptake.

In summary, if an individual is relatively insulin sensitive, larger amounts of glucose will need to be infused (≈6-12 mg/kg/min) for a given amount of insulin to keep
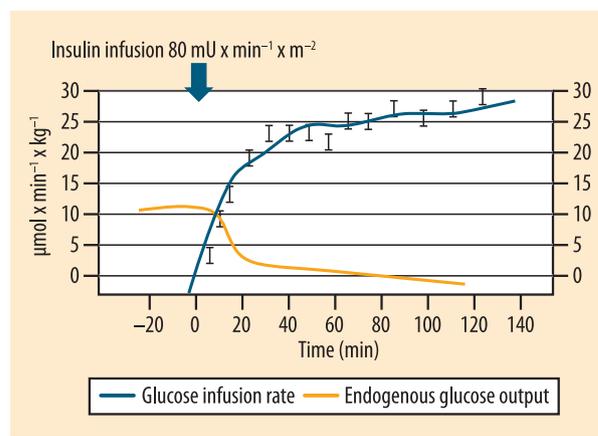

Figure 1. Exogenous glucose infusion and endogenous output during a standard euglycemic-hyperinsulinemic clamp. It must be noted that exogenous glucose infusion rate equals whole-body glucose disposal only when endogenous glucose output is nil; otherwise, total glucose disposal is the sum of endogenous and exogenous glucose entry. When the hyperinsulinemic clamp is established at high doses (80 mU $\times$ min^{-1} $\times$ m^{-2}), the hepatic glucose production is suppressed within about 40 min of starting the insulin infusion.

the individual's blood glucose in a euglycemic range. If an individual is relatively insulin resistant, smaller amounts of glucose will need to be infused for a given amount of insulin to achieve euglycemia (≈1-5 mg/kg/min).

Limitations/variations
The problems of clamp techniques include their artificiality, the supra-physiological plasma levels of insulin achieved, the limitations of clamp stability, the difficulty of repeated studies, and the relatively high coefficient of variation of the outcome measures. Overall, the original EHC and its variations offer exquisite precision for the parameters that it measures. In addition, the clamp tests are all labor intensive, technically difficult to perform, and expensive. A researcher needs to gain experience by working with someone proficient in administering clamp tests before attempting to use this method. Another limitation of the EHC is that insulin sensitivity is measured only under a steady-state condition, and therefore, the test does not realistically portray dynamic conditions such as those occurring after normal meals.

Anthropometry and body composition
Stature and weight were measured twice and the mean of the paired values was computed. Stature will be measured to 0.1 cm on a stadiometer and weight will be measured to 0.1 kg on a balance scale. Bioelectrical impedance will be

Figure 2. Indirect calorimetry coupled during the steady-state of the euglycemic hyperinsulinemic clamp. The substrate disappearance and energy production rate should be evaluated in fasting and steady-state during euglycemic clamp. Nonoxidative glucose metabolism will be determined by subtracting the glucose disappearance (oxidation) rate, measured by indirect calorimetry, from the whole insulin-mediated glucose intake during the EHC4

measured with a multi-frequency bioelectrical impedance analyzer on the second day of the experiment in fasting state with empty bladders. This method exploits the difference in the electrical conductivity of fat vs. fat-free tissue to estimate lean body mass (LBM). Body fat mass will be determined by subtracting LBM from body weight.[6]

Indirect calorimetry

The energy production (EP) and respiratory quotient (Rq) should be measured by continuous indirect calorimetry using a computerized, flow-through canopy gas analyzer system. The system will be calibrated with standard gas mixtures and calibration verified at intervals throughout the collection periods. These systems have a precision of less than 1% measuring the rate of oxygen consumption (VO_2) or carbon dioxide production (VCO_2). Energy expenditure will be measured after an equilibration period of 10 min. The gas-exchange rate will be recorded for 30 min in the fasting state and during the last 30 min of the euglycemic clamp, between 150 and 180 min (figure 2). From knowledge of the VO_2 and VCO_2 (L/min) and the urinary nitrogen (N, g/min), glucose (G), lipid (L), and protein (P) disappearance rates (g/min) can be calculated according to calorimetric formulas.[7] The protein oxidation rate was estimated from urinary nitrogen excretion (1 g nitrogen= 6.25 g protein) in 12 h urine collections from the over night previous to

Table 3. The equations used in the indirect calorimetry calculations are listed below
1. Glucose oxidation $(g \cdot kg^{-1} \cdot d^{-1}) = [(4.55 \times \dot{V}CO_2) - (3.21 \times \dot{V}O_2) - (2.87 \times$ urinary N$)$
2. Fat oxidation $(g \cdot kg^{-1} \cdot d^{-1}) = [(1.67 \times \dot{V}O_2) - (1.67 \times \dot{V}CO_2) - (1.92 \times$ urinary N$)$ Where $\dot{V}CO_2$ and $\dot{V}O_2$ are in $L \cdot kg^{-1} \cdot d^{-1}$ and urinary N is in $g \cdot kg^{-1} \cdot d^{-1}$
3. Protein oxidation $(g \cdot kg^{-1} \cdot d^{-1}) =$ urinary N $\times$ 6.25
Where urinary N is equal to nitrogen excretion in $g \cdot kg^{-1} \cdot d^{-1}$ and 6.25 is the conversion factor for converting nitrogen to protein.

study. In general, the equations are nearly exact for the calculation of net lipid, glucose, and protein oxidation rates and for the calculation of net lipid synthesis from glucose. These equations must be interpreted differently whenever net lipogenesis from carbohydrate is known to occur.

When non-protein respiratory quotients are >1.0, ie, when net lipogenesis is thought to occur, fat oxidation is a negative value and thus, measured carbohydrate oxidation and true carbohydrate oxidation differ by the glucose equivalent of the fat synthesized. Under these conditions, true carbohydrate oxidation is less than measured carbohydrate oxidation. It is also known that lipogenesis can occur in some tissues at a non-protein RQ of <1.0. Thus, the calculated carbohydrate oxidation rate represents the true carbohydrate oxidation rate, the amount of glucose converted to fat during lipogenesis, and an unknown but negligible contribution from gluconeogenesis[8] (table 3).

Hyperglycemic clamp

The hyperglycemic clamp (HC) is a technique used primarily to measure β-cell secretory function, but some groups have also used it to estimate insulin resistance. Glucose is infused intravenously at a variable rate to achieve and maintain a predetermined plasma glucose concentration (e.g. 12 mmol/l [216 mg/dl]). Blood or plasma glucose concentration is measured every few minutes so that the rate of infusion of glucose may be adjusted on a moment to moment basis to establish a steady concentration or 'clamp'. An estimate of insulin resistance is obtained from the plasma samples towards the end of the clamp which are assayed for insulin: the relationship between insulin and glucose is sigmoidal
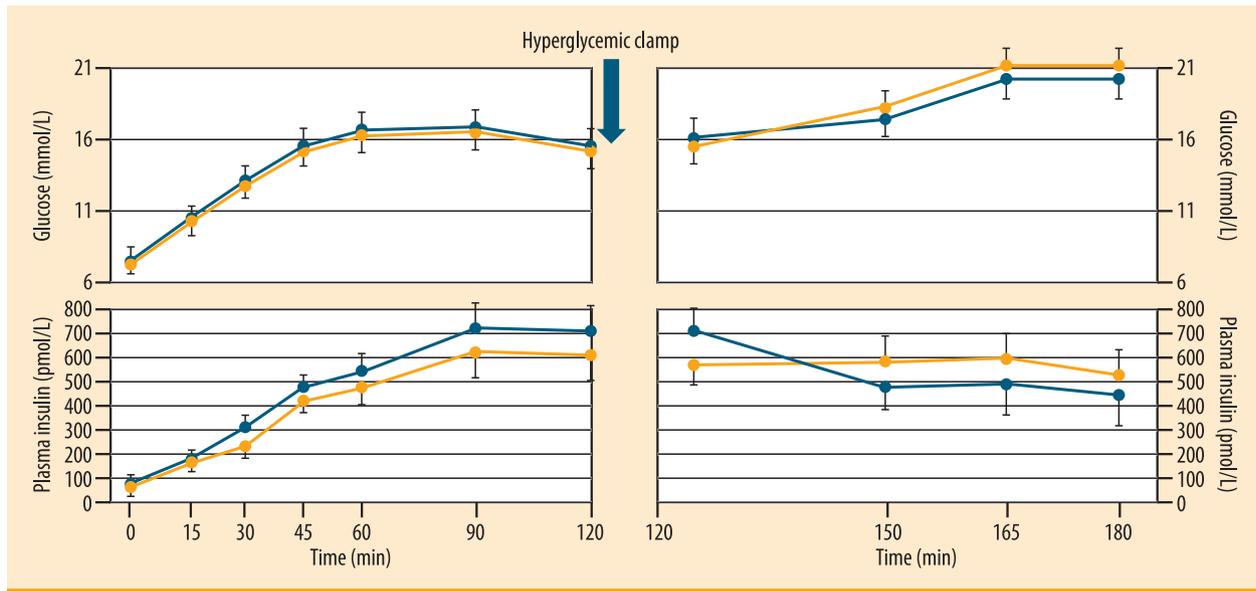
Figure 3. Hyperglycemic Clamp study. Serum glucose concentration (top) and plasma insulin responses (bottom) during OGTT (left) and Hyperglycemic Clamp study (right). Several indices could be obtained from HC studies as the amounts of glucose perfusion necessary to maintenance the prefixed glucose levels. The incremental insulin and c-Peptide area under the curve during the hyperglycemic clamp, and the plasma C-peptide/plasma insulin ratio will be considered to be the index of hepatic insulin extraction in the fasting state and during the HC. Finally, could be obtained and insulin sensitivity index, calculated by dividing the average glucose infusion rate by the average plasma insulin concentration[4]

(figure 3) and is approaching maximal in this situation. Insulin sensitivity is obtained by dividing the required glucose infusion for the maintenance of a prefixed hyperglycemia by the mean insulin concentration over the last 20-30 min of the test.[4] Plasma C-peptide/plasma insulin ratio could be considered to be the index of hepatic insulin extraction in the fasting state and during the HC (mean values at 150, 165, and 180 min).

The hyperglycemic clamp offers the advantage of allowing the assessment of insulin resistance and β-cell function from a single test. The problem with hyperglycemic clamps is that prevailing glucose is higher than that found in normal physiology. Thus the glucose infusion is an integrated measure of the insulin-mediated glucose disposal, glucose-mediated glucose disposal and, sometimes, renal glycosuria. Euglycemic clamps aim to minimize the latter two aspects, although there is a good correlation between the two methods.

Conclusions

Reduced glucose responses to insulin constitute an underlying feature of type 2 diabetes. In addition, the importance of the insulin-resistant phenotype for the assessment of cardiovascular risk and response to intervention is increasingly being recognized. Insulin resistance cannot be measured separately, and terminology describing its occurrence must specify the circumstances under which it was assessed as well as the method.

The euglycemic hyperinsulinemic clamp (EHC) is currently the gold standard method available for the determination of whole glucose uptake in response to insulin, from which several derived indices of insulin sensitivity are obtained. A standard (6 mU × min^{-1} × kg^{-1} insulin) version will shut off endogenous glucose release in the great majority of subjects, thus yielding a direct, clean and fully "usable" estimate of peripheral insulin sensitivity. The clamp is also the technique of choice to be combined with other experimental methods in more specific studies. Therefore, this technique is frequently used in combination with indirect calorimetry to evaluate net lipid, glucose, and protein oxidation rates, and with bioelectrical impedance to limit at lean body mass the glucose uptake. Finally, in physiological and pathophysiological investigations aimed at improving the understanding of insulin biology, with the used of tracers it might not be long before a clinically usable test to investigate the complete kinetics of insulin action *in vivo* simultaneously with the dynamics of insulin release.

Acknowledgements

Key points
• The euglycemic hyperinsulinemic clamp is currently the gold standard method available for an accurate and reproducible measurement of insulin resistance in vivo. • The glucose clamp technique is labor intensive, technically difficult to perform, and expensive. This technique should be used in combination with indirect calorimetry to evaluate net lipid, glucose, and protein oxidation rates, and with bioelectrical impedance to limit at lean body mass the glucose uptake. • The hyperglycemic clamp (HC) is a technique used primarily to measure β-cell secretory function. It has been used also by some groups to the assessment of insulin sensitivity, although several problems related to the high glucose concentrations has limited its widespread use.

Declaration of potential conflict of interest

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