IN VIVO INSULIN ACTION AND RESISTANCE

THE HYPERINSULINAEMIC, EUGLYCAEMIC CLAMP TECHNIQUE IN CONSCIOUS RATS

Sietse Jan Koopmans and Jasper Katrinus Rudder
Department of Endocrinology and Metabolic Diseases, University Hospital Leiden, Leiden, The Netherlands

SUMMARY

• After almost 75 years of research and four Nobel prizes awarded for insulin-related investigations, we still do not understand how insulin works. Of course, substantial progress has been made and there are some clues. We review in vivo insulin action from the moment of insulin biosynthesis in the pancreatic β-cell to insulin action on effector systems in target tissues. The mechanism of insulin action at the whole body, tissue and cellular level is discussed. In addition, factors and conditions influencing insulin action and events leading to insulin resistance are summarized. Finally, the reader is introduced into the principles of the hyperinsulinaemic, euglycaemic clamp technique which is considered the "golden standard" for measurement of insulin action and resistance in vivo. An example is given how to clamp conscious rats in a proper way.

INTRODUCTION

• Insulin is a 6 kD, double chain 51 aminoacid polypeptide which is produced in the pancreatic β-cell, the first protein formed being preproinsulin (Fig. 1). It is converted by proteolytic steps to proinsulin by removing the signal peptide. Proinsulin is finally cleaved into insulin and a C-chain, also called connective peptide (C-peptide) (1-3). Insulin is secreted into the portal circulation, in parallel with an equimolar amount of C-peptide and a small amount (±5%) of unconverted proin- sulin. Metabolic, hormonal, and neural factors modulate the insulin secretion (4-7). This modulation is necessary to ensure that the adequate amount of insulin is present in the blood to meet with the changing metabolic demands. The main meta-
Figure 1. Preproinsulin consisting of a signal peptide, a C-chain (C-peptide) and insulin. Insulin consists of an A- and B-chain held together by two disulfide bonds. The proteolytic processing sites are indicated by lines between some amino acid residues. Amino acids are designated by their one-letter abbreviations. Adapted from Ebberink et al (3).

The first target tissue which insulin encounters after its secretion is the liver. The liver removes approximately 50% of the amount of insulin which is present in the vascular portal system (13-16). First passage hepatic extraction of insulin from the plasma is a variable process and the liver can change the plasma clearance rate of insulin under certain conditions (17). It has been described that hepatic insulin removal strongly depends on the prevailing plasma glucose levels (18,19). Hyperglycemia would reduce hepatic insulin degradation which then leads to higher peripheral plasma insulin levels. This in turn results in a stronger insulin signal in muscle and fat tissues which leads to an increase in cellular glucose uptake and a subsequent reduction in plasma glucose levels. This implies that the liver determines for a substantial part to which plasma insulin levels the peripheral tissues are exposed to.

Under normal conditions, the half-life of insulin in the blood is less than 5 minutes (2,20). Insulin binds to the plasma membrane receptor. Some insulin is then partially degraded on the membrane by insulin degrading enzymes and some insulin can be released intact. Receptor-bound insulin migrates by endocytosis towards various intracellular sites, including endosomes and lysosomes, and intact insulin and partially degraded insulin reach the nucleus (13). The major part of insulin degradation occurs in the liver and, a lesser one, in the kidneys. Receptor-mediated insulin internalization in muscle and fat cells plays a role in the removal process of insulin from the blood (13,21). Internalization of the insulin-receptor complex is one of the processes that terminate the signal transduction (22).

When insulin has passed the liver, it can exert its actions in peripheral tissues. A summary of the actions of insulin is given in Fig.2 (10). Less known is the effect of insulin in the central nervous system (23,24). Insulin is able to increase the net uptake of glucose into the brain and it stimulates brain glycogen and amino acid synthesis (25,26). Insulin may arise from blood via receptor-mediated transcytosis through the brain capillary endothelial cells (26) and/or insulin may migrate from the blood via the cerebrospinal fluid to the central nervous system (27,28). Since insulin binding sites have been found throughout the central nervous system (29), it is conceivable that insulin modulates certain central nervous functions (30). The fact that hyperinsulinemia is able to increase sympathetic nervous system activity (31,32), is in support of this statement.

- **Mechanism of insulin action**

The cellular action of insulin starts by binding to its membrane receptor. The receptor is able to recognize the hormone with high affinity and a high degree of specificity. Next, the receptor produces a transmembrane signal that governs intracellular metabolism (33-35). The insulin receptor is a heterotetrameric glycoprotein consisting of two extracellular α-subunits with a molecular mass of 130 kD and two transmembrane β-subunits with a molecular mass of 95 kD which are linked to one another by disulfide bonds (36). The receptor recycles between the plasma membrane and an intracellular pool (37). Approximately 10% of the insulin receptors are intracellular in the basal state in rat adipocytes and this increases to a steady state of about 30% after insulin exposure (38). There are some 200 000 insulin receptors in rat adipocyte and hepatocyte plasma membrane. Theoretically, each receptor could bind two insulin molecules but ligation of one binding site decreases the affinity of the other site almost 100 fold. Insulin is internalized with the receptor and is passed to
**figure 2. A selection of major effector systems governed by insulin.**

endosomes leaving the receptor to be recycled to the plasma membrane.

Transmembrane signaling starts with binding of insulin to the oc-subunit which activates a tyrosine kinase in the cytoplasmic domain of the fi-subunit. The B-subunit then autophosphorylates on tyrosine residues 1146,1150 and 1151 and this autophosphorylation activates the receptor tirosine kinase to phosphorylate various substrates in the cell which then in turn would further transduce the insulin binding signal (34,39). Upon maximal insulin binding, approximately one-third of the total receptor pool becomes autophosphorylated (40). Recently, intermolecular phosphorylation of insulin receptors was described after insulin binding which could be a mechanism for amplification of the insulin binding signal (41). On the other hand, inactive insulin receptors (no kinase activity upon insulin binding) can inhibit the function of normal receptors, which could be a mechanism for weakening the insulin binding signal (34).

Only 10% of the entire insulin receptor pool has to bind insulin in order to reach a 100% biological effect in the rat. Thus 90% of the receptors are spare. They function normally and it is a random event to which particular receptor insulin is binding. This phenomenon is called the spare-receptor concept and was first described by Kono & Barham (42). The insulin concentration dependencies for insulin binding to its receptor and for the stimulation of glucose transport were compared by Simpsonand Cushman (43). The insulin concentration needed for a half-maximal response (ED$_{50}$) is 0.14 nM for glucose transport, whereas the ED$_{50}$ for insulin binding to its receptor is 10 fold higher (1.33 nM). Recently, it was shown that a similar phenomenon is applicable for the relationship between insulin activation of insulin receptor kinase and insulin stimulation of glucose uptake in rat adipocytes. Glucose uptake was half-maximally or maximally stimulated, respectively, when only 4% or 14% of the maximal kinase activity had been reached. This could now be designated as "the spare receptor-kinase concept" (44).

The signal transduction after the receptor is still an enigma (45). The link between the activated receptor, target enzymes and effector systems remains obscure. Progress in this area has been complicated by the pleitropic nature of insulin action. Well described mechanisms of signal transduction, like cyclic nucleotides and ion channels, appear not to be central to insulin action (45,46). Another second messenger specific for insulin has not yet been found and it may not even exist. It appears that numerous intracellular factors interact with one-anothers to transduce the insulin signal depending on specific conditions in the cell (47). After interaction of insulin with its receptor and subsequent tyrosine kinase activation, one signal transduction...
pathway involves a cascade of serine/threonine kinases also interacting with phosphatases which are regulated by phosphorylation. This phosphorylation/dephosphorylation pathway is thought to control insulin-mediated glycogen metabolism (48). Another signal transduction pathway may involve the polar head group of an insulin-sensitive glycosphospholipid which selectively inhibits lipolysis (49,50). The independent character of glucose and glycogen metabolism and lipolysis is reflected in the sensitivities of these effector systems for insulin. Antilipolysis is 3-5 fold more sensitive to insulin than glucose transport (51-53).

It is now widely accepted that insulin stimulates glucose metabolism in its target tissues via recruitment of glucose transporters (GLUT) from a trans-Golgi pool to the plasma membrane (43). The action of insulin involves a transporter translocation (54) and transporter activation (55). Recently, a number of GLUT subtypes with diverse tissue distributions and relative abundances have been identified by molecular cloning and expression (56): (i) GLUT1 derived from human HepG2 cells and from rat and rabbit brain, also called the erythrocyte-type GLUT, is predominantly present in insulin-insensitive tissues, 00 GLUT2 is present in human and rat liver, and pancreatic islet cells, (Hi) GLUT3 derived from human fetal muscle is expressed in all tissues, (iv) GLUT4 derived from human and rodent skeletal muscle and adipocytes, also called the insulin-regulatable GLUT, is present in insulin-sensitive tissues, (v) GLUTS derived from small intestine, and (v/) GLUT6 derived from small intestine, also called the NaVglucose cotransporter.

- **Insulin resistance, sensitivity and responsiveness**

Insulin resistance, sensitivity and responsiveness were described and defined more than a decade ago (2,57,58). These terms used to indicate changes in the action of insulin still remain a source of misunderstanding. To recapitulate, see Fig. 3 (57): (i) insulin resistance exists whenever a certain concentration of insulin produces a less than normal biologic response, 00 insulin sensitivity is defined as the concentration of insulin necessary for a half-maximal effect (ED50), i.e. the relative position (left-right shift) of the dose-response curve, and (Hi) insulin responsiveness is defined as the maximal response (Vmax) to insulin.

To fully characterize insulin action, a dose response curve has to be constructed. From Fig. 3 it is clear that alterations in insulin sensitivity and responsiveness can only then be evaluated. In general, it can be said that insulin sensitivity provides information to what extent the insulin triggered transmembrane (receptor) and/or intracellular (receptor-coupled second messengers) signal transduction is functioning. Insulin responsiveness provides information about the maximal capacity of the effector system to execute a certain biological effect.

When the dose-response relationship between insulin and a certain biological effect is studied, an increase in insulin levels result in an increase both in receptor ligation and biological effect, until the crucial number of receptors (± 10% of the total receptor pool under normal conditions in the rat) (42) required to reach a maximal biological effect is occupied. Higher insulin levels lead to a further increase in insulin receptor binding but this is without any further effect on the biological response. Some postreceptor step(s) are now rate-limiting for the measured biological effect.

When insulin sensitivity and responsiveness are investigated in combination with an insulin receptor study, a detailed knowledge is obtained concerning the mechanism of insulin action.

In general, it is accepted that a change in insulin sensitivity is partly influenced by a change in receptor number, affinity and function, whereas a change in insulin responsiveness is more likely to be influenced by postreceptor processes. Taken the spare receptor-(kinase) concept in mind, it is clear that a reduction in the number and functionality of insulin receptors has to exceed the 90% level before receptor-mediated signal transduction becomes rate-limiting for insulin responsiveness. This 90% reduction is limited to a few rare genetic disorders.
In vivo, insulin has to be transferred across capillary endothelium when it is travelling from the blood to interstitial fluid and target tissues. This transfer seems to be conducted by a receptor-mediated process (61). In support of this observation is the fact that receptor-mediated transport of insulin across capillaries can be downregulated by exposure to physiological levels of insulin (62). The transfer of insulin from blood to target tissue is described to be restricted (61) and can be rate-limiting for insulin action under certain conditions (63). For instance, half-maximal response to insulin occurs in perfused fat tissue at 100 (IU/ml, whereas it occurs at 30 uU/ml in incubated tissue and at 8 uU/ml in incubated adipocytes (61). In addition, the time required for maximal response to insulin is longer in perfused tissue than in incubated cells and tissues. This is consistent with the finding that insulin is transported across vascular endothelial cells with a delay of 5-10 min (62) and that it takes approximately 20 minutes before lymph insulin levels reach a steady state situation when plasma insulin levels are already in steady state (63).

When studying insulin action on hepatic glucose production, at least two independent mechanisms seem to play a role. It is believed that insulin inhibition of glucose release by the liver is due to inhibition of glycogenolysis and/or gluconeogenesis. However, insulin may also attenuate hepatic glucose production through indirect action on peripheral tissues by restricting the flux of gluconeogenic substrates like lactate, alanine, glycerol (64,65) and by restricting the flux of stimulators of gluconeogenesis (66,67) to the liver. When the latter mechanism plays an important role and insulin action is studied in vivo, different results will be obtained regarding hepatic glucose production when compared to liver perfusion studies. In this particular situation, comparison of in vivo and in vitro data may lead to conflicting conclusions when the mechanism of the studied biological process is not completely understood. The phenomenon just described might play an important role in the hepatic action of the recently discovered pancreatic p-cell hormone amylin, also called islet amyloid polypeptide (68-70).

In vitro experiments enable us to study a certain tissue or cell type in detail without interference of other tissues or cells. Apart from that, each individual tissue or cell type can respond in its own specific way to certain stimuli or metabolic alterations. Although the metabolic and hormonal environment in vivo is roughly similar to all tissues (with the exception of the liver, which is exposed to higher hormonal and metabolite levels), each individual tissue can undergo specific alterations under several metabolic conditions (39,71). In general, insulin binding, receptor autophosphorylation and tyrosine kinase activity can differ from one tissue to another. During a change in the metabolic state of the organism, like long-term hyperinsulinaemia, diabetes, starvation and fat feeding, it has been reported that muscle, fat and liver each can undergo their own specific alterations in insulin signal transduction (60,72-77).

It is often stated that the use of a well defined medium leads to an experimental set-up which allows the investigator to study one separate factor and/or condition at the time of insulin action on a certain tissue or cell type. However, it is a misunderstanding that in vitro experiments can be considered free from disturbing factors and that the environment is constant during the experiment. Focused on insulin action, it is well known that an accumulation of free fatty acids in the incubation medium released by adipocytes after lipolysis influences insulin stimulated glucose uptake in a negative way (78). Without adding albumin to the incubation medium which binds free fatty acids, a time dependent suppression of insulin-stimulated glucose uptake will occur. Also, cells rapidly release adenosine, a potent antiprolifcatory agent, in the medium (52,79). In addition, a similar phenomenon has been described for prostaglandins which are also released by cells (80).
• Events leading to insulin resistance

In general, modulation of insulin action can occur from the moment of insulin synthesis until the ultimate biological effect is executed by a particular insulin-mediated effector system. The modulation of insulin action can be subdivided as follows: (I) P-cell insulin synthesis: (a) an abnormal insulin molecule, and (b) an incomplete conversion of proinsulin to insulin (2), (II) p-cell secretion: (a) a change in the amount (60,81-84) and pattern (20,85,86) of insulin release, and (b) a change in the release of amylin (68-70), (Hi) circulating insulin agonists and antagonists: (a) a change in the levels of hormones, neuropeptides, and cytokines, e.g. glucagon (87,88), catecholamines (89,90), corticosteroids (91), growth hormone (92), insulin-like growth factors (93), gestational hormones (94), calcitonin gene-related peptide (95), adenosine (96), prostaglandins (80), and interleukines (97), and (b) antibodies to insulin receptors (2), (iv) circulating metabolites: a change in the levels of glucose (98-100), ketone bodies (101), and free fatty acids (78,88,102), and (v) target tissue alterations: (a) a change in hepatic insulin extraction (13-19), (b) a change in insulin receptor function (103), (c) a change in postreceptor signal transduction (104,105), and (d) a change in insulin-mediated effector system, i.e. the end of the signal transduction pathway, like changes in tissue glucose metabolites (106), enzyme activity (107), and GLUT (55).

Several metabolic conditions have been reported to induce changes in insulin action and most of them induce insulin resistance. Obesity, non-insulin dependent diabetes mellitus, insulin dependent diabetes mellitus, disorders associated with increased production of counterregulatory (stress) hormones, hypertension, atherosclerotic cardiovascular disease, uremia, aging, and even bedrest are characterized by insulin resistance (108-115). Only physical exercise is reported to improve insulin action (116-117). The data for body weight reduction, fasting and starvation are controversial (60,72,118-123). Each tissue is able to react in its own specific way when the entire body is subject to a change in metabolic state. Therefore, one tissue might not reflect the whole body situation, and when two tissues are compared they might show contradictory results (39,72-76,124).

MEASUREMENT OF IN VIVO INSULIN ACTION BY MEANS OF THE GLUCOSE Clamp TECHNIQUE IN RATS

• Evaluation of insulin action in vivo, using isolated tissues or cells, may reflect in vivo action of the hormone, although there can be a dissociation between insulin action and/or receptor binding in vitro and the action of insulin in the intact organism. Therefore, to measure insulin-mediated metabolism in vivo, it is important to develop a technique which is able to accurately quantify insulin action in the intact organism (125,126).

In 1936, Himsworth was the first investigator who introduced a standartized method to measure insulin action in vivo in the intact organism (127). By using the oral glucose tolerance test in conjunction with intravenous injections of insulin he was able to measure in vivo insulin action and showed that some diabetic patients were resistant to the action of insulin (128). For correct quantitation of insulin action however, one needs to know the relationship between stable plasma insulin levels and a certain specific insulin-mediated metabolic process. After the development of the radioimmunoassay for insulin (129), investigators were able to measure plasma insulin concentrations. Since then many techniques have been introduced to assess insulin action in vivo. These techniques have critically been reviewed (126). One of these methods, the glucose clamp technique, is now the most widely accepted approach to quantify insulin action in vivo and is considered the "golden standard".

We will briefly describe the method to measure insulin action in vivo by means of the glucose clamp technique in undisturbed, freely moving rats (60,130).

• Principle of the hyperinsulinaemic, euglycaemic clamp technique

The plasma insulin concentration is acutely raised and maintained at a constant level by means of a primed-continuous systemic infusion of insulin at a constant rate. When insulin starts to act, by inhibiting hepatic glucose production and stimulating peripheral tissue glucose uptake, plasma glucose levels decrease. This is detected by frequent measurement (every 5-10 min) of plasma glucose levels and counteracted by a simultaneous variable infusion of glucose to maintain euglycemia. After 30-60 min, when full action of insulin is achieved, a steady-state situation is reached during which plasma insulin and glucose levels and glucose infusion rate are constant. In this situation, the exogenous glucose infusion rate is a rough reflection of whole body glucose uptake at that particular plasma insulin level. An example of the clamp in normal and diabetic rats is presented in Fig.4. This example shows the dynamics in glucose infusion rate and plasma glucose levels during a 4-step sequential hyperinsulinaemic euglycaemic clamp. The sequential, dose-response clamp allows discrimination between insulin sensitivity and insulin responsiveness in one single experiment. Because the body also contains a potential endogenous source of glucose appearance, namely the liver, an exact quantization of whole body glucose uptake is not possible during the clamp. Precise measurement of glucose uptake can only be achieved when the clamp technique is conducted in conjunction with a continuous infusion of radiolabeled glucose (60,130). Thus, by measuring plasma glucose specific activity, exact calculation of glucose turnover is possible. Glucose turnover is a direct re-
Figure 4. Insulin infusion rates, plasma glucose concentrations, and glucose infusion rates in normal and streptozotocin-induced diabetic rats during insulin/glucose clamp. A 4-step sequential (dose response) hyperinsulinaemic, euglycaemic clamp is shown with insulin infusion steps of 90 min each. The final 25 min of each individual insulin infusion step, when steady state is present, is used for quantitation of insulin action.
clamping is that a systematic error occurs if rats are compared at equivalent venous glycaemia and insulinemia when their sensitivities to insulin are not identical. The more sensitive to insulin, the greater the arteriovenous glucose difference across the peripheral tissues. If venous glycaemia is identical, arterial glycaemia is highest in the rat most sensitive to insulin. In this situation, whole body glucose uptake is not compared at matched glycaemia and glucose uptake will be overestimated in the rat with a high insulin sensitivity since the clamp is performed at higher arterial glycaemia.

**Anesthesia**

It has repeatedly been shown that anesthesia exhibits a great impact on glucose metabolism. Pentobarbital anesthesia induces an inhibition of the central nervous system which is reflected in a disbalance of plasma catecholamine levels, body temperature, heat rate and blood pressure (131,132). This results in a changed glucose kinetics. Glucose turnover, using \(^3\)H-glucose as a tracer, show that a transient increase in hepatic glucose production occurs and peripheral tissue glucose uptake decreases by 30% as compared with conscious rats (133). Pentobarbital anesthesia increases hepatic synthesis of glycogen from the gluconeogenic precursors alanine and pyruvate in rats (134). From these data it is clear that the hyperinsulinemic, euglycaemic clamp technique should be performed in conscious rats. Since carbohydrate metabolism is under direct control of the central nervous system, an undisturbed awake rat model would provide a more reliable physiological reflection of hepatic glucose production and peripheral tissue glucose uptake as measured during the clamp. The experimental set-up for clamping conscious rats is depicted in Fig. 6, by using a modification of techniques previously described (135,136).

**Compensation of blood loss**

During a 7.5 hour lasting 5-step sequential clamp, ±2 ml of blood is sampled during each individual insulin infusion step. Since a rat of 350 g contains approximately 20 ml of blood, it is obvious that long-term clamping must be accompanied by blood transfusion which compensates for blood loss. To achieve this, fresh prewarmed (38°C) donor blood obtained by heart puncture from littermates is returned to the experimental animal via the arterial blood sampling cannula. To prevent clotting, donor blood is supplemented with 25 IU/ml heparin or 3 mg/ml citrate. During the first 15 min of each individual insulin infusion step, 3 samples of 0.7 ml donor blood are transfused into the rat to avoid disturbances and dilution of plasma \(^3\)H-glucose specific activity during the steady state period (70-90 min) of the clamp, when insulin action is quantitated. An alternative way of transfusing donor blood to the experimental animal is by continuous infusion through the venous cannula. When insulin is infused during the clamp procedure, insulin can be mixed with saline-diluted donor blood instead of pure saline. High frequency blood sampling and transfusion of donor blood in awake rats have been shown not to influence the secretion of stress hormones like prolactin and corticosterone. Blood composition remains normal and no metabolic alterations could be detected (137-139).
Figure 6. Experimental set-up for clamping conscious rats. The rat is placed in a metabolic cage allowing the collection of urine and faeces.
REFERENCES


12. Foster DW. From glycogen to ketones-and back. Diabetes 1984; 33: 1188-1199


46. Mooney RA, Swicegood CL, Marx RB. Coupling of adenylate cyclase to lipolysis in permeabilized adipocytes: direct evidence that an antilipolytic effect of insulin is independent of adenylate cyclase. *Endocrinology* 1986; 119: 2240-2248


49. Kelly KL, Mato JM, Merida I, Jarett L. Glucose transport and antilipolysis are differentially regulated by the polar head group of an insulin-sensitive glycoprophospholipid. *ProcNatAcadSci USA* 1987; 84: 6404-6407


53. Wieringa T, Krans HMJ. Reduced glucose transport and increased binding of insulin in adipocytes from diabetic and fasted rats. BiochimBiophysActa 1978; 538:563-570


67. Kruszynska YT. The role of fatty acid metabolism in the hypertriglyceridaemia and insulin resistance of Type 2 (non-insulin dependent) diabetes. In: SM Marshall, PD Home, RA Rizza, editors. The Diabetes Annual/9 1995; 107-139


78. Kruszynska YT, McCormack JG, McIntyre N. Effects of non-esterified fatty acid availability on insulin stimulated glucose utilisation and tissue pyruvate dehydrogenase activity in the rat. Diabetes 1990; 33: 396-402


82. Cerasi E. Insulin deficiency and insulin resistance in the pathogenesis of NTDDM: is a divorce possible? Diabetologia 1995; 38: 992-997


92. Davidson MB. Effect of growth hormone on carbohydrate and lipid metabolism. Endocr Rev 1987; 8: 115-131


135. Steffens AB. A method for frequent sampling of blood and continuous infusion of fluids in the rat without disturbing the animal. Physiol Behav 1969; 4: 833-836


138. Wiersma J, Kastelijn J. A chronic technique for high frequency blood sampling / transfusion in the freely behaving rat which does not affect prolactin and corticosterone secretion. JEndocrinol 1985; 107: 285-292

139. Wiersma J, Kastelijn J. Haematological, immunological and endocrinological aspects of chronic high frequency

blood sampling in rats with replacement by fresh of preserved donor blood. Labor Anim 1986; 20: 57-66

Received 18 March 1996
Accepted 25 April 1996

Address for correspondence:
Dr Sietse Jan Koopmans
Department of Endocrinology and Metabolic Diseases,
University Hospital Leiden,
Building 1, C4R
POBox9600
NL-2300 RCLeiden
The Netherlands
Tel: 31 (71) 5263082
Fax: 31 (71) 5248136

Biomed Rev 5,1996