HEPATIC PARASYMPATHETIC NERVES ARE ESSENTIAL FOR INSULIN EFFECTIVENESS

By

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BY

HONGSHENG XIE

A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Hepatic parasympathetic nerves are essential for insulin effectiveness

Hongsheng Xie

Abstract

This thesis work demonstrated the role of hepatic parasympathetic nerves on insulin effectiveness, based on the hypothesis that hepatic parasympathetic neuropathy may account for insulin resistance in some disease states (Lautt 1979).

A modified insulin sensitivity test was established to provide sensitive, reproducible and multiple tests for insulin effectiveness. This technique tests whole body glucose disposal in response to a bolus of exogenous insulin infusion. A five-step time control Insulin test in cats produced quantitatively and qualitatively similar results with a mean coefficient of variance of 3.0 ± 0.5% and without changes in glucoregulatory hormones.

Surgical denervation by cutting the hepatic anterior nerve plexus in cats reduced the hypoglycemic response to insulin (100 mU/kg) infusion by 37.2 ± 9.8%. Extensive surgical hepatic denervation with phenol or bilateral vagotomy failed to further alter the insulin resistance produced by anterior plexus denervation, suggesting that hepatic innervation exerts a synergistic hypoglycemic effect with insulin and that the relevant nerves reach the liver via the anterior plexus.

Blockade of hepatic parasympathetic nerves with intraportal administration of muscarinic antagonists inhibited insulin effectiveness in a dose-dependent manner. The ED_{50} of atropine was about 1 mg/kg with maximum inhibition of 40.3 ± 11.6%. Atropine 3 mg/kg inhibited insulin sensitivity by 33.6 ± 3.4%, similar to that produced by surgical hepatic denervation. Doses greater than 3 mg/kg did not alter the insulin resistance caused by atropine 3 mg/kg administration. The results indicate that hepatic cholinergic nerves are responsible for the observed insulin resistance produced by surgical denervation. M1 muscarinic receptors may mediate the synergistic effect of hepatic nerves with insulin.

By measuring changed glucose gradients across the liver, extra-hepatic splanchnic organs and hindlimbs, insulin resistance produced by hepatic parasympathectomy was localized mainly in the skeletal muscle, rather than in the denervated liver or portal vein-drained organs. It is hypothesized that the liver, with functional parasympathetic nerves, releases a circulating factor in response to insulin, which can exert a synergistic effect with insulin to increase glucose transport in skeletal muscle. The denervated liver loses this ability.

Insulin resistance produced by surgical denervation was treated with administration of acetylcholine in rats. Acetylcholine 2.5 ug/kg/min intraportal infusion can totally reverse the insulin resistance generated by hepatic

denervation, while intravenous administration did not produce this reversal. Acetylcholine intraportal infusion did not alter the insulin effectiveness in normal rats, indicating that acetylcholine intraportal administration is a specific and efficient therapy for insulin resistance produced by hepatic parasympathetic dysfunction.

In rats with chronic bile duct ligation, insulin sensitivity was inhibited to a similar degree to that produced by surgical denervation, which was not further altered with atropine 3 mg/kg intraportal infusion. Insulin resistance in these bile duct ligated animals was completely reversed with acetylcholine (2.5 ug/kg/min) intraportal administration, demonstrating that insulin resistance caused by hepatic damage can be treated by cholinergic muscarinic agonism.

Based on linear regression analysis, insulin effectiveness is composed of two components: insulin effect dependent on (about 2/3) and independent of (about 1/3) the tone of hepatic parasympathetic nerves. The variability of insulin sensitivity may be accounted for by the variability of tone of hepatic cholinergic nerves in daily glucose metabolism.

In these primary investigations, a new theory for treating insulin resistance in non-insulin dependent diabetes, hepatic diseases and hypertension is experimentally proposed. A powerful means for testing insulin sensitivity is established. The traditional theory about liver's function in

glucose metabolism is enriched.

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Section I. Hepatic innervation

I. 1. Hepatic nerve distribution

I. 1. 1. Hepatic efferent nerves

Hepatic efferent nerves innervate the liver mainly through two pathways, the anterior and posterior nerve plexuses (Fig. 1.), although the nerve fibres have been seen infrequently in the hepatic ligaments [Alexander 1940]. The anterior plexus, also known as the periarterial plexus, forms a sheath surrounding the common hepatic artery, while the posterior plexus ramifies around the portal vein and bile duct and freely mingles with the anterior plexus in the region where the hepatic artery, portal vein and bile duct pass in close apposition [see Lautt 1983]. The anterior nerve plexus contains both sympathetic and parasympathetic components, as shown by hepatic glucose responses to direct electrical stimulation of the anterior plexus with different methods [Lautt and Wong 1978a and 1978b]. Both the anterior and posterior plexuses contain sympathetic nerves, indicated by hepatic vascular responses to reflexly induced sympathetic stimulation [Lautt 1981].

Intrahepatic distribution of hepatic nerves is highly species-dependent. The variation may originate from evolution of different species. Birds have evolved originally as herbivores, which consume almost continuously a carbohydrate-rich food and are faced with hyperglycemia. The liver of birds contains a dense network of parasympathetic nerve endings. The most effective glucose uptake inducer in birds is acetylcholine, rather than insulin

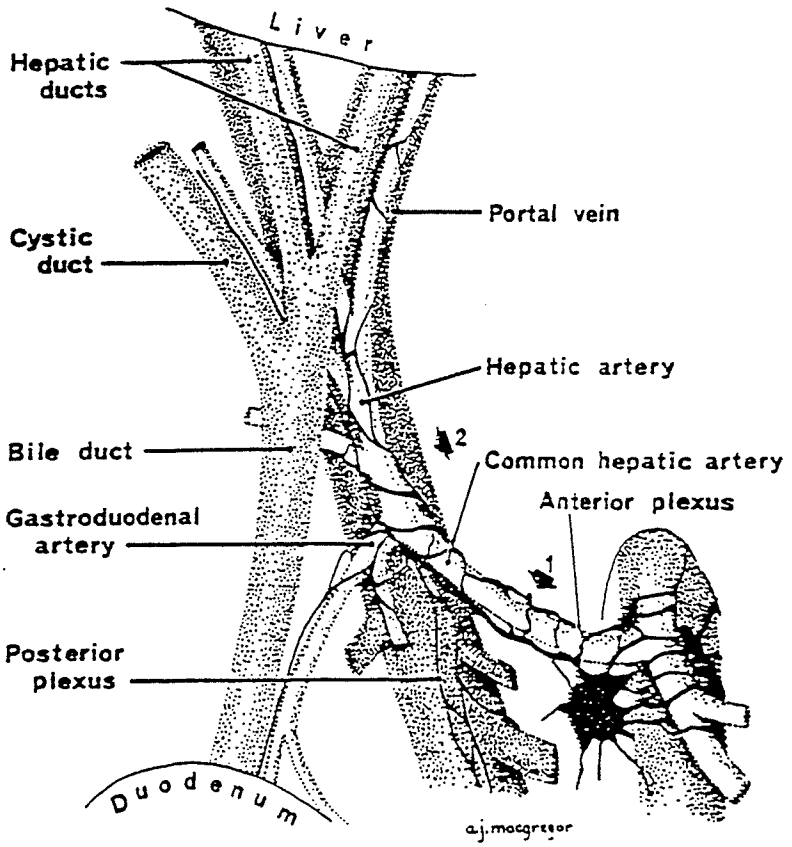


Fig. 1. Ventral review of the anterior hepatic plexus running from the celiac ganglion along the common trunk of the hepatic artery, and the posterior hepatic plexus passing to the liver along the portal vessel. The two plexuses freely mingle in the region where the hepatic artery and the portal vein run in close apposition. The biliary tracts are shown leading from the liver at the top of the illustration (the lobes of which are elevated for better access during surgery) to the duodenum at the bottom of the illustration. The arrows show the site at which the anterior plexus (1) can be conveniently isolated for ablation or stimulation and the site, along the portal vessel (2), at which full hepatic denervation can be performed. The latter site cannot be as conveniently used for stimulation since the plexus is very diffuse. Phenol (90%) applied to site 2 results in rapid denervation that lasts for at least two weeks. Reprinted from Lautt. W. W. Can. J. Physio. Pharmacol. 59:1014, 1981.

(see Pilo and Verma 1985). The latter is secreted either sluggishly or only during acute hyperglycemic conditions [Hazewood 1973]. However, in rat liver, the nerves are mainly restricted to the portal space and the hilus with only a limited innervation of both cholinergic and adrenergic nerves to the preterminal hepatic artery and the portal venule [Reilly et al. 1978; Metz and Forssman 1980].

Adrenergic innervation of hepatic parenchyma is observed for a variety of higher species, such as monkeys, rabbits, guinea pigs and pigeons, while lack of adrenergic contact to hepatic parenchyma is noticed for rats, golden hamsters, amphibians and fishes [see Lautt 1983]. Human liver shows a quite rich hepatocyte innervation, and contact of adrenergic endings to Kupffer cells, endothelial cells and Ito cells [Forssmann and Ito 1977]. It was suggested that the human, guinea pig, cat and tree shrew possess similar nerve distribution. No evidence showed that hepatic parenchyma is innervated by cholinergic nerve terminals in rats and rabbits [Hans-Rudolf et al.1992; Akiyoshi 1989; Ballantyne 1978].

Although the adrenergic nerve distribution in the livers of guinea pig and tree shrew is quite different from that in the liver of rat, and the content of norepinephrine in liver tissue and the amount of norepinephrine released upon nerve stimulation were much higher in livers of guinea pig and tree shrew than those in the liver of rat, the hepatic carbohydrate metabolism response to nerve stimulation is similar in all these animals [Beckh et al. 1990]. The mechanism for these livers to achieve the similar response to

stimulation is best explained by the gap junction theory. In those liver with sparse innervation, the nerve signals appear to be propagated through gap junctions via electrical coupling.

The density of gap junction is inversely related to density of innervation in the liver, suggesting a compensatory mechanism whereby the rare innervated area requires more gap junctions for signal transmission [Forssman and Ito 1977]. In the isolated liver perfused in situ, gap junction inhibitors can block the metabolic and hemodynamic effects in response to hepatic nerve stimulation [Florian et al. 1992]. The content of gap junction protein, connexin 32, was known to be transiently reduced in the regenerating liver after partial hepatectomy. In this regenerating liver, the increase in glucose output upon hepatic nerve stimulation was almost completely inhibited in the early stage (48-72 h) after partial hepatectomy, while norepinephrine outflow by nerve stimulation was not reduced and the content of hepatic glycogen was not significantly reduced [Iwai et al, 1991]. However, hepatic glucose output in response to exogenous norepinephrine infusion was not influenced even in this early stage of regeneration, since the infused norepinephrine could readily reach the majority of the hepatocytes through sinusiods, and act directly on almost all hepatocytes [Iwai et al. 1991]. Hypothetically, norepinephrine released by adrenergic nerves binds to α1-adrenoceptors on the preportal parenchymal and non-parenchymal cells. In the proximal hepatocytes, the binding enhances glucose release, and triggers a signal, perhaps Ca^{++} or inositol 3, 4, 5-triphosphate (IP_3), which spreads via

gap junction to its distal hepatocytes and increases their glucose release. In the proximal non-parenchymal cells (vascular sphincter), norepinephrine elicits constriction and reduces the sinusoidal flow. It can also cause the release of prostaglandins (F_{2a}) into the space of Disse. In the proximal cells, prostaglandins (PGs) further increase glucose release. PGs autocatalytically re-enforce constriction and reduces sinusoidal flow. PGs can not reach the distal cells, since its degradation proceeds very quickly [Florian et al. 1992]. However, how the signals of hepatic parasympathetic nerves spread over the liver is totally unknown.

I. 1. 2. The afferent nerves of the liver

In rats, hepatic afferent fibres originate from the left (about 90%) and right (about 10%) nodose ganglia and are contained in the hepatic branches, which are loosely associated with the hepatoesophageal artery. Near the junction of this artery with the hepatic artery proper, the afferent nerves run into two directions. Some of them turn towards the individual liver lobes, while the others join the anterior nerve plexus. Approximately two thirds of the afferent fibres contained in the hepatic branch bypass the liver hilus [Hans-Rudolf et al. 1990]. Therefore, hepatic denervation by interrupting the anterior plexus or near the hilum at the position 2 in figure 1 may leave the hepatic afferent supply intact.

The liver contains ten times more vagal afferents than efferents [Prechtl and Powley 1990]. Studied with the anterograde technique in rat, vagal afferent fibres form dense terminal-like structures surrounding the hepatic bile

duct, portal vein and paraganglia. The afferent nerve terminals run deep within the collagen fiber-rich adventitia and form fine terminal arborization in the wall of the portal vein. No fibers and terminals are seen in hepatic parenchyma [Hans-Rudolf et al. 1990]. These observations in rat make it unlikely that the glucose receptors are located in the hepatic parenchyma.

The liver is extremely active in regulation of physical and chemical hemostasis. The hepatic afferent nerves monitor the fluctuations of the temperature and pressure of hepatic blood, ionic and nutrient content of the portal blood and is involved in the regeneration of hepatic parenchyma. The sensory signals are transmitted to the central nervous system through the afferent nerves in the vagus. The lateral hypothalamic area (LHA, parasympathetic center) and ventromedial hypothalamic nuclei (VMH, sympathetic center) integrate and reciprocally regulate the behaviour and activity of their effectors to maintain internal hemostasis [see Lautt 1983; Yamaguchi 1992].

I. 1. 3. Neural reflex arch for glucose regulation

The neural reflex arch for glucose regulation has undergone substantial studies in recent years. The action of this neural pathway is to counterregulate the hypo- or hyperglycemia under physiological and pathological conditions to maintain glucohemostasis. The dysfunction of any limb in this neural pathway will result in disturbance of glucose metabolism.

I. 1. 3a. The afferent limb

Glucose receptors are mainly located in the hepatic portal system. The

function of the glucoceptors is to detect the glucose concentrations in the portal flow. The firing rate of the hepatic afferent nerves was inversely proportional with the glucose concentrations of the portal blood, even after the associated liver lobes were removed [Niijima 1980]. The particular relationship of hepatic afferent discharge with portal glucose concentrations could not be duplicated by using other hexoses or pentoses [Niijima 1969]. Intraportal infusion of the glucose metabolic inhibitor, 2-deoxy-D-glucose, caused an increase of the vagal afferent discharge rate [Niijima 1981]. Morphological study indicated that afferent nerves terminate in the walls of the portal system, rather than in the hepatic parenchyma [Hans-Rudilf 1990]. Using a local euglycemic clamp technique at the liver and at the brain, Casey et al. (1991) indicated that hepatic, rather than cerebral, hypoglycemia is essential to produce the counterregulatory response upon insulin-induced hypoglycemia, and that the glycemic threshold for sympathetic responses was a function of hepatic glycemia and not arterial glycemia, suggesting that the liver glucoceptors play a main role in the counterregulatory response. The concept that hepatic glucoceptors detect portal glucose concentrations but not systemic concentrations, is extensively used to study the neural reflex arch for glucoregulation by comparing effects of intraportal glucose administration with those of intravenous administration [Lautt 1984; Adkins-Marshall et al. 1992; Casey et al. 1994].

However, the glucoceptors may be present in other locatios. Intravenous injection of glucose produced a similar recording on the

pancreatic afferent nerves to that on the hepatic afferents in response to intraportal glucose administration [Niijima 1981], suggesting the presence of glucoceptors in the pancreas which monitor systemic, rather than portal, glucose concentrations. The extra-hepatic glucoceptors may also participate in glucoregulation, since an incomplete suppression of the sympathoadrenal response to moderate systemic hypoglycemia was produced when the liver was clamped at euglycemia [Casey et al. 1994]. In the rat with CCl_4-induced hepatic cirrhosis, section of the hepatic branch of the vagal nerves did not affect either plasma insulin or glucose concentrations after intraperitoneal glucose administration, in contrast to the control rats [Hiroshi et al. 1993], suggesting that the function of the glucose receptors in the cirrhotic liver is disturbed.

The decrease in the firing rate of hepatic afferents caused by intraportal glucose administration appears to be produced by the activation of the energy dependent sodium pump. After intraportal administration of ouabain, the pump inhibitor, the change of glucose-induced firing rate was totally blocked [Niijima 1981]. Hyperpolarization of the liver cell membrane is not a glucose-sensitive mechanism, since both metabolizable and nonmetabolizable glucose can cause hyperpolarization of hepatocytes [Meyer and Scharrer 1991]. The hepatic glucoceptors can sense the range of portal glucose concentrations from 100-500mg/dl, and are specific to D-glucose [Niijima 1982].

The signals generated by the glucoceptors are transmitted to the hypothalamus through hepatic capsaicin-sensitive afferent C-fibres in vagal

nerves. Section of the hepatic branch of the vagal nerves blocked the effect of intraportal infusion of glucose [Niijima 1981]. Capsaicin treatment inhibited the adrenal response upon insulin-induced hypoglycemia [Donnerer 1988]. However, the neurotransmitter of the hepatic afferent nerves is unclear.

I. 1. 3b. The central components

The site in the central nervous system involved in glucose-sensitive neural control is not very clear. It appears to be the left side of the solitary tract nucleus (NTS) [Adach et al. 1984] or the lateral hypothalamic area (LHA) [Shimazu et al, 1983]. The information on glucose concentrations in portal blood is conveyed from the liver to NTS via the afferent vagus and projected further to the LHA with modulation of neural activity [Shimazu et al 1983]. Changes in firing rate of lateral hypothalamic neurons have been recorded with injection of glucose into the portal vein of the rat [Schmitt 1973]. It was morphologically and functionally identified that intrahypothalamic neural connections exist between the LHA and the ventromedial hypothalamic nuclei (VMH) [Zaborsky and Makara 1979; Pankseep et al. 1979]. By recording electronic activity of hypothalamic neurons, Schmitt (1973) found that after intraportal administration of hypertonic glucose, the neurons in the LHA could respond by either an increase or a decrease in firing rate, while those in VMH always responded with an increase in discharge rate, suggesting that interneurons in this region may exert inhibitory or facilitative influence. The VMH and LHA appear to control metabolism primarily through reciprocal modulation of the two efferent limbs of the autonomic nervous systems. The

cholinergic system originates in the LHA with efferent fibres in the vagus, while the sympathetic system originates in the VMH and, via the splanchnic nerves, reaches the target organs [Szabo et al. 1983]. Electrical stimulation of the LHA or VMH increases the outflow of the corresponding nervous system [Shimazu and Fujimoto 1971]. However, microinjection of insulin into the VMH resulted in rapid decrease in hepatic glucose output, which was abolished by vagotomy or pretreatment with atropine but not with adrenal blockers [Szabo et al. 1983], suggesting that the VMH may not be a pure adrenergic center, from which the glucoregulatory impulses can also travel through the cholinergic fibres of the vagal nerves. The biochemical messages involved in neural transmission within this central region remain to be confirmed, although serotonin, dopamine, norepinephrine, γ aminobutyric acid (GABA), cholecystokinin (CCK), substance P and others may be the candidates [see Bray 1985].

I. 1. 3c. Target organs of the neural reflex arch

The autonomic nerves innervate all the visceral organs. It is clear that three principal peripheral organs including the pancreas, the adrenal gland, and the liver are closely interrelated to maintain the glucose hemostasis under the control of the autonomic nervous system.

Intraportal glucose injection increases pancreatic vagal efferent discharge [Nagase et al. 1993].The pancreas has four types of endocrine cells, which are richly innervated by autonomic nerves. The B-cell is for insulin secretion, A-cell for glucagon, D-cell for somatostatin, and F-cell for

pancreatic polypeptide. The sympathetic nerves innervate the pancreas through the splanchnic nerves travelling along the pancreaticoduodenal artery and enter this organ as a part of the mixed autonomic nerves. Sympathetic nerves terminate to individual islet cells in close proximity [Miller 1981]. The parasympathetic nerves enter the pancreas through the vagus and innervate the islet via parasympathetic ganglia within the pancreas [Miller 1981]. It was supposed that these ganglia integrate neural signals and control the activity of the intrapancreatic postganglionic nerves that innervate endocrine cells. The intrapancreatic parasympathetic ganglia are innervated by both noradrenergic and preganglionic cholinergic nerves [Miller 1981].

Direct stimulation of splanchnic nerves produced a decrease of insulin release and an increase of glucagon release [Dunning et al. 1988]. Parasympathetic stimulation increased the secretion of both insulin and glucagon [Ahren and Taborsky 1986]. Local norepinephrine infusion could not reproduce the inhibitory effect of sympathetic stimulation on basal insulin secretion, but did reproduce an increase in basal glucagon secretion similar to that seen with pancreatic sympathetic nerve stimulation [Ahren et al. 1987], suggesting that different neurotransmitters may be involved in the inhibitory response. Inhibition of insulin secretion appears to be mediated by α_2-adrenoceptors and activation of β_2-adrenoceptors increases insulin secretion. Glucagon secretion is enhanced by both α- and β-adrenoceptor activation, likely to be α_2- and β_2-adrenoceptors [Miller 1981].

The enhancement of insulin release in response to vagal stimulation

is also produced by local infusion of acetylcholine and blocked by atropine in some species [Miller 1981]. However, in cat and pig, atropine failed to block the insulin response to vagal stimulation [Miller 1981], implying that other neurotransmitters may be involved in the regulation of pancreatic insulin release. Glucagon release was increased by vagal stimulation and acetylcholine infusion in the dog and this response could be blocked by atropine [Kaneto and Kosaka 1974]. However, in humans, atropine administration did not inhibit release of glucagon in response to 2-deoxy-D-glucose stimulation [Hedo et al. 1978]. Cholinergic agonists, atropine, or vagotomy failed to alter the release of glucagon in response to insulin-induced hypoglycemia [Palmer et al, 1979], showing that noncholinergic mechanisms may also play a role in vagal stimulation-induced glucagon release. Neuropeptide Y and galanin may also be involved in the endocrine of pancreas [see Yamaguchi 1992].

Intra-islet cellular interactions seem to be a regulatory mechanism for islet hormone release. In the rat pancreas, a directionality of blood flows from the β-cell core to the A-cell and then to the D-cell. Amylin (islet amyloid polypeptide), co-secreted with insulin from the β-cell at a molecular ratio of 1: 100, is proposed to be a modulator of pancreatic paracrine function [Fehmann et al, 1990]. It was assumed that amylin inhibited the release of glucagon from the A-cell, and that the inhibitory action of amylin on the D-cell was through its inhibitory effect on the A-cell [Fehmann et al, 1990]. However, no significant change of glucagon release from the cultured monolayer of rat

pancreaes was indicated when the release of both amylin and insulin was stimulated by hypertonic glucose [Kahn et al, 1990].

The effect of amylin on insulin release is controversal. Both stimulatory [Fehmann et al, 1990] and inhibitory [Parlapiano et al, 1995] actions were reported. In the isolated rat islets of Langerhans, the presence of 10^{-6} M amylin and 5.5 mM glucose showed no effect on insulin mRNA levels, the rate of proinsulin biosynthesis, the rate of coversion of proinsulin to insulin and the secreation of insulin, indicating that amylin is not a physiological modulator of insulin secretion [Nagamatsu et al, 1990]. Amylin in concentrations from 5 x 10^{-12} to 10^{-7} M had no significant effects on insulin secretion [O' Brien et al, 1990]. The levels of amylin under normal physiological conditions are lower than 10^{-8} M. The observed effects of amylin on insulin secretion in islet are likely to be pharmacological rather than physiological [Nagamatsu et al, 1990].

Amylin was proposed to be a hormone that antagonized the effects of insulin in peripheral tissues [Molina et al, 1990]. The results with amylin overproduction in transgenic mice indicated that amylin overproduction did not induce peripheral insulin resistance [Oosterwijk et al, 1995].

Somatostatin was originally found in hypothalamic extract as an inhibitor of growth hormone secretion, which is also synthesized and secreted by theD-cells of the pancreatic islets. Somatostatin circulates in peripheral plasma in a concentration of 60 pg/ml with a half-life of 2 minutes. The secretion of this hormone from the D-cells is stimulated by meals, by

gastrointestinal hormones, by β-adrenergic and cholinergic stimuli, and by glucagon, while the secretion is inhibited by α-adrenergic stimulation and insulin [Berne and Levy, 1988].

Somatostatin is an inhibitor of insulin, glucagon and growth hormone secretion. This effect was used in the insulin suppression test [Ratzmann et al, 1982, Harano et al, 1978]. Somatostatin also reduces the assimilation rate of all nutrients from the gastrointestinal tract. It is assumed that somatostatin participates in a feedback regulation, in which entrance of food into the gut stimulates the release of the hormone so as to prevent rapid nutrient overload [Berne and Levy, 1988].

Catecholamine output in the right innervated adrenal gland increased in response to insulin-induced hypoglycemia, while the output in the left denervated gland remained unchanged [Lamarche et al. 1992], indicating that sympathetic nerve impulse is necessary for the adrenal gland to participate in counterregulation of hypoglycemia. The principal innervation of the chromaffin cells in the adrenomedulla is preganglionic and the nerve fibres terminate among the medullary cells without the interposition of postganglionic neurons. It seems that vagal nerves are absent in the adrenal glands. The adrenomedulla is embryologically considered as a sympathetic ganglia and is an integral component of the peripheral sympathetic neuroeffector system. Modulation of transmitter release by presynaptic α_2- and β_2-adrenoceptors is a general characteristic of neuroeffector and synaptic transmission in the sympathetic nervous system [Starke et al. 1989], which

should be involved in adrenal catecholamine release. Available observations are contradictory in this regard [Sharma et al. 1987]. The modulation of catecholamine release from the adrenal gland by adrenoceptors is complex and needs further study. It appears that adrenoceptors may be involved in the local regulation of adrenal catecholamine release under some experimental conditions, which is different from those operating in peripheral sympathetic nerve terminals. It is likely that Ca^{++} antagonists, 1,4-dihydropyridine derivatives in particular, inhibit adrenal catecholamine release in vitro, but this has not been confirmed in vivo. Peptidergic neurons containing substance P and VIP may also be involved in the modulation of adrenal catecholamine release [see Yamaguchi 1992].

The liver is innervated by sympathetic and parasympathetic nerves as mentioned before. However, in the sympathetic side, it should be noted that the metabolic and hemodynamic activities of the liver are redundantly controlled by the hepatic sympathetic nervous system and by circulating catecholamines released from the adrenal medulla. Bilateral adrenaectomy or hepatic denervation alone did not reduce, but both the percedures together eliminated almost all the hyperglycemic response to hemorrhage [Lautt et al. 1982]. It is possible that hepatic sympathetic dysfunction may be compensated by a supplementary enhancement of adrenal catecholamine release, since the depletion of epinephrine with adrenodemedullation was compensated by a higher plasma norepinephrine level [Ricordi et al. 1988]. The hepatic glycogenolysis in exercised rats is dominately mediated by

adrenal catecholamine release [Sonne, et al. 1985], suggesting different mechanisms exist under different experimental conditions. On the hepatic parasympathetic side, the neural activity provided by the vagal nerves is the only cholinergic input. If hepatic parasympathetic dysfunction happens, no other system can compensate. It can be assumed that hepatic denervation may only produce the hepatic parasympathetic dysfunction.

Electrical stimulation of VMN enhanced glucose utilization in the heart and brown adipose tissue. Local sympathetic denervation totally abolished the increase of glucose uptake in brown adipose tissue [Takahashi et al. 1992]. This observation indicated that the effectors of VMN may be extensively present in the whole body, not limited to the organs discussed above. Takahashi et al [1992] also pointed out that VMN stimulation increased glucose uptake by increasing apparent functional activity of the glucose transporters, while insulin acted by both recruitment and activation of glucose transporters.

I. 2. The methods for hepatic nervous research

To adequately study the functions of the hepatic nerves, typical neurobiological techniques of neural interruption, stimulation and electrical recording have been used.

I. 2. 1. Neural interruption

Hepatic nerve interruption can be achieved by surgical, chemical and pathological methods. The isolated liver perfused in situ is a fully denervated organ, which can be used to study the neural functions with selective nerve

stimulation [Jungermann et al. 1987]. The results obtained by this method should be interpreted with caution, since controversies exist in comparing the observations from whole animal studies [Lindfieldt et al. 1987].

Surgical denervation in many studies has been carried out by cutting anterior nerve plexus. Elimination of 0-100% (mean 59%) of the arterial vascular constriction in response to reflexly sympathetic activation elicited by bilateral carotid arterial occlusion was achieved by cutting the anterior plexus with the remaining 41% being abolished by dissection of the posterior plexus and hepatic ligaments [Lautt 1981], suggesting that section of the anterior plexus provides only a partial denervation and that the proportion of hepatic sympathetic nerve fibres travelling in the anterior plexus is highly variable. Microsurgical denervation can be performed by severing the liver ligaments and attachments and dissecting the portal vein, hepatic artery and the common bile duct free under the microscope [Cucchiaro et al. 1990]. This method appears to be effective and induces complete and long-lasting denervation. However, This process is tedious and can be complicated by lesion of the vascular and biliary structures and its reproducibility may be not very reliable, as shown by a 17.2% variation in the liver norepinephrine content one week after the denervation [Cucchiaro et al. 1990]. Full denervation can be also carried out by combining the surgical with chemical denervation at the point 2 in figure 1. The liver ligaments are severed. The nerve bundle and the connective tissues around the hepatic artery, portal vein and bile duct are separated and cut. A phenol solution (90%) applied on a

swab is painted around each of these structures [Lautt 1984]. The potential lesion of the vasculature, as occurred with microsurgical denervation, is avoided. The performance is simplified, and no obvious untoward effects are noticed by such denervation [Lautt and Carroll 1984].

Chemical sympathectomy can be achieved about one week after intraportal administration of 6-hydroxydopamine. Responses of the liver blood volume and portal pressure to direct hepatic nerve stimulation were almost completely prevented by this pretreatment. The responses to infused exogenous norepinephrine was not affected, and femoral arterial response to systemic sympathetic stimulation did not change [Lautt and Cote 1976], indicating a lack of extrahepatic denervation. Using this sympathectomy model, Lautt and Wong [1978] first successfully demonstrated hepatic parasympathetic effect on glucose metabolism by stimulating the remaining parasympathetic nerves in the anterior plexus of the sympathectomized cat.

Norepinephrine outflow or tissue norepinephrine content is a sensitive and extensively used test for assessing the effectiveness of organ denervation or monitoring the degree of reinnervation. A quantitative relationship of hepatic norepinephrine with the degree of denervation was established [Cucchiaro 1990]. After hepatic denervation, if norepinephrine level of hepatic tissue was undetectable, the electrical stimulation of the hepatic nerves did not modify the systemic arterial pressure. Modification of arterial pressure by 21% occurred with 3% of normal norepinephrine content. The normal arterial response to hepatic nerve stimulation could be seen with

only 28-30% of the liver norepinephrine content [Cucchiaro 1990], suggesting that adrenoceptors became supersensitive as a result of chronic denervation. Functional assessment, such as hepatic hemodynamic responses to hepatic nerve stimulation, is another way to reach the same purpose [Lautt 1978, Lautt and Cote 1976]. By determination of norepinephrine content and mean arterial pressure in response to hepatic nerve stimulation, Cucchiaro et al (1990) found that surgical denervation with phenol (90%) application is as effective and reliable as liver transplantation with undetectable norepinepherine contents in the liver tissue and unmodified hemodynamic response to hepatic nerve stimulation in the first week after these interventions in rats. Intraportal administration of 6-hydroxydopamine does not completely eliminate the liver norepinephrine content, and reinnervation was faster than the other methods. Special attention should be paid if chronic denervation is performed with this method.

One week after liver transplantation in rats, liver norepinephrine content was almost undetectable. At 8 weeks after surgery, norepinephrine content was significantly increased to 20.2% of normal, suggesting that reinnervation occurred after chronic hepatic denervation [Cucchiaro 1990].

Vagotomy at the cervical level may eliminate vagal effects on all the splanchnic organs, affecting gastrointestinal secretion and movement, pancreatic endocrinal and exocrinal functions, bile secretion and afferent nerve function. Section of the hepatic branch of the vagal nerves produces more selective hepatic parasympathectomy and may not result in the

untoward effects. Severing the hepatic branch did not change the portal and peripheral plasma insulin and glucagon, or peripheral epinephrine and norepinephrine in either resting or exercising rats [Cardin et al. 1991; Lavoie et al. 1989]. Whether the hepatic vagotomy affects bile secretion is unclear. It should be noted that section of the hepatic branch of vagal nerves also interrupts the hepatic afferent supply.

A marked degeneration of vagal fibres and no evidence of spontaneous reinnervation were observed in the wall of the stomach six months after gastric truncal vagotomy. The gastric movement and acid secretion in response to vagal stimulation is seriously impaired [Nanobashvili et al. 1994]. No similar observation was noticed in hepatic vagotomy, although a significant sympathetic reinnervation of the liver has been observed 8 weeks after hepatic transplantation [Cucchiaro 1990].

Pathological hepatic nerve dysfunction can be observed in animal models and human diseases. Two weeks after bile duct ligation in cat, the response of portal venous pressure and lobar venous pressure, but not the femoral arterial pressure, was significantly reduced in response to anterior plexus stimulation. The responses were unchanged to exogenous norepinephrine infusion. Increase of systemic arterial pressure responding to norepinephrine administration was not impaired, indicating that chronic bile duct ligation-induced nerve dysfunction is limited to hepatic nerves, rather than the hepatic or systemic vasculatures [d'Almeida and Lautt 1994]. In guinea pig, nerve fibres clearly branched off the perivascular fibres and

entered the liver parenchyma. However, six weeks after bile duct ligation, Ach-E positive nerve fibres disappeared from the parenchymal cells [Akigoshi 1989]. In humans, hepatic parenchymal nerves were absent in cirrhotic nodules and were depleted in the fibrotic strands of alcoholic cirrhotic liver. However, in alcoholic hepatitis without portal hypertension, nerve fibres were increased around the portal vein branches. Innervation showed an inverse relationship with fibronectin deposition and a direct relationship to luminin content [Juskiewicz 1994; Lee et al. 1992], suggesting that the degree of hepatic nerve damage is stage-dependent in liver diseases. Autonomic neuropathy is present at the earliest stage of diabetes and parasympathetic abnormality is the earliest manifestation of diabetic autonomic neuropathy [Hosking et al. 1978], which is detailed in Section III, 3. Hepatic parasympathetic neuropathy in non-insulin dependent diabetes and hepatic diseases was proposed, which may account for reduction of insulin effectiveness in glucose metabolism [Lautt 1979].

Pathological nerve dysfunction has not been evaluated by the criteria of norepinephrine content. However, according to the quantitative relationship of vascular response with the liver norepinephrine content [Cucchiaro 1990], it can be assumed that the liver norepinephrine content should be about 3% two weeks after bile duct ligation in cat, since the systemic arterial pressure response to hepatic nerve stimulation was about 21% of those in control cats [d'Almeida and Lautt 1994], suggesting that bile duct ligation is very effective in producing hepatic nerve dysfunction.

I. 2. 2. Stimulation of hepatic nerves

Nerve stimulation is conveniently done using the anterior plexus. It is tedious to attempt stimulation the entire nerve plexus near the hilum. Although innervation of the hepatic sympathetic nerves via the anterior plexus is variable from animal to animal [Lautt 1981], electrical stimulation of the anterior plexus appears to produce qualitatively and quantitatively similar hemodynamic responses to those produced by stimulation of the combined nerve bundle around the hilum [see Lautt 1983]. This may be, again, an example showing electronic coupling through gap junctions in the liver.

Stimulation of the anterior plexus was suspected to activate the pancreatic nerves and further alter pancreatic hormone release, since the nerves along the gastroduodenal artery appears to communicate with the anterior plexus and to branch off to the pancreas. However, in cat, insulin and glucagon levels were not affected by hepatic nerve stimulation with either nerves along the gastroduodenal artery intact or interrupted [Lautt et al. 1982]. The plasma levels of glucagon and catecholamines were not altered by hepatic denervation in insulin-induced hypoglycemic rats [Mikines et al. 1985]. It is surprising that atropine administration did not affect the glucose output and hepatic vascular constriction in response to mixed nerve stimulation [Lautt and Wong 1978; Greenway et al.1967]. Stimulation of the mixed hepatic nerves produced a similar response to stimulation of the splanchnic nerve [Edwards 1972]. One week after selective sympathectomy by intraportal 6-hydroxydopamine infusion, the parasympathetic response

induced by stimulating remaining parasympathetic nerves can be overwhelmed by any remaining sympathetic nerves [Lautt and Wong 1978]. The mechanism for the dominant sympathetic effects is not fully understood. However, in the terms of glucose metabolism, it has been proposed [Lautt 1979c] that activation of glycogen phosphorylase a form by sympathetic nerves prevents the activation of glycogen synthetase that is activated by hepatic parasympathetic nerves. This results in a pure sympathetic type of effect when both sympathetic and parasympathetic nerves are stimulated.

Stimulation of the hepatic parasympathetic nerves can be done either by accurate localization of the hepatic branch of the vagus or by selective stimulation of the parasympathetic component in the mixed hepatic nerve plexus. Pretreatment of the animals by intraportal administration of 6-hydroxydopamine (25mg/kg) can selectively destroy hepatic sympathetic nerves. One week after pretreatment, stimulation of the remaining nerve fibres in the mixed nerves can specifically produce a parasympathetically induced response. The effect of hepatic parasympathetic nerves on glucose metabolism has been elicited by this animal model [Lautt and Wong 1978]. The extra-hepatic sympathetic system remained functional and the normal hypertensive response was produced by tyramine administration [Allman et al.1982].

I. 3. Carbohydrate metabolism

I. 3. 1. Glucose metabolism

I.3.1a. Effects of hepatic sympathetic nerves

Hepatic glycogenolysis is under direct control of hepatic sympathetic activity. Direct stimulation of the hepatic anterior nerve plexus increased hepatic glucose output rapidly, with onset by one minute and reaching a maximum 3-4 minutes after stimulation and decreasing thereafter. The degree of increase in glucose output was largely determined by the basal levels measured immediately prior to stimulation. The basal plus stimulation-induced glucose output kept relatively constant, suggesting that sympathetic stimulation-induced increase in glucose output was added to the basal activity of hepatic enzymes. Hepatic parasympathetic nerve did not show measurable influence on this response, since atropine administration did not alter the response [Lautt and Wong 1978]. Stimulation of hepatic adrenergic nerves activated hepatic glycogenolytic enzymes (phosphorylase and glucose-6-phosphatase) with a concurrent decrease in the liver glycogen content [Shimazu and Amakawa 1968]. Adrenalectomy did not affect hepatic glucose response to nerve stimulation, indicating a local effect of sympathetic stimulation in the liver [Edwards 1971].

Adrenergic activity-induced increase in hepatic glucose output is dominantly mediated through α_1-adrenoceptors in the liver of cat [Lautt 1979b], rat [Kunos 1984],and mouse [Seydoux et al. 1979] . Interaction of catecholamine with cytomembrane α_1-adrenoceptors leads to changes in cellular Ca^{++} fluxes which apparently results in a rise in cytosolic Ca^{++} released from intracellular organelles such as mitochondria and endoplasmic reticulum [Exton 1982]. Epinephrine, norepinephrine and α-adrenergic

agonists cause an increase of IP_3 in the liver, which may serve as an internal signal messenger to mobilize Ca^{++} from intracellular stores [Berridge 1986]. In the isolated liver perfused *in situ*, norepinephrine infusion causes an increase of glucose output accompanied by a time-dependent increase of inositol mono-, bi- and triphosphate, while hepatic sympathetic stimulation leads to a similar increase of glucose output without enhancement of inositol mono-, bi- and triphosphate [Puschel and Jungermann 1988]. Infused norepinephrine might reach every hepatocyte and enhances their inositol phosphate formation, while the neurally released norepinephrine might directly reach only a few perivascular hepatocytes, thus resulting in less inositol phosphate production [Puschel and Jungermann 1988]. In cat, α-adrenoceptor blockade with phentolamine eliminated both glycogenolytic and hemodynamic responses upon hepatic nerve stimulation, while propranolol , an unselective β-blocker, was without any inhibitory effect [Lautt 1979b]. However, the mobilization of hepatic glucose following administration of exogenous catecholamines appeared to be mediated through both α- and β-adrenoceptors, as shown by the effects of α- and β-adrenergic agonists [Lum et al. 1980].

In dog, the hyperglycemic effect following administration of catecholamines appears to be mediated through β-adrenoceptors [Hjemdahl et al. 1979]. β-adrenoceptor agonists and glucagon are linked to the plasma membrane enzyme adenylate cyclase, causing an increase in the intracellular messenger cyclic AMP. The increase of cAMP activates cAMP-dependent

protein kinase, which in turn, phosphorylates many intracellular enzymes involved in the regulation of glycogen metabolism, glyconeogenesis and glycolysis [Exton 1987].

I. 3. 1b. Effect of parasympathetic nerves

Effect of hepatic parasympathetic nerves on glucose regulation has not been extensively studied. Some important mechanisms are still unclear, for example, how hepatic cholinergic impulse spreads in the liver, why stimulation of the mixed hepatic nerve produces a dominant sympathetic effect.

In the isolated liver perfused *in situ*, insulin or acetylcholine alone, in the presence of α- and β-adrenoceptor blockers, produced only an insignificant increase of hepatic glucose uptake. However, hepatic parasympathetic stimulation in the presence of insulin resulted in a pronounced enhancement of glucose extraction from the perfusant [Monton an Burton 1971; Gardemann and Jungermann 1986]. The addition of prednisolone did not enhance the effect of insulin on glycogen deposition after three hours perfusion, suggesting that the action of glucocorticoids on hepatic glycogen synthase is a delayed effect [Monton and Burton 1971]. These observations on the isolated liver indicate a synergistic effect of insulin with the hepatic parasympathetic nerves in hepatic glucose uptake.

Electrical stimulation of the LHA on rabbits produced a decrease in blood glucose level [Shimuzu et al. 1966]. Direct stimulation of the vagal nerves markedly increased (5-fold) the rate of incorporation of glucose into

hepatic glycogen. This effect was not impaired by previous pancreatectomy, suggesting that a direct hypoglycemic effect by hepatic cholinergic stimulation. Vagal stimulation did not affect incorporation of glucose into muscle glycogen [Shimazu and Fujimoto 1971], suggesting that vagal stimulation only produces a local effect, which is limited to the liver. Net hepatic glucose uptake after oral glucose was significantly reduced in atropine-treated dogs [Chap et al 1985]. Acute vagotomy resulted in a marked reduction of glycogen deposition and elevated portal vein glucose concentration in rats [Mondon and Burton 1971]. One week after chronic hepatic sympathectomy with intraportal administration of 6-hydroxydopamine, electrical stimulation of the remaining parasympathetic nerves produced a rapid and sustained decrease of glucose release from the liver, with onset almost immediately after the stimulation, and reaching zero by 10 minutes [Lautt and Wong 1978b]. These animal studies indicated that hepatic cholinergic nerves may possess important action in hepatic glucose trapping and glycogen synthesis.

In humans, direct evidence for effect of hepatic cholinergic activity on glucose balance was observed [Boyle et al. 1988]. Administration of the muscarinic cholinergic agonist, bethanechol, caused direct inhibition of hepatic glucose production when the islet clamp technique was used to keep glucagon, insulin, pancreatic polypeptide and growth hormone levels constant. Muscarinic stimulation resulted in a sharp initial decrease in hepatic glucose output and a consequent fall (25%) in the plasma glucose

concentration [Boyle et al. 1988], suggesting that hepatic cholinergic function is mediated through muscarinic receptors.

I. 3. 2. Effect of hepatic nerves on lipid metabolism

In the Syrian hamster, hepatic vagotomy completely abolished the circadian variation in insulin-stimulated lipogenesis and reduced the magnitude of the daily lipogenic peak by 65%. Body fat stores were severely reduced (40%) 8 weeks after hepatic vagotomy, suggesting that hepatic vagal activity regulates the daily interval of lipogenic responsiveness to insulin [Martin et al. 1990]. These observations are consistent with the hypothesis that hepatic parasympathetic activity and insulin exert a synergistic effect in glucose metabolism [Mondon and Burton 1971; Lautt 1979a; Gerdemann and Jungermann 1986]. Severing the celiac nerves resulted in reduction of cholesterol synthesis and decrease in plasma level, while vagotomy produced essentially the opposite effects [Shanygina et al. 1981]. Sympathectomy of rat liver decreased serum cholesterol levels and increased the activity of the enzyme 7-α-hydroxylase which converts cholesterol to bile acids [Rogers et al. 1979]. This area needs further study.

Section II. Glucose clearance

II. 1. Glucose metabolism

Maintenance of plasma glucose within a relatively narrow range is vitally important in the mammalian organism since the central nervous system depends upon a continuous supply of glucose which is the only energy substrate it can use at a significant rate. Prolonged hypoglycemia can lead to coma and, if uncorrected, to death. Sustained hyperglycemia is also life-threatened, which can result in a loss of great amounts of sugar, water and electrolytes in the urine, leading to progressing dehydration, hypotension and coma [West 1985].

II. 1.1. The sources and storage of glucose

The exogenous source of plasma glucose is via intestinal absorption of glucose or its precursors. After a carbohydrate meal, polysaccharides (starch in plant foods and glycogen in animal foods) and disaccharides, such as sucrose, are hydrolyzed as glucose, galactose and fructose in the intestine [Berne and Levy 1988]. Glucose is actively taken up by the brush border epithelial cells of the small intestine through an active glucose transport system, as occurs in the renal proximal tubule. This transport system takes up glucose via cotransport with Na^+, deriving the required energy from a Na^+ pump, and accumulates glucose against higher glucose concentration in the epithelial cell. Glucose exits the epithelial cells through the antiluminal plasma membrane via a facilitated diffusion process [Bihler 1987]. Glucose and galactose compete for entry. Fructose transport in the intestine is not well

understood. The blood containing the absorbed sugars is drained into the portal vein and the liver is the first organ exposed to the higher concentration of sugar. This will favour the neural reflex of glucoceptor-CNS-effectors to handle the postdigestive hyperglycenia (see Section I. 1. 3.)

The liver serves as a central organ in carbohydrate metabolism between the dietary sources and the peripheral tissues. The liver takes up about 30% of glucose when it is readily available from dietary sources [Thurman et al. 1986]. Part of the glucose is converted to glycogen, which will later be available to sustain plasma glucose for many hours. This store has a limited capacity of about 75g per human. Part is metabolized to acetyl CoA, which in turn serves as the substrate for free fatty acid synthesis [Felig et al. 1975]. Extra carbohydrate can be more effectively stored as triglyceride in adipose tissues. About 70% of the exogenous carbohydrate escapes immediate hepatic uptake. This remaining carbohydrate is taken up by muscle as well as adipose tissue for storage as glycogen. Muscle glycogen can be hydrolyzed as glucose or returned to the liver as lactate [West 1985].

The liver can contribute glucose to plasma by two general mechanisms, by breakdown of stored glycogen (glycogenolysis) and by the formation of glucose from nonglucose precursors (glyconeogenesis). During early hours after postabsorption, the stored glycogen in the liver is broken down to provide glucose to plasma. Beyond this period of time, the most important source of glucose is glyconeogenesis in the liver, although the kidney can become a significant source of glucose [Owen et al. 1969]. Muscle and

adipose tissue contain glycogen, but that glycogen can not directly enter the plasma as glucose, since these tissues do not contain hexokinase that converts glucose 6-phosphate to glucose [West 1985]. From the magnitude of the arterial-venous glucose difference and simultaneous measurements of blood flow, the rate of delivery of glucose from the liver (or other organs) into the plasma can be calculated, showing the contribution of the liver or other organs to the plasma glucose pool [Laakso et al. 1992; Baron ea al. 1994].

II. 1. 2. Hepatic glucose metabolism

The liver, as a central organ, stores glucose by glycogen synthesis, produces glucose by glycogenolysis and glyconeogenesis and metabolizes glucose by oxidation and glycolysis. Glycolysis in the liver is closely related to glyconeogenesis and glycogen metabolism. These metabolic pathways are under the control of food availability, hormones, autonomic nervous system and physical activity.

II. 1. 2a. Glycogen metabolism in the liver

After glucose is transferred into hepatocytes via hepatic glucose transporters (see Section II. 2.), glucose-6-phosphate (G-6-P) is formed from glucose in a reaction with ATP catalyzed by glucokinase and hexokinase. G-6-P is in turn converted into glucose 1-phosphate (G-1-P) in a reaction catalyzed by phosphoglucomutase. Uridine diphosphate glucose (UDP-glucose) is further formed from G-1-P. UDP-glucose is the immediate precursor that donates a glucosyl group to a growing chain of glycogen. This reaction is catalyzed by glycogen synthase [Arias et al. 1994]. Conversion of

glucose into its polymeric form allows a relatively large amount of glucose to be stored without loss by diffusion and without the osmotic consequences that would attend the presence of high concentrations of the monosaccharide.

Glycogen synthase occurs in a nonphosphorylated active form, designed a, which can be converted to a phosphorylated inactive form, designated b. This conversion is catalyzed by protein kinases utilizing ATP. A phosphatase may cleave the phosphate group of b, restoring the enzyme to the a form [Arias et al. 1994]. In contrast, Phosphorylase, the enzyme that catalyzes glycogenolysis, occurs in an active phosphorylated form, called a, which can be cleaved hydrolytically by a phosphatase to an inactive, nonphosphorylated form, called b. The enzyme catalyzing the phosphorylation of glycogen phosphorylase is phosphorylase kinase, which exists as an inactive unphosphorylated b form and is converted into fully active a form by a cAMP-dependent protein kinase; phosphorylase kinase also phosphorylates glycogen synthase a to render it inactive. Therefore, a phosphorylation-dephosphorylation system controls the reactive directions towards glycogenesis or glycogenolysis [Arias et al. 1994].

In the cultured chicken embryo hepatocytes, less than 30% of the maximal storage of hepatic glycogen is stimulated by glucose *per se*, indicating that glucose is not a major factor to stimulate glycogen formation in the liver [Parkes and Grieninger 1985]. This hormone-free glycogenesis was characterized by a 30-min burst of glycogen deposition following glucose addition with no detectable change in glycogen synthase activity. The stored

glycogen degraded following removal of glucose but not following glucagon addition [Parkes and Grieninger 1985]. In the same preparation, the rate of insulin-induced glycogen deposition was comparatively slower but constantly increasing until maximal storage capacity was reached and was accompanied by a near 3-fold of glycogen synthase activity. Insulin-induced glycogen store degraded following removal of insulin or glucagon addition, even if glucose was present in higher concentration. These observations were consistent with those of Plas et al.(1982), indicating that hepatocytes require insulin, not glucose, to maintain and build up a maximal storage of glycogen and that glucose influences glycogen synthesis as a substrate by a mass action. The control of glycogen metabolism by insulin appears to involve regulation of phosphorylation-dephosphorylation through active tyrosine kinase, cAMP or Ca^{++} fluxes [Berne and Levy 1988; Radziuk 1991]. Glycogenesis is favoured by glucocorticoids, which cause changes in the activities of protein phosphatases and result in glycogen deposition. In addition, glucocorticoids cause a significant increase in phosphorylase-phosphatase activity, thereby, decreasing glycogenolysis [Stalmans et al. 1987]. In the liver, a variety of hormones and peptides can promote glycogenolysis. The transducing systems include the cAMP cascade that activates cAMP-dependent protein kinases, the Ca^{++}-calmodulin system that activates other protein kinases and the various phosphatidylinositol pathways producing phosphoinositals and diacylglycerols that activate a cAMP-independent protein kinase C. Glucagon increases intracellular cAMP and Ca^{++} concentration and promotes

glycogenolysis [Jelinek et al. 1993]. However, the actual output of glucose from the liver may be partially suppressed by the presence of insulin. Thus the ratio of glucagon to insulin may determine whether glycogenolysis results in hyperglycemia. Epinephrine, by binding to α-adrenoreceptors, may increase glycogenolysis primarily through changes in concentration of Ca^{++} and not through the cAMP system [Hems and Whitton 1980]. AMP activates phosphorylase b but also inhibits the phosphatase that inactivates phosphorylase a. Its overall effect is to maintain glycogenolysis. Under anoxic condition, AMP accumulates in the liver, and glycogenolysis is accordingly promoted.

II. 1. 2b. Glycolysis

Glycolysis is the only way by which glucose can be oxidized anaerobically with production of ATP. Under normal aerobic conditions, the liver primarily uses fatty acids as substrates for oxidation, and the glycolytic rate is low. Glycolysis occurs mainly from stored glycogen and little proceeds directly from glucose *per se*. In the other words, glycolysis depends on controls imposed on glycogen metabolism. For example, after a meal, the high concentration of glucose entering the liver activates glycogen synthase and diminishes the activity of phosphorylase, which favours glycogenesis over glycogenolysis and results in less substrate available for glycolysis [Hems 1980]. Control at the level of glycogen metabolism is coordinated by control of the irreversible reactions in the glycolytic pathway. The pathway has three irreversible reactions catalyzed by glucokinase or hexokinase, 6-

phosphofructokinase and pyruvate kinase, respectively. Hexokinase is inhibited by G-6-P in physiological concentration. 6-phosphofructokinase is inhibited by ATP, which is relieved by Pi, AMP, ADP and fructose 6-phosphate. All of the enzymatic reactions involved in glycolysis are physiologically irreversible, so that the conversions are unidirectional. Increase in concentration of cAMP results in decrease of flux of glucose through glycolysis and increase of activities of enzymes favouring glyconeogenesis. The third irreversible reaction in glycolysis is catalyzed by pyruvate kinase, which can be phosphorylated by cAMP-dependent protein kinase and results in inactivation. Dephosphorylation by a phosphatase results in reactivation [Engstrom et al.1980]. This enzyme is also inhibited by ATP and by some amino acids. In the liver, glycolysis is closely related to glycogen deposition, lipogenesis and glyconeogenesis. Normally, glycolysis and glyconeogenesis occur simultaneously. Each irreversible steps in glycolysis is matched by an irreversible step in glyconeogenesis.

II. 1. 2c. Glyconeogenesis

Under prolonged fasting condition, the liver obtains lactate, pyruvate, amino acids and glycerol from peripheral tissues, converts these precursors to glucose which redistributes to the peripheral tissues. The lactate is release from muscle, erythrocytes and renal medulla as a result of glycolysis. Skeletal muscle is responsible for 40% of lactate production in the fasting condition [Consoli et al 1992]. Specific lactate transporters have been described in skeletal muscle [Juel and Wibrand 1989]. Erythrocytes provide a relatively

constant but limited amount of lactate to glyconeogenesis. A major fraction of amino acids for glyconeogenesis is from degradation of functional muscle protein and protein in visceral organs during prolonged starvation. Some amino acids can give all of their carbon atoms to glyconeogenesis, after transamination to yield pyruvate. Others can contribute some proportion of their carbon atoms for glyconeogenesis. Alanine and glutamine are quantitatively the most important amino acids in glyconeogenesis during starvation. Glycerol released from adipose tissue during the mobilization of stored triglycerides can contribute, but is not an significant source, to glyconeogenesis. Glyconeogenesis is both an energy-consuming and a reductive process. Glyconeogenesis is catalyzed mainly by two enzymes. Pyruvate carboxylase has an almost complete dependence on the presence of acetyl-CoA for activity. Phosphoenolpyruvate carboxykinase is controlled most effectively by changes at the transcriptional level in its rate of synthesis, which is promoted by glucagon and glucocorticoids and inhibited by insulin [Nelson et al. 1980]. Amylin, coreleased with insulin, is considered to promote the hepatic recycling of lactate from the periphery into glucose [Cooper et al.1989] (also see Section I, 1, 3c). Fatty acids cannot make a net contribution to glyconeogenesis. In prolonged fasting or in diabetic ketoacidosis, ketoacids have a minor potential to be a source for glyconeogenesis.

II. 1. 3. Glucose balance in other tissues

All body tissues utilize plasma glucose. They are either obligatory or facultative users. Obligatory users, such as the central and peripheral nervous

systems, cannot use alternative substrates when glucose is not available. However, in a prolonged starvation (5-6 weeks), the brain undergoes a metabolic switch that allows the brain to use ketone bodies to substitute as much as 50% of its usual glucose requirement [Owen et al. 1967]. During such starvation, the plasma concentration of ketone bodies rises, since a large amount of fatty acids are continuously mobilized from the adipose tissue triglycerides, and a portion of them are converted to ketone bodys in the liver. A few other tissues utilize plasma glucose almost exclusively, such as red blood cells, the intestinal mucosa and the renal medulla. Glucose is largely metabolized via anaerobic glycolysis in these tissues. The facultative glucose users, such as muscle, use glucose as the first option, which substitutes for free fatty acids when plasma glucose concentration is high, and switch to use FFA as their primary metabolic fuel, when glucose source is stringent and plasma FFA levels rise. The switch from one metabolic state to another in the tissues is under the control of hormonal and nervous actions.

In muscle, a small portion of glucose undergoes glycolysis and oxidation, while a major fraction of glucose is specifically directed into storage of muscle glycogen. The increased store of muscle glycogen then is available for oxidative support for short-term exercise. The control of glycogen synthesis and glycogenolysis in skeletal muscle appears to be similar to in the liver with some notable differences in respect of hormone control. In addition to stimulation of glucose transport into muscle, insulin-stimulated protein kinase promotes phosphorylation of the protein phosphatase involved

in regulation of glycogen metabolism, thereby, enhancing the phosphatase activity toward the bound glycogen synthase and phosphorylase kinase. The respective rates of activation or inactivation of these enzyme substrates are responsible for insulin stimulation of glycogenesis. The most important effect of glucagon is to promote and sustain hepatic glucose output, which is dominant in the liver. Its action on muscle and adipose tissues is minor unless insulin is virtually absent. Catecholamines stimulate muscle glycogenolysis and enhance muscle glycolysis, which increases plasma lactate level and provides additional substrate to the liver. Epinephrine also inhibits insulin-stimulated glucose uptake of muscle [Berne and Levy 1988].

In adipose tissue, insulin also stimulates the transport of glucose into the adipose cells in a manner similar to the insulin effect on muscle. Some of this glucose is stored as glycogen, another portion is then metabolized to alpha-glycerophosphate, which is used in the esterification of fatty acids, permitting their storage as triglycerides. On subsequent lipolysis the glycerol released can serve as a gluconeogenic precursor in the liver.

In the kidney, glucose is rapidly filtered into the glomerular fluid, but it is normally reabsorbed very effectively by the renal tubules. If the glomerular filtration rate is normal (120ml/min), loss of glucose in the urine will only occur if the plasma glucose concentration is beyond 160-190mg/dl (renal threshold for glucose), therefore, the amount of glucose excreted in the urine is used as a rough indicator of the degree of hyperglycemia in advanced diabetes.

Glucose uptake in splanchnic organs was not affected by insulin, even

if plasma insulin levels increased to more than 1000uU/ml [DeFronzo et al. 1983]. However, elevation of the plasma glucose concentration by 125 mg/dl led to a greater than twofold increase in splanchnic glucose uptake. A basal insulin level was necessary for permitting this increase in splanchnic glucose uptake. The increase in splanchnic glucose uptake by hyperglycemia was the result of an increase in the delivery of glucose to the splanchnic region and not an increase in the intrinsic ability of the splanchnic tissues to remove glucose, since the splanchnic extraction ratio was not increased [DeFronzo et al. 1983]. The trapped glucose may be converted into lactate, which circulates to the liver and serves as the precursor for hepatic glycogen repletion [Radziuk 1979]. A large release of lactate by gut tissues following glucose administration has been observed [Windmueller and Spaeth 1978]. These observations suggest that the splanchnic bed may play a role in storage of fuel and supplement of hepatic glycogen when its content decreases.

II. 2. Glucose transporters

Although there is a type of transporter in small intestine and the renal tubules (section II.1.1), which mediates the Na^+-dependent active glucose transport against the glucose concentration gradient, the type of glucose transporters discussed here carries glucose into the cells by facilitated diffusion. The glucose transporters are a family of highly hydrophillic membrane proteins. The best characterized of these transporters is that of the human erythrocytes, since they are both abundant and readily available. This

isoform is designed E-transporter isoform (GLUT-1). Distinct but highly homogeneous cDNAs were also cloned from the liver, designated L-transporter isoform (GLUT-2) and fetal muscle (GLUT-3). Virtually identical cDNAs were also cloned from libraries of skeletal muscle and adipocytes, which was designed M-transporter isoform (GLUT-4) [Klip and Paquet 1990].

The glucose transporters possess all the characteristics of facilitated diffusion, which is saturable, stereospecific for D-glucose and competitively inhibited by glucose analogues. Nonmetabolizable glucose analogues, such as 3-O-methyl-glucose, are transported by the same system and show a competatitive inhibition with D-glucose. This advantage has been taken to study quantitatively the rate of glucose transport. The transport of glucose is inhibited by the fungal metabolite cytochalasin B in a kinetically competitive manner with glucose, which has been the most useful tool for the detection and quantitation of glucose transporter protein in various tissues, although cytochalasin B binds the L-transporter isoform less well. A novel and more sensitive probe for the glucose transporter, 3-$^{[125]}$iodo-4-azidophenethyl-amido-7-0-succinyldeacetyl forskolin, has been developed recently, which recognizes glucose transporters from insulin-responsive and -unresponsive tissues, and its binding is displaceable with cytochalacin B [Kasanicki and Pilch 1990].

E-transporter isoform is glycosylated and contains 12 membrane-spanning domains linked by a large highly hydrophillic cytoplasmic domain. The asparagine residue bearing the carbohydrate is positioned on the

extracellular face. A similar topology is predicted for the amino acid sequence of M-transporter isoform and L-transporter isoforms that are -65% homologous to the predicted amino acid sequence of E-transporter isoform [Mueckler et al. 1985]. Several amphipathic segments containing abundant hydroxyl and amide side chains could participate in glucose binding or line a transmembrane channel through which glucose can pass. A high degree of identity of the amino acid sequence is present in the membrane-spanning segments in these isoforms, which reflects a conserved functional importance in the movement of glucose across the plasma membrane [Kasanicki and Pilch 1990].

Two molecular mechanisms have been proposed for the translocation of glucose across the plasma membrane. An alternating conformation model predicts that the transport protein can exist in two conformations, with glucose binding in a fixed orientation. Translocation of the loaded or unloaded transport site through the membrane is determined by a conformational change in the transporter, which is induced by the occupancy of the binding site with glucose [Bihler 1987]. A second model predicts that there are two glucose-binding sites simultaneous present, implying a more complex mechanism. The kinetics of glucose transport are described well with the simple asymmetrical conformational model [Lowe and Walmsley 1986].

Glucose transporters found in different cell types appear geared to their functional characteristics. Epithelial absorptive cells transport glucose from the lumen to the plasma against a concentration gradient and fulfil their

primary role by active transport. This transport process is not regulated by insulin. Cells, such as red blood cells, are bathing in the closely regulated internal environment and do not need an accumulative system. An unregulated equilibrating system is adequate for these cells with a relatively stable biological function and a constant rate of glucose utilization. A modulated glucose transport system should be associated with cells whose function entails rapid and large changes in metabolic activity and glucose consumption, such as muscle [Bihler 1987; Kasanicki and Pilch 1990].

Both M- and E-transporter isoforms are present in human and animal skeletal muscle, although the E-transporter isoform is present at barely detectable levels. The M-transporter isoform is found almost exclusively in the tissues that typically respond to acute insulin stimulation [James et al. 1989]. It is hypothesized that the E-transporter isoform may exist mostly in the plasma membrane and is responsible for basal rate of glucose transport in the unstimulated state. The M-transporter isoform is primarily intracellularly stored. In whole body, 85% of glucose metabolism is insulin-mediated which mainly occurred in muscle [DeFronzo et al. 1983]. Upon insulin stimulation, increase of glucose uptake is produced by the recruitment of transporters from the low-density microsome to the plasma membrane, in which they are inserted by an exocytotic process [Wardzala et al. 1978; Deems et al. 1994]. The fractional rates of inward and outward transport of glucose in the forearm of healthy subjects have been studied by using the euglycemic clamp with hyperinsulinemia [Bonadonna et al. 1993]. The inward glucose transport was

dose-dependently stimulated by insulin, displaying an almost linear relationship at a low physiological range of insulin. At the higher range of insulin doses, the fractional outward transport was also stimulated by insulin in a dose-dependent manner, which was much less than the stimulation exerted on the inward transport. It was suspected that increase in fractional outward transport at higher insulin doses may be related to a build-up of intracellular free glucose in skeletal muscle [Bonadonna et al. 1993]. This hypothesis challenged the well-known concept that transmembrane glucose transport is a rate-limited step for glucose metabolism in muscle [Kasanicki and Pilch 1990].

Isoproterenol, a β-adrenergic agonist, can diminish glucose uptake without changes in transporter distribution, suggesting that the intrinsic activity of a preexisting number of glucose transporters can be regulated [Kuroda et al. 1987]. Agents that can alter membrane fluidity may also alter glucose transport rates in membrane vesicles [Pilch et al. 1980].

In adipose cells, E- and M-transporter isoforms are present, and both of them translocate in response to insulin stimulation. In skeletal muscle cells and adipocytes, circulating insulin can enhance the rate of glucose transport by 20 to 40 fold. This response does not need new protein synthesis and is fully manifest in 10-20 min [Kasanicki and Pilch 1990]. The rate-limiting step for glucose uptake in muscle and adipose tissues is suggested to be the glucose transport through the plasma membranes [Kasanicki and Pilch 1990].

In contrast to muscle and adipose tissue membrane, liver cell

membranes appear to be permeable to glucose, and the rate-limiting step for uptake is the rate at which the free glucose delivered into the cytoplasm can be phosphorylated. In the liver, glucose is phosphorylated by two major kinases: hexokinase, which is a relatively nonspecific enzyme and saturated at very low glucose concentrations (Km about 10-5M); glucokinase, a specific enzyme with a Km value close to the physiological region of glucose concentration (Km about 10-2M), so that in the physiological glucose range, glucose transport can increase when glucose levels rise and decrease when glucose levels fall. On the other hand, glucokinase is not inhibited by accumulation of glucose-6-phosphate. Therefore, G-6-P can build up and drive glycogen synthesis but does not inhibit the continuing hepatic glucose uptake when plasma glucose is high [Winehouse 1976].

Unlike the M-transporter isoform, the L-transporter isoform of hepatocytes is not translocated to intracellular membranous vesicles, but remains associated with the plasma membrane. This fact is consistent with the insensitivity of the L-transporters to insulin. This transporter in hepatocytes is symmetrical, i.e. the Km value for glucose entry and glucose exit are both 20 mM [Arias 1994]. Even though glucose transport through the hepatocyte membrane is not insulin responsive, insulin definitely stimulates hepatic glucose uptake and reduces hepatic glucose output. This effect depends on insulin inducing glucokinase and reducing 6-G-P levels by simulating 6-G-P conversion to glycogen, since 6-G-P is a feedback inhibitor of hexokinase. Reducing G-6-P concentration accelerates glucose

phosphorylation and glucose uptake [West 1988]. During prolonged starvation, hepatic glycogen breaks down and the glucose concentration in hepatocytes is higher than that of the plasma. A net glucose efflux from hepatocytes occurs. Therefore, the influx or efflux of glucose in the hepatocytes is dictated by the gradients of free glucose between plasma and intracellular glucose concentrations, rather than by a direct insulin-mediated system.

II. 3. Regulation of glucose uptake

II. 3. 1. Glucose autoregulation

In both insulin-sensitive and insulin-insensitive tissues, glucose concentration availability is an important factor determining rate of glucose uptake. Insulin enhances uptake at any glucose concentration. Even in the complete absence of insulin, glucose uptake continues in all tissues, but the rate of uptake is much lower because of the lesser efficiency of the transport system. In the perfused liver, at glucose levels above 150mg/dl, the liver shows a net glucose uptake. In human, acute somatostatin-induced hypoinsulinemia left at least 85% of glucose transport activity and 70% of glucose uptake in skeletal muscle unaffected in the postabsorptive state [Bonadonna et al. 1993]. The glucose uptake by the muscle cells may occur through the E-transporter isoform, which mediates an insulin-independent uptake in muscle. This effect exists not only in the basal state but also in exercising conditions. Muscle contraction induced by electrical stimulation of the hindquarters perfused in the presence of insulin antiserum increased

glucose uptake by 5-fold in both diabetic and normal rats [Ploug et al. 1984]. In pancreatectomized and nephrectomized rats and dogs, contraction of muscle increased the space of nonmetabolizable sugar [Goldstein et al. 1953].

II. 3. 2. Hormonal effects

II. 3. 2a. Insulin effects

Insulin is a key factor in regulating glucose uptake. In muscle, adipose tissue and other insulin-sensitive tissues, glucose transporters are translocated from intracellular store to the plasma membrane after exposure to insulin. The uptake response to insulin stimulation *in vivo* is detectable within 5.5 ± 1.5 min, reaches half maximum at 20-30 min and peak at 35-40 min and is maintained at plateau level for several hours in rabbit thigh muscle [Mossberg and Taegtmeyer 1992], with a similar time course to that of the *in vitro* response [Walker et al 1989]. Insulin causes a dose-dependent stimulation of transmembrane inward glucose transport in human skeletal muscle. Within the physiological region of daily plasma insulin concentrations (up to -600pM), glucose transport *in vivo* displays almost linear sensitivity to insulin [Bonadonna et al. 1993]. Most commonly, insulin causes a modest (50%) to dramatic (>30 fold) increase in the rate of glucose uptake, depending on the cell types. A maximal stimulation of glucose transport (approximately four-fold above the basal) has been observed with plasma insulin concentration of 10000 pM in human forearm skeletal muscle [Bonadonna et al. 1993]. Insulin-stimulated transport activity was increased

by 3.2 fold in the isolated skeletal muscle, whereas the number of cytochalasin B-binding sites only doubled [Sternlicht et al. 1988], suggesting that mechanisms other than recruitment may participate in elevating uptake. Whether a potential elevation of intrinsic activity of the glucose transporters occurs as a posttranslocational modification or is accounted for by differences in intrinsic activity of the recruited transporters remains unclear.

It has been suggested that insulin-stimulated glucose uptake is partially ascribed to increase in muscle blood flow after insulin administration. Insulin dose-dependently lowers vascular resistance in skeletal muscle and specifically augments perfusion in that tissue. This vascular action occurs over the physiological range of insulin concentrations and was found to be impaired in insulin-resistant states, such as obesity, non-insulin-dependent and insulin-dependent diabetes and hypertension [Laakso et al. 1990; Baron and Brechtel 1993]. Enhanced muscle perfusion by insulin-stimulation appears to increase capillary surface area available for glucose exchange, which probably occurs via a muscle capillary recruitment mechanism. Capillary recruitment would have the net effect to also expose previously underperfused tissue to insulin [Baron et al. 1994]. Since insulin, similar to inulin, exhibits a gradient across the capillary wall [Holmang et al. 1992; Yong et al. 1989]. Recruitment of functional capillaries within the same tissue volume would enhance the insulin action. It has been suggested that there was an optimal coupling rate between capillary flow, capillary surface area for exchange, and permeability to achieve the most efficient glucose uptake

[Baron et al. 1994]. Whether any vasodilator can have the same effect on glucose uptake is unclear. In normotensive human, hyperinsulinemia increased both cardiac output and leg blood flow. However, rates of insulin-mediated glucose uptake and the individual increment of leg blood flow in response to insulin were inversely correlated with the mean arterial pressure, suggested that attenuated insulin-mediated skeletal muscle blood flow appears to be a substantial cause of insulin resistance in the subjects with elevated mean arterial pressure [Baron et al. 1993].

Pancreatic insulin reaches the liver first. Alteration of this normal route by surgery to drain pancreatic venin into the systemic circulation produced an increase in the peripheral insulin levels and glucose clearance under basal conditions, a decrease in insulin sensitivity (more than twofold) and a potentiation of hepatic glucose production [Barron et al. 1994]. The improvement of glucose clearance is at the price of reduction of hepatic function in glucose metabolism. Hyperinsulinemia may also induce insulin receptor downregulation. Although the author suggested that this surgery may be useful for the complete normalization of glucoregulation in diabetes [Barron et al. 1994], a long-term observation is required for this conclusion. This model is similar to that seen in cirrhotic liver, in which the clearance of insulin is reduced and hepatic function is diminished by cirrhotic liver and portosystemic shunting.

II. 3. 2b. Effects of catecholamines

In healthy lean men, epinephrine alone (0.002mg/kg) did not change

whole body glucose uptake, but decreased arterial-venous glucose gradients and increased leg blood flow, indicating epinephrine has no effect to modulate non-insulin-dependent mechanisms of glucose uptake. In the presence of epinephrine, insulin dose-response curves for insulin-mediated glucose uptake in whole body and leg muscle and for leg arterial-venous glucose differences and leg blood flow were shifted to the right. Epinephrine reduced insulin-mediated skeletal muscle glucose extraction at all insulin concentrations (10-1200 $mU/M^2/min$). The ED_{50} for the effect of insulin to stimulate whole body glucose uptake was about twofold higher with epinephrine administration. Rates of whole body insulin-mediated glucose uptake at maximally effective insulin concentration were not changed by epinephrine infusion, indicating no change in maximal insulin responsiveness but a reduction in sensitivity to the effect of insulin to stimulate glucose uptake in whole body [Laakso et al. 1992]. Acute epinephrine infusion elevated hepatic glucose output (hepatic glycogen breakdown 50%), increased glycogenolysis in the peripheral tissues and decreased insulin-mediated glucose uptake in skeletal muscle. These effects were prevented by β-adrenergic blockade with propranolol and potentiated by α_1-adrenergic blockade with prazosin [James et al 1986]. Although the major effect of catecholamines is not on glucose uptake but on hepatic glucose production, epinephrine reduces insulin-mediated glucose uptake [Deibert and DeFronzo 1980; Chiasson et al. 1981], which is mediated through β-adrenoceptors, while norepinephrine potentiates this effect via α_1-adrenoceptors.

One week after adrenodemedullation of the rats, a significant increase in insulin sensitivity was observed with the euglycemic clamp at a low-dose (6.0 mU/kg/min) insulin infusion [Oshida et al. 1993]. Adrenodemedullation causes a decrease in plasma epinephrine concentration and compensatory increase in plasma norepinephrine concentration [Ricordi et al. 1988]. Chronic norepinephrine infusion (10 days) did not change basal glucose and insulin concentrations. However, basal hepatic glucose output was enhanced by norepinephrine treatment. During a euglucemic hyperinsulinemic clamp study, the glucose disposal rates were significantly elevated. This response was reversible within 36 hours after norepinephrine infusion was stopped [Lupine et al. 1990].

Adrenergic hormones show similar intracellular effects (stimulating glycogenolysis and gluconeogenesis), which counterbalance hypoglycemia induced by insulin. However, in terms of glucose transport, epinephrine suppresses but norepinephrine potentiates insulin-mediated glucose uptake in the peripheral tissues. The physiological significance has not been verified.

II. 3. 2c. Effect of glucocorticoids and growth hormone

Glucocorticoids decrease glucose uptake in peripheral tissues, both basal and insulin-stimulated. Part of this effect is associated with a decrease in the number of insulin receptors. In addition, cortisol plays a permissive role in hormone-stimulated release of free fatty acids which tends to reduce glucose transport. The major effect of cortisol is to increase gluconeogenesis [West 1988]. Growth hormone shows biphasic effects on glucose uptake. The

short-term effect of this hormone (1-2 hours) is insulin-like, increasing glucose uptake. Long-term elevation of growth hormone levels reduces glucose uptake in muscle and adipose tissues, and induces a form of secondary diabetic state. Insulin sensitivity is reduced and is partially compensated for by increased insulin release [West 1988]. During puberty, the increased growth hormone secretion was accompanied by a reduction (25%) of insulin-stimulated glucose disposal with a high rate of fat oxidation [Arslanian and Kalham 1994].

II. 3. 3. Free fatty acids

High plasma levels of free fatty acids reduce glucose uptake in skeletal muscle at any given insulin concentration. Physiologically, FFA levels are elevated during fasting since adipose tissue triglycerides are rapidly mobilized. The utilization of glucose by peripheral tissues is inhibited in order to conserve the limited glucose supply for obligatory users. The levels of free fatty acids altered both muscle insulin-mediated and non-insulin-mediated glucose uptake. After overnight fasting in normal human, non-insulin-mediated glucose uptake in forearm was 71% (66-85%) of the total muscle glucose uptake. The rates of non-insulin-mediated uptake showed inverse changes with alterations of plasma FFA levels in euglycemia. A much greater degree of inverse changes in the rates of muscle insulin-mediated glucose uptake was observed with FFA level changes in both euglycemic and hyperglycemic studies. However, the rates of muscle non-insulin-mediated glucose uptake was independent of FFA levels in hyperglycemic studies, suggesting that

during hyperglycemia, glucose uptake is mainly insulin-mediated [Piatti et al. 1991]. In addition to glucose uptake, elevated plasma FFA levels impaired the effect of insulin to suppress hepatic glucose output and reduced the rate of total glucose oxidative metabolism and increased lipid oxidation [Saloranta et al. 1993].

II. 3. 4. Working muscle

At any given glucose concentration, the rate of glucose transport into muscle is enhanced by muscle contraction. Whether glucose uptake in contracting muscle needs insulin present is controversial. In the early studies, a so-called permissive concentration of insulin was shown to be necessary for increased glucose uptake in contracting muscle [Berger et al. 1975; Vranic and Berger 1979]. However, when hindquarters from diabetic and nondiabetic rats were perfused with a perfusate containing insulin antiserum or after the muscles were sufficiently washed out with non-insulin medium, electrical stimulation increased glucose uptake of the contracting muscles fivefold, indicating that an insulin permissive effect is not necessary for an increase in muscle glucose uptake during contraction. This response to contraction only occurred in the muscle fibres that were severely glycogen depleted but not in the fibres in which glycogen was not depleted, suggesting that glycogen content is a determinant of glucose transport in response to muscle contraction [Ploug et al. 1984; Wallberg-Henriksson 1984]. Additive recruitment of glucose transporters by insulin and exercise was not observed. The same increase in glucose transporter number was caused by either

exercise or exercise followed by insulin [Sternlicht et al. 1989]. However, plasma membrane glucose transporters translocated by insulin stimulation were significantly reduced in skeletal muscle after physical restriction, as measured by cytochalasin B binding sites [Fushiki et al. 1991].

However, in addition to increasing glucose transport independently of insulin, exercise potentiates insulin action by increasing both insulin responsiveness and sensitivity. These effects outlast the stimulation of glucose transport by exercise alone in the absence of insulin [Cartee et al. 1989]. Combination of exercise and insulin infusion showed a synergistic effect on glucose disposal. Exercise decreased the half-maximal doses (ED_{50}) and increased the maximal responses for insulin-dependent glucose disposal [Wasserman et al.1991]. The additivity in glucose transport that is seen in the exercise and insulin-stimulated muscle is not produced by a further increase in the number of glucose transporters in the cell membrane, which appears to result from an increase in the intrinsic activity of the recruited transporters.

When glucose transport has returned to basal levels 2.5 hours after exercise, insulin-stimulated glucose transport is much higher than that occurring in the resting muscle. Half-maximal activation of glucose transport shifted from 60 uU/ml of insulin in muscle from sedentary rats to 10 uU/ml in muscle incubated for 3 hours after exercise. In muscle from sedentary rats, insulin at 30 uU/ml induced only -25% of glucose transport stimulated by the same dose of insulin 3 hours after exercise. Insulin did not change the amplitude and the time course of increased glucose transport seen after

exercise, since this effect exist in the absence of insulin after 3 hours of exercise. High glucose and insulin levels after exercise reduced the enhancement of insulin sensitivity. The enhanced insulin sensitivity after exercise may be caused by rapid resynthesis of muscle glycogen which was depleted during exercise [Gulve et al. 1990; Wallberg et al. 1988].

II. 3. 5. Route of glucose administration

Although hyperglycemia and hyperinsulinemia may serve as the primary signal responsible for the switch of the liver from glucose production to glucose uptake, their effects are small compared to the effect of the route of glucose administration, i.e. oral versus intravenous. Despite the presence of supraphysiologic hyperglycemia and hyperinsulinemia, net hepatic glucose uptake was always less than the postprandial rate of glucose uptake. In dogs and humans, net hepatic glucose uptake was much greater after an oral glucose load than with comparable loading by peripheral intravenous glucose administration despite controlled insulin and glucagon levels [Bergman et al. 1982; Ishida et al. 1983]. Delivery of a glucose load intraportally elicited net hepatic glucose uptake as large as that seen with oral glucose delivery and larger than that seen during delivery of glucose by a peripheral vein [Ishida et al. 1983]. At arterial insulin level of 35uU/ml and hepatic glucose load of 55 mg/kg/min, net hepatic glucose uptake was 1.16 mg/kg/min during peripheral glucose delivery and 3.77 mg/kg/min during portal glucose delivery [Adkins-Marshall et al. 1989].

The importance of the route of glucose delivery in determining net

hepatic glucose uptake has been demonstrated, but the mechanism was not totally understood. It was postulated that this gut effect is mediated by the release of some unidentified gastrointestinal hormone [DeFronzo et al. 1978] and in part by the increase in hepatic blood flow that accompanies oral glucose ingestion [Felig et al. 1975]. These hypotheses failed to explain why intraportal glucose administration produced as great an hepatic glucose uptake as oral digestion [Ishida et al. 1983]. Lautt (1979) suggested that the hepatic parasympathetic nerves are capable of fulfilling this role, in which a reflex parasympathetic effect on the liver may be mediated through the central nervous system and the activated vagus can also mediate release of insulin. The elevated insulin levels can then induce a further glucose uptake activation in the liver. The importance of this dual activation is obvious, since hepatic glycogen synthetase is maximally activated within 5 minutes of vagal stimulation but only after 30 minutes after infusion of insulin [Shimazu 1967]. This hypothesis is strongly supported by the experimental observations in dogs that surgical hepatic denervation completely abolished the differences between hepatic glucose uptakes during intraportal and intravenous glucose administration with no change in the hepatic blood flow. With infusion of somatostatin, exogenous insulin and glucagon to maintain arterial plasma insulin level (about 35 uU/ml) and glucagon level (50 pg/ml) constant, the rates of glucose uptake by the denervated liver during peripheral and portal glucose delivery were 2.13 and 2.19mg/kg/min, respectively, which were less than the rate seen in the normal liver during portal glucose delivery (3.77

mg/kg/min) and greater than that seen in the normal liver during peripheral glucose delivery (1.16 mg/kg/min) [Adkins-Marshall et al. 1992]. The lack of additive insulin activity may be an explanation [Lautt 1979], indicating, once again, a synergistic effect of insulin with hepatic parasympathetic nerves in glucose metabolism.

II. 3. 6. Circulating factors to stimulate glucose uptake in skeletal muscle

Although muscle stimulated to contraction *in vitro* after 15 min washout did not develop increased insulin sensitivity, muscle stimulated to contract in situ and then allowed to recover *in vitro* after being dissected showed a significant enhancement in insulin sensitivity like that which occurred after exercise. The apparent discrepancy between the effects of *in situ* and *in vitro* muscle contraction on muscle insulin sensitivity showed that a serum factor and an effect of contractile activity is necessary for increased insulin-sensitive glucose transport [Gao et al. 1994]. A similar observation was reported by Richter et al (1984). This factor is not produced during exercise but is present in serum obtained from sedentary rats and humans. The factor does not stimulate glucose uptake directly but plays a role in the increase in muscle insulin sensitivity that develops after contractile activity. This factor appears to be a protein with a molecular weight of more than 10000, which is not insulin, insulin-growth-factor-I, serum albumin, or a serum α-, β-, or γ-globulin. Muscle glycogen resysthesis after exercise cannot explain the observed increase in insulin sensitivity in vivo, since the marked muscle glycogen depletion after exercise in vitro is not followed by an increase in

insulin-sensitive glucose transport [Gao et al. 1994].

The liver may release a factor into circulation, which selectively enhances insulin sensitivity in skeletal muscle. The glucose utilization in the hind limbs of eviscerated dogs during maximal stimulation by either insulin or hyperglycemia was only 40% of that of controls. The liver was assumed to have the ability to stimulate peripheral glucose uptake. Cross-circulation with an intact dog restored the glucose disposal of the eviscerated animal to normal [Lang et al. 1955]. The rates of glucose uptake in eviscerated rats was decreased by half. An extract corresponding to 25mg of fresh liver per 100g rat restored the reduction in glucose uptake rate to normal. Smaller and large doses of this extract impaired the maximal effect, suggesting a limited quantitative relationship. Extracts from the spleen and kidney had no effect [Mertz and Schwarz 1962]. Glucose uptake in the isolated hindquarter was measured during perfusion with or without inclusion of the isolated liver and before and after addition of liver extract to the perfusate. Inclusion of the liver in the perfusion system increased the glucose uptake about 2-fold. Extracts from liver and kidney showed a similar effect, with an increase of 1.6-fold of glucose uptake. During perfusion of the hindquarter alone following perfusion of the hindquarter and the liver together, glucose uptake decease gradually with a half life about 30 min. The resistance of heat treatment and precipitation with perchloric acid appears to exclude it being a protein with high molecular weight [Petersen and Tygstrup 1994]. These observations indicate that a glucose uptake-stimulating factor is produced in the liver,

which can be released into circulation and enhance glucose uptake in skeletal muscle. However, this factor may differ from the factor described by Gao et al (1994), since the latter stimulates glucose uptake only after muscle contraction, and its nature is a protein. Although the factor observed by Petersen and Tygstrup (1994) is quite similar in the nature and functions to the one observed by Mertz and Schwarz (1962), the former is present in both liver and kidney and the latter present only in the liver. These observations suggest, perhaps, that multiple factors may be present in mammalian organism to stimulate glucose uptake in skeletal muscle.

II. 3. 7.. Adenosine effects

Adenosine inhibits insulin sensitivity in skeletal muscle, however, it increases insulin sensitivity in adipose tissue. Insulin stimulates the rate of glycolysis and glycogen synthesis in muscle. Adenosine influences only insulin sensitivity of muscle glycolysis, but not that of muscle glycogen synthesis. In adipose tissue, adenosine changes both lipolysis and glucose utilization. The lack of effect of adenosine on glycogen synthesis suggests that adenosine may reduce insulin sensitivity of muscle glycolysis via a post-receptor mechanism [Espinal et al, 1983]. Adenosine is continuously produced and utilized by muscle. A decrease in its rate of formation, an increase in its rate of utilization or a change in the number of adenosine receptors can account for the change in insulin sensitivity. Insulin sensitivity of muscle glycolysis is markedly improved by adenosine deaminase, an enzyme converting adenosine into inosine, or by the adenosine-receptor

antagonist, 8-phenyltheophylline [Espinal et al. 1983; Budohoski et al. 1984]. The effect of adenosine deaminase is completely abolished by the presence of an adenosine analogue, N^6-phenylisopropyladenosine, which is not metabolized by the deaminase. The adenosine-receptor agonist, 2-chloroadenosine, decreases the sensitivity of muscle glycolysis to insulin [Budohoski et al. 1984]. Adenosine deaminase is effective in increasing the sensitivity of glycolysis of insulin. The concentration of insulin in the incubation medium required to produce half-maximal stimulation of muscle glycolysis was reduced by 90% in the presence adenosine deaminase [Espinal et al. 1983].

II. 4. The methodology for insulin sensitivity study *in vivo*

This measurement has provide a great deal of useful information for understanding the pathogenesis and therapeutics of metabolic disorders. Assessment of insulin sensitivity *in vivo* is based on a quantitative relationship between plasma insulin concentration and the measurable insulin dependent metabolism. The optimal criteria for assessing insulin sensitivity has been proposed to be: time-saving, safe for the subject, accurate, independent of the glycemia, sensitive in the physiological range of insulin action, allowing assessment of insulin independent glucose clearance and providing insights into the cellular mechanism for alteration of insulin sensitivity [Bergman et al. 1985]. Although none of the measurements available satisfy every aspect, the methodology has been improving in the last half century.

II. 4. 1. Glucose tolerance test

Oral glucose tolerance test (OGTT) has long been used to evaluate the effectiveness of glucose homeostasis mechanism. In OGTT, a 75-100g dose of glucose is given after overnight fasting. Plasma glucose levels are determined in venous blood samples taken before and at 1, and 2 hours after the load. In the normal subject, the glucose levels should be below 115, 200 and 140mg/dl in the three measurements, respectively. If both the 1- and 2-hour values are more than 200mg/dl, the test is definitely abnormal. Intermediate test results indicate impaired glucose tolerance [West 1985]. The peak value of glucose level is influenced in part by the gastric emptying time and the absorption rate in the intestine. The role of the liver in OGTT is obvious, since it is bathing in the portal blood and is exposed to both the newly absorbed glucose and the newly secreted insulin at a concentration higher than that in peripheral tissues. After the introduction of the radioimmunoassay for analysis of insulin levels, inappropriately elevated plasma insulin levels with a normal or elevated plasma glucose level during the glucose tolerance test has been accepted as evidence for insulin resistance [Reaven et al. 1983].

An alternative method is the intravenous glucose tolerance test (IGTT), in which a 25-g dose of glucose is given rapidly by vein. Plasma glucose levels are measured at 10 min intervals for 1 hour, and the concentration is plotted on semilogarithmic graph paper. The slop of the disappearance curve gives a rate constant, k, which is proportional to the efficiency of glucose

uptake. In the normal subject, the k value is 1.2 or greater. The IGTT is independent of intestinal glucose absorption. This may be useful in evaluating subjects with gastrointestinal disorders [West 1985].

During the OGTT, a closed loop exists between the β cells of the pancreas and the insulin-sensitive tissues. Increase in insulin release will quickly enhance the rate of glucose disappearance. Conversely, changes in glucose production and utilization will cause a compensatory secretion of β cells. In this closed loop, glucose utilization, production and insulin secretion are all variables. Hyperglycemia induced by OGTT should enhance the glucose autoregulation effect, which may have substantial influence on insulin sensitivity. The existence of insulin insensitivity increases the difficulty of detecting all but a profound β cell defect [Bergman et al. 1985]. Therefore, the OGTT is unable to verify the contributions of insulin dependent and insulin independent glucose utilization and β cell responsiveness.

II. 4. 2. Insulin suppression test

To break the closed loop in OGTT, endogenous insulin secretion is inhibited by epinephrine infusion (6 ug/min) and propranolol-induced β-adrenergic blockade (5mg in bolus then 0.08mg/min continuous infusion) [Shen et al. 1970] or by somatostatin (125-250 ug/hour) infusion [Harano et al. 1978] during exogenous insulin (80mU/min) and glucose (6mg/kg/min) infusion. Glucose achieves a steady-state level more than 90 min after the beginning of the combined infusion. Steady-state plasma glucose is considered a measure of insulin effectiveness [Shen et al. 1970]. In the

suppression test, hepatic glucose output is assumed to be totally suppressed by the combined infusion, and the infused glucose represents the total glucose uptake in the whole body. The infused insulin raises the plasma level to about 100 uU/ml and increases glucose uptake by insulin-sensitive tissues. The steady-state plasma glucose level will be determined by the degree of hepatic glucose output suppression, insulin-independent and insulin-dependent glucose uptake.

Conflicting evidence exists as to whether hepatic glucose output is totally suppressed by insulin and glucose infusion during the insulin suppression test. Hepatic glucose output assessed by 3-H^3-glucose method was suppressed by only 80% in normal individuals [Kolterman et al. 1979] and suppressed by 69% in nondiabetic obese subjects and suppressed less in non-insulin dependent diabetic subjects [Davis et al. 1979]. The incomplete suppression is itself a manifestation of insulin resistance. Insulin insensitivity will be overestimated if hepatic glucose output is only partially suppressed.

A significant fraction of glucose utilization in the fasting state is insulin-independent, which changes as plasma glucose and insulin change. Since some pathophysiologic states shows changes in insulin-independent glucose utilization [Best et al. 1981], the model established by Gergman et al. (1985) indicates a highly and unpredictablely nonlinear function of insulin independent glucose clearance and insulin resistance. The decrease in insulin-independent glucose utilization exerts a substantial influence on the measured insulin effectiveness secondary to effects on the steady-state

glucose level. If insulin-independent glucose utilization decreases, increased steady-state plasma glucose will exaggerate insulin resistance.

The combined infusions may themselves alter tissue insulin effectiveness. Epinephrine and propranolol infusion increased the steady-state glucose level, but to the same degree in normal and diabetic subjects [Ginsgerg 1977]. One possible explanation is related to an effect of α-adrenergic stimulation on the liver. Glucose intolerant and diabetic subjects are resistant to insulin inhibition of hepatic glucose output and fasting hepatic output is higher in these subjects. During combined infusion,α-adrenergic stimulation of hepatic glucose output may exacerbate the inability of insulin to inhibit the liver [Exton 1980]. Another possibility is increase in secretion of growth hormone, which has been found to be increased by 4 to 7-fold during the insulin suppression test [Flier et al. 1976]. A slow glucose decline during the steady-state period was observed either with infusion of epinephrine and propranolol or somatostatin [Ratzmann et al. 1982; Harano et al. 1978].

II. 4. 3. Glucose clamp

A glucose clamp was introduced to maintain euglycemia by a variable exogenous glucose infusion during a constant exogenous insulin infusion [DeFronzo et al. 1979]. According to the glucose levels maintained, there are hypo-, eu- and hyper-glycemic clamps. Insulin is administered intravenously either by injection plus infusion, to achieve plasma insulin equilibrium quickly, or by infusion alone. The majority of clamp studies are performed at hyperinsulinemia. Blood samples are taken at frequent intervals, the glucose

levels are analyzed, and the rate of glucose infusion is altered to maintain plasma glucose at the target value. After a steady-state level for both plasma insulin and glucose is reached, the rate of glucose infusion is considered an indication of insulin action [DeFronzo et al. 1979]. The doses of infused insulin can be increased in separate study to establish a dose-response relationship relating plasma insulin concentration to glucose utilization.

Arterial sampling for glucose analyses should be used, since venous glucose concentration can be influenced by tissue glucose uptake in different abnormal states. In human study, arterialized venous blood was taken from veins in hands warmed to 68°C [McGuire et al. 1976]. Sampling frequency is often at 5-min intervals, but this time interval may not be sufficient for the plasma glucose to equilibrate in the extracellular fluid. A 10-min sampling is also used. The rate of glucose infusion is determined either by manual, algorithm, or automatic controls. There are both advantage and disadvantages for all these procedures [Bergman et al. 1985]

The glucose clamp is superior to the insulin suppression test in that increased endogenous insulin secretion can be avoided without pharmacological agents and the complexities in interpretation of the steady-state glucose could be minimized by comparison of subjects at similar glycemia. However, the main problems faced by the insulin suppression test, such as incomplete inhibition of hepatic glucose production, unpredictable insulin-independent glucose utilization and difficult to achieve steady-state level, have not been appropriately improved by the glucose clamp method

[Bergman et al. 1985].

Glucose clamp defines steady-state as a period during which the change of the rate of glucose infusion was not different from zero. Only under this condition is the rate of glucose infusion equal to glucose disappearance. However, when the glucose levels were tested after 120 min clamping, during the sequential 60 min, plasma glucose declined 18%, indicating that steady-state had not been achieved at 120 min. The rate of glucose infusion at a constant insulinemia continued to increase for 5 hours. Therefore, no actual steady-state rate of glucose infusion existed even after 8 hours clamping [Doberne et al. 1981]. Insulin itself needs a long time to equilibrate between the plasma pool and the remote compartment where it acts [Holmang et al. 1992]. The levels of counterregulatory hormones were analyzed during a graded hyperinsulinemic study [Koopmans et al. 1993]. Plasma glucagon levels were insulin dose-dependently inhibited, while norepinephrine levels were increased (by 50% at peripheral insulin level above 100uU/ml), which may play an important role in modulating blood flow and glucose uptake in skeletal muscle [Oshida et al. 1993; Lupine et al. 1990]. Significant metabolic changes may occur during steady-state period. Chronic hyperinsulinemia may induce rate-limiting metabolic enzymes [West 1985]. The steady-state glucose level maintained by glucose infusion at constant hyperinsulinemia may have important metabolic effects, including induction of glucose transporters, time dependent filling of glycogen storage sites, and intracellular metabolic effects [Wolfe 1984]. In an incubation study of rat epitrochlearis

muscle, prolonged exposure (1-5 h) to a moderate concentration of insulin (50-100 uU/ml) showed a progressive activation of glucose transporter over a long time so that glucose transport increased 1- to 2-fold after 3 h and 2- to 4-fold after 5 h compared with 1 h in the incubated muscle. In the presence of low glucose concentration, further activation of glucose transport was mediated through a protein synthesis-dependent mechanism. High glucose concentration inhibited its own transport [Young et al. 1986].

In addition, a marked increase ($P<0.02$) in glucose clearance was observed in all 14 human subjects except one, who showed a 31% decrease. When insulin effectiveness was tested repeatedly on different days in the same subjects without any intervention, the change range was as large as -31% to +33% in the absence of any other differences, including mean plasma glucose concentrations and steady-state insulin concentration [Greenfield et al. 1981]. The poor reproducibility is a serious problem.

Hepatic glucose output is assumed to be totally inhibited in clamping studies. Only under this assumption, exogenous glucose infusion can be equal to whole body glucose disappearance. However, whether hepatic glucose output is completely suppressed during glucose clamping is controversial. First, the tracer methods used to determine endogenous glucose production is questionable, since it was often noted that after insulin administration, the calculated rate of glucose appearance was less than the known rate of glucose infusion [Rizza et al. 1982]. Under this condition, endogenous glucose production was assumed to be zero, since the

calculated production was a negative value (tracer-determined glucose uptake - glucose infusion). A negative glucose appearance is physiologically impossible. A number of explanations are possible, including 1) recycling of the isotopic label, 2) impurities in the tracer 3). isotope discrimination and 4) model error [Molina et al 1990]. It was using this method that endogenous glucose production was estimated to be fully suppressed. In glucose clamps utilizing a single dose of insulin infusion to attain a plasma insulin of 100 uU/ml, endogenous glucose production was reported to be entirely suppressed in normal and abnormal subjects [DeFronzo et al. 1982]. Less effectiveness of insulin in suppression of hepatic output was observed in subjects with impaired glucose tolerance and nonobese and obese insulin-independent diabetes [Kolterman et al.1981]. In addition, hepatic glucose output differs between healthy subjects and noninsulin-dependent diabetics. To determine the impacts of hepatic insulin resistance in insulin resistance states, the measurement of hepatic glucose production is mandatory during euglycemic clamping to define total glucose availability, which consists of glucose infusion and hepatic output. In the study of pathogenesis of insulin resistant states, determination of hepatic glucose output is of great importance, since there may be an association between the elevated hepatic glucose output and the impaired suppression by insulin [Waldhausl 1993].

Section III. Profiles of glucose intolerance in human diseases

III. 1. Cause or result ?

Normal glucose tolerance depends on the simultaneous occurrence of several processes in response to glucose load: 1) insulin secretion, 2) suppression of hepatic glucose output and stimulation of hepatic glucose uptake and 3) stimulation of peripheral glucose uptake [Shmueli et al. 1993] (see Section II). Fasting hyperglycemia (glucose intolerance), tissue insulin insensitivity and hyperinsulinemia are the common features of non-insulin dependent diabetes mellitus (NIDDM), hepatic diseases and hypertension. With insulin-dependent diabetes, an additional inherent pancreatic defect is characterized. The primary pathogenesis of glucose intolerance in human diseases is not clearly understood, since in glucose metabolism, the actions of the liver, peripheral tissues and the pancreas connect tightly as a closed loop. Alteration of a single component will be almost simultaneously reflected by corresponding changes in two other components. Defects at β-cells, liver or peripheral cells can start a chain response and eventually lead to diabetes. If insulin release in response to glucose load is slightly inadequate or delayed, the rise in plasma glucose concentration will be excessive and prolonged. This hyperglycemia will persistently stimulate insulin secretion and bring the fasting plasma glucose concentration to normal. Fasting euglycemia will be maintained but at the expense of an increase in the plasma insulin concentration. The enhanced insulin secretion serves as a regulatory signal for insulin receptors, which will be downregulated and cause insulin

resistance in the tissues. If the insulin response to glucose load is more markedly deficient, as an initial defect or as a result of exhaustion of a predisposed pancreas, fasting hyperglycemia will eventually ensue. The clinical picture would be that of typical NIDDM [DeFronzo and Ferrannini 1982]. If hepatic glucose uptake is primarily impaired, excessive amounts of glucose will enter the systemic circulation, which will stimulate an increase in pancreatic insulin secretion. The fasting plasma glucose concentration may be maintained but only with increased insulin release, which will in turn downregulate insulin receptors and lead to insulin resistance in the tissues. A peripheral defect in glucose uptake will result in hyperglycemia, which stimulates the pancreatic secretory activity. Chronic hyperinsulinemia eventually exacerbates insulin insensitivity secondary to insulin receptor adaptation. Although both receptor and postreceptor steps could be the sites of impairment in tissue glucose uptake, it seems likely that receptor changes seen in human subjects with chemical diabetes is the only abnormality, whereas a postreceptor deficit would characterize more serious degrees of glucose intolerance. The liver, the peripheral tissues and possibly β-cells all show the insulin resistance so that the entire range of metabolic alterations could be related to the same primary defect [DeFronzo and Ferrannini 1982].

Insulin secretion, hepatic glucose uptake and production, and peripheral glucose uptake are all under direct control of autonomic nerves. Abnormal activities of the autonomic nervous systems have substantial effects on glucose balance. Parasympathetic dysfunction will result in a

reduction insulin secretion [Ahren and Taborsky 1986; Miller 1981], a decrease in hepatic glucose uptake and increase in hepatic glucose output [Shimazu et al.1966; Shimazu and Fujimoto 1971; Lautt and Wong 1978b; Boyle et al 1988; Adkins-Marshall et al. 1992;], even though the influence of parasympathetic abnormality on peripheral glucose disposal is unclear. Sympathetic deficit may be compensated by overdriving the adrenal gland. Sympathetic overaction, as seen in hypertension, results in an increase in hepatic glucose production and a decrease in peripheral glucose uptake [Shimazu and Amakawa 1968; Lautt and Wong 1978; Puschel and Jungermann 1988].

III. 2. Insulin resistance in hepatic diseases

Since glucose intolerance was noted by Creutzfeld et al. in liver cirrhosis in 1906, the pathogenesis of this metabolic disturbance has undergone extensive investigation. In terms of glucose metabolism, hyperinsulinemia and impaired glucose tolerance are frequently seen in the patients with chronic liver diseases. Basal hepatic glucose production is normal or insignificantly reduced in the patients with liver cirrhosis and could be suppressed to a similar degree as in control subjects: by 85 vs 90%, 97 vs 85% and 95 vs 95% at plasma insulin levels of 30-60uU/ml, 80-90uU/ml and 40-60 uU/ml, respectively, suggesting insignificant hepatic insulin resistance in cirrhotic liver [Proietto et al.1980; Cavallo-Perin et al. 1985; Petrides et al. 1991].

Although there is no clear-cut consensus regarding its mechanism,

peripheral insulin resistance may play an important role for the development of glucose intolerance [Megyesi et al. 1967]. The insulin dose-response curves of glucose clearance rates were shifted to the right and downward in the patients with liver cirrhosis, indicating a reduced sensitivity and responsiveness to insulin. ^{125}I-insulin binding to its receptors on erythrocytes was reduced, which was due to the reduction in the receptor sites, rather than in affinity [Miyamoto et al. 1992]. These observations suggested that the reduced receptor binding may be partly responsible for the rightward shift of the insulin dose-response curve. However, the downward shift may be caused by a defect beyond the receptor binding. In these cirrhotic subjects, insulin-stimulated maximal glucose clearance was correlated with fasting plasma insulin levels ($r = 0.57$, $P<0.01$) [Miyamoto et al. 1992]. Therefore, insulin resistance in cirrhotic patients may be associated with a down-regulation of insulin receptors produced by hyperinsulinemia. In a study using positron-emission tomography in liver cirrhotic patients, insulin-stimulated glucose transport into skeletal muscle was diminished by 69% in the cirrhotic patients. Glucose oxidation determined by indirect calorimetry and phosphorylation were normal and whole body nonoxiditive glucose disposal (glycogen synthesis) was markedly depressed in these patients. The kinetic constants for glucose transport into muscle were reduced, but those for transport out of muscle were enhanced with a marked decrease in accumulation of [^{18}F]fluorodesoxyglucose activity in muscle tissue [Selberg et al. 1993], indicating a marked reduction of skeletal muscle glucose transport.

Insulin resistance in CCl_4-induced cirrhosis in rat was found to be due to reduction in whole body glucose disposal, which was mainly accounted for by a decrease in insulin-stimulated glycogen disposal in muscle and lipid tissues, while insulin-mediated tissue glucose uptake and phosphorylation were unchanged [Meyer-Alber et al. 1992]. Impaired insulin-stimulated nonoxidative glucose metabolism was also observed in patients with non-insulin dependent diabetes [Shulman et al. 1990], suggesting a posttransporter abnormality. In the chronic CCl_4-induced cirrhotic model (10 weeks), unchanged glucose transport into muscle was noted. In cirrhotic patients, reduced peripheral glucose transport is a main feature. This apparent discrepancy is unclear, but may related to the different pathogeneses or species.

In alcoholic cirrhotic subjects, insulin degradation, indicated by the molar ratio of C-peptide to insulin was significantly reduced in the cirrhotic subjects, explained at least partially by decreased hepatic function and portal-systemic shunting [Petrides and DeFronzo 1989], The diminished hepatic insulin clearance led to an elevated peripheral insulin level. Total glucose utilization and forearm glucose uptake were reduced by more than 50% and 200%, respectively, in cirrhotic subjects during hyperglycemic clamping. However, insulin-independent glucose uptake, studied by somatostatin infusion, and splanchnic glucose uptake was unchanged [Shmueli et al. 1993].

The causes of the hyperinsulinemia in cirrhosis has been extensively studied in order to discern whether the hyperinsulinemia is due to increased

pancreatic β-cell secretion, impaired hepatic insulin clearance or impaired nonhepatic insulin clearance. An impaired hepatic insulin clearance could be due to intrinsic hepatic disability or to portal-systemic shunting. Low or normal insulin levels in the portal vein have been demonstrated in cirrhotic patients with peripheral hyperinsulinemia [Greco et al. 1979], indicating normal or impaired pancreatic insulin secretion. Low C-peptide to insulin ratios have been observed in cirrhotic patients [Johnston et al. 1977; Kruszynska et al. 1991], suggesting decreased hepatic degradation, since C-peptide and insulin are secreted at a molar ratio of 1 to 1 and C-peptide is primarily cleared by the kidney [Henriksen et al. 1987]. Hepatic parenchymal damage has been found to be mainly responsible for peripheral hyperinsulinemia [Johnston et al. 1977]. Transposition of pancreatic venous drainage to the systemic circulation [Barron et al. 1994], in which pancreatic insulin escapes from the first pass effect of the liver, produced peripheral hyperinsulinemia and insulin insensitivity, and less suppression of endogenous glucose production.

Insulin resistance was suspected to be induced by high plasma growth hormone levels. Cirrhotic patients showed a variable elevation of plasma growth hormone levels after glucose loading [Saaman et al. 1969]. Growth hormone administration diminished whole body and forearm glucose uptake [Fineberg and Merimee 1974]. Although the hypothesis has been extensively studied, the correlation between elevated growth hormone and impaired insulin sensitivity has not been confirmed [Shmueli et al. 1993]. When growth hormone was suppressed by somatostatin administration, insulin resistance

could be still demonstrated [Shmueli et al. 1993; Greco et al. 1979]. It is likely that elevated growth hormone may not be the main cause in the pathogenesis of cirrhotic insulin insensitivity.

Glucagon levels were high in the cirrhotic subjects under basal and hyperglycemic conditions. It is unlikely that glucagon altered whole body or forearm glucose uptake, since this hormone has no direct effect on peripheral glucose uptake and hepatic glucose uptake can be normally suppressed during hyperglycemic clamping [Pozefsky et al. 1976]. It has been suggested that cirrhotic patients are more dependent on glucagon for their hepatic glucose production. However, the evidence is controversial. Suppression of glucagon secretion with somatostatin infusion either decreased glucose production with a consequent fall in plasma glucose level [Keller et al. 1982] or maintained it unchanged [Shmueli et al. 1993].

Another explanation for diminished glucose uptake in cirrhosis is that oxidation of FFA can reduce muscle glucose utilization [Randle et al. 1963]. Elevated plasma FFA levels are an ubiquitous phenomena in cirrhosis, which is explained as adipocyte insulin resistance [Petrides et al. 1991]. However, the confirmed evidence argues against a role for FFA in the insulin resistance in cirrhosis. Increase or decrease in the plasma FFA levels did not affect whole body or muscle glucose uptake, neither insulin-dependent nor insulin-independent [Shmueli et al. 1993; Walker et al. 1991; Baron et al. 1989].

Impairment of hepatic autonomic nerves in cirrhotic liver has been demonstrated histologically and functionally in animal models [Akigoshi

1989; Schafer et al. 1993; d'Almeida and Lautt 1994] and in humans [Lee et al. 1992; Juskiewicz 1994]. In long-term bile duct ligation-induced cirrhosis in cats, hemodynamic studies indicated that the basal systemic arterial and central venous pressures were unchanged; responses of liver volume and venous resistance to infused norepinephrine were normal, but these responses to hepatic nerve stimulation were reduced; reflex arterial blood pressure response to carotid occlusion was normal [Schafer et al. 1993]. These observations suggested that the impairment of hepatic sympathetic nerves may be compensated by overdriving the adrenal gland, as indicated by an increase in the plasma catecholamine levels in cirrhotic patients [Petrides et al. 1991; Campillo et al. 1990]. Hepatic parasympathetic nerve dysfunction would be maintained, since no supplementary mechanism exists. The cirrhotic liver may also change in release of some blood-borne factors, which can selectively influence glucose uptake by peripheral tissues (see Section II. 3. 6.).

III. 3. Non-insulin dependent diabetes (NIDDM)

Although the pathogenesis of non-insulin dependent diabetes remains controversial, NIDDM is characterized by postprandial hyperglycemia, glucose intolerance and hyperinsulinemia. Deficiency of insulin secretion in response to OGTT and IGTT in NIDDM patients with fasting hyperglycemia is often observed, especially during the early phase of the tolerance test. There is a progressively greater deficit with increasing severity of fasting hyperglycemia. If the fasting plasma glucose levels are higher than 140-

160mg/dl, the insulin secretion tends to be flat [DeFronzo and Ferrannini 1982]. The contradictory observations exist, which indicated that even if the fasting plasma glucose concentrations were in the 140-160 mg/dl range, the insulin response could be enhanced during early and late tolerance test [Jackson et al. 1972]. Therefore, a defect of insulin secretion cannot entirely explain the fasting hyperglycemia. In chemical diabetes (normal fasting glycemia with an abnormal OGTT), insulin secretion was increased in absolute terms [Jackson et al. 1973; Reaven and Olefsky 1977]. However, if both OGTT and IGTT were abnormal, the early phase insulin release was diminished [Seltzer et al. 1967]. The plasma insulin response in chemical diabetes is quiet variable. Whether such subjects with chemical diabetes are truly diabetics or simply represent one extreme distribution of the normal glucose tolerance is uncertain [DeFronzo and Ferrannini 1982]. It should be noted that the observed impairment of insulin secretion in NIDDM is compared to normal subjects. The decreased insulin secretion does not mean hypoinsulinemia. Compensatory hyperinsulinemia is common in patients with NIDDM, since the β-cell is under constant stimulation of hyperglycemia, unless the secretory capacity of the β-cell is exhausted.

Tissue insulin resistance plays an important role in the impaired glucose clearance in NIDDM. *In vivo*, a blunted decline in plasma glucose concentration following intravenous insulin administration was observed in NIDDM with fasting hyperglycemia [Alford et al. 1971]. By using the isolated perfused forearm technique, an impairment in insulin-stimulated glucose

uptake was demonstrated [Zierler and Rabinowitz 1963]. Insulin resistance was indicated with the insulin suppression test in chemical diabetes and patients with fasting hyperglycemia [Reaven and Farquhar 1969; Reaven and Olefsky 1977]. Despite fasting hyperglycemia, insulin-stimulated glucose utilization was reduced by 30%, as indicated with the glucose clamp technique [DeFronzo et al. 1979]. Therefore, tissue insensitivity to insulin is invariably associated with glucose intolerance in non-insulin dependent diabetes. The cellular mechanism of tissue insulin resistance has been hypothesized to occur both at the insulin receptor and post-receptor levels. In both chemical diabetes and in diabetes with postprandial hyperglycemia, the total specific binding of radioactive insulin was reduced by approximately 30-40% with a decrease in total binding capacity but unchanged affinity. A positive correlation between the decrease in insulin binding to monocytes and insulin resistance was observed [Olefsky and Reaven 1977].

Whole body insulin resistance could result from augmented hepatic glucose output, impaired hepatic glucose uptake or reduced glucose uptake in peripheral tissues. In NIDDM, the liver and skeletal muscle are both insulin insensitive. Physiologically, fasting hyperglycemia *per se* and hyperinsulinemia in NIDDM exert an inhibitory action on hepatic glucose production. However, hepatic insulin resistance results in increased glucose production, even though there is an appropriate compensatory increase in insulin secretion [DeFronzo et al. 1982; Bogardus et al. 1984]. Hepatic glucose production is increased in subjects with NIDDM in the postabsorptive

state, and is directly correlated with fasting hyperglycemia [DeFronzo 1988]. Therefore, the hepatic insulin resistance can overcome the inhibitory effect of hyperglycemia and hyperinsulinemia to produce glucose. Hepatic glucose production in NIDDM has been emphasized by Firth et al. (1986), since they observed that glucose uptake by muscle was actually not reduced in their NIDDM subjects due to fasting hyperglycemia and the consequent glucose autoregulation. An increase in hepatic glucose production can be considered to be a major factor responsible for postabsorptive hyperglycemia in NIDDM [Consoli 1992]. Increased gluconeogenesis has been suggested to be the predominant source of glucose production [DeFronzo et al. 1985]. Applying a new isotopic technique, Consoli et al. observed about a three fold increase (12.7 ± 1.4 vs. 3.6 ± 0.4 umol/k/min) of gluconeogenesis in diabetic subjects, compared with that in nondiabetics. No significant change in glycogenolysis was seen [Consoli et al. 1989]. However, the sources of gluconeogenic precursors have not been fully identified [Consoli 1992]. Adipose tissue [Consoli 1992] and the splanchnic bed [DeFronzo et al. 1983] have been speculated to be the source. Some hepatic autoregulatory mechanisms must be defective in NIDDM, since in nondiabetic subjects, increase in gluconeogenic precursors failed to produce an increase in hepatic glucose output [Jahoor et al.1990]. Elevated glucagon levels may contribute to the increased rates of hepatic glucose production in NIDDM. The activity of key enzymes in the gluconeogenesis pathway is stimulated by glucagon (see Section II).

Following oral glucose administration, approximately 60% of the glucose load is retained in the splanchnic bed, 25% is utilized by the brain, and only 15% is available for insulin-mediated uptake by peripheral tissues [Felig et al. 1975]. Therefore, the liver is the major organ responsible for the disposition of oral glucose. Under the postprandial state, both hyperglycemia and hyperinsulinemia are the primary signals to switch the liver from glucose production to uptake. The route of glucose administration plays a great role for hepatic glucose uptake (see Section II. 3. 5.). Intravenous glucose administration results in much less hepatic glucose uptake than occurs after that oral or portal administration [Donovan et al. 1994; Adkins-Marshall et al. 1992]. However, in patients with NIDDM, whether hepatic glucose uptake in the postprandial state is impaired or unchanged remains unanswered, since the observations from the same laboratories were inconsistent [Ferrannini et al. 1980, 1988]. Felig et al. reported (1978) that over 3 hours after oral glucose was administered, 2-fold more glucose escaped from splanchnic uptake in diabetics with fasting hyperglycemia than in normal subjects. In contrast, an increased initial splanchnic glucose sequestration was observed in NIDDM patients [Butler and Rizza 1991].

Although intravenous insulin and/or glucose induce a normal decline in hepatic glucose production in diabetes with fasting hyperglycemia [Kalant et al. 1963], a defect in suppression of hepatic glucose production following oral glucose cannot be excluded. Decreased suppression of hepatic glucose output in postprandial state was invariably observed in NIDDM [Ferrannini et

al. 1988; Mitrakou et al. 1990; Butler and Rizza 1991], indicating that endogenous glucose production plays an important role in postprandial hyperglycemia. The 5 hour integrated postprandial rate of hepatic glucose release was 46 g in NIDDM, while it was only 26 g in nondiabetic subjects, so that 20 g more glucose was released into the peripheral circulation in NIDDM [Mitrakou et al. 1990]. Since the ED_{50} of the plasma insulin levels for suppressing hepatic glucose production is much lower than the ED_{50} for stimulating muscle glucose uptake [Campbell et al. 1988], it was suggested that hepatic insulin resistance is more severe than peripheral insulin resistance [Consoli 1992]. Therefore, although decreased hepatic glucose uptake may affect glucose disposal, impaired suppression of hepatic glucose production may be the main cause of postprandial hyperglycemia in NIDDM.

Intravenous and oral glucose administration shows an apparent discrepancy in suppression of hepatic glucose output as well as in stimulation of hepatic glucose uptake. This effect has been suggested to be related to hepatic parasympathetic neural regulation [Lautt 1979], to the release of some unidentified gastrointestinal hormones [DeFronzo et al. 1978] or to the increase in hepatic portal flow that accompanies oral glucose ingestion [Felig et al. 1975]. The hypotheses proposed by DeFronzo et al. and Felig et al. failed to explain an important fact that portal glucose administration, escaping this gut effect, induced a similar degree of hepatic glucose uptake to that induced by oral administration [Adkins-Marshall et al. 1992]. In the same study, surgical hepatic denervation totally abolished the apparent discrepancy

in hepatic glucose uptake between intravenous and intraportal glucose administration. Atropine administration produced a similar effect on hepatic glucose uptake after oral glucose in conscious dogs [Chap et al. 1985], indicating that normal activity of hepatic parasympathetic nerves is vital for the normal hepatic glucose uptake and output in response to intraportal glucose delivery.

Autonomic neural disruption is extremely common and accounts for many of the less severe symptoms of diabetes. At the earliest stage of diabetes, autonomic neuropathy exists and parasympathetic impairment is the earliest manifestation of diabetic autonomic neuropathy [Hosking et al. 1978]. The early peripheral nerve functional abnormalities in human diabetes occurred after a few days of severe metabolic derangement and included a slight decrease in nerve conduction velocity and an increased resistance of electric impulse propagation. The histological changes are dramatic even in asymptomic patients, including a severe fibre loss, axonal degeneration and demyelination. The prolonged diabetes mellitus resulted in peripheral nerve damage that included sensory loss, weakness, pain and absent tendon reflexes. It has been suggested that neural biochemical abnormalities result from exposure of this tissue to hyperglycemia, which might relate to the deranged nerve function in diabetics. However, reversible biochemical abnormalities can not explain the constant functional impairment of the nerves, since restoration of normoglycemia does not reverse the decreased nerve conduction to normal [Pietri et al. 1980]. Plasma catecholamine levels

in response to hypoglycemia in patients with a slight or severe diabetic neuropathy was insignificantly changed [Polonsky et al. 1982; Bolli et al. 1983]. As far as plasma norepinephrine responses were concerned, no differences in diabetes with and without symptoms of autonomic neuropathy occurred [Bolli et al. 1983]. Even though sympathetic neuropathy is unconfirmed, parasympathetic impairment exists at the beginning of the disease. Whether autonomic nerve dysfunction is a cause or a result of NIDDM remains unclear.

III. 4. Hypertension

Borderline hypertension, a condition in which the blood pressure oscillates between normal and high value, is a predictor of future more severe hypertension. Extensive studies have been carried out in this early state of hypertension with the intention of providing to give an insight into the primary mechanism of hypertension [Julius et al.1971; Sannerstedt et al 1971]. Borderline hypertension is characterized by an increased cardiac output and an elevation of heart rate [Lund-Johansen 1967]. It is clear that the elevated cardiac output and heart rate in borderline hypertension is entirely neurogenic [Julius and Esler 1975], in which the sympathetic overactivity is coupled with a decrease in parasympathetic tone [Julius and Gudbrandsson 1992]. After Intravenous administration of 0.2mg/kg of propranolol, resting cardiac output and heart rate decreased more in patients with the borderline hypertension, but still maintained significantly higher than normal subjects [Sannersted et al. 1971]. Only following injection of 0.04mg/kg of atropine, did the residual

difference of cardiac output and heart rate between two groups disappear [Julius et al. 1971], indicating that the residual elevation of cardiac output and heart rate in the borderline hypertension after propranolol injection was produced by inhibition of parasympathetic nerve system. Parasympathetic neural abnormalities in borderline hypertension is widely spread over the whole body. The production of saliva, which is regulated by the parasympathetic nervous system, was tested by placing cotton-wool cylinders in the mouths of the borderline hypertensive patients and normal subjects [Rahn et al. 1983; Bohm et al. 1985]. Saliva production in the patients with borderline hypertension was significantly reduced. The reciprocal changes in the tone of the two branches of the autonomic nervous system was assumed to be regulated by central nervous system [Julius 1991]. However, this explanation is controversial, since sympathetic tone restores to normal level in more advanced hypertension, but parasympathetic tone remains diminished in established hypertension [Korner et al. 1973; Esler et al. 1986]. Although the mechanism is unclear, sympathetic overactivity seems likely to be reversible, but parasympathetic nervous dysfunction may last as long as the entire course of essential hypertension. If parasympathetic impairment is never able to restore to the normal level for the rest of the patients' life, it is reasonable to assume that a parasympathetic neuropathy exists in hypertensive patients. This hypothesis needs further study. Reduced parasympathetic activity may have a substantial influence on glucohemeostasis, and may result in a decreased insulin secretion and

hepatic glucose uptake and an unsuppressed hepatic glucose output in postprandial state and impaired glucose intolerance (see Section III. 1.).

Patients with hypertension, treated or untreated, are insulin resistant, hyperglycemic and hyperinsulinemic [Reaven 1991]. In young lean normotensive healthy offspring (24 ± 1 years old) of essential hypertensive and nondiabetic parents, insulin resistance, subtle impairment of glucose tolerance and hyperinsulinemia have been shown to occur with normal fasting plasma glucose concentration before any evidence of hypertension [Allemann et al.1993]. These offspring developed hypertension more frequently during 5 years of follow-up, suggesting that metabolic alteration may precede the onset of hypertension. High blood pressure itself apparently does not produce insulin resistance, since insulin sensitivity has been reported to be normal in human renovascular hypertension [Marigliano et al. 1990] and in renal vascular or deoxycorticosterone-salt hypertensive rats, but not in spontaneously hypertensive rats [Burztyn et al. 1992]. Hyperinsulinemia itself appears not to be a primary determinant of hypertension, since the patients with insulinoma have hyperinsulinemia without hypertension [Tsutsu et al. 1990]. In insulinoma, hyperinsulinemia is always accompanied by a low blood concentration, while most other hyperinsulinemia conditions are associated with normal or high plasma glucose levels. These observations suggested that there may exist a common pathogenesis, perhaps autonomic nervous dysfunction, behind all the phenomena, including insulin resistance, hyperinsulinemia, hypertension, high triglycerides and cholesterol and lower

high-density lipoprotein values.

The changes in glucose and insulin metabolism may play a role in the etiology of hypertension. Rats developed insulin resistance and hyperinsulinemia within one week and a consistent increase in blood pressure within ten days after substituting fructose for carbohydrate in rat chow. Infusion of somatostatin into fructose-fed rats significantly reduced both plasma insulin concentration and blood pressure [Hwang et al. 1987]. In obese subjects, weight loss, which enhanced insulin sensitivity and lowered plasma insulin concentrations, also decreased blood pressure in patients with hypertension [Reisin et al. 1978]. Blood pressure could be lowered in obese individuals by physical training without any change in weight, but only in those individuals who were hyperinsulinemic before the training program was begun [Rosenthal et al. 1983]. A significant correlation between plasma insulin concentration and blood pressure has been indicated [Lucus et al. 1985]. Therefore, changes in insulin metabolism may play a role in the increase in blood pressure. Insulin increases blood pressure perhaps through enhancement of sympathetic activity and promotion of renal tubular sodium reabsorption [DeFronzo et al.1975]. Autonomic neural imbalance appears to be the probable cause of hyperinsulinemia and both overactivity of sympathetic nerves and hyperinsulinemia tend to raise the blood pressure [Reaven 1991]. Therefore, endogenous hyperinsulinemia is a risk factor for coronary heart disease. The fact that antihypertensive treatment has not focused on correcting these metabolic abnormalities may explain why it has

been difficult to show that lowering blood pressure decreases the risk of coronary heart disease [Reaven 1991].

Section IV. Hypotheses and objectives of this study

Although hepatic parasympathetic nerves have been shown to have direct effects on hepatic glucose metabolism in isolated liver and in animals (see Section I. 3. 1b), the pathological impacts of dysfunction of these nerves is unclear. Insulin and hepatic parasympathetic nerves have been reported to have a synergistic effect in glucose uptake in the isolated perfused animal liver. However, this has not been demonstrated in whole animals. Extensive autonomic neuropathy is common in patients with chronic hepatic diseases, non-insulin dependent diabetes and hypertension. These disease states also show progressive insulin resistance (see Section III). As early as in 1979, it was proposed that hepatic parasympathetic neuropathy seen in non-insulin dependent diabetes may account for insulin resistance occurring in these patients [Lautt 1979]. Therefore, an *in vivo* study to clarify the role of hepatic parasympathetic nerves in insulin-mediated glucose metabolism is imperative.

The hypotheses to be tested are: (1) normal functional hepatic parasympathetic nerves are essential for insulin-mediated glucose uptake; insulin sensitivity is hepatic parasympathetic tone-dependent; (2) hepatic parasympathetic interruption produces insulin resistance; compensatory supply of cholinergic agonists reverses it; (3) insulin insensitivity occurring in hepatic disease is also accounted for by hepatic parasympathetic neuropathy.

The objectives of these studies are (1) to quatitate the effectiveness of hepatic parasympathetic nerves on insulin-stimulated glucose uptake; (2) to

localize the site where insulin resistance is produced after hepatic parasympathectomy; (3) to reverse this insulin resistance by means of cholinergic agonist; (4) to reveal the dependent relationship of insulin effectiveness on the activity of hepatic parasympathetic nerves. As a peripheral issue, it also became necessary to develop a new insulin sensitivity test that could facilitated these objectives.

V. Insulin resistance produced by surgical hepatic denervation

V. 1. Materials and methods

Cats of either gender (3.75 ± 0.16 kg, n=22) were fed with standard laboratory cat chow and subjected to controlled lighting (light on 0800-2000) for at least three days. Prior to experiment, the cats were fasted over night (18 hours), but were allowed free access to water, and were anesthetized with intraperitoneal injection of sodium pentobarbital (32.5 mg/kg). Anesthesia was maintained throughout the whole experiment by supplemental doses (6.5 mg/kg) of the anesthetic with an intravenous drop through a branchial vein cannula. The anesthesia level was checked frequently and adjusted according to disappearance of the corneal reflex to touching. Body temperature was maintained at 37.5° C by means of a rectal probe and a thermal control unit that regulated heating rods in the surgical table.

Systemic arterial blood pressure was monitored from a catheter in the right femoral artery. Central venous pressure was monitored from a cannula inserted via the right femoral vein, into the inferior vena cava. The arterial blood samples (0.2 ml) were taken from a catheter in the left femoral artery. Plasma glucose concentration was analyzed by the glucose oxidase method with a YSI model 27 glucose analyzer (Yellow Spring Instrument Co., Yellow Spring, Ohio). The left femoral vein was cannulated for insulin infusion. A tracheal cannula was inserted to prevent aspiration of gastric contents.

After laparotomy, by midline incision from the xiphoid plexus to the umbilicus, the cat was allowed to recover from the surgical interventions for

45 min before any procedures were carried out. Arterial blood samples were taken at 10 min intervals until three successive stable glucose concentrations were obtained. The mean of these three concentrations is referred to as the basal glucose level. As soon as the basal glucose level was determined, insulin (beef and pork) (Novo Laboratories Ltd., Willowdale. Ontario) (100 mU/kg in 1 ml saline) was intravenously infused over 5 min. Plasma glucose concentrations were measured at 15, 30, 45, and 60 min after insulin infusion.

In the tested group (n=13), sequential hepatic denervation was performed in each cat in the following order after having determined the control hypoglycemic response to infused insulin.

Hepatic anterior nerve plexus and associated connective tissue were carefully separated from the common hepatic artery and cut. Arterial glucose levels were allowed to stabilize, as determined by analyzing plasma glucose concentration at 10 min intervals. As soon as three successive stable concentrations were obtained, the second dose of insulin (100 mU/kg) was intravenously infused over 5 min. Arterial blood samples were taken at 15, 30, 45, and 60 min after insulin administration, and the glucose concentrations were analyzed.

An extensive hepatic denervation was carried out following hepatic anterior plexus denervation. The hepatic artery, portal vein, and bile duct were separated using blunt dissection with forceps at the level of the mixed anterior and posterior nerve plexus near the hilum of the liver. A 90% aqueous phenol solution was applied around each of these vessels and adjacent tissues with

a cotton swab on a stick. Phenol was also painted along the hepatic ligaments (falciform, left and right triangular ligaments) to ablate any nerves that may have passed to the liver via these routes. Care was taken not to leave any excess phenol in the abdomen. This method of denervation was previously described and evaluated [Lautt and Corroll 1984]. A stabilization period was allowed as previously described and the third dose of insulin was administered.

Following this extensive hepatic denervation with phenol, vagi were cut bilaterally at the cervical level. The stabilization process was repeated and the fourth dose of insulin infusion was administered, and glucose levels were determined at 15 minute intervals over 1 hour.

All experimental conditions were the same in both control (n=9) and test groups (n=13). The sham hepatic anterior plexus denervation was performed by only separated the hepatic anterior nerve plexus from the common hepatic artery, but leaving it intact. The sham phenol denervation was performed by applying saline in the same manner as used for the phenol. The sham vagotomy was conducted by exposing and manipulating the vagi. Stabilization, sampling and insulin infusion were carried out as described for the test group.

Thus each animal received four infusions of insulin (beef and pork, 100 mU/kg in 1 ml saline), with the test group receiving insulin prior to denervation and following each progressive denervation procedure. The sham-denervated group represented a time control, where no denervation was actually

performed but insulin was infused before any manipulation and after each of the three sham denervation procedures.

The data were analyzed with unpaired t-test between control and test groups and with blocked ANOVA for comparison of responses within the same group. The data were expressed as mean ± S.E throughout. Basal glucose levels prior to insulin infusion were taken as the mean of the three consecutive stable values taken 10 min apart.

V. 2. Results

The index used in this study to describe the magnitude of insulin sensitivity was the area under the glucose response curve (AUC) expressed as the percentage of the basal area under the curve (BAUC), as shown in Fig. 2. The reasons for using this index are that plasma glucose responses to infused insulin showed a positive linear relationship with basal glucose level in both control (n=9) and test (n=13) groups (r^2=0.37, P< 0.003, n=22) and that the area under the curve can more exactly depict the change of the measured parameters over a given period of time. Comparing the percent change of glucose response after dnervation with that before denervation showed the influence of hepatic denervations on the effect of insulin in glucose balance.

Figure 3 shows %AUC/BAUC after each procedure in both groups. Sham hepatic denervations did not significantly alter responses compared with their control. The control responses to insulin infusion in both control and test groups were not different (P=0.84). In the test group, anterior plexus denervation significantly reduced the hypoglycemic response to insulin

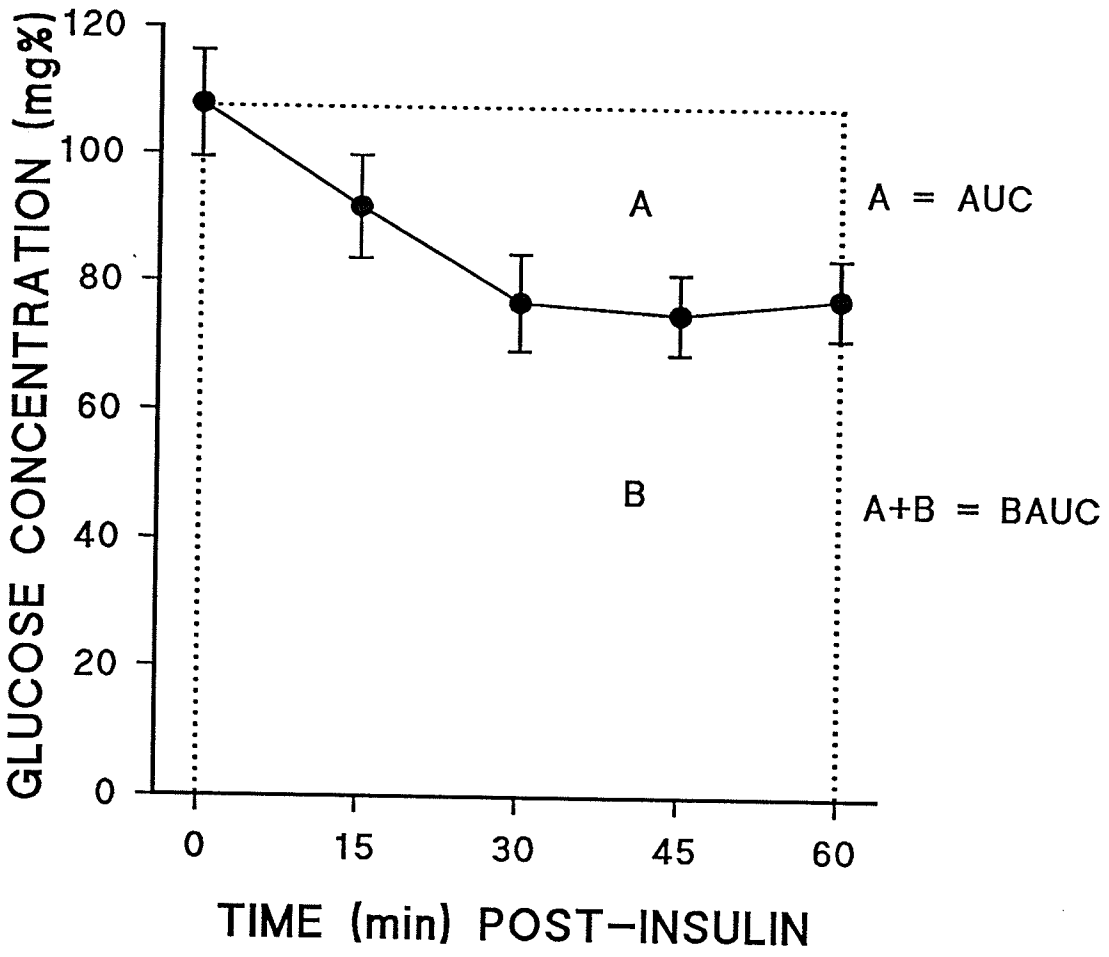


Fig. 2. Hypoglycemic responses (mg%) to control insulin (100mU/kg) infusion pooled for the sham and test groups (n=22). A + B = BAUC: basal area under the curve. The control glucose level is the mean of three stable glucose concentrations measured 10 min apart. A = AUC: area under the glucose response curve. The response to insulin is expressed as AUC/BAUC x 100%. Reprinted from Xie et al. Can. J. Physiol. Pharmacol. 71:177, 1993.

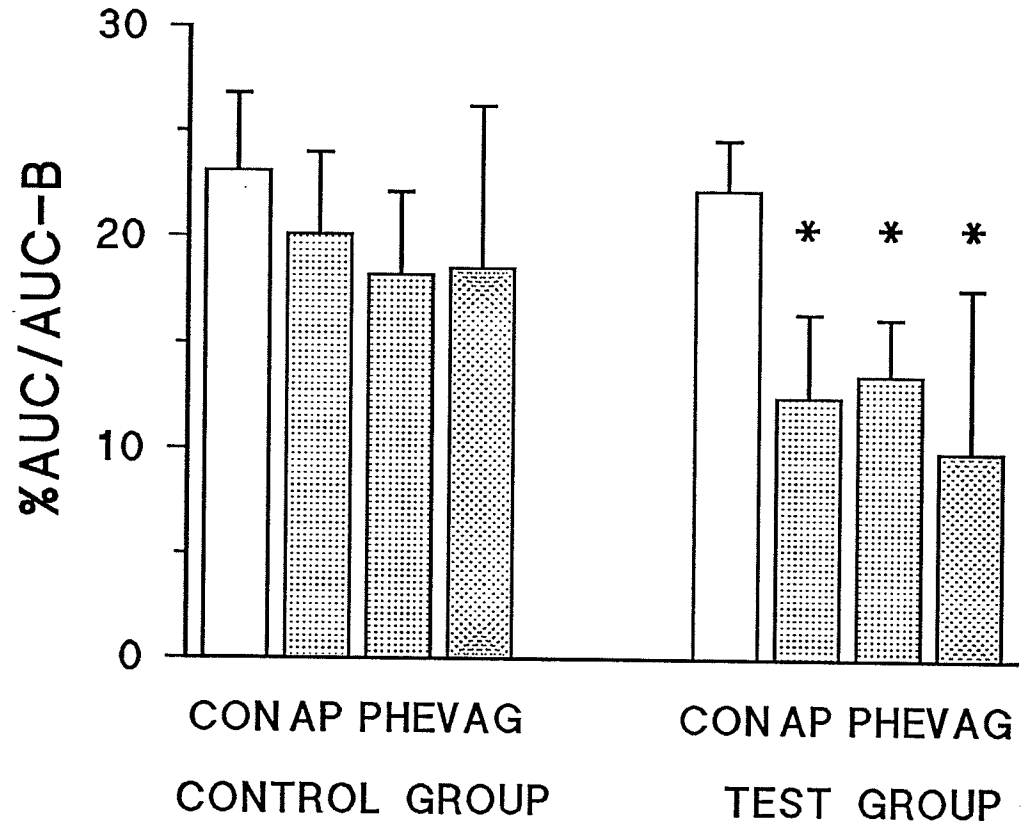


Fig. 3. Reduction in glucose levels, expressed as area under the curve (%AUC/BAUC), in response to insulin infusions in both groups. Control (CON) responses are compared with responses after anterior plexus denervation (AP), total hepatic denervation using phenol (PHE), and bilateral vagotomy (VAG) or the sham operations. There are no differences of response between control and any sham denervation. There is very significant difference between control and hepatic anterior plexus denervation ($P < 0.006$, $n=13$), but no differences between hepatic anterior plexus denervation and phenol denervation, or bilateral vagotomy. Reprinted from Xie et al. Can. J. Physiol. Pharmacol. 71:177, 1993.

(inhibition of 37.2 ± 9.8%, P< 0.006, n=9) and subsequent denervation procedures produced no further significant changes.

Figure 4 shows the time course of absolute glucose levels after insulin infusion before and after anterior plexus denervation. The maximal decrease in glucose levels occurred 30 min after insulin infusion and the temporal pattern of insulin effectiveness was not altered by the denervation.

V. 3. Discussion

The present study quantified the whole body insulin-mediated hypoglycemic response before and after hepatic denervation at different levels in anesthetized cats. Surgical anterior nerve plexus denervation caused 37.2 ± 9.8% inhibition of the hypoglycemic response over 60 min to insulin (100 mU/kg) infusion (P<0.006, n=9). The maximal decrease in the glucose level (20.9 ± 2.8%) occurred at 30 min after insulin infusion. Phenol-induced total hepatic denervation and bilateral vagotomy failed to further significantly alter the degree of insulin resistance established by anterior plexus denervation. These observations demonstrate that insulin-stimulated glucose balance is, at least partially, dependent on an intact and normal functional hepatic anterior nerve plexus. Insulin sensitivity in glucose uptake is reduced by about one-third with hepatic anterior plexus innervation.

The liver receives its innervations via the anterior plexus, forming a sheath along the common hepatic artery, and the posterior plexus, ramifying around the portal vein and bile duct. Both nerve plexuses freely communicate and carry sympathetic and parasympathetic fibres [Lautt 1983]. Reduction of

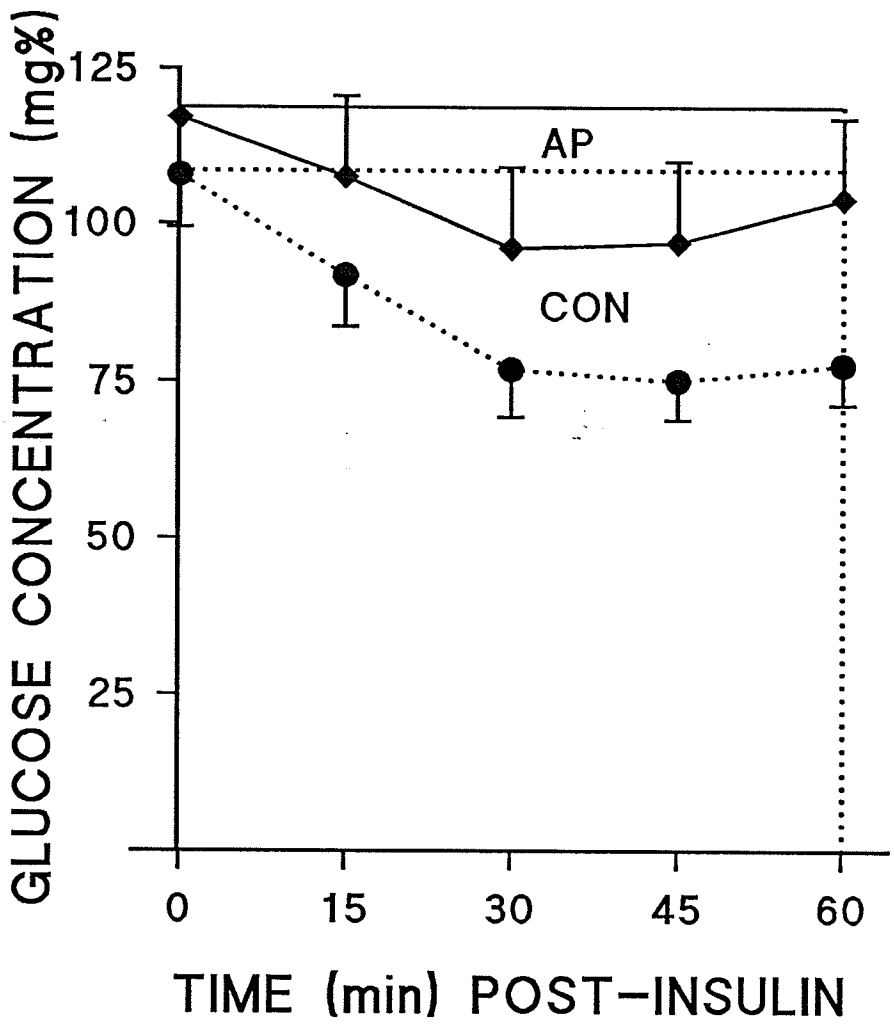


Fig. 4. Comparisons of time course of glucose concentration after insulin infusion before (CON) and after anterior plexus denervation (AP). Denervation changed the magnitude of the response to insulin but not the time course. ● represents the hypoglycemic response over 60 minutes in control insulin test; ✦represents the response to the insulin test after hepatic anterior plexus denervation.

insulin sensitivity developed by anterior plexus denervation can not, with certainty, be ascribed to hepatic parasympathetic denervation. However, several lines of evidence implicate the parasympathetic nerves. Bilateral vagotomy reduces hepatic glycogen deposition and decreases hepatic glucose uptake in rats. Administration of acetylcholine restores the deposition rate to normal. Furthermore, the lack of dramatic effect of insulin on the isolated perfused liver can be overcome by concomitant administration of acetylcholine [Mondon and Burton 1971]. Electrical stimulation of the lateral hypothalamic area (parasympathetic area) of rabbits results in a decrease of blood glucose level [Shimazu et al. 1966]. In cats pretreated with 6-hydroxydopamine to selectively destroy hepatic sympathetic nerves, electrical stimulation of the remaining efferent nerve fibres of the anterior plexus leads to a rapid and sustained decrease of hepatic glucose release [Lautt and Wong 1978]. Direct electrical stimulation of the vagus nerve increases the activity of hepatic glycogen synthase and hepatic glycogen deposition in the absence of the pancreas and decreases glucose output from the liver [Shimazu 1967, 1971; Shimazu and Fujimoto 1971]. Insulin-induced glucose uptake is potentiated and the glucagon-induced glucose release is antagonized by concomitant stimulation of hepatic nerves in the presence of α- and β-adrenoreceptor antagonists [Gardemann and Jungermann 1986]. These observations demonstrate a role of hepatic parasympathetic nerves in glucose deposition in the liver and are consistent with the hypothesis that insulin resistance produced by surgical denervation is dependent on interruption of

the hepatic parasympathetic nerves.

It is unlikely that the anterior plexus denervation produces insulin resistance secondary to sympathetic denervation because stimulation of the sympathetic nerves result in a rapid glycogenolysis and glucose release [see Lautt 1983; Shimazu 1971; Jungermann et al. 1987; Yamaguchi 1992]. Pancreatic and adrenal hormones were unchanged by hepatic denervation [Doiron et al. 1990; Sonne et al. 1985], suggesting that the efferent nerve activity involved in glucose regulation was unaltered. Therefore, although a possible role of afferent nerves cannot be assessed from this study, the responses appear to best be accounted for by interactions between insulin and hepatic parasympathetic nerves. This hypothesis is consistent with the suggestion that hepatic parasympathetic neuropathy may contribute to insulin resistance in the non-insulin dependent diabetes [Lautt 1979]. In addition, the insulin resistance known to occur with hepatic cirrhosis [Collins et al. 1970] may be partly accounted for by the hepatic neuropathy that is seen in the cirrhotic liver [Lee et al. 1992]..

V. 4. Conclusions and further study

Hepatic anterior plexus denervation reduces the insulin-mediated hypoglycemic response by one-third. The extensive hepatic denervation or bilateral vagotomy fails to alter the degree of insulin resistance caused by anterior plexus denervation, suggesting that the hepatic anterior nerve plexus is the main pathway for the denervation-induced insulin resistance. This study did not confirm which autonomic nervous system interruption is responsible

for the observed insulin resistance. From the literature, the hepatic parasympathetic nervous system seems to be the better candidate.

Insulin sensitivity was tested by estabolishing insulin-induced hypoglycemic responses in this study. Although there were no significant differences, the hypoglycemic responses to insulin tended to reduce over the experiment in the control group. It is likely that hypoglycemia influences subsequent insulin responses to some degree. Severe hypoglycemia may stimulate strongh counterregulatory mechanisms, as indicated by the observation that 250 mU/kg insulin in rats produced a marked increase in plasma catecholamines and glucagon concentrations [Kari et al. 1985]. As reviewed in Section II, 4., the insulin suppression test and the classic euglycemic technique are unsatisfactory insulin sensitivity tests for multiple determinations in the same animal. A satisfactory euglycemic technique was required to overcome these disadvantages. Development of this methodology in both rats and cats became the next priority.

Section VI. Insulin sensitivity tested with a modified euglycemic technique in cats and rats

VI. 1. Materials and methods

VI. 1. 1. Modified euglycemic clamp in rats

Male Sprague-Dawley rats (368.8 ± 6.2 gm, n=19) were fed with standard laboratory rat chow and subjected to controlled lighting (light on 0800-2000) for at least three days. The rats were not fasted and were anesthetized with an intraperitoneal injection of pentobarbital sodium (65 mg/kg) at 8:00 a.m. Constant anesthesia was maintained throughout the experiment by continuous infusion of pentobarbital (1 mg/100gm/hour, 1.5 mg/ml saline) through a cannula (polyethylene tubing, PE50) in the right jugular vein, supplemented with 3.25 mg (0.05 ml) pentobarbital intravenous injection if required. The rats were placed on their back. The trachea was cannulated (polyethylene tubing, PE240). The body temperature was monitored with a rectal thermometer (HI 8875, Hanna Instruments) and temperature was maintained at 38 ± 0.3°C by means of a heated surgical table and a heat lamp above the table. The rats were heparinized with 200 IU/kg heparin (200 IU/ml saline).

Arterial blood pressure was monitored through a cannula (polythelene tubing, PE50) in the left femoral artery and central venous pressure was monitored through a cannula, inserted via the left femoral vein, into the inferior vena cava. The left jugular vein was cannulated with the same size tubing for glucose infusion.

The arterial blood samples (25 µl) for glucose analysis were obtained through a right femoral arterial-venous loop shown in Figure 5. We designed this sampling technique specifically for this purpose. The circuit consisted of one piece of silicon tubing to act as a sleeve (#14, 8 cm in length) and two pieces of PE50 tubing (12 cm long each). The two pieces of PE50 tubing were tightly connected to the ends of the sleeve with double ties. A 3-way connector was inserted into the middle of the silicon sleeve. The third opening of the connector was connected to a piece of PE90 tubing (20 cm long) with a stop cock at its end. The whole system was filled with heparin/saline (200 IU/ml) solution and the ends of the circuit were inserted into the right femoral artery and vein, respectively. Arterial blood, with higher pressure, continuously flows through the circuit into the venous side. Arterial blood samples were taken and insulin was administered intravenously by puncturing the sleeve. To check the effectiveness of the circuit, a small amount of saline (0.1 ml) was injected and a diluted blood stream could be seen flowing through the loop. If clotting occurred in the circuit, the clot was removed through the connector.

The rats were allowed to recover from the surgical interventions for 45 min before any procedures were carried out. Arterial blood samples were taken at 5 min intervals until three successive stable glucose concentrations were obtained. The mean of these three concentrations is referred to as the basal glucose level. When a basal glucose level was established, insulin (Insulin-Toronto, beef and pork, connaught Laboratories Ltd,. Willowdale, Ontario) (100 mU/kg in 11 rats and 50 mU/kg in 8 rats) was intravenously

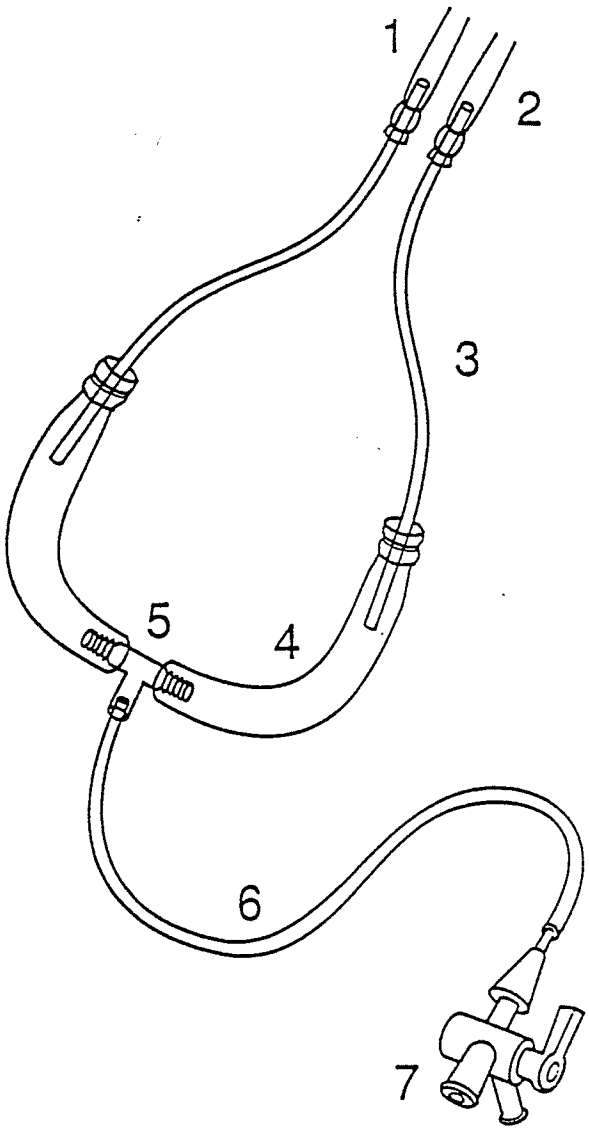


Fig. 5. The femoral arterial-venous loop showing the right femoral artery (1), the right femoral vein (2), PE50 tubing (3), silicon sleeve (4), three-way connector (5), PE90 tubing (6), and stopcock (7). Arterial samples or intravenous drug administration are done via direct needle puncture of the silicon sleeve.

infused over 5 min.

A glucose solution (100 mg/kg in saline) was infused by a continuous variable infusion pump (Harvard Apparatus) with a 60 ml syringe. Arterial blood samples (25µl) were taken at 2 min intervals over the first 20 min and at 4 min intervals over the next 40 min. Arterial glucose concentrations were immediately analyzed by the oxidase method with a glucose analyzer (see Section IV. 1.). Based on the arterial glucose concentration measured at each time point, the infusion rate of the glucose pump was adjusted whenever required, to clamp the arterial glucose levels as close to the basal value as possible. To avoid an early decline in arterial glucose levels, the glucose infusion was commenced immediately after the insulin administration at a rate of 5 mg/kg/min for the 50 mU/kg dose of insulin and 8 mg/kg/min for the 100 mU/kg dose. The initial infusion level was based on pilot studies and was used in all animals. No theoretical calculations were required to estimate infusion rates required. The adjustment of the infusion rate of the glucose pump may be as small as ± 1 mg/kg/min and assessment of suitable euglycemia can be made and adjusted in as short a time as 2 minute intervals. The total volume of blood withdrawn was 0.5 ml, and the volume of fluid infused was about 5.5 ml per insulin test.

VI. 1. 2. The modified euglycemic clamp in cats

Cats of either gender (3.48 ± 0.2 kg, n=13) were prepared as described in Section IV.1.. A closed sampling system was designed for blood sampling. Two lengths of polythelene tubing (PE90, 30 cm and 5 cm long) were tightly

connected to the two ends of a piece of silicone tubing (#14, 10 cm long) to act as a puncture sleeve. The other end of the 5 cm piece of tubing was connected to a 3-way stop cock with a 5 ml syringe. The whole system, including 2 ml in the syringe, was filled with the heparin-saline solution (200 IU/ml). The open end of the 30 cm catheter was placed into the left femoral artery. Before sampling, at least 2.5 ml of blood was drawn through the system into the syringe to avoid dilution within the sleeve. Blood samples for glucose analysis (25 µl) were obtained by directly puncturing the silicon sleeve. If blood was needed for other samples, it was obtained through the stop cock. This procedure allows sterile technique. However, since sterile technique was not required, the 2.5 ml blood in the syringe was returned to the animal after sampling. The modified system for sampling from the rat (Figure 5) would also be useful in the cat.

The cat was allowed to stabilize after the surgical interventions for 45 min before any procedures were carried out. The process of arterial glucose stabilization was performed as in rats. When the basal glucose level was determined, insulin 100 mU/kg was infused over 5 min. The euglycemic clamp technique was performed as in the rats, except that glucose infusion (50 mg/ml saline) was started with 2 mg/kg/min and arterial glucose levels were tested 3 min apart over 60 min after insulin administration. The adjustment of glucose infusion rate was as small as ± 0.5 mg/kg. The total volume of blood withdrawn was 0.5 ml.

VI. 1. 3. Five-step time control insulin sensitivity tests in cats

In order to determine if the insulin effect, and insulin and glucagon levels, changed with time or with readministration of insulin, a five-step time control was carried out in 5 cats. The cats were prepared as described in Section V. 1. 2. Each cat was treated with the same dose of insulin five times. Between each insulin sensitivity test the arterial glucose level was allowed to stabilize and a new basal glucose level was determined prior to the next test.

Arterial blood samples (1.5 ml) were taken before and 15 min after each insulin infusion, plasma was separated and then stored at -20° C for later analyses of insulin and glucagon. Plasma insulin and glucagon concentrations were analyzed by radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA).

The data were analyzed by unpaired t-tests in the two groups of rats with different doses of insulin. The data were analyzed by repeated measures ANOVA with Tukey-Kramer multiple comparisons test in the 5-step time control group. The data were expressed as mean ± S.E. throughout. Basal glucose levels prior to insulin infusion were taken as the mean of the three consecutive stable values taken 5 min apart.

VI. 2. Results

The index to express insulin effectiveness used in this new insulin sensitivity test (IST) was the sum of glucose (mg/kg) infused over a period of time after bolus insulin administration. After 45 min recovery from surgical interventions, a longer period of time was required to stabilize the arterial

glucose level in cats (46.3 ± 2.4 min, n=13) than in rats (27.4 ± 0.7 min, n=19, $P< 0.05$).

In rats, insulin 100 mU/kg administration required 493.6 ± 27.3 mg/kg glucose to keep the basal glucose level constant over 60 min, or 355.0 ± 14.3 mg/kg over the first 30 min and 138.6 ± 14.1 mg/kg for last 30 min. Insulin 50 mU/kg infusion required 269.3 ± 20.2 mg/kg over 60 min, or 198.7 ± 11.1 mg/kg over the first 30 min and 70.6 ± 11.4 mg/kg over the last 30 min. The IST showed different responses to the two doses of insulin ($P<0.05$). The curve of the glucose infusion rate showed a sharp increase in the first 10 min, reached a peak at about 10 minutes and rapidly dropped to a stable level around 25 minutes (Fig. 6 and 7). The insulin sensitivity teat could be completed in the rat over a period of only 30 min.

In fasted cats, insulin 100 mU/kg administration required 250.6 ± 4.6 mg/kg glucose to maintain arterial glucose level constant over 60 min, or 127.2 ± 3.2 mg/kg over the first 30 min, 123.6 ± 1.8 mg/kg for the last 30 min. A similar amount of glucose was required over the last 30 min to that for the first 30 min. The maximal infusion rate occurred around 30 min after insulin infusion (Fig. 8). At the end of the insulin test, about two-thirds of the peak glucose infusion rate was still needed. The curve of glucose infusion rate in cat is flat and smooth and decreases slowly. The deviation of arterial glucose level from the ideal baseline level was less than 3.5%.

In the five-step time control cats, insulin 100 mU/kg administration required 214.0 ± 23.6, 208.4 ± 27.1, 220.8 ± 30.1, 205.7 ± 24.1 (n=5,

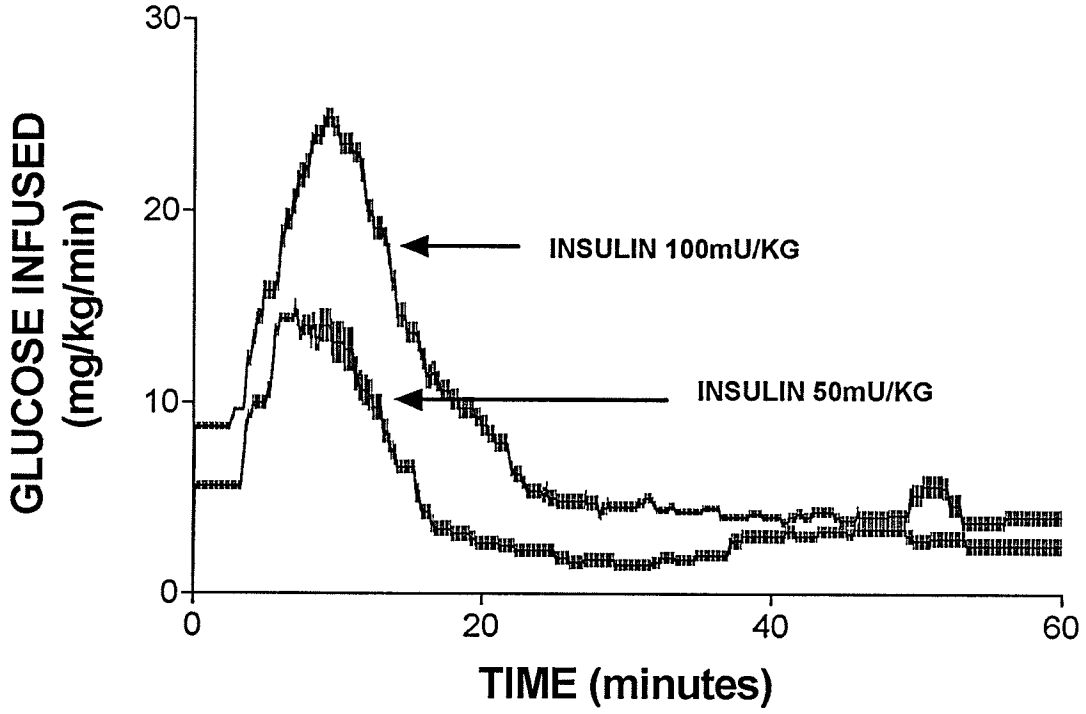


Fig. 6. The upper curve represents the rates of glucose infusion in rats (mg/kg/min) at each 0.1 min over 60 min after insulin 100 mU/kg administration (mean ± S.E., n = 11). The lower curve shows the corresponding rates of glucose infusion after insulin 50 mU/kg administration (n = 8).

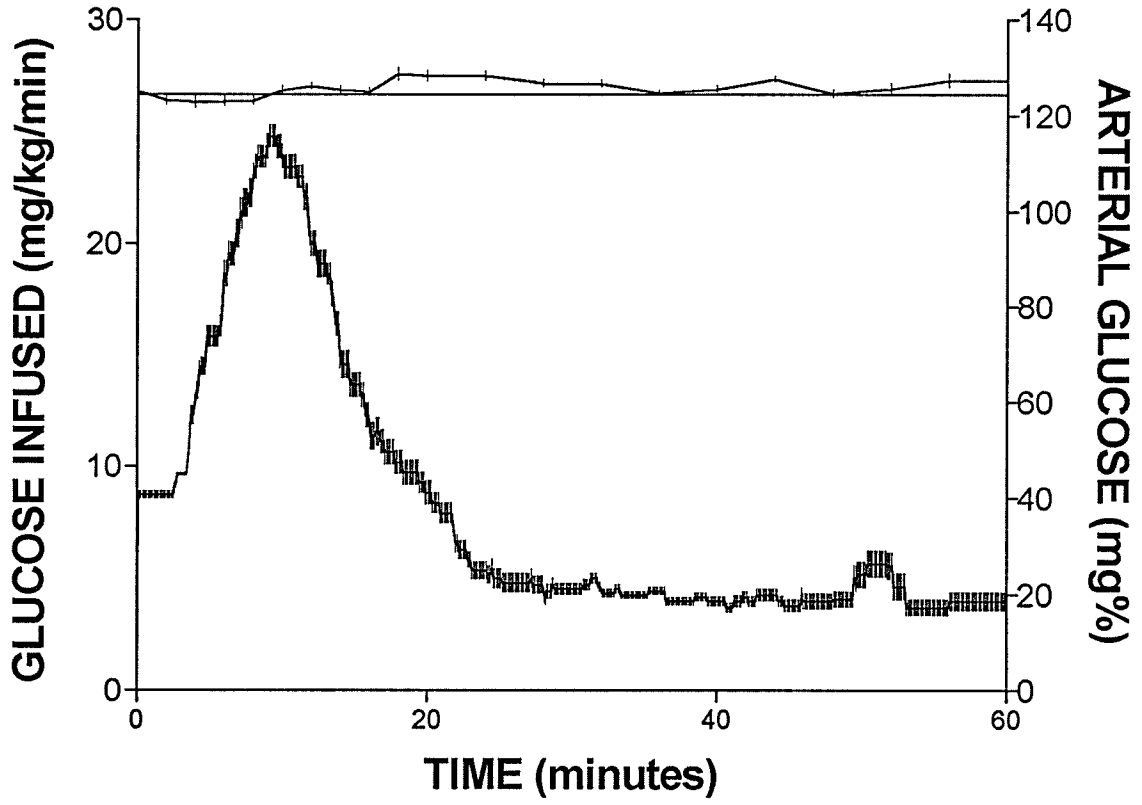


Fig. 7. The lower curve represents the rates of glucose infusion in rats (mg/kg/min) at each 0.1 min over 60 min after insulin 100 mU/kg administration (mean ± S.E., n = 11). The top straight line represents the basal arterial glucose level (mg%) determined by the mean of three successive stable arterial glucose concentrations. The upper line with S.E. shows the actual glucose levels (mg%) maintained by the modified euglycemic clamp after insulin 100 mU/kg administration.

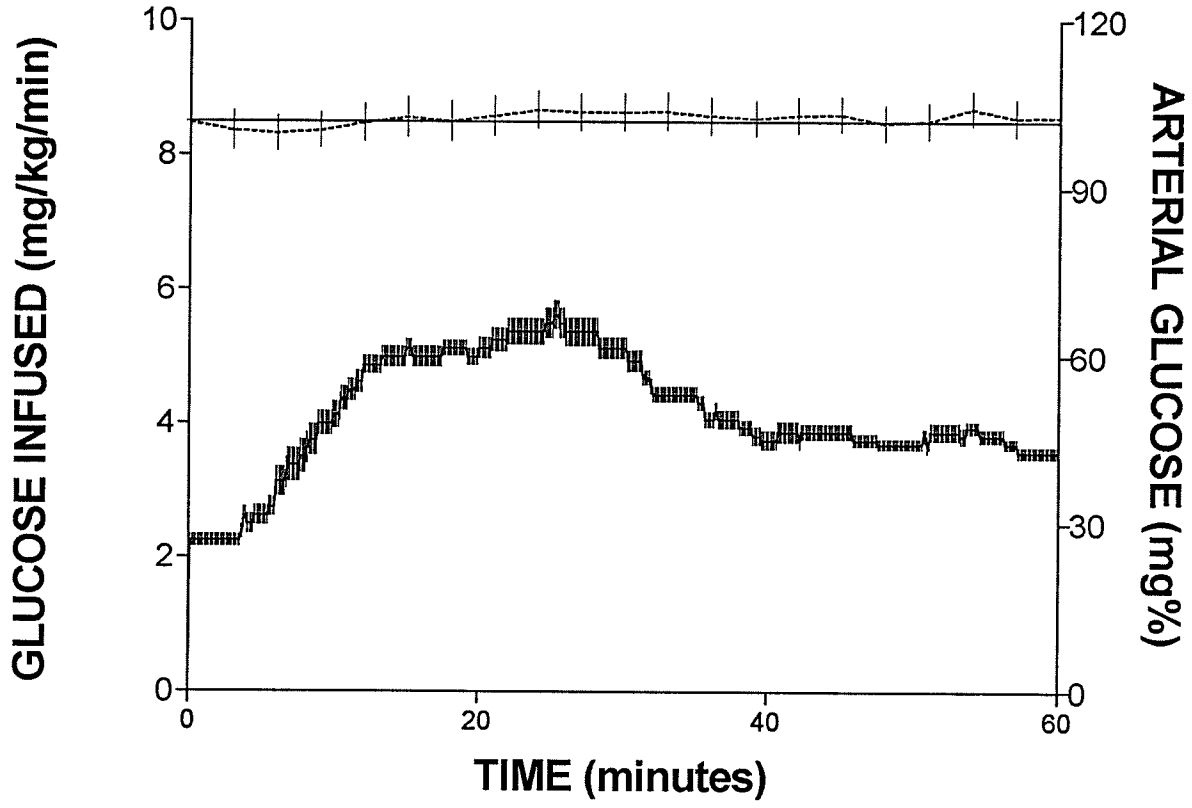


Fig. 8. The lower curve represents the rates of glucose infusion in cats (mg/kg/min) at each 0.1 min over 60 min after insulin 100 mU/kg administration (mean ± S.E., n=8). The top straight line represents the basal arterial glucose level (mg%) determined by the mean of three successive stable arterial glucose concentrations. The line with S.E. shows the actual glucose levels (mg%) maintained by the modified euglycemic clamp after insulin 100 mU/kg administration.

respectively) and 178.0 ± 23.6 mg/kg (n=4, one cat data was missed for technical reasons in the last step) (Table. 1.) glucose to maintain the arterial glucose level constant after five subsequent insulin infusions (Fig 9). No significant differences were observed in the amounts of glucose infused during clamping after insulin infusion in the five-step time control animals. The variability is mainly between animals with the control glucose requirment ranging from 121 to 266 mg/kg. The % coefficient of variance calculated as mean divided by standard error expressed as % for each animal ranged from 2.3 to 4.8 (mean 3.0 ± 0.5%). The five insulin tests were performed on the same animal over approximately a 12 hour period. Approximately 40 minutes was required between each subsequent test in order for glucose baseline stability to be re-established. The basal glucose level priro to commencing each IST was 97.4 ± 9.2, 107.1 ± 8.8, 114.7 ± 11.0, 112.0 ± 7.5, and 113.2 ± 9.9 mg% (Table. 1.). Basal levels did not change significantly throughout the experiment.

Basal insulin and glucagon levels returned to baseline prior to each test. The plasma insulin levels during the IST were significantly higher than the basal insulin levels ($P<0.05$, n=5). However, the net increase after insulin infusion was not significantly different at any test. The basal glucagon levels and the glucagon levels after each insulin infusion were not different throughout the experiment (Table. 2).

VI. 3. Discussion

The insulin sensitivity test (IST), using a modified euglycemic technique,

TABLE 1

The basal glucose levels (mg%) and the amount of glucose infused during clamping (mg/kg) in the five-step time control in cats

STEP-1	STEP-2	STEP-3	STEP-4	STEP-5
The basal glucose levels (mg%)				
94.7 ± 9.4	107.1 ± 8.8	114.7 ± 11.0	112.0 ± 7.5	113.2 ± 9.9
The amount of glucose infused during clamping(mg/kg)				
214.0 ± 23.6	208.4 ± 27.1	220.8 ± 30.1	205.7 ± 24.1	178.0 ± 23.6

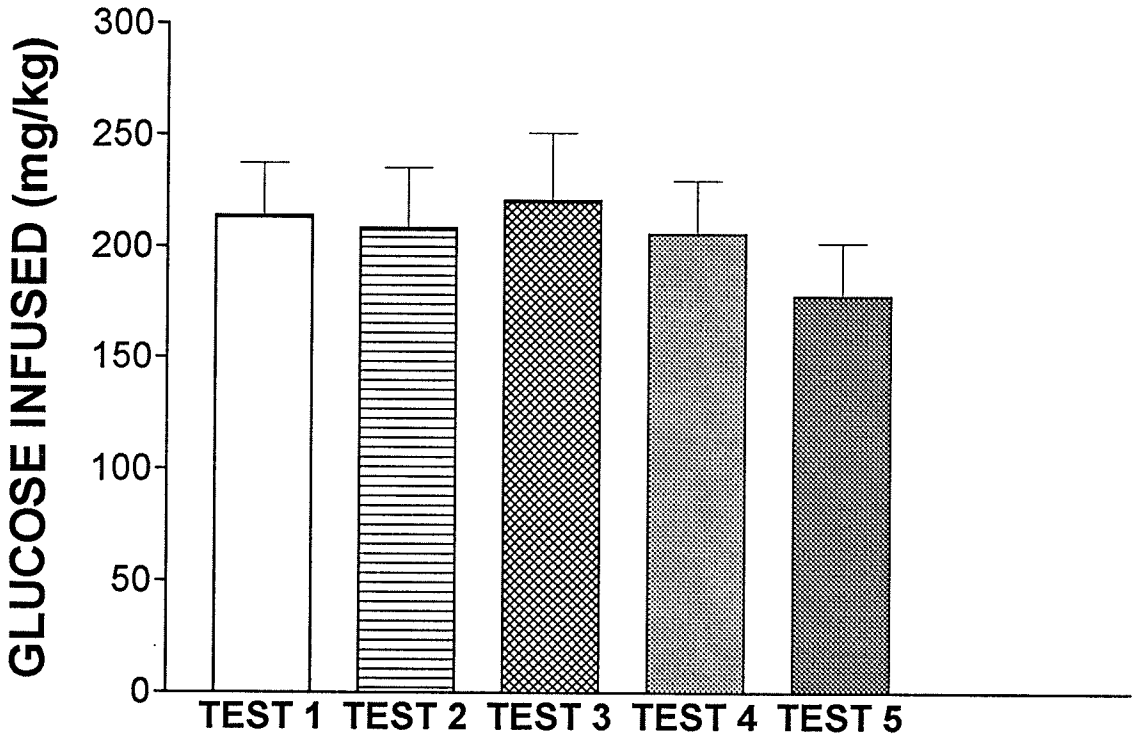


Fig. 9. The amounts of glucose (me/k) infused by the modified euglycemic technique over 60 min after insulin (100mU/k, i.v.) administration in the five-step time control insulin sensitivity tests in cats (n=5). Note: test 5 had n=4; coefficient of variance = 3.0 ± 0.5%.

TABLE 2

The plasma insulin and glucagon concentrations

in the five-step time control in cats

TIME-CONTROL	BEFORE INSULIN	AFTER INSULIN
INSULIN	μIU/ml	
STEP-1	28.4 ± 7.6	57.1 ± 18.6
STEP-2	42.2 ± 12.9	69.2 ± 8.7
STEP-3	29.2 ± 7.5	64.6 ± 20.6
STEP-4	42.0 ± 10.6	87.1 ± 14.4
STEP-5	38.7 ± 11.1	91.6 ± 26.2
GLUCAGON	pmol/L	
STEP-1	53.1 ± 13.8	66.6 ± 4.2
STEP-2	80.5 ± 16.4	69.6 ± 4.1
STEP-3	81.5 ± 22.8	77.9 ± 22.5
STEP-4	78.6 ± 23.8	75.7 ± 24.9
STEP-5	86.5 ± 25.4	72.2 ± 29.6

provided a novel means to quantitate insulin effectiveness in anesthetized cats and rats. There was a difference between the curves of glucose infusion rate obtained during the IST in the two species. The curve of glucose infusion rate in cats was flat and gradually decreased from a peak level at 30 min. By 60 min after insulin administration, the glucose infusion rate was still about two-thirds of the peak rate. The amount of glucose infused per kg body weight over 60 minutes after insulin 100 mU/kg administration in rats was almost 2-fold that in cats. The glucose infusion required after insulin administration reached a peak after 10 min and declined rapidly in rats. The response was essentially completed after 30 minutes. Glucose stabilization after surgical interventions took a shorter period of time in rats than in cats. Different mechanisms for insulin equilibration between intravascular space and extracellular space may explain this species difference in insulin sensitivity [McGuire et al. 1979; Sherwin et al. 1974]. The effect of feeding or fasting on the IST has not been tested. The cats had been fasted for 18 hours, whereas the rats had free access to food up to the time of anesthesia. The IST thus is effective in either the fed or fasted state. Although the effect of feeding on this IST has not been systematically evaluated, there may be some species-dependent differences. However direct comparisons of the present data cannot be made.

The IST was done only when a basal glucose level had been stabilized for at least 15 minutes. Arterial glucose concentrations were analyzed 3 min apart during the IST in cats, since the required infusion rate of glucose in cats

change slowly. The fluctuation of actual glucose level maintained by this technique was less than ± 3.5% of the basal glucose level. In the five-step time control study in cats, five insulin tests were performed on the same animal between 8:00 a.m and 8:00 p.m in the same day. The amounts of glucose infused over 60 min after insulin 100mU/kg administration did not change significantly, nor did the basal glucose levels prior to each IST (Table 3). The time control had a mean coefficient of variance of 3.0 ± 0.5%. Similar time controls have not been conducted in rats but our impression from later studies is that three and perhaps up to four consecutive tests are stable and reproducible (see Section VIII).

Two different blood sampling methods used in rats and in cats reduced the loss of blood to 0.5 ml per IST. Blood samples can be taken in just seconds, which made it possible for each feedback circle to be completed in as short as 2 minutes. Small fluctuations in arterial glucose level can be detected and glucose infusion can be varied to clamp arterial glucose concentration at the basal level. Although two different sampling methods were used, the A-V loop design shown in Figure 5 is recommended for use with both the cat and rat because of ease of use (no washout sample is required), speed of sampling, ability to take arterial samples and administer intravenous fluids via the same route and ability to rapidly assure patency and lack of clotting of the loop.

This modified euglycemic technique quantified the effectiveness of a bolus amount of insulin, rather than a steady-state hyperinsulinemia, as used

in the classical euglycemic clamp technique (DeFronzo et al. 1979]. Since only a peak of insulin concentration transiently appeared in blood, insulin sensitivity was not changed in this modified technique as shown by the ability to generate the same response several times in the same animal. In contrast, glucose utilization during prolonged euglycemic glucose clamp studies was significantly increased over time [Doberne et al. 1981]. A steady-state glucose level cannot be maintained at a constant glucose infusion rate using constant insulin infusion [Doberne et al. 1981; Bergman et al. 1985]. Prolonged hyperinsulinemia may induce rate-limiting metabolic enzymes and downregulate insulin receptors and alter receptor affinity [Defronzo et al. 1978; Insel et al. 1980; Nuttall 1972]. During prolonged hyperinsulinemia, plasma glucagon levels were reduced by insulin in a dose-dependent manner, while norepinephrine levels were increased [Koopmans et al. 1993]. A marked increase in glucose clearance was observed when insulin effectiveness was tested repeatedly on different days in the same patients without any intervention [Greenfield et al. 1981]. Thus, the classical methodology causes a wide range of acute and long-term biological effects not directly related to the insulin sensitivity that exists at the onset of the test. With the modified IST technique, five successive stable insulin tests have been obtained in the same animal on the same day.

Glucose metabolism is affected by anesthetica [Clark et al. 1990]. It is not known if insulin sensitivity measured using the IST is affected by anesthesia but the IST should be readily adaptable to a conscious preparation

if the blood sampling method reported here is used in the carotid artery and jugular vein.

Unlike the classical euglycemic clamp, in which hepatic glucose production and the release of endogenous insulin were theoretically totally suppressed by the steady-state hyperinsulinemia [DeFronzo et al. 1979], this modified technique tests the effect of a bolus of exogenous insulin added to the endogenous level at a steady state. The glucose infused to maintain the basal glucose level is assumed to reflect the biological response to the added insulin. Plasma insulin and glucagon concentrations were analyzed before and after insulin 100mU/kg administration in this study in cats. Plasma insulin concentration significantly increased from 28.4 ± 7.6 μU/ml (the basal level) to 57.1 ± 18.6 μU/ml ($P<0.05$) 15 min after insulin administration. The basal level and the change in response to the insulin administration were not different in five subsequent tests despite experimental manoeuvres over 10-12 hours. Since the main stimulation for glucagon secretion is the plasma glucose level [Berne and Levy 1988], which was maintained constant by euglycemic clamping in this study, plasma glucagon remained unchanged after each insulin administration. Hepatic glucose production is able to respond to infused insulin in this modified technique. If hepatic glucose balance is one target for studies of insulin sensitivity, this modified technique may prove useful, since the traditional method assumes that hepatic glucose production is totally inhibited [Defronzo et al. 1979]. There have been no studies to directly compare the classical euglycemic clamp and the present

model for their ability to detect changes in insulin sensitivity. The effect of feeding and fasting has not been evaluted although the test works in both conditions as shown here. The IST has not been tested in conscious animals or other species.

The modified IST described provides a sensitive and reproducible test for measuring insulin sensitivity in anesthetized rats and cats.

VI. 4. Conclusions and further study

The modified insulin sensitivity test works well in both the cat and rat to provide reproducible, sensitive and multiple tests for insulin effectiveness. No changes in basal plasma insulin, glucagon and basal glucose levels were produced by this IST in five repeated insulin tests over a 12 hour experimental period. Since this technique satisfies our requirement well, it will be used in all later studies.

The next abjective was to test the hypothesis that dysfunction of hepatic parasympathetic nerves produces the insulin resistance observed in Section V. The approch was to block hepatic cholinergic muscarinic nerves with intrapotal administration of atropine. Insulin sensitivity was quantified with the new insulin sensitivity test (IST).

VII. Insulin resistance produced by cholinergic blockade with atropine in cats

VII. 1. Materials and methods

Cats (either gender, 3.48 ± 0.2 kg, n=18) were handled and prepared as described in Section VI. 1. 2. In addition, following laparotomy, the portal vein was cannulated via the cecal vein to allow atropine infusion. The tip of the cannula was located about 2 cm from the hilum of the liver. A microsphere distribution study indicated no streamlining of the blood flow to the liver in cats cannulated in this manner [Lautt et al. 1992]. A tracheal tube was inserted and artificial respiration was maintained throughout the experiment and was adjusted according to carbon dioxide partial pressure in expired air which was monitored with a Medical Gas Analyzer, Model LB-2 (SensorMedics, Anaheim, CA). The cats were allowed to recover from the surgical interventions for 45 min before any procedures were carried out. Insulin sensitivity tests were performed as described in Section VI. 1. 2. Arterial blood samples (1.5 ml) were taken before and 15 min after insulin infusion, plasma was separated and then stored at -20° C for later analyses of insulin and glucagon. Plasma insulin and glucagon concentrations were analyzed as described in Section VI. 1. 3. The total volume of blood withdrawn was 3.5 ml per insulin test. Replacement fluids were administered at the time of sampling and throughout as required to maintain arterial and central venous pressure.

After the control IST was obtained, atropine, 1 mg/kg, was infused intraportally over 5 min. Arterial plasma glucose level was allowed to stabilize,

the same dose of insulin (100 mU/kg) was intravenously infused and the glucose level was clamped at the basal glucose level over 60 min. The same procedure was followed after each sequential dose of atropine (1, 2, 4 and 8 mg/kg, n=8).

In another group of cats (n=10), after control insulin sensitivity was tested in each cat, an M_1-selective antagonist, pirenzepine (10 nmol/kg, followed by 100nmol/kg, n=5), or an M_2-selective antagonist, methoctramine (100 nmol/kg, followed by 1000 nmol/kg, n=5), was intraportally infused over 5 min. After each treatment, an IST (100mU/kg insulin) was done. Finally, the IST was repeated after atropine intraportal administration (3mg/kg, equal to 4 umol/kg) in each animal. The amounts of glucose infused during clamping were calculated and compared.

The data were analyzed by repeated measures ANOVA with Tukey-Kramer multiple comparisons test. The data were expressed as mean ± S.E. throughout. Maximum inhibition (I_{max}) and the dose required to produce 50% of I_{max} (ED_{50}) for the atropine dose-response curve were established by non-linear regression (GraphPAD INPLOT, ISI Software).

VII. 2. Results

Insulin effectiveness was expressed by the amount of glucose infused over 60 min after insulin (100mU/kg) administration. Insulin resistance was expressed as the percentage of reduction of glucose amount in comparison with that infused in the control state. Atropine (1 mg/kg intraportal infusion) decreased glucose requirement over 60 min after insulin (100 mU/kg) infusion

by 40.5 ± 16.8 mg/kg and caused an 18.1 ± 5.8% inhibition of insulin effectiveness. Atropine at an accumulated dose of 3 mg/kg produced a decrease in glucose requirement of 85.5 ± 10.5 mg/kg and a significant inhibition of insulin effectiveness (33.6 ± 3.4%, $P<0.05$, n=8). Elevating atropine doses beyond 3 mg/kg failed to cause further significant inhibition of insulin sensitivity. The accumulated atropine-% inhibition glucose requirement curve (Fig.10) showed that the ED_{50} of atropine is 0.99 mg/kg (1 mg=1.5 μmol) with the maximal estimated inhibition of 40.3 ± 11.6 %.

Both the ratio of insulin and glucagon and absolute plasma concentration of both glucoregulatory hormones were compared. The basal insulin levels were not changed over the whole experiment. The insulin levels during each IST were significantly increased ($P<0.05$, n=8) to a similar extent in each IST in the atropine series. The basal as well as the level of glucagon after insulin infusions were not significantly changed (Table 3).

The two highest doses of atropine were added only to confirm that the maximal insulin resistance had been achieved. The dose of 3 mg/kg (cumulative) clearly indicates a maximal response (Fig. 10). The higher doses are suspected of producing other systemic effects since these doses are very high for in vivo studies. For full atropine-induced insulin resistance the dose of 3 mg/kg is clearly all that is needed and results in full resistance without involvement of insulin or glucagon changes in basal state or during the IST (Table 3).

The peak level of glucose infusion during clamping occurred around 30

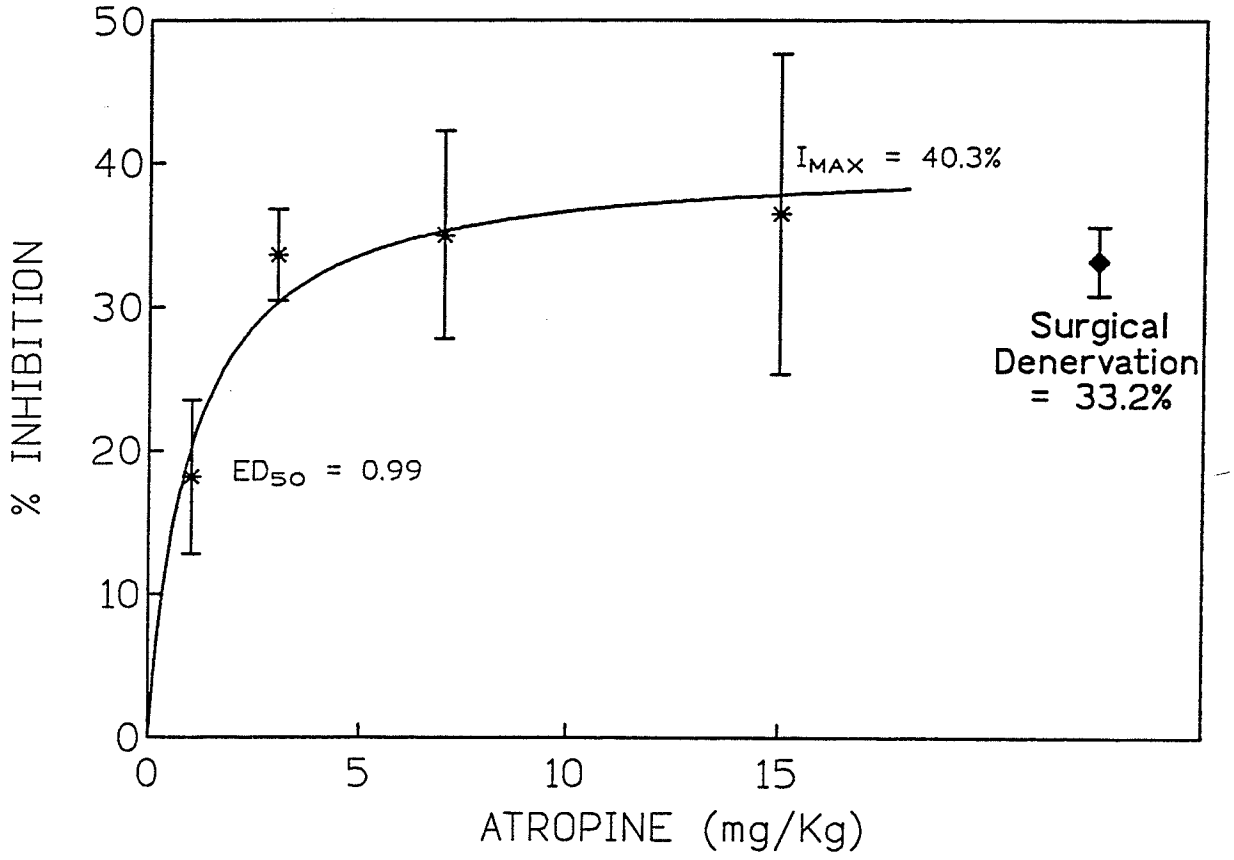


Fig. 10. Atropine cumulative doses plotted against % inhibition of glucose requirement after insulin infusion (100 mU/kg). ED_{50} of atropine is 0.99 mg/kg (1 mg/kg = 1.5 umol/kg) in cats. The maximal calculated inhibition (I_{max}) of glucose requirement by atropine is 40.3 ± 11.6%, which is very similar to that produced by surgical hepatic anterior plexus interruption (◆, 33.2 ± 2.4%).

TABLE 3

The plasma insulin and glucagon concentrations

ATROPINE mg/kg	BEFORE INSULIN 0 min	AFTER INSULIN 15 min
INSULIN	μIU/ml	
0	26.7 ± 4.0	65.5 ± 10.6
1	28.0 ± 5.1	65.9 ± 14.1
3	24.3 ± 4.4	82.1 ± 13.7
7	36.6 ± 15.2	132.1 ± 25.2
15	24.9 ± 3.9	104.9 ± 28.0
GLUCAGON	pg/ml	
0	70.0 ± 22.9	54.9 ± 12.9
1	87.9 ± 33.3	70.8 ± 15.9
3	74.5 ± 18.6	69.9 ± 10.3
7	82.3 ± 21.1	77.1 ± 17.6
15	84.7 ± 42.2	63.4 ± 26.9

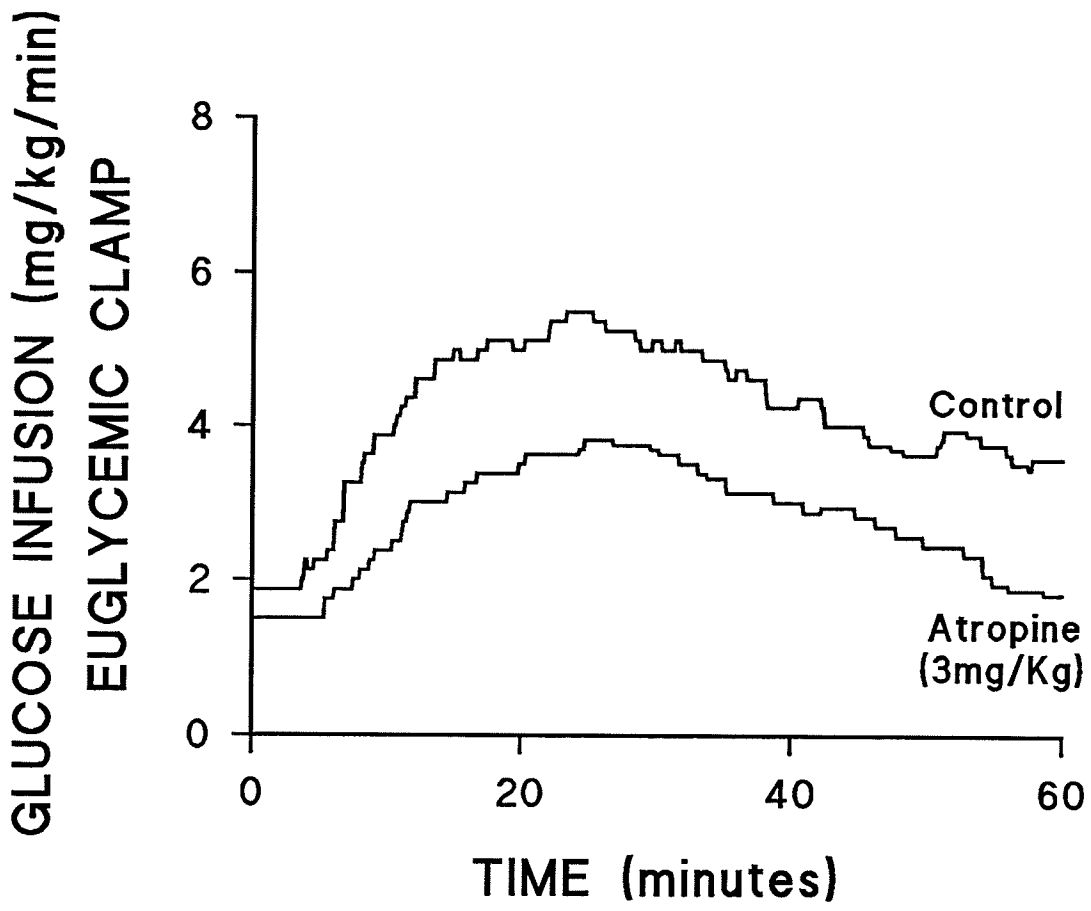


Fig. 11. Comparison of the amount of glucose (mg/kg/min) infused with the modified euglycemic clamp averaged at 0.1 min time points over 60 min after insulin infusion in the control state with that after atropine (cumulative dose 3 mg/kg). Atropine changed the magnitude of the rate of glucose infusion but not the time course. The maximal infusion rate occurred around 30 min after insulin infusion.

min after infusion of insulin in both control and treated states (Fig. 11). The temporal pattern of insulin effectiveness was not altered by atropine administration.

In the cats treated by selective M_1 or M_2 blockade, the amounts of glucose infused over 60 min clamping period after insulin administration were not significantly different in the control insulin tests of both treated groups. The amounts of glucose required after low doses of pirenzepine (10 nmol/kg, intraportal, n=5) or methoctramine (100 nmol/kg, intraportal, n=5) showed no difference from those in the control insulin test. Treatment with the high doses of pirenzepine (100 nmol/kg, intraportal, n=5) or methotramine (1000nmol/kg, intraportal, n=5) produced a significant reduction ($P<0.05$) in the amount of glucose needed to hold arterial glucose levels steady (Fig. 12, 13). Atropine administration in both treated groups failed to alter further the glucose amount infused after administration of pirenzepine (100 nmol/kg) or methoctramine (1000nmol/kg), resulting in the same degree of insulin resistance, expressed as % of the inhibition produced by the high dose of each selective antagonist (Fig. 14). Thus the high dose of each selective muscarinic antagonist produced full insulin resistance.

VII. 3. Discussion

Surgical hepatic anterior plexus denervation, as described in Section V, causes 37.2 ± 9.8% inhibition of the hypoglycemic response over 60 min following insulin (100 mU/kg) infusion, indicating that glucose regulation is at least partially dependent on an intact and functional hepatic anterior nerve

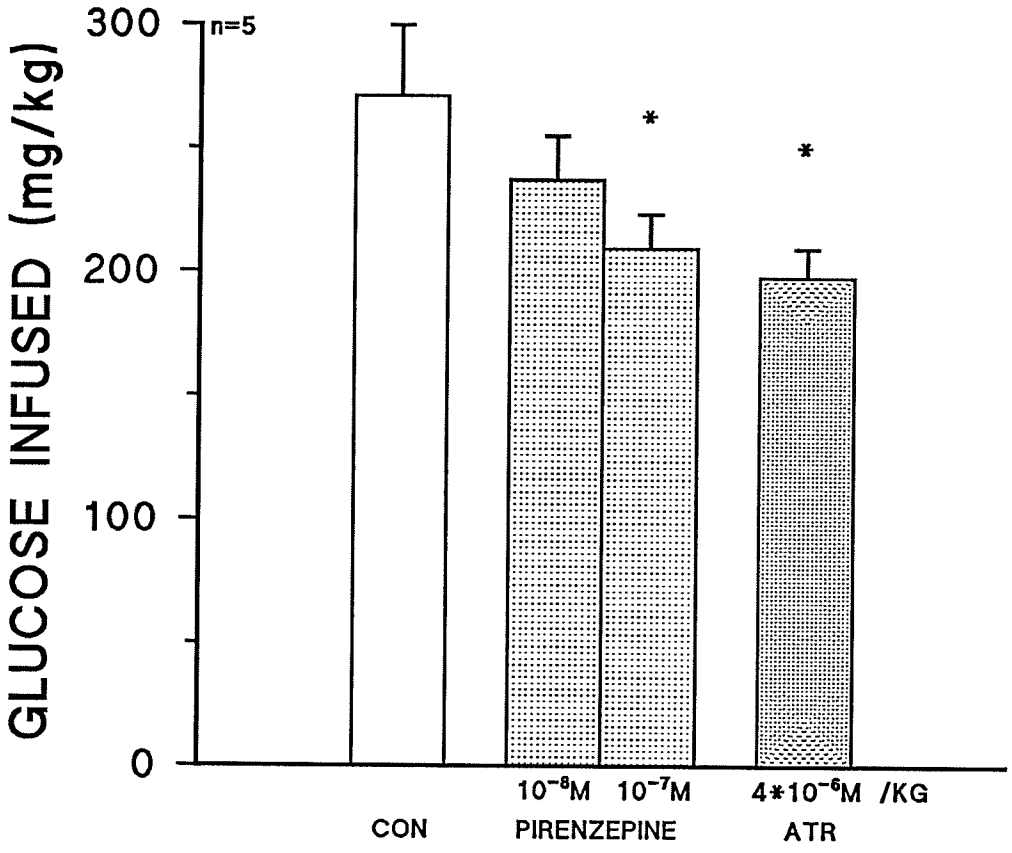


Fig. 12. The amount of glucose (mg/kg, n=5) infused with the modified euglycemic clamp in the control (CON) insulin test and in the insulin tests after treatments with pirenzepine (10^{-8}mol/kg, followed by 10^{-7}mol/kg) and after atropine (ATR) ($4*10^{-6}$mol/kg) administration. * represents $P < 0.05$, compared with the amount of glucose infused in control insulin test.

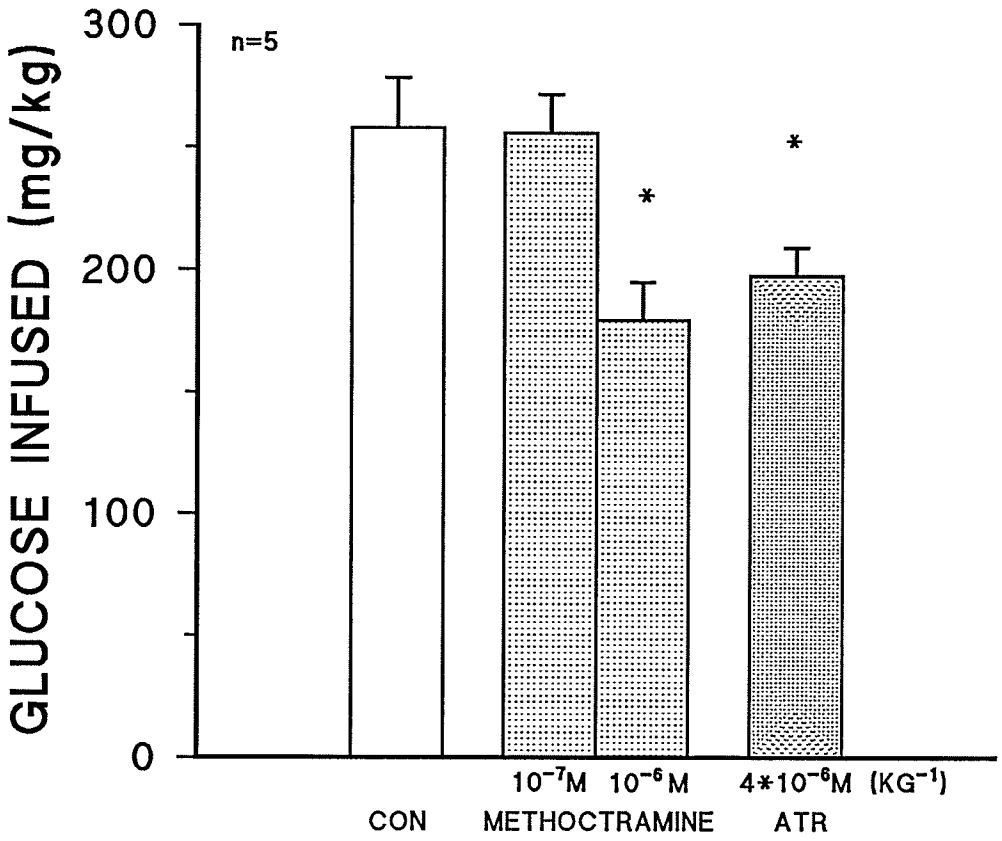


Fig. 13. The amount of glucose (mg/kg, n=5) infused with the modified euglycemic clamp in the control (CON) insulin test and in the insulin tests after treatments with methoctramine (10^{-7}mol/kg, followed by 10^{-6}mol/kg) and after atropine (ATR) ($4*10^{-6}$mol/kg) administration. * represents $P < 0.05$, compared with the amount of glucose infused in control insulin test.

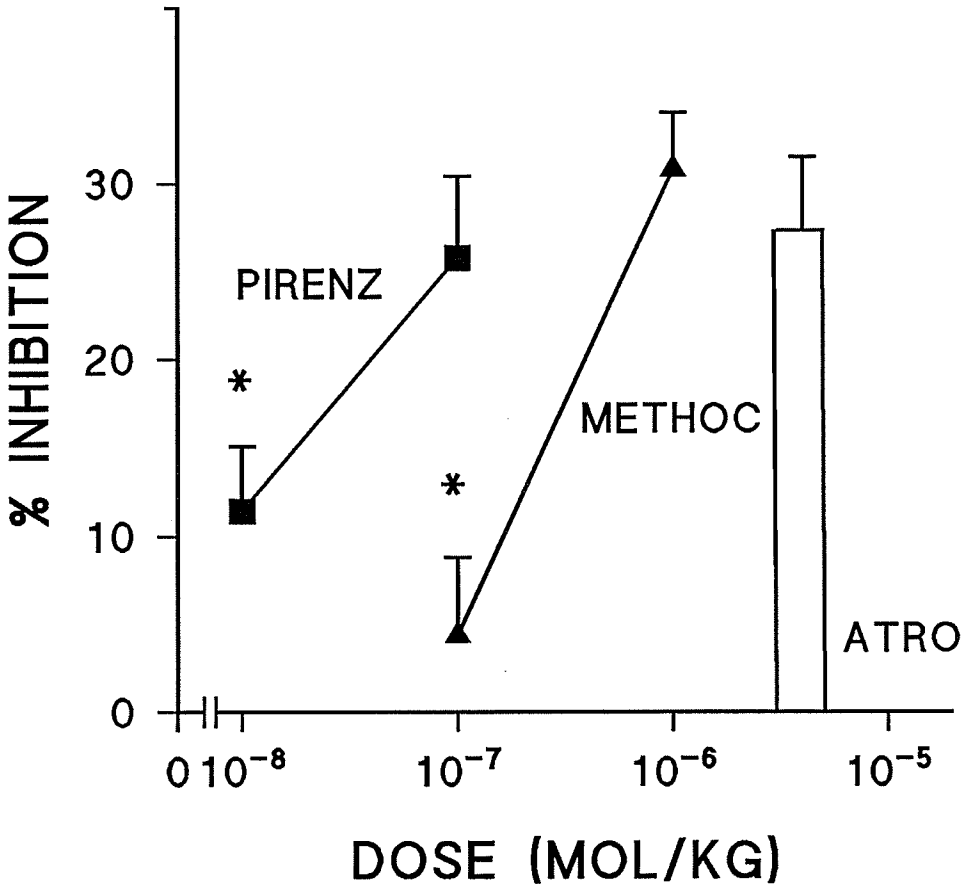


Fig. 14. % inhibition, referred to the amount of glucose infused in the control insulin test produced by both doses of pirenzepine (Pirenz) and methoctramine (Methoc), was compared with that produced by atropine (ATR $4*10^{-6}$M/kg) administration. * represents $P < 0.05$.

plexus. This study shows that antagonism of cholinergic muscarinic receptors using atropine resulted in a similar reduction of insulin effectiveness as produced by surgical denervation of the liver. Atropine (3 mg/kg) produced 33.6 ± 3.4% inhibition of glucose requirement, similar to the degree of insulin resistance produced by surgical denervation. The ED_{50} of atropine is approximately 1 mg/kg (1.5 umol/kg) with maximal inhibition of insulin sensitivity calculated to be 40.3 ± 11.6%. In the previous study, insulin was allowed to produce a hypoglycemic response and insulin sensitivity was calculated based on the degree of hypoglycemia. In this study, we introduced the modified euglycemic technique as an insulin sensitive test and showed that the resistance produced by denervation was similar using both types of insulin sensitivity test. The IST proved to be a reliable tool in quantitative study of insulin effectiveness, because this technique is sufficiently sensitive to allow a dose-related effect of atropine to be determined from five successive responses in the same animal. This method offers a unique tool to demonstrate and study responsiveness to insulin.

The observation that the degree of insulin resistance produced by hepatic denervation was similar to that produced by atropine is compatible with, but does not prove, the hypothesis that the mechanism of insulin resistance produced by hepatic denervation and muscarinic receptor blockade is the same. This study is consistent with the hypothesis that the insulin resistance produced by surgical hepatic denervation is a result of loss of parasympathetic muscarinic activation. The hepatic parasympathetic nerves

thus appear to act synergistically with insulin, either directly or indirectly, and the absence of cholinergic activation leads to insulin resistance.

Electrical stimulation of the hypothalamic parasympathetic area [Shimazu et al, 1966], vagi [Shimazu, 1967, 1971; Shimazu and Fujimata 1971], and hepatic parasympathetic nerves [Lautt and Wong 1978] increase hepatic glycogen synthesis and decrease hepatic glucose output. Net hepatic glucose uptake after oral glucose was reduced in atropine-treated dogs [Chap et al. 1985]. Those animal studies suggested a direct effect of the parasympathetic nervous system on hepatic glucoregulation. Insulin effect on glucose uptake by the liver is dramatically reduced in the isolated liver. Acetylcholine administration alone does not enhance the deposition of glycogen in the isolated liver; however, administration of acetylcholine in the presence of insulin markedly enhances the uptake of glucose and deposition of hepatic glycogen by the isolated liver [Mondon and Burton 1971]. Stimulation of the hepatic nerves in the presence of α- and β-adrenoreceptor antagonists potentiates the insulin-induced glucose uptake by the isolated liver [Gardemann and Jungermann 1986; Jungermann et al. 1987]. These results from the isolated liver are consistent with our observations that the muscarinic cholinergic blockade or hepatic anterior plexus denervation significantly reduce insulin effectiveness on glucose uptake.

A similar degree of insulin resistance was achieved with the M_1-selective antagonist, pirenzepine, or 10-times higher dose of the M_2-selective antagonist, methoctramine. Atropine added to these treated groups did not

produce a further degree of insulin resistance. The data suggest that the M_1 receptor subtype is responsible for the synergistic effect of the parasympathetic nerves and insulin. This conclusion is consistent with the concept that the M_1 receptor is a neural muscarinic receptors activated mainly by vagal stimulation [Pagani et al. 1984; Stockbruegger et al. 1984].

One recent report [Bevan et al. 1991] showed that pirenzepine produced an improvement in glucose tolerance in humans resulting in reduced plasma glucose and insulin levels. Atropine administration in the dog reduced hepatic glucose uptake after oral glucose [Chap et al. 1985]. Whether these effects involved altered glucose absorption, effects on the central nervous system or other mechanisms is not known, but the results are clearly different from what was observed in our atropine or pirenzepine-treated cats. The difference is unlikely to be species-dependent since other studies are consistent with the present data.

In humans, it was reported that the muscarinic cholinergic agonist, bethanechol, in conjunction with the islet clamp technique to hold pancreatic glucoregulatory hormones constant, induces a reduction in glucose production and in the plasma glucose concentration with no change in glucose utilization. The data suggest a direct inhibitory effect of cholinergic muscarinic activation on hepatic glucose production in human. Atropine administration, however, caused no change in the plasma glucose level in humans [Boyle et al. 1988] or in the present study (Table 4). These results suggested that muscarinic cholinergic antagonism, no matter how large the dose is, has no net systemic

TABLE 4

The basal glucose levels (mg%) and the amount of glucose infused during clamping (mg/kg)

CONTROL	ATROPINE 1 mg/kg	ATROPINE 3 mg/kg	ATROPINE 7 mg/kg	ATROPINE 15 mg/kg
The basal glucose levels (mg%)				
101.8 ± 8.0	109.8 ± 6.9	119.9 ± 9.8	108.6 ± 6.5	101.4 ± 5.2
The amount of glucose infused during clamping (mg/kg)				
250.6 ± 11.5	197.1 ± 8.8	164.7 ± 6.1	157.5 ± 14.7	150.9 ± 23.4

glucoregulatory effect in terms of the plasma glucose concentration. However, our study indicated that cholinergic blockade reduces the insulin effect on glucoregulation by one-third, showing that the full insulin effect depends on the normal functional hepatic parasympathetic nervous system. Combination of muscarinic cholinergic activation with insulin, rather than either factor alone, is essential for normalization of glucose metabolism.

The present data are consistent with the hypothesis that the denervation and atropine are acting directly on the liver rather than through endocrine effects. This is supported by the lack of difference in insulin and glucagon levels either before insulin injection or 15 minutes after injection and after all doses of atropine (Table 3). The affinity or sensitivity of the these hormone receptors should not be altered in thses acute test. The insulin and glucagon levels in the basal state and during the insulin test show no tendency for changes to the cummulative dose of atropine of 3 mg/kg which also effectively shows full insulin resistance. The higher doses of atropine do not show statistically significant changes in these pancreatic hormones but variability is clearly greatly increased and the higher doses of atropine must be accepted here only for demonstration that doses higher than 3 mg/kg do not lead to greater insulin resistance. This is a crucial demonstration, since Boyle et al. (1988) showed that the muscarinic agonist, bethanechol, increased glucagon secretion in the human to offset the direct effect of cholinergic activation on hepatic glucose production.

Although atropine was administered directly into the portal vein and the

doses were slowly infused over 5 min to minimize systemic concentrations, some degree of extrahepatic muscarinic blockade is likely, especially at the higher doses. The site of action of atropine cannot be assumed since atropine has access to the central nervous system and it remains possible that the hepatic denervation procedure could also produce its effect secondary to elimination of a neural connection to the CNS. Further studies are needed to differentiate central from peripheral site of action.

Surgical hepatic denervation and intraportal atropine result in insulin resistance of rapid (within 1 hour) onset in the absence of altered insulin and glucagon levels. The data support the hypothesis that hepatic parasympathetic nerves, possibly mediated by muscarinic subtype receptors, M_1, have a synergistic effect with insulin.

VII. 4. Conclusions and further study

Intraportal administration of atropine produced a dose-dependent inhibition of insulin sensitivity in glucose metabolism. The ED_{50} of atropine was 0.99 mg/kg (1mg=1.5μmol) with maximum inhibition of 40.3 ± 11.6%. Atropine 3 mg/kg reduced insulin sensitivity to a similar degree (33.6 ± 3.4%) to that produced by hepatic surgical denevation (37.2 ± 9.8%). Doses greater than 3 mg/kg failed to further alter the insulin resistance produced by atropine 3 mg/kg administration. M_1 receptors may mediate the synergistic effect of hepatic nerves with insulin. Atropine reduced insulin sensitivity without changes in pancreatic hormone release and clearance.

The next objective was to to localize the site where insulin resistance

is produced by hepatic cholinergic nerve interruption, since it has been reported that insulin insensitivity of cirrhotic patients was mainly seen in extra hepatic tissues (see Section III. 1). The approch is to measure glucose gradients across the liver, splanchnic organs and skeletal muscle during the modified euglycemic clamping before and after both surgical denervation and atropine treatment in cats.

VIII. Insulin resistance of skeletal muscle produced by hepatic parasympathetic interruption

VIII. 1. Materials and method

Cats (either gender, 3.4 ± 0.2 kg, n=8) were treated as described in Section VI. 1. 2.. Hepatic venous samples were obtained as previously described [Lautt 1982]. Briefly, following laparotomy, a snare was put around the inferior vena cava at the level above both renal and adrenal veins. A cannula was inserted via the right femoral vein into the inferior vena cava, the tip of which passed the snare and was located near the diaphragm. After blood flow in the inferior vena cava was stopped by constricting the snare, the only source of blood flow in the vena cava above the snare was from the hepatic vein. The hepatic venous blood sample was taken after 8 seconds and, when samples were not being taken, central venous pressure was monitored through the same cannula.

The inferior vena cava was cannulated through the left femoral vein. The tip of the cannula was located above the branching point of both femoral veins. Insulin was infused through the same cannula. The portal vein was cannulated via the cecal vein for portal sampling and atropine infusion. The right jugular vein was cannulated for glucose infusion. Insulin sensitivity was tested by using the modified euglycemic technique described in Section IV. 2.. Arterial blood samples (1.5 ml) were taken before and 15 min after insulin administration, plasma was separated and then stored at -80° C for later analysis of plasma norepinephrine and insulin concentration. In the study

described in Section VI. plasma insulin and glucagon concentration were analyzed under the same experimental conditions, showing that the insulin level increased after insulin administration but was not altered in the pre-injection basal state following atropine and that glucagon remained unchanged at all time points. Plasma catecholamines were eluted using the ESA Plasma Catecholamine Analysis Kit (Scientific Products & Equipment Ltd., Bedford, MA) and the levels were determined on a HPLC system consisting of a Shimadzu LC-10AD pump, Shimadzu SIL-9A Auto Injector, an ESA Catecholamine HR80 colum, ESA Model 5020 guard Cell, ESA Model 5011 Analytical Cell, and a ESA Coulochem II detector. Data were acquired and integrated with Maxima 820 Chromatography Software (Waters Associates, Milford, MA). CAT-A-Phase (ESA) Delivery at a flow rate of 1.4 ml/min was used as the mobile phase. The detection limit of analysis was 2-5 pg injected on the column. Insulin was determined by the same way as described in Section VI. 3.

Protocol: Glucose levels in the blood from the femoral artery, hindlimb vein, portal vein and hepatic vein were analyzed simultaneously before and 30 min after insulin infusion, a time when previous studies indicated a peak glucose uptake. The total volume of blood withdrawn was 3.7 ml per insulin test. Replacement of fluids were administered as described in Section VI. 1.

In eight cats, an insulin (100 mU/kg) sensitivity test was carried out in the control state and following atropine and surgical denervation, with the order of the two treatments randomized. After the control insulin effectiveness

was tested, the nerve bundles and the connective tissues around the common hepatic artery, the portal vein and the bile duct were separated, ligated and cut between two ties. A 90% phenol solution was painted on the surface of these vescles to assure completely hepatic denervation, as described in Section V. 1.. Another IST was then determined. Atropine effect on insulin sensitivity was assessed before denervation in four cats and after denervation in four cats. Atropine 3 (mg/kg) was infused intraportally over 5 min. The IST was conducted as in the control test. Arterial-venous glucose differences were determined during each IST.

The data for the amounts of glucose infused over 60 min after insulin infusion were analyzed by repeated measures ANOVA followed by Tukey-Kramer multiple comparisons test. The basal and changed glucose gradients before treatment and after the last intervention were analyzed by paired t-test.

VIII. 2. Results

Insulin resistance: In the animals treated by hepatic denervation followed by atropine administration, denervation produced a reduction of the glucose amount required leading to a 33.2 ± 2.4% inhibition of insulin effectiveness. The combination of atropine in the denervated group resulted in a 34.9 ± 2.4% inhibition of insulin effectiveness. No significant difference was observed between insulin resistance produced by surgical denervation alone or after additional atropine administration (Fig. 15).

In the animals treated with atropine followed by surgical hepatic denervation, atropine administration produced a reduction of insulin

effectiveness by 29.0 ± 4.3%. The denervation combined with atropine resulted in reducing the insulin effectiveness by 35.0 ± 5.5%. There was no difference between insulin resistance produced by atropine administration alone or after addition of hepatic denervation, nor was there a difference in the inhibition of insulin effectiveness regardless of which intervention was performed first (Fig. 15). These comparisons validated pooling the results from the randomized ordering of the treatments and considering comparisons between control responses and the responses after the combination of atropine and surgical denervation.

Site of resistance: Glucose gradients were calculated by arterial minus hindlimb venous (A-L), arterial minus hepatic venous (A-H), arterial minus portal venous (A-P) and portal venous minus hepatic venous glucose concentrations (P-H) before (the basal gradient) and 30 min after insulin administration in each insulin test. The hepatic glucose gradient was weighted by (A-H) * 0.33 + (P-H) * 0.67, since about one-third of hepatic blood supply originates from the hepatic artery and the rest from the portal vein [Greenway and Lautt 1989]. Different proportions (20%-50%) of hepatic arterial supply were weighted. The change in the calculated data was negligible so all hepatic data reported here used the 0.33:0.67 weighted factor. The responses in the control insulin sensitivity tests are shown (Fig. 16).

There was a significant increase in the basal glucose output after combined atropine and nerve interruption ($P<0.01$), indicated by comparing the basal weighted hepatic glucose gradients in the control with those after

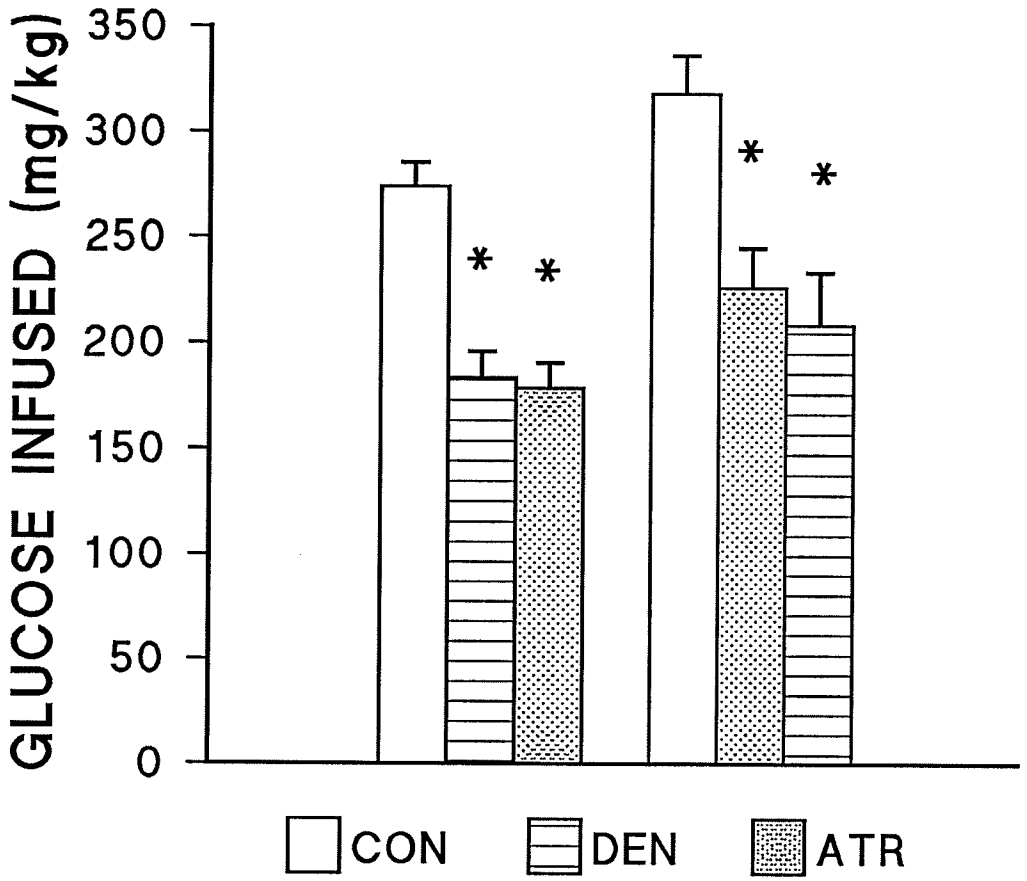


Fig. 15. The amount of glucose infused (mg/kg) with the modified euglycemic clamp over 60 min after insulin 100 mU/kg intravenous administration before (CON) and after surgical hepatic denervation (DEN) followed by atropine (ATR) 3 mg/kg intraportal administration (n=4) or in the reversed sequence (n=4). * represent $P < 0.001$.

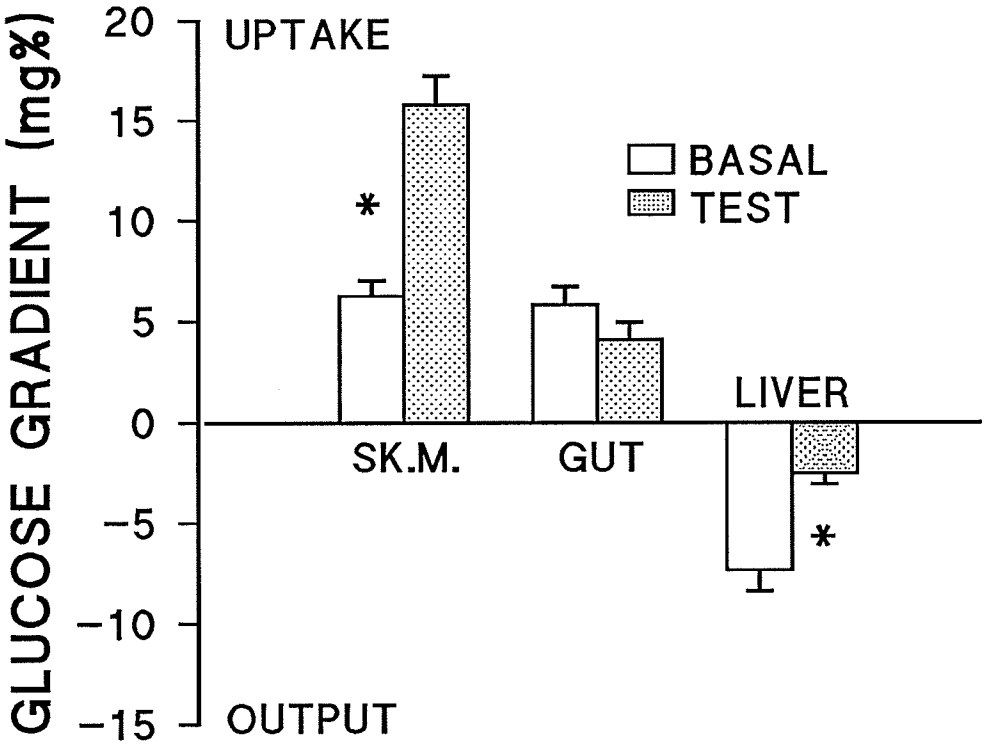


Fig. 16. The glucose gradients (mg%) across skeletal muscle (Sk.M), splanchnic organs (Gut) and liver before (Basal) and 30 minutes after insulin (100 mU/kg) at the mid-point of the control euglycemic insulin sensitive test (Test). * represents $P < 0.05$, n=8.

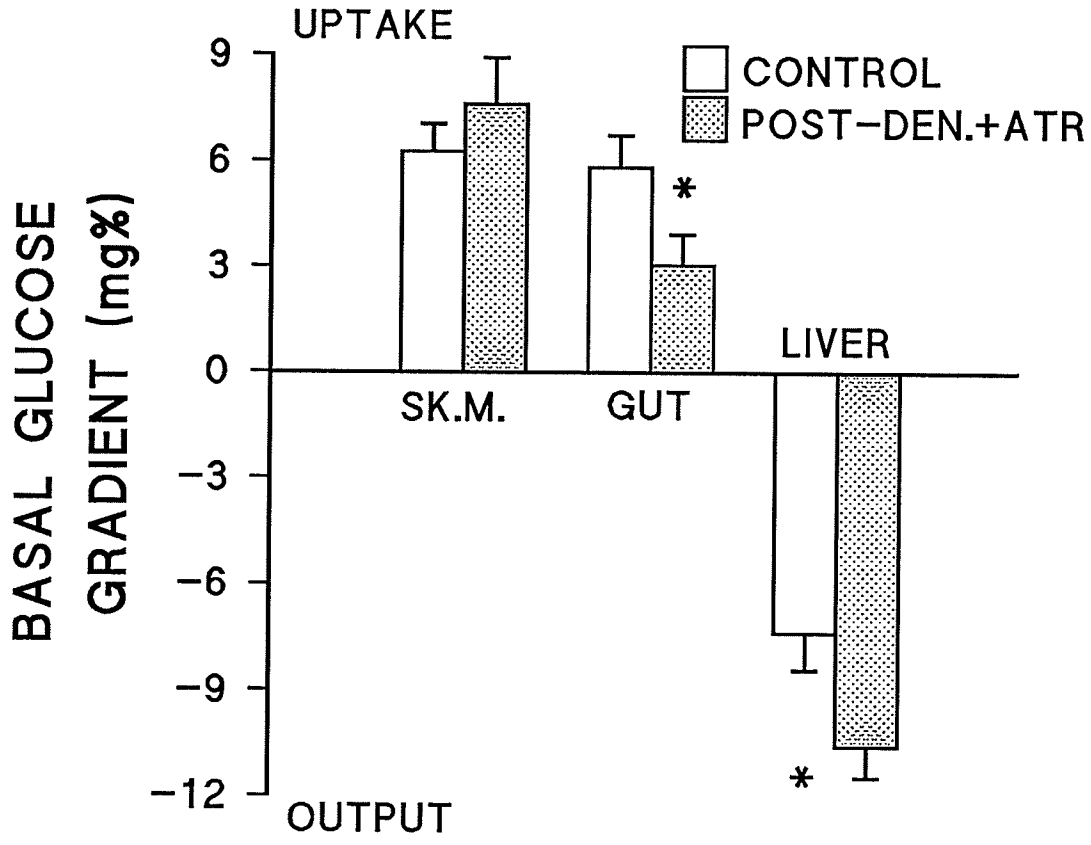


Fig. 17. The basal glucose gradients (mg%) across skeletal muscle (Sk.M), splanchnic organs (Gut) and the liver before (Control) and after combined surgical denervation and atropine 3 mg/kg administration (post-DEN. + ATR). Positive gradients represent glucose uptake and negative gradients represent output. * represents $P < 0.01$, control versus post-treatment for liver, $P < 0.05$ for gut (n=8).

both interventions (Fig. 17). These was a small but significant ($P<0.05$) reduction in glucose uptake across the guts.

The insulin effect on glucose gradients was assessed from comparisons of the gradient before and after insulin administration. Gradients are expressed as mg% (mg glucose per 100ml blood). Changes in glucose gradient were calculated by subtracting each basal glucose gradient from its corresponding gradient at 30 min in the control insulin tests and after both interventions. The changes in gradient across the gut and liver showed no differences between the control insulin test and the insulin test after both treatments. A significant reduction of glucose uptake by the hindlimb in response to the bolus of insulin was observed ($P<0.02$, $n=8$) (Fig. 18). Changes in plasma insulin level for this series was reported in Section VI. The basal insulin levels in this three-step IST were unchanged, but the levels 30 min after exogenous insulin infusion during the IST were significantly increased. The plasma norepinephrine level remained unchanged at basal states and during each IST (Table 5).

VIII. 3. Discussion

Surgical denervation of the liver or atropine (intraportal) administration resulted in insulin resistance that was localized to skeletal muscle with no effect on the insulin sensitivity in the liver or the organs draining to the portal vein. We suggest that the hepatic parasympathetic nerves are involved with causing the release of some factor from the liver in response to the injected insulin and that this factor selectively potentiates the effect of insulin in

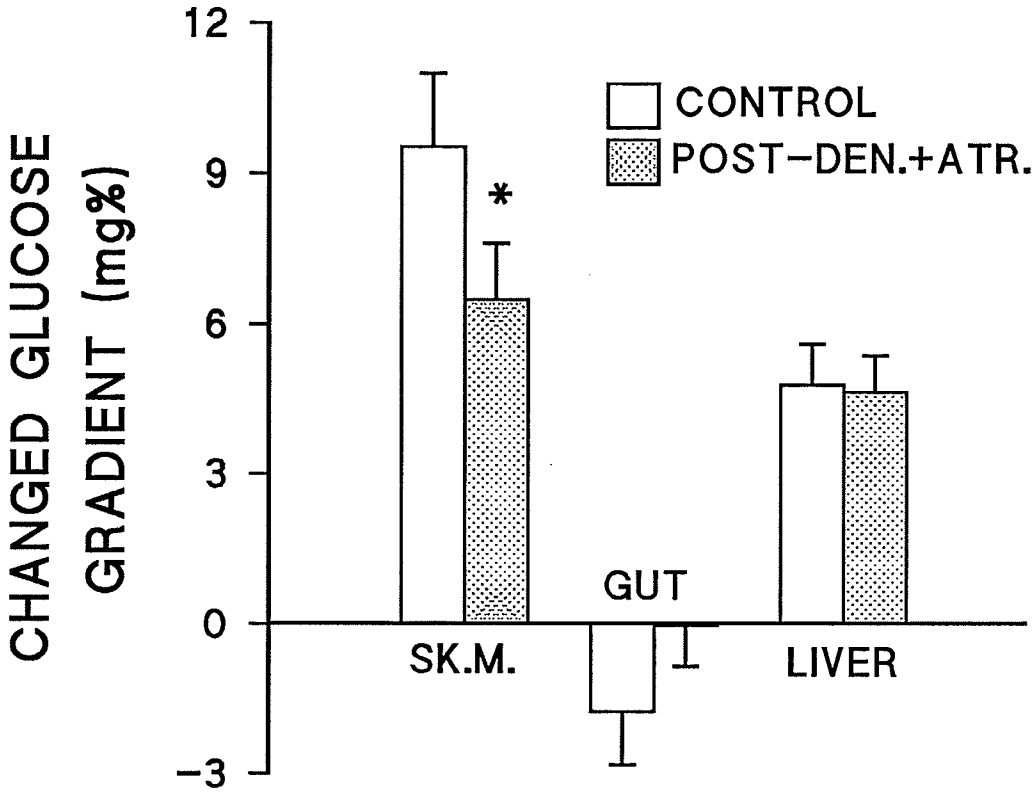


Fig. 18. The changed glucose gradients (mg%) across skeletal muscle (Sk.M), splanchnic organs (Gut) and the liver before (Control) and after combined surgical denervation and atropine 3 mg/kg administration (post-DEN. + ATR). * represents $P < 0.02$, control versus post-treatment (n=8).

TABLE 5

The plasma insulin (μIU/ml) and norepinephrine concentration (pg/ml)

	CONTROL	DENERVATION	ATROPINE
INSULIN (μIU/ml)			
BEFORE INSULIN	25.2 ± 3.2	30.5 ± 4.8	22.9 ± 2.7
AFTER INSULIN	79.2 ± 4.5	71.9 ± 3.8	76.2 ± 3.4
NOREPINEPHRINE (pg/ml)			
BEFORE INSULIN	113.3 ± 26.7	90.4 ± 22.0	97.3 ± 15.0
AFTER INSULIN	105.8 ± 23.1	111.4 ± 31.5	92.5 ± 13.5

skeletal muscle.

In the present study, we used the modified euglycemic clamp, denervation procedure, and atropine dose (3 mg/kg) as in the section V and VII and confirm that denervation results in an insulin resistance of 33.2 ± 2.4% and atropine resulted in a resistance of 29.0 ± 4.3%. Addition of the second intervention did not significantly alter the degree of resistance produced by either intervention alone. This is consistent with the hypothesis that the insulin resistance produced by intraportal atropine or hepatic denervation are via the same mechanism. Previous data indicated that the insulin resistance produced by atropine was not dependent on altered plasma glucagon and insulin levels. In this study we confirmed that the combination of denervation and atropine did not alter insulin levels nor did it result in altered circulating norepinephrine levels. The similarity of basal, pre-test, norepinephrine and insulin and the observation that insulin levels rose to the same level upon insulin administration indicates that hepatic extraction of these compounds was unaltered by the procedures. Hepatic blood flow is not significantly altered by atropine or hepatic denervation [Greenway and Lautt 1989]. This is consistent with a lack of effect of sectioning of the hepatic branch of the vagus nerve in rats on plasma levels of glucagon, insulin, catecholamines, and corticosterone [Doiron et al. 1990; Sonne et al. 1985].

Arterial-venous glucose gradients were taken in order to assess in which tissues insulin resistance was produced. The changes were similar after either atropine or denervation alone and the effects seen after combined

treatment did not alter the pattern, therefore the data are reported for the pooled group, half of which had denervation performed first and half of which had atropine administered first. The combined interventions produced minor changes in baseline A-V gradients although the liver generated a significantly greater output of glucose and the gut produced a reduced uptake following the combined interventions. The liver gradient was increase after denervation or atropine and was somewhat (not significantly) greater with the combined therapy. The mechanism of the small baseline changes are not clear but the increased output from the liver could relate to elimination of the hepatic parasympathetic nerves since these nerves are known to reduce glucose output when stimulated [Lautt and Wong 1978]. The response to insulin as assessed by changes in glucose gradient were not, however, changed in the liver or gut by the combined therapy.

In response to injection of insulin, glucose output in the liver was inhibited, as shown by the change in glucose gradient (Fig. 16). There was a stimulation of uptake across the hindlimbs (mainly skeletal muscle). In contrast, the splanchnic organs draining into the portal vein showed no stimulation of glucose uptake by insulin. After the combined treatment of denervation and atropine, the splanchnic organs and the liver showed unaltered responses but the hindlimb showed a significant decrease in insulin-stimulated glucose uptake. The insulin resistance produced by either intervention directed at the liver was accounted for by resistance at extrasplanchnic sites, probably largely representative of skeletal muscle (Fig.

18).

Our working hypothesis to account for these observations is that the insulin bolus administration led to parasympathetic-mediated release of a factor from the liver that augments insulin effectiveness in selective tissues including (or perhaps exclusively) skeletal muscle. Whether intact hepatic parasympathetic nerves are responsible for a maintained basal release of circulating factor or whether the nerves are involved with producing a transient release cannot be assumed based on the present data. An alternate hypothesis is that the hepatic denervation or atropine interrupt a reflex neurogenic regulation of glucose uptake in skeletal muscle. This possibility is rejected in subsequent studies (section IX).

The lack of effect of insulin on net glucose balance across the extrahepatic splanchnic region is consistent with previous reports. Neither hyperglycemia nor hyperinsulinemia had an effect on the splanchnic glucose uptake even at insulin levels greater than 1000 mU/ml [DeFronzo et al. 1983]. Our data show that the net effect of insulin on organs draining into the portal vein is insignificant and that the putative potentiating factor is without effect on these organs.

A role of the liver in regulation of skeletal muscle responsiveness to insulin has precedent. Insulin resistance commonly occurs in patients with chronic liver disease and the resistance appears to be restricted to skeletal muscle and may be related to reduced transport, reduced non-oxidative glucose metabolism (glycogen formation) but normal oxidative metabolism

and phosphorylation of glucose [Muller et al. 1992; Selberg et al. 1993]. Hepatic sympathetic nerve dysfunction is seen in the chronic bile duct ligation model of liver disease [Schafer et al. 1993] and, although not demonstrated, it is unlikely that the nerve disruption would be restricted to the sympathetic branch of the autonomic nerves. The mechanism by which liver affects peripheral glucose metabolism is unclear but our data are compatible with the speculation that a factor is released from the liver that is dependent upon intact hepatic parasympathetic nerves; and that liver disease may cause parasympathetic dysfunction leading to suppression of release of the putative factor.

The existence of a hepatic factor affecting insulin sensitivity in skeletal muscle is suggested by studies showing a 60% reduction in hindlimb glucose utilization in eviscerated dogs that could be reversed by cross perfusion with an intact dog [Lang et al. 1955]. A similar resistance in eviscerated rats was reversed by administration of an extract from 25 mg/100 g body weight of fresh rat liver [Mertz and Schwarz 1962]. Inclusion of an isolated liver with an isolated hindlimb preparation increased glucose uptake by the hindlimb and the rate of glucose uptake declined after elimination of the liver in the circuit with a half-life of about 30 minutes [Petersen and Tygstrup 1994]. Deproteinized liver or kidney extract also had a similar effect. Whether these factors are related to the putative factor that we suggested is dependent upon hepatic nerve action is unclear.

Direct electrical stimulation of hepatic parasympathetic nerves has, on

the other hand, been suggested to release a factor that produces the opposite effect, that is, it leads to a reduced peripheral glucose uptake [Lautt and Wong 1978]. This factor was invoked to account for the fact that stimulation of hepatic parasympathetic nerves in cats [Lautt and Wong 1978] or of the vagus in pancreatectomized rabbits enhances glycogen storage and increases glucose uptake in the liver [Shimazu and Fujumoto 1971] but does not decrease blood glucose levels. Although speculative, it seems possible that the hepatic parasympathetic nerves produce a variety of effects secondary to direct and indirect effects on the liver and indirect effects on peripheral tissues and that the specific conditions may dictate the net effect. An additional complication arises from the observation that glucose delivered to the liver via the portal vein is much more effectively taken up than when administered via the peripheral route and that this difference disappears upon hepatic denervation [Adkins-marshall et al. 1992]. Whether parasympathetic nerves have any role in this response is unknown. Clearly additional studies are required to resolve the physiological role of hepatic parasympathetic nerves in glucose metabolism under these varied conditions.

VIII. 4. Conclusion and further study

The data reported in this section showed that whole body insulin resistance can be produced rapidly by interference with the hepatic parasympathetic nerves by surgical denervation or atropine administration. These interventions do not result in an altered hepatic or splanchnic response to a bolus of insulin but produce a deceased response in the hindlimb,

probably representing an effect primarily on skeletal muscle. It is speculated that the liver, with functional parasympathetic nerves, releases a factor that potentiates insulin effectiveness in selective tissues including skeletal muscle. Absence of normal hepatic parasympathetic nerve functions leads to insulin resistance in skeletal muscle and may account for the insulin resistance seen in non-insulin dependent diabetics [Lautt 1979] and in patients with chronic liver disease.

It is clear that hepatic cholinergic interruption reduces insulin effectiveness. It is possible that reestablishing the activity of hepatic parasympathetic nerves by administration of a cholinergic muscarinic agonist in the surgically denervated animal may restore the reduced insulin sensitivity to normal. This hypothesis was the objective of the following section.

IX. Insulin resistance caused by hepatic cholinergic interruption and reversed by acetylcholine administration

IX. 1. Materials and methods

Male Sprague-Dawley rats (316.4 ± 3.6 g, n=21) were used. Animal handling, surgical performance and insulin sensitive test were induced as described in Section VI. 1. 1., except that, after laparotomy, the superior mesenteric vein was punctured with a JELCO™ I. V. catheter (24G, 3/4"). The tip of the catheter was located at 1 cm from the hilum of the liver. Intraportal acetylcholine or atropine was infused through this catheter. The dose of insulin used in these insulin sensitivity tests was 50 mU/kg.

Protocols:

IX. 1. 1. Insulin sensitivity in control, with Ach and after atropine intraportal infusion

After the control IST, Ach 2.5 ug/kg/min was continuously infused into the portal vein. A stable basal glucose concentration was determined as previously described. The same dose of insulin was intravenously infused. Arterial glucose level was clamped over 60 min as in the control test. The second control IST was conducted in four of the seven tested rats. Finally, atropine 3 mg/kg was intraportally infused over 5 min. Glucose stabilization, insulin administration and euglycemic clamping were performed as in the control test.

IX. 1. 2. Insulin sensitive tests in control, after surgical denervation, and with different doses of Ach

After the control IST was determined, the nerve bundles and the connective tissue around the common hepatic artery were separated, double ligated and cut between two ties. The common hepatic artery was painted with 95% phenol solution using a cotton swab to destroy any remaining nerve fibres. Insulin sensitivity was tested in all rats after denervation. Another two ISTs were conducted in each animal with Ach 1.0, 2.5 or 5.0 ug/kg/min continuous intraportal delivery in a random order. Therefore, five ISTs were carried out with Ach 1.0 ug/kg/min, six with 2.5 ug/kg/min and five rats with 5.0 ug/kg/min in these nine denervated rats.

IX. 1. 3. Insulin sensitivity with Ach intraportal or intravenous infusion in denervated rats

After surgical denervation, insulin effectiveness was tested, while Ach (2.5 ug/kg/min) was continuously infused either intraportally or intravenously in random sequence. In two of these five rats, a final insulin sensitivity was also tested without Ach.

IX. 2. Results

The reduction of insulin sensitivity after surgical denervation or atropine administration was expressed as % inhibition in comparison with the amount of glucose infused in the control insulin test. The recovery of insulin effectiveness with different doses of acetylcholine after surgical denervation was expressed as % reversal.

IX. 2. 1. Insulin sensitivity with acetylcholine and after atropine intraportal infusion

In the control IST, 328.3 ± 52.3 mg/kg glucose was infused. Acetylcholine 2.5 ug/kg/min intraportal infusion, did not significantly alter the glucose amount required after insulin administration (303.3 ± 52.6 mg/kg) with nerves intact. The glucose amount required in the second control IST showed no difference from that in the corresponding first control IST (254.8 ± 58.8 vs 210.4 ± 44.8, P=0.65, n=4). Atropine, 3 mg/kg intraportal infusion, significantly reduced the amount of glucose infused to 159.2 ± 28.2 mg/kg (P< 0.001). A 49.4 ± 5.8% inhibition of insulin effectiveness was produced (Fig. 19).

IX. 2. 2. Insulin sensitivity after surgical denervation and with different doses of acetylcholine

In the control insulin test, a glucose amount of 442.0 ± 24.7 mg/kg was infused to maintain the basal glucose level constant. Surgical denervation markedly reduced the amount of glucose required to 124.4 ± 22.8 mg/kg (P<0.001, n=9). A 70.7 ± 10.8% inhibition of insulin sensitivity was produced. Acetylcholine, 2.5 ug/kg/min, infusion brought the reduced insulin effectiveness back to the normal level. A glucose amount of 402.1 ± 30.9 mg/kg was infused to maintain the glucose level constant (P<0.001, n=6). A 95.4 ± 7.9% reversal was obtained. A lower and higher dose of acetylcholine was tested in five animals. Acetylcholine, 1 ug/kg/min, infusion restored the glucose amount required to 325.6 ± 39.1 mg/kg (P< 0.001, n=5). A 73.5 ± 11.8% reversal of insulin resistance was observed. Acetylcholine, 5 ug/kg/min, intraportal administration increased the glucose amount required to 319.7 ± 48.5 mg/kg (P<0.001, n=5). A 74.7 ± 6.2% reversal was produced (Fig. 20).

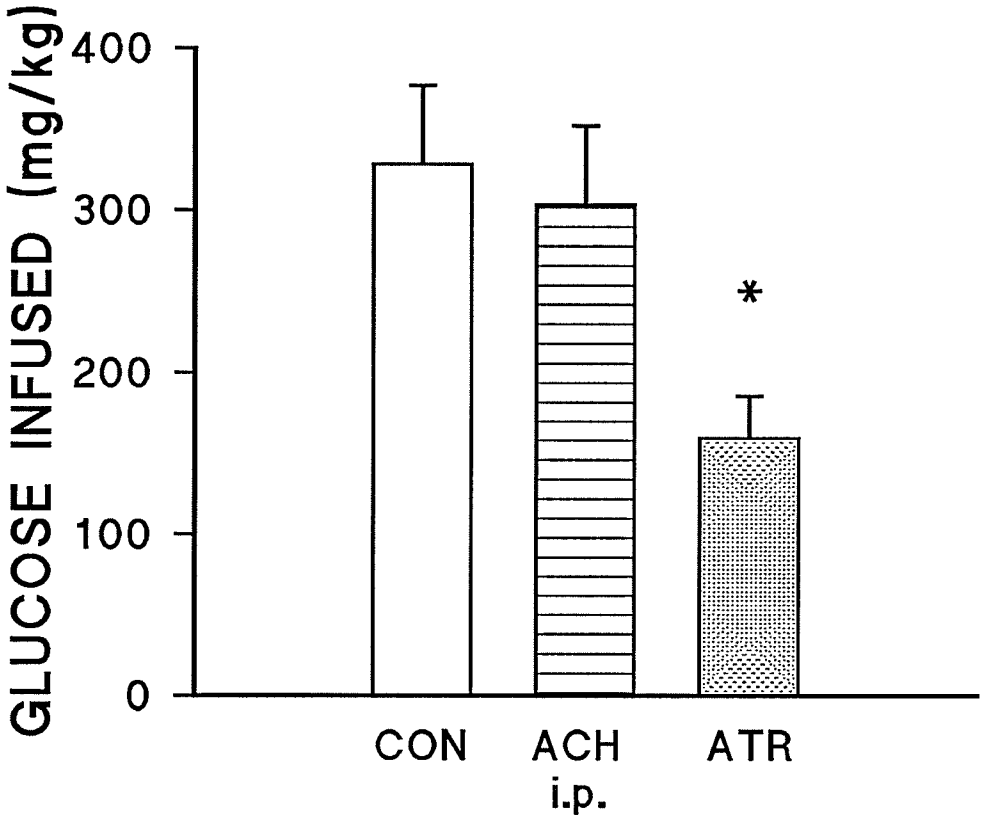


Fig. 19. The amounts of glucose infused (mg/kg) over 60 min after insulin (50 mU/kg, i.v.) administration in control state (CON), with Ach (2.5ug/kg/min) continuous intraportal infusion (ACH i. p.) and after atropine 3 mg/kg intraportal administration (ATR). * represents $P < 0.001$, (n=7).

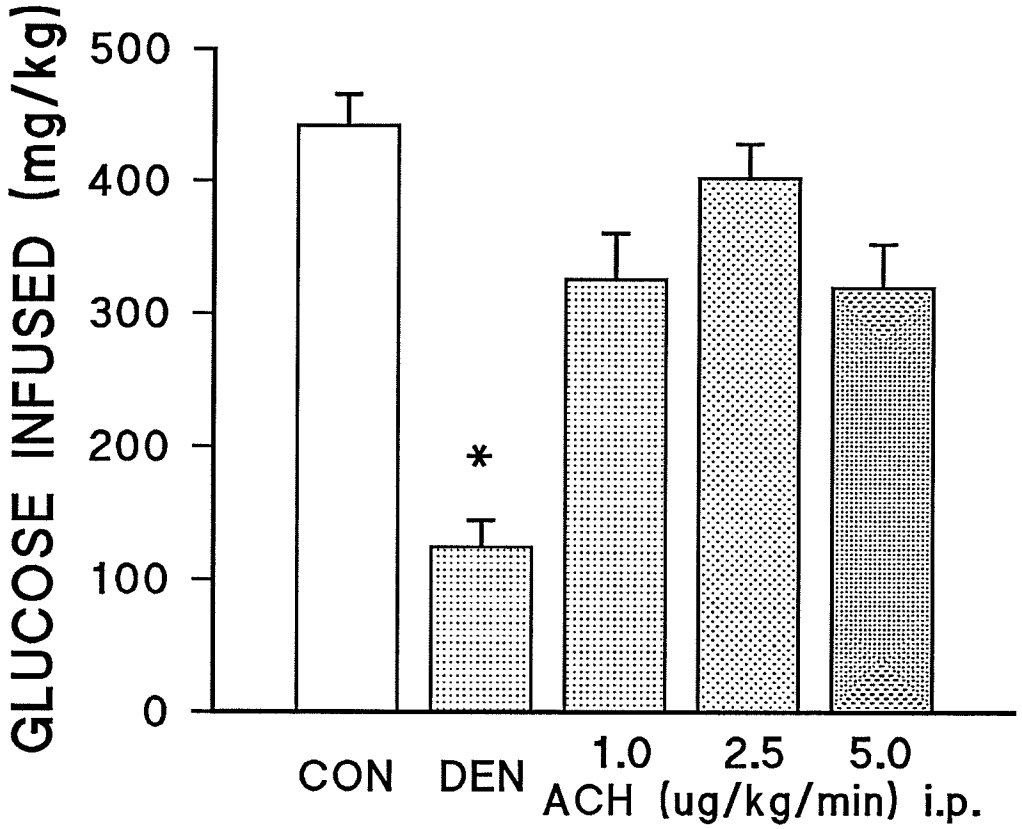


Fig. 20. The amounts of glucose infused (mg/kg) over 60 minutes after insulin (50mU/kg, i.v.) administration in control state (CON), after surgical hepatic denervation (DEN) and with Ach (1.0, 2.5 and 5.0 ug/kg/min continuous intraportal infusion (Ach i.p.). * represents $P < 0.001$.

IX. 2. 3. Insulin sensitivity with acetylcholine intraportal or intravenous infusion in the denervated rats

After surgical denervation, acetylcholine 2.5 ug/kg/min intraportal infusion required 536.4 ± 83.4 mg/kg glucose to maintain the basal glucose level constant after insulin 50 mU/kg administration. However, acetylcholine intravenous infusion at the same dose reduced glucose amount required to 222.9 ± 37.2 mg/kg. In three of these denervated animals, the following insulin administration without acetylcholine needed 213.5 ± 12.1 mg/kg glucose to keep the basal glucose level unchanged. There was no difference of this glucose amount from that required with acetylcholine intravenous infusion (Fig 21). The responses with intravenous acetylcholine in the denervated animals were similar to the responses obtained in rats with interrupted hepatic nerves. Acetylcholine intraportal, but not intravenous, infusion reversed insulin resistance produced by surgical hepatic denervation.

IX. 2. 4. Parasympathetic tone dependent and independent insulin sensitivity

Because the hepatic denervation appeared to produce a somewhat greater degree of insulin resistance (70.8 ± 5.8%) compared to atropine (49.4 ± 5.8%), we further examined the relationship between basal insulin sensitivity and the effect of denervation or atropine by doing regression analysis of the control response versus the decrease in response produced by either intervention (Fig. 22).

The slope of the regression curve is 0.84 (0.70 to 1.03, 95% confidence

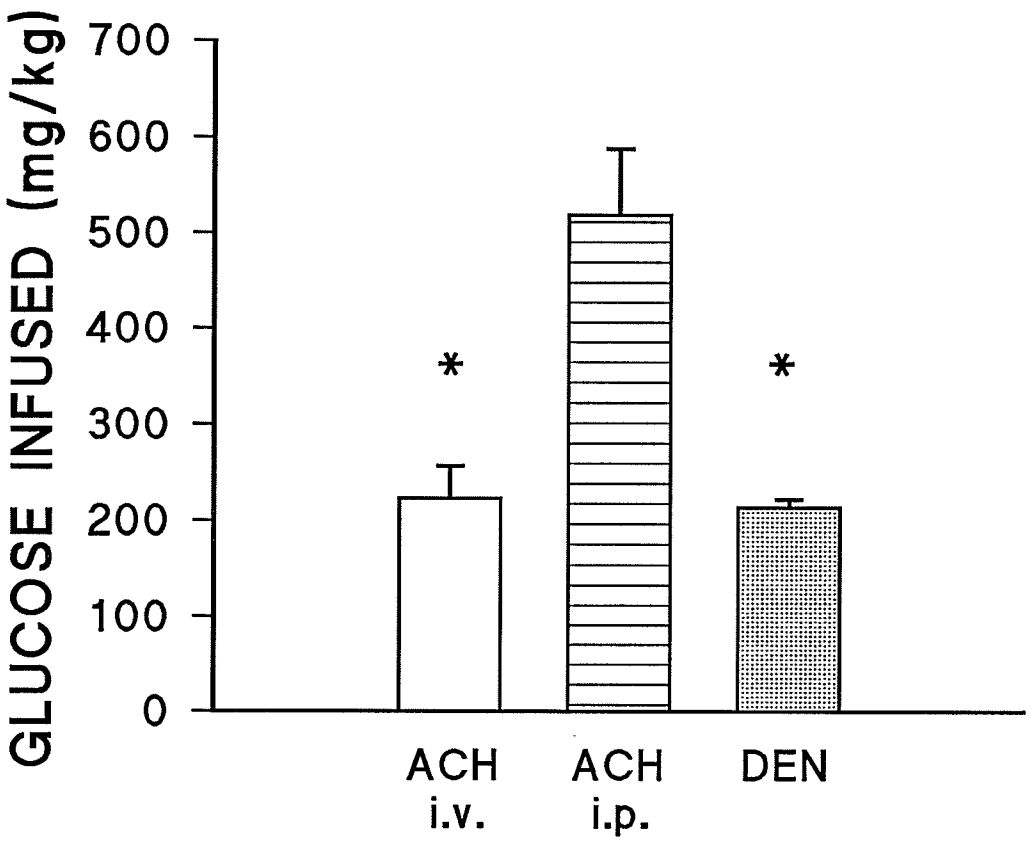


Fig. 21. The amounts of glucose infused (mg/kg) in the denervated rats over 60 min after insulin (50mU/kg) administration with Ach 2.5 ug/kg/min continuous infusion into the peripheral vein (ACH i.v.) or into the portal vein (ACH i.p.) and in insulin test without treatment (DEN). * represents $P < 0.01$ (n=5).

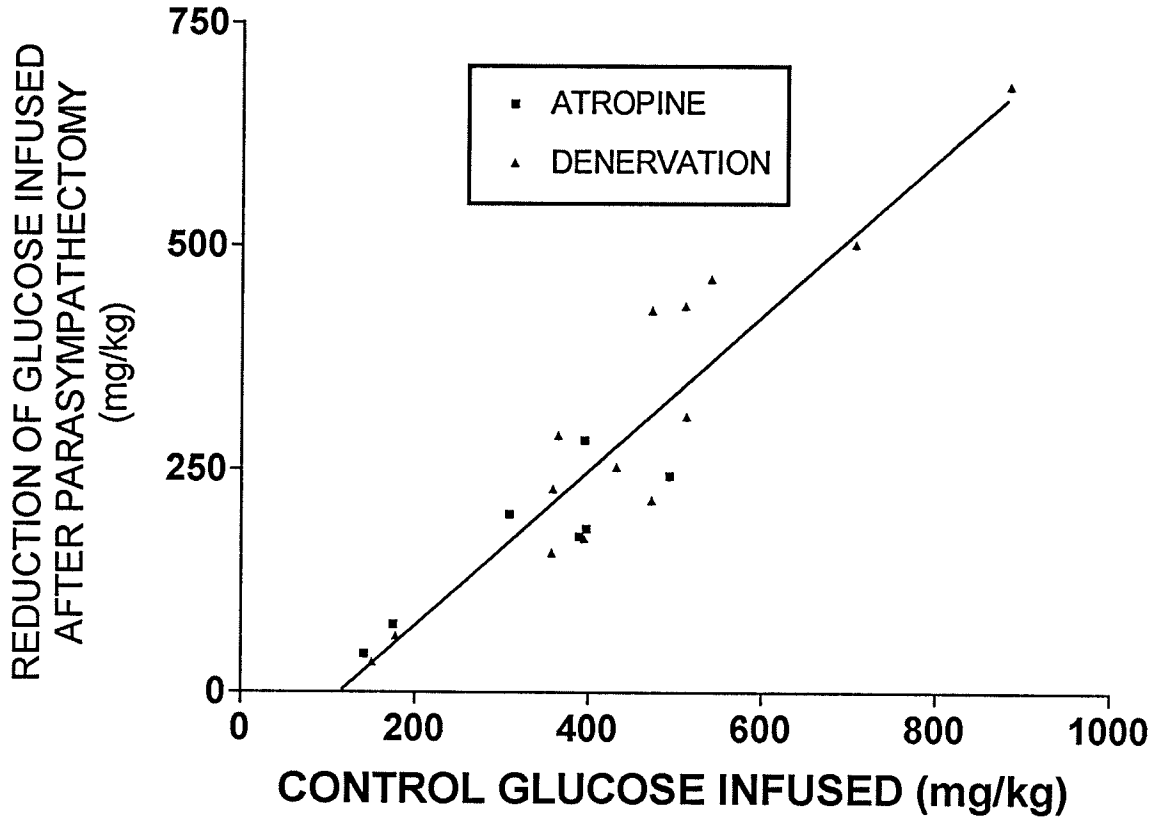


Fig. 22. Linear regression of glucose amounts (mg/kg) infused in control insulin tests (n=21) against the reduced glucose amounts (mg/kg, n=21), calculated by subtracting the amounts infused after surgical denervation or atropine administration from the corresponding amounts in the control insulin tests. Slope equals to 0.86. Intercept on X axis is 112.4 (mg/kg). $r^2 = 0.87$ ($P < 0.0001$).

limits) which is not significantly different from a slope of 1. The intercept at the X axis is 112.6 mg/kg, indicating that the responses lower than this level are independent of hepatic parasympathetic tone. The intercept is similar to the amount of glucose required to maintain euglycemia in response to insulin bolus after denervation (123.8 ± 22.3 mg/kg) or atropine (159.2 ± 28.2 mg/kg).

IX. 3. Discussion

Insulin resistance produced by surgical denervation was totally reversed by intraportal acetylcholine 2.5 ug/kg/min and a dose of acetylcholine of 5.0 ug/kg/min failed to further increase insulin effectiveness. The optical tone of hepatic cholinergic nerves for full expression of insulin effect may thus be mimicked by acetylcholin2.5 ug/kg/min administration.

Acetylcholine administration into the peripheral vein in the denervated rats showed no effect on insulin resistance. This observation strongly indicated that the site of action of acetylcholine was the liver and that reversal of the insulin resistance is through mimicking hepatic parasympathetic nerve function. Our previous study of arterial-venous glucose gradients in the surgical denervated cats indicated a reduction of insulin-mediated glucose uptake by skeletal muscle, rather than in the liver. It is hypothesized that hepatic nerve blockade interrupted a signal system delivered from the liver. A neural signal delivered through hepatic afferent nerves to the central nervous system was initially considered since skeletal muscle shows partial dependence on efferent nerve input to regulate glucose metabolism [Booth et al. 1982; Burant et al. 1984; Buse and Buse 1959]. However, the possibility

that the hepatic-skeletal muscle link is neural is disproved by the observation that the resistance produced by denervation can be reversed by intraportal but not intravenous acetylcholine.

Because the insulin resistance produced by hepatic nerve dysfunction results in resistance of skeletal muscle (see Section VIII.), we assume that the nerves result in regulation of some blood-borne factor. In the cat, the test procedure did not result in alteration of basal glucagon or norepinephrine concentrations. Interruption of nerve function also did not alter the level of insulin in the basal state or the peak level of insulin produced by the exogenous administration, as described in Section VI, VII and VIII. Sectioning of the hepatic branch of the vagus nerve in rat did not result in alteration of plasma levels of insulin, glucagon, catecholamines, or corticosterone [Dioron et al. 1990; Sonne et al. 1985]. The possible identify of the putative factor cannot be assessed but appears not to be explainable by altered insulin, glucagon, or catecholamine secretion or clearance, or altered basal levels of corticosterone.

Infusing acetylcholine into the portal vein of the innervated liver did not significantly alter the insulin sensitivity. Maximally effective parasympathetic effect thus appears to occur with intact nerves using this test system. The unfasted rat used in this study would be anticipated to possess a high tone of hepatic parasympathetic nerves.

Although the rats and the cats (see Section VI ,VII and VIII) were tested under the same protocol, a large degree of insulin resistance was able to be

produced in rats. Surgical hepatic denervation in cat reduced insulin (100 mU/kg) effectiveness by 33.2 ± 2.4%, while a 70.8 ± 5.8% inhibition of insulin (50 mU/kg) sensitivity was observed in rats. Atropine 3 mg/kg intraportal administration produced 33.6 ± 3.4% reduction of insulin (100 mU/kg) effectiveness in cats, but 49.4 ± 5.8% in rats (insulin dose 50 mU/kg). The maximum inhibition of insulin effectiveness caused by atropine administration in cats was 40.6 ± 11.9, which was not significantly different from that produced by surgical denervation (see Section VII). Either a higher tone of hepatic parasympathetic nerves or a greater dependency of insulin effectiveness on this nerve activity in rats might account for the species difference. The cats were fasted but the rats were not. This seems to be the most probable difference to account for the greater dependence on nerve function in rats. Further study is required to resolve the impact of feeding pattern on the nerve-dependent insulin sensitivity.

The magnitude of insulin resistance produced by surgical denervation or atropine administration is predictable from the control insulin test with hepatic nerves intact. This relationship is indicated by the linear regression (Fig 22, $P<0.0001$, $n=21$) of the control response plotted against the reduction in glucose required (difference between control and post-treatment response) after nerve interruption by denervation or atropine administration. The intercept on the X axis (112.3 mg/kg) divides insulin responsiveness into two parts, the cholinergic tone-independent effect (0-112 mg/kg), which accounts for less than one-third of total insulin effectiveness in the unfasted rats, and

hepatic cholinergic tone-dependent insulin effect (beyond 112 mg/kg).

The dominant role of cholinergic dependence suggested by this regression analysis was unexpected and requires further confirmation. However, our interpretation of the results is that one component of insulin response is independent of the hepatic cholinergic nerve related synergism as shown from the intercept on the X axis (112.3 mg/kg). This is similar to the glucose required to be infused to maintain euglycemia after denervation (123.8 ± 22.3 mg/kg) or atropine (159.2 ± 28.2 mg/kg). Insulin effectiveness greater than this level appears to be dependent upon hepatic cholinergic tone. A slope of 1 would suggest that all of the variability in insulin sensitivity under these test condition is accounted for by variability in the cholinergic nerve-dependent synergism. The slope of 0.84 (not significantly different from 1) therefor suggests a very strong, perhaps complete, dependency.

Thus, whole body insulin responsiveness appears to be dramatically dependent upon some function of hepatic parasympathetic nerves. Data from the cat indicated that the synergistic action of hepatic nerves on insulin response occurs in skeletal muscle. Acute elimination of the nerve function results in insulin resistance with the insulin responsiveness being dramatically reduced to a baseline value that is nerve independent. Acetylcholine delivered directly to the liver can fully return the insulin effectiveness to normal. Most, if not all, of the variability in insulin effectiveness in normal rats appears to be due to variable hepatic parasympathetic effect. Therefore, the tone of hepatic parasympathetic nerves may serve as a variable factor to regulate insulin

sensitivity in the peripheral tissues.

IX. 4. Conclusions and further study

Intraportal acetylcholine administration reverses the insulin resistance produced by surgical denervation, while intravenous administration does not. Acetylcholine 2.5 ug/kg/min intraportal infusion can totally reverse the insulin resistance generated by hepatic denervation. Acetylcholine intraportal administration does not alter the insulin effectiveness in normal rats. There is a linear relationship between basal and the reduced insulin effectiveness produced by hepatic cholinergic interruption. This relationship divides insulin sensitivity into hepatic parasympathetic tone-dependent and independent components. Most of the variability of insulin sensitivity in normal rats appears to be caused by variable hepatic parasympathetic effects.

It is hypothetized that hepatic parasympathetic nerve damage caused by liver pathological changes may also reduce insulin effectiveness. This resistance may be reversed with acetylcholine intraportal administration. Insulin sensitivity and the effect of intraportal acetylcholine was tested in a chronic hepatic damage model in next study.

X. Insulin resistance two weeks after bile duct ligation; reversal with acetylcholine intraportal infusion and unaltered by atropine

X. 1. Materials and methods

Male rats (n=9) were unfasted and anesthetized with intraperitoneal injection of pentobarbital 65 mg/kg. Ampicillin (62.5 mg) was given intramuscularly into the hindlimb muscle prior to surgery. Under sterile conditions, a mid-line incision from the xiphoid process to the umbilicus was performed and a laparotomy conducted. A 5 mm length of the common bile duct, approximately 1 cm from the duodenum, was cleared and separated from the surrounding tissues. The bile duct was ligated twice (6.0 silk sutures) and cut between the ties. The abdominal muscles were closed with 6.0 chromic gut sutures using a continuous running stitch. The skin layer was closed with 6.0 chromic gut sutures using a continuous hidden mattress stitch.

The sham-operated rats underwent the identical surgical procedure except that the bile duct was not ligated. Animals were transferred to a recovery room where they were individually housed in separated cages until they had recovered from the anesthesia. Animals were checked frequently during the recovery period. No animal died during this time. Each animal received another 62.5 mg of ampillicin the day following surgery.

On the surgical day (10-14days after bile duct ligation), the rats were prepared as described in Section VI. 1. 1. After laparotomy, the superior mesentery vein was cannulated as described in Section IX. 1. for administering acetylcholine or atropine. Animals were allowed 45 min to

stabilize after the surgical interventions before any procedure was carried out.

The IST was carried out as described in Section VI. 1. 1 with an insulin dose of 50 mU/kg. The IST was determined in control state, then during intraportal continuous infusion of acetylcholine, 2.5 ug/kg/min, and again after intraportal administration of atropine 3 mg/kg.

X. 2. Results

In the sham-ligated rats (n=7), 483.3 ± 98.2 mg/kg glucose was required to keep the basal glucose level constant 10 days after sham operation. With acetylcholine (2.5 ug/kg/min) intraportal infusion, the insulin sensitivity was not altered (476.0 ± 85.6 mg/kg). After atropine (3 mg/kg) administration, the glucose amount required was reduced to 240.1 ± 85.5 mg/kg, producing a 50.3 ± 7.9% inhibition of insulin effectiveness, similar to a 49.4 ± 5.8% inhibition in normal rats (Section IX. 2. 1.). Since the insulin sensitivity seen in these sham-ligated animals was similar to that observed in Section IX. 2. 1, we compared the data from the bile duct ligated (BDL) animals (n=7) with the pooled control data from sham operated and normal rats.

In BDL group, 199.4 ± 37.0 mg/kg glucose was required during IST 10 days after bile duct ligation. With the treatment of acetylcholine 2.5 ug/kg/min, the glucose amount required was 363.8 ± 65.1 mg/kg ($P<0.001$, n=7). After intraportal atropine (3 mg/kg) the IST was not different from what was seen in the BDL control IST. The insulin resistance in the bile duct ligated animals was thus not altered by atropine administration (Fig. 23).

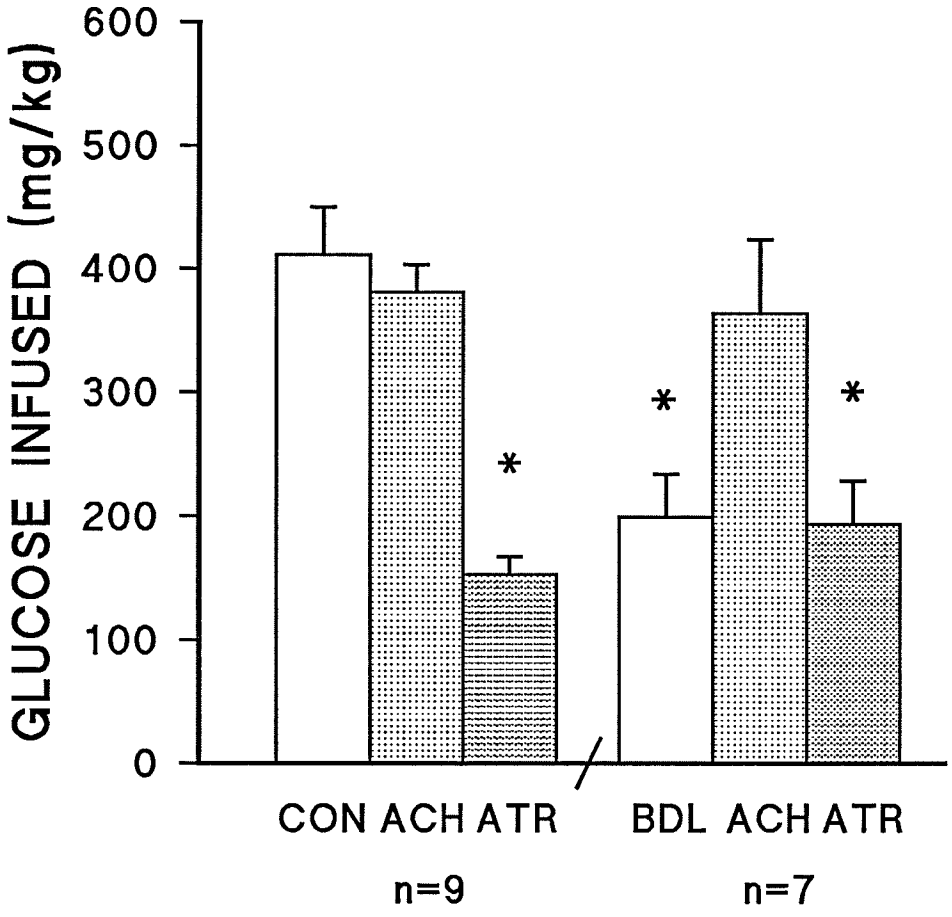


Fig. 23. The amounts of glucose (mg/kg) infused over 60 min after insulin (50 mU/kg, i.v.) administration in the control or 10 days after bile duct ligated rats, with Ach (2.5 ug/kg/min) continuous intraportal infusion and after atropine (3 mg/kg) introportal administration in both control (the left panel, n=9, pooled from two sham-operated and seven normal rats) and bile duct ligated rats (the right panel, n=7).

X. 3. Discussion

The sham rats did not show any obvious alterations in behavior throughout the postoperative period. These animals had a weight gain of 8.5 ± 1.2 g/day. The bile duct-ligated rats were inactive and sick. They gained only 3.0 ± 0.6 g/day in weight, which was significantly less than in the sham animals. In all cases, the sham animals did not show any signs of liver or biliary system pathology. The ligated animals displayed general jaundice, a hard, swollen and yellow-coloured liver and dilated biliary system. Bacterial infection was not observed in any animals.

The hepatic damage induced by bile duct ligation produced a qualitatively and quantitatively similar insulin resistance to that caused by surgical hepatic denervation (70.8 ± 5.8%) or atropine intraportal administration (49.4 ± 5.8%), as indicated in Section IX. Additive atropine treatment failed to further alter the bile duct-ligation-induced insulin resistance. Chronic hepatic disease using the BDL model significantly reduced insulin-mediated glucose deposition, and the insulin resistance produced by this pathology is ascribed to the dysfunction of hepatic parasympathetic nerves.

Although the degree of damage of hepatic parasympathetic nerves was not assessed in the bile duct ligated rats, impairment of hepatic sympathetic nerves has been demonstrated in 10 day bile duct ligated cats in our laboratory [Schafer et al. 1993, d' Almeida 1994]. The norepinephrine content of the bile duct ligated liver was less than 3% of the normal liver [Cucchiaro 1990]. In long-term bile duct ligated guinea pigs, acetylcholine-E positive

nerve fibres disappeared from the parenchymal cells [Akigoshi 1989]. In humans, hepatic nerves were absent in cirrhotic nodules and in the fibrotic strands of alcoholic cirrhotic liver [Juskiewicz 1994; Lee et al. 1992]. These observations indicate a loss of hepatic parasympathetic innervation in the cirrhotic liver and are consistent with our functional observations that chronic bile duct ligation reduces insulin effectiveness. This conclusion is supported by the results that cholinergic agonism totally restored insulin sensitivity to normal and muscarinic antagonism did not cause extra insulin resistance in the damaged liver.

Acetylcholine infusion totally reversed the bile duct ligation-induced insulin resistance; a similar reversal was seen in the surgically denervated rats. This observation lends support to the hypothesis that the insulin resistance occurring in some disease states typified by autonomic nerve dysfunction, including hepatic diseases, non-insulin dependent diabetes or hypertension, can be restored to normal level by treatment with cholinergic agonism. Although chronic bile duct ligation can result in an extensive intra- and extra-hepatic tissue damage [d' Almeuda and Lautt 1994; Schafer et al.1993], acetylcholine intraportal administration completely reverses insulin resistance in this animal model, suggesting that this treatment is specific and that the insulin resistance in these animals is mainly accounted for by hepatic parasympathetic neuropathy. The other intrahepatic or systemic impairments appear not to contribute significantly to the produced insulin resistance.

Long-term bile duct ligation in rats induces a similar degree of insulin

resistance produced by surgical denervation, which is not significantly different from the resistance produced by atropine (3 mg/kg) intraportal administration (see Section IX), suggesting that 10 day bile duct ligation in rats is effective to produce a full hepatic parasympathetic dysfunction in terms of insulin sensitivity in glucose deposition.

Acute insulin resistance is produced by surgical hepatic denervation and cholinergic muscarinic blockade. Chronic and prolonged insulin resistance is produced by bile duct ligation-induced liver damage. Both the acute and chronic insulin resistance is qualitatively and quantitatively similar. Acetylcholine intraportal administration reverses the produced insulin resistance completely and immediately, indicating that hepatic parasympathetic nerves are essential for insulin effect and can be mimicked by blood-borne cholinergic agonism.

X. 4. Conclusions

In 10 day's bile duct ligated rats, insulin sensitivity was reduced to the same level as produced by surgical hepatic denervation, which can not be further altered by atropine 3 mg/kg intraportal administration. Intraportal Acetylcholine (2.5 ug/kg/min) completely reverses this insulin resistance.

XI. General conclusions and significance

This thesis work, for the first time, demonstrates the role of hepatic parasympathetic nerves in insulin-mediated glucose metabolism in vivo. A sensitive and reproducible euglycemic clamp technique was developed, which can provide multiple insulin sensitivity tests in cats and rats, in fed and fasted state.

Significant reduction of insulin effectiveness was produced by different methods. Surgical hepatic denervation produced a 37.2 ± 9.8% and a 70.7 ± 10.8% inhibition of insulin effectiveness in fasted cats and in unfasted rats, respectively. Pharmacological blockade of hepatic cholinergic muscarinic nerves with atropine produced a dose-dependent insulin resistance in cats. Atropine 3 mg/kg reduced insulin effectiveness by 33.6 ± 3.4%, which was similar to that produced by hepatic denervation. Cholinergic M_1 receptor may mediate this hepatic function. Pathologically, bile duct ligated rats showed a significant inhibition of insulin effectiveness 10 days after the surgery, which is quantitatively and qualitatively similar to that produced by surgical denervation or atropine administration.

The insulin resistance produced by surgical hepatic denervation and pathological hepatic damage has been completely reversed with acetylcholine 2.5 ug/kg/min intraportal infusion. Acetylcholine administered into the portal vein but not the systemic venous ststem can produce this effect but does not alter insulin effect in animals with normal hepatic parasympathetic function. This treatment is specific and extremely effective.

The insulin resistance produced by hepatic cholinergic nerve interruption is produced in skeletal muscle, rather than in the liver. It is hypothesized that a normal liver may release a blood-borne factor in response to insulin, which can exert a synergistic effect with insulin to facilitate glucose transport in skeletal muscle. The denervated liver does not release this putative factor.

Insulin effectiveness can be divided into two components: insulin effects independent and dependent on the tone of hepatic cholinergic nerves. In normal unfasted rats, the nerve independent effect is less than one-third of total insulin effectiveness. The variability of insulin sensitivity may be totally accounted for by the variability of tone of hepatic parasympathetic nerves in this situation.

We speculate that insulin resistance occurring in patients with hepatic diseases, non-insulin dependent diabetes or hypertension might be accounted for by hepatic parasympathetic neuropathy, and may be diminished by treatment with cholinergic muscarinic agonism.

Through these primary studies, the dependence of insulin-mediated glucose uptake on hepatic parasympathetic nerves was demonstrated. How to reverse the insulin resistance caused by hepatic parasympathectomy was also indicated. Insulin effectiveness can be tested by using the modified euglycemic technique with high reproducibility. A new theory for treating insulin resistance in non-insulin dependent diabetes, hepatic diseases and hypertension is experimentally proposed. The classical theory that the liver is

a central organ in carbohydrate metabolism is enriched. The liver not only regulates plasma glucose level by storage and release of glucose, but also may actively control peripheral glucose metabolism by releasing a blood-borne factor which synergizes with insulin to regulate glucose uptake in skeletal muscle.

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