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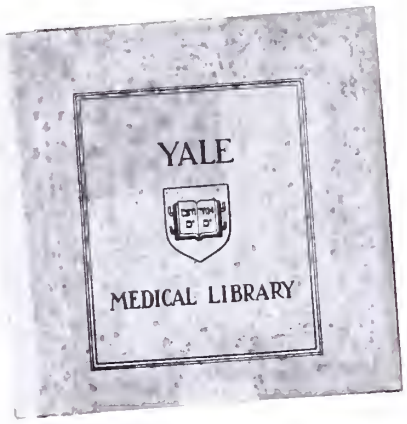

THE EFFECTS OF IN VIVO HYPERINSULINEMIA ON 2-DEOXYGLUCOSE TRANSPORT IN RAT SOLEUS MUSCLE

BY

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Short Introduction

The initiation of insulin action first involves its binding to a specific receptor on the cell membrane and this is followed by the stimulation of glucose transport and intracellular glucose metabolism. Although the acute effects of insulin on glucose transport have been well characterized, the effects of more chronic exposure to insulin are less well defined. These effects are of general interest in understanding insulin physiology, and assume clinical importance in understanding what role hyperinsulinemia plays in the insulin resistance associated with obesity (1-4), maturity-onset diabetes (5-11), uremia (12-14), acidosis (15), and other insulin resistant states.

Recently, strong evidence has been provided to support the concept that prolonged insulin exposure, both in vitro (15-18) and in vivo (16-20), is capable of leading to a decrease in the number of insulin receptor sites on the cell surface. However, the metabolic consequence of this decrease in receptor binding has yet to be established.

Much less is known about the effect of prolonged in vitro or in vivo hyperinsulinemia on glucose transport and metabolism. Olefsky et. al. have documented an increase in 2-deoxyglucose (2-DOG) transport by adipocytes obtained from rats chronically made hyperinsulinemic with a 14 day protocol of insulin injections (21, 22). Employing the insulin clamp technique in man, Soman and DeFronzo have demonstrated a small but significant increase in overall glucose metabolism following 3-5 hours of hyperinsulinemia (20). However, other studies have failed to document an increase in cellular glucose

transport following a prolonged in vivo (19) or in vitro (18) exposure to insulin. Instead, these studies (18, 19) found a decrease in tissue sensitivity to insulin and have attributed this to the decrease in receptor number which followed prolonged insulin exposure (18, 19).

The present study was undertaken: 1) to examine the rate of overall in vivo glucose metabolism during sustained, physiologic hyperinsulinemia and 2) to evaluate glucose transport by rat soleus muscle following prolonged in vivo hyperinsulinemia. To accomplish this goal, the euglycemic insulin clamp technique (23) was appropriately modified to allow study of the unanaesthetized rat. With this technique a steady-state plateau of hyperinsulinemia can be achieved while maintaining the plasma glucose concentration constant at the basal level.

Introduction

I. Glucose Transport

Following the binding of insulin to a cell surface receptor, glucose transport is activated and this represents the first step in the cellular metabolism of glucose. Stimulation of glucose transport is extremely sensitive to insulin and occurs at hormone concentrations well within the physiologic range. Most importantly, insulin-stimulated glucose metabolism in vivo appears to represent a primary effect on glucose transport rather than a stimulation of intracellular glucose metabolism (24, 25).

Early studies of glucose transport were performed by examining glucose disappearance from the media (26) or by the chemical determination of non-metabolized glucose analogues taken up by the cell (27, 28). More recently, radioactively-labelled non-metabolized glucose analogues, including 3-0-methylglucose (3-0-MG) and 2-deoxyglucose (2-DOG), have been employed to study cellular glucose transport. 3-0-MG is transported into the cell but not further metabolized (27). However, there is also a significant efflux of 3-0-MG from the cell and uptake actually represents the sum of influx and efflux. The intracellular accumulation of 3-0-MG proceeds until a steady-state equilibrium is reached. Thus, the uptake of 3-0-MG reflects glucose transport only if initial rates of uptake are measured, i.e., before significant intracellular accumulation of 3-0-MG has occurred and the rate of efflux becomes quantitatively important. Consequently, special methodology (29) is necessary to analyze the initial rapid rates of 3-0-MG uptake and this feature

makes the study of 3-O-MG transport difficult. In contrast to 3-O-MG, 2-deoxyglucose (2-DOG) is transported into the cell and phosphorylated by the enzyme hexokinase (28). However, 2-deoxyglucose-phosphate (2-DOG-P) is not further metabolized (28). Under conditions where transport of 2-DOG is rate-limiting and phosphorylation is rapid, little free 2-DOG accumulates within the cell and there is no significant efflux of 2-DOG from the cell. Thus, uptake of 2-DOG into the cell is linear with time and this feature greatly facilitates study of glucose transport with this analogue.

Early studies by Kipnis and Cori (28) demonstrated that 2-DOG was transported by rat diaphragm and that glucose and 2-DOG had similar affinities for the transport system. Furthermore, uptake was found to be linear even under conditions of high rates of 2-DOG transport (28). All intracellular deoxyglucose was present as the phosphorylated derivative indicating that the hexokinose enzyme was not rate-limiting. Thus, 2-DOG uptake appeared to provide a good measure of glucose transport into the cell.

Most of the initial studies on glucose transport employed rat diaphragm. However, diaphragm is a constantly contracting muscle and subsequent studies have shown that it does not reflect typical skeletal muscle (30). Therefore, more recent studies have employed soleus muscle which is composed mainly of slow-twitch red fibers and is more representative of whole body skeletal muscle mass. The soleus muscle can be isolated as an intact preparation without damage to the muscle fibers (26). Uptake of 2-DOG has been shown to be linear at a 2-DOG concentration of 0.5 mM for prolonged periods of time (31). Transport of 2-DOG in soleus muscle is

thought to be rate-limiting since: 1) the Km for hexokinase is 10x less than for transport, and 2) intracellular concentrations of free 2-DOG are very small (31).

II. Insulin-Stimulated Glucose Transport

Insulin stimulation of glucose transport is thought to involve three steps: 1) insulin binding to specific plasma membrane receptors, 2) coupling between occupied receptors and glucose transport units, and 3) activation of glucose transport units.

Insulin Binding

The importance of insulin receptor binding in the activation of cellular glucose transport was first suggested by the observation that when adipocytes were treated with trypsin insulin binding was diminished and this was parallelled by a decrease in cellular glucose transport (32, 33). More recently, the relationship between insulin receptor binding and glucose transport activation has been examined in adipocytes (34-37) and soleus muscle (31).

Radioligand binding has been used to define insulin receptors on the plasma membrane (38). Such receptors are assumed to be present if the binding of ^{125}I-insulin can be shown to be 1) saturable and 2) competitively inhibited by the addition of unlabelled insulin. Using such radioligand binding systems several investigators have attempted to examine the relationship between insulin binding and insulin-mediated activation of the glucose transport system (31, 34-37). Insulin stimulation of glucose transport has been shown throughout the range of physiological insulin concen-

trations (31, 34-37). However, half-maximal stimulation of glucose transport occurs at insulin concentrations where only 5% receptor occupancy is present, and maximal stimulation occurs where approximately 20% of receptors are bound by insulin (31, 34-37). The term spare receptors (31, 34-37) has been used to describe the remaining 80% of the receptors beyond those necessary to elicit a maximal biologic response.

Interpretation of spare receptors is complicated by the early observation that receptor binding affinity is not uniform but decreases with receptor occupancy (38, 39). This observation is compatible with the existence of 1) two or more distinct populations of receptors with different fixed binding affinities or 2) a uniform population of receptors which display site to site interactions with modification of binding affinity as receptor occupancy changes. Distinct receptor populations might suggest that high affinity receptors are physiologically active while low affinity receptors are inactive. However, De meyts et. al. (39) demonstrated the conversion of high affinity to low affinity sites when receptor occupancy was increased. These experiments provided evidence that a uniform population of binding sites exists and is capable of negative cooperativity in the binding of insulin. Thus, insulin receptors may represent a uniform population of sites that are physiologically equivalent. It is postulated that occupancy of an equal number of any random insulin receptors will produce an equivalent response.

Glucose Transport Activation

The mechanism of insulin stimulation of glucose transport has been examined by Olefsky et. al. (34-37) employing 2-DOG uptake in isolated rat adipocytes. Transport is a function of extracellular 2-DOG concentration. By analogy to an enzymatic model, insulin activation can be viewed as representing an increase in Vmax for the system rather than an alteration in the Km or affinity for 2-DOG (34). Based on similar activation energies, for basal and insulin-stimulated transport, the insulin induced increase in Vmax may represent a recruitment of new transport units rather than enhanced mobility of already active units (34).

Recently, cytochalasin-B binding has been employed to study glucose transport activation by insulin (40, 41). Cytochalasin-B binding sites are thought to be closely related to the glucose transport system (40, 41) since: 1) low concentrations of cytochalasin-B inhibit glucose transport and 2) the effectiveness of sugars in inhibiting cytochalasin-B binding is stereo-specific and related to their affinity for the glucose transport system. An increase in plasma membrane cytochalasin-B binding and a stoichiometric decrease in microsomal membrane cytochalasin-B binding are correlated with insulin activation of glucose transport in rat adipocytes (41, 42). Thus, these results suggest that insulin activation of glucose transport may involve the recruitment of new transport units into the plasma membrane from an intracellular microsomal pool.

III. Effects of Prolonged Insulin Exposure

The acute stimulating effects of insulin on glucose transport, glycolysis, and glycogen synthesis have been well studied in vitro in adipocytes (34-37) and skeletal muscle (31). Similarly, the acute stimulation of glucose utilization by peripheral tissues has recently been examined in vivo during experimental hyperinsulinemia in man (23). In contrast, the effects of prolonged in vitro or in vivo insulin exposure on glucose metabolism have not been well defined. These effects are important not only in understanding the physiology of insulin, but also in understanding what role chronic hyperinsulinemia plays in insulin resistant states.

Difficulty in studying the effects of prolonged insulin exposure has stemmed from problems 1) in maintaining tissue viable in vitro for extended periods of time and 2) in developing a suitable model of hyperinsulinemia which maintains the plasma glucose concentration constant. Although improved tissue culture techniques have made it possible to study the effects of chronic in vitro insulin exposure, the study of chronic in vivo hyperinsulinemia has proven difficult. Experimental models of chronic hyperinsulinemia have been developed in the rat (19, 21, 22) but have been associated with wide fluctuations in plasma glucose levels. Development of the euglycemic insulin clamp technique (23) has been important in the study of hyperinsulinemia in man, although studies to date have examined hyperinsulinemia for only 3-5 hour periods (20).

Hyperinsulinemia and decreased receptor binding

Interest in the effect of changes in circulating insulin levels on its own receptor were first raised by the frequently observed inverse correlation between fasting plasma insulin concentrations and insulin receptor binding. This inverse correlation has been demonstrated in normal man (5), in subjects with obesity (1-4), maturity-onset diabetes (5, 6) and following fasting (1, 43-45) and carbohydrate feeding (1, 43, 46). Similar observations have been made in animal models of obesity (31, 47-50) streptozotocin-induced diabetes (51-54), and following fasting (53-55).

Both in vitro (16-18) and in vivo (19, 20, 56) experiments have established that hyperinsulinemia leads to a decrease in insulin receptor binding. Down-regulation of insulin receptors was first demonstrated in vitro by Gavin et. al. (16) who found a decrease in insulin receptor binding in cultured lymphoblastoid cells following a 5-16 hour exposure to supraphysiologic concentrations (10 mM) of insulin (16). This effect was time, concentration, and temperature dependent (16). The decrease in insulin receptor binding was due to a decrease in receptor number without any change in receptor binding affinity (16). Decreased receptor binding has also been demonstrated at more physiologic insulin concentrations in cultured rat hepatocytes following a 16 hour exposure to 1 nM of insulin (17). Down-regulation of insulin receptor binding in vitro has also been shown in rat adipocytes following exposure to pharmacologic doses of insulin (10.5 nM), but not to physiologic doses (18).

Down-regulation in vivo has also been observed following experimental hyperinsulinemia. Rat adipocytes show a 40% decrease in insulin receptor binding in animals maintained on a chronic 14 day regime of NPH insulin injections (19). The plasma insulin level was reported to be 65 ± 5 μU/ml 12 hours after the last dose of NPH insulin (19). Thus, it is likely that much higher plasma insulin levels, in excess of 150-200 μU/ml were reached during this experiment. The interpretation of these changes is also somewhat obscured by the large fluctuations in plasma glucose levels that occured during this study. Less prolonged exposure to in vivo hyperinsulinemia has also been shown to decrease insulin receptor binding in man (20, 56). Sustained hyperinsulinemia of 3-5 hours duration produced by the euglycemic insulin clamp technique resulted in a 25-30% decrease in monocyte binding at insulin levels of 31 ± 2 μU/ml (20). A greater decrease (40%) in insulin receptor binding was observed with plasma insulin levels of 102 ± 6 μU/ml (20). Hyperinsulinemia produced by the quadruple infusion technique (insulin, glucose, epinephrine, and propranalol) also resulted in decreased insulin receptor binding with steady-state insulin levels of 108-118 μU/ml (56). When a lesser degree of hyperinsulinemia (27-31 μU/ml) was created with concomitant hyperglycemia, a decrease in insulin receptor binding was also observed after 5 hours (56). This was attributed to a decrease in receptor binding affinity for insulin (56).

Thus, the phenomenon of down-regulation (38, 57, 58) has been demonstrated for insulin receptors following both in vitro and in vivo exposure to insulin, both in man and animals. Most importantly,

the phenomenon of down-regulation has been demonstrated with insulin concentrations in vivo which are well within the physiologic range and which have commonly been observed in the post-prandial state and in insulin resistant states. As previously discussed, present receptor theory would predict that a decrease in insulin-mediated glucose metabolism should accompany a decrease in insulin receptor binding. More specifically, one would expect a decrease in "sensitivity" (59) to insulin, i.e., an increase in the concentration of insulin required to elicit half-maximal stimulation of the full insulin response. In fact, any concentration of insulin below that which achieves a maximal effect would be expected to have a decreased effect. In contrast, one would not expect a decrease in "responsiveness" (59) to insulin, i.e., a change in the maximal biological response that can be achieved at any concentration of hormone, because at increasing concentrations of insulin, binding of spare receptors should achieve a sufficient receptor occupancy to elicit the maximal biological response. Thus, a rightward shift in the dose response curve with normalization at maximally effective insulin concentrations is the predicted consequence of a decrease in insulin receptor binding.

Hyperinsulinemia and glucose metabolism

Definition of the effects of prolonged insulin exposure on glucose metabolism requires the study of tissues active in glucose metabolism following either in vitro or in vivo exposure to insulin. Livingston et. al. studied rat adipocytes following a 17 hour in vitro exposure to 3.5-10.5 nM of insulin (18). This in vitro

exposure to supra-physiologic doses of insulin produced a rightward shift in the insulin dose response curve, but no change in either basal or maximal insulin-stimulated rates of glucose transport.

Similar findings have been reported by Olefsky et. al. who studied 2-DOG transport in adipocytes isolated from rats made chronically hyperinsulinemic in vivo with a 14 day regime of twice daily NPH insulin injections (19). However, subsequent studies from the same lab have demonstrated a significant increase in both basal and maximal insulin-stimulated 2-DOG transport in rats similarly treated (21, 22). Although these results are inconsistent, the latter data suggest that chronic in vivo hyperinsulinemia may increase cellular glucose transport. However, the failure to maintain euglycemia in these animals complicates the interpretation of these results. This model of hyperinsulinemia (19, 21, 22) in fact represents the combined effects of both prolonged insulin exposure and changes in plasma glucose concentration. Despite free access to 5% glucose drinking water and sugar cubes, the hyperinsulinemic animals were markedly hypoglycemic compared to controls which were both hyperglycemic (plasma glucose = 152 ± 5 mg/dl) and hyperinsulinemic (plasma insulin = 29 ± 3 μU/ml). Plasma glucose concentration was measured only at the time of sacrifice which was 12 hours after the last insulin dose. The results indicate that many, if not all, of the hyperinsulinemic rats were frankly hypoglycemic throughout a significant period of the 2 week study. The authors attempted to negate the effects of hypoglycemia by treating another group of rats with injections of 20% glucose given 4 times daily to prevent hypoglycemia (19, 21, 22). However, these animals were subject to large fluctuations in plasma

glucose during the day with periods of marked hyperglycemia. Although these authors have concluded that chronic in vivo hyperinsulinemia results in an increase in cellular glucose transport (21, 22), significant changes in plasma glucose concentration were clearly present and it is also likely that changes in counter-regulatory hormones also occurred. Both of these factors could have potentially contributed to the findings in this model.

The development of the euglycemic insulin clamp technique (23) has made it possible to study the effects of prolonged hyperinsulinemia on in vivo glucose metabolism, while maintaining plasma glucose concentration constant at the basal level and avoiding the release of counter-regulatory hormones (20). With this technique, a prime-continuous insulin infusion is administered intravenously to rapidly achieve steady-state hyperinsulinemia while euglycemia is maintained by a variable glucose infusion based on a negative feed back principle (23). Since hyperinsulinemia suppressed hepatic glucose production and since urinary glucose loss is negligible under these conditions (23), the glucose infusion rate required to maintain euglycemia represents a measure of the amount of total in vivo insulin-stimulated glucose metabolism. Using this technique, Soman and DeFronzo (20) have demonstrated a small but significant increase in in vivo glucose metabolism during the 3-5 hour period of hyperinsulinemia at two different plasma insulin levels (30 and 100 μU/ml). The increase in insulin-stimulated glucose metabolism occurred despite a 30-40% decrease in insulin receptor binding to circulating monocytes (20). However, since the monocyte is not a target tissue for insulin-mediated glucose metabolism, studies

examining changes in cellular glucose transport or metabolism could not be performed. Similar results have been reported using the quadruple infusion technique in man (56), but again changes in cellular glucose transport and metabolism could not be evaluated, since circulating erythrocytes were employed and this tissue is also not a target tissue for insulin-mediated glucose metabolism.

V. Clinical significance of hyperinsulinemia

Interest in the effects of chronic in vivo hyperinsulinemia on glucose metabolism has been prompted by a clinical interest in what role hyperinsulinemia might have in insulin resistant states. An inverse relationship between fasting plasma insulin concentration and insulin receptor binding, and a positive correlation between decreased insulin binding and decreased insulin action in vivo, have been observed in insulin resistant states including obesity (1-3) and maturity-onset diabetes (5, 6) in man.

The data discussed above provide strong evidence that hyperinsulinemia per se leads to a decrease in insulin receptor binding (16-20, 56) and suggest that hyperinsulinemia may also play an important role in the decrease in insulin receptor binding observed in insulin resistant states. However, the metabolic consequence of decreased receptor binding has not been directly established. Decreased receptor binding has been shown to correlate with decreased insulin sensitivity in vivo in obese man (1, 2, 3). In obese rodents, a decrease in receptor binding has also been correlated with a decrease in insulin sensitivity in vitro in adipocytes (50) and skeletal muscle (31). However, in addition obese animals also demonstrate a decrease in tissue responsiveness to insulin both

in adipocytes (50, 60) and in skeletal muscle (31). Although hyperinsulinemia may be responsible for the receptor-mediated decrease in insulin sensitivity, what role, if any, hyperinsulinemia plays in the decreased tissue responsiveness to insulin remains to be defined.

The relationship between insulin resistance, hyperinsulinemia, and decreased receptor binding in maturity-onset diabetes is even less clear. Although a decrease in insulin receptor binding has been correlated with a decrease in in vivo insulin sensitivity (5), those diabetics did not manifest fasting hyperinsulinemia. Furthermore, other studies have failed to correlate decreased receptor binding with decreased in vivo insulin sensitivity (6). Thus, what role hyperinsulinemia plays, and the mechanism of insulin resistance, in maturity-onset diabetes is unclear.

Streptozotocin-treated diabetic rats are hypoinsulinemic and demonstrate an increase in both insulin receptor binding and in vitro tissue sensitivity to insulin (51, 52, 54). However, in addition, adipocytes from these animals show a decrease in both basal and maximal insulin-stimulated glucose transport (51, 52) and glucose oxidation (51). In contrast, soleus muscles from similar animals showed only an increase in tissue sensitivity to insulin (54). At least in adipocytes, these hypoinsulinemic animals appear to have a defect in the glucose transport system per se. Similar results have been observed in hypoinsulinemic animals following prolonged fasting (53-55). It has not yet been established whether these "post-receptor" defects are a direct effect, a secondary effect, or independent of decreased circulating insulin levels. Although other explanations are possible, these

findings are compatible with a decrease in circulating insulin levels per se leading to a decrease in cellular glucose metabolism.

In summary, although the acute effects of insulin have been well studied, the effects of prolonged insulin exposure on glucose metabolism are not well defined. The effects of chronic hyperinsulinemia are of importance not only in the understanding of insulin physiology, but also in the understanding of insulin resistance in hyperinsulinemic states.

Methods

Animals

Fifty-five male Sprague Dawley rats (156 ± 3 grams body weight) were maintained in a constant temperature room with a 12 hour light cycle. All studies were performed in the post-absorptive state after a 12-14 hour overnight fast. Animals were allowed free access to drinking water until the start of the study.

Four days prior to study, chronic indwelling catheters (PE-50 polyethylene tubing) were inserted into the jugular vein and carotid artery for infusions and withdrawal of blood samples respectively. Animals were anesthetized with Nembutol (0.08 cc/100 grams body weight, IP) and a vertical midline incision was made to expose the right internal jugular vein and carotid artery. The venous catheter was inserted into the jugular vein and passed via the superior vena cava into the right atrium. The carotid artery catheter was passed to the origin of the right brachiocephalic artery. The catheters were secured in place with 4-0 silk and the distal ends were tunnelled subcutaneously to the dorsum of the neck where they exited via a skin incision. The animals recovered quickly after the surgical procedure and were eating and drinking within 12 hours. Catheters were flushed daily with 0.2 cc of heparinized saline (0.9% saline + 100 U/ml heparin) to maintain patency.

All animals were studied 4-5 days following catheter placement. At the time of study, they were restrained in a plexiglass cage which allowed them to rest comfortably but limited them from moving

about. The catheters exited through a hole in the top of the cage.

Euglycemic Insulin Clamp

To produce sustained physiologic elevations of plasma insulin, a priming infusion of insulin was administered during the first 8 minutes and this was followed by a continuous insulin infusion at the rate of 1.75 μU/gram body weight per minute (Table 1). The continuous insulin infusion was maintained throughout the three hour study period and raised steady-state levels by approximately 30 μU/ml above fasting plasma insulin. The insulin infusion was prepared from regular crystalline porcine insulin (Lilly Co., Indianapolis, Ind.) in 0.9% saline; 300 mg of albumin were added to each 100 ml of normal saline to prevent adherence of the insulin to the glassware and plastic tubing.

During the 3 hour period of hyperinsulinemia, the plasma glucose concentration was maintained constant at the basal fasting glucose level by the infusion of a 5% glucose solution. The glucose infusion is started 4 minutes following initiation of the insulin infusion. The initial glucose infusion was empirically set at 7 μg/gram body weight per minute. At 8 minutes the glucose infusion rate was increased to 10 μg/g·min., and at 11 minutes to 14 μg/g · min. Subsequent alterations in the glucose infusion rate were based on determinations of arterial plasma glucose drawn at 10-15 minute intervals. The first glucose determination was obtained 15 minutes after initiation of insulin infusion. In the initial experiments, the glucose infusion rate was adjusted using

TABLE 1. The Prime Continuous Insulin Infusion Employed During Euglycemic Insulin Clamp

Time (Min.)	Insulin Infusion Rate (μU/g Body Wt. Min.)
0-2	3.36
2-8	2.40
8-180	1.75

a negative feed back formula as previously described by DeFronzo et. al. (23). This formula takes into account changes in the rate of glucose metabolism as well as changes in the size of the glucose pool. After the first few studies all changes in the glucose infusion rate were made empirically based on experience gained from the initial studies.

Plasma insulin concentration was determined in the basal state and at 60, 120, and 180 minutes during insulin infusion. Plasma epinephrine was determined at 180 minutes.

Twenty-three rats served as a control group. These animals were treated similarly to the above group except that they received a 3 hour infusion of normal saline instead of the glucose-insulin infusions. Blood samples for insulin and glucose determinations were obtained as described above.

Computations

Glucose Metabolism (M). Under steady-state conditions of euglycemia, the glucose infusion rate (INF) must be equal to the amount of glucose taken up and metabolized by all tissues and provides a measure of insulin-stimulated glucose metabolism. This assumes that hepatic glucose production (HGP) is complete suppressed. To the extent that HGP is not suppressed, the rate of endogenous glucose production must be added to the glucose infusion rate to determine the actual rate of tissue glucose metabolism. In the present study HGP was not directly determined. These studies are presently

being performed and until completed it should be noted that the rates of glucose metabolism presented in Table 3 may be a slight underestimate of the true rate of glucose metabolism.

During the insulin clamp study, small changes in plasma glucose do occur. These changes represent additions of glucose to, or subtractions from, the glucose space caused by a change in the rate of tissue glucose metabolism. Thus, the infusion rate (INF) must be corrected by a space correction (SC) to arrive at the true rate of glucose metabolism (M). Therefore,

$$M = INF - SC$$

The space correction is given by the formula:

$$SC = \frac{(G_2-G_1) \times 10 \times (0.19 \times \text{body weight})}{(T_2-T_1) \times \text{body weight}}$$

Where (G_2-G_1) is the change in plasma glucose in mg/dl, $(G_2-G_1) \times 10$ is the change in mg/l, (0.19 x body weight) was used to approximate the glucose space, and $(T_2 - T_1)$ is the time interval. In this study glucose metabolism (M) was calculated for 40 minute time intervals and in this case the space correction formula reduces to:

$$SC = (G_2-G_1) \times 0.0475$$

Units for SC are expressed as μg/gram body weight per minute of glucose added or subtracted.

The metabolic clearance rate (MCR) for insulin is calculated as described previously (23).

$$MCR = \frac{\text{insulin infusion rate}}{\text{increase in plasma insulin above basal}}$$

MCR is expressed in ml/gram body weight per minute when the insulin infusion rate is in μU/gram body weight per minute and the increase

in steady-state insulin levels above basal is in μU/ml. This assumes that endogenous insulin secretion remains constant during insulin infusion. To the extent that elevation of plasma insulin leads to a decrease in basal insulin secretion (23), the metabolic clearance rates given in Table 3 may be slightly overestimated.

Statistical analyses were done by the unpaired student's t-test (61). All data are presented as mean ± SE.

Deoxyglucose Transport

Following 180 minutes of insulin-glucose or saline infusion animals were sacrificed. Nembutol (0.2 cc/100 grams body wt.) was administered through the venous catheter and within 10-15 seconds both legs were removed. Soleus muscle was exposed, tendons ligated with 4-0 silk, and excised intact. Muscles were tied taught onto stainless steel rings (3/4 inches diameter), rinsed with 0.9% saline, and placed in glass incubation vials. Incubations were conducted in a shaking water bath at 20°C under a 95% O_2 5% CO_2 atmosphere.

Muscles were initially preincubated for 15 minutes in a 2 mM pyruvate Krebs bicarbonate albumin buffer: Krebs ringer (120 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$ 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4) with 24mM $NaHCO_3$ and bovine serum albumin (2 g/100 cc). Following the 15 minute preincubation period, muscles were removed and the final incubation was carried out for 3 hours in Krebs bicarbonate albumin buffer (pH 7.4). One muscle from each animal was incubated in the absence of insulin, while the other muscle was incubated with insulin at one of the following concentrations: 0.5, 0.75, 1.0,

5.0, 10.0 mg/ml.

Following the 3 hour incubation period, glucose transport was determined by the addition of 0.2 mM ^{14}C-2-deoxy-D-glucose (1 μCi/ incubation vial) (New England Nuclear, Boston, MA) for 15 minutes. Muscles were removed and washed 6 times for 5 minutes in 5 cc of an ice-cold 0.9% NaCl + 500 mg/100cc human serum albumin solution. Sutures were then removed, and the tendons were trimmed. The muscles were dried at 120°C for 24 hours, weighted, and dissolved in 1 ml of 1 N NaOH for 12 hours. Following dilution to 10 ml with distilled water, a 1 ml aliquot was taken for counting.

Analytic Methods

Plasma glucose determinations were made on 50 μl samples, obtained from the carotid artery, using the glucose oxidase method (Yellow Springs Instruments, Model 23A, Yellow Springs, OH). Plasma insulin and epinephrine determinations were performed by Dr. Rosa Hendler. Insulin was measured on pooled samples by radioimmunoassay (62). Epinephrine was measured on blood samples obtained at sacrifice by a radioenzymatic assay (63). ^{14}C-2-DOG radioactivity in dissolved muscles was determined in duplicate following the addition of aquasol (New England Nuclear, Boston, MA) and counting with a Packard spectrometer (Packard Instruments, Model 3320, Palo Alto, CA). ^{14}C-2-DOG uptake is expressed as pmoles of 2-DOG/mg dry wt of tissue·15 min.

Results

Euglycemic Insulin Clamp

Basal plasma insulin concentration in the post-absorptive state prior to infusion of insulin averaged 23 ± 2 μU/ml (Table 2). The steady-state plasma insulin concentration during the insulin clamp study was 55 ± 2 μU/ml (Table 2). Steady-state levels were 54 ± 3 μU/ml at 60 minutes, 55 ± 2 μU/ml at 120 minutes, and 55 ± 2 μU/ml at 180 minutes (Figure 1). The mean increment in plasma insulin concentration during the 180 minute study period was 31 ± 2 μU/ml (Table 2). The metabolic clearance rate of insulin was 0.065 ± 0.007 ml/gram body wt · min. (Table 2).

Basal arterial plasma glucose was 100 ± 1 mg/dl in the post-absorptive state following a 14-16 hour fast, and was maintained at 97 ± 2 mg/dl during the 180 minutes of insulin infusion (Table 2). Mean plasma glucose varied between 91 ± 2 mg/dl and 108 ± 4 mg/dl (Figure 1). The mean coefficient of variation of steady-state plasma glucose was 11.9 ± 1.3% (Table 2).

The amount of glucose metabolized (M) during the 3 hour study period of hyperinsulinemia averaged 15.49 ± 0.80 μg/g gram body wt· min. The rate of glucose metabolism increased rapidly during the first 40 minutes of insulin infusion and remained constant thereafter (Figure 1 and Table 3).

In the control animals, the fasting plasma insulin averaged 22 ± 1 μU/ml and glucose averaged 113 ± 3 mg/dl, and remained unchanged during the 180 minute saline infusion (Table 4). Plasma glucose concentrations were 10-15 mg/dl higher in control animals than in animals receiving insulin infusions (Table 4).

TABLE 2. Summary of Individual Animals During the Euglycemic Insulin Clamp

Rat (Number)	Fasting Plasma Glucose (mg/dl)	Steady-State Plasma Glucose* (mg/dl)	C.V. of Plasma Glucose** (%)	Glucose Metabolism Rate (M) + (μg/g/min.)	Fasting Plasma Insulin (μU/ml)	Steady-State Plasma Insulin++ (μU/ml)	Increment in Plasma Insulin (μU/ml)	Metabolic Clearance Rate of Insulin (ml/g/min.)	Final Plasma Epinephrine (pg/ml)
1	108	117	20.5	13.50	29	61	32	.056	-
2	105	96	17.0	16.31	17	50	33	.053	-
3	91	112	12.7	16.34	36	61	26	.068	-
4	100	121	15.4	14.36	33	42	10	.182	24
5	99	110	15.9	10.91	13	58	45	.039	145
6	104	104	15.6	8.45	18	36	18	.097	447
7	93	95	9.4	14.55	14	41	26	.066	325
8	99	108	13.6	11.62	14	46	32	.055	-
9	85	85	9.2	20.65	-	58	-	-	36
10	100	93	4.2	14.64	21	53	32	.054	134
11	90	84	7.9	18.76	30	57	27	.065	69
12	93	87	7.6	15.71	42	64	22	.081	10
13	93	97	14.8	12.03	27	63	36	.049	52
14	109	108	23.4	9.72	26	60	34	.052	317
15	106	90	7.9	17.68	18	59	41	.043	260
16	103	83	3.7	19.58	29	58	29	.060	193
17	98	97	10.1	14.34	-	61	-	-	551
18	106	90	5.6	18.53	30	48	18	.097	555
19	100	85	8.1	21.67	18	65	47	.037	66
20	106	89	8.2	20.53	22	63	41	.042	27
21	101	81	25.5	-	22	53	31	.057	93
22	108	101	6.6	15.33	26	60	33	.053	188
Mean	100	97	11.9	15.49	23	55	31	.065	194
±SE	±1	±2	±1.3	±0.79	±2	±2	±2	±.007	±42

* Represents mean glucose concentration from 20-180 minutes during insulin clamp.
** Coefficient of variation of steady-state plasma glucose concentration during insulin clamp.
\+ Represents mean rate of glucose metabolism from 20-180 minutes during insulin clamp.
++ Represents mean insulin concentration from 60, 120, and 180 minute samples during insulin clamp.
\- Data not available.

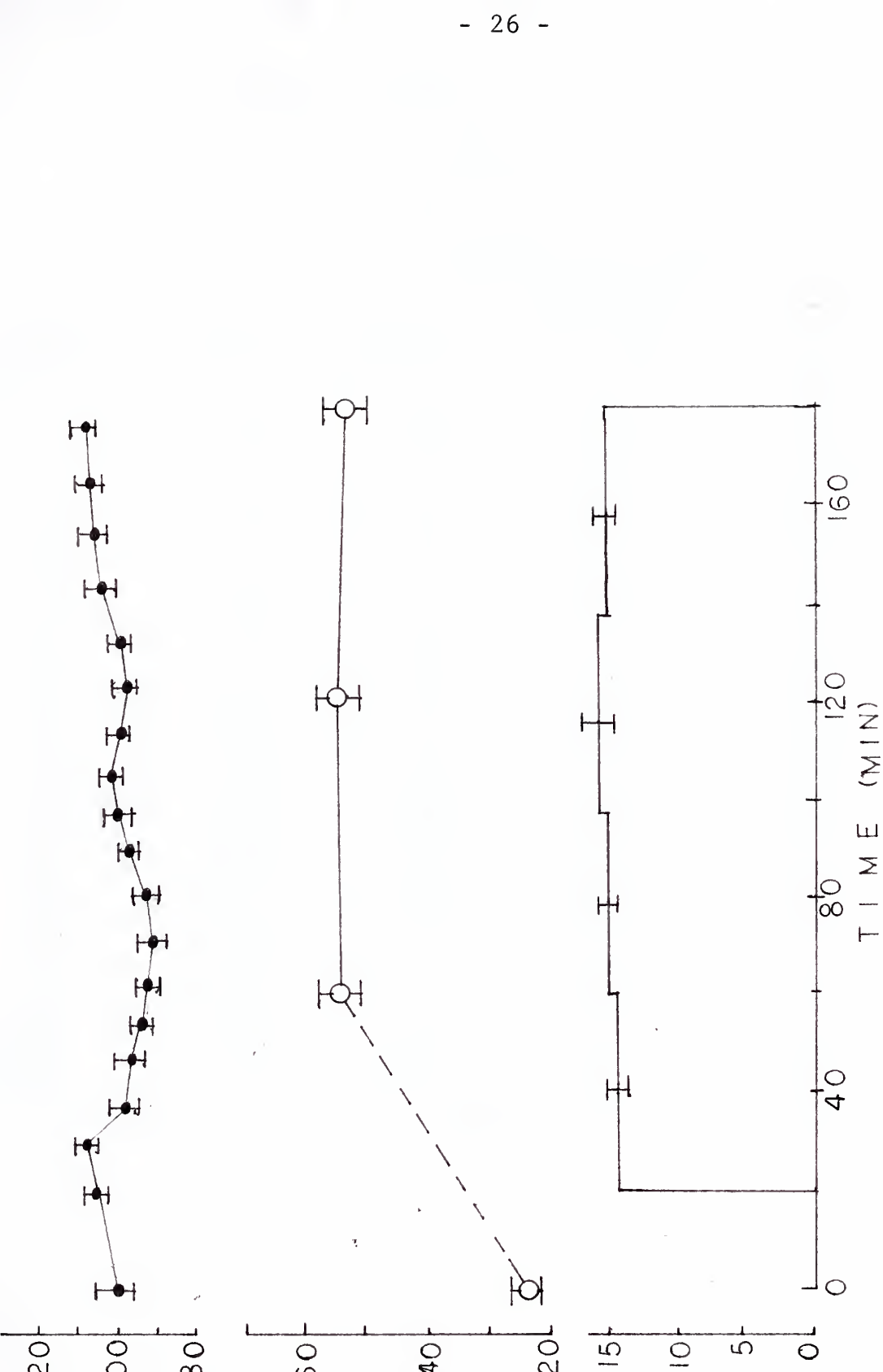

Figure 1. Summary of steady-state plasma glucose and insulin concentrations and glucose metabolism during the euglycemic insulin clamp. The first insulin determination after initiation of the primed insulin infusion was at 60 minutes. The time course of increase to steady-state insulin levels was not studied as indicated by the dotted line. Values for glucose metabolism were calculated for 40 minute intervals starting 20 minutes after initiation of the insulin infusion. All values represents means ± SE.

TABLE 3. Summary of Glucose Metabolism in Individual Animals During Insulin Clamp

Subject	Glucose Metabolism Rate During Insulin Clamp (μg/g/min.) Time (Min.) 20-60	60-100	100-140	140-180	20-180
1	14.89	13.44	13.78	11.88	13.50
2	18.62	16.72	16.47	13.41	16.31
3	14.53	15.98	17.70	17.13	16.34
4	19.44	14.68	12.75	10.55	14.36
5	11.95	11.65	9.57	10.42	10.91
6	8.57	7.81	9.22	8.21	8.45
7	12.73	13.84	15.92	15.69	14.55
8	11.36	11.44	12.88	10.79	11.62
9	17.00	15.81	-	21.69	20.65
10	14.13	14.91	15.04	14.48	14.64
11	16.03	17.67	20.74	20.61	18.76
12	15.28	15.16	15.96	16.48	15.71
13	10.70	12.65	12.47	12.30	12.03
14	9.69	11.41	8.65	9.13	9.72
15	15.76	16.89	17.33	20.73	17.68
16	17.43	18.59	20.06	22.25	19.58
17	14.08	13.95	14.35	14.98	14.34
18	15.73	18.46	19.90	20.04	18.53
19	17.71	25.11	22.18	21.69	21.67
20	19.53	20.79	20.92	20.80	20.53
21	17.07	-	-	-	-
22	14.62	14.18	15.31	17.22	15.33
Mean ±SE	14.64 ±0.98	15.12 ±0.79	15.34 ±1.01	15.44 ±1.00	15.49 ±0.80

TABLE 4. Characteristics of Insulin Infused and Saline Infused Animals*

	Body Wt. (g)	Fasting Plasma Glucose (mg/dl)	Steady-State Plasma Glucose** (mg/dl)	Fasting Plasma Insulin (μU/ml)	Steady-State Plasma Insulin*** (μU/ml)
Saline Controls (n = 23)	151 ± 4	113 ± 3+	109 ± 2++	22 ± 1	21 ± 1
Insulin Infused Animals (n = 22)	161 ± 4	100 ± 1	97 ± 2	23 ± 2	55 ± 2

* All values are expressed as means ± SE.

** Represents mean glucose concentrations from 20-180 minutes.

*** Represents mean insulin concentrations from 60-180 minutes.

+ $P < 0.001$

++ $P < 0.005$

Deoxyglucose Transport

Basal 2-deoxyglucose (2-DOG) transport was increased by 42% in hyperinsulinemic animals (113 ± 7 pmoles/mg day wt · 15 min, n = 21) vs. controls (79 ± 5 pmoles/mg day wt · 15 min, n = 24) ($p < 0.005$). Basal transport in hyperinsulinemic animals (mean plasma insulin concentration = 2.2 ng/ml) was comparable to insulin-stimulated transport in soleus muscles from control animals incubated with 0.75 ng/ml of insulin in vitro.

When soleus muscle from hyperinsulinemic animals was incubated in vitro with progressively increasing concentrations of insulin (Figure 2), insulin-stimulated 2-DOG transport was also increased. The insulin dose response curve in muscles from hyperinsulinemic animals was parallel but raised upwards. The increase in insulin-stimulated 2-DOG transport was statistically significant at insulin concentrations of 0.5 ng/ml ($p < 0.05$) and 5 ng/ml ($p < 0.02$). Although insulin-stimulated 2-DOG transport was also increased at insulin concentrations of 0.75, 1.0, and 10 ng/ml, this difference did not reach statistical significance. When data from the 5 ng/ml and 10 ng/ml points were pooled to represent a maximally-stimulated value, this difference was found to be statistically significant ($p < 0.05$). Dose response curves in muscles from hyperinsulinemic and control animals were similar when basal 2-DOG transport was subtracted from insulin-stimulated 2-DOG transport (Figure 3).

Epinephrine levels prior to sacrifice were somewhat elevated in hyperinsulinemic animals (197 ± 42 pg/ml) vs. controls (100 ± 23 pg/ml). However, this difference was not statistically significant due to the large amount of scatter in both groups. Moreover, there was no correlation between epinephrine levels and basal 2-DOG transport ($r = -0.20$).

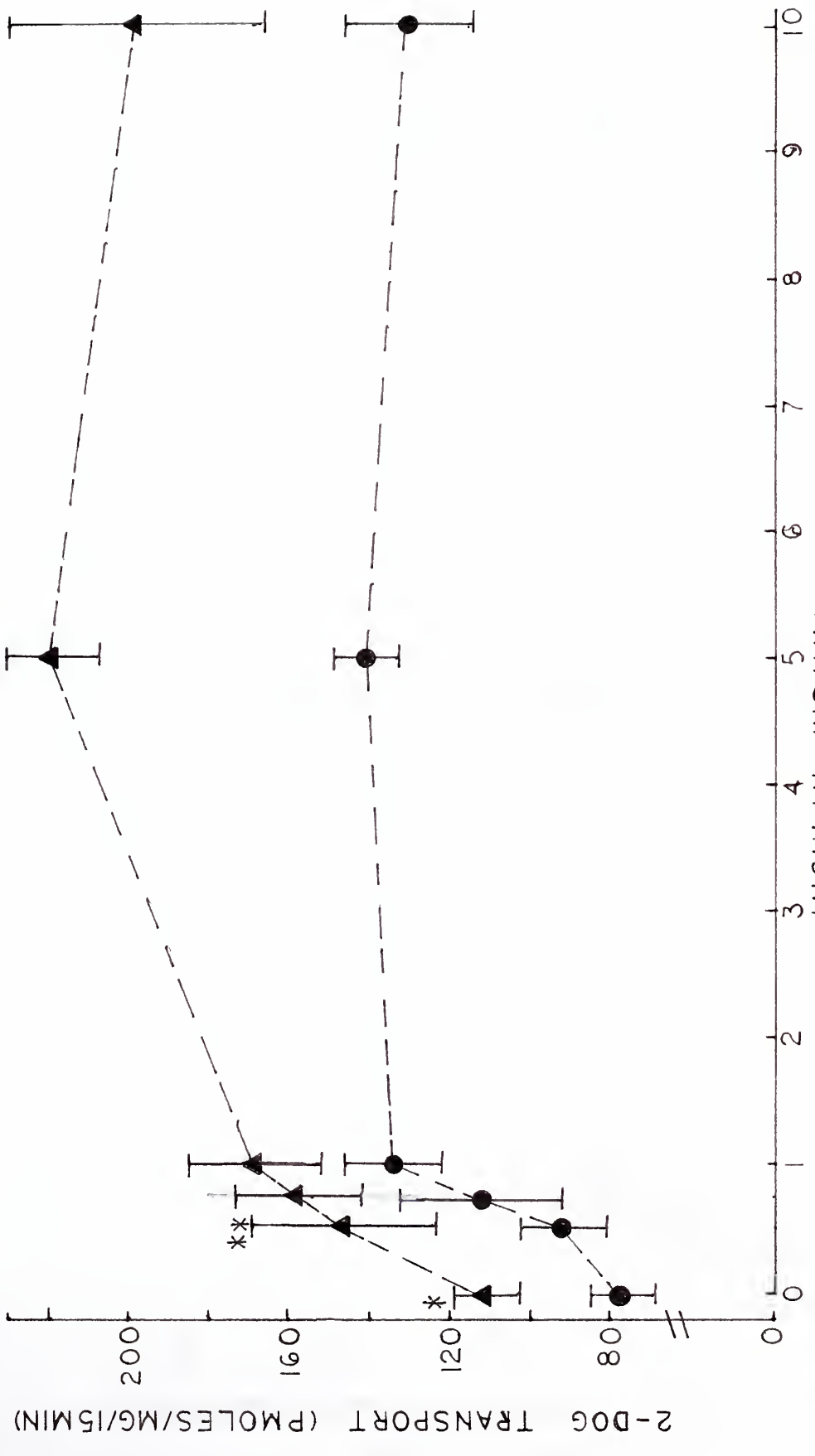

Figure 2. 2-Deoxyglucose (2-DOG) transport in vitro in rat soleus muscle from animals receiving insulin (▲--▲) or saline (●----●) infusions. Insulin was infused at a rate of 1.75 mU/g · min while plasma glucose concentration was maintained constant at the basal level employing the euglycemic insulin clamp technique. In vitro deoxyglucose transport was studied following a 3 hour in vitro incubation with 0-10 ng/ml insulin. 2-DOG uptake is expressed in pmoles of 2-DOG/mg dry weight of soleus muscle · 15 minute assay period. All values are expressed as means ± SE. *$p < 0.005$, **$p < 0.05$, ***$p < 0.02$.

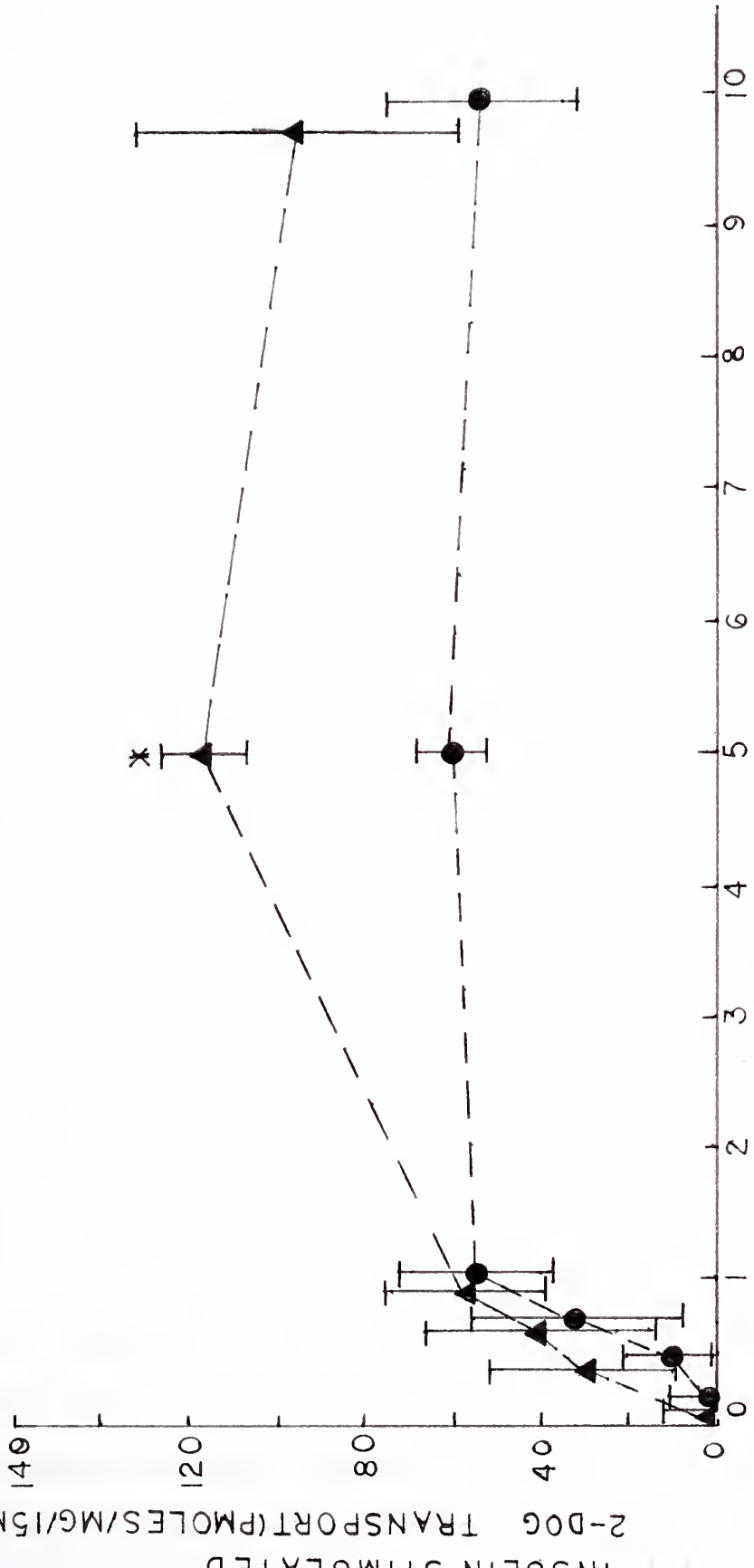

Figure 3. Insulin-stimulated 2-deoxyglucose (2-DOG) transport in vitro in rat soleus muscles from animals receiving insulin (▲--▲) or saline (●--●) infusions. Basal transport, in the absence of added in vitro insulin, has been subtracted from all values. Values represent insulin-stimulated transport above basal. All values are expressed as mean ± SE. *$p < 0.05$.

Discussion

The present studies have examined the effects of prolonged (3 hours) hyperinsulinemia on in vivo glucose metabolism and in vitro glucose transport in rat soleus muscle. Modification of the euglycemic insulin clamp technique (23) to the rat has enabled us to perform these studies under conditions where changes in plasma glucose concentration are prevented.

Studies by Olefsky et. al. (21, 22) have suggested that chronic in vivo hyperinsulinemia leads to an increase in basal and maximally insulin-stimulated glucose transport in rat adipocytes. However, earlier studies (19) by the same group failed to demonstrate this effect in a similar model. In these earlier studies, an increase in glucose transport may have been prevented by the failure to maintain euglycemia in these hyperinsulinemic animals. Both changes in plasma glucose and counter-regulatory hormones have been shown to effect tissue glucose metabolism (64-70). Alternatively, an increase in glucose transport may have already been present in the control animals, which were both hyperinsulinemic (29 ± 3 μU/ml) and relatively hyperglycemic (152 ± 5 mg/dl). An increase in glucose transport in the control group may have masked the difference between the two groups.

The present experiments demonstrate an increase in skeletal muscle glucose transport following 3 hours of physiologic hyperinsulinemia in vivo. This increase in glucose transport was observed both in the basal state and after stimulation with insulin in vitro (Figure 2). Similarly, an increase in maximal insulin-stimulated glucose transport was also observed, although data from the 5 ng/ml and 10 ng/ml points had to be pooled to establish

statistical significance. On the other hand, insulin stimulation of glucose transport above basal was unchanged (Figure 3). Thus, in vivo hyperinsulinemia causes an activation of basal glucose transport but appears to have no effect on the ability of the soleus muscle to respond to additional insulin in vitro.

It could be argued that a carry over of insulin from the in vivo insulin clamp study into the in vitro transport assay would account for an increase in basal glucose transport. This possibility seems unlikely for the following reasons. 1) Several steps are involved which would dilute out insulin from the in vivo study, including washing the muscles, a 15 minute preincubation, and a 3 hour incubation in insulin free buffer prior to assay. Previous studies have also shown that washing procedures are effective in removing in vivo insulin which has stuck to the plasma membrane before assaying for insulin binding (71). 2) Physiologically, insulin carried over from the in vivo study would be additive with insulin added during the in vitro study and would shift the dose response curve to the left. However, maximally stimulated glucose transport would remain the same and this was not the case.

We also considered the possibility that an increase in basal and insulin-stimulated glucose transport could be attributed to the ability of insulin in the plasma during the insulin clamp study to have access to a compartment which was not accessible during in vitro exposure to insulin. This seems unlikely since muscles were incubated with insulin for 3 hours in vitro and diffusion of insulin into rat soleus muscle does not appear to be rate-limiting under these conditions (26, 31). One would expect equilibration of buffer and interstitial insulin concentrations during the 3 hour incubation.

Persistent activation of the glucose transport system by in vivo exposure to insulin appears to be the most likely explanation for the increase in basal 2-DOG transport observed in vitro. Basal transport 3 hours after exposure to 55 μU/ml (2.2 ng/ml) of insulin in vivo was 40% greater than in control animals, but was less than that observed in muscles from control animals exposed to an equivalent concentration of insulin (2.2 ng/ml) in vitro. This finding may reflect a time-dependent deactivation of insulin-stimulated glucose transport. Basal transport immediately following exposure to insulin in vivo may in fact have been similar to transport observed with an equivalent concentration of insulin in vivo.

In this regard, the time course of deactivation of insulin-stimulated glucose transport has been studied in the rat adipocyte following a 60 minute in vitro exposure to 1 ng/ml of insulin (36). Deactivation of insulin-stimulated 3-O-MG transport had a $t_{\frac{1}{2}}$ of 43 minutes at 37°C and 73 minutes at 24°C in the presence of 1 mM glucose. Persistant activation of glucose transport continued despite a reversal of insulin receptor binding (36). Furthermore, insulin-stimulation of glucose transport persisted unchanged when deactivation was studied for 2 hours in the absence of both insulin and glucose (36). Thus, these results are consistent with the present experiments which demonstrate a persistent activation of 2-DOG transport in rat soleus muscles 3 hours after in vivo exposure to insulin.

In addition to an increase in basal 2-DOG transport, insulin-stimulated 2-DOG transport was also increased following 3 hours of in vivo hyperinsulinemia. An increase in maximal stimulated transport must represent activation or recruitment of new transport

units from a compartment not accessible to a 3 hour exposure to insulin in vitro. If in vivo exposure to insulin caused activation of transport units from a fixed compartment, then further in vitro insulin-stimulation would not have resulted in the apparent increase in maximal transport observed in these studies.

The ability of combined in vivo hyperinsulinemia and in vitro insulin to produce a greater activation of glucose transport than normoinsulinemia in vivo plus in vitro insulin could have several explanations. This effect may be time-dependent and require 6 hours of exposure to increased insulin concentrations, or alternatively an additional 3 hours may be required following the 3 hour in vivo exposure to insulin for the enhancing effect on glucose transport to develop. If either of these explanations were the case, one would expect that 6 hours of exposure to either in vivo or in vitro insulin alone should produce a similar effect. While these possibilities cannot be excluded, we think that it is most likely that some effect of in vivo as opposed to in vitro exposure to insulin contributes to the observed increase in 2-DOG transport. Perhaps the acceleration of glucose metabolism achieved during in vivo hyperinsulinemia is involved in this effect.

We also considered the possibility that other in vivo factors unrelated to insulin might have an effect which persists through the 3 hour in vitro incubation prior to assay of glucose transport. Since the plasma glucose concentration was maintained constant at the basal level, it is unlikely that changes in glucose concentration could have contributed to the increase in 2-DOG transport observed. Similarly, although fasting levels of plasma glucose were slightly lower in hyperinsulinemic animals (100 vs 113 mg/dl)

we would not expect that this small difference in plasma glucose would account for the increase in 2-DOG transport observed, especially since lower plasma glucose in the normal range has been correlated with a lower rate of in vivo glucose metabolism. (R. A. DeFronzo, personal communication). It is also unlikely that changes in counter-regulatory hormones contributed to the increase in 2-DOG transport observed since plasma epinephrine levels were not significantly elevated in hyperinsulinemic animals and there was no correlation between epinephrine levels and basal transport. Furthermore, increases in counter-regulatory hormones, e.g., epinephrine, would have been expected to have an opposite effect, namely a decrease in cellular glucose transport, based on previous in vitro and in vivo studies (64-70).

Skeletal muscle has been shown to be the principle tissue responsible for glucose utilization during euglycemic hyperinsulemia (23, 72). In the present study, an increase in insulin-stimulated 2-DOG transport by soleus muscle was documented following a combined exposure to 3 hours each of in vivo and in vitro insulin. Nonetheless, the rate of glucose metabolism during the 140-180 minute period of the insulin clamp study (15.44 μg/g/min.) was similar to that observed during the 20-60 minute period (14.64 μg/g/min.). Several explanations may account for this "apparent discrepancy".

The failure to observe an increase in glucose metabolism during the 3 hour insulin clamp study may indicate that the increase in glucose transport is time-dependent and a 6 hour combined in vivo and in vitro exposure to insulin is required to produce the increase in 2-DOG transport observed. Alternatively,

an increase in glucose metabolism in vivo may have been prevented by other changes which led to a counter-balancing decrease in peripheral glucose utilization, but whose effects were dissipated during the 3 hour incubation period prior to assay of 2-DOG transport. In this regard, a concomitant reversible decrease in receptor binding could off-set an increase in glucose transport and prevent an increase in in vivo glucose utilization. Although insulin binding to soleus muscle was not examined in the present study, decreased insulin binding to monocytes has been demonstrated by Soman and DeFronzo (20) following a 3 hour euglycemic insulin clamp at 100 μ U/ml in man. In addition, regeneration of insulin receptor binding in adipocytes within 3 hours after chronic in vivo hyperinsulinemia has been demonstrated by Kobayaski and Olefsky (19). If these results are applicable to soleus muscle, then a decrease in insulin receptor binding may have counter-balanced an increase in the glucose transport system and prevented an increase in in vivo glucose utilization. Alternatively, an increase in plasma epinephrine during the last period of the insulin clamp could prevent an increase in glucose metabolism, but have been dissipated during the 3 hour incubation prior to assay of 2-DOG transport. This seems unlikely since epinephrine levels were not significantly elevated and there was no correlation between epinephrine levels at sacrifice and the rate of glucose metabolism during the final period of the insulin clamp study.

Recently, Cushman et. al. (41, 42) have proposed that insulin activation of glucose transport involves the recruitment of glucose transport units from an intracellular microsomal compartment

into the plasma membrane. Based on the present experiments, an extension of this model can be proposed. Prolonged in vivo insulin stimulation may lead to the recruitment of transport units from an intracellular pool and the persistent activation of these units in the plasma membrane, and a subsequent regeneration of the microsomal pool, so that acute insulin-stimulation will again elicit a full augmentation of glucose transport and the additive effect will produce an increase in maximal glucose transport. The time-dependence of the increase in maximal glucose transport postulated in this discussion may in fact represent the synthesis of new glucose transport units.

In conclusion: in vivo hyperinsulinemia achieved by the euglycemic insulin clamp technique results in 1) prolonged activation of glucose transport in skeletal muscle manifest as an increase in basal transport 3 hours after in vivo exposure to insulin and 2) an augmentation of insulin-stimulated glucose transport.

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