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CAPILLARY-MUSCLE FIBER MORPHOMETRY IN FEMALE DIABETIC & IMPAIRED GLUCOSE TOLERANT RATS

BY

ANNA E. ENGEL

A thesis submitted in conformity with the requirements for the degree of Master of Science Graduate Department of Community Health University of Toronto 1998

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Capillary-muscle fiber morphometry in female diabetic & impaired glucose tolerant rats.
MSc. Thesis 1998 by Anna E. Engel, Department of Community Health, University of Toronto, Toronto, Ontario M5S 1A1.

Muscle morphology was investigated in diabetic (D; n=8) and impaired glucose tolerant (IGT; n=7) female rats. Size changes were assessed by measuring fiber area (FA), and perimeter (P). Capillary supply was quantified using number of capillary contacts (CC); individual capillary:fiber ratio (C:Fi); FA/CC; and P/C:Fi. FA and P were significantly (p<.05) decreased in D and IGT rats compared to the controls. The capillary supply was significantly (p<.05) reduced in the D and IGT groups compared to the controls. The decrease in fiber area in Type I and II fibers was disproportionate to the decrease observed in the capillary supply. FA/CC (µm²) in D (I:757±35.5; II:405±15.0) and IGT (I:780±29; II:538±28) rats were significantly reduced versus controls (C1 I:1336±40.5; II:776±28; C2 I:1033±28; II:641±18), indicating a decrease in diffusion distance from the capillary to muscle fiber. In contrast to FA, muscle fiber perimeter was maintained in relation to the capillary supply, such that P/C:Fi (µm) was not significantly different between the four groups. The data suggest that: 1) decreased diffusion distance may be an adaptive response to optimize the delivery of hormones and 2) the maintenance of perimeter in relation to capillary supply may be related to diabetes-induced changes in insulin responsiveness.
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Another key individual was Dr. Marius Locke, whom I thank for all of his time and encouragement. My thanks also go to Kirby Pilch, whose assistance during the glucose tolerance tests was greatly appreciated. As well, I would like to thank two doctoral students, Heather Smith and Russell Hepple, who took the time to introduce me to the image analysis software. Lastly of special note is Mr. Mike DeRocher, who always came to the rescue when there were computer problems - thank you Mike!
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Glossary of Terms

CC  Capillary contacts. A quantitative description of the absolute number of capillaries appearing around the perimeter of individual muscle fibers. An increase in CC represents a measure of new capillary development, but a decrease in CC indicates a loss in the absolute number of capillaries supplying individual muscle fibers.

CD  Capillary density. This relative measurement is derived by counting the number of capillary profiles (cross sections) per mm² in a transverse section of muscle. It describes the capillary supply of the muscle as a whole, and provides little information on the capillary supply of individual fibers. CD is sensitive to perturbations in muscle fiber size.

C:F  Capillary-to-fiber ratio. This measurement negates the effect of changes in fiber size, as it is derived by dividing the capillary density (CD) by the fiber density for the same muscle cross section. It is a population-based descriptor. It measures the global ratio of the number of capillaries relative to the number of muscle fibers, but ignores diffusion distance.

C:Fi  Individual capillary-to-fiber ratio. This index provides a quantitative description of capillaries for an individual muscle fiber. Because it accounts for the sharing factor (see below), it provides a standard measurement of the number of capillaries on the perimeter of a muscle fiber in relation to the number of fibers in contact with each capillary.

FA/CC  Fiber area supplied by each capillary contact. FA/CC is derived as the quotient of the cross-sectional area of a fiber (FA), and the number of capillary contacts (CC) situated around the perimeter of the fiber. This measurement reflects alterations in both the capillary supply and the size of the individual muscle fiber. FA/CC does not account for the sharing factor.

GLUT 1 One isoform of a family of tissue-specific glycoproteins, called glucose transporters. It is responsible for the facilitated diffusion of glucose across plasma membranes in a basal state (non-insulin-stimulated). It is regulated by glucose, and may or may not respond to insulin. It is widely distributed in mammalian tissue, but its expression in skeletal muscle appears to be restricted to the plasma membrane.
GLUT 4 Another isoform of the glucose transporter family, expressed in insulin-sensitive tissues, namely skeletal and heart muscle, and adipose tissue. It is present in the plasma membrane of muscle, but exists in a higher yield in the intracellular membrane fraction of muscle cells. Responds to insulin, and is therefore important in maintaining glucose homeostasis. GLUT 4 protein is also regulated by levels of glycemia.

TYPE 1 A type of diabetes characterized by insulin deficiency (hypoinsulinemia) and high levels of blood glucose (hyperglycemia). Also referred to as insulin-dependent diabetes mellitus (IDDM).

IGT Impaired glucose tolerance. Defective metabolism of glucose. Measured by following blood glucose concentrations over time after an administration of a glucose load.

IMGU Insulin-mediated glucose uptake. Represents one mechanism of glucose uptake into skeletal muscle, whereby insulin stimulates glucose uptake by increasing the cellular permeability to glucose via recruitment and activation of glucose transporter proteins (see above).

NIMGU Non-insulin-mediated glucose uptake. Represents a second mechanism of glucose uptake into both non-insulin sensitive (central nervous system, peripheral nerves, splanchnic bed, endothelial cells, blood cells) and insulin sensitive tissues. Occurs in the absence of insulin, and involves facilitative diffusion of glucose by GLUT 1 (see above). Designated as basal glucose uptake.

P Perimeter of a transversely sectioned muscle fiber. Represents a 2-dimensional estimate of the fiber surface area (3-dimensional variable).

P/C:Fi Perimeter-to-capillary supply index. It is a measure which describes the capillary supply relative to the perimeter of an individual muscle fiber. It is calculated as the quotient of the fiber perimeter (P) and the individual capillary-to-fiber ratio (C:Fi) for each muscle fiber. The P/C index provides a means of quantitating alterations in carrier- or receptor-mediated aspects of blood tissue exchange.

SF Shape factor. SF is a representation of fiber area in relation to fiber perimeter, FA/P. Proportional changes in area and perimeter will result in no change in fiber shape.

STZ Streptozotocin. A cytotoxic chemical which selectively destroys the ß cells of the islet of Langerhans in the pancreas.
Glucose tolerance is the body's response to the influx of glucose from the diet, or in the case of a glucose tolerance test (GTT), the body's response following an oral glucose load of 50 to 100g after an overnight fast (Linder, 1991). Glucose tolerance represents a continuum ranging from the normal state to the pathological state, e.g., Type I diabetes or severe impaired glucose tolerance. The regulation of plasma glucose concentration by insulin following a glucose challenge is a dynamic process, which involves a coordination between the amount of insulin secreted and the subsequent action of insulin to stimulate glucose disposal in target tissues (Kohrt and Holloszy, 1995). In some cases of IGT, the insulin response is lower than normal, whereas in other cases of IGT, the insulin response is normal or higher. The latter represents a disorder of insulin action, and is associated with hyperinsulinemia. Thus, insulin deficiency and/or insulin resistance are important factors in the development of impaired glucose tolerance (Yki-Jarvinen, 1990; Matsumoto et al., 1997). Type 1 diabetes is associated with a deficiency in insulin production (Ignatavicius and Bayne, 1991) and severe glucose intolerance (Zeman, 1991). When insulin is absent, the cells are unable to metabolize glucose effectively (Zeman, 1991). With glucose transport into most cells at about one fourth of normal (Guyton, 1966), the cells are in a state of starvation, while an excess of glucose is present in the blood (hyperglycemia) (Ignatavivius and Bayne, 1991).

Skeletal muscle is the major site of glucose uptake (Krotkiewski, 1994; Kohrt and Holloszy, 1995). Insulin and glucose uptake are determined by their delivery via capillaries (Hespel et al., 1986; Lillioja et al., 1987; Holmang et al., 1992; Baron, 1996; Utrainen et al., 1996). Theoretically, the more capillaries supplying a muscle fiber, the better the potential delivery of insulin and glucose (Krogh, 1919).
However, the capillary supply to individual skeletal muscle fibers and the functional implications of this parameter during impaired glucose tolerance (IGT) and Type I diabetes have rarely been described. Obesity and/or body mass have been the main focus of studies investigating muscle capillary fiber morphology during IGT. In addition, the structural changes in the capillary vasculature during Type I diabetes have largely been limited to hemodynamic disturbances, i.e., thickening of the capillary basement membrane. Of the studies which have reported on muscle capillary-fiber morphology during Type I diabetes, much of the data regarding the capillary supply has been described by morphometric estimates, which are insensitive to perturbations in fiber size, ignore diffusion distance completely, or provide a global index of capillarization, and therefore, yield little information on the capillary supply and the diffusion characteristics of individual muscle fibers. More recently, measurements have been developed to quantitate the capillary supply to individual muscle fibers to reflect alterations in the capillary supply in relation to changes in muscle fiber area, and to describe the capillary supply in terms of the perimeter of the muscle fiber as an index of the surface area available for exchange. These indices, then, may provide significant information for interpreting the effects of an altered capillary-fiber morphometry in relation to insulin delivery and insulin receptor alterations.

Furthermore, much of the previous data in the literature regarding the capillary supply of skeletal muscle during IGT and diabetes has pertained to males. Gender differences are clearly apparent with respect to many factors related to carbohydrate metabolism; these differences have been attributed to sex hormones, i.e., estrogens and androgens. For example, women have substantially smaller muscle fibers than men (Zumstein et al., 1983; Brooks et al., 1996), and estrogen has been reported to play an inhibitory role in the development of Type II muscle fibers (Suzuki and Yamamuro, 1985).

Importantly, variations in the microvasculature are also evident between females and males. The basement membrane of muscle capillaries thickens with age, but at a faster rate in males, resulting in the thickness of the capillary basement membranes of
males being significantly greater than that of females (Williamson and Kilo, 1976). Holmang et al. (1990) reported that capillary density is diminished in female rats after androgen administration. With testosterone treatment, capillary density and blood flow in muscle are diminished in female rats (Bjorntorp, 1995). There is also considerable evidence that estrogen exerts direct effects on the vasculature. For example, estrogen has been demonstrated to diminish vascular constriction, in part, by stimulating the release or production of vasodilators, such as nitric oxide in the endothelial cell (Sowers, 1998). Lieberman et al. (1994) investigated the effect of estrogen replacement therapy on endothelium-dependent vasodilation in post-menopausal women, and found that sustained (9 weeks) estrogen replacement therapy improved endothelium-dependent vasodilation in post-menopausal women.

Finally, distinct differences between males and females with respect to the susceptibility and prevalence of chemical diabetes have been documented in the literature. One hundred percent of male mice given multiple low doses of injections of streptozotocin (STZ) developed severe and permanent insulin dependent hyperglycemia, but less than fifteen percent of the females treated identically developed the syndrome (Paik et al., 1980). Rossini et al. (1978) demonstrated that testosterone administration to female mice lowered their sensitivity to the diabetogenic effect of STZ, and castration of male mice lowered their sensitivity to STZ-induced diabetes. Paik et al. (1982) examined the effects of both androgens and estrogens in mice which have been shown to be highly susceptible to STZ-induced diabetes. They found that estrogen administration to males completely suppressed their susceptibility to diabetes, while androgen administration to female mice made them as highly susceptible to diabetes as males (Paik et al., 1982). More recently, Shi et al. (1994) reported that the increased incidence of diabetes seen in ovariectomized rats was abolished by estrogen treatment. Thus, disparities between females and males, suggests that sex steroid hormones, such as estrogens and androgens, have a significant influence on carbohydrate metabolism.

Taken together, the impetus for the current study was the lack of information regarding muscle capillary fiber morphology in females during altered states of glucose
metabolism. Therefore, the current study was undertaken to investigate the capillary supply of individual muscle fibers in female rats during conditions of Type I diabetes and IGT. Given the effect of female sex hormones on capillary and muscle fiber geometry and blood flow, it was hypothesized that females with Type I diabetes and IGT would demonstrate changes in the capillary supply to skeletal muscle fibers, with IGT females demonstrating less severe changes.

1.2 DIABETIC MICROANGIOPATHY: THE HEMODYNAMIC HYPOTHESIS

Diabetes mellitus, a serious and common metabolic disease (Stryer, 1988), represents a significant risk factor for microangiopathy. Diabetic microangiopathy affects arterioles, venules, and in particular capillaries (Ditzel, 1976).

Diabetic microangiopathy is a chronic complication of hyperglycemia (Zeman, 1991; Tooke, 1995). The effects of the disease on the small vessels of the retinal and glomerular microcirculation are well established (Ditzel, 1976; Zatz and Brenner, 1986). Although retinopathy and nephropathy constitute two important clinical symptoms of diabetic microangiopathy, diabetes produces numerous degenerative changes in other regional capillary networks. A narrowing or complete occlusion of capillary lumens has been reported in several peripheral tissues, including the extremities, skin and subcutaneous tissue, skeletal muscle, myocardium, and nervous system (Zatz and Brenner, 1986). Alterations to the capillary basement membrane in muscle may be the earliest means of diagnosing diabetes (Bradley, 1975).

It has been postulated that the pathogenesis of diabetic microangiopathy originates from early hemodynamic disturbances, followed by a period of structural adaptation and remodeling of the microvasculature, ultimately leading to microvascular failure (Parving, et al., 1983; Tooke, 1995). Early insulin-dependent diabetes is characterized by an increased blood flow in the renal, retinal, and peripheral
microcirculation. This state of generalized capillary hyperperfusion appears to be related to a lack of glycemic control and is largely reversible if normal blood glucose values can be achieved (Zatz and Brenner, 1986; Tooke, 1993; 1994). The increased flow is the result of a reduced vascular resistance, mainly precapillary in nature, with an increased hydrostatic pressure (Parving et al., 1983). Capillary hypertension, in turn, results in an enhanced leakage of plasma proteins through, as well as their deposition into, the walls of the arterioles and capillaries (Parving et al., 1983). The increased flux of plasma proteins (IgG and albumin) into the arteriolar and capillary wall is a decisive, initial event in the morphogenesis of microvascular sclerosis, i.e., arteriolar hyalinosis and capillary basement membrane thickening (Parving, et al., 1983; Tooke, 1995; Fagrell and Intaglietta, 1997). Over time, microvascular sclerosis, limits the capacity of the microvasculature to dilate in response to the demands of increased flow (impaired vasodilation) and interferes with the process of autoregulation (Bohlen and Niggl, 1979; Tooke 1993, 1994, 1995). In contrast to an early occurring hyperemia, insulin-dependent diabetes of long duration is characterized by underperfusion (Tooke, 1993; Williamson et al., 1996), with the progressive sclerosis often inducing microvascular occlusion (Parving, et al., 1983). Although capillary basement membrane thickening is generally accepted as the ultrastructural hallmark of diabetic microangiopathy (Williamson and Kilo, 1976), it is probably not the rate-limiting event in the transcapillary transport of hydrophilic substances in diabetic subjects (Leinonen et al., 1982). Therefore, the microvascular hemodynamic changes play a role in the pathogenesis of diabetic microangiopathy, and together constitute what is referred to as the “hemodynamic hypothesis” (Tooke, 1993, 1994).
1.3 MORPHOMETRIC ANALYSIS OF CAPILLARIES

Historically, most data used to quantitate the capillary supply of skeletal muscle has been based on "global" estimates of various structural aspects of the capillary bed, e.g., capillary density (CD), i.e., the number of capillary cross-sections per mm$^2$ in a transverse section of muscle (Hudlicka, et al., 1992; Mathieu-Costello, 1993; Plyley, 1990). CD is strongly influenced by changes in fiber size, which makes it a useful index when calculating changes in the maximal diffusion distance, i.e., the average distance to the center of a fiber that nutrients and metabolites must diffuse (Mathieu-Costello, 1993; Plyley, 1990). However, being sensitive to any perturbation in fiber size, means that changes in CD may result from any process which alters fibre size, including animal growth or wasting, experimental procedure, or tissue preparation, in addition to changes in the actual number of capillaries supplying the muscle. (Mathieu-Costello, 1993).

A second global quantitative description of muscle capillarization, is the capillary-to-fiber (C:F) ratio (Mathieu-Costello, 1993). Unlike CD, the CF corrects for changes in fiber size by quantitating the capillary density in terms of the fiber density (i.e., CF= CD/FD) for the same muscle cross-section (Plyley, 1990). While the C:F ratio is not affected by changes in fiber size, it cannot account for changes in diffusion distance (Plyley, 1990; Mathieu-Costello, 1993). Similar to CD, the C:F ratio is more of a global index of capillarization and yields little information on the capillary supply of individual muscle fibers (Plyley, 1990).

Plyley and Groom (1975) proposed the concept of capillary supply to an individual muscle fiber by measuring the sharing factor (SF). SF represents the number of fibers being supplied (shared) by a given capillary (Mathieu-Costello, 1993), and is related to the number of capillary contacts (CC) and the C:F ratio by the equation: SF=CC/C:F (Hepple, 1997). The sharing of capillaries by two or more muscle fibers, except at the juncture between the edge of a fascicle and a single fiber, emphasizes the two-dimensional geometric arrangement between capillaries and muscle fibers. Alterations in any aspect of the capillary-fiber arrangement (e.g., SF), as might occur
during angiogenesis or capillary degeneration, has implications for the size of the domain supplied by a given capillary (Hepple, 1997), and therefore, will affect blood-tissue exchange. However, others have disagreed, suggesting that SF is relatively insensitive to adaptive changes (Hudlicka et al., 1992).

Another index used to describe the capillary supply is the fiber area supplied by each capillary contact (i.e., FA/CC). Since FA/CC incorporates both the capillary supply and the fibre area of individual fibers (Hepple, 1997), it represents an ideal quantitation of capillary supply. For example, changes in CC represents a measure of new capillary development (angiogenesis), and changes in FA/CC reflect alterations in the capillary supply itself and/or fiber size, and therefore, accounts for changes in the diffusion distance (Plyley, 1990). A decrease in FA/CC implies “more” capillaries supply a given fiber volume or area, which in turn, suggests a decrease in diffusion distance. Conversely, an increase in FA/CC suggests an increase in diffusion distance as “fewer” capillaries are now supplying a given fiber area. A reduced mitochondrial density in fast-glycolytic fiber of diabetic rats has been reported (Chao et al., 1976), which would suggest that diffusion distance may be important in describing the capillary supply of individual muscle fibres, and therefore, an area-based index such as FA/CC may be important. Although it appears essential that one must use an index which accounts for diffusion distance, it is not directly evident whether the critical distance is to the center of the muscle fiber, or to the most distant point between capillaries along the periphery of a fiber (Saltin and Gollnick, 1983). In otherwords, since mitochondrial density is largely subsarcolemma, with fewer mitochondria being found centrally, (Saltin and Gollnick, 1983; Brooks et al., 1996), it may be more appropriate in terms of substrate uptake to be concerned with an index that is sensitive to changes in fibre size in terms of fibre perimeter. For instance, glucose transport into a muscle cell occurs via the process of facilitated diffusion, i.e., glucose is transported through the membrane by means of specialized glucose transporters, which are activated when insulin binds to receptors on the muscle cell (Guyton, 1966; Kahn and Folli, 1993; Bjorntorp, 1995). Since insulin and glucose are dependent upon receptor- and transporter-mediated events respectively, a
better representation of the capillary supply might be given on the basis of fiber perimeter rather than fibre area (Hepple, 1997).

Hepple (1997) suggested a new measurement to describe the capillary supply of individual muscle fibers, the capillary-to-fiber perimeter exchange (CEPE) index, which provides a means of quantitating potential alterations in any carrier- or receptor-mediated aspect of blood-tissue exchange between the capillaries and muscle fibers. The CEPE is a two-dimensional measurement which describes the capillary supply in relation to the perimeter (surface area in three dimensions) of the muscle fibre. Therefore, this index may well be useful in assessing the effects of altered muscle fiber-capillary morphometry in relation to alterations in glucose transport and insulin binding to skeletal muscle fibres (Hepple, 1997).

To appreciate the limits and application of the various indices utilized in the morphometric analysis of capillaries, it is important to recognize the physical principles that affect the size of the capillary bed, and therefore the validity and accuracy of such indexes.

### 1.4 MUSCLE CAPILLARY-FIBER ARCHITECTURE

On the whole, the capillaries in skeletal muscle are oriented parallel to the fiber axis, which allows for materials to diffuse from capillary blood to the muscle fibers (Hudlicka et al., 1992; Brooks et al., 1996). When sectioned perpendicular to the long axis of the fibers, the appearance of the section is a mosaic of small ellipsoids (capillaries) interspersed amongst large ellipsoids (muscle fibers). Because of this particular arrangement, CD reflects diffusion distance, and as such will be directly affected by the changes in the diameter of muscle fibers (Brooks et al., 1996); in most situations where a change in capillary supply has been reported, some change in mean fiber size has also been evident (Hudlicka et al., 1992). Further, the process of diffusion occurs not only along a line joining adjacent capillaries, but throughout the surrounding tissue (Hudlicka et al., 1992). Therefore, during muscle hypertrophy, the capillaries are “pushed” further
apart, thereby reducing the capillary density and increasing the diffusion distance. Likewise during atrophy, capillaries are “shifted” closer together, which increases CD and decreases diffusion distance (Brooks et al., 1996; Hudlicka et al., 1992).

In skeletal muscle then, CD is inversely related to fiber diameter (Hudlicka et al., 1992). This is further illustrated by the fact that the smaller, slow-twitch muscle fibers have a higher CD than the larger fast-twitch glycolytic fibers, which have a lower capillary supply (Hudlicka, 1996; Hudlicka et al., 1992). Specifically, the mean diameter of the red (slow-twitch) muscle fibers is only 50-60% of that observed in the white (fast-twitch) fibers, but the ratio of capillary number to fiber number is larger (Holmang et al., 1992).

### 1.5 ANGIOGENESIS

Angiogenesis is the development of new capillaries from pre-existing blood vessels (Pintucci, et al., 1996), and can be induced via a number of physiological and pathological conditions.

Long-term exposure to hypoxia has been cited as a key factor for capillary growth in skeletal muscle. Further, it is believed that hypoxia increases the activity of the oxidative enzymes and the volume density of mitochondria in the muscle fibers (Saltin and Gollnick, 1983; Hudlicka et al., 1992, 1996). While hypoxia may be an important trigger for capillary proliferation, a higher capillary density, in circumstances of low oxygen, has been attributed to atrophy of the muscle fibers without real capillary growth (Hudlicka et al., 1996). Perhaps comparable in importance to the oxidative capacity of skeletal muscle, is the fact that capillarization is much more closely related to fiber size (Hudlicka et al., 1992).

Other factors besides a greater demand for oxygen, (e.g., insulin level and glucose uptake) have been considered as important determinants of capillary density. For
example, capillary density has been shown to be correlated with fasting plasma glucose and insulin concentrations (Lillioja et al., 1987; Eriksson et al., 1994; Marin et al., 1994).

Angiogenesis appears to be initiated by disruption of the capillary basement membrane, followed by migration and proliferation of endothelial cells (Pintucci et al., 1996; Hudlicka et al., 1992, 1996). Capillary proliferation may result from the damage connected with the mechanical consequences of increased blood flow, such as the shear stress that results from the increased red blood cell velocity and increased capillary wall tension as a consequence of an increased capillary pressure (Hudlicka et al., 1992).

**1.6 HEMODYNAMIC ACTIONS OF INSULIN**

Insulin plays a physiological role in the vasodilation of skeletal muscle vasculature (Baron et al., 1990; Edelman et al., 1990; Laakso et al., 1990a, 1992; Baron and Brechtel, 1993; Baron, 1994, 1996; Spraul et al., 1996; Tack et al., 1996b; de Haan et al., 1997). In healthy men and women, insulin administered locally in physiological concentrations induced vasodilation in the skeletal muscle of the forearm (Tack et al., 1996b). In lean insulin sensitive subjects, insulin caused a twofold increase in leg resting blood flow (Baron, 1994); leg skeletal muscle has been shown to be reflective of the various muscle beds in the rest of the body (Baron et al., 1991). Changes in peripheral hemodynamics are accompanied by similar proportional changes in cardiac output. However, the magnitude of the changes in cardiac output which occur in response to physiological hyperinsulinemia do not cause significant increases in arterial pressure (hypertension) as there is a commensurate decrement in peripheral vascular resistance (Baron, 1994). In healthy subjects, as well as in patients with hypertension, insulin does not increase systemic blood pressure (Baron, 1994; Tack et al., 1996b). Baron and Brechtel (1993) observed that insulin decreases leg vascular resistance more than systemic vascular resistance, and via this mechanism essentially redistributes cardiac output to insulin-sensitive tissues. Thus, it appears that insulin not only regulates
vascular resistance in a differential fashion, it preferentially causes dilatation of skeletal muscle vasculature (Baron, 1994).

A relaxation of the resistance vessels would, in turn, lead to an increased blood flow through the capillary microcirculation (McNally et al., 1995). It should be noted however, that measurements of the total blood flow into skeletal muscle beds do not address whether any change in blood flow distribution actually occurs within the muscle (Rattigan et al., 1997). Measurements of mean blood flow do not provide any information on the functional or anatomical blood flow heterogeneity, ie. capillary flow (Utrainen et al., 1997). Capillary perfusion can occur without changes in total flow (Rattigan et al., 1997; Utrainen et al., 1997), or alternatively, a decreased capillary flow can occur even with an increase in total flow (Rattigan et al., 1997). Further, insulin’s action to increase glucose uptake appears to depend on its direct effect on blood flow heterogeneity (capillary perfusion) rather than on the increase in total blood flow (Utrainen et al., 1997). Using the perfused rat hindlimb, skeletal muscle metabolism has been altered positively or negatively by vascular activity which redistributed flow, while total flow to the muscle was constant (Rattigan et al., 1997). Rattigan et al. (1997) reported that insulin in rats caused an increase in blood flow as well as a recruitment of additional capillary flow. Insulin has also been reported to increase capillary recruitment in human skeletal muscle (Baron et al., 1993). In 36 normotensive healthy males, insulin within the physiological range, increased peripheral blood flow by vasodilating skeletal muscle resistance arterioles.

Insulin’s dilatory action on the skeletal muscle vasculature is dose dependent (Laakso et al., 1990, 1992; Baron, 1994) with a slow onset (Baron, 1994; de Haan et al., 1997; Raitakari et al., 1996). Insulin infusion into the femoral artery has resulted in a significant increase in femoral blood flow after 20 minutes, but not after 10 minutes of infusion (Tack et al., 1996b). The time to reach half-maximal rates of blood flow has been reported to be 40 to 60 minutes, which is similar to the time course of whole body insulin-mediated glucose uptake (Baron, 1994). Tack et al. (1996b) reported that insulin-induced vasodilation in the forearm muscle (during euglycemic systemic
hyperinsulinemia) took at least three hours to obtain its maximal effect at physiological concentrations.

Proposed mechanisms for insulin-mediated skeletal muscle vasodilation include: 1) a direct effect of insulin to relax vascular smooth muscle, 2) an indirect effect coupled to metabolic activity, and/or 3) an indirect effect via the release of an endothelial or vascular wall mediator. A direct insulin-effect on vascular smooth muscle cells has been reported. As well, insulin is known to stimulate NaKATPase and may cause vasodilation by hyperpolarizing vascular smooth muscle, and reducing calcium influx (Baron, 1994). Because insulin stimulates tissue glucose metabolism, the increase in perfusion may occur in response to this metabolic need (Baron, 1996). In exercising muscle, metabolic demands are met with a 5- to 10-fold increase in skeletal muscle flow and enhanced capillary recruitment, suggesting that these two actions are coupled (Baron, 1994). The stimulation of metabolic activity in intracellular pathways of glucose metabolism, such as glucose oxidation or glycolysis, may induce an increase in blood flow (Laakso et al., 1990a). However Baron (1996) found that the insulin concentration rather than the rate of carbohydrate oxidation was the primary determinant of the degree of vasodilation. Tack et al. (1996b) have shown that insulin-mediated glucose uptake precedes the slow vasodilator response to insulin, but does not appear to be an important determinant of insulin-induced vasodilation in the forearm. Raitakari et al. (1996) quantitated blood flow and glucose uptake into leg muscle, and reported no relation between these two variables. Further, the stimulation of muscle glucose extraction by insulin, occurred within minutes after an intravenous insulin administration, and appears to involve a different mechanism, namely, the stimulation of glucose transport and phosphorylation via translocation of glucose transporters and an increase in hexokinase enzyme activity, respectively (Raitakari, et al., 1996). Thus, it has been suggested that insulin’s hemodynamic and metabolic effects in skeletal muscle are not closely coupled (Raitakari et al., 1996; Tack et al., 1996). Current evidence strongly indicates that insulin directly stimulates the production of endothelium-derived nitric oxide (EDNO) (Steinberg et al., 1994; Baron, 1996; de Haan et al., 1997), which is also termed endothelium-derived relaxing factor (Baron, 1996). EDNO is not only a potent endogenous
vasodilator, it also has a cardiovascular protective role whereby it reduces, or inhibits, platelet adhesiveness and aggregation, smooth muscle cell proliferation, and lipoprotein peroxidation (Baron, 1996).

Since insulin is known to stimulate muscle sympathetic nerve activity, the sympathetic nervous system may also play a role in the modulation of skeletal muscle blood flow in response to insulin (Baron, 1994). An interaction of insulin with the autonomic nervous system seems likely in that an insulin-induced stimulation of the sympathetic nervous system does not lead to an increase in blood pressure (Tack et al., 1996a). Although, it has been reported that hyperinsulinemia is a trigger for sympathetic activation (Anderson et al., 1991; Vollenweider et al., 1993; Tack et al., 1996a), Spraul et al. (1996) found that during insulin infusion the increase in muscle sympathetic nerve activity occurred more rapidly than the insulin-induced vasodilation in leg skeletal muscles. Further, the increase in muscle sympathetic nerve activity did not appear to increase arterial pressure or peripheral vascular resistance regardless of the effects of insulin on the vasculature. It was concluded that insulin-mediated sympathetic nerve activity in the muscle of male subjects was neither a response to, nor a cause of, the vasodilation observed in insulin-sensitive men. Tack et al. (1996a) also reported that the vasodilation effect of insulin to increase forearm blood flow was not related to the modulation of α- or β- responsiveness. When either α- or β-blockers were infused, insulin-mediated vasodilation was not observed, which also suggests that an adrenergic mechanism does not mediate insulin vasodilation (Baron, 1994). The effect of insulin in stimulating the sympathetic nervous system in muscle appears to be dissociated from its acute haemodynamic action, and likely occurs at the level of the central nervous system (Spraul et al., 1996).
1.7 THE PHYSIOLOGICAL ROLE OF INSULIN-MEDIATED VASODILATION: EFFECTS OF SKELETAL MUSCLE PERFUSION ON INSULIN ACTION

Besides modulating vascular resistance and blood pressure, insulin’s vasodilatory action also plays a role in the modulation of fuel disposal by increasing substrate and hormone delivery (Baron, 1996). Via capillary recruitment, the specific action of insulin to vasodilate the skeletal muscle vasculature may be integral to the delivery of both hormone and substrate (Baron, 1994). Since 70-80% of insulin-mediated glucose uptake occurs in skeletal muscle, increased rates of insulin and glucose delivery (blood flow) to this tissue may represent an important mechanism of insulin’s overall metabolic action (Baron et al., 1991; Baron, 1994). During euglycemic clamp studies, Lillioja et al. (1987) reported that the capillary density of skeletal muscle was directly correlated to the rate of insulin-mediated glucose uptake or insulin sensitivity. The ratio of capillaries per muscle fiber is significantly correlated with both basal and insulin-stimulated blood flow (Utrainen et al., 1996), and blood flow to insulin sensitive tissues is an important determinant of whole body glucose uptake (James et al., 1986; Edelman et al., 1990; Laakso et al., 1990b; Baron et al., 1993, 1994). Changes in leg blood flow have been reported to correlate positively with the rate of insulin-mediated glucose uptake (Steinberg et al., 1994). Differences in insulin-mediated glucose uptake appear to be largely accounted for by the magnitude of the change in muscle blood flow, and not by differences in skeletal muscle glucose extraction (Baron et al., 1993). An increment in dynamic exercise presumably increases the rate of insulin and glucose delivery, and the enhanced glucose delivery stimulates muscle glucose uptake (Hespel et al., 1996). By modulating the rate of substrate delivery (perfusion) in an isolated rat hindlimb preparation at constant insulin and glucose concentrations, glucose uptake was increased (Baron, 1994). A given increment in glucose delivery to the muscle stimulated the glucose uptake rate, moreso with higher insulin concentrations, indicating that insulin delivery is also important with respect to the regulation of the rate of muscle glucose uptake (Hespel et al., 1996). Yang et al. (1989) have shown that transcapillary insulin
transport is rate limiting for action of insulin on target tissues. It has also been hypothesized that the diffusion distance from the feeding capillary to the interstitium may be limiting to the action of insulin (Lillioja et al., 1987). Similar to glucose, an insulin gradient exists between the capillary and the plasma membrane as a result of one or more of the following: 1) a transendothelial barrier to insulin transport/diffusion, 2) a constitutively low capillary density, and/or 3) a reduced capillary recruitment i.e., a low functional capillary density (Baron, 1994; Hespel et al., 1996). Another mechanism by which insulin-mediated vasodilation/capillary recruitment could enhance glucose uptake is via an enhanced insulin delivery; an increased insulin delivery would reduce the plasma-to-interstitium insulin gradient (Baron, 1994). Raising the capillary insulin supply via an increase in arterial insulin concentration has been shown to facilitate capillary insulin transport (Hespel et al., 1996). Further, insulin binding to the capillary endothelium occurs instantly with exposure to insulin, and is proportional to muscle capillary density and blood flow (Björntorp, 1995). Insulin uptake into muscle shows a positive association with the degree of tissue capillarization (Holmang et al., 1992).

Taken together, it appears that an insulin-mediated vasodilation plays a role in amplifying the metabolic actions of insulin via a mechanism of capillary recruitment (Baron et al., 1995; Rattigan et al., 1997). Moreover, perfusion (insulin and glucose delivery) may be viewed as an independent determinant of glucose uptake into skeletal muscle (Baron, 1994). Baron (1994) has proposed a model for flow/perfusion-modulated glucose uptake. He suggests that in the basal state, the feeding arteriole is relatively constricted, and muscle blood flow/perfusion, and thus glucose and insulin delivery, is low. At this time, both the functional capillary density and capillary perfusion are reduced, and insulin and glucose exhibit large gradients from the capillary to the interstitium, leaving some muscle fibers understimulated. In an insulinized state (i.e., after a meal), insulin causes precapillary arteriolar vasorelaxation. As a result of the vasodilation, skeletal muscle vascular tone is reduced and a greater proportion of cardiac output is directed to skeletal muscle. Arteriolar flow increases, which results in capillary recruitment and augmented capillary perfusion, leading to a greater delivery of both insulin and glucose. With an increased capillary recruitment, and the consequent
increase in functional capillary density, the plasma-to-interstitium insulin concentration gradient is reduced (Baron, 1994). Recruitment of new capillaries increases the mass of muscle tissue available for insulin-mediated glucose metabolism, and thus the overall action of insulin is amplified (Baron et al., 1990; Baron, 1994).

1.8 SKELETAL MUSCLE GLUCOSE UPTAKE

Because it is one of the main consumers of glucose in the body, skeletal muscle is regarded as an essential component of the glucose homeostatic system. Glucose uptake is readily stimulated by insulin, a hormone whose main function is to facilitate glucose transport (Sasson and Cerasis, 1986; Klip and Paquet, 1990; Krotkiewski, 1994). A family of tissue-specific glycoproteins, called glucose transporters are responsible for the facilitative diffusion of glucose across the plasma membranes (Edelman et al., 1990). Insulin’s stimulation of glucose uptake in both human and rat skeletal muscle is primarily mediated through translocation of GLUT 4 (Galante et al., 1995; Guma et al., 1995). The expression of this glucose transporter isoform is confined to insulin sensitive tissues, namely skeletal and heart muscle, and adipose tissue (Miele et al., 1997). Although GLUT 4 transporters are present in the plasma membrane of muscle, they exist in a higher yield in the intracellular membrane fraction, which is responsive to acute exposure to insulin (Handberg, 1995). For this reason, this isoform has been dubbed the major “insulin-regulatable” glucose transporter (Guma et al., 1995; Garvey and Birnbaum, 1993), and as such, is extremely important in the maintenance of glucose homeostasis (Handberg et al., 1996).

1.8.1 FIBER-TYPE SPECIFIC DIFFERENCES IN GLUCOSE UPTAKE

Skeletal muscle exhibits heterogeneity with regard to muscle fiber types (Table 1.1). Muscle is composed of predominantly three distinct fiber types, each with unique metabolic and contractile characteristics (Hickey et al., 1995; Noble and Ianuzzo, 1985).
“Red” muscle is composed predominantly of fibres which are oxidative, slow twitch (SO; Type I) or oxidative, glycolytic, fast twitch (FOG; Type IIa), and generates energy for ATP resynthesis predominantly by aerobic energy transfer; fatty acids are the major fuel source. “White” muscle is mainly comprised of fibres which are glycolytic, (FG; Type IIb), and depend almost entirely on anaerobic metabolism for energy; glycogen is utilized as the major fuel (Holloszy, 1982; McArdle et al., 1991; Krotkiewski, 1992; Kainulainen et al., 1994; Hickey et al., 1995;). The color classification of muscle corresponds to the mitochondrial supply and levels of myoglobin present in the fibers. While the “white”, fast twitch, glycolytic fibers contain few mitochondria and lower levels of myoglobin, the “red” slow twitch, oxidative and fast-twitch, oxidative-glycolytic fibers contain large numerous mitochondria and high levels of myoglobin (Brooks et al., 1996; Krotkiewski 1992).

A difference in glucose transport capacity has been demonstrated between the various fiber types. The SO and FOG muscle fibers have higher basal rates of glucose uptake than do the FG fibers (Barnard and Youngren, 1992; James et al., 1985). Since GLUT 1 is involved with basal glucose supply, and because GLUT 1 has a low expression in the muscle membrane, little research has examined the relationship between levels of GLUT 1 and fiber type composition (Handberg, 1995). However, it has been reported that GLUT 1 levels are lower in FG muscle fibers (Barnard and Youngren, 1992) than in SO fibers (Han et al., 1995b). Marette et al. (1992) found that red muscle contains a higher amount of GLUT 1 and GLUT 4 in both the basal and insulin-stimulated states. Insulin-stimulated glucose uptake appears to be much higher in the SO and FOG fibers compared to the FG fibers (Barnard and Youngren, 1992). Hickey et al. (1995) have reported that the percentage of type I muscle fibers is related to the insulin-stimulated glucose transport rate. The difference in the IMGU may be attributed to greater insulin binding in the SO fibers (Bonen et al., 1981), and/or a lower number of insulin receptors in the FG fibers (Barnard and Youngren, 1992).

A larger intracellular pool of glucose transporters has been observed in the more insulin responsive fibers (Barnard and Youngren, 1992), suggesting that the differences
in maximally stimulated glucose transport activity amongst the various fiber types may be related to differences in their content of GLUT 4 protein (Henricksen et al., 1990). There is some uncertainty as to the relative level of GLUT 4 in fast twitch compared to slow twitch fibres, but within each fibre type, GLUT 4 has been reported to be more abundant than GLUT 1 (Johansson et al., 1996). Concentration of GLUT 4 appears to be higher in red (FOG) compared to white (FG) muscle (Handberg, 1995; Barnard and Youngren, 1992; Henricksen et al., 1990). Han et al. (1995b) have observed higher GLUT 4 mRNA and protein levels in the SO fibers compared with the FG fibers. Furthermore, the difference in GLUT 4 expression among metabolically heterogeneous muscles can be explained on the basis of a high correlation between the oxidative capacity of the muscle fibers and their GLUT 4 content (Marette et al., 1992; Handberg et al., 1996). The process of GLUT 4 translocation however, does not differ between fiber types though (Marette et al., 1992). GLUT 4 expression among different muscle fibers also seems to follow the level of hexokinase activity, another protein which plays an important role in regulating glucose utilization (Handberg, 1995).

Glucose uptake varies proportionately with glucose delivery (Schultz et al., 1977a; 1977b). The capacity for blood flow to muscle has been reported to be proportional to the differences in oxidative capacity among fiber types (Terung and Engbretson, 1988), as indicated by the high, medium, and low blood flow to the SO, FOG and FG fibers, respectively (James et al., 1985). It is well known that red muscle, subsequently characterized as having a highly oxidative metabolism, has a much more dense capillary network compared to white muscle with its predominantly glycolytic metabolism (Hudlicka et al., 1992). Furthermore, based on the relationship between blood flow and glucose metabolism (James et al., 1985; Bousekela et al., 1997), it seems likely that glucose transport capacity among muscles of varying fiber type
Table 1.1 Biochemical and physiological features of muscle fiber types in rats

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Slow-twitch, Oxidative</th>
<th>Fast-twitch, Glycolytic-Oxidative</th>
<th>Fast-twitch, glycolytic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexokinase</td>
<td>High</td>
<td>Medium</td>
<td>Low</td>
</tr>
<tr>
<td>Oxidative Enzymes</td>
<td>High</td>
<td>Medium</td>
<td>Low</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>High</td>
<td>Medium</td>
<td>Low</td>
</tr>
<tr>
<td>Insulin Binding</td>
<td>High</td>
<td>?</td>
<td>Low</td>
</tr>
<tr>
<td>Insulin Receptors</td>
<td>?</td>
<td>?</td>
<td>Low</td>
</tr>
<tr>
<td>Glucose Uptake</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) basal :GLUT 1</td>
<td>High</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>GLUT 4</td>
<td>High</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>b) Insulin-stimulated:</td>
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<tr>
<td>GLUT 1</td>
<td>High</td>
<td>High</td>
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<tr>
<td>GLUT 4</td>
<td>High</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Insulin Sensitivity</td>
<td>High</td>
<td>Medium</td>
<td>Low</td>
</tr>
<tr>
<td>Maximal insulin response at rest</td>
<td>High</td>
<td>Medium</td>
<td>Low</td>
</tr>
<tr>
<td>Blood Flow</td>
<td>High</td>
<td>Medium</td>
<td>Low</td>
</tr>
<tr>
<td>Resting Contractile Activity</td>
<td>High</td>
<td>Medium</td>
<td>Low</td>
</tr>
</tbody>
</table>

Adapted from: James et al., 1985; Holloszy, 1982; Marette et al., 1992

is not only the result of differential expression, membrane distribution or translocation of glucose transporters, but also influenced by substrate delivery as determined by the capillary supply and muscle blood flow (James et al., 1985).

1.9 HEMODYNAMIC CHANGES IN DIABETES

Insulin stimulation of glucose uptake is achieved by increasing both glucose extraction and blood flow (Raitakari et al., 1996). In Type I diabetes, insulin resistance i.e., a decreased insulin-stimulated glucose uptake, has been attributed to chronic hyperglycemia-induced defects in glucose extraction (i.e., secondary to defects in
transport and post-transport steps of glucose metabolism), rather than a vascular mechanism (Baron et al., 1991; Makimattila et al., 1996). In vivo IMGU, which occurs primarily in skeletal muscle, is determined by the arteriovenous glucose difference (AVGd) or glucose extraction across muscle, and the rate of blood flow (BF) into muscle (Baron et al., 1990). Disposal of a glucose load occurs by the combined effects of endogenous insulin to stimulate tissue glucose uptake and by the prevailing serum glucose concentration via mass action to promote glucose uptake; both of these effects lead to an increase in glucose extraction (AVGd) across skeletal muscle (Baron et al., 1990). Insulin stimulation of muscle glucose uptake could also result from post-prandial increases in blood flow (insulin and glucose delivery) to skeletal muscle (Baron et al., 1990). Baron et al. (1995) have reported that ≈20-30% of the overall rate of insulin-mediated glucose uptake in lean men is dependent on increments in muscle perfusion. This occurs at both submaximally effective insulin concentrations (insulin sensitivity) and maximally effective insulin concentrations (insulin responsiveness) (Baron et al., 1995). Additional reports (Baron et al., 1993; Baron, 1994) have suggested that the differences in insulin responsiveness between lean non-diabetic normotensive individuals is largely determined by differences in the hemodynamic response to insulin, rather than insulin’s effect of increasing muscle glucose extraction. The relative contribution of tissue (AVGd) and hemodynamic (BF) actions of insulin determine the overall rate of glucose uptake. Therefore, defects in one or both components can result in decreased rates of glucose uptake, and thus insulin resistance (Baron et al., 1990).

Vasodilation and increased blood flow are characteristic early vascular responses in Type I diabetes due to acute hyperglycemia and tissue hypoxia (Williamson et al., 1993). The lack of insulin in IDDM may also lead to a generalized vasodilation via tissue hypoxia. From the early onset of the disease, particularly during poor metabolic control, there is evidence of a decreased release of oxygen from the erythrocytes (Ditzel, 1976), as well as an increased basal oxygen consumption in diabetics. Williamson et al. (1993) have reported that vascular changes in hypoxic tissues are linked to metabolic imbalances associated with an impaired oxidation of NADH to NAD+, and the resulting increased ratio of NADH/NAD+. A reduced oxygen tension, as determined by oxygen consumption
and delivery, would cause an increase in capillary blood flow and dilatation, in an effort to ensure adequate delivery of oxygen to the local tissues (Ditzel, 1976; Zatz and Brenner 1986).

Elevated blood glucose levels may also induce vascular growth. Prolonged incubation in high glucose levels has been shown to up-regulate transcription for genes coding for the basement membrane components of endothelial tissue (Shoemaker and Bonen, 1995).

An increased metabolism of glucose via the sorbitol pathway is an important mechanism related to hyperglycemia. Hyperglycemia also induces a redox imbalance which results from an increased oxidation of sorbitol to fructose coupled to the reduction of NAD+ to NADH from a later step in the sorbitol pathway. This imbalance is a characteristic feature of poorly controlled diabetes, and is referred to as pseudohypoxia because the partial pressure of tissue oxygen is normal. Pseudohypoxia mimics the effects of true hypoxia with respect to increased vascular flow, but also plays a role in the pathogenesis of diabetic complications (Williamson et al., 1993). Williamson et al. (1993) predicts that at any arterial blood pressure, microvascular blood flow will be higher in complication-prone tissues of diabetic subjects compared to non-diabetic subjects. The functional consequences which are linked to pseudohypoxia include: 1) an increased blood flow in diabetic rat tissues which are prone to late complication (without an increase in systemic blood pressure), 2) a dilation of the resistance arterioles and impaired smooth muscle contractile function in the affected tissues 3) a transmission of systemic blood pressure further downstream causing microvascular hypertension, 4) a stimulation of vascular collagen production and an increased oxygen production by hypertension, which leads to 5) sclerosis and stiffening of the vessel wall (Williamson et al., 1993). As mentioned previously, Type I diabetes of long duration is characterized by underperfusion (Tooke; 1993; Williamson et al., 1996), with the progressive sclerosis often inducing microvascular occlusion (Parving, et al., 1983). The functional changes in diabetic tissues therefore, involve an increased blood flow after the early onset of
diabetes, and a decreased blood flow later in the course of the disease (Williamson et al., 1993).

There is also evidence for hyperglycemia to induce vascular contraction. An acute elevation of glucose activates protein kinase C in endothelial tissue, which in turn stimulates production of vasoconstrictive prostaglandins. As well, high glucose levels have resulted in the production of a potent vasoconstrictor, endothelin, by aortic endothelial cells (Shoemaker and Bonen, 1995). Endothelial dysfunction, as measured by the vasodilatory response of forearm resistance vessels to endothelium-dependent vasoactive agents, such as acetylcholine, is impaired during chronic hyperglycemia in Type I diabetes (Makimattila et al., 1996). The high glucose levels associated with hyperinsulinemia may also be toxic to vascular endothelial cells (Shoemaker and Bonen, 1995).

Changes in insulin resistance can be found when the relationship between skeletal muscles and their nutritional blood supply is altered. Aging and hypertension have a high prevalence of insulin resistance and lead to vascular rarefaction, i.e., a decreased number of blood vessels in skeletal muscle (Julius et al., 1991). Julius et al. (1991) have hypothesized that pressure-induced restriction of the microcirculation limits nutritional flow, and thereby, impairs glucose uptake in skeletal muscle. An increased insulin sensitivity with exercise training can be readily explained by hemodynamic factors, such as an increased capillary density and blood flow (Julius et al., 1991; Pratley et al., 1995; Shoemaker and Bonen, 1995). However, in Type I diabetic subjects without, or having only mild, clinical evidence of diabetic microangiopathy, Wallberg-Henriksson et al. (1984) have observed that the capillary response to training was altered, and that glycemic control was not improved. Capillary density (expressed as the number of capillaries per unit muscle cross sectional area) increased in controls but failed to do so in the diabetic patients. As well, the control group exhibited an increase in the number of capillaries per muscle fiber, but muscle capillarization did not change in the diabetic group (Wallberg-Henriksson et al., 1984). It was proposed that the microvascular lesions of the muscles resulted in an impaired capacity to form new capillaries (Wallberg-
Henriksson et al., 1984). Mandroukas et al. (1986) have also reported a deficient proliferation of capillaries in patients with Type I diabetes, even though normal central (e.g., maximal oxygen uptake) and peripheral (i.e., mitochondrial enzyme) adaptations to the physical training were observed. Suppression of the proliferation of capillaries partly explains the lack of improvement of glucose homeostasis during physical training in diabetes (Krotkiewski, 1994). Further, in poorly controlled Type I diabetic patients, Baron et al. (1991) have reported that muscle glucose extraction (AVGd) is normal compared to controls. Decreased rates of in vivo IMGU were accounted for by a decreased blood flow to skeletal muscle, which suggests that the reduced insulin and glucose delivery or blood flow to skeletal muscle is an important mechanism in insulin resistance in Type I diabetes (Baron et al., 1991). Insulin mediated-glucose transport into muscle could be limited if insulin and glucose delivery were inadequate (Mijares and Jensen, 1995).

Similarly, in a wide variety of insulin resistant states with hyperinsulinemia (i.e., obesity, Type II diabetes, and hypertension), there is strong evidence that reduced rates of glucose uptake are largely due to the lack of an increase in blood flow to the insulin sensitive tissues (Baron et al., 1990; Baron, 1994; Tack et al., 1996b). Insulin-mediated skeletal muscle vasodilation significantly contributes to the disposal of glucose (Tack et al., 1996b), such that the magnitude of vasodilation is proportional to the degree of insulin sensitivity (Baron, 1996). It follows that a diminished vasodilator capacity explains, in part, the decreased insulin sensitivity in these insulin resistant states (Tack et al., 1996b). Baron et al. (1990) have observed that peak muscle glucose uptake is lower in obese subjects compared to a lean group, which may be the result of insulin’s inability to dilate the skeletal muscle vasculature. In obese subjects, insulin’s actions to increase cardiac output and reduce skeletal muscle vascular resistance were blunted, resulting in a diminished insulin-mediated skeletal muscle blood flow, a resistance to carbohydrate metabolism, and a risk for the development of hypertension (Baron and Brechtel, 1993). In other studies (Baron, 1996; Steinberg et al., 1996), it has been reported that obese subjects have an impaired endothelium-dependent vasorelaxation, and are resistant to insulin’s physiological action to modulate EDNO release. In subjects with elevated blood
pressure, reduced rates of IMGU were reported as secondary to an impairment in the action of insulin to increase skeletal muscle blood flow (Baron et al., 1993). Finally, in rats with both elevated blood pressure and decreased skeletal muscle perfusion, marked reductions in the rate of IMGU were observed (Baron et al., 1995).

### 1.10 DIABETES AND MUSCLE PROTEIN LOSS

Insulin is probably the single most influential factor for regulating and maintaining positive protein balance in skeletal muscle via its anabolic effect and significant inhibitory effects on protein degradation (Tischler, 1981). In diabetes, defects in the normal effects of insulin significantly alter hormonal control of protein metabolism. For instance, in Type I diabetes, a pathological defect in insulin is characterized by considerable disturbances at the level of skeletal muscle. A lack of insulin in diabetes is known to contribute to a significant increase in muscle protein turnover (Rutschmann et al., 1984), a decrease in protein synthesis (Rutschmann et al., 1984) and an accelerated rate of proteolysis (Rutschmann et al., 1984; Tischler, 1981). An increased proteolysis can result in the production of amino acids which with their subsequent conversion to glucose, can provide an important fuel source for the organism during pathological conditions such as diabetes (Tischler, 1981). Glycosuria, a consequence of reduced carbohydrate catabolism, is known to cause a substantial loss of calories and a decrease in protein synthesis in the absence of insulin (Han et al., 1995a). Taken together, diabetes is accompanied by a reduction in weight gain, and severe muscle atrophy (Han et al., 1995a) through the loss of protein (Rutschmann et al., 1984).

Insulin regulates a myriad of metabolic processes which can affect protein synthesis in skeletal muscle. The exact mechanisms of insulin’s stimulatory effect on protein synthesis is not known, but there is evidence that insulin increases the uptake of amino acids into cells (Balon et al., 1990; Han et al., 1995a), as well as their incorporation into protein (Armstrong and Ianuzzo., 1977). Insulin also appears to increase the synthesis of both sarcoplasmic and ribosomal proteins by accelerating both
the translation and transcription of mRNA (Balon et al., 1990). It is also possible that insulin regulates the availability of high energy phosphates, ribosomes, tRNA and mRNA, and the activity of enzymes and factors which catalyze peptide formation, chain initiation, elongation and termination. In diabetes, protein synthesis in skeletal muscle appears to be limited at the level of peptide-chain initiation, which represents the rate-limiting step for protein synthesis (Balon et al., 1990; Jefferson, 1980).

The reduced rate of protein synthesis has been attributed to a loss of tissue RNA and a reduced translational efficiency (Jefferson, 1980). The acute effects (2 day) of diabetes on the rates of protein synthesis, peptide-chain initiation and the level of RNA have been examined in rat red muscle (soleus) and white muscle (gastrocnemius). This short-term diabetes resulted in a reduced rate of protein synthesis in the gastrocnemius, which was due to a loss of tissue RNA and a reduced translational efficiency (Jefferson, 1980; Flaim et al., 1980). In the soleus, the reduction in protein synthesis was due entirely to a reduced concentration of RNA. No decrease in translational efficiency was detected, indicating that muscles with more oxidative fibers show little impairment in the initiation of protein synthesis (Flaim et al., 1980) and are less susceptible to the block in peptide-chain initiation caused by hypoinsulinemia in diabetes (Jefferson, 1980). Insulin deficiency of longer duration (7 days) caused no further reduction in RNA, but resulted in the development of an additional impairment in protein synthesis in the soleus, i.e. exhibited an impaired translational efficiency (Flaim et al., 1980; Jefferson, 1980). Some factors which protect oxidative muscle in short-term diabetes from the inhibition of protein synthesis caused by insulin deficiency include high levels of circulating free fatty acids and glucose-6-phosphate. It has been proposed that the high levels of fatty acids observed in diabetes can maintain the translation efficiency. In the heart muscle of rats with diabetes, the presence of a fatty acid (palmitate) was as effective as insulin in maintaining the rates of protein synthesis. Provision of lactate, pyruvate, acetoacetate, B-hydroxybutyrate, or oleate have also been shown to be effective in maintaining protein synthesis. A role for glucose-6-phosphate in the regulation of peptide-chain initiation has also been speculated. In the hearts of diabetic rats, the maintainance of peptide-chain initiation was associated with an elevated level of glucose-6-phosphate (Jefferson, 1980).
It is also apparent from studies of muscle protein degradation in STZ diabetes that muscle catabolism is abnormal. For example, small and basic non-glycoproteins, which are stable intracellular components are degraded more rapidly (Jakobsen and Reske-Nielsen, 1986). Increases in the overall activities of alkaline proteinases and aminopeptidases have also been observed (Rothig et al., 1978; Jakobsen and Reske Nielsen, 1986), as well as an elevated excretion of metabolites of degraded actomyosin (3-methylhistidine) (Tischler, 1981; Jakobsen and Reske-Nielsen, 1986). An increased activity of these enzyme systems associated with the myofibrillar fraction, may be responsible for the breakdown of structural proteins (Jakobsen and Reske-Nielsen, 1986).

A reduced protein synthesis and an increased proteolysis may largely explain the reduced metabolic potential that exists in diabetic skeletal muscle (Ianuzzo et al., 1974). In STZ-diabetic rats, the gastrocnemius muscle was found to have a significantly lower myoglobin content, succinate dehydrogenase activity, as well as a reduced capacity to oxidize pyruvate and palmitate than observed in non-diabetic sedentary animals. A lower than normal concentration of mitochondrial protein in these animals was also probably a manifestation of the effects of hypoinsulinemia on protein synthesis (Ianuzzo et al., 1974). Similarly, Chao et al. (1976) observed that the FG fibers of diabetic rats had a reduced number of mitochondria. In contrast, the number of mitochondria did not change in either the SO or FOG fibers, although numerous morphological changes were observed (Chao et al., 1976). A reduced content of mitochondria has also been reported in the cardiac myocytes of STZ-diabetic rats (Warley et al., 1995).

### 1.11 ALTERATIONS IN MUSCLE CAPILLARY-FIBER MORPHOLOGY WITH DIABETES

Specific fiber type alterations in muscle fiber size have been reported in diabetic male animals. Armstrong et al. (1975), have reported that the FG fibers in the diabetic gastrocnemius muscle of male rats had a smaller mean cross-sectional fiber area than the respective fibers in normal muscle. However, when fiber size was adjusted for body
mass, there was no difference between the diabetic and normal animals. The FOG fibers from the diabetic rats were also smaller than normal, but the difference was not significant, and the SO fibers were larger than normal. No differences in muscle capillary:fiber ratios were observed between the normal and diabetic rats, although the FG fibers tended to have fewer adjacent capillaries (Armstrong et al., 1975).

Other studies have reported a differential effect of STZ-diabetes on the various muscle fiber types. Ogoh et al. (1995) found that in male rats, the SO muscle fiber area of diabetic rats was significantly increased, in contrast to the area of the FOG fibers, which decreased. In male rats, diabetes (10-60 days) resulted in type I fiber hypertrophy, and type II fiber atrophy in the rectus femoris muscle. The decrease in fiber area was greater in the type IIb fibers than in the type IIa fibers (Medina-Sanchez et al., 1991). More recently, Klueber and Feczko (1994), reported a decrease in both type IIa and IIb mean fiber area of the EDL from STZ male mice. Again, the shift toward smaller myofibers was more pronounced in the type IIb fibers (Klueber and Feczko, 1994).

Hegarty and Rosholt (1981) have investigated the effect of STZ-induced diabetes on the biceps brachii, sternomastoideus, and soleus muscles of male and female rats. They found that after 79 days of diabetes, the diabetic rats incurred a significantly greater reduction in body mass than the control group, and that this reduction was greater in males than in females. The effect of diabetes on the biceps brachii and sternomastoideus in the male rats included the development of smaller muscle fibres with smaller diameters than those of the control rats; however, diabetes had no effect on the diameter of the soleus muscle. A similar result, i.e. the development of smaller muscle fibers were observed in biceps brachii, sternomastoideus and soleus of diabetic female rats compared to control groups, but the difference was not significant (Hegarty and Rosholt, 1981). Another study, this one involving female diabetic animals, reported a significant decrease in fiber area and perimeter of the plantaris muscle. Following six to eight weeks of diabetes, female rats exhibited a 25% decrease in the capillary:fiber ratio, indicating a net loss of capillaries. However, because the degree of fiber atrophy exceeded the degree of capillary loss, the capillary density was increased in the diabetic
plantaris muscle (Sexton et al., 1994). This is in contrast to the reduced muscle mass and decrease in capillary number observed in the cremaster muscle of diabetic male mice (Bohlen and Niggl, 1980).

A synopsis of the various findings from the research literature are presented in Appendix A. Discrepancies between the results of various studies can be attributed to variations in: 1) the diabetogenic dosage of STZ, 2) the duration of diabetes, 3) the response to diabetes among male and female animals and/or 4) the morphological assessment utilized, including whole muscle or individual muscle fiber analysis.

1.12 CAPILLARY-FIBER MORPHOLOGY IN IGT

No published studies have been found which specifically addressed muscle morphology in females during IGT. Moreover, as previously mentioned, muscle capillary-fiber changes in conditions of IGT are not well defined, and have been related to obesity (body mass), a factor which directly influences skeletal muscle morphology (Marin et al., 1994). Lithell et al. (1981) reported that glucose tolerance was correlated with muscle morphology measurements, i.e., the mean fiber area per capillary. In male subjects with relatively large body weights, the enlargement of muscle cells caused a relative decrease in the density of capillaries. Furthermore, because each capillary supplied a larger muscle area, the diffusion distance would be increased. It was postulated that a low capillary density in skeletal muscle contributes to the reduced glucose tolerance and the developed insulin sensitivity found in conditions of IGT (Lithell et al., 1981).

Lindgarde et al. (1982) reported that after weight reduction, insulin sensitivity and glucose tolerance was improved in men with IGT. Weight loss resulted in a reduction in muscle fiber size, and although the average number of capillaries around each fiber was unchanged, the decrement in fiber size led to a decreased diffusion distance from capillaries to muscle fibers (Lindgarde et al., 1982). An increased capillary diffusion...
distance may help explain: 1) the delayed activation of glucose uptake, as well as 2) the
defect in the rate and amplitude of the response to insulin in obesity (Lillioja et al., 1987). It
should be noted that IGT is also a characteristic of non-obesity (Matsumoto et al., 1997).

### 1.13 SUMMARY OF THE REVIEW OF LITERATURE

Glucose tolerance (the body’s response to a glucose load), represents a continuum ranging from a normal state to a pathological state, e.g., Type I diabetes or severe impaired glucose tolerance. There is evidence that muscle morphology, particularly the size of muscle fibers and the capillary supply to each fiber, are associated with varying degrees of glucose tolerance and insulin sensitivity (Lithell et al., 1981).

However, few studies have reported on the geometry of muscle capillaries and fibers during Type I diabetes and impaired glucose tolerance (IGT), and those which have, pertain almost exclusively to males. However, distinct differences between males and females with respect to the susceptibility and prevalence of diabetes have been documented in the literature. As well, women have substantially smaller muscle fibers than men, and estrogen has been reported to play an inhibitory role in the development of Type II muscle fibers. The structure of muscle capillaries also tends to differ between males and females with aging. Finally, estrogen has a direct effect on the microvasculature, with females exhibiting a decrease in the capillary supply of muscle after treatment with male hormones. Thus, in view of the impact of sex hormones on factors related to glucose metabolism, differences in capillary-fiber morphology of skeletal muscle may be expected between females and males.

Furthermore, with respect to the reported alterations in muscle capillary-fiber morphology during Type I diabetes, most of the descriptions of the capillary supply, i.e., capillary density and capillary-to-fiber ratio, have rather limited scientific value because they represent global measurements, and as such, yield little information pertaining to the
capillary supply of individual muscle fibers. However, the fibre type specific differences in substrate utilization, glucose transporter number and capillary supply, together with the known effects of diabetes on the glucose transporters and muscle morphology, strongly suggest that quantification of the capillary supply in terms of individual muscle fibers may be more meaningful.

Studies reporting the changes in muscle morphology during IGT are also limited because obesity (body mass), a factor which influences muscle morphology, has been the major focus of such work.

Over the last decade, insulin has been recognized for its hemodynamic action. By decreasing muscular vascular resistance to a greater extent than systemic vascular resistance, insulin preferentially causes vasodilation of the skeletal muscle vasculature. Insulin redirects blood flow to areas where it stimulates the uptake of glucose (Utrainen et al., 1997), which suggests the purpose of insulin’s vasodilatory action is, in large part, related to the delivery of hormones and substrate. Insulin-mediated vasodilation appears to facilitate the delivery of insulin and glucose to skeletal muscle via capillary recruitment, thereby amplifying its overall effect to stimulate glucose metabolism (Baron et al., 1994). Insulin’s dilatory action on skeletal muscle vasculature is dose-dependent with a slow onset.

Early in the development of Type I diabetes, generalized capillary hyperperfusion occurs, and appears to be related to a lack of glycemic control. In addition to hyperglycemia, there is evidence that pseudohypoxia and hypoxia perhaps stimulate blood flow to muscle, and possibly contribute to the hyperperfusion observed after the early onset of diabetes. Conceivably, a hyperglycemia-induced increase in blood flow, together with the known effects of high glucose levels on vascular growth, could potentially influence capillary growth (angiogenesis) in skeletal muscle during the early stages of diabetes. An increase in the extent of the capillary bed would increase the size of the capillary endothelial surface area available for diffusion and augment the capability of the tissues for taking up insulin and glucose (Edelman et al., 1990). However, in contrast to early hyperemia, decreased blood flow or underperfusion is evident later in the
course of this disease. Thus, angiogenesis in Type I diabetes of long duration is questionable.

Insulin stimulation of glucose uptake is achieved by increasing both glucose extraction and blood flow. In poorly controlled Type I diabetes, muscle glucose extraction appears to be normal, while blood flow to skeletal muscle is decreased; the decrease in blood flow to skeletal muscle accounts for the insulin resistance, i.e., a decreased insulin-stimulated glucose uptake. Further, insulin-mediated blood flow/capillary recruitment is reduced in conditions of insulin resistance, i.e., during Type I and II diabetes, obesity and hypertension.

With a lack of insulin in Type I diabetes, the insulin effect on blood flow would be impaired, thereby reducing insulin and glucose delivery. As a result, it is conceivable that structural changes would occur in skeletal muscle, which may exacerbate or compensate for the reduced hormone and substrate delivery. Lillioja et al. (1987) has suggested that the diffusion distance from the capillary to the interstitium may be limiting to insulin action. Within this context, changes in muscle capilarization commensurate with muscle fiber atrophication in Type I diabetes, has functional implications for diffusion distance, which in turn, can influence insulin and glucose delivery. Further, the capillary supply in terms of the perimeter of the muscle fiber has implications for the muscle surface area available for exchange between capillaries and fibers.

Likewise, as a result of an insulin deficiency associated with IGT, changes in muscle capillary-fiber morphology may also occur. In which case, both the size of muscle fibers and the capillary supply to a given fiber, will modify diffusion distance and thus insulin and glucose delivery to skeletal muscle.

Therefore, alterations in the geometric arrangement of the capillaries in relation to the muscle fibers in skeletal muscle are of particular importance during altered states of glucose metabolism.
The purpose of this study was to examine the capillary supply and dimensional changes of individual muscle fibers from the plantaris muscle in diabetic and impaired glucose tolerant rats using several indices of capillary quantification.

The scientific hypotheses to be evaluated include:

- The impaired glucose tolerant female rats will demonstrate no changes in the capillary supply of individual muscle fibers of the plantaris muscle compared to the control animals.

- The diabetic female rats will demonstrate no changes in the capillary supply of individual muscle fibers of the plantaris muscle in comparison to the control rats.
The animals utilized in this study were obtained from a previous study investigating the responsiveness and susceptibility of female rats to a diabetogenic agent (streptozotocin-STZ) during various phases of the estrus cycle. Briefly, Rodgers et al. (in press) injected female, Sprague-Dawley rats (n=20; Charles River Laboratory) intravenously with a dosage of 65 mg kg$^{-1}$ mass of STZ dissolved in sterile saline. This process involved a single injection into the tail vein of each animal during specific phases of the estrus cycle (proestrus, metaestrus, diestrus I, diestrus II). The effectiveness of streptozotocin varied according to the phase of the estrus cycle in which the female rat had been injected (Appendix 6.2).

2.2 DIABETES AND IGT DIAGNOSIS

The major diagnostic test for diabetes is the measurement of plasma or blood glucose concentration (Zeman and Hansen, 1991). Thus, to determine which animals had become diabetic, blood samples were obtained (via tail vein pricks) seventy-two hours post-STZ injection, and the blood glucose concentrations were analyzed using a glucometer. Rodgers et al. (in press) observed that all the females, except those in the metaestrus phase, exhibited a significantly elevated blood glucose level.

In addition to the plasma glucose concentration, a glucose tolerance test (GTT) is recommended in order to further establish a diagnosis of diabetes or severe impaired glucose tolerance (Zeman and Hansen, 1991). Glucose tolerance is the body's response to the influx of glucose from the diet, or in the case of a GTT, the body's response following an oral glucose load of 50-100g after an overnight fast. The extent of glucose tolerance is determined by the rate at which the various inherent mechanisms for
removing excess glucose from the blood perform their functions. The degree of insulin release and its effectiveness determine how soon blood glucose reaches its peak, the magnitude of the peak, and how long it takes blood glucose to return to normal, or baseline levels. An impairment in any aspect of the insulin pathway will alter the glucose tolerance, and will result in a glucose curve which deviates from the normal curve (Linder, 1991). Characteristic abnormalities in the glucose tolerance curve that are indicative of glucose intolerance are a higher than normal and/or delayed peak, and a delay in returning to normal. Diabetes is characterized by an abnormal glucose curve which is represented by: 1) a high fasting glucose level, 2) a higher than normal and/or delayed peak, and 3) a delay in returning to normal (Linder, 1991).

Glucose tolerance is measured by following the blood glucose concentration over time after administration of a glucose load (Linder, 1991). Fourteen days post-STZ injection, Rodgers et al. (in press) injected female rats (interperitoneal) with 3ml of a 30% glucose solution. Blood glucose concentration was then measured at time 0, 30 minutes, 60 minutes and 120 minutes using a blood glucose meter (Glucometer Elite; Bayer Inc.). Based on these results, 50% of the females injected with STZ demonstrated a typical diabetic response with significantly elevated blood glucose levels, while the remaining 50% of the rats demonstrated only an impaired glucose tolerance, with normal basal glucose concentrations. Refer to Appendix A.

The current phase of the study therefore began with one group of STZ-induced diabetic female animals (D), and one group of female animals which exhibited impaired glucose tolerance (IGT). The D group (n=8) had a mean weight of 243 grams, and the IGT group (n=7) had a mean weight of 274 grams. Both groups of animals were approximately 13 weeks in age.

The animals were fed and watered ad libitum and housed in a temperature- and humidity-controlled room with a 12h dark:light cycle. The body mass of each female animal was recorded on alternate days for twelve weeks. The survival of diabetic rats with a significant degree of hyperglycemia is usually not more than 20-25 weeks (Knudsen et al., 1991). For this reason, 12 weeks post-STZ injection was chosen as the
longest observation period for this study. The diabetic animals were maintained without insulin therapy for the entire length of the study to ensure an equilibration of the diabetic condition (Hegarty and Rosholt, 1981).

A second glucose tolerance test was administered twelve weeks post-STZ injection, prior to sacrifice, to verify the metabolic status, i.e., diabetes and impaired glucose tolerance, which had been established in the prior study. All female rats were again given an intraperitoneal injection with 3ml of glucose solution (30%). Blood samples were then obtained from each animal via tail vein pricks at time 0, 30 minutes, 60 minutes and 120 minutes in order to determine the blood glucose concentrations over time.

Because the STZ-induced diabetic animals failed to gain weight at the same rate as the non-responsive group of animals, two additional weight-matched groups of female, Sprague-Dawley rats (Charles River Laboratory) were added to the study. Weight-matched animals were added to the study to control for changes in growth patterns and to establish "true" controls groups: Control 1 (C1; n=5) and Control 2 (C2; n=5) for the diabetic and impaired glucose tolerance groups, respectively. Similar care (i.e., housing and feeding), as employed for the diabetic and non-responder groups, was provided for the control female rats. The body mass of each animal was also recorded on alternate days until the desired weight was achieved which was a mean of 259 grams for C1 and a mean of 328 grams for C2. A glucose tolerance test, following the same procedure as described above, was administered to these females twenty-one days post-arrival.

The major criterion used to evaluate the response to a glucose tolerance test was the measurement of the area under the glucose concentration versus time curve (A_GTT). The A_GTT was calculated for the four groups of animals (D; IGT; C1; C2) using the following equation: $A_{GTT} = a + d/2 + b + c$,

where, a, b, c, d = the mean blood glucose concentration at various times during the GTT where, a = 0 min., b = 30 min., c = 60 min., d = 120 min.
2.3 MORPHOMETRIC METHODS

Preceding surgery, each animals was anaesthetized with 1 cc kg\(^{-1}\) body mass of sodium pentobarbitol. The plantaris muscle was then exised from one hindlimb of each animal. A cross-section of the muscle was removed from the mid belly of the plantaris muscle, mounted on a specimen holder with embedding compound (O.C.T. compound), immediately immersed in liquid isopentane, chilled in liquid nitrogen, and following freezing stored at -80° C for subsequent histochemical analysis. Sections of each muscle were cut transversely at 8 \(\mu\)m, in a cryostat at -20° C, and were stained simultaneously for capillaries and fibre type (Type I and II only) according to the method of Rosenblatt et al. (1987). Initially, the sections were fixed in a Guth and Samaha fixative for five minutes at 4° C, and then incubated for one hour at 37° C in a lead-based ATPase stain at a pH 7.2. Tissue samples from the D and IGT groups were processed with the same batches of fixative, incubation medium and developing solution. Tissue from the control animals was processed on two separate occasions using different batches of fixative, incubation medium and developing solution. Following staining, coverslips were applied to the sections via Permount, and stored in slide boxes until morphometric analysis. Refer to a detailed procedure in Appendix A.

2.4 MORPHOMETRIC ANALYSIS OF CAPILLARIES AND MUSCLE FIBERS

All morphometric analyses were performed blindly by one investigator, and were performed using the method established by Hepple (1997). A light microscope, on-line with a microcomputer and video image analysis software (Mocha 1.2, Jandel Inc.), were utilized to view the stained sections of muscle. Muscle fibers and their respective capillaries were quantitated manually from the microscopic image. From the serial sections derived from each animal, 25 muscle fibers of each type from the total pool of Type I and Type II muscle fibers were randomly selected. From each group of animals,
the total number of muscle fibers counted were as follows: D 200 Type I, 200 Type II; IGT 175 Type I, 175 Type II; C1 125 Type I, 125 Type II; C2 125 Type I, 125 Type II.

The capillary supply was estimated for each muscle fiber, using several indices for quantitation of the capillary supply (Plyley, 1990; Hepple, 1997): 1) the number of capillaries around each fiber (capillary contacts; CC), 2) the capillary:fiber ratio assessed on an individual fiber basis (individual capillary:fiber ratio; C:Fi), and 3) the number of fibers sharing each capillary (sharing factor; SF). Changes in CC represent an absolute measure of new capillary development, and hence the potential for an increased ability for the delivery of insulin and glucose. The individual capillary:fiber ratio (C:Fi) was determined by taking the sum of the fractional contribution of each capillary supplying an individual fiber using the following equation (Hepple, 1997): C:Fi= [CC in contact with 2 fibers x .50]+[CC in contact with 3 fibers x .33+[CC in contact with 4 fibers x .25]+[CC in contact with 5 fibers x .2].

The fiber area (FA;µm²), fiber perimeter (P;µm), and fiber shape (SF) for each fiber were measured from images captured by the image analysis software. Each muscle fiber was traced using a mouse pointing device, and the area, perimeter, and shape of the fiber outlined by the tracing, was determined by the commercial software. The software was calibrated which involved capturing an image of a stage mirometer (with x and y axis gradations) under the microscope at the same magnification as the muscle sections. A three-point calibration was required to facilitate the transformation of pixels into meaningful measurement units, i.e., square microns and microns. This method of calibration accounts for discrepancies between the microscope image and the captured image of a muscle section. Area is determined by summing the numbers of pixels contained in the muscle fiber, and the calibration allows the area to be given in the units specified in the calibration, i.e., square microns. Perimeter is determined by summing the contributions of all pixels forming the boundary of a muscle fiber, and similarly, with the calibration, this boundary pixel number is converted to the calibrated units (i.e. microns).

Shape factor is a measure of how nearly circular a given object is (Shape Factor= 4Π x Area/Perimeter). A perfect circle has a shape factor of 1.0, and a line has a shape
factor close to 0. Thus, the software compared the area of each muscle fiber to that of a circle with the same perimeter. Theoretically less circular muscle fibers, and therefore fibers which are more oblong or elliptical, would have a smaller area relative to their perimeter. Compared to a circle having the same perimeter, the shape factor of an elliptical muscle fiber would be less than than of a circle (i.e., <1.0).

In order to establish a measure which reflects the potential for blood-tissue exchange and includes an assessment of diffusion distance, the amount of fiber area supplied by each capillary contact (FA/CC; μm²) was determined for each fiber by dividing a fiber's area by its capillary supply in terms of the number of capillary contacts.

To examine potential alterations in the carrier- or receptor-mediated aspects of blood-tissue exchange between the capillaries and muscle fibers, such as glucose transport across the sarcolemma, and to assess the relative surface area of the capillary supply of a fiber compared to the fiber's surface area available for diffusion/transport, the ratio of a fiber's perimeter to its individual capillary:fiber ratio (P/C:Fi; μm) was determined for each fiber.

### 2.5 STATISTICAL ANALYSIS

All data are presented as means ± standard error (SEM). A students's t-test was utilized to compare the differences in the mean values between: 1) the D group and C1, 2) the IGT group and C2, and 3) the D group and IGT group. The significance of differences between the groups was based on a probability level of p≤0.05.

The sequence of methods used in the current study are presented in a flow chart (Appendix 6.4).
CHAPTER 3

RESULTS

3.1 CHARACTERISTICS OF DIABETIC, IGT & CONTROLS RATS

Table 3.1 summarizes parameters characterizing the long-term effects of streptozotocin-induced diabetes and impaired glucose tolerance in female rats. Diabetes caused a pronounced decrease in body mass. Diabetic rats weighed, on average, 21% less than the impaired glucose tolerant group. By experimental design, the weight of the D and IGT groups was not significantly different from their corresponding control groups, C1 and C2 respectively.

<table>
<thead>
<tr>
<th>Weight (g)</th>
<th>Age (wk)</th>
<th>Blood Glucose (mmol L⁻¹)</th>
<th>Area Under Curve (min.mmol L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIABETIC</td>
<td>259±6.1cd</td>
<td>24.1±4.2bcd</td>
<td>84.8±1.0bcd</td>
</tr>
<tr>
<td>CONTROL 1</td>
<td>260±1.6cd</td>
<td>5.6±0.2a</td>
<td>27.2±1.9ac</td>
</tr>
<tr>
<td>IGT</td>
<td>328±8.5ab</td>
<td>5.9±3a</td>
<td>42.0±4.6abd</td>
</tr>
<tr>
<td>CONTROL 2</td>
<td>316±16.9ab</td>
<td>5.7±2a</td>
<td>29.8±1.1ac</td>
</tr>
</tbody>
</table>

Table 3.1 Physical Characteristics and Blood Glucose Parameters of Female Rats

<table>
<thead>
<tr>
<th></th>
<th>DIABETIC</th>
<th>CONTROL 1</th>
<th>IGT</th>
<th>CONTROL 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>259±6.1cd</td>
<td>260±1.6cd</td>
<td>328±8.5ab</td>
<td>316±16.9ab</td>
</tr>
<tr>
<td>Age (wk)</td>
<td>22</td>
<td>14</td>
<td>22</td>
<td>28.5</td>
</tr>
<tr>
<td>Blood Glucose (mmol L⁻¹)</td>
<td>24.1±4.2bcd</td>
<td>5.6±0.2a</td>
<td>5.9±3a</td>
<td>5.7±2a</td>
</tr>
<tr>
<td>Area Under Curve (min.mmol L⁻¹)</td>
<td>84.8±1.0bcd</td>
<td>27.2±1.9ac</td>
<td>42.0±4.6abd</td>
<td>29.8±1.1ac</td>
</tr>
</tbody>
</table>

Table 3.1 and 3.2: statistically significant p<0.05 a=diabetic b=control 1 c=IGT d=control 2

Twelve weeks after the streptozotocin injection, the fasting plasma glucose concentration was significantly elevated in the diabetic rats compared with IGT and controls animals (Table 3.1). Furthermore, although the fasting blood glucose levels (Time 0) in the IGT rats appeared to be normal, the results of the glucose tolerance test showed that the IGT rats displayed an impaired glucose tolerance, i.e., a higher than normal peak at 30 and 60 minutes following administration of a glucose load (Figure 3.1). The IGT group however, returned to baseline values within the time frame of the glucose tolerance test. Area measurements under the glucose curve also showed that both the D and IGT rats differed significantly from each other and control animals in their response to a glucose load (Table 3.1 and 3.2)
FIGURE 3.1 Response after administration of a glucose load. The values are means ± SE. p ≤ 0.05 a = versus D, b = versus IGT, c = versus C1, d = versus C2.

D: 24.1±1.5  30.0±.01  29.55±.45  26.35±1.0
IGT: 5.90±.32  19.0±1.5  16.4±3.2  7.15±.71
C1: 5.62±.15  13.3±.85  6.86±.64  6.12±.13
C2: 5.68±.22  15.2±.58  8.52±.54  6.06±.17
Table 3.2 Matrix Defining Glucose and IGT Status of Groups

<table>
<thead>
<tr>
<th>BLOOD GLUCOSE CONCENTRATION</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>AREA UNDER CURVE - D</td>
<td>-</td>
<td>C1,C2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>NR</td>
</tr>
</tbody>
</table>

where (+) = normal value (-) = abnormal value

3.2 DIABETES- AND IGT-INDUCED ALTERATIONS IN FIBER MORPHOLOGY

The data in Figure 3.2 and Figure 3.3 demonstrate that the STZ-diabetes and IGT results in significant muscle fiber atrophy in both the Type I and Type II muscle fibers. Fiber area (μm²) of both type I and type II fibers was reduced (p<0.05) in the D rats (type I:2786±54.2; type II 1363±41.5) and IGT rats (type I:3274±84; type II 1769±58) compared with the control rats (control 1 type I :7522±185; type II: 3647±97: Control 2 type I 5803±149; type II 3230±107). Similarly, the perimeter (um) of both the Type I and Type II muscle fibers from the D (type I 235±2.2; type II 164±2.4) and IGT (type I:252±3.6; type II:186±3.1) rats was significantly less (p<0.05) than the control animals (Control 1 type I 375±4 type II 261±3.3; Control 2 type 1 316±4.4; type II 236±3.9). The reduction in muscle fiber area and perimeter in the D animals was significantly greater (p<0.05) than that of the IGT rats, a potential impact of the severity of the metabolic state in the D animals.

The shape factor of type I and II muscle fibers was significantly different (p<0.05) between D (type I .63±.006; type II .61±.005) and IGT (type I .64±.006; type II .62±.006) rats versus controls (Control 1 type I .67±.007; type II .66±.006: Control 2 type I .72±.006; type II .71±.006). The shape of muscle fibers in the D and IGT groups was not significantly different. (Figure 3.4).
Figure 3.2 Fiber area in Type I and II muscle fibers. The values are means ± SE. 

p < 0.05; a = versus D, b = versus IGT, c = versus C1, d = versus C2.
FIGURE 3.4 Fiber perimeter in Type I and II muscle fibers. The values are means ± SE.

p≤.05  a = versus D, b = versus IGT, c = versus C1, d = versus C2.
SHAPE FACTOR

FIGURE 3.4 Shape factor in Type I and II muscle fibers. The values are means ± SE.

p < 0.05 a = versus D, b = versus IGT, c = versus C1, d = versus C2.
3.3 CAPILLARY CHANGES DURING DIABETES AND IGT

With respect to both type I and type II muscle fibers, the mean capillary contacts of a fiber (CC) were significantly reduced (p<0.05) in the D and IGT females compared to the control animals (D type I: 4.4±1.0 type II:3.9±1.2; IGT type I:4.6±.12 type II:3.9±.12; Control 1 type I:5.9±1.1 type II:5.0±.11; Control 2 type I:5.7±.11; type II 5.1±.11). No significant differences were observed in the number of capillary contacts between the D and IGT females. CC did not differ significantly between the C1 and C2 groups. (Figure 3.5).

In contrast to the control rats (Control 1 type I:1.95±.05 type II:1.72±.05; Control 2 type I:1.89±.04 type II:1.72±.05), the "shared" number of capillaries supplying individual muscle fibers (C:Fi) was significantly lower (p≤0.05) in the D (type I:1.48±.04 type II:1.36±.05) and IGT (type I:1.63±.05 type II:1.41±.05) rats. (Figure 3.6). A significant difference in C:Fi of Type I fibers (p≤0.05) was also observable between the D and IGT animals.

3.4 EFFECT OF DIABETES AND IGT ON CAPILLARY-FIBER MORPHOLOGY

Figure 3.7 and 3.8 display the significant differences (p<0.05) in FA/CC and FA/C:Fi between the D and IGT rats versus controls, respectively. FA/CC (type I:757±35.5 type II:405±15.0) and FA/C:Fi (type I:2226±99 type II:1201±47) in the D and NR rats (FA/CC type I:780±29 type II:538±28; FA/C:Fi type I:2348±106 type II:1622±100) were decreased compared to both the Control 1 (FA/CC type I:1336±40.5 type II:776±28; FA/C:Fi type I:4158±145 type II:2379±111) and Control 2 (FA/CC type I 1033±28 type II:641±18; FA/C:Fi type I:3222±102 type II:1953±64) animals. When FA/CC and FA/C:Fi were compared between the D and IGT rats, they were found to be significantly reduced (p≤0.05) only in the type II muscle fibers of the D rats.
FIGURE 3.5: Capillary contacts in Type I and II muscle fibers. The values are means ± SE.

P = 0.05 a = versus D, b = versus IGT, c = versus C1, d = versus C2.
FIGURE 3.6: Individual capillary: fibre ratio in Type I and II muscle fibres. The values are means ± SE.

a = Versus D, b = Versus C1, c = Versus C1, d = Versus C2.
FIGURE 3.7 FA/CC in Type I and II muscle fibers. The values are means ± SE.

p≤.05  a = versus D, b = versus IGT, c = versus C1, d = versus C2.
FIGURE 3.8 FA/C:Fi in Type I and II muscle fibers. The values are means ± SE. 
p < .05  a = versus D, b = versus IGT, c = versus C1, d = versus C2.
P/C:Fi did not significantly differ amongst the four groups (D type I:190±7.8 type II:155±6.4; IGT type I:187±8.5 type II:169±9.8; Control 1 type I:208±6.1 type II:172±7.5; Control II type I:176.5±4.4 type II:147±3.8). (Figure 3.9). When P/CC was analyzed, the D group (type I:61.7±2.22 type II 52.0±1.98), did not significantly differ from IGT (type I: 61.8±2.29 type II 58.0±2.54) or the controls (Control 1 type I:66.8±1.57 type II 55.8±1.60; Control 2 type I:56.6±1.08 type II:48.0±.911). No differences were observed in P/CC between the IGT rats (type I: 61.8±2.29 type II 58.0±2.54) and the C1 animals (type I 66.8±1.57 type II 55.8±1.60). In type I muscle fibers, P/CC did not differ between the IGT group (61.8±2.29) and C2 group (type I 56.6±1.08), but a difference was observed in the type II fibers (NR type II 58.0±2.54 C2 type II 48.0±.91).

3.5 SUMMARY OF FINDINGS

The findings of this study have been summarized in Table 3.3.
FIGURE 3.9 P/C:Fi in Type I and II muscle fibers. The values are means ± SE. No significant differences exist between groups.
<table>
<thead>
<tr>
<th>TABLE 3.3 SUMMARY OF EXPERIMENTAL RESULTS</th>
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<tr>
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<tr>
<td><strong>WEIGHT (g)</strong></td>
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<td>DIABETIC</td>
</tr>
<tr>
<td>259±6.1&lt;sup&gt;cd&lt;/sup&gt;</td>
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<tr>
<td>CONTROL 1</td>
</tr>
<tr>
<td>260±1.6&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>NON-RESPONDER</td>
</tr>
<tr>
<td>328±8.5&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>CONTROL 2</td>
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<tr>
<td>316±16.9&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>BLOOD GLUCOSE (min·mmol L&lt;sup&gt;-1&lt;/sup&gt;)</strong></td>
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</tr>
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<td>24.1±4.17&lt;sup&gt;bcd&lt;/sup&gt;</td>
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<tr>
<td>CONTROL 1</td>
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<td>5.6±15&lt;sup&gt;*&lt;/sup&gt;</td>
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<td>NON-RESPONDER</td>
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<tr>
<td>5.9±32&lt;sup&gt;*&lt;/sup&gt;</td>
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<td>CONTROL 2</td>
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<tr>
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</tr>
<tr>
<td><strong>AREA UNDER CURVE (mmol L&lt;sup&gt;-1&lt;/sup&gt;)</strong></td>
</tr>
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<td>CONTROL 2</td>
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<td>1.95±.05&lt;sup&gt;abcd&lt;/sup&gt;</td>
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<td>1.72±.05&lt;sup&gt;abcd&lt;/sup&gt;</td>
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<td>FA/CFi (um&lt;sup&gt;2&lt;/sup&gt;)</td>
</tr>
<tr>
<td>2226±99&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>1201±47&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>4158±145&lt;sup&gt;cd&lt;/sup&gt;</td>
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<td>3.04±0.3&lt;sup&gt;ab&lt;/sup&gt;</td>
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<td>3.07±0.3&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.99±0.3&lt;sup&gt;ab&lt;/sup&gt;</td>
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<tr>
<td>2.94±0.3&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.92±0.05&lt;sup&gt;ab&lt;/sup&gt;</td>
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<tr>
<td>3.06±0.4&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>3.02±0.3&lt;sup&gt;ab&lt;/sup&gt;</td>
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</table>

The values are the means ± SE. a = p<.05 versus D, b = p<.05 versus NR, c = p<.05 versus C1, d = p<.05 versus C2. *Sharing Factor = CC/C:Fi
CHAPTER 4
DISCUSSION

To our knowledge, the present study is the first to describe individual muscle fiber and capillary morphology in diabetic (D) female rats. The present study is also unique in that it describes changes in the capillary-muscle fiber morphology in females with demonstrated impaired glucose tolerance (IGT), i.e., a condition in which the fasting level of glucose is normal, but the response to a glucose load is abnormal, as evidenced here by the IGT group.

With respect to fiber area and perimeter, the muscle fibers of the females in both the D and IGT groups were significantly smaller than those from the weight-matched controls. The decrement in muscle fiber area was greater than that observed in muscle fiber perimeter, indicating that a change in fiber shape has occurred. Interestingly, the number of capillaries around the corresponding muscle fibers were also significantly reduced in the experimental animals; however, the reduction in the capillary supply was not to the magnitude as the reduction in muscle fiber area, resulting in a decreased fiber area per capillary. On the other hand, muscle fiber perimeter in relation to the capillary supply did not change across the groups of animals.

4.1 COMPARISON WITHIN THE LITERATURE

When comparing the changes in skeletal muscle morphology among the various animal models of diabetes, it is important to consider such factors as the dosage of STZ used to induce diabetes, the duration of the diabetic state, the particular muscle examined, the level of assessment (i.e., whole muscle tissue vs. individual fibers), and gender differences. It should be noted that male animals were not investigated in the present study. Therefore, the following discussion includes a comparison between the findings from the current study and literature values of male animals.

The primary cause of the abnormal metabolism in the STZ-injected animals was a deficiency of insulin. The severity of the STZ-induced hypoinsulinemia varies depending
on the dosage of STZ used (Junod et al., 1969). In rats, diabetes is induced using doses of STZ ranging from 45 to 70 mg kg\(^{-1}\) body mass. In the present study, diabetes was induced in the female rats utilizing an STZ dose of 65 mg kg\(^{-1}\) body mass. Rats receiving 60-70 mg kg\(^{-1}\) body mass of STZ exhibit a marked hyperglycemia (Ar'Rajab and Ahren, 1993). Permanent hyperglycemia may be detected as early as 18 hours after a diabetogenic dosage of 60 mg kg\(^{-1}\) of STZ, and has been shown to increase progressively 18-27 hours post-STZ injection (Wong and Wu, 1994). A significantly lower pancreatic insulin content was found after 28 days, when compared to that seen after 24 hours and 7 days with a dose of 65 mg kg\(^{-1}\) body mass of STZ (Junod et al., 1969). Streptozotocin administration at dose levels exceeding 40 mg kg\(^{-1}\) body mass results in a long-term, stable hyperglycemia with no insulin response to glucose at 3 months, and a marked derangement of islet morphology, i.e., few insulin cells (Ar'Rajab and Ahren, 1993). Lower doses (30-40 mg kg\(^{-1}\) body mass) are less effective (Wong and Wu, 1994), inducing only a transient diabetes, which frequently results in spontaneous recovery (Junod et al., 1969; Ar'Rajab and Ahren, 1993). Higher doses (i.e., 80 or 100 mg kg\(^{-1}\) body mass STZ) can place the life of the animal at risk (Junod et al., 1969; Wong and Wu, 1994), but most often elicits the cellular alterations over a shorter period of time.

The dose of STZ used in most studies to date ranges from 60 to 200 mg kg\(^{-1}\) body mass in male animals (Armstrong et al., 1975; Medina-Sanchez et al., 1991; Kleuber and Feczko, 1994), and 65 to 70 mg kg\(^{-1}\) body mass in female animals (Hegarty and Rosholt, 1981; Sexton et al., 1994). Thus, the administration of STZ in a dose of 65 mg kg\(^{-1}\) body mass, as in the present study, permits a valid comparison, at least in terms of the STZ-dosage, between the current results and the findings from previous reports. However, two reports on muscle morphology in male mice following the administration of STZ utilized a dose of 200 mg kg\(^{-1}\) body mass. This dose of STZ resulted in muscle atrophy similar to that observed in the male rats in other investigations. The observed alterations in muscle morphology during the STZ-induced diabetes are in agreement with the previous findings indicating alterations to both Type I and II fibers, with major changes occurring in the Type II fibers. Although we did not differentiate between the FG and FOG fibers in the present study, we found a significant overall decrease in Type
II fiber area in diabetic females, similar to that which has been observed previously in male diabetic animals (Armstrong et al., 1975; Medina-Sanchez et al., 1991; Klueber and Feczko, 1994). In contrast to the observations in male animals (Armstrong et al., 1975; Medina-Sanchez et al., 1991), both the fiber area and perimeter of the Type I fibers in the diabetic female rats in this study were significantly reduced compared to the weight-matched controls. The smaller fibers reported here for female diabetic rats are in agreement with the findings of Sexton et al. (1994), who also observed an overall decrease in both fiber area and perimeter in the plantaris muscle of diabetic female rats. On the other hand, Hegarty and Rosholt (1981) reported finding no difference in the diameters of fibers from the biceps brachii, sternomastoideus and soleus muscles of diabetic females rats. It must be noted however, that both Sexton et al. (1994) and Hegarty and Rosholt (1981) reported data on the complete fiber population, with no differentiation between fiber types.

The duration of the diabetic state could also be responsible for some of the differences in results observed here compared with the findings of other studies. The variation in the duration of the diabetes between the various studies ranged from just over 4 weeks to 11 weeks in the male animals (Armstrong et al., 1975; Medina-Sanchez et al., 1991; Klueber and Feczko, 1994), and from 6 to 14 weeks in the female animals (Hegarty and Rosholt, 1981; Sexton et al., 1994). Nonetheless, similar changes in muscle morphology were noted between the shorter and longer periods of diabetes in the male animals. The results from the current study, in which the plantaris muscle in female rats was examined following 14 weeks of diabetes, were in general agreement with the alterations reported by Sexton et al. (1994) in the plantaris muscle of female rats after 6-8 weeks of STZ-induced diabetes. On the other hand, the morphological changes observed in the present study are in conflict with the results of Hegarty and Rosholt’s (1981), who evaluated biceps brachii, sternomastoideus and soleus muscles of female rats following 7 weeks of STZ-diabetes. The diversity of the muscles examined may also help to explain the variable results reported in the literature concerning morphological alterations with STZ-induced diabetes. For example, in the present study, capillary-fiber morphology was analyzed in the plantaris muscle; this muscle was selected for analysis because it reflects
the muscle fiber composition of the rat hindlimb better than any other individual muscle (Sexton et al., 1994). The plantaris muscle contains 7% slow-twitch, oxidative (SO) fibers, 40% fast-twitch oxidative-glycolytic (FOG) fibers, and 53% fast-twitch glycolytic (FG) fibers in comparison to the overall composition of the rat hindlimb, which is composed of 5% SO fibers, 19% FOG fibers, and 76% FG fibers (Sexton et al., 1994). Two studies involving male rats investigated morphological changes in the lateral and medial heads of gastrocnemius muscle (Armstrong et al., 1975) and the rectus femoris muscle (Medina-Sanchez et al., 1991). The fiber-type compositions for these muscles are as follows: 1) the lateral head of the gastrocnemius is comprised of 16% FOG, 84% FG and 0% SO, 2) the medial head contains 62% FOG, 8% FG, and 30% SO, and 3) the red and white portions of the rectus femoris consist of 53% FOG, 40% FG and 7% SO, and 25% FOG, 74% FG and 1% SO, respectively (Armstrong and Phelps, 1984). The extensor digitorum longus muscle, with a fiber-type profile of 42% FOG, 56% FG and 2% SO (Armstrong and Phelps, 1984) was examined in male mice by Klueber and Feczko (1994). Hegarty and Rosholt (1981) analyzed changes in several muscles including the soleus muscle (13% FOG; 0 % FG; 87 % SO) in both male and female diabetic rats (Armstrong and Phelps, 1984). Sexton et al. (1994) also chose to analyze diabetes-induced changes in the plantaris muscle.

Few studies have reported changes in fiber and/or muscle morphology during STZ-induced diabetes in relation to the capillary supply. Armstrong et al. (1975) reported a trend for fewer capillaries only in the FG fibers of male rats. Bohlen and Niggl (1980) observed a decrease in the capillary density of the cremaster muscle from male mice. Sexton et al. (1994) observed a decrease in the global capillary-to-fiber ratio and an increase in the capillary density of plantaris muscle from diabetic female rats. These results are different than the changes in capillary-fiber morphology observed in the current study, i.e., a decrease in capillary contacts and a reduction in the fiber area per capillary contact. Bear in mind that the indices for capillary quantification and the level of morphometric assessment varied between this study and the Sexton et al. (1994) study.

The capillary density (CD) is assessed by counting the number of individual capillary profiles per unit area (usually per mm²) that appear in a transverse section in
muscle tissue. The global capillary-to-fiber ratio (C:F) is derived from the quotient of the CD and the corresponding fiber density (FD) from the same transverse section of muscle tissue (Hepple, 1997). However, both of these measurements provide only a global index (i.e., a population mean) of capillarization in the muscle as whole, and yield little information on the status of the capillary supply of individual muscle fibers.

The level of assessment at which the observations were made, i.e., on the basis of individual muscle fibers (this study) versus on the basis of the muscle as a whole (Armstrong et al., 1975; Bohlen and Niggl, 1980; Sexton et al., 1994), not only provides a means of explaining the variations in capillary-fiber morphology reported, but is of practical importance in interpreting the physiological alterations in function. Specifically, changes in the capillary supply at the level of individual muscle fibers may not be evident when assessed over the whole muscle as is the case with global descriptors of capillary quantification. In addition, it is known that skeletal muscle fibers differ in a number of physiological properties, including those related to glucose uptake and/or metabolism. Type I fibers have been reported to have a denser capillary network (Hudlicka et al., 1992), a greater capacity for blood flow (James et al., 1985), and contain a higher amount of glucose transporters (GLUT 1 and GLUT 4) in both the basal and insulin-stimulated states (Henricksen et al., 1990; Barnard and Youngren, 1992; Marette et al., 1992; Han et al., 1995b; Handberg, 1995). Thus, Type I fibers exhibit a greater insulin-stimulated glucose uptake, which may in part, be due to a greater insulin binding in the Type I fibers (Bonen et al., 1981), or to a lower number of insulin receptors in the Type II fibers (Barnard and Younggess, 1992). The designation of muscle fibers as being insulin sensitive (Type I) and insulin-insensitive (Type II) warrants the description of capillary-fiber morphology on an individual fiber basis, and provides a link between any adaptive changes in muscle fiber morphology and changes in metabolism during an altered metabolic state.

In male rats, chronic streptozotocin-induced diabetes differentially affects the various types of muscle fibers in skeletal muscle (Armstrong et al., 1975; Medina-Sanchez et al., 1991). Armstrong et al. (1975) were the first to show that in chronic STZ-induced diabetes, distinct alterations occur in the various fiber types of skeletal
muscle from diabetic male rats compared to controls. These observations were later confirmed by Medina-Sanchez et al. (1991) who reported that insulin deficient diabetes had a negative effect on the fast-twitch muscle fibers (FG; FOG), viz., the fast-twitch fiber area was decreased, while the slow-twitch muscle fibers (SO) were less affected by this condition. In the female rats in the current study, the STZ-induced diabetes did not appear to differentially affect the two types of muscle fibers per se. No distinction was seen in fiber type alterations between the D, IGT and weight-matched control rats. The observed differences in the present study were only notable between the D and NR rats, and not in the control animals, where the C:Fi was lower only in the Type I fibers, and the FA/CC was reduced only in the Type II fibers of the D females. The effect of diabetes on the various muscle fibers types in female rats has not been reported previously.

Differences between the female data from the present study and literature reported male data may also be due to gender differences. Accumulating evidence supports this view. Female animals are less susceptible to experimentally-induced diabetes (Rossini et al., 1978; Paik et al., 1980; Paik et al., 1992; Shi et al., 1994), male animals have a greater propensity for the development of glucose intolerance (Kava et al., 1989), and testosterone treatment of female animals results in a diminished capillary density and blood flow with an associated insulin resistance (Holmang et al., 1990; Marin et al., 1994; Bjorntorp, 1995). More recently, there is evidence that: 1) estrogen has a direct effect on vasodilation (Lieberman et al., 1994; Sowers, 1998), 2) estrogen binds to skeletal muscle and heart muscle (Godsland, 1996), and 3) estrogen induces increases in insulin sensitivity (McKerns et al., 1958; Rushakoff and Kalkhoff, 1981; Lindheim et al., 1993; Shi et al., 1994; Godsland, 1996). The basis for sexual dimorphism clearly appears to be related to sex steroid hormones, such as estrogen and androgen (Paik et al., 1982; Kava et al., 1989; Rodgers et al., in press). Taken together then, the current data point to the possibility that female animals adapt differently to diabetes and that capillary-fiber morphology may be another reflection of this difference. This observation warrants future investigation.

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4.2 LIMITATIONS OF THE CURRENT STUDY

4.2.1 EXPERIMENTAL MODEL

The female rats utilized in this study were obtained from a previous study in which experimental diabetes was induced by injecting 65 mg kg$^{-1}$ body mass of STZ during various phases of the rat estrous cycle (Rodgers et al., in press). Rodgers et al. (in press) found that the STZ failed to induce diabetes in all the females injected with the drug. The IGT group represents those animals which did not become diabetic, yet exhibited an impaired glucose tolerance. It has been reported that STZ sensitivity is determined by estrogen (Paik et al., 1982). The diabetogenic effect of STZ involves a selective interaction of the toxin with the islet beta-cells through binding to the cell surface or via uptake into the cell, eventually leading to pancreatic damage (Paik et al., 1982). Paik et al. (1982) proposed that estrogen inhibits STZ transport by the islet beta-cells, thereby resulting in a reduction in the diabetogenic effects of the toxin. Earlier studies have also reported that estrogen exerts a cytotropic effect on the pancreatic beta-cell, thereby increasing insulin secretion (Spellacy et al., 1972). The increase in insulin content of the pancreas appears to be associated with an increase in islet tissue and hyperactivity of the beta-cell (Spellacy et al., 1972).

Insulin levels were not measured in the present study. This poses a limitation to interpreting the response of the experimental animals, particularly with respect to the IGT group. Further, an important question in any study investigating diabetes is whether the abnormalities observed in the diabetic state can be reversed with insulin (Mueller, 1984). Because of the longevity of this study (12 weeks), no attempt was made to treat any of the females with insulin. Insulin treatment however would have distinguished whether the changes observed were due to an insulin effect.

Ideally, to determine muscle morphological characteristics for females with Type I diabetes and IGT consideration must be given to the factors which influence muscle morphology, i.e., gender, mass and age (Marin et al., 1994). Male rats were not introduced in this study and as a result, direct inferences cannot be made with regard to
the role played by gender in the observed capillary-fiber changes of the D and IGT rats.

There was also a difference, although not significant, between the body mass of the IGT group and their weight matched controls (Control 2 - C2). Two of the control animals never did reach 300 grams, as their weight stabilized at 28.5 weeks to 285 grams. The mean life expectancy of Sprague-Dawley rats is about 27-30 months, with many surviving longer than 3 years (Burek and Hollander, 1980). Thus, in terms of biological age, the C2 females would be considered the equivalent of young adults and not aged animals. Therefore, the discrepancy in mass was likely a result of the breeding of these animals. Specifically, the female Sprague-Dawley rats, which we received, were outbred, meaning that they carried different genetic profiles. Consequently, even with exposure to the same environmental conditions, these animals experienced different growth patterns, and while some will be expected to exceed 300 grams, others may stabilize at a lower weight (Couture, 1998). Even accounting for the discrepancy in body mass, there were still significant differences in capillary-fiber morphology, which suggests that the differences in mass represented only a minor limitation in the present study.

It is well known that the mean fiber area is positively correlated with relative body mass (Lithell et al., 1981). For this reason, we chose weight-matched controls versus age-matched controls, even though muscle changes have been reported with aging (Brooks et al., 1996; Aspnes et al., 1997). Furthermore, it is clear that the age range of the rats utilized in the present study (i.e., D and IGT at 22 weeks of age vs C1 at 14 weeks of age vs C2 at 28.5 weeks of age) includes animals from what would be collectively considered young adulthood. Thus, the relevance of these issues to the current study lies in the understanding that body mass is a more important consideration than age with respect to our investigation of capillary muscle-fiber morphology.
4.2.2 CAPILLARY QUANTIFICATION

Our attempt to provide the most accurate and meaningful measures of the capillary supply to individual muscle fibers has involved the utilization of specific quantitative indices of capillary-fiber morphometry.

We refrained from using conventional estimates, such as capillary density (CD), primarily because such measures express the capillarization globally, i.e., as a population average. Because CD is sensitive not only to changes in the number of capillaries, but also to factors that affect fiber size, such as hypertrophy and atrophy, changes in CD are difficult to interpret. In addition, muscle fiber shrinkage can result from histochemical processing, which can also affect the measurement of fiber area, and thus, haphazardly decreases the estimate of diffusion distance. With the staining method applied in this study, the degree of fiber shrinkage is negligible (Hepple, 1997).

Another conventional index of muscle capillarization, the global capillary-fiber ratio (C:F), was also not used in the present study. While the C:F accounts for any changes in fiber size, it is another global index of muscle capillarization, and has limited value as it does not account for diffusion distance.

The individual fiber-based measurements (i.e., CC; C:Fi; FA/CC; P/C:Fi) used in this study have been shown to be more sensitive expressions of changes in the capillary supply, and may be useful in evaluating alterations in capillary-fiber geometry during altered metabolic states (Hepple, 1997).

It should be pointed out that the two relative measurements used here, FA/CC and P/C:Fi, incorporate estimates of both the capillary supply and fiber size, namely fiber cross sectional area (= \( \Pi \) radius\(^2 \)) and fiber surface area (= \( 2 \Pi \) radius \( \times \) length), with both estimates being made on 100-200 individual fibers from each animal. It is important to note that any estimation of the capillary supply to individual muscle fibers may be viewed as limiting, because it requires the assumption that all capillaries have a comparable potential for perfusion, and an equal capacity for diffusion.

It should also be noted that an elliptical fiber shape would alter the distance from the perimeter to the center of a muscle fiber (i.e., diffusion distance). Thus, an additional
drawback may be that we are unable to define the extent to which these various factors might contribute to the changes in the overall diffusion potential between the capillaries and the muscle fibers.

4.2.3 INFLUENCE OF THE "TIMING" OF THE ANALYSIS

In spite of the limitations associated with the current study, capillary-fiber morphology in diabetes remains a question of considerable importance, particularly as it relates to the influence which hemodynamics and diffusion have on insulin-mediated glucose uptake in skeletal muscle.

In the present study, capillary-fiber morphology was analyzed in the plantaris muscle following 12 weeks of STZ-induced diabetes. In general, there was a significant fiber atrophy, and a loss of capillaries supplying individual fibers.

It has been suggested that an increase in blood flow results in an increased mechanical distortion of the capillary lumen, which, in turn, is believed to be an important stimulus for capillary proliferation (Hudlicka et al., 1992). Early in the development of the insulin-dependent diabetic state, there is an increased blood flow in the renal, retinal and peripheral microcirculation, which appears to be related to glycemic control (Zatz and Brenner, 1986; Tooke, 1993, 1994; Williamson et al., 1993). The increased flow results from a reduced vascular resistance and leads to an increased hydrostatic pressure (Parving et al., 1983). As a result, capillary proliferation would be expected as a result of the damage connected with the mechanical consequences of the increased shear stress that results from both the increased red blood cell velocity, and the increased capillary wall tension, which is a consequence of an increased capillary pressure (Hudlicka et al., 1992). Since permanent hyperglycemia can be detected as early as 18 hours after a diabetogenic dosage of 60 mg kg⁻¹ body mass of STZ (Wong and Wu, 1994), and given that diabetes in the present study was induced using a similar dosage of STZ, it may be presumed that the D rats became hyperglycemic early in the process. The high glycemic levels may in turn, have produced an increase in capillary blood flow. Thus, our analysis of capillaries in the diabetic rats at only one time point (i.e., at the end
of the study) is a limitation to the study in that we have missed any potential increase in the capillary supply which may have been observed in the diabetic females earlier in the study.

Similarly, an acute exposure to a high insulin concentration may inhibit vascular smooth muscle contractility, which in turn, leads to: 1) a decreased response of the resistance vessels to any degree of neurohormonal stimulation, 2) an increased blood flow to the microcirculation, 3) an increase in hydrostatic pressure, and 4) an increase in vascular permeability (McNally et al., 1995). Short-term (7 days) hyperinsulinemia has been shown to increase the capillary density in female rats (Holmang et al., 1993). In addition, following an acute exposure to moderate physiological hyperinsulinemia, Holmang et al. (1996) reported an increase in the number of mitoses in capillary endothelial cells in the soleus and extensor digitorum longus muscle of female rats after 3 days, and a moderate increase in the number of capillaries per unit muscle surface area in the extensor digitorum longus muscle at 7 days, and in the extensor digitorum longus and soleus muscles at 7 weeks. If the IGT group were indeed insulin resistant and exhibited hyperinsulinemia, an analysis of capillary supply at only one time point, as in the current study, may exclude any potential increases in skeletal muscle capillaries which may have occurred during the earlier stages of adaptation. Given the damaging effect of STZ on the pancreas, hyperinsulinemia in the IGT group is questionable. As a result, an increase in the number of capillaries in the IGT females at other time points during this study would have been unlikely.

### 4.3 Insulin Deficiency in the Experimental Rats

It is recognized that no direct comparisons can be made between human diabetes and experimental diabetes in animals. However, the experimental model of Type I diabetes used here, the streptozotocin (STZ) model, mimics insulin deficiency and hyperglycemia observed in human diabetes. A permanent diabetic phase is established within 24 hours after STZ administration (Junod et al., 1969; Wong and Wu, 1994). The diabetic state results primarily from the specific cytotoxic action of STZ on the β-cells of
the islets of Langerhans. The acute response to STZ is illustrated by a triphasic blood glucose curve comprised of the following segments: (a) an early hyperglycemia, which may be attributed to an adrenaline response, (b) a hypoglycemic phase, possibly due to the release of insulin from necrotizing pancreatic beta-cells, and (c) a precipitous fall in pancreatic insulin content with no further release of insulin into the circulation and hyperglycemia (Junod et al., 1969). STZ acts in a dose-dependent fashion (Junod et al., 1969; Wong and Wu, 1994), and at dosages of 50 to 70 mg kg\(^{-1}\) body mass, STZ induces a stable diabetes (AR’Rajab and Ahren, 1993).

An STZ dosage of 65 mg kg\(^{-1}\) body mass was utilized in the current study. This dose would result in the metabolic perturbations indicative of Type I diabetes, in particular insulin deficiency. Although insulin levels were not measured in the present study, we assume that an insulin deficiency occurred in the diabetic females following the STZ administration. This assumption is indirectly supported by the significant decrease in body mass which occurred in the animals of this group. At 22 weeks, the D female rats weighed significantly less than the IGT females. Normally, insulin activates the transport systems and enzymes involved in the intracellular use and storage of glucose, fatty acids and amino acids, and inhibits the catabolic processes involved in the breakdown of glycogen, fat and protein (Kahn and Folli, 1993). In Type I diabetes, an adequate level of insulin is not available to facilitate the movement of glucose into the cells, resulting in a shift in fuel utilization from glucose to fatty acids and protein (Ignatavicius and Bayne, 1991). An insulin deficiency in the D animals would have resulted in a decrease in fat and protein synthesis, commensurate with an increase in fat and protein breakdown; this would explain the significantly lower body mass of these animals. Moreover, it has been suggested that the degree of muscle atrophy is directly proportional to the severity of the diabetic state, which, in turn is reflected in the fasting levels of plasma glucose (Sexton et al., 1994). Considering that the D females in the current study demonstrated a marked hyperglycemia compared to both the IGT rats and control animals, a greater muscle atrophy (with respect to fiber area and perimeter), and thus, a reduced body mass, was to be expected in these animals.
The fasting blood glucose levels in the IGT rats was not significantly different from that observed in the control animals, which suggests the presence of similar circulating levels of endogenous insulin. However, the normoglycemic IGT rats exhibited an elevated plasma glucose level at 30 and 60 minutes during the glucose tolerance test, indicating a condition of altered insulin-mediated glucose uptake, i.e., glucose intolerance. One reason for the irregular or slower physiological response to the glucose load in the IGT rats may include an insulin deficiency, although not of the same magnitude as that found in the D group. The assumption of an insulin deficiency in the IGT rats is based on the fact that STZ was injected into all females in the IGT and D groups. Because STZ decreases by more than 60% within 10 minutes of injection, it would seem that only a small portion of the in vivo dose was actually responsible for damaging the pancreatic cells, and that the rate of STZ degradation in rat plasma is very rapid (Paik et al., 1982; Lee et al., 1993). However, the degradation products of STZ (i.e., the methyl nitroso moiety of STZ), which have been observed in the plasma, may be biologically active and capable of inducing diabetes (Lee et al., 1993). As well, Kroncke et al (1995) and Turk et al. (1993) have reported that the intracellular formation of nitric oxide during STZ degradation in the islets, contributes significantly to the induced cytotoxicity of STZ. Thus, it is reasonable to assume that the pancreas from the IGT rats incurred moderate damage, resulting in a decreased capacity for insulin secretion. An abnormal glucose tolerance and insulin response have been observed when pancreatic insulin secretion was depleted by about one-third, while a fasting hyperglycemia and gross glycosuria was developed when insulin was depleted by two-thirds and three-quarters, respectively (Junod et al., 1969). Moreover, with similar alterations in capillary-fiber morphometry being observed between the D and IGT rats, it’s reasonable to assume that a deficiency of insulin secretion was also evident in the IGT females. Insulin deficiency in the IGT rats however, would not be of the same magnitude as that predicted for the D group.

The efficiency with which insulin depresses the fasting blood sugar level, and with which it suppresses hyperglycemia following an oral or intravenous administration of glucose, is a function of both the amount of insulin released, as well as the sensitivity
of the body to insulin (Rizza et al., 1981). Disposal of an intravenous or oral glucose load is largely mediated by insulin-stimulated glucose transport into muscle tissue (Pollet, 1983). Because we did not measure insulin levels in the IGT group (or in any group for that matter), we also have to consider the possibility that the reduced insulin-mediated glucose uptake observed during the glucose tolerance test occurred as a result of a reduced sensitivity to insulin. In the latter case, a higher concentration of insulin would be needed to promote the same level of glucose transport (Sinacore and Gulve, 1993). In conditions, such as IGT, mild non-insulin dependent diabetes, and obesity, the primary metabolic lesion is a resistance to the action of insulin, and the physiologic response to the resistance is to increase insulin secretion in an attempt to minimize hyperglycemia (Reaven et al., 1976). Therefore, depending on the severity of insulin resistance in the IGT rats, it may be possible that these animals maintained a greater than normal level of circulating insulin in the post-absorptive state. In view of the partial β-cell destruction by STZ in the IGT group, high insulin levels would seem unlikely.

### 4.4 Changes in Muscle Fiber Morphology During Diabetes and IGT

#### 4.4.1 Muscle Fiber Distribution

No changes were observed in the muscle fiber type distribution of the plantaris muscle in either the diabetic or glucose intolerant groups in the present study. However, a decrease in the percentage of Type I muscle fiber population has been reported for diabetic muscle (Holmang et al., 1993; Marin et al., 1994), with a loss of Type I fibers being associated with hyperinsulinemia in Type II diabetes (Holmang et al., 1993; Marin et al., 1994).

The rats in the present study were 14 to 28.5 weeks of age. A decrease in the percentage of Type I fibers has been reported with aging (Cartee, 1995). On the other hand, Eddinger et al. (1985) have reported that a decrease in Type I muscle fibers is not consistently observed in all muscles with aging. Eddinger et al. (1985) reported that there
were no differences in the percent of Type I fibers among four groups of rats, aged 3, 9, 28 and 30 months, and that there was no apparent trend for an increase in the percent of Type IIa and IIb muscle fibers between the four age groups.

**4.4.2 MUSCLE FIBER SIZE**

Because the muscle fibers area and perimeter from the D rats were significantly reduced in size when compared to the fibers from the weight-matched controls, it may be assumed that the muscle fiber changes were due to the diabetic state. As mentioned previously (see Section 1.10), insulin is probably the single most influential factor involved in regulating and maintaining a positive protein balance in skeletal muscle by increasing protein synthesis and inhibiting protein degradation (Tischler, 1981). A lack of insulin is known to contribute to a significant increase in muscle protein turnover (Rutschmann et al., 1984), a decrease in protein synthesis (Rutschmann et al., 1984), an accelerated rate of proteolysis (Rutschmann et al., 1984; Tischler, 1981) and a shift in fuel utilization from glucose to fatty acids and protein (Ignatavicius and Bayne, 1991). In fully developed Type I diabetes, little or no insulin is produced (Zeman and Hansen, 1991), and as a result the diabetes is accompanied by a reduction in weight gain, and severe muscle atrophy (Han et al., 1995a) through the loss of protein (Rutschmann et al., 1984). The significant reduction in both muscle fiber area and fiber perimeter are indicative of muscle atrophy in the D rats, which is consistent with the diabetic state.

The fiber morphometry data confirm the foregoing supposition in that the muscle fibers of the D females were significantly reduced in size with respect to fiber area and perimeter when compared to the muscle fibers from the impaired glucose tolerant (IGT) group. This finding suggests that the metabolic status of the D rats was much more severe than that of the IGT group. Interestingly, both the muscle fiber area and fiber perimeter of the IGT group were significantly less than the area and the perimeter of the fibers from the weight-matched controls. In most cases of impaired glucose tolerance, the data on fiber morphology have been limited to studies where body mass and/or obesity was a factor (Lithell et al., 1981; Lingarde et al., 1982; Lillioja et al., 1987).
Given that body mass and fiber size are highly correlated (Lithell et al., 1981), and weight-matched controls were utilized in the present study, it may be speculated that the changes in the muscle of the IGT rats represent a transition from IGT to Type I diabetes. Diabetes mellitus can be viewed as a disease characterized by a continuous spectrum with varying degrees of glucose intolerance (Reaven et al., 1976). Earlier reports have suggested that IGT may represent a stage in the development of Type I diabetes, and that the majority of persons with IGT remain in this class for many years or return to normal glucose tolerance (National Diabetes Data Group, 1979). A study involving 200 children with IGT reported that only 10% of these subjects progressed to insulin dependence during the 1 to 17 years of observation (Reaven et al., 1976). More recently, Eriksson et al. (1994) followed 29 subjects with IGT continuously for 15 years; muscle biopsies were taken and analyzed. The results from the study suggested that the muscle changes were early features of the diabetic process, preceding the disease by more than one decade (Eriksson et al., 1994). A scheme describing the potential transition from IGT to diabetes is discussed later in section 4.7.

4.4.3 MUSCLE FIBER SHAPE

We observed a decreased fiber area in relation to fiber perimeter in the muscle fibers from both the D and NR groups, as indicated by a significant change in the fiber shape factor. However, the shape factor also differed between the two groups of control animals. The mean age of Control Group 1 (C1) was approximately 14 weeks, while the mean age of Control Group 2 (C2) was 28.5 weeks. Many mammalian species, including humans and rats, exhibit age-associated changes in skeletal muscle, such as a decline in muscle mass (Brooks et al., 1996; Aspnes et al., 1997). In humans, muscle fiber diameter remains relatively constant, or shows only a modest decrease, through the age of 70 years. Thereafter, significant changes are more common and in very old rats, a 20 - 30% decline in muscle fiber diameter has been reported (Brooks et al., 1996). As mentioned previously (Section 4.2.1), the C2 females would be considered, in terms of biological age, the equivalent of young adults, no where near an aged animal. It is possible that the
changes in the shape factor we demonstrated resulted from a lack of precision in our technique i.e., different blade angles utilized during tissue slicing, etc. Although no study has yet to show a change in fiber shape, it is conceivable for muscle fibers to change from a round to a more elliptical shape in order to achieve a more optimal packing for transport activities. Furthermore, a change in shape would help to maximize the three-dimensional arrangement of the capillary network.

4.5 CAPILLARY LOSS IN DIABETIC AND IGT FEMALE RATS

In the present study, in both the D and IGT rats, the mean number of capillary contacts (CC) was significantly reduced. This indicates a loss in the absolute number of capillaries supplying individual muscle fibers in these females. In addition, the capillary supply of individual muscle fibers, as assessed by the individual capillary:fiber ratio, was diminished in the D and IGT rats compared to the controls. The present study did not address the mechanisms by which the capillaries in the D and IGT rats were reduced, but some speculation is relevant to the discussion.

Angiogenesis is the development of new capillaries from pre-existing blood vessels (Pintucci et al., 1996), and can be induced via a number of physiological and pathological conditions. As discussed in Section 1.6, an increased blood flow may be a stimulus for capillary proliferation (Hudlicka et al., 1992). In contrast to early hyperemia, the later stages of diabetes are characterized by underperfusion (Section 1.2) or a reduced blood flow (Tooke, 1993; Williamson et al., 1993; Sexton et al., 1994; Williamson et al., 1996), which would remove a potential initiator of capillary growth. Moreover, in poorly controlled Type I diabetic patients, the decreased rate of IMGU has been accounted for by the decreased blood flow to skeletal muscle (Baron et al., 1990). In view of the likelihood of an insulin deficiency in the D rats, and the subsequent decrease in the insulin-mediated uptake of glucose, the data are consistent with the observed changes in the capillary supply, i.e., an absence of capillary development and a reduced number of capillaries around individual muscle fibers. Certainly, these changes would
result in the potential for a reduced blood flow and capillary exchange, and therefore a decreased delivery of insulin and glucose delivery to the muscle fibers in these animals.

In adult STZ-diabetic mice, the decreased number of capillaries has been attributed to a loss of perfused capillaries (Bohlen and Niggl, 1980). In keeping with this line of thought, it seems likely that the reduced rate of IMGU in the IGT females would be associated with a lower number of capillaries and a reduced blood flow through existing capillaries. As mentioned previously, insulin increases the blood flow in muscle tissue via the recruitment of previously closed, already existing capillaries (Section 1.7 and 1.8). Secondly, a rapid formation of capillary endothelial cells occurs in rat skeletal muscle after exposure to insulin (Section 4.2.3). Lastly, hyperinsulinemia leads to the proliferation of capillaries (Section 4.2.3). However, positive effects of the insulin on both capillarization and blood flow are abolished in the state of insulin resistance following long-term hyperinsulinemia (Krotkiewski, 1994). Holmang et al. (1992) reported that insulin uptake shows a positive association with the degree of tissue capillarization. Blood flow is also an important determinant of the rate of glucose uptake (Laakso et al., 1990a, 1990b; Edelman et al., 1990). The low capillary density observed in such conditions as physical inactivity, obesity, and Type II diabetes, closely parallels a diminished insulin sensitivity (Bjorntorp, 1995). Further, in a wide variety of insulin resistant states (eg., obesity, Type II diabetes, and hypertension), reduced rates of insulin-mediated glucose uptake is largely the result of a decreased vasodilation of the skeletal muscle vasculature by insulin (Baron et al., 1990; Baron and Brechtel, 1993; Baron, 1994, 1996; Baron et al., 1995; Tack et al., 1996b). Refer to section 1.8.3. The increase in muscle blood flow as a result of the action of insulin is blunted to a degree commensurate with the magnitude of insulin resistance in hypertensive, NIDDM, and obese patients (Baron, 1993; Baron et al., 1993; Laakso et al., 1990a, 1990b). It follows then, that insulin and glucose delivery may be limiting in the IGT group.
4.6 CAPILLARY-FIBER MORPHOLOGY IN THE D AND IGT ANIMALS

4.6.1 ALTERATIONS IN AREA-BASED MEASUREMENTS

Morphometric estimates of the capillary supply of muscle fibers are indicative of the maximum structural potential for exchange (Mathieu-Costello, 1993). It is important however, to interpret the reported microvascular changes in relation to the change in muscle fiber morphology in the D and IGT rats. Both models of disturbed glucose homeostasis, i.e., Type I diabetes and IGT, were associated with muscle fiber atrophy, as both the mean fiber area and fiber perimeter were significantly reduced in the D and IGT animals compared to the controls. By assessing the capillary supply of a given fiber relative to the fiber area, or in relation to the perimeter of the fiber, we were able to derive a more complete and accurate interpretation of the ramifications of the capillary changes and fiber alterations during adaptation in the D and IGT groups. A comparison between the D and IGT females revealed that the FA/CC and FA/C:Fi in the type II fibers of the diabetic rats were significantly less than that of the IGT females, with no differences being observed in the type I fibers.

In the present study, diabetes and IGT resulted in a disproportionate decrease in the cross-sectional area of a muscle fiber in relation to the number of capillaries supplying that fiber. The morphological imbalance between the changes in fiber area and the loss of capillaries may simply be the result of capillary diminution lagging behind fiber atrophy. It is believed that in muscles undergoing atrophy due to denervation, tenotomy, or immobilization, degeneration of the muscle fibers proceeds much faster than the changes in the vascular bed (Hudlicka et al., 1992). The length of the present study, i.e. 12 weeks should reduce this discrepancy. Further, as it pertains to the D and IGT females in the current study, we speculate that the changes in capillary-fiber morphology occurred as a means of compensating for the altered states of insulin-mediated glucose metabolism during conditions of diabetes and impaired glucose tolerance. Alterations in glucose transport (both basal and insulin-stimulated) in denervated muscle have been
observed (Handberg et al., 1996; Johannsson et al., 1996; Sinacore and Gulve, 1993), which suggests that any changes in capillary-fiber morphology in this state may follow a similar pattern. Further, it has been suggested that there is a close coupling between the structural and exchange potential of capillaries and the extent of muscle atrophy in STZ-induced diabetic female rats (Sexton et al., 1994).

4.6.2 SURFACE AREA/VOLUME-BASED MEASUREMENTS

In contrast to fiber area, the reduction in fiber perimeter paralleled the reductions in muscle capillarization, i.e., no significant changes were observed in the P/C:Fi in the D and IGT rats. In other words, the perimeter (indicative of fiber surface area) of the muscle fibers remained constant in relation to the capillary supply.

4.7 MORPHOLOGICAL ADAPTATIONS IN DIABETES AND IGT: AN INTEGRATED VIEW

The functional corollary of the morphological changes indicated by the decreased FA/CC and FA/C:Fi in the D and IGT rats is that there was a decrease in the diffusion distance from the capillary lumen to the center of the muscle fiber, and a decrease in the required fiber area supplied per capillary. In view of the link between hemodynamics and insulin-mediated glucose metabolism (Baron et al., 1990; Baron, 1994), it seems likely that the observed changes in capillary-fiber morphology in the experimental animals are of significant physiological importance.

After plasma insulin increases, a cascade of sequential steps occurs, culminating in the enhancement of glucose disposal, including insulin-stimulated blood flow. The extent to which skeletal muscle is perfused can be a determinant of insulin-mediated glucose uptake (Baron et al., 1994). The most likely mechanism by which the insulin-stimulated increase in skeletal muscle blood flow produces an increased muscle IMGU is via the opening of previously under-perfused or non-perfused capillaries (i.e., via capillary recruitment). This increase in capillary perfusion leads to an increased delivery of insulin and glucose, and in addition, directly exposes more muscle cells to insulin and
glucose, thereby amplifying the effect of insulin on glucose transport (Laakso et al., 1990a, 1990b; Baron, 1994; Krotkiewski, 1994). After insulin binds to the capillary endothelium (Bjorntorp, 1995), transcapillary transport takes place, i.e., insulin is transported through the endothelial cells (Yang et al., 1989), after which insulin diffuses from the plasma to the interstitium and/or muscle cell surface (Bjorntorp, 1995). Because the insulin-mediated blood flow and capillary recruitment are reduced in both diabetes and insulin resistant states (Section 1.10), a decrease in insulin (and glucose) delivery would be expected in the D and IGT rats. It follows that a decrease in diffusion distance would help to offset the limited insulin delivery associated with a reduced blood flow. Therefore, it may be hypothesized that the observed decrease in diffusion distance from the capillary to the centre of each muscle fiber in the D and IGT female rats constitutes an adaptive strategy by the muscle to compensate for the ineffective delivery of insulin. A decrease in the diffusion distance for hormones and nutrients may be viewed as a metabolic advantage through the provision of added potential for increased function (Kanabus and Merola, 1985; Krotkiewski, 1994).

Furthermore, the diffusion distance in skeletal muscle has been linked to different degrees of glucose tolerance and insulin sensitivity (Lithell et al., 1981; Lindgarde et al., 1982; Lillioja et al., 1987). In insulin-dependent diabetes, a diminished hormonal response is accompanied by an increased number of surface insulin receptors (Blecher and Bar, 1981; Carpentier et al., 1984). In hepatocytes obtained from hypoinsulinemic STZ-induced diabetic rats, receptor mediated endocytosis is decreased (Carpentier et al., 1984, 1986; Krischer et al., 1993) as a result of a decreased concentration of clathrin coated pits on the cell surface (Krischer et al., 1993). This alteration concerns not only the uptake of the receptors, but also reduces the amount of insulin internalized, the intracellular processing of insulin, and insulin degradation. Although the liver is the major site for the degradation of insulin, the rate and magnitude of the internalization and degradation of insulin could be altered in other tissues (Kuzaya et al., 1983). A reduced internalization of the insulin-receptor complex has also been reported in peripheral blood monocytes from patients with Type I insulinopenic diabetes (Carpentier et al., 1986)
It has been suggested that the reduced endocytosis represents a mechanism to preserve insulin and its receptor on the cell surface in order to amplify the muted insulin signal caused by a deficient insulin secretion (Carpentier et al., 1986; Krischer et al., 1993). Receptor occupancy appears to be the major determinant for the activation of the glucose transport system. As it is only necessary to occupy a small fraction of the receptors to elicit a submaximal or near-maximal effect, the presence of an excess of receptors may be regarded as a means for making the cells sensitive to insulin (Gliemann et al., 1985; Heidenreich and Olefsky, 1985). One could argue that this post-binding defect (i.e., a decreased receptor-mediated endocytosis) is not necessarily disadvantageous. The affinity and capacity of the insulin receptors also changes in response to the cellular environment (Kobayashi et al., 1983). During a hypoinsulinemic state, insulin binding to the cell-surface receptors is increased due to an enhanced receptor capacity and affinity (Kobayashi and Olefsky, 1979; Carpentier et al., 1986). Kobayashi and Olefsky (1979), in their study of insulin receptors and various aspects of glucose metabolism in isolated adipocytes from insulin-deficient STZ-treated diabetic rats, found a 105% increase in insulin binding. They reported that this increase was due to an enhanced receptor affinity and capacity, and was quantitatively comparable to the increase in insulin sensitivity, despite impairments in the glucose transport system and subsequent steps of glucose metabolism (i.e., glucose oxidation) (Kobayashi and Olefsky, 1979), or the decrease in insulin responsiveness. Just as these alterations occur to compensate for the insulin deficiency, it is conceivable that a decrease in diffusion distance is another predictable step of the adaptation process during diabetes. In other words, a decrease in diffusion distance may be an adaptive strategy to optimize insulin delivery. Moreover, because the cellular insulin resistance of insulin-deficient diabetes is due to multiple defects in the effector systems distal to the insulin receptor, i.e., glucose transport (Yki-Jarvinen et al., 1990; Kobayashi and Olefsky, 1979) and/or phosphorylation (Cline et al., 1997), the processes described above would be essential to offset these perturbations. It has been postulated that a growing diabetic animal positively acclimates to diabetes (Mueller, 1984). It should be noted that a decrease in diffusion distance does not suggest that diabetes-induced alterations in glucose transport and
phosphorylation will be alleviated or "fixed". Instead, a reduced diffusion distance is an adaptation by the body to compensate for these perturbations which occur further along the insulin action pathway.

In addition to the above, further evidence to support this view is derived from the morphometric measurements estimating the capillary supply in relation to the fiber perimeter. Insulin deficiency leads to an up-regulation of the insulin receptors, as well as a reduced internalization of the insulin-receptor complex or receptor-mediated endocytosis. Because no significant change was observed in the P/C:Fi in the D rats, it is possible that the P/C:Fi of an individual muscle fiber plays a role in the regulation of the insulin receptors, and/or their function during hyposinulinemia. In other words, the cell surface not only accommodates the increased number of insulin receptors during this condition, it's also a direct reflection of the reduced receptor-mediated endocytosis.

Recruitment of unoccupied receptors to the cell surface compensates for receptors which are internalized during the uptake of insulin (Kaplan, 1986). Membrane recycling provides a major mechanism for the conservation of cell surface and cell surface receptors during receptor-mediated endocytosis (Morre, 1985). Thus, this process allows the cells to maintain a relatively constant surface area/volume ratio during high levels of endocytotic activity (Kaplan, 1986; Damke, 1996). Conceivably, the up-regulation of insulin receptor density, together with a decreased receptor-mediated endocytosis, would result in an altered cell-surface/volume ratio. Our data support this theory since fiber shape was altered in the D rats compared to controls. A change in muscle fiber shape favors a more optimal packing, which could also contribute to an enhanced insulin delivery in the D animals.

The fiber-specific, morphological alterations observed in the diabetic rats also support this line of reasoning. A comparison between the D and IGT females revealed that both the FA/CC and FA/C:Fi of the Type II fibers of the diabetic rats were significantly less than that observed in the IGT females, with no differences being observed in the Type I fibers. Thus, the Type II muscle fibers in the D animals had a lower capillary supply in relation to fiber area (i.e., the decrease in FA/C:Fi was less), and the distance from capillary to the center of the muscle fiber was greater in these fibers.
(i.e., the decrease in diffusion distance was less). Richardson et al. (1991) have demonstrated that "white" skeletal muscle down-regulates the GLUT 4 protein and mRNA at a significantly lower rate. While a decreased glucose uptake has been reported in both the oxidative Type I and IIa fibers, the Type IIb fibers maintained a normal rate of glucose uptake in spite of a reduced level of total cellular glucose transporters (Kainulainen et al., 1994). Hence, it is possible that the Type II muscle fibers require less alteration in diffusion distance because intracellular defects in the action of insulin distal to the cell membrane are less severe.

An improvement in insulin sensitivity and glucose tolerance has been reported to coincide with a reduced capillary diffusion distance after weight reduction in men with IGT (Lindgarde et al., 1982). Based on these data, a larger diffusion distance in the muscle fibers of the IGT females would be expected; however, this was not found, as there was a decrease in the diffusion distance between the capillaries and the muscle fibers in the IGT group when compared to the controls. Further, the decrease in diffusion distance was more prominent in the IGT females than in the D rats. In insulin-resistant obese Zucker rats, the transcapillary delivery of insulin, which is normally rate-limiting for insulin uptake and metabolism in muscle, was reported to be less rate limiting for insulin uptake (Holmang et al., 1997). This change, together with a decrease in the diffusion distance as indicated in the present study, suggests that favorable changes are also part of the adaptive process during conditions of IGT or insulin resistance. An alternative hypothesis is that the observed changes may be related to the eventual progression of the IGT rats to a diabetic state. As mentioned in Section 4.4.1, Eriksson et al. (1994) followed 29 subjects with IGT continuously for 15 years to determine whether an increased number of capillaries in the men with IGT was an early marker of diabetic progression. The subjects who developed diabetes exhibited a lower mean fiber area per capillary contact, indicative of a lower diffusion distance from the capillary to the center of the muscle fiber, long before the onset of the disease (Eriksson et al., 1994). Warram et al. (1990) have also reported that a reduced glucose clearance is present one to two decades before Type II diabetes is diagnosed. Although there is a strong association between the presence of obesity and the development of Type II diabetes, Type II
diabetes may also manifest in lean individuals (Horton, 1995). Horton (1995) suggests that a diagnosis of IGT is a strong predictor for the development of Type II diabetes. Since diabetes is a continuum of varying degrees of glucose intolerance (Reaven et al., 1976), it follows that the observed changes in capillary-fiber morphology in the IGT rats may also represent early features of the diabetic process. Thus, similar to the trend observed in the study by Eriksson et al. (1994), it may well be that the IGT rats will eventually progress to a state of diabetes, as indicated by the changes in their muscle capillary-fiber morphology.

**4.8 SUMMARY AND PERSPECTIVES**

In summary, the results from the present study indicate that STZ-diabetes and impaired glucose tolerance results in a generalized remodeling of muscle from female rats. We observed an overall reduction in muscle fiber size from both the D and the IGT rats. Specifically, both the muscle fiber area and the perimeter were significantly reduced in the experimental animals, in contrast to the control animals. A notable change in the muscle of the D and IGT rats compared to the control animals was that the reductions in capillary supply in relation to muscle fiber area and fiber perimeter were not as prominent, or did not change, respectively.

The physiological role of the alterations in the muscles of the D and IGT rats, i.e., a decrease in muscle fiber area relative to the capillary supply, and a constant muscle fiber perimeter in relation to the capillary supply, are unknown. We believe that the functional implications of these morphological changes are important to our understanding of the disease processes associated with diabetes and impaired glucose tolerance. We have interpreted these data in terms of an adaptive response, whereby the changes in the capillary-fiber morphology occur in response to other changes taking place as a result of the various environmental challenges resulting from the diabetes and impaired glucose tolerance. Because insulin-mediated blood flow and capillary recruitment are important in insulin's action on glucose uptake, and because both are reduced in diabetes, it is reasonable to postulate that capillary muscle-fiber morphology is
altered to compensate for the impaired hemodynamic actions during this disease; in this case, the decrease in diffusion distance would facilitate the delivery of insulin and glucose. Moreover, through this favorable adaptation, it is possible that muscle fiber perimeter in relation to capillary supply plays a role in insulin receptor regulation. Lastly, the similar morphological changes in the rats which presented with impaired glucose tolerance, may be a reflection of their progression towards a diabetic disease state.

In conclusion, we hypothesized at the outset of this research that the capillary supply to individual muscle fibers in the D and IGT female rats would not change. However, in relation to muscle fiber area, the capillary supply was increased, and as a result, the null hypotheses in terms of FA/CC were rejected. However, the capillary supply in relation to the perimeter of the muscle fibers did not change, and therefore in terms of P/C:Fi the null hypotheses were accepted. Indeed the variable changes in the capillary supply relative to muscle fiber area and fiber perimeter suggest that the process of adaptation during diabetes and impaired glucose tolerance is complex, and may involve a complicated interplay among various factors which are known to be altered during these conditions, including insulin deficiency, reduced insulin delivery as a result of decreased insulin-mediated blood flow and capillary perfusion, increased insulin receptor number and insulin binding, and decreased receptor-mediated endocytosis.
CHAPTER 5
FUTURE WORK

One important consideration for future work is whether the capillary muscle-fiber changes during diabetes and impaired glucose tolerance are gender dependent. Additional concerns for future studies include: 1) to determine whether the changes in the capillary supply in relation to the muscle fiber area are directly related to hemodynamic defects, 2) to examine the capillary supply in relation to the muscle fiber perimeter as an accurate predictor of insulin-receptor up-regulation, and 3) to assess whether the morphological changes observed during impaired glucose tolerance directly reflect a progression to diabetes. This information will more clearly define the physiological role or adaptive function of the alterations in muscle-capillary fiber morphology during diabetes and impaired glucose tolerance.
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### Table 6.1 Alterations in muscle morphology in STZ-diabetes

<table>
<thead>
<tr>
<th>Reference</th>
<th>Animal</th>
<th>Gender</th>
<th>STZ Dosage</th>
<th>Duration of Diabetes</th>
<th>Muscle</th>
<th>Fiber Types</th>
<th>Whole Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Armstrong et al. (1975)</td>
<td>Rat</td>
<td>Male</td>
<td>60mg/kg</td>
<td>10 weeks body wt.</td>
<td>Gastrocnemius</td>
<td>↑FA</td>
<td>FG; Gastrocnemius</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>body wt.</td>
<td></td>
<td></td>
<td>↓FA</td>
<td>fewer capillaries</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10 weeks</td>
<td></td>
<td></td>
<td>~C:F</td>
<td></td>
</tr>
<tr>
<td>Medina-Sanchez et al. (1991)</td>
<td>Rat</td>
<td>Male</td>
<td>60mg/kg</td>
<td>30 &amp; 60 days body wt.</td>
<td>Rectus femoris</td>
<td>*↑</td>
<td>*↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>body wt.</td>
<td></td>
<td></td>
<td>*↓↓</td>
<td>*↓↓</td>
</tr>
<tr>
<td>Klueber et al. (1994)</td>
<td>Mice</td>
<td>Male</td>
<td>200mg/kg</td>
<td>42 days body wt.</td>
<td>EDL</td>
<td>*↓</td>
<td>*↓↓</td>
</tr>
<tr>
<td>Hegarty &amp; Rosholt (1981)</td>
<td>Rat</td>
<td>Male</td>
<td>70mg/kg</td>
<td>79 days body wt.</td>
<td>Biceps brachii</td>
<td>*↓</td>
<td>Diameter</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td></td>
<td>70mg/kg</td>
<td></td>
<td>Sternomastoideus</td>
<td>*↓</td>
<td>Diameter</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>body wt.</td>
<td></td>
<td>Soleus</td>
<td>~ Diameter</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>70 days</td>
<td></td>
<td>Biceps brachii</td>
<td>~ Diameter</td>
<td></td>
</tr>
<tr>
<td>Sexton et al. (1994)</td>
<td>Rat</td>
<td>Female</td>
<td>65mg/kg</td>
<td>6-8 weeks body wt.</td>
<td>Sternomastoideus</td>
<td>*↓</td>
<td>Diameter</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>body wt.</td>
<td></td>
<td>Soleus</td>
<td>*↓</td>
<td>Diameter</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6-8 weeks</td>
<td></td>
<td>Plantaris</td>
<td>*↓</td>
<td></td>
</tr>
<tr>
<td>Bohlen and Niggl (1980)</td>
<td>Mice</td>
<td>Male</td>
<td>200mg/kg</td>
<td>8-10 weeks body wt.</td>
<td>Cremaster</td>
<td>*↓</td>
<td>Muscle Wt.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>body wt.</td>
<td></td>
<td></td>
<td>↑</td>
<td>CD</td>
</tr>
</tbody>
</table>

* significant  ~ no difference  FA: fiber area  P: fiber perimeter  C:F: capillary-to-fiber ratio  CD: capillary density
6.2 EXPERIMENTAL GROUPS BASED ON PHASE OF ESTRUS CYCLE INJECTION

<table>
<thead>
<tr>
<th>Phase</th>
<th>n=6</th>
<th>n=3</th>
<th>n=3</th>
<th>n=3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proestrus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metaestrus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diestrus 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diestrus 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D: Diabetic  IGT: Impaired Glucose Tolerance

It should be noted that streptozotocin (STZ) is almost 100% effective in inducing diabetes in male rats, but the typical response of females to STZ is 50%, regardless of the phase of the estrus cycle in which the female rat has been injected (Rodgers et al., in press).
6.3 HISTOCHEMICAL ANALYSIS

Using the method of Rosenblatt et al. (1987), capillaries and fibers were simultaneously demonstrated in the plantaris muscle of the female rats. Sections of each muscle were cut transversely at 8 μm, in a cryostat at -20°C, and mounted on slides. The slides were placed in Coplin jars and refrigerated at 4°C for 30 minutes prior to staining. Sections were then fixed for 5 minutes at 4°C in the buffered fixative (pH 7.6) described by Guth and Samaha (1970), which contains: Na Cacodylate (.144M; 23.054g L⁻¹), CaCl₂ (.068M; 9.997g L⁻¹) and sucrose (.336M; 115.013g L⁻¹) in a 5% formalin (v/v) solution. Following fixation, the sections were rinsed 15 times in distilled water by successively filling the Coplin jars.

Fixed sections were then incubated in a medium for 60 minutes at 37°C in a shaker bath. The incubation medium was prepared as follows: 75 mg of gelatin was added to 60 ml of .1M (4.74 g tris(hydroxymethyl)aminomethane maleate/100ml) Tris-Maleic Acid Buffer and heated in order to dissolve the gelatin. Added to this solution were 9 ml of .06M (2.00g/100 ml) lead nitrate, 15 ml of .068M (1.00g/100 ml) calcium chloride, 60 ml of distilled water and 75 mg of ATP (crystalline disodium salt, Sigma Chemical Co., St. Louis, MO). The pH of this acidic medium was adjusted from 3.55 to 7.2 with NaOH prior to use.

Following incubation, slides were rinsed in distilled water through 15 successive fillings in the Coplin jars. The stain was developed in 1% ammonium sulfide (v/v) for 1 minute, after which the slides were rinsed another 15 times with distilled water.

The slides were removed from the Coplin jars and patted dry. Cover slips were applied to the sections via Permount, so as to permanently fix the tissue sample onto each slide. Slides were then stored in slide boxes until the morphometric analysis.
6.4 FLOW CHART OF METHODS & MATERIALS

TIME: 0

EXPERIMENTAL ANIMALS

FEMALE SPRAGUE-DAWLEY RATS (N=20)

STZ-65 MG/KG\(^{-1}\) BODY MASS

72 HOURS DIABETES & IGT DIAGNOSIS

BG: D (N=8) NR (N=7)
GTT: BG@ TIME 0, 30, 60, 120 MIN.

13 WEEKS CURRENT PHASE OF STUDY

D (N=8) IGT (N=7)
BODY MASS: 243g 274g

22 WEEKS DIABETES & IGT DIAGNOSIS

REPEAT OF GTT: BG @ TIME 0, 30, 60, 120 MINUTES

D (N=8) IGT (N=7)
BODY MASS: 259 g 328 g

*BG: BLOOD GLUCOSE  *GTT: GLUCOSE TOLERANCE TEST
6.4 FLOW CHART OF METHODS & MATERIAL

TISSUE PREPARATION

PLANTARIS MUSCLE

\[ \downarrow \]

CROSS SECTION (MIDBELLY)

\[ \downarrow \]

TISSUE SLICING (8\( \mu \)m)

\[ \downarrow \]

CAPILLARY & FIBER TYPE STAINING (Rosenblatt et al., 1987)

MORPHOMETRIC ANALYSIS

LIGHT MICROSCOPE MICROCOMPUTER + VIDEO IMAGE ANALYSIS SOFTWARE

\[ \downarrow \]

RANDOM SELECTION OF TYPE I AND TYPE II MUSCLE FIBERS (25 OF EACH)

\[ \downarrow \]

ASSESSMENT OF FIBER SIZE & CAPILLARY SUPPLY VARIABLES IN 25 TYPE I AND TYPE II MUSCLE FIBERS:

\[ \begin{align*}
FA & \quad P & \quad SHAPE & \quad CC & \quad C:Fi & \quad FA/CC & \quad P/C:Fi \\
\downarrow & & & & & & \\
\downarrow & & & & & & \\
\downarrow & & & & & & \\
\end{align*} \]

STATISTICAL ANALYSIS

T-TEST BETWEEN: \( D \) AND \( C1 \); \( IGT \) AND \( C2 \); \( D \) AND \( IGT \)