The effect of testosterone on skeletal muscle energy metabolism in diabetic and non-diabetic endurance trained rats

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The effect of testosterone on skeletal muscle energy metabolism in diabetic and non-diabetic endurance trained rats

cover: Claude Bernard (1813-1878) contributed much to the European experimental physiology in general. His famous work: "*An Introduction to the Study of Experimental Medicine*" (1865) was the first clear exposition of the theory and practice surrounding physiological and medical experiments. He also contributed much to the understanding of diabetes at that time (storage of sugar as glycogen in the liver).

**The effect of testosterone on
skeletal muscle energy metabolism in
diabetic and non-diabetic
endurance trained rats**

Proefschrift

ter verkrijging van de graad van doctor
aan de Rijksuniversiteit Limburg te Maastricht,
op gezag van Rector Magnificus, Prof. dr. H. Philipsen,
volgens het besluit van het College van Dekanen,
in het openbaar te verdedigen
op donderdag, 24 februari 1994 om 16.00 uur

door

ERIC VAN BREDA

geboren op 25 april 1959 te Schiedam

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Opgedragen aan:

Alba en Farouk, twee trouwe viervoeters, voor kilometers heerlijk wandel plezier.

Mijn schoonouders, niet alleen voor hun belangstelling maar vooral voor hun hulp daar waar ik vaak verzaakte, het huishouden. Bovendien zou ik dit proefschrift, en met name in de laatste fase, niet hebben kunnen voltooien zonder hun heerlijke culinaire inbreng.

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Tenslotte Charlotte, onze "hartewens", alleen voor jou al was alles de moeite waard. Helaas ik kan je nooit de vele uren en dagen teruggeven die ik in het laatste jaar van dit proefschrift tevens het eerste jaar van jouw leven, niet thuis was. Ik kan alleen hopen dat je er op een dag begrip voor zult hebben.

Ter herinnering aan : Karel Dolmans †

"The hurried scribbles in our laboratory notebooks are intelligible only to ourselves, and the seminar and lecture have only a temporary and narrow influence. The published record however, is permanent, it is there for all time as a source of pride or shame as the case may be".

H.B. Vickers, J. Biol. Chem. 233, 1958

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1

Introduction and objectives of the thesis

1.1 Historical background

Though scientists have speculated about the mechanisms by which biological systems are **controlled** for many centuries, it was not until the past decade that technological progress allowed us to evaluate these speculations accurately. The significance of the nervous system, comprising the central and peripheral nervous system, was appreciated long before the awareness that special glands throughout the body produce chemical messengers (endocrine factors) that are conveyed via the circulation to their target cells. The concept that endocrine factors play a significant role in substantiating and maintaining physiological equilibrium is now generally accepted. This ability of living beings to maintain a fairly constant internal environment illustrates the superb design of nature. The idea that regulatory factors exist which are ready to counteract a perturbed organism is found in the papers of distinguished early physiologists such as Bernard (Bernard, 1878), Frederiq (Frederiq, 1885), Pflüger (Pflüger, 1877) and Richet (Richet, 1900). To Walter B. Cannon, however, belongs the credit of being the first to give the name "homeostasis"¹ to the concept of the regulating factors which operate to achieve a steady state in the living being (Cannon, 1925; Cannon, 1926). He was also among the first to demonstrate the influence of the endocrine system on metabolism (Cannon, 1925). Nevertheless, to date information regarding the effects of the endocrine system on muscle metabolism is scarcely available.

1.2 Aim and rationale of this thesis

Since skeletal muscle represents 35-45% of body mass it plays an important role in whole body glucose and lipid metabolism. In this thesis we endeavour to answer questions associated with endocrine control of muscle carbohydrate and lipid metabolism. Despite the rapidly growing number of studies that, at present, mushroom from laboratories throughout the scientific world, most aspects regarding the control and/or integration of both metabolic pathways remain poorly understood. Although the role of insulin and counter regulatory hormones have received a great deal of scientific attention, the role of sex-steroids on muscle carbohydrate and lipid metabolism has not. This is somewhat surprising, since hyperandrogenism has

¹In 1926, Walter Bradford Cannon (1871-1945) outlined for the first time his classic concept of 'homeostasis' based on the concept of the 'milieu interieur' by Claude Bernard (1813-1878). For more details see "Homeostasis: origins of the concept" (Langley, 1973).

been described to be associated with pathological changes in carbohydrate metabolism (Achard and Thiers, 1921; Poretsky, 1991), whereas even low-dose oral contraceptives are able to impair glucose metabolism as well (Godsland *et al.*, 1992). Nevertheless, the precise role of the reproductive hormones (testosterone and estradiol-17 β) in the control of muscle carbohydrate and lipid metabolism remains speculative and conflicting (for more detailed information see chapter 2 and Gillespie and Edgerton 1970; Bergamini 1974; Max and Toop, 1983; Kendrick *et al.*, 1987; Holmång *et al.*, 1990; Kendrick and Ellis, 1991; Holmång *et al.*, 1992).

Testosterone acts on skeletal muscle by cytoplasmic and/or nuclear receptors. In muscle fibres of different species testosterone receptors have been identified (Krieg, 1976; Michel and Baulieu, 1980). Since steroid hormones easily cross the lipid bilayer of the cell membrane, the intracellular steroid hormone concentration is directly related to its plasma concentration (Bartsch *et al.*, 1983). Therefore, it is reasonable to suggest that changes in plasma testosterone concentration will result in changes in the intra-cellular concentrations which, in turn, may alter the metabolic state.

Furthermore, physical exercise, and more specifically endurance training, has been reported to have a profound influence on muscle metabolism as well (Bouchard, 1990). However, information regarding the therapeutic effects of training under defined pathological conditions is limited.

Therefore, the central aim of the present study was to investigate the intracellular effects of testosterone on skeletal muscle carbohydrate and lipid metabolism in both sedentary and trained diabetic and non-diabetic animals.

In **chapter 2** of this thesis we will provide the interested reader a global overview of the current concepts of muscle carbohydrate and lipid metabolism during rest, training and exercise and its endocrine control. Furthermore, we will provide today's point of view of the pathological derangements of carbohydrate and lipid metabolism in non-insulin-dependent diabetes mellitus.

Chapter 3 of this thesis describes two approaches to study the specific aims of this thesis, namely:

(i) a mice skeletal muscle model *in vitro*, described earlier by other investigators (Le Marchand-Brustel *et al.*, 1978; Bonen *et al.*, 1984; MacDonald *et al.*, 1990), to study the effects of hormones uncomplicated by side effects of other substances that might occur *in vivo*. Careful evaluation of this *in vitro* model, however, showed that the isolated mouse skeletal muscle preparation partly lost its viability and, therefore, is of only limited value for investigations of the effects of hormones on muscle metabolism. Therefore we switched to the rat model *in vivo* (**model ii**). In this model we have investigated the effects of testosterone, either separately or in combination with training on glycogen and lipid metabolism of two types of muscles (oxidative and glycolytic) in diabetic and non-diabetic female rats.

In the **chapters 4 and 5** studies are described on the effects of high pharmacological doses of testosterone and endurance training in intact non-diabetic female rats to explore whether these doses elicit any effect.

In chapter 6 the effects of mild hyper-testosteronemia [as occurring in acanthosis nigricans (AN) and polycystic ovary syndrome (PCOS)] and endurance training, either separately

or in combination on various aspects of carbohydrate and lipid metabolism were investigated in non-insulin-dependent diabetes mellitus female rats. Furthermore, since endurance exercise has a profound influence on muscle carbohydrate and fat metabolism as well, we investigated whether regular exercise could influence some of the metabolic derangements in non-insulin-dependent diabetes mellitus rats .

To unravel the underlying mechanisms of the testosterone induced effects on skeletal muscle carbohydrate metabolism we investigated in **chapter 7** whether the effects of testosterone could be mediated by estradiol-17 β rather than to testosterone itself. For this purpose, we measured the activity of the aromatase enzyme complex in the two types of muscles examined. Furthermore, we investigated whether inhibition of the aromatase enzyme complex by means of an aromatase inhibitor could abolish the testosterone induced effects.

Finally, the results of this thesis will be discussed and some final remarks are made and some perspectives for future research are given (**chapter 8**).

For a detailed reference list see chapter 2.

2

Muscle carbohydrate metabolism and its endocrine control

2.1 Introductory remarks

To be able to cope with the daily routines of live, to eat, to digest, to move, and to rest, a continuous supply of energy-rich substrates like carbohydrates and lipids is essential for life. The predominant carbohydrate presented as metabolic fuel to the cells is glucose. Indeed, this molecule, belonging to the class of hexoses, is of vital importance for the organism since some cell types, e.g. erythrocytes, exclusively, and the highly specialized cells of the brain predominantly utilize this substrate as metabolic fuel. Other cells, such as muscle cells and adipocytes are able to utilize glucose substantially, but they use fatty acids as well. Muscle cells, which comprise approximately 35-45% of total body mass, can modify their glucose uptake considerably, and thus play, concurrently with the liver, an important role in maintaining whole body glucose homeostasis. The amount and kind of the substrates consumed by individual cells depend on the plasma concentration, the energetic demands, the mobilization of the substrates from their specific storage sites, and the availability of enzyme systems to catabolize these substrates. Since the maintenance of normal glucose homeostasis is essential for survival, it is very well preserved even after the severe perturbation of prolonged exercise. The key consequence of physical exercise is the disturbance of the milieu intérieur. The acute cellular, organ and systemic changes that occur during a single bout of physical exercise disappear soon after the termination of the exercise bout. In contrast to a single exercise bout, however, repeated bouts of physical exercise on consecutive days (training) are designed to minimize disturbances to the milieu intérieur to the same single exercise bout and thus improve the functional effectiveness of the body. The distinctive features, then, persist for a longer period of time and are called adaptive responses (Fischer, 1958; Proser, 1964).

During exercise, the regulation of energy metabolism in general and in skeletal muscle cells in particular, builds on processes that act in a coordinated fashion. A subtle and accurate interplay between the endocrine- and nervous system not only assures the right quantity of each substrate being used as metabolic fuel but also guarantees whole body glucose homeostasis.

Before we will discuss the concepts of muscle fuel selection during rest, acute exercise and training the terms exercise and training need to be defined conscientiously.

Acute exercise can be defined as bodily movements produced by skeletal muscles which, depending on intensity and duration, result in a certain amount of energy expenditure, whereas training can be defined as repetitive bouts of exercise, conducted over periods of weeks, months or years (Bouchard *et al.*, 1990).

From these very general definitions, it emerges that we have to distinct between short-term adaptations, i.e. after a single work bout, and long-term adaptation, i.e. after a period of training. Furthermore, we also have to differentiate between effects of several types of exercise, i.e. endurance, strength, speed etc. Each type of exercise will elicit a specific effect. For example, endurance training (the exercise model used in this thesis) will generally result in increased muscle glycogen stores, relatively decreased reliance on carbohydrate oxidation at a given workload and increased activity of muscle oxidative enzymes.

The term acute exercise in this thesis refers to a single bout of moderate intensity exercise, whereas training refers to the repetitive bouts of acute exercise given in the

experimental period.

Finally, it is important to realize that there are several types of muscle in the body, and that, according to the function they must perform, they are composed of a mixture of fast (fast contractions) and slow (slow contraction) muscle fibres, and fibres which lie between these two ultimates. Table 2.1 gives the structural and biochemical characteristics of the major categories in which mammalian skeletal muscle fibres are divided.

Table 2.1 Structural and biochemical classification of skeletal muscle

Feature	Fast-twitch glycolytic (FG)	Fast-twitch oxidative-glycolytic (FOG)	Slow-twitch oxidative (SO)
Structural			
- fiber diameter	large	moderate	small
- number of capillaries	few	many	many
Biochemical			
- rate of fatigue	fast	moderate	slow
- myosine ATPase activity	high	high	low
- major ATP source	anaerobic glycolysis	oxidative phosphorylation	oxidative phosphorylation
- glycolytic enzyme activity	high	moderate	low
- oxidative enzyme activity	low	moderate	high
. number of mitochondria	low	moderate	high
. glycogen content	high	high	high
. glucose uptake capacity	moderate	moderate	high
. fatty acid-binding protein content	low	moderate/high	high
. capacity to metabolize fatty acids	low	moderate	high
. triacylglycerol content	low	moderate	high
Functional			
- major functional role	rapid, powerful movements	medium endurance	endurance
Examples (rat muscles)	extensor digitorum longus (EDL)	vastus lateralis	soleus

2.2 Muscle fuel selection during rest, acute exercise and training

Background

Once glucose is released into the circulation either after absorption from the intestine or production by the liver it can serve as a major energy source for all cells. It is essential that the blood glucose concentration is maintained within narrow limits (in man ca. 5 mmol/liter; range 3.9-8.5 mmol/liter) (Williams, 1992). The factors responsible for maintenance of the glucose homeostasis are summarized in Table 2.2. Skeletal muscle glucose uptake and metabolism accounts for the major portion of whole body glucose homeostasis. It is poised against the responsible factors mentioned in Table 2.2, against adipocyte lipolysis and against skeletal muscle fatty acid utilization. Once taken up glucose can be either oxidized to yield energy for various cellular processes, or stored as glycogen (glycogenesis) for later use. Once glucose is taken up by the muscle cell it is immediately phosphorylated to glucose-6-phosphate. Unlike the liver, muscle cells lack the enzyme glucose-6-phosphatase that dephosphorylates glucose-6-phosphate back to glucose. For this reason muscle glycogen does not play a role in maintaining whole body glucose homeostasis.

Table 2.2 Major factors responsible for maintenance of plasma glucose homeostasis

Intestinal absorption (dietary supply)	
Pancreatic insulin secretion	
Hepatic, muscle and adipocyte glucose :	uptake
	storage
	utilization
Hepatic glucose production :	
	glycogenolysis from glycogen
	gluconeogenesis from amino acids,
	glycerol and lactate

Adapted and modified from DeFronzo RA (1988)

Under resting conditions fatty acids are considered to be the predominant metabolic fuel for slow-twitch and fast-twitch oxidative-glycolytic (FOG) fibres. In plasma, fatty acids are bound to albumin in a concentration of about 0.4 mM (range 0.2-0.6 mM). Its turnover rate is extremely rapid and every few minutes half of the plasma fatty acids are replaced by new fatty acids. The main source of fatty acids emerges from the hydrolysis of triacylglycerols in adipose tissue. Apart from the fatty acids derived from the blood, muscle cells also contain a pool of triacylglycerols which can be used when necessary (Oscari *et al.*, 1990; van der Vusse, 1992).

Fuel metabolism during acute exercise

Skeletal muscle is truly remarkable in the sense that it is capable of enlarging its metabolic rate more than thirty-fifty times the resting level (Asmussen *et al.*, 1939). The exercise intensity is the prime factor determining which substrate will be catabolized for ATP resynthesis (McGilvery, 1975). Since in the present study we only used moderate-intensity exercise, in the next paragraph we will concentrate on this type of exercise only.

During the initial stages of submaximal exercise (ca. 10 minutes) the major part of energy is derived from muscle glycogen while blood glucose accounts for 8-14% of the total oxidative metabolism (Wahren *et al.*, 1971). It became apparent, that exhaustion is highly correlated with depletion of muscle glycogen stores (Bergström *et al.*, 1967). At about 30 minutes after the initiation of exercise, blood glucose accounts for approximately 20-30% of total energy supply and during prolonged exercise (ca. 90-180 minutes) for as much as 35-40% (Ahlborg *et al.*, 1974). In order to meet the enlarged metabolic fuel demands of the exercising muscle and at the same time maintain plasma euglycemia, metabolic processes within the liver have to be accelerated as well. The increased hepatic glucose production from the degradation of its glycogen pool is closely matched to the increase in glucose uptake by exercising muscle. The significance of this coupling was recently illustrated by Wasserman and Cherrington (Wasserman and Cherrington, 1991). Simple calculation indicated that if the liver would not respond synchronously in response to moderate-intensity exercise, plasma glucose levels would decrease at a rate of approximately 0.1 mmol/liter per minute resulting in severe hypoglycaemia soon after the initiation of exercise (Wasserman and Cherrington, 1991).

Christensen and Hansen (Christensen and Hansen, 1939) were among the first to demonstrate that fat is utilized as energy substrate during long-term exercise. As exercise proceeds for several hours, the utilization of fatty acids increases progressively. It has been assumed that an increased supply of fatty acids decreases glycogen degradation during exercise and, as a consequence, enhances endurance performance (glucose-fatty acid cycle) (Randle *et al.*, 1963). Several lipid pools (i.e. plasma fatty acids, plasma triacylglycerols, muscle triacylglycerol pool) have been identified but the exact contribution of each of these pools in the oxidation of fatty acids in muscle is unknown. It has been calculated that approximately 50% of the fatty acids used during exercise are derived from the intramuscular pool (Reitman *et al.*, 1973; Ahlborg *et al.*, 1974). Other studies have shown that approximately 50% of the lipids oxidized originates from plasma fatty acids (for a detailed review see (Romijn and Wolfe, 1992). The relative contribution of the latter source, however, remains unclear.

Although in man enough energy is stored in the body's triacylglycerol pools to run approximately 30 subsequent marathons the maximal rate of fat oxidation appears to be too low to provide the energy required for the power output during a single marathon (Guezennec, 1992). On the other hand, the rate of glucose oxidation can provide enough energy for the power output during a marathon, but the reserves for the duration of the run are inadequate (Newsholme and Leech, 1991). Thus both glucose and fatty acids are utilized and oxidized in parallel during exercise. Thus, a highly specialized control system which regulates the subtle interplay between glucose and fatty acid oxidation is essential.

Fuel metabolism following training

As mentioned earlier, training provides a stimulus for adaptive physiological responses of the body. The responses are not only tissue-specific but they are also highly integrated among the tissues and physiological systems involved. The adaptive responses to skeletal muscle affect gene expression and are of morphological (Schantz *et al.*, 1987), and biochemical (Holloszy and Booth, 1976; Saltin and Gollnick, 1983; Pette and Düsterhöft, 1992) origin. The adaptation of skeletal muscle to training depends strongly on the type of training stimulus. Endurance training, which includes frequent contractions for extensive periods of time, is primarily designed to increase muscle capacity for oxidative processes. The major metabolic adaptation of endurance training is an increased reliance on fatty acids, and a decreased utilization of blood glucose at a given exercise intensity and a more gradual utilization of an increased muscle glycogen pool.

2.3 Muscle glucose metabolism

Background

As mentioned above circulating glucose contributes considerably to muscle metabolism during prolonged exercise. Since the lipid bilayer, which is the basic structure of the muscle cell membrane, repels watersoluble substances like glucose, this substrate is taken up by muscle cells through carrier-mediated facilitated diffusion rather than simple diffusion (Widdas, 1988). Once taken up, the process is unidirectional due to fast intracellular irreversible conversion of glucose to glucose-6-phosphate. Subsequently, glucose-6-phosphate is utilized either to yield energy for contraction or to be stored as glycogen.

Muscle glucose uptake

The trans-membrane transport of glucose is the rate-limiting event in glucose utilization. Glucose uptake by muscle cells is dramatically increased by insulin and by contractile activity (Levine *et al.*, 1950; Wallberg-Henriksson, 1987). To date, a family of six

mammalian glucose transporters (GLUT's) has been identified (Table 2.3) (Bell *et al.*, 1990; Burant *et al.*, 1991).

Table 2.3 Isoforms of glucose transporters and their physiological functions

Isoform	Major sites of expression	Function
Na⁺-dependent glucose co-transporters		
SGLT1	small intestine and kidney	active uptake of dietary glucose from the lumen of the small intestine, and reabsorption of filtered glucose in the proximal tubule of the kidney
Facilitative glucose transporters		
GLUT1	placenta, brain, kidney, skeletal muscle	basal glucose uptake
GLUT2	liver, kidney, small intestine, pancreatic β -cells	uptake and release by hepatocyte, glucose sensor for β -cells
GLUT3	brain, placenta, kidney, liver	basal glucose uptake by almost all cells in humans; uptake of glucose by cells of the brain in other species
GLUT4	skeletal muscle, cardiac muscle white and brown adipose tissue	insulin-stimulated glucose uptake
GLUT5	small intestine, skeletal muscle	uptake from the lumen of the small intestine function in muscle (?)
GLUT7	liver	releases glucose from the endoplasmatic reticulum coupled to glucose 6-phosphate

Adapted and modified from Burrant CF et al. (1991) and Bell GI et al: (1990). GLUT6 is not shown because this isoform has been shown to encode a pseudogene.

More than one of these isoforms may be present in the same tissue, for reasons incompletely understood. Because they exhibit different K_m 's (K_m is the Michaelis Menten constant) for glucose binding it has been speculated that these GLUT isoforms may have different glucose transport capacities so as to support the physiological role of the tissue in which they predominate (Thorens *et al.*, 1990). Alternatively, the multiplicity of expression of some GLUT isoforms with different K_m 's indicates that they may have the same specific function in a number of different tissues (Bell *et al.*, 1990). This interpretation suggests that GLUT1 with a high affinity for glucose [low K_m (<2mM)] permits glucose to enter the cell efficiently even under conditions of low plasma glucose concentrations. It therefore most probably serves for basal glucose uptake. GLUT4 with a K_m of approximately 5 mM has a post-prandial role to channel glucose into the adipocytes and muscle cells. Since GLUT2, GLUT3, GLUT5 and GLUT7 are not involved in the facilitated glucose transport in skeletal muscle the functional role of GLUT1 and GLUT4 isoforms will be discussed only.

Figure 2.1. Cryosections of basal (A) and stimulated (not fasted, injected with 8 units/kg insulin, and 1 g/kg d-glucose and forced to exercise on a rodent treadmill (24 m/min) for 25 min.) (B) cardiac muscle, labeled for GLUT4 with 15-nm gold and for albumin 5-nm gold. The panels demonstrate the increase of GLUT4 labeling in the lateral (A and B) domains of the plasma membrane from basal state (A) to stimulated (B) state of the myocytes. GLUT4-positive tubulo-vesicular (T-V) elements occur more frequently near the lateral cell membrane in the basal state (A) than in stimulated (B) cells. m, mitochondria; f, myofilaments; z, Z discs; > , sarcolemma; —> , T-V elements; —> , junctional SR. (Bars = 200 nm).
 □ = artefact (Courtesy of J.W. Slot, University of Utrecht, the Netherlands).

In rat skeletal muscle the isoforms GLUT1 and GLUT4 have been identified. The level of GLUT's depends on the distribution of the various fibre types in metabolically heterogeneous skeletal muscles (Bonen *et al.*, 1992). Specifically, total GLUT4 protein content and GLUT4 mRNA levels are respectively five times and two times higher in rat oxidative muscle than in glycolytic muscle (Kern *et al.*, 1990). GLUT4 is remarkable because it is the only glucose transporter that, in response to insulin, translocates from intracellular domains to the cell surface and back (James *et al.*, 1988). Earlier studies, using the subcellular fractionation technique, showed that insulin and contractile activity increases the number of GLUT4 in the sarcolemma only two to three fold (Wardzala and Jeanrenaud, 1981; Klip *et al.*, 1987; Douen *et al.*, 1989;) which is not in agreement with the twenty to thirty fold increase in glucose transport. The difference between GLUT4 translocation and glucose transport rates may be an artefact due to the muscle fractionating technique to obtain muscle sarcolemma. In this approach the membrane fraction may be contaminated with intracellular material. More recently published data, using immunoelectron-microscopic techniques, reveal that during basal conditions only a fraction of the total GLUT4 pool is localized at the cell surface (Figure 2.1) whereas after stimulation a forty-fold increase in labelling was observed, which is entirely in accordance with the changes in glucose transport (Slot *et al.*, 1991; Rodnick *et al.*, 1992). A schematic representation of the glucose transporter and the subsequent cascade of glucose transport is depicted in Figure 2.2.

Effects of training and exercise on glucose uptake

Insulin binding and glucose uptake are increased with training (Bonen *et al.*, 1981; Tan and Bonen, 1984). These effects are probably related to the well-documented, training-induced increments in total GLUT4 transporter content (Friedman *et al.*, 1990; Ploug *et al.*, 1990; Wake *et al.*, 1991; Rodnick *et al.*, 1992), and possibly also in total GLUT1 transporter content (Ploug *et al.*, 1990). In addition, concurrent increments in GLUT4 mRNA (Ploug *et al.*, 1990; Rodnick *et al.*, 1990; Wake *et al.*, 1991), and GLUT1 mRNA (Ploug *et al.*, 1990) also occur with training. Thus training may be a regulator of GLUT gene expression. The benefit of this is a sustainable higher rate of glucose metabolism in muscles up to at least 4 days after the last training bout (Bonen *et al.*, 1992).

Increments in glucose transport during acute exercise appear to be due to an increase in the number of GLUT4 at the skeletal muscle cell membrane (Douen *et al.*, 1989; Fushiki *et al.*, 1989; Hirshman *et al.*, 1989; Goodyear *et al.*, 1990; Slot *et al.*, 1991). Slot *et al.* (Slot *et al.*, 1991) and Rodnic *et al.* (Rodnick *et al.*, 1992) showed that after exercise, translocation of GLUT4 entirely explains the increased glucose transport. GLUT1 was not affected by exercise (Douen *et al.*, 1990).

Cellular fate of glucose

The majority of glucose derived energy is conserved as high energy compounds, adenosine triphosphate (ATP) and Creatine Phosphate (CP) being quantitatively the most important in skeletal muscle. The pathway of cellular utilization of carbohydrates (Figure 2.3^a and 2.3^b) can be divided into three stages. In the first stage a glucose molecule, a 6-

Figure 2.2. Effects of insulin binding on the mechanisms and action of the insulin-regulatable-glucose-transporter (GLUT4). After binding to the α -subunit on the insulin receptor (1), a cascade of reactions takes place. The insulin receptor generates a signal which activates a second messenger system (2) which, among others, signals the redistribution of the GLUT4 (3) through the movement of transporter containing vesicles to the sarcolemma (4). The vesicles fuse with the sarcolemma accelerating the transport of glucose to the interior of the muscle cell (5). The transporters are retrieved from the sarcolemma by fission (6), and fuse with larger endosomes (7) where the transporter is stored in tubular extensions that pinch off to form new vesicles (8).

Note: This is a schematic and illustrative overview. Time course of the process and size of organelles are hypothetical.

Figure 2.3 a. Schematic representation of the cellular utilization of glucose. The number in the glycolytic pathway refer to the chemical reactions given on the right side of the figure. The enzymes that catalyze the reactions are given in *italic*.

*This reaction takes place when oxygen availability is insufficient (see text for more detailed information).

Figure 2.3^b. Citric acid cycle and the oxidative phosphorylation both of which occur inside the mitochondria. The formed ATP is transported from the inside of the mitochondria to the sarcoplasm where it serves as a high energy fuel for essentially all energy demanding processes within the cell.

F_p = flavoprotein; Q = ubiquinone (co-enzyme Q); Cyt = cytochromes
Adapted and modified from: Harper's Biochemistry (Murray et al. eds) (1990) Prentice-Hall International Inc. Englewood Cliffs, New Jersey, U.S.A.

carbon compound, is broken down through the glycolytic pathway into two molecules of pyruvate, a 3-carbon compound. This reaction produces enough energy to phosphorylate two molecules of ADP to ATP. In addition, two molecules of the coenzyme NAD^+ are reduced to produce NADH. When oxygen supply is sufficient, the pyruvate enters the mitochondrial matrix. This second stage involves the preparation and oxidation of acetyl coenzyme A in the citric acid cycle (also called "tricarboxylic acid cycle" or "Krebs cycle") to give CO_2 , H_2O and reduced coenzymes NADH and FADH_2 (see Figure 2.3^b). These reduced coenzymes enter the third and final stage of glucose oxidation which is called oxidative phosphorylation. This latter oxidation step is closely coupled to phosphorylation of ADP to ATP and produces most of the ATP for the cell. In muscle these pathways are responsible for about 95% of the ATP production. The efficiency to conserve energy in this manner is very high (ca. 60%). The other 5% ATP is produced outside the oxidative phosphorylation by means of substrate-linked phosphorylation. The major reaction sites of the substrate-linked phosphorylation are: (1) phosphoenolpyruvate to pyruvate; (2) bi-phosphoglycerate to 3-phospho-glycerate in the glycolysis, and (3) succinyl coenzyme A to succinate in the citric acid cycle.

Intracellular metabolism of glucose

Once glucose has entered the muscle cells it is immediately phosphorylated to glucose-6-phosphate by the cytoplasmic enzyme hexokinase. Except in renal tubular epithelium, intestinal epithelial and liver cells, which contain the enzyme glucose phosphatase, glucose-6-phosphate can be reconverted to glucose. Muscle cells lack this enzyme and glucose-6-phosphate is trapped inside the muscle cell. From this point on glucose can be utilized to yield energy or it can be stored as the glucose polymer glycogen. The transformation of the low molecular weight glucose molecules into the high molecular weight glycogen pool is extremely important in maintaining osmotic pressure of the intracellular fluids. It was calculated (Newsholme and Leech, 1991) that the total liver glycogen reserve, which equals 400 mM glucose, is stored as 0.01 μM glycogen, causing no osmotic pressure at all. In muscle cells up to three percent of their weight consists of glycogen, and in liver cells up to eight percent. However, since total muscle mass is substantially larger than total liver mass, there is twice as much total muscle glycogen.

Glycogenesis refers to the formation of glycogen; glycogenolysis to the degradation of glycogen. Glycogen synthase is the regulatory key enzyme of glycogenesis whereas glycogen phosphorylase regulates glycogenolysis. Both enzymes are known to exist in two forms. One is the active dephosphorylated "l"-form, the other the inactive phosphorylated "D"-form. Glycogen phosphorylase exists in an active phosphorylated "a"-form and an inactive dephosphorylated-"b" form. Note that glycogen synthase is active when dephosphorylated whereas glycogen phosphorylase is active when phosphorylated. Both processes are, as most metabolic intracellular processes, under strict control of the endocrine system. The metabolic pathway involved in the synthesis and degradation of glycogen is illustrated in a simplified manner in Figure 2.4.

Under conditions of insufficient oxygen supply, glycolysis represents an emergency-pathway to provide adequate ATP levels for a brief period of time. Since oxygen is not available to accept electrons in the respiratory chain cytoplasmic NADH cannot be

Figure 2.4. Pathway of glycogenesis and glycogenolysis in muscle. Mind that glycogen synthase is inactive in the phosphorylated state while glycogen phosphorylase is active in the phosphorylated state illustrating the close connection between these two enzymes.

* reactions are given in detail in left bottom panel of the figure.

oxidized in the mitochondria. To continue glycolysis the NAD^+/NADH ratio must be preserved at high values in the cytoplasm. This condition is met in the muscle cell by using NADH for the conversion of pyruvate to lactate. The lactate produced in the skeletal tissue can be reconverted to glucose in the liver (gluconeogenesis) (Bonen, 1989) or be directly used for energy, especially in cardiac muscle (de Groot, 1992; van der Vusse *et al.*, 1992).

Control of glucose metabolism by key enzymes

The control of metabolic flux through the glycolytic pathway is regulated by allosteric modulation of the glycolytic enzymes. Depending on the energy demands of the cell, this important way of modulation reduces the overall glycolytic flux. The most important regulatory enzymes of glycolysis are: (1) hexokinase; (2) fructose-6-phosphate kinase (FPK) also known as phosphofructokinase; and (3) pyruvate dehydrogenase, and these enzymes are inhibited by (1) glucose-6-phosphate; (2) an increased ATP to ADP ratio, and NADH to NAD^+ ratio, and citrate; and (3) an increased acetyl coenzyme A to CoA ratio, respectively.

Although still a matter of debate, the citric acid cycle as well as the oxidative phosphorylation are thought to be controlled by either the ATP : ADP ratio and/or by the intracellular level of calcium (Ca^{2+}). An increased ATP : ADP ratio reduces the overall velocity of the cycle(s).

2.4 Muscle fatty acid metabolism

Background

As mentioned earlier, under resting conditions and during prolonged submaximal exercise fatty acids are the predominant metabolic fuel for muscle cells with oxidative metabolic properties. Unlike glucose, fatty acids are poorly soluble in water. In plasma, the transport of lipids occurs as triacylglycerols as part of chylomicrons and very low-density lipoproteins (VLDL's), whereas in plasma fatty acids are also transported bound to albumin, or esterified as fatty acids.

Muscle fatty acid uptake

A schematic representation of uptake of fatty acids is depicted in Figure 2.5. The traditional view that the uptake of fatty acids occurs by simple diffusion has recently been challenged (for detailed reviews see Bass, 1988; Bassingthwaite, 1989; Glatz and van der Vusse, 1990; van der Vusse *et al.*, 1992). To date, the sequence of events leading to the absorption of fatty acids into the cell has been only partly elucidated. Prior to their uptake, blood-borne triacylglycerols have to be hydrolyzed by the enzyme lipoprotein

After van der Vusse et al., 1992.

The first step in the uptake of these fatty acids is their translocation through the luminal membrane of the endothelial cell, the cytoplasmic compartment of the endothelial cell and

finally through the abluminal membrane of the endothelial cell (see Figure 2.5.). Next, the fatty acids have to be transported through the interstitial space most probably bound to albumin (van der Vusse *et al.*, 1992), and finally, they are shuttled passively by diffusion or facilitated by plasma membrane fatty acid-binding proteins (FABP_{pm}) through the sarcolemma (van der Vusse *et al.*, 1992).

Figure 2.6. β -oxidation of fatty acids. This process takes place inside the mitochondria eventually leading to the formation of acetyl-CoA molecules which can be used in the citric acid cycle to form ATP. The numbers in the β -oxidative pathway refer to the chemical reactions given on the right side of the figure. The enzymes that catalyze the reactions are given in *italics*.

Abbreviations: see Figure 2.5.

Figure 2.7. Differences in fatty acid-binding protein (FABP) content between heart muscle (A) and oxidative soleus muscle (B) and glycolytic extensor digitorum longus muscle (C). Cryosections were labeled with 12-nm gold. (van Breda *et al.* unpublished results).

Effects of training and exercise on fatty acid transport

Since the transendothelial, interstitial and transsarcolemmal transport mechanisms of fatty acids have not yet been fully elucidated, information regarding the effects of training and exercise on these transport mechanisms is lacking as well. However, there are indications that training increases the lipoprotein lipase activity fraction at the luminal endothelial surface of muscle capillaries. This suggests that training increases the ability to hydrolyze VLDL-triacylglycerols and chylomicrons making more fatty acids available for uptake. Although during exercise a close correlation has been found between plasma fatty acid concentration and fatty acid uptake during exercise (Romijn and Wolfe, 1992), Turcotte *et al.* (Turcotte *et al.*, 1991) recently reported that palmitate uptake follow saturation kinetics.

Cellular fate of fatty acids

As mentioned before (see 2.3), there are marked differences between intracellular utilization of fatty acids and glucose. Whereas glycolysis takes place in the cytoplasm, fatty acids are metabolized in the mitochondria or peroxisomes by a process called β -oxidation under the formation of acetyl Coenzyme A (Figure 2.6) (van der Vusse *et al.*, 1992).

Intracellular metabolism of fatty acids

Upon entrance of the muscle cells fatty acids can be utilized to yield energy for metabolic processes, be incorporated into triacylglycerols or phospholipids for storage, or return to the circulation (backflux). To date, however, it is not clear whether fatty acids incorporated in the phospholipid pool are used for oxidative energy production (van der Vusse *et al.*, 1992).

Figure 2.8. Synthesis and degradation of triacylglycerol in muscle. Fatty acids are taken up from the vascular space as a result of the action of lipoprotein lipase on the circulating triacylglycerol of chylomicrons and very low density lipoproteins (VLDL's). The esterification of glyceral-3-phosphate, an intermediate of the glycolysis, with fatty acyl-CoA is accomplished by a set of enzymes (van der Vusse *et al.*, 1992). Intracellular lipases are responsible for the hydrolysis of triacylglycerol to fatty acids and glycerol. Since glycerol cannot be utilized in the muscle, it diffuses out into the plasma. Abbreviations: see Figure 2.5

Analogous to the albumin-bound fatty acid transport in the blood, intracellular transport of fatty acids to subcellular organelles probably requires or is facilitated by the presence of a carrier protein. Such a specific cytoplasmic carrier, the fatty acid-binding protein (FABP), was found 20 years ago by Ockner *et al.* (Ockner *et al.*, 1972). Although FABP may indeed function as a cytoplasmic fatty acid carrier its role, especially during training, has not yet been proven (Glatz and van der Vusse, 1990). Recently, FABP in skeletal muscle was found to be identical to the heart type FABP (H-FABP) (Peeters *et al.*, 1991). Vork *et al.* (Vork *et al.*, 1991) reported a positive relationship between the FABP content and the percentage of oxidative fibres in skeletal muscle, which suggests an important role for FABP in the rate of fatty acid oxidation (cf. Figure 2.7). The triacylglycerols are stored as cytoplasmic lipid particles. Since there is a continuous turnover of triacylglycerols, this storage is not the ultimate fate of the fatty acids. Although most of the triacylglycerols are stored in adipose tissue, skeletal and cardiac muscle also contain triacylglycerols, but only for local energy utilization. The sequence of reactions of the synthesis and degradation of triacylglycerol is depicted in Figure 2.8.

β -oxidation of fatty acids is an important source of acetyl coenzyme A formation, which can be readily used for degradation by the citric acid cycle of all muscle fibres with oxidative capacity. As can be viewed from Figure 2.6, the process can be divided into two stages: (1) an extra-mitochondrial activation step by which fatty acids are converted to acyl coenzyme A followed by carnitine-mediated translocation to the innermitochondrial space and (2) an intra-mitochondrial or intra-peroxisomal oxidative cycle by which the acyl coenzyme A re-enters the cycle finally leaving acetyl coenzyme A.

During stage 1 acyl coenzyme A is produced from fatty acids at the expense of ATP. The complete degradation and oxidation of fatty acids not only occurs inside mitochondria but also inside peroxisomes. The first step for utilization is the transport across the mitochondrial membrane to the mitochondrial matrix, where oxidation occurs. First fatty acids are activated by acyl-coenzyme A synthetase located at the outer mitochondrial membrane. Next, the acyl CoA-ester is converted at the innerface of the outer membrane into acyl-carnitine by carnitine-palmitoyl transferase I (CPT I) and reconverted at the matrix-site of the inner membrane by carnitine-palmitoyl transferase II (CPT II) into acyl-CoA. Subsequently, the acyl-CoA can be metabolized by the enzymes involved in β -oxidation. During stage 2 two carbons are removed from acyl coenzyme A leaving another acyl coenzyme A with two carbons less which re-enters the cycle until the final acyl coenzyme A is converted to acetyl coenzyme A.

Control of fatty acid metabolism by key enzymes

The control of the metabolic flux through β -oxidation is regulated by regulatory enzymes and is depicted in Figure 2.6. The enzymes of β -oxidation are: (1) acyl-CoA dehydrogenase, (2) enoyl-CoA hydratase, (3) 3-hydroxyacyl-CoA dehydrogenase and (4) 3-oxo-acyl-CoA thiolase. Furthermore, β -oxidation is fed by the substrate acyl-CoA. Therefore, the transport of acyl-CoA over the mitochondrial membrane by the enzymes CPT I and CPT II is the rate limiting event in controlling β -oxidation.

Figure 2.9. Schematic and simplified representation of the glucose-fatty acid cycle (Randle cycle). An increased uptake and/or oxidation of fatty acids is believed to inhibit glucose uptake and oxidation at several levels (dashed lines). An increased uptake of fatty acids inhibits the uptake of glucose (1), whereas an increased oxidation of fatty acids slows down the glycolysis and leads to an accumulation of glucose 6-phosphate which inhibits its own formation at the level of the enzyme hexokinase (2). Citrate diffuses out of the mitochondrion and inhibits one of the key enzymes of the glycolysis fructose 6-phosphate kinase (3). Inactivation of pyruvate dehydrogenase is facilitated by an high ATP/ADP, NADH/NAD⁺, and acetyl CoA/CoA ratio (4). ⊖ = inhibition

2.5 Integration of glucose and fatty acid metabolism

In the previous sections the regulation of glucose and fatty acid metabolism has been outlined separately. In this section the relation of the interaction between these pathways in muscle will be discussed.

Randle and coworkers (Randle *et al.*, 1963) were the first to demonstrate that the greater utilization of fat inhibits carbohydrate utilization via operation of the glucose-fatty acid cycle. According to their original hypothesis, they proposed that promotion of fatty acid oxidation leads to an increase in intracellular acetyl-CoA : CoA ratio and in NADH : NAD⁺ ratio which inhibits glucose oxidation at the level of pyruvate dehydrogenase (PDH). Furthermore, an increased acetyl-CoA concentration leads to an enhanced intramitochondrial citrate concentration. Some of the citrate escapes into the cytoplasm, where subsequently it inhibits the rate-limiting enzyme of glycolysis: fructose-6-phosphate kinase (FPK). This inhibition results in a rise of glucose-6-phosphate (G-6-P), a potent allosteric activator of glycogen synthase, and the diversion of glucose into glycogen synthesis. Accumulation of glucose-6-phosphate also inhibits cellular glucose utilization via inhibition of hexokinase (Randle *et al.*, 1964). A schematic overview of the Randle's cycle is depicted in Figure 2.9.

The operation of the glucose-fatty acid cycle was originally demonstrated to be active in isolated rat hearts and diaphragms (Randle *et al.*, 1963). To date, *in vivo* the cycle has been shown to be operative also in resting and exercised rat skeletal muscle (Dohm *et al.*, 1983; Kruszynska *et al.*, 1990; Koubi *et al.*, 1991; Kruszynska *et al.*, 1991).

As mentioned earlier (see 2.2) endurance-trained individuals rely less on carbohydrate and more on fat as an energy source during submaximal exercise. This decrease of carbohydrate utilization during prolonged exercise delays depletion of muscle and liver carbohydrate reserves (Jansson and Kaijser, 1987). Although attempts have been made to prove that substrate competition also occurs during exercise in man (Costill *et al.*, 1977; Décombaz *et al.*, 1983; Ravussin *et al.*, 1986; Hargreaves *et al.*, 1991), the results remain controversial and the precise biochemical mechanisms accounting for the glycogen sparing effect of endurance training remain largely unknown.

Besides the above mentioned catabolic integration between glucose and fatty acid metabolism, anabolic integration between glucose and fatty acids exists as well. In liver, but also in adipose tissue, acetyl-CoA derived from the degradation of glucose can be used for fatty acid synthesis. These fatty acids can be incorporated into triacylglycerols. The glycerol backbone of the triacylglycerol is supplied by glycerol-3-phosphate, a product derived from the glycolytic breakdown of glucose (see Figure 2.3^a).

A surplus of ingested carbohydrates can be stored as lipids, however, the storage of fatty acids themselves requires the presence of an active glycolytic pathway. These processes are of physiological importance because glucose storage as glycogen is limited; a few hundred grams in the human body, whereas kilograms of lipids can be stored. Furthermore, carbohydrates stored as lipids contain much more energy for each gram stored than carbohydrates stored as glycogen.

2.6 Endocrine regulation of fuel metabolism

Historical background

Although the effects of hormones and evidence for abnormal endocrine function are known since ancient times [the Georg Ebers papyrus (1550 BC) with the description of polyuria and the document of Pen Tsoa (2737 BC) with the recommendation of the use of semen of young men for the treatment of sexual weakness (Medvei, 1982)], it was not until the beginning of this century before it was realized that special glands discharge chemical messengers, which were later called hormones, into the circulation for action away from their production site. Hence, the term internal secretion was born. In the last decades it became apparent that a close relation and cooperation exists between the two most prominent control systems of the body, the central nervous system and the endocrine system.

In 1925 Walter Bradford Cannon was among the first to demonstrate the influence of the endocrine system on metabolism (Cannon, 1925). This interesting and exciting part of endocrinology is a rapidly expanding field and numerous experiments have been and will be carried out in attempts to unravel the complex concepts of the pathways of hormone action on metabolic processes. Especially the effects of endocrine function and its subsequent metabolic control during exercise is a relative new area of research.

Because new substances with endocrine characteristics are being discovered regularly, e.g. gastrointestinal hormones, Table 2.4. is given as an example and far from complete. Within the scope of this thesis, only the following aspects of hormonal control will be reviewed in this section:

Hormones and energy metabolism

- insulin
- glucagon
- growth hormone
- cortisol

Hormones with reproductive action

- testosterone
- estradiol-17 β

Before we will examine the endocrine systems involved in the control of carbohydrate and lipid metabolism, a brief general overview of hormone action will be provided.

Hormones and their actions

Hormones are usually classified, based on their molecular structures, in two basic categories: (1) lipophylic hormones (steroids), e.g. cortisol, estradiol-17 β , and testosterone and (2) hydrophylic hormones (peptides/proteins), e.g. follicle stimulating hormone (FSH),

Table 2.4 Listing of some of the principal hormones in the body

Hormone or source	Original gland	Target cells effect	Principal physiological
Steroids (lipophylic)			
- cortisol - corticosterone (in rats)	adrenal cortex	liver, muscle & adipose tissue	regulation of metabolism
- aldosterone	adrenal cortex	kidney	regulates Na ⁺ excretion
- progesterone	ovaries & placenta	uterus & breasts	cyclic glandular development of the endometrium; regulates metabolism of carbohydrates, proteins & lipids
- estradiol-17 β	ovaries & placenta; conversion from testosterone	reproductive tract; heart and skeletal muscle	stimulates growth and development; regulates muscle carbohydrate, lipid & protein metabolism
- testosterone	testes (males) in females primarily adrenal cortex	reproductive tract; heart & skeletal muscle	stimulates growth and development; regulates carbohydrate, lipid & protein metabolism
Amines (hydrophylic)			
- epinephrine	adrenal medulla	cardiovascular liver & adipose tissue	stimulates cardiovascular system; muscle; function; regulates energy metabolism
- thyroxine (T ₄)	thyroid gland	almost all cells	fetal development; regulates metabolism
Peptides/Proteins (hydrophylic)			
- calcitonin	parafollicular cells of bone thyroid gland		regulates plasma calcium & phosphate metabolism

continued on next page

Table 2.4 continued

- insulin	pancreatic β -cells	liver, muscle & adipose tissue	controls plasma glucose & regulates muscle, liver & adipose tissue energy metabolism
- growth hormone	anterior pituitary	bone, adipose tissue, & liver	stimulates growth of skeleton & muscle; skeletal muscle regulates muscle energy metabolism
- glucagon	pancreatic α -cells	liver	increases glycogenolysis and gluconeogenesis in the liver

calcitonin, and insulin (Table 2.4). Hormone action usually takes place away from the production site. The quantitative amount of hormones in the blood depend on the rate of secretion and the rate of removal from the blood [metabolic clearance rate (MCR)]. The amount of hormones in the blood is relatively small (range from a few pmol/l to nmol/l). Before hormones initiate their specific cellular response they have to be recognized by highly specialized proteins (receptors) which are localized on the surface of the cell membrane, in the cytoplasm or in the nucleus. The tissues that are affected by the hormone(s) apparently contain the appropriate receptor(s) for these hormone(s). Therefore, it can be easily understood that each cell contains a large number of different receptors. These receptors have to recognize a particular hormone and translate its receptor interaction into a signal which promotes a specific cellular response.

Once the hormone-receptor binding has been completed a cascade of events occurs that causes the hormonal effects. This can be achieved directly by the receptor itself (i.e. steroids) or indirectly via second messenger systems [cyclic AMP (cAMP); calmodulin; phosphoinositides (PI-cycle)].

Testosterone and estradiol-17 β , both belonging to the class of steroid hormones, are derived from cholesterol and have a lipophylic character. Because of this characteristic they easily cross the cell membrane and subsequently bind to cytoplasmic and/or nuclear receptors. In contrast to hormones with a hydrophylic character, the intracellular concentration of hormones with a lipophylic character is directly related to the plasma concentration (Bartsch *et al.*, 1983). Therefore, one can easily understand that changes in plasma hormone concentration may have a major impact on the intracellular action of hormones.

The small amounts of hormones in the blood require a highly sensitive assay system

and several decades ago it was almost impossible to measure the blood hormone concentrations. However, in the late fifties radioimmunoassays were developed and from then on it became possible to accurately measure hormone concentrations and their metabolic end products (Yalow and Berson, 1959).

Regulation of carbohydrate and lipid metabolism by hormones directly involved in energy metabolism

Hormones that regulate the maintenance of a normal plasma glucose concentration include insulin, glucagon, growth hormone and cortisol. Insulin is the main hormone with a blood glucose lowering effect. Under resting pre-absorptive conditions muscle tissue is almost impermeable to glucose and relies on fatty acids for energy. The rise of the plasma glucose concentration after the absorption of glucose from the intestine stimulates the β -cells of the pancreas to release insulin. The fact that the pancreatic vein drains directly into the liver makes insulin ideally suited to regulate glucose homeostasis by reducing glycogenolysis and gluconeogenesis in the liver. Furthermore, insulin causes the insulin-sensitive peripheral tissues, e.g. skeletal muscle, to absorb more glucose. During exercise, muscle cells depend largely on glucose as metabolic fuel (for a more detailed general view see chapter 2.2). Interestingly, however, during prolonged as well as during short-term intensive exercise muscle cells do not require a corresponding increase in plasma insulin concentration to meet an increased muscle glucose utilization. Even at very low plasma insulin concentrations, muscle cells become highly permeable to glucose by muscle contraction itself (Richter *et al.*, 1985; Wallberg-Henriksson, 1987). During the post-absorptive period the non-exercising muscle directs its increased glucose uptake into glycogen storage by increasing the insulin dependent key enzymes of the glycogenetic pathway.

In addition to the profound effects of insulin on carbohydrate metabolism are its effects on lipid metabolism. Since most of these effects occur in the liver and adipose tissue one can easily understand the close, insulin controlled, relation between carbohydrate and lipid metabolism in these and other organs. A lack of insulin has a profound effect on lipid metabolism. The important anti-lipolytic effect of insulin then has disappeared subsequently resulting in the activation of adipocytal hormone-sensitive lipase, which induces the release of large amounts of glycerol and fatty acids. This will cause on the site of the liver increased gluconeogenesis from glycerol and an increased ketone body production from fatty acids and at the site of skeletal muscle an inhibited glucose uptake.

Counteracting the glucose-lowering effect of insulin, is the glucose-raising effect of glucagon, growth hormone, and cortisol. Glucagon represents the humoral mechanism for the delivery of energy to the tissues between meals. It is synthesized in the α -cells of the islands of Langerhans in the pancreas. The main metabolic aim is to protect the organism from hypoglycemia by stimulating glycogenolysis, gluconeogenesis and ketogenesis in the liver.

Aside from its growth promoting effect on almost all tissues, growth hormone (GH) also has an important effect on carbohydrate metabolism. Unlike the other anterior pituitary hormones, GH is not a "glandotrope" hormone but elicits its effect directly on all cells of the body ("effector hormone"). GH decreases the utilization of glucose for energy by enhancing

glycogenesis and diminishing tissue glucose uptake. An interesting observation is that growth hormone fails to exert its growth promoting action in the absence of insulin. The main effect of GH on lipid metabolism is the enhancement of fatty acid utilization and, as a consequence, a decrease of carbohydrate metabolism (see 2.5 the glucose-fatty acid cycle).

Like glucagon, the liver seems to be the main target organ involved in the metabolic effects of cortisol. Cortisol is the principal glucocorticoid and is secreted by the zona fasciculata of the adrenal cortex. Cortisol increases hepatic glucose production by increasing gluconeogenesis and in turn increasing the availability of glucogenic amino acids from the peripheral tissues (Exton, 1979). Furthermore, cortisol inhibits peripheral glucose uptake and utilization by limiting the rate of glucose transport across the cell membrane (Fain, 1979). Like the other counter regulatory hormones, cortisol also promotes the release of fatty acids from adipose tissue which subsequently leads to an increased lipid metabolism. Thus, cortisol increases the utilization of lipids to conserve glucose and glycogen stores. Like GH, the cortisol counter regulatory mechanism requires several hours to become fully expressed and is by far not as effective as a similar percentual shift of plasma insulin.

Figure 2.10. Conversion of androgens by complex enzyme systems. A very important physiological and pathological conversion is that of testosterone to 5α-dihydrotestosterone or the conversion of testosterone to estradiol-17β. HSD = hydroxysteroid dehydrogenase

Regulation of carbohydrate and lipid metabolism by hormones with reproductive action

Unlike the effects of the above mentioned hormones, literature concerning the effects of hormones with reproductive action on carbohydrate and lipid metabolism, is rather limited. In males testosterone is the main testicular steroid hormone and is secreted by the interstitial cells of Leydig whereas in females the major part of the circulating testosterone is produced by the adrenal glands and the ovaries. A variety of biological functions of testosterone has been defined with the most important one being the development of the primary and secondary sexual characteristics (Mooradian *et al.*, 1987).

In female individuals the ovary is the major source of female sex-hormone production. Estradiol-17 β , the major female sex-hormone produced by the ovary, is synthesized from testosterone, which is produced by the theca cells in the ovaries, by a group of enzymes known as the aromatase enzyme complex (Figure 2.10). This aromatase enzyme system has also been shown to be operative in other tissues, including human skeletal muscle (Longcope *et al.*, 1976; Longcope *et al.*, 1978; Matsumine *et al.*, 1986; Doody and Carr, 1989). Estradiol-17 β and its related estrogens are required for normal maturation of the female. Among others, estradiol-17 β stimulates growth, the distribution of body fat, and the "secondary sexual characteristics" of the female individual.

Except for the effects of testosterone on protein metabolism (for detailed reviews see Heitzman, 1980 and Florini, 1987), information regarding the testosterone-induced effects on carbohydrate and lipid metabolism remains incomplete. Gillespie and Edgerton (Gillespie and Edgerton, 1970), Bergamini (Bergamini, 1974), and Guezennec *et al.* (Guezennec *et al.*, 1984) reported a testosterone-induced enhancement of glycogenesis in rat skeletal muscles. Support for these latter findings is provided by the results of Max and Toop (Max and Toop, 1983) and Adolfsen and Ahren (Adolfsson and Ahren, 1968), who reported a testosterone-induced enhancement of glucose uptake and glycogen synthase activity in skeletal muscle. In contrast with these latter findings are the recent results of Holmång *et al.* (Holmång *et al.*, 1990; Holmång *et al.*, 1992), who reported that testosterone mitigated the effect of insulin on muscle glucose uptake and glycogen disposal in ovariectomized female rats.

Since the introduction of the hormonal contraceptives in the late 1950s, studies concerning the metabolic effects of, especially, estradiol-17 β , rapidly evolved. Estradiol-17 β has been shown to induce glucose intolerance in oral contraceptive users (Godsland *et al.*, 1992), to stimulate glucose uptake in animal muscle (Roskoski and Steiner, 1967; Puah and Bailey, 1985; Meier and Garner, 1987), and spare cardiac and skeletal muscle glycogen of female rats (Gorski *et al.*, 1976; Kendrick *et al.*, 1987). From these studies it is apparent that estradiol-17 β has a profound effect on metabolism but the mechanisms by which these effects are generated remain incompletely understood.

Effects of acute exercise and training on plasma hormone concentrations

Exercise is a strong stimulus for changing plasma concentrations of many hormones. The exact mechanism by which the endocrine system regulates the complex pathways of metabolic changes under these conditions are only partly understood (Fotherby and Pal,

1985; Bouchard *et al.*, 1990). In males, for example, it is well known that after exhaustive exercise the disturbed endocrine system significantly lowers plasma testosterone levels for hours or even days (Janssen *et al.*, 1986). Since this is beyond the scope of this thesis, we will only provide a brief representation of the acute and chronic exercise-induced changes on the hormones mentioned above. For more detailed information the reader is referred to excellent reviews by Galbo, 1981, Boyden *et al.*, 1983; Fotherby and Pal, 1985, Bouchard *et al.*, 1990 and Keizer and Rogol, 1990.

To date, it is generally accepted that during prolonged exercise plasma insulin concentration decreases (Sutton *et al.*, 1990), and as mentioned before, this decrease of plasma insulin results in decreased hepatic glycogen synthesis, increased peripheral lipolysis and increased hepatic glycogenolysis (Wolfe *et al.*, 1986). Since the contraction process itself can regulate the utilization of glucose from the circulation, the continuous decrease of insulin does not limit the exercise performance itself, that is, glucose uptake can and will remain adequate for a given work load. Because numerous studies concerning the acute and chronic exercise-induced changes in plasma insulin have been published, the reader is referred to some detailed reviews (Galbo, 1981; Pruett, 1985; Viru, 1985). In brief, in humans it has been shown that in untrained individuals, a single bout of exercise increases insulin sensitivity (binding capacity of insulin to its receptor), which effect lasts for at least 48 hr but less than 5 days, to values similar to those found in trained subjects. However, insulin responsiveness (cellular action of insulin) was lower than in the trained individual (Horton, 1988; Mikines *et al.*, 1988; Mikines *et al.*, 1989a; Mikines *et al.*, 1989b). Interestingly, in trained individuals abstained from exercise for 5 days, a single bout of exercise decreased insulin sensitivity without alterations in insulin responsiveness (Mikines *et al.*, 1989a; Mikines *et al.*, 1989b). The latter findings could, as we shall see later on, have a great beneficial effect in individuals with a disturbed insulin action.

Plasma glucagon concentrations has been shown to increase during exercise. However, in humans the rise of plasma glucagon seems to be somewhat delayed following the onset of exercise. Since the exercise-induced effect is mandatory in order to prevent hypoglycemia, it is not known whether glucagon plays a role in hepatic glycogenolysis, ketogenesis or gluconeogenesis (Sutton *et al.*, 1990).

It is generally agreed that growth hormone secretion is increased during exercise in humans. As applies to all hormones, intensity and duration of the exercise bout seems to be the most important factor controlling its secretion. At the same relative workload, the peak response was the same for both untrained and trained individuals, whereas the duration of the exercise-induced secretion was prolonged in the untrained subjects (Sutton *et al.*, 1969; Bloom *et al.*, 1976).

Cortisol, together with the catecholamines, is the most essential hormone in preparing the body to cope with stress, and hence the name "stress hormone" (Seyle, 1956). Numerous studies of the effect of exercise upon adrenocortical function have emerged from the endocrine laboratories yielding different results due to difficulties in the methods used and differences in experimental design. Furthermore, due to differences in training status it is difficult to compare the results of most studies concerning the training induced effects on plasma cortisol concentration. Despite these difficulties some general accepted conclusions can be drawn: (1) in males after exercise increments in plasma cortisol have been found (Kuoppasalmi, 1980a); (2) at an intensity of more than 60% of the individual's maximal oxygen uptake ($\text{VO}_{2\text{-max}}$), cortisol starts to increase progressively as the

duration of the exercise increases (Davies and Few, 1973; Bonen, 1976); (3) at total exhaustion, cortisol concentrations have been shown to be completely suppressed (Dessypris *et al.*, 1976; Brandenberger, 1985) and (4) plasma cortisol did not change as a response to training (Brandenberger, 1985).

During the last decade a growing interest emerged regarding the effects of acute exercise and training on the plasma concentration of reproductive hormones (Fotherby and Pal, 1985; Keizer and van Soest, 1986; Keizer, 1987; Hackney, 1989; Keizer and Rogol, 1990). In males, after exhaustive exercise plasma testosterone levels were found to be decreased (Dufaux *et al.*, 1979; Kuoppasalmi, 1980a; Kuoppasalmi, 1980b; Janssen *et al.*, 1986). Furthermore, it has been shown that the longer the duration, the longer it takes before testosterone levels return to the pre-contest levels (Keizer, 1987). Unlike in males, in females testosterone levels have been shown to increase proportional to the applied exercise bout (Keizer, 1980b; Keizer, 1987; Keizer, 1987a). Training itself augments the basal testosterone levels in males (Kuoppasalmi *et al.*, 1976; Remes *et al.*, 1979; Kuoppasalmi, 1980a; Fotherby and Pal, 1985), whereas in females, no changes in plasma testosterone in response to training were found (Keizer, 1987a).

To date, to the best of our knowledge, no information is available regarding the exercise and/or training induced changes in plasma estradiol-17 β in males. In females, however, the inconsistencies between the results of exercise-induced changes in plasma estradiol-17 β are hard to explain. Confounding factors may be the phase of the menstrual cycle in which the test took place and the sensitivity of the radioimmunoassay. For instance, Keizer (Keizer, 1987a) reported an exercise induced increment in plasma estradiol-17 β in the follicular as well as in the luteal phase of the menstrual cycle, whereas Jurkowski *et al.* (Jurkowski *et al.*, 1978) and Bonen *et al.* (Bonen *et al.*, 1979) reported no change in plasma estradiol-17 β . The results of the studies investigating the training-induced changes in plasma estradiol-17 β are also inconsistent (Boyden *et al.*, 1984; Bullen *et al.*, 1984; Bonen and Keizer, 1987; Keizer, 1987a; Keizer *et al.*, 1987b). The major problem in studying highly trained female athletes, are the training-induced menstrual cycle disturbances, differences in energy balance and in body composition, which makes it very hard to decide in what phase of the cycle the women are.

In conclusion, the exercise and training-induced changes in plasma reproductive hormones might be sufficient to disturb the endocrine homeostasis which, in turn, might have implications for skeletal muscle metabolism, as was found in animal experiments (Gorski *et al.*, 1976; Guezennec *et al.*, 1984; Kendrick *et al.*, 1987; Holmäng *et al.*, 1990; Kendrick and Ellis, 1991; Holmäng *et al.*, 1992).

2.7 Pathological derangements of carbohydrate and lipid metabolism in non-insulin-dependent diabetes mellitus

Clinically overt (Type 2) non-insulin-dependent diabetes mellitus can be described as a disorder characterized by miscellaneous humoral and/or metabolic derangements. This type of diabetes mellitus accounts for 80-90% of all the diagnosed cases of diabetes

Figure 2.11. A scheme for the disturbances of metabolism in the late stage of non-insulin-dependent diabetes mellitus. Insulin resistance at the level of the liver (1) causes a decreased glycogenesis and an increased gluconeogenesis in the liver (2) and an impaired glucose uptake in skeletal muscle (3) due to insulin resistance (1), hyperglycemia develops. Chronically elevated plasma glucose levels decrease β -cell responsiveness and consequently decrease plasma insulin (4). At the level of the adipocyte insulin resistance (1) causes an increased lipolysis (5). In skeletal muscle the increased uptake and/or oxidation of fatty acids directly decrease glucose uptake (6), and reduces the glycolytic flux by inhibiting key enzymes of the glycolytic pathway (7). Defects of the insulin receptor (8) may cause an impaired insulin binding. In addition defects in postreceptor signal transduction causes an impaired glucose uptake and/or storage by decreasing the translocation of glucose transporter isoform 4 (GLUT4) (see Figure 2.2) (9) or impairing glycogen synthesis by inhibiting the synthesis of the key enzyme glycogen synthase (9). $\ominus$ = inhibition; $\oplus$ = stimulation. Adapted and modified from: Textbook of Diabetes (Eds. Pickup, J and Williams, G).

mellitus and the prevalence of persons suffering from this disease is still increasing (Verkley, 1990).

The full blown syndrome of non-insulin-dependent diabetes mellitus is characterized by the presence of peripheral insulin resistance, i.e. in skeletal muscle, liver, and adipose tissue, in combination with an impaired pancreatic insulin secretion (Figure 2.11). To date, the evidence is increasing that insulin resistance of skeletal muscle is the earliest and primary defect in the development of non-insulin-dependent diabetes mellitus (DeFronzo, 1988; de Boer *et al.*, 1990; Yki-Järvinen *et al.*, 1990; Häring, 1991; DeFronzo, 1992; Vaag *et al.*, 1992; Häring and Mehnert, 1993). Another important defect is the inability of the pancreatic β -cell to compensate for the insulin resistance, which, once overt diabetes develops, results in hypoinsulinemia (DeFronzo, 1988). It has also been suggested that some of the mechanisms responsible for insulin resistance are the consequence of multiple metabolic derangements such as hyperglycemia, and elevated plasma fatty acids (Widén *et al.*, 1992; Häring and Mehnert, 1993). Furthermore, epidemiological human and animal studies (Achard and Thiers, 1921; Stein and Leventhal, 1935; Poretsky, 1992). Especially in women with non-insulin-dependent diabetes mellitus and in non-diabetic women with hyperandrogenism due to polycystic ovaries and/or acanthosis nigricans (Achard and Thiers, 1921; Stein and Leventhal, 1935; Chang *et al.*, 1983; Dunaif *et al.*, 1987; Dunaif and Graf, 1989), hyperandrogenism has been suggested to play a significant etiological role in the development of the pathological condition. Although hepatic and pancreatic metabolic defects also play an important role in the pathogenesis of non-insulin-dependent diabetes mellitus (DeFronzo, 1988; Wolffenbuttel and van Haeften, 1993), in the present overview we will only focus on skeletal muscle as tissue involved in the pathogenesis of non-insulin-dependent diabetes mellitus.

Carbohydrate derangements in skeletal muscle of non-insulin-dependent diabetes mellitus

Both insulin receptor and/or post-receptor defects are possibly involved in skeletal muscle resistance to insulin (Häring and Mehnert, 1993). The insulin receptor itself seems to be the first candidate for the localization of a defect of intracellular insulin signalling, which subsequently could lead to insulin resistance. The existence of this receptor was postulated simultaneously by three independent investigators in the early 1970s (Cuatrecasas, 1971; Freychet, 1971; Kono and Barham, 1971). Insulin binding to its receptor results in a cascade of molecular reactions (for a detailed review see; Häring, 1991). In brief, binding of insulin to the α -subunit of the receptor leads to phosphorylation of another subunit which contains an intrinsic tyrosine kinase which subsequently undergoes autophosphorylation (Häring, 1991). Further signal transduction, i.e. activation of metabolism, occurs, among others, through tyrosine phosphorylation of cellular proteins. Results from several studies, which have been reviewed in detail by DeFronzo (DeFronzo, 1988) and Häring (Häring, 1991), have shown that no differences in insulin binding could be observed in the insulin resistant state and that a postbinding defect must be responsible for the insulin resistance. There is now strong evidence that insulin receptor kinase inactivity (postbinding step) contributes, in particular in skeletal muscle, to the insulin resistant state of skeletal muscle in non-insulin-dependent diabetes mellitus.

(Häring, 1991; Häring and Mehnert, 1993).

Defects beyond the receptor level affect insulin controlled metabolism of glucose and are called post-receptor defects. Defects at this level include the glucose transport system, defects in kinase activities, and the enzymes involved in the regulation of muscle glycogenesis (i.e. glycogen synthase) and muscle glucose oxidation (i.e. pyruvate dehydrogenase). As mentioned in section 2.3 glucose is absorbed by the muscle cells in the basal state through GLUT1 and after stimulation by insulin mainly through GLUT4. The literature concerning GLUT4-regulated glucose uptake in non-insulin-dependent diabetes mellitus skeletal muscle is now rapidly emerging. In streptozotocin (for action of streptozotocin see 3.3) induced-diabetic animals reduced glucose transport has been attributed to a reduction in the plasma membrane GLUTs (- 50%) and/or in the intracellular pool GLUTs (- 32%), although the insulin-induced GLUT-translocation from its intracellular pool to the plasma membrane was not impaired (Ramlal *et al.*, 1989). In contrast, the locus of impaired glucose transport in obese fa/fa Zucker rats (Sherman *et al.*, 1988), and ob/ob mice (Crettaz *et al.*, 1980), can not be related to concomitant changes in the GLUTs, since total GLUT4 levels are normal in these insulin resistant animals (Friedman *et al.*, 1990). Similar observations were made in patients with obesity or non-insulin-dependent diabetes mellitus (Pedersen *et al.*, 1990). However, from recent studies in rats it has become clear that GLUT4 availability from the intracellular domains (translocation) is responsible for the entire increase in glucose transport (Slot *et al.*, 1991a; Slot *et al.*, 1991b; Rodnick *et al.*, 1992). Although to date only a limited number of this kind of studies in non-insulin-dependent diabetic patients have been published, a defect in translocation rather than in total number of GLUTs seems to be responsible for the mentioned defects in glucose uptake.

Besides an impaired glucose oxidation at the level of the insulin regulated enzyme pyruvate dehydrogenase, the most important postreceptor defect caused by persistent hyperglycemia is the impaired glycogenesis due to decreases in the activity of the rate limiting key enzyme glycogen synthase (DeFronzo, 1988). In both non-insulin-dependent diabetic patients (Bogardus *et al.*, 1984; Felber *et al.*, 1987; Mandarino *et al.*, 1990) as well as in streptozotocin-induced diabetic rats (Schaffer *et al.*, 1986; Kruszynska, 1988; Kruszynska and Home, 1988) it has been shown that a reduction in activity of glycogen synthase contributes to the insulin resistant state.

Since the anaerobic glycolytic pathway (lactate production) is, to the best of our knowledge, not involved in the impaired glycogenesis as observed in non-insulin-dependent diabetes mellitus, we will not discuss this metabolic route.

Lipid derangements in non-insulin-dependent diabetes mellitus

Lipid aberrations are common among patients with non-insulin-dependent diabetes mellitus (Raeven, 1987; Wolffenbuttel, 1991). It has been suggested that the insulin resistance may be caused by substrate competition between glucose and fatty acids (Bevilacqua *et al.*, 1990; Widén *et al.*, 1992). This substrate competition was originally demonstrated to be operative in isolated rat hearts and diaphragms by Randle *et al.* (Randle *et al.*, 1963; Randle *et al.*, 1964). Although recently the Randle cycle has been

shown to be operative also in rat skeletal muscle (Kruszynska *et al.*, 1990; Kruszynska *et al.*, 1991), information regarding the effect of an increased substrate competition in patients with non-insulin-dependent diabetes mellitus and diabetic laboratory animals remains to be demonstrated (DeFronzo, 1988; Bevilacqua *et al.*, 1990; Widén *et al.*, 1992).

Exercise and non-insulin-dependent diabetes mellitus

Regular physical exercise was recognized as a principal therapy for the treatment of diabetes already centuries ago (Sushruta, 1938 (The original book was published in 500 BC); Horton, 1988;). Nevertheless, it was not until the beginning of this century that the metabolic response of exercise, i.e. a lower blood glucose concentration, was appreciated (Allen *et al.*, 1919). The increase in insulin sensitivity and glucose tolerance which accompanies exercise training can actually alleviate the symptoms of non-insulin-dependent diabetes mellitus. Indeed it has been shown that in patients with non-insulin-dependent diabetes mellitus the response to an oral glucose tolerance test (OGTT) was normalized after 12 months of training (Holloszy *et al.*, 1987). To date, our understanding of the beneficial cellular responses of exercise and/or training, especially in skeletal muscle, is rapidly increasing due to new techniques. For instance, it is known that contractile activity and insulin independently can stimulate glucose transport into muscle, and together they synergistically stimulate glucose transport (Bonen *et al.*, 1984; Nesher *et al.*, 1985; Bonen *et al.*, 1988; Bonen, 1989; Wallberg-Henriksson, 1989; Bonadonna *et al.*, 1990; Ploug *et al.*, 1990). Furthermore, recent animal studies have shown that the increased glucose transport capacity after exercise and training seems to be due to an increase in the number of total GLUTs in skeletal muscle (Douen *et al.*, 1989; Douen *et al.*, 1990; Ploug *et al.*, 1990; Wake *et al.*, 1991). Slot *et al.* (Slot *et al.*, 1991a) and Rodnick *et al.* (Rodnick *et al.*, 1992) have shown that in the rat translocation of GLUT4 to the sarcolemma entirely explains the increased exercise-induced glucose uptake. Furthermore, it has been shown that a single bout of exercise increases muscle sensitivity to insulin due to an increased nonoxidative glucose disposal (Devlin *et al.*, 1987). Therefore, it is clear that the effects of acute exercise on improving glucose tolerance do not last indefinitely. Within a day or two after the last training bout reversal of the improved glucose handling is already evident (Schneider *et al.*, 1984; Segal *et al.*, 1991). This once again underscores the need for regular physical activity.

2.8 References

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3 General Methods

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Steroid drug delivery systems: evaluation of three models

(submitted)

3.1 Rationale

In this chapter we describe the general methods used in the following chapters of this thesis. Furthermore we will describe the analytical methods and their validation in detail, whereas in the corresponding chapters we will refer to this chapter and give a brief summary of the methods and materials used only.

3.2 Introduction to the study of a model in vitro

It is well recognized that skeletal-muscle metabolism is under strict control of the endocrine system (Galbo, 1981; Florini, 1987). Unlike the situation in the intact animal, the isolated muscle in buffer makes it possible to study particular effects of hormones, uncomplicated by side effects of other substances which might occur in vivo. However, this model in vitro does not optimally reflect the situation in vivo. A major disadvantage of the model might be the absence of capillary flow, which could severely hamper substrate and O₂ diffusion into muscle cells. It has been shown that incubation of skeletal muscles of young rats (weighing 40-70 g) inevitably led to formation of a central core in which loss of glycogen occurred (Maltin and Harris, 1985). Several investigators (Le Marchand-Brustel *et al.*, 1978; Bonen *et al.*, 1981; Bonen *et al.*, 1984; Jones *et al.*, 1984; Tan and Bonen, 1984; Watson-Wright *et al.*, 1984; Puah and Bailey, 1985; Wallberg-Henriksson, 1987) assumed (but did not provide firm evidence) that mouse and other small-rodent skeletal muscles are thin enough to avoid diffusion problems into the inner part of the muscle. However, core formation and diffusion of O₂ are influenced, not only by the tissue thickness, but also by the metabolic rate of the donor animal (Maltin and Harris, 1985; Segal and Faulkner, 1985) and the temperature of the buffer solution. Therefore the aim of the present study was to evaluate the mouse skeletal muscle preparation in vitro as described by Le Marchand-Brustel *et al.* (Le Marchand-Brustel *et al.*, 1978) and Bonen *et al.* (Bonen *et al.*, 1981) in order to determine: (a) whether we could reproduce the results of glycogenesis as described by the aforementioned investigators, (b), if so, to determine what accounted for the variation in glycogenesis, and (c) to evaluate the viability of the incubated muscles by employing histochemical techniques.

3.3 Materials & Methods

Fifty-four male Swiss-Webster mice weighing 30-32 g were obtained from Charles River Wiga GmbH (Sulzfeld, Germany). The animals were housed in groups of eight in a constant-temperature (22 ± 1 °C) room on a 12 h-light (7:30-19:30) / 12 h-dark (19:30-7:30)

cycle and had free access to water and standard laboratory chow (Hope Farms, Woerden, The Netherlands). Procedures were performed as described by Le Marchand-Brustel *et al.* (Le Marchand-Brustel *et al.*) and Bonen *et al.* (Bonen *et al.*, 1981). Briefly, at the time of the experiment the mice were anaesthetized with phentany/fluanisone (0.05 ml/g body weight, subcutaneously) (Hypnorm; Janssen, Beerse, Belgium). From the hindlimbs both the soleus and the extensor digitorum longus muscles were carefully removed, weighed on a Mettler balance and mounted on a stainless steel V-shaped clip. Each muscle was individually immersed at 0 °C in an oxygenated ($O_2/CO_2 = 19:1$) Krebs-Ringer-Bicarbonate buffer, pH 7.4 (Bonen, 1981), containing 4% (w/v) BSA (essentially fatty acid-free, catalogue no. A-6003, Sigma Chemicals Co., St Louis, MO, USA). After the muscles of one animal were dissected free, requiring about 15 min, they were transferred to plastic vials (Poly Q; Beckman, Galway, Ireland) containing 1.5 ml Krebs-Ringer bicarbonate buffer supplemented with 2 mM-pyruvate and either 0, 0.4, 1.0 or 10.0 munits/ml of insulin (pig insulin, catalogue I 3505; Sigma Chemicals, Co.). The muscles were randomised so that, of each pair from one animal, one was used as test muscle and the contralateral as control muscle. All the muscles were then preincubated in a shaking water bath for 30 minutes at 37 °C. At the end of the preincubation period the muscles were transferred to other incubation vials containing 1.5 ml. Krebs-Ringer bicarbonate buffer supplemented with 5 mM D-[3- 3H]glucose (1 μ Ci/vial) (New England Nuclear, Dreichenheim, Germany) and insulin at the concentrations as indicated above. Incubation was then continued for 60 min at 37 °C. In order to keep both the pH and the PO_2 constant throughout the preincubation and incubation periods the gas in the vials was saturated with ($O_2/CO_2 = 19:1$) and refreshed every 15 minutes; in between, the vials were closed air-tight. In a previous study it turned out that O_2 -saturation was the same ($> 99.5\%$) whether the gassing was intermittently or continuously (van Breda, unpublished results). pH in medium was measured every 15 minutes by a standard pH meter (PHM 82 standard; Radiometer, Copenhagen, Denmark). Osmolality was measured by the method of freezing point depression. In a separate study creatine kinase activity was measured in samples withdrawn from the incubation medium every 15 minutes and in the muscle homogenates at the end of the incubation period by using a Boehringer Mannheim G.m.b.H. (Mannheim, Germany) standard method. Values were corrected for volume changes of the medium.

Washing and digesting was carried out as described by Le Marchand-Brustel *et al.* (Le Marchand-Brustel *et al.*, 1978). Labelled glycogen was measured as described previously (Bonen *et al.*, 1984), and protein was determined by the method of Lowry *et al.* (Lowry *et al.*, 1951), with BSA as standard.

Muscle samples for histochemical studies were taken from both pre-incubated [30 min ($n=6$)], incubated [60 min ($n=8$) and 90 min ($n=6$)] and non-incubated [0 min ($n=6$)] control muscles. They were mounted in an embedding medium (Tissue Tek; Miles Laboratory, Elkhart, IN, U.S.A.), frozen in isopentane cooled with liquid N_2 to its freezing point, and stored at -80 °C until analysis. Transverse sections (10 μ m) were taken from the middle 'belly' region of the muscles with a cryostat at -22 °C and made to react with hematoxylin/eosin (HE) for a general view and periodic acid/Schiff reagent for glycogen. Next the average maximal thickness was determined using video images of the sections and a Kontron MOP-videoplan digitizer system (Zeis, FRG) at the middle 'belly' region of the muscles. Because of the small absolute amount of glycogen in the muscles, we had to

Table 3.1. Parameters of incubation medium that are indicative of a stable muscle preparation *in vitro*

Time (min)	Creatine kinase release (% total muscle content)		pH	Osmolality (mOsm)	O ₂ saturation (% O ₂ in medium)
	Soleus	EDL			
0	0.20±0.9 (12)	0.12±0.2 (12)	7.37 ±0.02 (40)	288 ±3.2 (12)	99.7 ±0.05 (12)
15	4.19±1.2 (12)	2.39±1.8 (12)	7.38 ±0.02 (37)	290 ±4.0 (11)	99.7 ±0.08 (12)
30	5.20±2.2 (12)	2.89±1.4 (12)	7.37 ±0.03 (40)	289 ±2.2 (12)	99.6 ±0.11 (12)
45	6.78±1.6 (12)	3.68±1.9 (12)	7.40 ±0.10 (40)	300 ±5.8 (8)	N.D.
60	8.96±3.4 (12)	4.93±2.8 (12)	7.40 ±0.09 (40)	290 ±5.1 (12)	99.4 ±0.05 (20)
90	8.32±2.8 (12)	5.03±1.8 (12)	7.41 ±0.11 (40)	287 ±1.3 (12)	99.5 ±0.06 (20)
120	N.D.	N.D.	7.40 ±0.10 (20)	N.D.	N.D.

The values are expressed as means ± S.D., with the number of observations (n) in parenthesis. The values for pH, osmolality and O₂ saturation of medium for soleus and extensor digitorum longus muscle are combined. The first 30 min coincided with the preincubation period, whereas the period from 30 min to 90 min coincided with the incubation period (see Materials and methods section). The pH was checked a further 30 min after removal of the muscle from the incubation medium. Abbreviations: EDL, extensor digitorum longus; N.D., not determined.

muscles. Because of the small absolute amount of glycogen in the muscles, we had to prepare a fresh Schiff reagent solution every day.

3.4 Results

Mean muscle weight ($\pm$ S.D.) for soleus and extensor digitorum longus were 7.6 ± 1.3 mg and 9.4 ± 2.2 mg, respectively. The average largest diameter ($\pm$ S.D.) for soleus was 1.15 ± 0.45 mm and that for the extensor digitorum longus 1.35 ± 0.82 mm.

During incubation of the muscles under our experimental conditions, three important extracellular physiological parameters, namely pH, O_2 saturation and osmolality, appeared to be stable (Table 3.1). However, release of the enzyme creatine kinase (CK) (up to 10% the total muscle content for both muscles), which is indicative of a situation of metabolic stress caused by physical or metabolic damage (Jones, 1984), showed that during the incubation period damage might still have occurred (Table 3.1).

Incorporation of D-[3 H]-D-glucose into glycogen was linear at the lower insulin concentrations (0.4 and 1.0 munit/ml) but levelled off at a concentration considered to be supraphysiological (10 munits/ml) (Figure 3.1). The effect of insulin on glycogenesis of the soleus and extensor digitorum longus muscles is shown in Figure 3.1. Basal rate of glycogenesis (at 0 munits insulin/ml) was approximately 2-fold greater in the soleus than in the extensor digitorum longus muscle. In the presence of 0.4, 1.0 and 10.0 munits insulin/ml glycogenesis increased significantly in both muscles but remained 2-3 fold greater in the soleus than in the extensor digitorum longus muscle. At the lower (0.4 and 1.0 munits/ml) insulin concentrations there was a steep and progressive increase, then, thereafter, the curve levelled off, probably because of maximum insulin-receptor occupation.

Figure 3.2(b), stained with periodic acid/Schiff reagent, shows that the incubated soleus muscles (30 min preincubation and 60 min incubation) demonstrated a central loss of glycogen, in contrast with the non-incubated control muscles (Figure 3.2^a). The same was found for the extensor digitorum longus muscle (result not shown). It was further observed that the appearance of the core had already appeared after 30 min of preincubation (result not shown), indicating that core formation had already taken place during the preincubation period. Furthermore, the cells in the core lost their normal shape and positioning with respect to each other (HE staining). In contrast with the control (Figure 3.2^d) they had a rounded shape, and were swollen and darker (Figure 3.2^e).

3.5 Discussion

The aim of the present study was to evaluate a previously described intact skeletal-muscle model in vitro (Le Marchand-Brustel *et al.*, 1978; Bonen *et al.*, 1981; Bonen *et al.*,

1984; Tan and Bonen, 1984; Watson-Wright *et al.*, 1984). The incubated mouse skeletal muscles used in these studies were assumed to provide a suitable model in order to study various endocrinological effects on metabolism. Mouse soleus and extensor digitorum longus muscles, containing predominantly (75%) type I, slow-twitch fibres (soleus), and predominantly (99%) type II, fast-twitch fibres (extensor digitorum longus) (Ariano, 1973), have been considered thin enough to allow an adequate delivery of O_2 and substrates in vitro. However, this assumption is merely based on extrapolations of biochemical data on whole rat muscle or muscle-strips homogenates (Maltin and Harris, 1986; Newsholme *et al.*, 1986).

Figure 3.1. Effects of insulin on soleus and EDL muscles. Data are expressed as nmol [3-3H]-D-glucose incorporation into glycogen ($\bar{X} \pm \text{SEM}$). Muscles were incubated at 37°C for 1 h preceded by a 30 min pre-incubation period. One muscle of each animal was used as test muscle [0.4, 1.0 & 10.0 mU/ml insulin ($n=12$) while the contra-lateral muscle was used as control [0 mU/ml insulin ($n=36$).

In the present investigation the data on D-[3-³H]-D-glucose incorporation into muscle glycogen agreed well with those reported by other investigators using the same model (Le Marchand-Brustel *et al.*, 1978; Bonen *et al.*, 1981; Bonen *et al.*, 1984; Watson-Wright *et al.*, 1984) with the exception that [³H]-glucose incorporation into glycogen was slightly less pronounced. This is probably due to the different starting levels of glycogen in this study and the previous mentioned studies or due to different feeding states (fed versus fasted) of the animals (Le Marchand-Brustel *et al.*, 1978; Chiasson *et al.*, 1980). At all insulin concentrations studied, insulin-stimulated glycogenesis was found to be greater in the soleus than in the extensor digitorum longus (Figure 3.1), which is in line with previous investigations (Bonen *et al.*, 1981; Bonen *et al.*, 1984; Watson-Wright *et al.*, 1984). This difference in glucose uptake between both types of muscles is probably initiated by the different availability of glucose-transporter molecules, located at the muscle membrane (Simpson and Cushman, 1986). However, the results of the present study showed a wide variance, which seemed to be in keeping with other investigators (Le Marchand-Brustel *et al.*, 1978; Bonen *et al.*, 1981; Bonen *et al.*, 1984).

A check on parameters of the incubation medium indicated that O₂ saturation, osmolality, pH and creatine kinase efflux from the muscles could not account for the observed variation between the different studies. However, staining the muscles with heamatoxylin/eosin and periodic acid/Schiff showed the formation of large cores in both muscles, with swollen, darkened and isolated cells (Figure 3.2). This core formation was time dependent and was already observed after 30 min of preincubation. The core area differs markedly from one muscle to another, resulting in a wide variation of glucose incorporation. At this moment we have no explanation for this observation.

Despite the fact that a considerable amount of force is required to dissect the muscles free, the observed intact outer layer of cells (Figure 3.2^b) indicates that the surgical technique did not cause the high release of CK from the soleus and the extensor digitorum longus muscles. We hypothesize that core formation is largely responsible for the CK efflux. Core formation is probably caused by intramuscular hyperosmolality due to a hypoxic situation. As a consequence of the O₂ deficiency intracellular ATP decreases in the central core which leads to an increase in cytosolic Ca²⁺. This results in an increased phosphorylase activity and consequently in a rapid loss of glycogen on the one hand and of CK release on the other hand (Jackson *et al.* 1987). Recently, core formation was also observed by Maltin and Harris (Maltin and Harris, 1985; Maltin and Harris, 1986) in muscles of slightly larger animals (rats of body wt. 40-70 g). Our results support the findings of the latter investigators for even smaller animals (with higher metabolic rates), but contrast sharply with the observations and calculations of Segal and Faulkner (Segal and Faulkner, 1985) and Newsholme *et al.* (Newsholme *et al.*, 1986). They suggested a diameter of 1.2 mm or less for rat muscle to be sufficient for oxygenation during incubation *in vitro*. The results of the present investigation show this cannot be maintained for mice muscles incubated at 37 °C either, as already suggested by Maltin and Harris (Maltin and Harris, 1986). Hence, either muscle strips or isolated muscle fibres should be used. However, strips of mice muscle are hard to obtain because of the small size of the muscle. Although isolated individual muscle fibres treated with collagenase as described by Zuurveld *et al.* (Zuurveld *et al.*, 1985) could be maintained and used under controlled conditions for the study of muscle metabolism under a wide variety of experimental

Figure 3.2. Transverse sections through unincubated control soleus (3.2a) (Bar= 0.35 mm) and soleus muscle incubated (3.2b) (Bar= 0.30 mm) for 90 min (30 min pre- incubation and 60 min incubation) at 37° C, stained for glycogen (PAS). The central core (3.2c) (Bar= 0.03 mm) demonstrated darkened and swollen cells , in contrast to the control (3.2d) (Bar= 0.05 mm) probably caused by hypoxia.

interventions, this system has its drawbacks too. A major disadvantage of such a test system is the loss of the integrity of the tissue. However, it is not possible to evaluate the muscle viability by applying biochemical and histochemical techniques, such as in our study. Furthermore, it may be questioned whether the incubation (5.5 h) itself and/or treatment with collagenase will damage the muscle-fibre membrane, causing degradation of the insulin receptors.

At last, it has been shown that incubation of muscles at lower temperatures (20-24 °C) might offer a good alternative (Jones *et al.*, 1983; Jackson *et al.*, 1987).

In conclusion, the present study shows that the isolated mouse skeletal-muscle preparation is only of limited value for investigations of the effects of hormones (especially hormones with a less potent effect than insulin) on muscle metabolism, which is in line with the observations of Maltin and Harris (Maltin and Harris, 1985) for rat muscles. The viability of the muscle is not sufficiently preserved despite the stability of important biochemical parameters. Therefore viability of the muscle should not only be confirmed by biochemical assays but also with histochemical techniques to rule out any regional heterogeneity. Core formation seriously underestimates the rate of glycogenesis which, in turn, may lead to misinterpretations of other biochemical and physiological events. Results of in vitro studies carried out in vitro with intact muscles from mice or larger rodents should thus be interpreted with caution.

3.6 Introduction to the study of a model in vivo

As mentioned above, the in vitro model as discussed above, cannot be used for proper muscle endocrine research. In order to investigate the questions addressed in chapter 1, we decided to use a commonly used rat model in vivo. Although we are aware that in this model side effects of other substances might occur, it is our conviction that the results are of value to the endocrine scientific field.

Animal treatment

Female Brown-Norway rats were obtained from the breeding facility (C.P.V.) of the University of Limburg. The animals were, unless stated otherwise, obtained at 4 weeks of age and were housed in groups of 4 in a climate-controlled room (temperature 22 ± 1 °C, humidity 50%-60%) on a 12-h dark/12-h light cycle. A standard laboratory chow (54.2 en% carbohydrates, 7.3 en%, fat, 27.5 en% protein) and water were provided ad libitum. Brown-Norway rats were chosen because of their low body weight and their natural capability to run.

In all studies, rats were assigned at random to a sedentary group or a training group which were, depending on the experiment, subdivided in subgroups (for details see the corresponding chapters). During the first 2 weeks the animals from the training groups

were accustomed to handling and running on a rodent treadmill (pretraining). Subsequently they were trained for 9 weeks, 5 days/week, on a treadmill on a gradually increasing training schedule until they could run continuously for 60 min at a pace of 30 m/min during the final 3 weeks of the experiments.

To study the influence of testosterone each group of rats were randomly divided into control and testosterone treated groups as indicated in detail in the corresponding chapters. Testosterone was delivered during the last 2 weeks of the experimental period by means of a subcutaneously implanted silastic tube (1.8 cm or 0.4 cm) (Dow medical grading, inner diameter = 1.47 mm) containing crystalline testosterone (T1500, Sigma, St. Louis, U.S.A.). After implantation the rats were housed individually for 4 days until the incision was healed. To some subgroups of control and testosterone treated animals a new, highly specific, non-steroidal, aromatase inhibitor, R76713 (kindly provided by Dr. R. DeCoster, Janssen Research Foundation, Beerse, Belgium) was administered daily at a dose of 1 mg/kg body weight.

To validate the delivery of testosterone by the silastic tubes, we performed a study in which we compared three commonly used drug delivery systems (see below).

Non-insulin-dependent diabetic rat model

A rat model that displays some of the pathophysiology of human non-insulin-dependent diabetes mellitus is the high dose (100 mg/kg) neonatal streptozotocin (STZ) model developed by Portha *et al.* (Portha *et al.*, 1989). According to this model, pups received STZ (Boehringer, Mannheim, FRG) in 50 μ l citrate buffer (pH 4.5) intraperitoneally (i.p.) within 24-h after birth. Control litters received buffer alone. Animal housing and animal care were as described for the non-diabetic animals (see 3.1).

Streptozotocin is a 2-deoxymethyl-nitrosourea glycopyranose molecule that has a selective detrimental effect on the DNA of β -cells of the pancreas eventually leading to cell death (Portha *et al.*, 1989). The neonatal rats treated in this way exhibit plasma hyperglycemia and hypoinsulinemia, an abnormal response to a glucose tolerance test at 15 min, and insulin resistance at 10 weeks of age.

3.7 Introduction to the validation of the testosterone delivery model

Medium to long term treatment with exogenous steroids has been used in physiological endocrine research extensively. In order to achieve controlled fairly constant, low plasma steroid levels, elegant new drug dosing techniques have been developed in the past decade. These techniques should overcome the large fluctuations in plasma steroid levels, as have been observed after daily injections (Dohm *et al.*, 1992). Nowadays frequently used techniques are osmotic mini-pumps, depot injections, and silastic tubes.

Although these systems have been developed to achieve long term and constant drug delivery, the results presented in current literature have to be handled with care. One of the major draw-backs in current literature is that only endpoint concentrations, i.e. steroid concentrations at the final experimental day, are given without taken into consideration possible day to day fluctuations (Guezennec *et al.*, 1982; Guezennec *et al.*, 1984; Danhaive and Rousseau, 1988; Goudsmit *et al.*, 1988; Holmång *et al.*, 1990; Holmång *et al.*, 1992). To approach this problem, we decided to investigate whether medium-term small elevations of testosterone within the (patho)physiological range can be maintained for 14 days by using (1) the osmotic mini pump, (2) the depot injections or (3) the silastic tube.

3.8 Materials and Methods

Animal treatment and experimental procedures

A total of 36 adult female Brown-Norway rats were obtained at 14 weeks of age.

In order to study the day to day variation of the three different drug delivery systems, the rats were randomly divided into 6 groups; control depot injection, control osmotic mini pump, control silastic tube, testosterone depot injection, testosterone osmotic mini pump, and testosterone silastic tube. Matched groups of rats were treated for 14 days with testosterone (experimental rats) or the vehicle only (sham treated control rats). Blood was sampled once every two days by orbital puncture. At the end of the experimental period, the animals were killed and visually checked for inflammatory areas at the injection or implantation site.

Technical procedures, drugs and solutions

Depot testosterone. Testoviron® Depot (100 mg/ml) containing testosterone-propionate 25 mg, and testosterone-enanthate 110 mg in oil was purchased commercially (Schering, Berlin, FRG). This drug has been proven to be active for 2-3 weeks in humans. After making a small incision in the skin we injected 20 µl of the solution in the right or left hindlimb muscle (quadriceps) by means of a Hamilton syringe.

Osmotic mini pumps. Mini-osmotic pumps model 2002 were purchased from Alzet (Alza, USA). The lot (#041103) has been tested to have a mean pumping rate of 0.46 ± 0.02 µl/hr and has a mean fill volume of 227 ± 10 µl. Since steroids do not dissolve in aqueous solutions we dissolved crystalline testosterone (T1500, Sigma, St. Louis, USA) in the solvent polyethylene glycol 400. The stock solution was prepared as described previously (Will *et al.*, 1980). Next the pumps were filled according to the filling instructions of the manufacturer and implanted subcutaneous in the scapular area of the ether

anaesthetised rat. After the mini pump was inserted, the incision was closed with two sutures. The entire surgical procedure lasted ± 5 min.

Silastic tubes. Silastic tubes were used as described previously (van Breda *et al.*, 1992; van Breda *et al.*, 1993). The tubes were purchased commercially, (Dow Medical, USA) and were of medical grade. The delivery rate of this system is dependent on inner surface of the tube. A pilot study was performed in order to determine the length of the tube which achieved a constant testosterone plasma concentration of ± 5 nmol/l. It turned out, that a length of 0.4 cm was effective (data not shown). Next the tubes were filed with crystalline testosterone (T1500, Sigma St. Louis, USA) and closed at both ends with silicone. The surgical procedure and implantation was similar to that of the mini pump.

Control animals. Polyethylene glycol 400 was used as control vehicle in osmotic mini pump. The pumps were filled exactly as described erstwhile. In the control depot injection animals vegetable oil was injected in a similar manner as the drug. For the silastic tube-treated animals, an empty tube served as control.

Analytical procedures. Analysis of plasma testosterone concentration was performed in duplicate using a commercially available radioimmunoassay kit (Diagnostic Products Corporation, Ca., USA). The intra-assay coefficient of variation, as calculated from the replicates, was 7.5 % at 5 nmol/l and 7.1 % at 8 nmol/l. The inter-assay coefficient of variation was 5.5%.

3.9 Results & Discussion

All animals except 1 recovered within 5 minutes from the surgical procedure and remained healthy for the remaining experimental period. At the time of the killing, no infection was observed neither in the subcutaneous pouch area of the osmotic mini pumps or silastic tubes nor at the Testoviron® Depot injection side.

The validity of the osmotic mini pump, the Testoviron® Depot injection and the silastic tube was verified by measuring plasma testosterone concentrations once every two days. Plasma testosterone levels in testosterone treated animals was significantly higher ($P < 0.005$) in comparison with the control animals whereas between the control groups no differences were observed (data not shown). From panel A (Figure 3.3) it can be concluded that polyethylene glycol 400 is an effective solvent for testosterone in osmotic mini pumps. However, as can be concluded from the same panel, drug delivery through the osmotic mini pumps, caused severe fluctuations in plasma testosterone levels. The same is true for animals treated with the depot preparation (Figure 3.3 panel B). Interestingly, the daily plasma testosterone levels of animals treated with a silastic tube implant fluctuated considerable less in comparison with the other delivery systems (Figure 3.3 panel C).

Although all three methods deliver exogenous testosterone into the circulation of the rat, the day to day fluctuations as observed by the osmotic mini pumps and depot injections might be of considerable significance in the choice of the drug delivery system. In fact, the coefficient of variation of plasma testosterone was considerably smaller using the silastic

Figure 3.3. Day to day fluctuations of plasma testosterone concentrations (nmol/l) between osmotic mini pumps, Testoviron® Depot injections and silastic tubes. Data are represented as means $\pm$ SD for 6 animals.

tubes compared with the osmotic mini pumps and depot injections of the drug.

In conclusion the present study gives evidence that silastic tubes offer a practical and economic alternative for medium-term (14 days) steady state delivery of exogenous testosterone in comparison with osmotic mini pumps and depot injections.

3.10 General analytical procedures

Surgical methods

On the final experimental day, (i.e at approximately 16 weeks after birth) the rats were anaesthetised under ether narcosis. The extensor digitorum longus (EDL), and soleus, and/or the white and red gastrocnemius muscles were dissected free from the right or left hindlimb, an operation requiring about 2 min. Next, 2 -4 ml blood was withdrawn from the animal and the heart and liver were quickly excised. To avoid differences due to diurnal variation all rats were killed between 9.00 a.m. and 10.00 a.m.

Analytical methods of plasma parameters

Analysis of plasma testosterone, estradiol-17 β and insulin was performed using commercially available radioimmunoassay kits (Diagnostic Products Corporation, Ca., USA) For testosterone the intra-assay coefficient of variation, as calculated from the replicates, was 8.8% at 2.5 nmol/l, 7.5% at 5 nmol/l, 7.1% at 8 nmol/l, and 4% at 22 nmol/l. The inter-assay coefficient of variation was 5.5%.

Analysis of estradiol-17 β was carried out by modifying the radioimmunoassay according to Kai Kwong Yeung and Menheere (Kai Kwong Yeung and Menheere personal communication). Applying this modified radioimmunoassay, however, we found levels below the detection level of 20 pmol/l and a coefficient of variation between 10-35% in control rats.

Analysis of insulin was performed using a commercial kit with rat insulin as standard. The intra-assay coefficient of variation, as calculated from the replicates, was 3.5% at 55.4 μ U/ml. The inter-assay coefficient of variation was 8%.

Plasma glucose (Boehringer Mannheim, FRG), plasma fatty acids (Wako Chemicals G.m.B.H., Neus, FRG), plasma glycerol (Boehringer Mannheim, FRG) and plasma triacylglycerols (Boehringer Mannheim, FRG) was analysed on a centrifugal analyser (Cobas Bio or Cobas Fara, Hoffman-La Roche, Switzerland) using commercially available kits. Concentrations are expressed as mmol/l (glucose and triacylglycerols) and as μ mol/l (fatty acids and glycerol).

Analytical methods of muscle parameters

One set of hindlimb muscles from each animal was used for the determination of glycogen, and/or fatty acid-binding protein (FABP) while the muscles from the contralateral hindlimb were used for the preparation of a fresh homogenate (50 mg/ml) in sucrose (0.25 M)/EDTA (0.002 M)/TRIS (0.01 M) buffer at pH 7.4. The homogenates were sonicated and stored at -80 °C and were used within 3 weeks for analyses of the activities of glycogen synthase (GS), glycogen phosphorylase (GP), fructose-6-phosphate kinase (FPK), 3-

hydroxyacyl-CoA dehydrogenase (HAD), palmitoyl-CoA synthetase (PCS), citrate synthase (CS), or cytochrome c oxidase (Cyt ox). From a subset of sedentary animals the extensor digitorum longus and soleus muscles were used for the determination of the activity of the aromatase enzyme system. Since it is physically impossible to measure all parameters in a single run, we used a pool homogenate from previous studies to check the intra-assay variation during each analytical run.

Glycogen was assayed in freeze-dried muscles, heart and liver according to the method of Passoneau and Lauderale (Passoneau and Lauderale, 1974) and were expressed as μmol glycosyl units/g dry weight of tissue. This method measures total glycogen (i.e. the acid soluble fraction and the alkali soluble fraction).

H-FABP in heart and skeletal muscle was determined with a newly developed enzyme-linked immuno-sorbent assay of the sandwich type with a detection limit of $0.2 \mu\text{g/l}$ (Vork *et al.*, 1991). Since at the time of the experiments no information was available of FABP contents in skeletal muscle, we decided to validate the assay in extensor digitorum and soleus by incorporating heart muscle samples in the assay.

In brief, PVC polyvinyl chloride plates were coated with $50 \mu\text{l}$ 0.1 M carbonate buffer (pH 9.6) containing 100 ng anti-(heart FABP)_c IgG at 37°C for 2 hours. Thereafter the plates were washed five times with PBT (phosphate-buffered saline + 0.1% bovine serum albumin + 0.05% Tween 20, pH 7.4). All other steps were performed at room temperature. FABP, either in standards or biological samples, was allowed to bind to immobilized IgG for 90 minutes. After five fold washing, 200 ng biotinylated IgG (*b*-IgG) in $50 \mu\text{l}$ PBT was added and incubated for 90 minutes. The concentration of both IgG and *b*-IgG used in the assay were in large excess to ensure maximum binding of FABP. Streptavidin and biotinylated horseradish peroxidase (*b*-HRP) were mixed in PBT 30 min prior to use, with a molar ratio of 1:3 and a constant streptavidin concentration of 12 nM . After washing, $50 \mu\text{l}$ of the streptavidin/*b*-HRP solution was added to the plates and incubated for 30 minutes. Excess streptavidin/*b*-HRP complex was removed by multiple washing with PBT. Unless indicated otherwise, $100 \mu\text{l}$ 0.1 M citrate buffer (pH 5.0), containing 20 mM *o*-phenylenediamine and 6 mM H_2O_2 , was added to each well and incubated for 10-20 minutes. Finally, the enzyme reaction was terminated with $50 \mu\text{l}$ 4 M H_2SO_4 and absorbance was measured with a Titertek Multiskan MK 11 at 492 nm . With this assay, the recovery of pure FABP, added to a muscle homogenate, amounted to $97 \pm 6\%$ (mean $\pm$ SD for 4 experiments), indicating that the binding of FABP to the catcher-IgG was independent of the presence of other proteins in the sample.

Aromatase activity was assayed as described previously (Krekels *et al.*, 1990). In brief, activity of the aromatase enzyme complex was determined by measuring the amount of $^3\text{H}_2\text{O}$ formed during the aromatization of [^3H]testosterone. Aliquots of the homogenate ($700 \mu\text{l}$) were incubated for 1 h at 37°C with an energy-regenerating system (consisting of 0.2 mM NADPH, 1 U glucose-6-phosphate dehydrogenase, 2.5 mM glucose-6-phosphate, and 2 mM MgCl_2) in the presence of substrate or its solvent. In the blanks the tissue homogenate was replaced by an equal volume of 0.1 M Tris-HCl, pH 7.4. The total volume of the incubation was always 1 ml . The substrate consisted of a mixture of [1β , 2β - ^3H] testosterone and unlabeled testosterone. For [1β , 2β - ^3H] testosterone the 3H distribution was 13.7% 1α , 42% 1β , 10.7% 2α , 31.2% 2β , and 2.3% others. Following the incubation,

the mixture was extracted with 10 ml of chloroform by brief agitation. The layers were separated by centrifugation for 10 min at 688 x g (2000 rpm). A 500 μ l aliquot of the aqueous layer was treated with 500 μ l of a charcoal suspension (0.25 g dextran and 2.5 g active charcoal in 100 ml of water) and centrifuged for 10 min at 1360 g (3300 rpm). A total of 700 μ l of the final supernatant was added to 12 ml of Formula 989 (Dupont) and counted for radioactivity in a Beckman LS3801 scintillation analyser.

The active portion of glycogen synthase (GSi) was assayed in the muscle homogenate at 30 °C in the absence of glucose-6-phosphate (G6P), while total glycogen synthase (GSi+d) by including 10 mmol/l G6P. The reaction was started by the addition of UDP-glucose and formation of UDP was measured spectrophotometrically according to Danforth (Danforth, 1965). The active form of glycogen phosphorylase (GP_a) was assayed at 30 °C in the absence of AMP and total phosphorylase activity (GP_a+b) by including 5 mmol/l AMP. The formation of hexosephosphates was measured spectrophotometrically according to Harris *et al.* (Harris *et al.*, 1974). Both methods were described in detail previously (Chasiotis, 1983) with activities expressed as μ mol/min per g wet weight (WW).

Fructose-6-phosphate kinase (FPK) was assayed as described in detail before (Glatz *et al.*, 1992). During the course of the experiment it was found that the absolute values of this assay were not correct (large variations and fluctuations). Since the data in chapter 4 were analyzed in the same run with a pool homogenate as standard the relative changes most likely reflect the physiological changes and could thus be used. 3-Hydroxyacyl-CoA dehydrogenase (HAD) was assayed at 37 °C, according to Bergmeyer (Bergmeyer, 1974) while palmitoyl-CoA synthase (PCS) was assayed according to Bar-Tana *et al.* (Bar-Tana *et al.*, 1971). Citrate synthase (CS) was assayed according to Shepherd and Garland (Shepherd and Garland, 1969) and cytochrome c oxidase (Cyt ox) was assayed according to Gohil *et al.* (Gohil *et al.*, 1981). Enzyme activities are expressed as μ mol/min per g wet weight or nmol/min per g wet weight.

Histological methods

Muscle samples for histological studies of the rat *in vivo* experiments were taken from extensor digitorum longus and soleus muscles. They were mounted in an embedding medium (Tissue Tek; Miles Laboratory, Elkhart, IN, U.S.A.), frozen in isopentane cooled with liquid N₂ to its freezing point, and stored at -80 °C until analysis. Transverse sections (10 μ m) were taken from the middle 'belly' region of the muscles with a cryostat at -22 °C and stained for routine ATP-ase after pre-incubation at pH 4.3 and pH 4.6. Fibre counting was performed using video images of the sections and a Kontron MOP-videoplan digitizer system (Zeiss, FRG) on whole muscle cross sections and the percentages of type I and II fibres were determined. At least 1000 fibres were counted per muscle.

3.11 General statistical procedures

All data were analyzed on a Macintosh computer using the SuperAnova® and/or the StatView® statistical packages. Data were analysed using analyses of variance unless stated otherwise. The Newman-Keuls post hoc test of multiple comparisons was used subsequently to compare significant differences among groups. The level of significance was set at $P < 0.05$. Data are expressed as mean values $\pm$ S.D. unless indicated otherwise.

3.12 References

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4

Modulation of glycogen metabolism of rat skeletal muscle by endurance training and testosterone treatment

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4.1 Introduction

Muscle and liver glycogen stores both serve as a dominant fuel source during exercise. To date, it is well documented that sex-hormones like estradiol-17 β and testosterone play a substantial role in regulating glycogen metabolism (Villar-Palasi and Lerner, 1960; Bergamini, 1974; Gorski *et al.*, 1976; Max and Toop, 1983; Puah and Bailey, 1985; Kendrick *et al.*, 1987; Haffner *et al.*, 1988; Holmång, 1990; Kendrick and Ellis, 1991). For example, both physiological and pharmacological doses of estradiol-17 β administered to gonadectomized or immature male or female rats have been shown to spare myocardial, liver, and skeletal muscle glycogen during submaximal exercise (Gorski *et al.*, 1976; Kendrick *et al.*, 1987; Kendrick and Ellis, 1991). This estradiol-17 β -induced glycogen sparing might be due to an enhanced lipid metabolism (Kendrick *et al.*, 1987; Kendrick and Ellis 1991) and/or to an increased glycogen synthesis (Villar-Palasi and Lerner, 1960). Results of previous studies are in favor of the former hypothesis (Gorski *et al.*, 1976; Palmer and Davies, 1982; Goldberg *et al.*, 1984; Kendrick *et al.*, 1987; Kendrick and Ellis, 1991).

Unlike the effects of estradiol-17 β , literature concerning the effects of testosterone on glycogen metabolism, particularly in female animals, is limited and partly conflicting. Holmång *et al.* (Holmång *et al.*, 1990) reported a substantial decrease of insulin-stimulated 2-deoxy-glucose uptake and glycogen synthesis in both glycolytic and oxidative muscles of testosterone-treated, ovariectomized female rats in comparison with sham-treated control animals. These findings might have clinical significance since hyper-androgenecity, as reported to occur in women with polycystic ovaries or with diabetes (Achard and Thiers, 1921; Stein and Leventhal, 1935; Chang *et al.*, 1983; Rebuffé-Scrive *et al.*, 1989), is believed to induce impaired glucose uptake of skeletal muscle by increasing insulin resistance. In contrast to the findings of Holmång *et al.* (Holmång *et al.*, 1990), Max and Toop (Max and Toop 1983) reported that after testosterone injections in the rat 2-deoxy-glucose uptake was enhanced in the glycolytic extensor digitorum longus (EDL) but not in the oxidative soleus muscle. This is in line with the findings of Guezennec and co-workers (Guezennec *et al.*, 1984) who reported an increased glycogen content in soleus muscles of both fed and fasted sedentary male animals after testosterone infusion, whereas after exercise no glycogen sparing could be observed. However, muscle samples were obtained after exhaustion only (7 hour running), i.e. after complete glycogen depletion. Increased glycogen synthesis in male animals is suggested by the findings of Adolfsson and Ahren (Adolfsson and Ahren, 1968) who reported a testosterone-induced increase in glycogen synthase activity in. Whether this can also be demonstrated during exercise in female animals has not yet been investigated. Furthermore, we might hypothesize that testosterone elicits quantitatively different effects on glycogenesis in oxidative (type I) and glycolytic (type II) muscle fibres. Thus, despite the above mentioned findings of Holmång *et al.* (Holmång *et al.*, 1990) testosterone-induced glycogen sparing by increased glycogenesis in female animals cannot definitely be ruled out.

Therefore, the aim of the present study was threefold: 1) to determine the effects of pharmacological-testosterone administration and endurance training on the glycogen

levels of a predominantly oxidative skeletal muscle (soleus), of a pre-dominantly glycolytic muscle (extensor digitorum longus, EDL) and of liver in intact female rats, 2) to determine the effect of testosterone administration on glycogen utilization in EDL and soleus muscles and liver of female rats, subjected to submaximal exercise for 1 hour, and 3) to determine in both EDL and soleus the effects of testosterone administration and endurance training on glycogen synthase activity, as marker for glycogenesis, on glycogen phosphorylase activity as marker for glycogenolysis and on fructose-6-phosphate kinase activities, as marker for glycolytic activity.

4.2 Materials & Methods

Animal treatment and experimental procedures.

Unless stated otherwise animal treatment, and analytical procedures are exactly as described in details in part 2 chapter 3. In brief, a total of 63 female Brown—Norway rats were obtained at 4 weeks of age. The rats were assigned to a training group ($n=39$) or a sedentary group ($n=24$). During the first two (pre-training) weeks the animals from the training group were accustomed to handling and running on a rodent treadmill. Subsequently, they were trained for 9 weeks, 5 days/wk, on a rodent treadmill on a gradually increasing trainingschedule until they could run continuously for 60 minutes at a pace of 30 m/min during the final 3 weeks of the experiment. At that time their body weights were 153 ± 3 g.

To study the influence of testosterone on selected parameters during the final two weeks of the experimental period, the group of sedentary (S) rats was randomly divided into two subgroups i.e. control (sedentary control [SC]) and testosterone-treated (sedentary testosterone [ST]), and the group of trained (Tr) rats was randomly divided into four subgroups, i.e. control (trained [TrC]) and exercised [TrExC]) and testosterone-treated (trained [TrT] and exercised [TrExT]) (Figure 4.1). Testosterone was delivered by means of a subcutaneously implanted 1.8-cm silastic tube (Dow medical grading, inner diameter 1.47 mm), containing 0.013 g crystalline testosterone (T 1500 Sigma, St.Louis, USA). The controls were sham treated with an empty implant.

On the experimental day, the exercised rats (TrExC and TrExT) ran for 60 min (45 min 26 m/min 0° elevation followed by 15 min 30 m/min and 10° inclination). Immediately after exercise, the rats were anaesthetized under ether narcosis. The soleus and extensor digitorum longus (EDL) muscles then were dissected free from the right or left hindlimb, requiring about 2 min. Thereafter, 2 ml blood was withdrawn from the animal and the liver was quickly excised. To avoid possible differences due to diurnal variation, rats from the other subgroups were killed on different days at the same time (between 9:00 and 10:00 am). Of each animal one set of hindlimb muscles were used for the determination of glycogen while the muscles from the contra-lateral hindlimb were used for the preparation of a fresh homogenate (50 mg/ml) in Sucrose (0.25 M)—EDTA(0.002 M)—Tris(0.01 M)

Figure 4.1. Schematic representation of study design.

buffer at pH 7.4. The homogenates were sonicated and stored at -80°C and were used within three weeks for analyses of the activities of glycogen synthase, glycogen phosphorylase, and fructose-6-phosphate kinase.

Analytical methods and statistical analysis.

Both are described in detail in Chapter 3.

4.3 Results

During the final three weeks of the training period, all trained rats were capable of running continuously for 60 min at a pace of 30 m/min (see (van Breda *et al.*, 1992; van Breda *et al.*, 1993) for further details). Body (10-20%) and muscle weight gain (10-15%) were significantly higher in the testosterone-treated animals compared with the body (2-5%) and muscle weight (3-8%) gain in the control animals. Plasma testosterone levels in the non-testosterone-treated groups were mutually not different (0.9 ± 0.3 nmol/l, 0.8 ± 0.2 nmol/l, and 1.1 ± 0.3 nmol/l for SC, TrC, and TrExC, respectively) (Table 4.1). In the testosterone-treated animals plasma testosterone levels were significantly higher ($25.8 \pm$

3.9 nmol/l, 23.6 ± 4.1 nmol/l, and 21.7 ± 2.9 for ST, TrT, and TrExT, respectively; $P < 0.05$). Plasma glucose concentrations were not significantly different between any of the groups (Table 4.1).

Table 4.1. Plasma testosterone and glucose concentrations

subgroups	n	Plasma T (nmol/l)	Plasma Glucose (mmol/l)
SC	16	0.9 ± 0.3	7.4 ± 0.4
ST	8	$25.8 \pm 3.9^*$	7.8 ± 0.8
TrC	8	0.8 ± 0.2	7.8 ± 0.9
TrT	8	$23.6 \pm 4.1^*$	7.7 ± 0.9
TrExC	12	1.1 ± 0.3	7.0 ± 0.7
TrExT	11	$21.7 \pm 2.9^*$	6.5 ± 2.0

Values are means $\pm$ SD; SC, sedentary control; ST, sedentary plus testosterone; TrC, trained control; TrT, trained plus testosterone; TrExC, trained and exercised; TrExT trained and exercised plus testosterone.

* Significantly different from appropriate control.

Response of muscle and liver glycogen to training and testosterone treatment

Glycogen contents ($\mu\text{mol/g}$ wet weight) of EDL and soleus are shown in Figures 4.2 and 4.3 and in Table 4.2. Neither training nor testosterone treatment altered the glycogen content in the EDL significantly (SC versus ST and SC versus TrC). However, in the testosterone-treated trained animals a significantly higher glycogen content was observed (TrC versus TrT; $P < 0.05$). The exercise induced relative decrement in glycogen content was in the testosterone-treated group significantly less (27%) than in the trained control group (34%; $P < 0.05$).

In contrast to the EDL, the glycogen content of soleus muscle increased significantly as a response to training (SC versus TrC; $P < 0.05$), but in the sedentary as well as in the trained animals glycogen levels were not increased upon testosterone treatment (SC

versus ST, and TrC versus TrT). In line with our findings in the EDL we also observed a sparing of glycogen upon exercise after testosterone treatment. The relative decrements were also lower in the TrExT group (76%) than in the TrExC group (87%; $P < 0.05$).

Figure 4.2. Glycogen content in *m. extensor digitorum longus* in the experimental groups. Data represent means $\pm$ SD. SC, sedentary control ($n=12$); ST, sedentary plus testosterone ($n=8$); TrC, trained control ($n=8$); TrT, trained plus testosterone ($n=8$); TrExC, exercised (60 min) control ($n=12$); TrExT, exercised (60 min) plus testosterone ($n=11$). * $P < 0.05$.

Figure 4.3. Glycogen content in *m. soleus* in the experimental groups. Data represent means $\pm$ SD. SC, sedentary control ($n=12$); ST, sedentary plus testosterone ($n=8$); TrC, trained control ($n=8$); TrT, trained plus testosterone ($n=8$); TrExC, exercised (60 min) control ($n=12$); TrExT, exercised (60 min) plus testosterone ($n=11$). * $P < 0.05$.

Table 4.2. Glycogen in *m. extensor digitorum longus* (EDL) and *m. soleus*

Subgroups	EDL ($\mu\text{mol g}^{-1}$ dry weight)	Soleus ($\mu\text{mol g}^{-1}$ dry weight)
SC	117 $\pm$ 17	45 $\pm$ 16
ST	119 $\pm$ 9	51 $\pm$ 8
TrC	123 $\pm$ 6	81 $\pm$ 18 ²
TrT	135 $\pm$ 12 ³	82 $\pm$ 8
TrExC	84 $\pm$ 14	11 $\pm$ 3
TrExT	102 $\pm$ 10 ⁴	20 $\pm$ 9 ⁴

Values are means $\pm$ SD and are expressed as $\mu\text{mol (g dry weight)}^{-1}$

¹ $P < 0.05$ SC versus ST; ² $P < 0.05$ SC versus TrC; ³ $P < 0.05$ TrC versus TrT; and ⁴ TrExC versus TrExT

In the liver only training increased the glycogen content significantly (29%; data not shown), while neither in the sedentary nor in the trained groups glycogen content was affected upon testosterone treatment. Also in the exercised subgroups no differences were found (data not shown).

Response of muscle glycogen synthase, glycogen phosphorylase, and fructose-6-phosphate kinase activities to training and testosterone treatment

The activities of glycogen synthase and glycogen phosphorylase in both EDL and soleus are shown in Tables 4.3 and 4.4. In the EDL neither testosterone treatment nor training changed the active portion of glycogen synthase (G_{Si}) significantly (SC versus ST and SC versus TrC). Interestingly, in concert with the increased glycogen content in the testosterone-treated trained animals, we observed a significantly higher G_{Si} activity (TrC versus TrT; $P < 0.05$) in this subgroup of animals. Remarkably, total glycogen synthase activity (G_{Si}+d) was significantly less in the sedentary testosterone-treated group, than in the training group (SC versus ST, and SC versus TrC; $P < 0.05$). Furthermore, in the EDL testosterone treatment in the sedentary as well as in the trained animals decreased the active portion of glycogen phosphorylase (G_{Pa}) significantly (SC versus ST and TrC versus TrT; $P < 0.05$). G_{Si} activity increased significantly more in the testosterone-treated exercised subgroup compared with the control group. Interestingly, G_{Pa} activities increased significantly less in the testosterone-treated subgroup. No significant changes were observed in total glycogen phosphorylase activities (G_{Pa}+b) (SC versus ST, and SC versus TrC, TrC versus TrT, respectively).

Table 4.3. The activities of glycogen synthetase, and phosphorylase in extensor digitorum longus (EDL)

Subgroups	Glycogen Synthetase (GS)			Glycogen Phosphorylase (GP)		
	I + D ($\mu\text{mol min}^{-1} \text{g}^{-1}$)	I ($\mu\text{mol min}^{-1} \text{g}^{-1}$)	I (%)	a+b ($\mu\text{mol min}^{-1} \text{g}^{-1}$)	a ($\mu\text{mol min}^{-1} \text{g}^{-1}$)	a (%)
SC	2.80 $\pm$ 0.3	0.79 $\pm$ 0.1	27.8 $\pm$ 2.0	44.5 $\pm$ 16.4	5.0 $\pm$ 1.3	11.6 $\pm$ 1.7
ST	2.09 $\pm$ 0.3 ^{*1}	0.73 $\pm$ 0.2	36.1 $\pm$ 12.7	49.2 $\pm$ 5.5	3.5 $\pm$ 0.8 ^{*1}	7.4 $\pm$ 2.2 ^{*1}
TrC	1.21 $\pm$ 0.1 ^{*2}	0.94 $\pm$ 0.1	77.6 $\pm$ 8.9 ^{*2}	55.4 $\pm$ 1.7	5.4 $\pm$ 0.6	8.6 $\pm$ 0.3
TrT	1.32 $\pm$ 0.1	1.23 $\pm$ 0.2 ^{*3}	92.5 $\pm$ 4.8 ^{*3}	60.2 $\pm$ 5.1	4.7 $\pm$ 0.2 ^{*3}	9.0 $\pm$ 0.4
TrExC	1.48 $\pm$ 0.2	1.03 $\pm$ 0.1	70.0 $\pm$ 6.1	62.6 $\pm$ 8.8	7.5 $\pm$ 1.0	12.2 $\pm$ 2.1
TrExT	1.70 $\pm$ 0.2 ^{*4}	1.41 $\pm$ 0.2 ^{*4}	82.9 $\pm$ 10.3 ^{*4}	61.8 $\pm$ 4.4	6.7 $\pm$ 1.4	10.8 $\pm$ 2.1

Values are means $\pm$ SD and are expressed as $\mu\text{mol min}^{-1} (\text{g wet weight})^{-1}$ and as percentage of the total activity.

^{*1} $P < 0.05$ SC versus ST; ^{*2} $P < 0.05$ SC versus TrC; and ^{*3} $P < 0.05$ TrC versus TrT; ^{*4} TrExC versus TrExT

Table 4.4. The activities of glycogen synthetase, and phosphorylase in soleus muscle

Subgroups	Glycogen Synthetase (GS)			Glycogen Phosphorylase (GP)		
	I + D ($\mu\text{mol min}^{-1} \text{g}^{-1}$)	I ($\mu\text{mol min}^{-1} \text{g}^{-1}$)	I (%)	a+b ($\mu\text{mol min}^{-1} \text{g}^{-1}$)	a ($\mu\text{mol min}^{-1} \text{g}^{-1}$)	a (%)
SC	4.34 $\pm$ 0.3	1.38 $\pm$ 0.2	31.9 $\pm$ 4.3	16.9 $\pm$ 1.7	1.1 $\pm$ 0.2	6.8 $\pm$ 0.4
ST	4.00 $\pm$ 0.3	1.24 $\pm$ 0.1	33.8 $\pm$ 6.9	16.5 $\pm$ 3.8	1.2 $\pm$ 0.1 ^{*1}	7.2 $\pm$ 0.3
TrC	2.09 $\pm$ 0.2 ^{*2}	1.26 $\pm$ 0.1	60.5 $\pm$ 7.3 ^{*2}	16.6 $\pm$ 1.7	0.7 $\pm$ 0.3 ^{*2}	3.9 $\pm$ 1.6 ^{*2}
TrT	2.22 $\pm$ 0.1 ^{*3}	1.38 $\pm$ 0.2	62.3 $\pm$ 8.1	17.2 $\pm$ 1.2	0.8 $\pm$ 0.2 ^{*3}	5.5 $\pm$ 3.2 ^{*3}
TrExC	1.66 $\pm$ 0.3	0.72 $\pm$ 0.1	43.9 $\pm$ 6.3	12.2 $\pm$ 3.0	1.7 $\pm$ 0.2	14.5 $\pm$ 3.5
TrExT	1.91 $\pm$ 0.3 ^{*4}	0.96 $\pm$ 0.2 ^{*4}	49.9 $\pm$ 5.8 ^{*4}	10.4 $\pm$ 3.6	0.8 $\pm$ 0.3 ^{*4}	8.2 $\pm$ 2.1 ^{*4}

Values are means $\pm$ SD and are expressed as $\mu\text{mol min}^{-1} (\text{g wet weight})^{-1}$ and as percentage of the total activity

^{*1} $P < 0.05$ SC versus ST; ^{*2} $P < 0.05$ SC versus TrC; and ^{*3} $P < 0.05$ TrC versus TrT; ^{*4} TrExC versus TrExT

In the soleus, GSi activity decreased significantly *less* in the testosterone-treated exercised group in comparison with its control ($P < 0.05$). Interestingly, GP_a activity in the testosterone-treated exercised group was not increased whereas, in the non-testosterone treated animals it increased significantly. In addition to the EDL, in the soleus total glycogen synthase activities (GSi+d) were increased in the trained animals, and in the trained and testosterone-treated animals (SC versus ST, and TrC versus TrT; $P < 0.05$).

Significant reductions in fructose-6-phosphate kinase activity (FPK) occurred in the sedentary EDL and soleus after testosterone treatment (SC versus ST; $P < 0.05$), and after training (SC versus TrC; $P < 0.05$), whereas also significant reductions were found in the exercised control EDL muscles (TrExC versus TrExT; $P < 0.05$) (Table 4.5).

Table 4.5. Fructokinase 6-phosphate kinase activities

Subgroups	Soleus ($\mu\text{mol min}^{-1} \text{g}^{-1}$)	EDL ($\mu\text{mol min}^{-1} \text{g}^{-1}$)
SC	25.8 $\pm$ 6.3	58.8 $\pm$ 11.8
ST	19.7 $\pm$ 1.2 ^{*1}	33.1 $\pm$ 6.2 ^{*1}
TrC	41.5 $\pm$ 10.6 ^{*2}	92.0 $\pm$ 27.2 ^{*2}
TrT	41.5 $\pm$ 6.5	76.0 $\pm$ 6.5 ^{*3}
TrExC	55.2 $\pm$ 10.2	99.5 $\pm$ 12.7
TrExT	35.9 $\pm$ 5.4 ^{*4}	85.5 $\pm$ 19.9 ^{*4}

Values are means $\pm$ SD and are expressed as $\mu\text{mol min}^{-1} (\text{g wet weight})^{-1}$.

^{*1} $P < 0.05$ SC versus ST; ^{*2} $P < 0.05$ SC versus TrC; ^{*3} $P < 0.05$ TrC versus TrT; and ^{*4} $P < 0.05$ TrExC versus TrExT.

4.4 Discussion

The present study was conducted to investigate whether testosterone administration affected the glycogen content of either glycolytic or oxidative skeletal muscles and of liver of sedentary, endurance trained and/or submaximal exercised intact female rats. An important finding of the present study is that the selected muscles responded differently to these interventions, each showing distinct alterations in glycogen storage and/or glycogen breakdown enzyme activities in concert with alterations in glycolytic key enzyme activities.

Effects of training and testosterone treatment on muscle glycogen content and glycogen synthase and glycogen phosphorylase activities

To the best of our knowledge, findings that testosterone treatment of trained rats increases the resting glycogen content of EDL, but not of soleus muscle, in concert with an increase of active glycogen synthase and a decrease of active glycogen phosphorylase activities, has not yet been reported before. It could suggest, that the glycolytic EDL is more sensitive to the concerted effects of testosterone and training than the oxidative soleus. Support for this latter hypothesis is provided by the results of a study by Max and Toop (Max and Toop, 1983), who found an increased 2-deoxy-glucose (2-DOG) uptake by the EDL, but not by the soleus of testosterone-treated immature male rats. Ostensibly in contrast with the results of the aforementioned authors are the results of a study of Holmäng *et al.* (Holmäng *et al.*, 1990), who reported a marked decrease in [^{14}C]-glucose incorporation into glycogen of both EDL and soleus muscles of intact mildly hyper-androgenetic female rats. This was not due, however, to a decreased glucose uptake, since the 2-DOG uptake was increased (not significantly in EDL and soleus, significantly in the oxidative gastrocnemius). It was salient, however, that in intact (sham operated) rats, testosterone increased glucose uptake, whereas in ovariectomized rats, 2-DOG uptake was severely deteriorated. Thus, the results of the study of Holmäng *et al.*, (Holmäng *et al.*, 1990) not only suggest that the ovaries provide some protecting factor, but also that testosterone per se is not causally related to impaired glucose uptake by skeletal muscle.

Glycogen sparing effect of testosterone during submaximal exercise and responsible mechanisms

Another interesting observation in the present study was the glycogen sparing effect of testosterone in the EDL as well as in the soleus muscle after a submaximal exercise bout. The available literature on the effects of testosterone on glycogen metabolism during exercise is limited. As mentioned earlier, only Guezennec and co-workers (Guezennec *et al.*, 1984) investigated this issue and failed to find a glycogen sparing effect in adult, castrated male rats after a treadmill run. Once again it should be noted however, that muscle samples were obtained after complete glycogen depletion (7 hrs. of running). In other investigations, it was found that other sex hormones, namely estradiol-17 β , elicited a glycogen sparing effect during exercise. For example, Gorski *et al.* (Gorski *et al.*, 1976), and Kendrick and Ellis (Kendrick, 1987; Kendrick and Ellis, 1991) found noticeably less glycogen depletion after exercise in heart and skeletal muscles of ovariectomized female and intact male rats. Although it is now reasonable to suggest that testosterone and/or estradiol both could spare glycogen after exercise, the responsible mechanism remains largely unknown. Most investigators (Gorski *et al.*, 1976; Palmer and Davies, 1982; Goldberg *et al.*, 1984; Kendrick *et al.*, 1987; Kendrick and Ellis 1991) suggest a greater availability and oxidation of lipids, but an increased glycogenesis during submaximal exercise cannot be excluded (Kuipers, 1989). The results of a study of Guzmán and Castro

(Guzmán and Castro, 1988) are in line with increased fat oxidation upon steroid treatment. These investigators observed in male rats a marked increase in the activity of the mitochondrial outer carnitine palmitoyltransferase after treatment with anabolic steroids. Interestingly, no additional effect of endurance training could be observed. These findings are, in part, in line with the results of a recent study from our laboratory in which we found an increased fatty acid-binding protein (FABP) content and a concomitant increase in cytochrome c oxidase activity of heart and soleus muscle after training plus testosterone administration (van Breda *et al.*, 1992). These data suggest that after testosterone treatment, the trans-cytoplasmic flux of fatty acids is increased, and, together with the decreased FPK activities of the present study, support the concept of the fatty acid-glucose cycle of Randle *et al.* (Randle *et al.*, 1963; Randle, 1964). One has to keep in mind, however, that the glycogen sparing effect of the fatty acids-glucose cycle of Randle is operative only during prolonged submaximal exercise (Guezennec *et al.*, 1984). The preferential use of fatty acids over glucose can also be found from the results of a recent study of Kendrick and Ellis (Kendrick and Ellis, 1991), who observed a higher plasma fatty acid concentration after exercise in estradiol-17 β -treated rats compared to control rats, although plasma glycerol concentrations were not changed accordingly. Despite the relative abundance of indirect prove, which favor increased fatty acid oxidation after sex steroid treatment, other mechanisms for the glycogen sparing effect during exercise may also apply.

The results of the present study show that in both muscles studied, the ratio between glycogen synthase "I" and glycogen phosphorylase "a" increased more in the testosterone treated exercised groups compared with the non-testosterone treated groups. This may indicate a relatively greater glycogenesis and provides an alternative explanation for the glycogen sparing effect during exercise in testosterone treated animals. Evidence for this such a dual pathway has previously been reported (Coderre *et al.*, 1991). Because liver glycogen is depleted without synchronous decrements of blood glucose concentrations it is still feasible that the uptake of glucose is enhanced, as has been reported earlier (Max and Knudsen, 1980). Thus, instead of being channelled through the glycolysis pathway, glucose is channelled through the glycogen synthesis pathway. This suggestion is consistent with our findings of a decreased fructose-6-phosphate kinase activity in both muscles. Furthermore, the glycogen sparing effect of testosterone in endurance trained rats, at a given workload, may also be caused by a shift from anaerobic breakdown (lactate formation) to the complete aerobic breakdown (CO₂) of glycogen. At this moment we have no explanation for the fact that the percentage of active glycogen synthase and glycogen phosphorylase is not congruent with the changes in the ratio of absolute activities of both enzymes.

Concluding remarks

According to the findings of the present investigation, the combination of endurance training and testosterone treatment is not only capable to prevent the undesired effects of hyper-androgenicity (i.e. decrease of muscle glycogen content), but also potentiates the effect of endurance training on the oxidative capacity of skeletal muscle. An explanation

for these phenomena is not easy to provide. We hypothesize that testosterone and exercise training interact with glucocorticoid action. The basis for this concept was provided by Mayer and Rosen (Mayer and Rosen, 1977), who showed that glucocorticoids and androgens compete for the same intracellular receptor in muscle. Later-on, Danhaive and Rousseau (Danhaive and Rousseau, 1988) reported that testosterone could exhibit anabolic action as a result of its anti-glucocorticoid properties. Furthermore, they showed that female rats responded better than male rats and that the action of androgens probably requires the presence of estrogens. In agreement with these results, Falduto *et al.* (Falduto *et al.*, 1989) reported that exercise training suppresses glucocorticoid action as well. Therefore, we might hypothesize that the effect of testosterone potentiates the effect of training at least in part, by suppressed glucocorticoid action.

Our present findings provides further evidence that pharmacological levels of testosterone acts on muscle metabolism. Especially in trained intact female animals we found that testosterone potentiates the training induced effects, including a sparing of muscle glycogen after submaximal exercise. These effects seem to result from an increased glycogen synthesis, a reduced glycolytic flux probably related to an increased fatty acid oxidation or a more complete aerobic breakdown of glycogen. Thus, although isolated hyper-androgenicity seems to be a strong stimulus for increasing the insulin resistance in muscle, the combination of endurance training and hyper-androgenicity potentiates the effect of training instead of deteriorating it.

4.5 References

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5

Modulation of fatty acid-binding protein content of rat heart and skeletal muscle by endurance training and testosterone treatment

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5.1 Introduction

To date it is well documented that cytoplasmic fatty acid-binding proteins (FABPs) are expressed in a highly tissue-specific manner (Glatz and Veerkamp, 1985; Bass, 1988; Glatz and van der Vusse 1989). In heart FABP (H-FABP) comprises about 5% of the cytosolic protein mass (Glatz and Veerkamp, 1985). In view of its relatively low molecular weight (15 kDa), this means that in heart FABP represents a remarkable 25-35% of the number of soluble proteins (Glatz and van der Vusse, 1989). Reported values for the FABP content of rat skeletal muscle vary from 4 (white muscle) to 30 $\mu\text{g}/\text{mg}$ cytosolic protein (red muscle) (Bass, 1988). Recently, it has been shown that the type of FABP found in skeletal muscle is identical with H-FABP (Peeters *et al.*, 1991). Although the structure and long chain fatty acid-binding capacities of several FABP types have now been reported, their control mechanism(s) and physiological role remain only partly understood (Glatz and van der Vusse, 1989). FABPs most likely play an important role during the transcytoplasmic movement and metabolism of the poorly soluble fatty acids (Glatz and Veerkamp, 1985; Bass, 1986). It has been reported that dietary, hormonal and pharmacological manipulations may change FABP content (Bass and Manning 1986). For example, Ockner *et al.* (Ockner *et al.*, 1980) have shown that in liver, L(iver)-FABP is controlled, among others, by sex hormones. These investigators found higher levels of L-FABP in the livers of male and female animals treated with 17- β estradiol than in animals treated with testosterone (Ockner *et al.*, 1980).

Under normal conditions (rest) and during submaximal exercise heart and skeletal muscle consume, among others, preferentially fatty acids for ATP production. In addition, endurance training causes an increased muscular utilization of fatty acids (Guzmán and Castro, 1988; Abernethy *et al.*, 1990;). Guzman and Castro (Guzmán and Castro, 1988) have shown that endurance training increased carnitine palmitoyltransferase 1 activity in rat heart and skeletal muscle mitochondria. These metabolic adaptations during exercise are, at least partially, controlled by the endocrine system (Galbo, 1981). However, information on manipulation of FABP content in muscle, especially by training or testosterone treatment, is lacking. Therefore, the aim of the present study was to investigate the influences of endurance training and testosterone administration, either separate or in combination, on FABP content and the mitochondrial marker, cytochrome c oxidase, of heart and skeletal muscles.

In the present experiments, we raised the plasma testosterone levels of both sedentary and trained, adult intact female rats for two weeks. FABP contents were measured in heart muscle, a predominantly glycolytic skeletal muscle (extensor digitorum longus) and a predominantly oxidative (soleus). Furthermore, the activity of the mitochondrial marker enzyme cytochrome c oxidase was measured.

5.2 Materials and methods

Animal treatment, experimental procedures, analytical methods and statistical analysis

Animal treatment is exactly as described in Chapter 4. The analytical methods are described in detail in Chapter 3.

Because at the moment of this study no information was available about fatty acid-binding protein content in skeletal muscle, samples of heart muscles were run along for validation.

Since all data were from different animals, data was analyzed by the unpaired Students t-test. The level of significance was set at $P < 0.05$. The data are expressed as means $\pm$ SD.

5.3 Results

During the last 3 weeks of the training period, all trained rats were capable of running continuously for 60 min at a pace of 30 m/min (see chapter 4.2 for further details and Figure 5.1 for schematic representation of study design). Body (10-20%) and muscle weight gain (10-15%) were significantly higher in the testosterone treated animals compared with the body (2-5%) and muscle weight (3-8%) gain in the control animals.

Plasma testosterone levels in the non-testosterone treated animals were mutually not different (0.9 ± 0.3 nmol/l, 0.9 ± 0.2 nmol/l, and 0.8 ± 0.2 nmol/l for SC, SCA, and TrC, respectively). In the testosterone treated animals plasma testosterone levels were significantly higher (25.8 ± 3.9 nmol/l, 21.3 ± 2.2 nmol/l, and 23.6 ± 4.1 nmol/l for ST, STA, TrT, respectively; $P < 0.05$).

Figure 5.1. Schematic representation of study design.

Response of FABP to training and testosterone treatment

Fatty acid-binding protein (FABP) contents ($\mu\text{g/g}$ wet weight) in heart, soleus and extensor digitorum longus (EDL) are shown in Figures 5.2 and 5.3 and Table 5.1. In untreated sedentary animals FABP levels were the highest in heart followed by soleus and EDL muscles. During either training or testosterone treatment the FABP content in the heart increased by 28-31%, whereas together these manipulations produced an 53% higher FABP level (Figure 5.2; Table 5.1).

In contrast to the training induced increments of cardiac FABP levels, the FABP content in soleus did not respond to training, but in sedentary as well as trained animals increased by about 20% upon testosterone treatment (Figure 5.3). In the EDL muscle neither training nor testosterone treatment had any significant effect on the muscular FABP content (Figure 5.3; Table 5.1).

Figure 5.2. FABP content in heart in the experimental groups. Data represent mean $\pm$ SD for 8 animals. SC = sedentary control; ST = sedentary plus testosterone; TrC = trained control and TrT = trained plus testosterone.

* = $P < 0.05$.

Response of cytochrome c oxidase to training and testosterone treatment

The activities of the mitochondrial marker enzyme cytochrome c oxidase are depicted in Table 5.2. In the untreated sedentary animals the enzyme activity was in heart greater than in soleus and in soleus greater than in EDL. Training increased the cytochrome c oxidase activity in heart by 64% and in soleus by 14% but did not affect that in EDL. In the heart and soleus of sedentary animals testosterone treatment did not increase the activity

of cytochrome c oxidase, but in the EDL, it resulted in a small (17%) but significantly higher enzyme activity as compared with its control (ST versus SC and TrT versus TrC; $P < 0.05$). Interestingly, testosterone treatment potentiated the training induced effects in heart and particularly soleus, resulting in 85% and 60% higher cytochrome c oxidase activities, respectively, when compared with untreated sedentary animals. In the EDL the increments were less pronounced (27%).

Figure 5.3. FABP content in *m. soleus* and *m. extensor digitorum longus* in the experimental groups. Data represent mean $\pm$ SD for 8 animals. SC = sedentary control; ST = sedentary plus testosterone; TrC = trained control and TrT = trained plus testosterone. * = $P < 0.05$.

Table 5.2. Fatty acid-binding protein content in heart, m. extensor digitorum longus (EDL), and m. soleus

Subgroups	Heart ($\mu\text{g/g. wet weight}$)	EDL ($\mu\text{g/g. wet weight}$)	Soleus ($\mu\text{g/g. wet weight}$)
SC	497 $\pm$ 32	58 $\pm$ 12	255 $\pm$ 50
ST	663 $\pm$ 58* ¹	48 $\pm$ 9	294 $\pm$ 18* ¹
TrC	639 $\pm$ 28* ²	50 $\pm$ 18	255 $\pm$ 21
TrT	759 $\pm$ 59* ³	55 $\pm$ 17	295 $\pm$ 15

Values are means $\pm$ SD and are expressed as $\mu\text{mol (g dry weight)}^{-1}$. *¹ $P < 0.05$ SC versus ST; *² $P < 0.05$ SC versus TrC; *³ $P < 0.05$ TrC versus TrT.

Table 5.2. Cytochrome c oxidase activities in heart, m. soleus and m. extensor digitorum longus

Subgroups	n	Heart (U/g wet weight)	EDL (U/g wet weight)	Soleus (U/g wet weight)
SC	8	168 $\pm$ 23	14 $\pm$ 3	26 $\pm$ 5
ST	8	165 $\pm$ 29	16 $\pm$ 2 * ¹	24 $\pm$ 4
TrC	8	276 $\pm$ 61 * ¹	12 $\pm$ 3	29 $\pm$ 5 * ¹
TrT	8	315 $\pm$ 35 * ²	15 $\pm$ 3	40 $\pm$ 9 * ²

Values are means $\pm$ SD and are expressed as Units/g wet weight. SC = sedentary control; ST = sedentary plus testosterone; TrC = trained control and TrT = trained plus testosterone.

*¹ $P < 0.05$ versus SC group and *² $P < 0.05$ versus TrC group.

FABP-cytochrome c oxidase relation

Earlier, a linear relation has been observed between FABP content and mitochondrial activity of various tissues (Glatz *et al.*, 1988). When the FABP contents measured in the present study are expressed against the corresponding cytochrome c oxidase activities, a non-linear relation between the mean values of the heart, soleus and EDL of all subgroups is seen (Figure 5.4), as the soleus shows a greater ratio of FABP content to cytochrome c oxidase activity (10-12 $\mu\text{g/U}$) than do heart and EDL (both 2.5-3.0 $\mu\text{g/U}$). Figure 5.4 further illustrates that in heart and soleus muscle both the FABP content and

cytochrome c oxidase activity increase by training and training plus testosterone administration with comparable relative changes, but in the EDL, there is only an effect of training on cytochrome c oxidase activity. Interestingly, in the sedentary groups, testosterone treatment significantly increased the FABP content without a concomitant increase in cytochrome c oxidase activity, in both heart and soleus muscle.

5.4 Discussion

The aim of the present study was to investigate whether endurance training and/or testosterone administration affects the cytoplasmic FABP contents in heart and oxidative or glycolytic skeletal muscles of intact female rats. The results demonstrate that these muscles, which show a wide variation in physiological and/or biochemical properties, responded differently to these interventions.

In accordance with the findings of other investigators (Miller *et al.*, 1988; Paulussen *et al.*, 1989; Glatz and van der Vusse, 1990) we found that FABP is present in all three types of muscle. It should be noted, that in tissues of sedentary rats we found noticeable lower

Figure 5.4. Relation between FABP and cytochrome c oxidase activity in heart (□), soleus (○) and EDL (Δ) muscles. Data represent mean ± SD for 8 animals. SC = sedentary control; ST = sedentary plus testosterone; TrC = trained control and TrT = trained plus testosterone.

FABP concentrations as compared with other studies (Penpargkul *et al.*, 1978; Bass, 1988). Probable causes of this discrepancy might be the use of male instead of female animals, but also a less accurate and/or less specific assay for FABP in those earlier studies. In view of the measured recovery of pure FABP of $97 \pm 6\%$ (see Materials & Methods) we conclude that previously reported contents may have been overestimated.

The results of the present study reveal that the cardiac FABP content is increased after training. Furthermore, testosterone treatment similarly increased the cardiac FABP content in both the sedentary and trained animals, which indicates that training and testosterone treatment operate independently on cardiac FABP levels. FABP content in soleus was not affected by training alone, but testosterone treatment in both the sedentary as well as in the trained animals provoked identically enhanced FABP levels. In the EDL neither training nor testosterone treatment had any effect on FABP content. The low FABP content in this muscle type is in line with the notion that fatty acid utilization is minimal in fast-twitch glycolytic muscle fibres.

Although several contradictory results have been observed, we found, akin to other investigators, that endurance training increased cytochrome c oxidase activity in heart (Penpargkul *et al.*, 1978; Beyer *et al.*, 1984) and soleus (Gohil *et al.*, 1981; Marcus and Tipton, 1985) but failed to do so in EDL muscle fibres. Interestingly, testosterone administration potentiated the training induced increments in cytochrome c oxidase activity in heart and soleus and it also slightly increased cytochrome c oxidase activity in the EDL of sedentary and trained rats. In part, our results are in agreement with the findings of Koenig *et al.* (Koenig *et al.*, 1980) who reported 23% higher cytochrome c oxidase activities in the white (glycolytic) part of the m. gastrocnemius and 17% in soleus of sedentary male compared with sedentary female mice. This sex difference was abolished after orchiectomy, but was restored after testosterone administration (Koenig *et al.*, 1980). In the present study, however, we could find a testosterone mediated increase in cytochrome c oxidase activity only in the fast-twitch EDL and not in the soleus. Currently we have no explanation for the different findings regarding the heart, soleus and EDL muscles.

Earlier we have described (Glatz *et al.*, 1988; Glatz and van der Vusse, 1989) the existence of a quantitative relation between mitochondrial fatty acid oxidation capacity and FABP content of several tissues. By studying the palmitate oxidation versus the cytochrome c oxidase activity in cardiomyocytes, endothelial and fibroblast-like cells isolated from rat hearts we also found such a linear correlation (Linssen *et al.*, 1990). The results of the present study do not entirely confirm these findings when comparing heart muscle and oxidative and glycolytic skeletal muscle fibres, as a non-linear increase in the FABP content versus cytochrome c oxidase activity was found. In the soleus, we noticed a relatively higher FABP content versus cytochrome c oxidase activity as in heart and EDL. Furthermore, it is interesting to note that in heart as well as in soleus, testosterone alone (ST groups), was capable of inducing increments only in FABP content and not in cytochrome c oxidase activity (Figure 4). Although the results of the present study extend our previous findings (Glatz *et al.*, 1988; Glatz and van der Vusse, 1989) by further indicating that FABP might play a role in muscular oxidative metabolism, the precise physiological meaning of the similarities and differences found among these various muscular tissues remains to be clarified.

In conclusion, the results of the present study indicate that the FABP contents and mitochondrial activities of heart and skeletal muscle are affected by training and testosterone and that these effects are different for heart and skeletal muscles. Although certainly testosterone seem to be involved, it remains uncertain whether the aromatization of testosterone to estradiol-17 β could also account for the findings in the present study. Furthermore, we have shown that among the three muscles studied the FABP content is related to the cytochrome c oxidase activity in a non-linear way. Future research on the differences in the relative content of FABP versus cytochrome c oxidase activity among these muscles will help to define the precise physiological significance of FABP in muscle.

5.5 References

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6

Modulation of skeletal muscle energy metabolic parameters of streptozotocin-induced diabetic rats by endurance training and testosterone treatment

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(submitted)

6.1 Introduction

Non-insulin-dependent diabetes mellitus can be described as a disorder characterized by miscellaneous humoral and/or metabolic derangements. To date, two primary defects are believed to be responsible for the pathogenesis of non-insulin-dependent diabetes mellitus. In some patients the primary defect manifests itself as an impairment in pancreatic (β -cell) insulin secretion, whereas in other non-insulin-dependent diabetic patients the primary defect starts as an impairment of peripheral (mainly skeletal muscle) sensitivity to insulin (Rossetti *et al.*, 1987; DeFronzo, 1988; Lisato *et al.*, 1992; Henry, 1992; Brichard *et al.*, 1993). In clinically overt non-insulin-dependent diabetes mellitus, both defects are found, leading to the emergence of persistent hyperglycemia (DeFronzo, 1988).

At the cellular level, insulin resistance can be attributed to a defect of the insulin receptor and/or post-receptor defects (DeFronzo, 1988). The post-receptor defects appear to reside in both the oxidative (primarily pyruvate dehydrogenase complex) and non-oxidative (primarily glycogen synthesis) metabolic pathways of glucose (DeFronzo, 1988). Acute hyperglycemia has been shown to increase the non-oxidative glucose disposal, by virtue of both an enhanced glucose supply (mass-action effect) (Revers *et al.*, 1984; Capaldo *et al.*, 1986; Hager *et al.*, 1991; Vaag *et al.*, 1992), and through an increase in activity of glycogen synthase, the rate limiting key enzyme of glycogen synthesis (Kelley and Mandarino, 1990). However, persistent hyperglycemia has been shown to reduce glycogen synthase activity (Lisato *et al.*, 1992; DeFronzo, 1988). Furthermore, based upon the findings in epidemiological human and animal studies, hyperandrogenism has been suggested to be related to the insulin resistant state as well (Achar, 1921; Stein, 1935; Holmång *et al.*, 1990; Boden *et al.*, 1991; Widén *et al.*, 1992). Especially in non-diabetic women with polycystic ovaries and acanthosis nigricans a close relationship between insulin resistance and mild hyperandrogenism (plasma testosterone 4-5 nmol/l) is well established (Boden *et al.*, 1991; Poretsky, 1991; Widén *et al.*, 1992; Holmång *et al.*, 1992). However, it is far from clear whether hyperandrogenism is the primary event leading to insulin resistance or is secondary to hyperinsulinemia (Stein and Leventhal, 1935; Chang *et al.*, 1983; Dunaif *et al.*, 1987).

In studies with female rats, Holmång *et al.* (Holmång *et al.*, 1990) recently showed that hyperandrogenism impairs glucose disposal in skeletal muscle. This androgen-induced insulin resistance, however, could not be confirmed in subsequent studies from our laboratory in intact (non-ovariectomized) testosterone treated female rats. In these investigations we found that a pharmacological dose of testosterone (ca 25 nmol/l) increases glycogen content of both oxidative (m. soleus) and glycolytic (m. extensor digitorum longus, EDL) trained skeletal muscles. In addition, we found that testosterone diminishes glycogen breakdown during acute submaximal exercise. Since impaired glycogenesis is the first sign of developing insulin resistance, our previously reported findings do not support a possible etiological role of testosterone in the development of insulin resistance. Conversely, we found that testosterone had a potentiating effect on training induced increases in the glycogen content of muscle.

Besides defects in carbohydrate metabolism, derangements in fat metabolism have also been described in non-insulin-dependent diabetes mellitus (DeFronzo, 1988). Specifically, plasma fatty acid levels are increased, probably due, among others, to resistance of adipocytes to the anti-lipolytic action of insulin (van Breda *et al.*, 1992). This, in turn, may lead to an increased fatty acid uptake and oxidation by peripheral tissues (mainly skeletal muscle), and decreased basal glucose uptake and oxidation as governed by the glucose-fatty acid cycle (see: van Breda *et al.*, 1993). The increased fatty acid uptake may lead to an increased content of fatty acid-binding protein (FABP), a 15 kD protein most likely involved in the transcytoplasmic flux of fatty acids, and abundantly present in heart and skeletal muscle (van Breda *et al.*, 1992). Previously, we found that tissue FABP contents were increased in oxidative but not in glycolytic muscle of hyperandrogenetic endurance-trained female rats (van Breda *et al.*, 1992), whereas glycogen content was increased as well (van Breda *et al.*, 1993). Taken together, these findings suggest that a combination of endurance training and testosterone treatment leads to adaptations in muscle carbohydrate and fat metabolism of intact non-diabetic rats (although oxidative and glycolytic muscle react differently), that would be favourable especially in diabetic animals. However, it is unknown whether testosterone elicits similar effects in diabetic (insulin resistant) animals as well.

Therefore, the aims of the present study were to investigate in both glycolytic (EDL) and oxidative (soleus) skeletal muscles of streptozotocin-induced non-insulin-dependent diabetic female rats whether testosterone administration, either alone or in combination with endurance training affects (a) glycogenesis and/or glycogenolysis; (b) selected markers of fatty acid utilization, and (c) glycogen depletion during acute exercise. For this purpose we assayed for (a) markers for glucose storage and breakdown (glycogen content and activities of glycogen synthase and glycogen phosphorylase), for (b) a marker for transcytoplasmic fatty acid trafficking (fatty acid-binding protein, FABP) and marker enzymes of fatty acid oxidation (3-hydroxyacyl-CoA dehydrogenase, HAD, and palmitoyl-CoA synthase, PCS); and for (c) markers of mitochondrial density (citrate synthase, CS, and cytochrome c oxidase, Cyt ox).

6.2 Materials & Methods

A total of 72 female Brown—Norway rats were included in this study. Animal treatment is exactly as described in Chapter 4, whereas the analytical methods are described in detail in Chapter 3.

To study the influence of testosterone on selected metabolic parameters, during the last two weeks of the training period the groups of non-diabetic sedentary (S) and diabetic sedentary (DS) rats were randomly divided into two subgroups each, i.e. non-diabetic sedentary control [SC], non-diabetic testosterone-treated [ST], diabetic sedentary control [DSC], and diabetic testosterone-treated [DST], while the group of trained diabetic rats was randomly divided into four subgroups, i.e. diabetic trained control [DTrC], diabetic

trained testosterone [DTrT], diabetic trained exercised control [DTrExC], and diabetic trained exercised testosterone [DTrExT] (Figure 6.1).

In order to investigate the effect of acute exercise on the utilization of muscle glycogen stores the rats from the diabetic trained exercised control [DTrExC] and diabetic trained exercised testosterone [DTrExT] subgroup ran for 60 min (45 min 26 m/min 0° elevation and 15 min 30 m/min 10° elevation).

Figure 6.1. Schematic representation of study design.

6.3 Results

Table 6.1 shows the baseline endocrine and metabolic characteristics at the end of the experimental period of the non-diabetic and streptozotocin-diabetic rats. Both the sedentary and trained diabetic rats demonstrated a significantly lower body weight, a higher fasting plasma glucose concentration in combination with a lower fasting plasma insulin concentration, and therefore a lower fasting plasma insulin to glucose ratio. Furthermore, the diabetic animals had a depressed (ca 22%) insulin response to an oral dose of glucose (1 g/kg body weight) at 15 and 30 minutes (data not shown). Finally, the sedentary diabetic rats showed increased plasma concentrations of fatty acids and glycerol. After 8-10 weeks, all streptozotocin-treated animals showed hypoinsulinemia, hyperglycemia, and peripheral insulin resistance, which is in agreement with previous observations (Portha *et al.*, 1989; Lisato *et al.*, 1992).

Plasma testosterone levels in the untreated rats were not different in the non-diabetic and the diabetic animals (0.9 ± 0.2 nmol/l, 0.9 ± 0.2 nmol/l, 1.0 ± 0.1 nmol/l, and 1.1 ± 0.2 nmol/l, for SC, DSC, DTrC and DTrExC, respectively). In the testosterone-treated animals plasma testosterone levels were significantly increased (5.7 ± 1.6 nmol/l, 4.9 ± 1.3 nmol/l

Table 6.1. Endocrine and metabolic characteristics of non-diabetic and STZ-diabetic rats at the end of the experimental period

Parameters	Non-diabetic rats	STZ-diabetic rats	
		sedentary	trained
Age(weeks)	15 (20)	15 (18)	15 (34)
Body weight (g)			
-before testosterone treatment	153±3 (20)	139±8(18)*	132±12(34)*
-after testosterone treatment	172±13 (8)	163±9(8)*	164±20(17)*
Plasma testosterone concentration (nmol/l)			
-before testosterone treatment	0.9±0.2 (20)	0.9±0.2(18)	1.0±0.1(34)
-after testosterone treatment	5.7±1.6 (8)	4.9±1.3 (8)	5.0±1.3(17)
Fasting plasma concentration			
-glucose (mmol/l)	7.4±0.4 (12)	9.8±1.4 (18)*	9.4±0.8(34)*
-insulin (μU/ml)	65.4±2.0 (8)	50.7±4.7 (8)*	49.8±6.0(8)*
-insulin/glucose ratio (U/mol)	8.8±0.6 (8)	5.2±0.6 (8)*	4.74±1.0(8)*
-fatty acids (μmol/l)	153±67 (8)	247±96 (8)*	183±68(8)
-glycerol (μmol/l)	79±12 (8)	153±44 (8)*	112±27(8)*
-triacylglycerols (mmol/l)	1.38±0.2 (8)	1.46±0.2 (8)	1.38±0.1(8)

Values are means ± SD of number of animals indicated within parentheses. * $P < 0.05$ non-diabetic versus streptozotocin-diabetic rats.

Note: The mean ± SD values age, body weight, and plasma testosterone concentrations from the trained diabetic rats were derived from the DTrC, DTrT, DTrExC, and DTrExT groups. The mean ± SD values for plasma glucose, insulin, fatty acids, glycerol, and triacylglycerols were randomly selected from a subset of animals from the non-diabetic SC group and from the diabetic DSC, and DTrC groups.

Figure 6.2. Glycogen content in *m. extensor digitorum longus* (upper panel) and *m. soleus* (lower panel) in the experimental groups. Data represent means $\pm$ SD. SC, sedentary control ($n=12$); ST, sedentary plus testosterone ($n=8$); DSC, diabetic sedentary control ($n=10$); DST, diabetic sedentary plus testosterone ($n=8$); DTrC, diabetic trained control ($n=10$); DTrT, diabetic trained plus testosterone ($n=9$); DTrExC, diabetic trained exercised (60 min) control ($n=9$); DTrExT, diabetic trained exercised (60 min) plus testosterone ($n=6$). * $P < 0.05$.

l, 4.8 ± 1.5 nmol/l, and 5.1 ± 1.1 nmol/l, for ST, DST, DTrT and DTrExT, respectively; $P < 0.005$) from the respective control animals.

During the final three weeks of the 9-week training period, all trained rats were capable of running continuously at a pace of 30 m/min for 60 min (cf. van Breda *et al.* 1992). Body weight gain (12-16%) (Table 6.1) was significantly higher in the testosterone-treated animals compared with the body weight gain (2-5%) in the control animals.

Table 6.5. Glycogen content in m. extensor digitorum longus (EDL) and m. soleus

Subgroups	EDL ($\mu\text{mol g}^{-1}$ dry weight)	Soleus ($\mu\text{mol g}^{-1}$ dry weight)
SC	117 $\pm$ 17	45 $\pm$ 16
ST	105 $\pm$ 9	70 $\pm$ 7*1
DSC	105 $\pm$ 7*2	39 $\pm$ 5*2
DST	123 $\pm$ 8*3	71 $\pm$ 16*3
DTrC	116 $\pm$ 6*4	65 $\pm$ 11*4
DTrT	124 $\pm$ 5	75 $\pm$ 13
DTrExC	72 $\pm$ 11	9 $\pm$ 4
DTrExT	107 $\pm$ 5*5	18 $\pm$ 3*5

Values are means $\pm$ SD and are expressed as $\mu\text{mol (g dry weight)}^{-1}$. *1 $P < 0.05$ SC versus ST;

*2 $P < 0.05$ SC versus DSC; *3 $P < 0.05$ DSC versus DST; *4 $P < 0.05$ DTrC versus DSC; and

*5 DTrT versus DTrExC

Response of muscle glycogen to diabetes, training and testosterone treatment

Glycogen contents ($\mu\text{mol/g dry weight}$) of EDL and soleus are shown in Figure 6.2 and Table 6.2. Glycogen contents of EDL and soleus muscles were significantly lower in sedentary diabetic rats than in sedentary control rats ($104.4 \pm 5.9 \mu\text{mol/g dw}$ versus $113.3 \pm 12.1 \mu\text{mol/g dw}$ for EDL and $38.5 \pm 4.7 \mu\text{mol/g dw}$ versus $48.9 \pm 15.1 \mu\text{mol/g dw}$ for soleus, respectively)(DSC versus SC). In diabetic rats the glycogen contents of both EDL and soleus muscles increased significantly in response to training ($116.3 \pm 5.9 \mu\text{mol/g dw}$ versus $104.4 \pm 5.9 \mu\text{mol/g dw}$ for EDL and $64.9 \pm 11.1 \mu\text{mol/g dw}$ versus $38.5 \pm 4.7 \mu\text{mol/g dw}$ for soleus, respectively)(DTrC versus DSC), and in response to testosterone treatment ($122.9 \pm 7.5 \mu\text{mol/g dw}$ versus $104.4 \pm 5.9 \mu\text{mol/g dw}$ for EDL and $70.7 \pm 16.3 \mu\text{mol/g dw}$ versus $38.5 \pm 4.7 \mu\text{mol/g dw}$ for soleus, respectively) (DST versus DSC). Together these manipulations (training and testosterone treatment) resulted in glycogen contents that were not different from these reached by separate manipulations ($124.3 \pm 4.9 \mu\text{mol/g dw}$ versus $122.9 \pm 7.5 \mu\text{mol/g dw}$ and $75.5 \pm 12.9 \mu\text{mol/g dw}$ versus $70.7 \pm 16.3 \mu\text{mol/g dw}$ for EDL and soleus, respectively)(DTrT versus DTrC).

In the testosterone-treated non-diabetic animals, however, only in the soleus a significantly higher glycogen content was observed as compared to the muscle in control rats ($105.3 \pm 8.6 \mu\text{mol/g dry weight}$ versus $113.3 \pm 12.1 \mu\text{mol/g dry weight}$ and $48.9 \pm 15.1 \mu\text{mol/g dry weight}$ versus $69.5 \pm 7.4 \mu\text{mol/g dry weight}$ for EDL and soleus, respectively)(ST versus SC).

Response of muscle glycogen to acute exercise

In the EDL the exercise induced absolute reduction in glycogen content was significantly less in the testosterone treated rats (16.0 $\mu\text{mol/g dw}$; DTrT versus DTrExT) than in the non-testosterone treated rats (44.5 $\mu\text{mol/g dw}$; DTrC versus DTrExC). However, in the soleus the absolute reduction in glycogen content was not different between the testosterone-treated and non-testosterone treated groups (58.3 $\mu\text{mol/g dw}$ versus 56.3 $\mu\text{mol/g dw}$).

Response of muscle glycogen synthase, and glycogen phosphorylase activities to diabetes, training and testosterone treatment

The activities of glycogen synthase and glycogen phosphorylase in EDL and soleus are shown in Tables 6.2 and 6.3. In EDL and soleus muscles of sedentary diabetic rats total glycogen synthase activity (GSi+d) and the active portion of glycogen synthase (GSi) were significantly lower compared with the sedentary non-diabetic rats (SC versus DSC). The GSi+d and GSi activities of both muscles increased significantly as in response to training (DSC versus DTrC). GSi and GSi+d activities in EDL and soleus muscles of sedentary non-diabetic and sedentary diabetic animals were significantly increased upon testosterone treatment (SC versus ST and DSC versus DST). In the soleus muscle of non-diabetic sedentary animals and in both muscles of diabetic sedentary animals testosterone reduced glycogen phosphorylase (active form) (GPa) activities significantly (SC versus ST and DSC versus DST). In the soleus muscle of the trained diabetic animals testosterone treatment increased GSi+d and GSi activity in concert with decreased total glycogen phosphorylase activities (GPa+b) and decreased activities of the active portion of glycogen phosphorylase (GPa) (DTrC versus DTrT).

Response of muscle glycogen synthase, and glycogen phosphorylase to acute exercise

In the EDL muscle of testosterone treated animals we observed a significant larger percentual enhancement of the active portion of glycogen synthase (GSi) immediately after exercise (+ 48%; DTrT versus DTrExT) than in the control animals (+ 21%; DTrC versus DTrExC). Interestingly, this was paralleled by a smaller percentual increase in the active portion of glycogen phosphorylase (GPa) activities (+ 29% for DTrT versus DTrExT and + 49% DTrC versus DTrExC). In the soleus muscle, however, we observed a decrease in the active portion of glycogen synthase as a response to exercise, which was not different between the testosterone treated animals and the controls (- 41% for DTrC versus DTrExC and - 39% for DTrT versus DTrExT). Remarkably, in the soleus muscle of testosterone treated rats we observed a significant decrease in the active portion of glycogen phosphorylase (- 40%), whereas in the control animals an increase was observed (+ 28%). As discussed in chapter 4, we do not have an explanation for the fact that the percentage of active glycogen synthase and glycogen phosphoarylase is not congruent with the changes in absolute activities of both enzymes.

Table 6.3. The activities of glycogen synthase, and glycogen phosphorylase in extensor digitorum longus (EDL)

Subgroups	Glycogen Synthase (GS)			Glycogen Phosphorylase (GP)		
	I + D ($\mu\text{mol min}^{-1} \text{g}^{-1}$)	I ($\mu\text{mol min}^{-1} \text{g}^{-1}$)	I (%)	a+b ($\mu\text{mol min}^{-1} \text{g}^{-1}$)	a ($\mu\text{mol min}^{-1} \text{g}^{-1}$)	a (%)
SC	2.80 $\pm$ 0.3	0.79 $\pm$ 0.1	27.8 $\pm$ 2.0	44.5 $\pm$ 16.4	5.0 $\pm$ 1.3	11.6 $\pm$ 1.7
ST	2.38 $\pm$ 0.3	0.90 $\pm$ 0.1	48.2 $\pm$ 16.3	55.4 $\pm$ 4.4	4.9 $\pm$ 0.2	8.8 $\pm$ 0.9
DSC	0.79 $\pm$ 0.1 ^{*1}	0.56 $\pm$ 0.1 ^{*1}	72.2 $\pm$ 17.3 ^{*1}	37.5 $\pm$ 2.4	6.9 $\pm$ 1.1	18.5 $\pm$ 2.0
DST	1.58 $\pm$ 0.2 ^{*2}	0.67 $\pm$ 0.1 ^{*2}	43.2 $\pm$ 7.5 ^{*2}	35.3 $\pm$ 6.8	3.4 $\pm$ 1.1 ^{*2}	9.4 $\pm$ 1.7 ^{*2}
DTrC	1.38 $\pm$ 0.1 ^{*3}	0.94 $\pm$ 0.1 ^{*3}	68.4 $\pm$ 7.0 ^{*3}	44.6 $\pm$ 5.5	4.7 $\pm$ 0.9 ^{*3}	10.6 $\pm$ 2.2
DTrT	1.69 $\pm$ 0.2	0.95 $\pm$ 0.2	51.3 $\pm$ 3.7	49.8 $\pm$ 4.3	4.5 $\pm$ 0.5	9.0 $\pm$ 0.4
DTrExC	1.50 $\pm$ 0.2	1.14 $\pm$ 0.1	76.6 $\pm$ 9.4	65.3 $\pm$ 5.3	7.0 $\pm$ 1.5	10.6 $\pm$ 1.9
DTrExT	2.28 $\pm$ 0.2 ^{*4}	1.41 $\pm$ 0.1 ^{*4}	62.3 $\pm$ 6.2	46.6 $\pm$ 4.0 ^{*4}	5.8 $\pm$ 1.1	12.5 $\pm$ 2.7

Values are means $\pm$ SD and are expressed as $\mu\text{mol min}^{-1} (\text{g wet weight})^{-1}$ and as % of the total activity.

^{*1} $P < 0.05$ SC versus ST; ^{*2} $P < 0.05$ SC versus DSC; ^{*3} $P < 0.05$ DSC versus DST; ^{*4} $P < 0.05$ DTrExC versus DTrExT.

Table 6.4. The activities of glycogen synthase, and glycogen phosphorylase in soleus muscle

Subgroups	Glycogen Synthase (GS)			Glycogen Phosphorylase (GP)		
	I + D ($\mu\text{mol min}^{-1} \text{g}^{-1}$)	I ($\mu\text{mol min}^{-1} \text{g}^{-1}$)	I (%)	a+b ($\mu\text{mol min}^{-1} \text{g}^{-1}$)	a ($\mu\text{mol min}^{-1} \text{g}^{-1}$)	a (%)
SC	4.34 $\pm$ 0.3	1.38 $\pm$ 0.2	31.9 $\pm$ 4.3	16.9 $\pm$ 1.7	1.12 $\pm$ 0.1	6.8 $\pm$ 0.4
ST	4.84 $\pm$ 0.3	1.84 $\pm$ 0.2 ^{*1}	7.8 $\pm$ 3.1 ^{*1}	17.0 $\pm$ 4.4	0.94 $\pm$ 0.2 ^{*1}	6.0 $\pm$ 2.2
DSC	1.51 $\pm$ 0.1 ^{*2}	0.69 $\pm$ 0.2 ^{*2}	45.6 $\pm$ 10.5 ^{*2}	14.2 $\pm$ 1.9	1.52 $\pm$ 0.3 ^{*1}	0.7 $\pm$ 1.8 ^{*2}
DST	2.18 $\pm$ 0.2 ^{*3}	0.85 $\pm$ 0.1 ^{*3}	39.5 $\pm$ 8.4	11.2 $\pm$ 1.7	0.89 $\pm$ 0.2 ^{*3}	7.9 $\pm$ 2.5
DTrC	1.86 $\pm$ 0.1 ^{*4}	1.11 $\pm$ 0.1 ^{*4}	59.7 $\pm$ 6.1 ^{*4}	17.4 $\pm$ 1.2	1.74 $\pm$ 0.4	9.9 $\pm$ 2.0
DTrT	2.21 $\pm$ 0.2 ^{*5}	1.25 $\pm$ 0.2 ^{*5}	57.0 $\pm$ 9.7	13.3 $\pm$ 1.2 ^{*5}	1.40 $\pm$ 0.1	10.6 $\pm$ 0.6
DTrExC	1.35 $\pm$ 0.1	0.65 $\pm$ 0.1	48.2 $\pm$ 10.0	25.1 $\pm$ 7.8	2.23 $\pm$ 0.6	8.9 $\pm$ 0.7
DTrExT	1.75 $\pm$ 0.2 ^{*6}	0.76 $\pm$ 0.1 ^{*6}	43.7 $\pm$ 6.5	15.6 $\pm$ 3.3 ^{*6}	0.84 $\pm$ 0.3 ^{*6}	5.5 $\pm$ 1.8 ^{*6}

Values are means $\pm$ SD and are expressed as $\mu\text{mol min}^{-1} (\text{g wet weight})^{-1}$ and as % of the total activity.

^{*1} $P < 0.05$ SC versus ST; ^{*2} $P < 0.05$ SC versus DSC; ^{*3} $P < 0.05$ DSC versus DST; ^{*4} $P < 0.05$ DSC versus DTrC;

^{*5} $P < 0.05$ DTrC versus DTrT; ^{*6} $P < 0.05$ DTrExC versus DTrExT.

Response of fatty acid-binding protein (FABP) content and enzymes of fatty acid oxidation to diabetes, training and testosterone treatment.

Fatty acid-binding protein (FABP) contents ($\mu\text{g/g}$ wet weight) of EDL and soleus are shown in Figure 6.3 and Table 6.5. FABP content was increased by about 2-fold upon inducement of diabetes in the soleus and by about 1.5-fold in the EDL (SC versus DSC). Neither training nor testosterone treatment, either alone or in combination, increased the FABP content in EDL and soleus of diabetic animals significantly as compared to their respective controls (DSC versus DTrC, DSC versus DST, and DTrC versus DTrT; respectively). In the non-diabetic animals, however, testosterone treatment resulted in an increase of FABP concentrations to a level as high as observed in muscles from diabetic animals (SC versus ST) (Figure 6.3; Table 6.5).

Figure 6.3. FABP contents in *m. extensor digitorum longus* (upper panel) and *m. soleus* (lower panel) in the experimental groups. Data represent means $\pm$ SD. SC, sedentary control ($n=8$); ST, sedentary plus testosterone ($n=6$); DSC, diabetic sedentary control ($n=10$); DST, diabetic sedentary plus testosterone ($n=6$); DTrC, diabetic trained control ($n=9$); DTrT, diabetic trained plus testosterone ($n=9$). * $P < 0.05$.

Table 6.5. Fatty acid-binding protein content in *m. extensor digitorum longus* (EDL), and *m. soleus*

Subgroups	EDL ($\mu\text{g/g. wet weight}$)	Soleus ($\mu\text{g/g. wet weight}$)
SC	58 $\pm$ 12	255 $\pm$ 50
ST	96 $\pm$ 6*1	478 $\pm$ 34*1
DSC	85 $\pm$ 12*2	503 $\pm$ 49*2
DST	83 $\pm$ 29*2	515 $\pm$ 31*2
DTrC	84 $\pm$ 9*2	577 $\pm$ 94*2
DTrT	83 $\pm$ 10*2	533 $\pm$ 38*2

Values are means $\pm$ SD and are expressed as $\mu\text{mol (g dry weight)}^{-1}$ *1 $P < 0.05$ SC versus ST;

*2 $P < 0.05$ Diabetic (D) versus SC.

Interestingly, in concert with the increased FABP concentrations in both muscles, 3-hydroxyacyl-CoA dehydrogenase (HAD) activity was also increased significantly upon inducement of diabetes (SC versus DSC), whereas in these diabetic animals neither training nor testosterone treatment alone, had an effect on the activity of HAD. In the soleus, the combined effect of training and testosterone treatment, however, increased HAD activity significantly as compared to the diabetic trained non-testosterone treated animals (DTrC versus DTrT) (Table 6.6). In the non-diabetic sedentary animals testosterone treatment also increased the activity of HAD significantly as compared with the non-diabetic non-testosterone treated animals (SC versus ST).

Palmitoyl-CoA synthase (PCS) activities were not different among any of the subgroups although in the soleus the "PCS" activity tended to be higher in the diabetic than in the non-diabetic animals.

Response of mitochondrial marker enzymes to diabetes, training and testosterone treatment

In the soleus but not in the EDL muscle of diabetic sedentary animals citrate synthase (CS) was significantly increased as compared to the sedentary non-diabetic animals (SC versus DSC) (Table 6.6). In comparison with the soleus muscle of untrained diabetic animals, testosterone treatment in the trained diabetic rats resulted in significantly higher CS activity (DTrC versus DTrT). In both muscles of non-diabetic sedentary animals testosterone treatment also enhanced CS activity significantly compared to the non-testosterone treated non-diabetic sedentary animals (SC versus ST).

Cytochrome c oxidase (Cyt ox) activities in the soleus and EDL muscles were not

Table 6.6. The activities of 3-hydroxyacyl-CoA dehydrogenase (HAD), palmitoyl-CoA synthetase (PCS), citrate synthase (CS) and cytochrome c oxidase (Cyt C) in extensor digitorum longus (EDL) and soleus muscle

Subgroups	m. extensor digitorum Longus			
	HAD	PCS	CS	Cyt ox
SC	5.6 ± 1.5	2411 ± 156	7.1 ± 0.5	14 ± 3
ST	11.8 ± 0.8*1	2687 ± 145	8.8 ± 0.4*1	22 ± 6*1
DSC	9.4 ± 1.8*2	2408 ± 223	7.7 ± 1.9	18 ± 5
DST	9.3 ± 1.4	2538 ± 225	8.5 ± 0.8	28 ± 6*3
DTrC	8.3 ± 1.0	2393 ± 200	8.8 ± 0.6	19 ± 8
DTrT	10.2 ± 0.9	2446 ± 221	9.8 ± 0.7	20 ± 6
m. soleus				
	HAD	PCS	CS	Cyt ox
SC	11.0 ± 0.8	4447 ± 199	14.3 ± 1.2	26 ± 5
ST	21.8 ± 6.3*1	4744 ± 242	22.2 ± 2.9*1	53 ± 13*1
DSC	17.4 ± 1.8*2	5119 ± 786	22.9 ± 2.6*2	25 ± 14
DS	18.0 ± 3.5	4876 ± 486	18.7 ± 2.4	49 ± 19*3
DTrC	20.2 ± 2.6*4	5438 ± 450	22.7 ± 1.4	50 ± 13*4
DTrT	24.5 ± 2.0*5	5664 ± 395	29.8 ± 2.7*5	70 ± 14*5

Values are means ± SD and are expressed as $\mu\text{mol min}^{-1} (\text{g wet weight})^{-1}$ for HAD, CS, and Cyt ox.

Significantly different ($P < 0.05$) *1 SC versus ST; *2 SC versus DSC; *3 DSC versus DST;

*4 DSC versus DTrC; *5 DTrC versus DTrT.

changed upon inducement of diabetes (SC versus DSC) (Table 6.6). However, Cyt ox was significantly increased in the soleus but not in the EDL of the trained diabetic animals (DSC versus DTrC). Furthermore, testosterone treatment resulted in significantly higher Cyt ox activities in EDL (SC versus ST and DSC versus DST) and soleus (SC versus ST, DSC versus DST, and DTrC versus DTrT).

6.4 Discussion

The present data of decreased muscle glycogen contents and glycogen synthase activities in streptozotocin-induced non-insulin-dependent diabetic rats support previously reported findings in studies using the same animal model (Schaffer, 1986; Kruszynska, 1988a; Kruszynska, 1988b). In addition, the findings of the present study in rats, as well as recent findings in humans (Farrace and Rossetti, 1992; Fransilla-Kallunki *et al.*, 1992), indicate that an enhanced glucose supply (mass-action effect of hyperglycemia) is not sufficient to compensate for the impaired glycogen synthesis in the sedentary diabetic rats. Finally, the results of the present study are not supportive for a primary role of testosterone in the development of the insulin resistant state.

Effects of training and testosterone treatment on muscle glycogen content and metabolism

Previously, we were able to show that in non-diabetic trained female rats, treated with pharmacological doses of testosterone (ca. 25 nmol/l), glycogen content in EDL, but not in soleus muscle was increased, in concert with an increase of the active portion of glycogen synthase compared to the non-testosterone treated rats (van Breda *et al.*, 1993). In contrast with the aforementioned study are the results of the present study in trained and testosterone-treated diabetic rats, in which we found a higher glycogen content of both muscles in concert with a significant increase in total glycogen synthase, but only in the soleus muscle with a significant increase in the active portion of glycogen synthase. An explanation for the increased glycogen content in the diabetic EDL without a concomitant increase in the active portion of glycogen synthase activity is hard to provide. It may, however, relate to the fact that, to avoid interference from the training session itself, the animals were killed three days after the last training bout. Support for this explanation is provided by the results of Allenberg *et al.* (Alленberg *et al.*, 1983), who reported that repletion of muscle glycogen takes place in the first hours after exercise. Furthermore, they showed that the percentage of active glycogen synthase was inversely related to the muscle glycogen content. Thus, at the time of muscle sampling in our study (DTRC and DTRT groups), glycogen content may have already been completely repleted and glycogen synthase activity be decreased to the pre-exercise levels.

Glycogen sparing effect of testosterone during submaximal exercise and responsible mechanisms

Another striking finding in the present study is the glycogen sparing effect of testosterone in EDL, but not in the soleus, after a submaximal exercise bout in concert with an increase in the active portion of glycogen synthase and a reduction in the active portion of glycogen phosphorylase. The fact that we could not observe a glycogen sparing effect in the soleus (which is more involved in running than the EDL) may be caused by the higher body weight of the testosterone treated animals. This glycogen sparing effect of testosterone during acute exercise is, in part (EDL), in line with our findings in non-diabetic animals (van Breda *et al.*, 1993).

The mechanism by which testosterone regulates glycogen metabolism during exercise of diabetic rats, however, remains obscure. The most plausible explanation is substrate competition, by which increased availability of one substrate (glucose, fatty acids) restrains consumption of the other. This mechanism, originally demonstrated by Randle *et al.* (Randle *et al.*, 1963; Randle *et al.*, 1964), has been demonstrated to be operative in skeletal muscle (Kruszynska and Home, 1988; Kruszynska *et al.*, 1988). Since androgens have been shown to increase lipolytic activity (Xu-X *et al.*, 1990), it can be argued that the glycogen sparing effect of testosterone, as observed in our study, is caused by an increased fatty acid supply and subsequent oxidation. According to the glucose-fatty acid cycle (Randle *et al.*, 1963; Randle *et al.*, 1964), the preferential use of fatty acids over glucose leads to an accumulation of citrate, a potent inhibitor of fructose-6-phosphate kinase. Inhibition of this regulatory key enzyme of the glycolysis results in a rise in glucose-6-phosphate and the diversion of substrate into glycogen synthesis. Although we did not actually measure glucose-6-phosphate and/or citrate concentrations, we did measure an increased citrate synthase activity and muscle glycogen deposition, which is in keeping with Randle's glucose-fatty acid cycle. In addition, we found an increase in fatty acid-binding protein (FABP) content in concert with increased activities of 3-hydroxyacyl-CoA dehydrogenase (HAD) and cytochrome c oxidase. These data suggest that in testosterone-treated trained diabetic animals, the trans-cytoplasmic flux of fatty acids is increased in concert with an increased capacity of β -oxidation. Further support for the preferential use of fatty acids over glucose can also be derived from the study of Kendrick and Ellis (Kendrick and Ellis, 1991), who reported higher plasma fatty acid concentrations after exercise in estradiol-17 β -treated non-diabetic rats compared to control rats. In our study, however, no differences in plasma fatty acid concentration could be observed in the testosterone-treated exercised streptozotocin-diabetic rats compared to the untreated animals. Our findings, therefore, might support the hypothesis that trained skeletal muscle utilizes more of its endogenous triacylglycerol pool (Jansson and Kaijser, 1987; Coggan *et al.*, 1993). Furthermore, as we have already mentioned in chapter 4, the glycogen sparing effect of testosterone in endurance trained rats, at a given workload, may also be caused by a to the complete oxidation of glycogen to CO₂ rather than the aerobic breakdown to lactate.

Differences between glycolytic and oxidative muscle fibres

An important finding of the present study is that testosterone elicits quantitatively different effects on energy metabolic properties in glycolytic (m. extensor digitorum longus, EDL) and oxidative (m. soleus) muscle fibres (Figure 6.4). In trained female diabetic animals we found that testosterone potentiates the training induced effects, and causes a sparing of glycogen after submaximal exercise in the EDL.

Figure 6.4. Schematic representation of the differences between glycolytic (EDL) and oxidative (soleus) muscles of diabetic female rats.

Symbols used: ↑ increase, → no change, ↓ decrease

The glycogen sparing effect in the EDL of female diabetic rats, after training plus testosterone treatment, extends our previous findings in non-diabetic female rats treated with a pharmacological doses of testosterone (ca. 25 nmol/l) (van Breda *et al.*, 1993). In the soleus muscle no absolute differences in glycogen depletion could be observed between the testosterone treated animals and the control animals upon exercise. We have to keep in mind, however, that the testosterone treated animals were heavier, which under normal conditions should have caused a greater reliance upon glycogen breakdown. Thus, from the latter assumption we cannot rule out the existence of glycogen sparing effect of testosterone in the soleus muscle as well. Besides, in keeping with the glucose-fatty acid cycle of Randle *et al.* (Randle *et al.*, 1963) it can also be argued that the glycogen

sparing effect would result from an increased oxidation of fatty acids. In the soleus support for this idea is provided by a relatively larger increase (DSC versus SC) in the capacity of fatty acid handling (FABP content) than in the EDL, in combination with enhanced HAD activity.

Concluding remarks

The data of the present study demonstrate that mild hyperandrogenicity affects muscle metabolism in hyperglycemic-hypoinsulinemic streptozotocin-diabetic female rats. Especially in the trained diabetic animals we found that testosterone potentiates the training induced effects, and causes a sparing of muscle glycogen after submaximal exercise in the glycolytic muscle (EDL) but not in the oxidative muscle (soleus). These findings in the EDL seem to result from increased glycogen synthesis, decreased glycogenolysis, and an increased fatty acid handling and oxidation. Our data strongly suggest that hyperandrogenism is not a causative factor in the development of insulin resistance. Finally, our results support the idea (Devlin *et al.*, 1987; Wasserman and Abumrad, 1989; Eriksson and Lindgärde, 1991) that regular physical exercise, especially in combination with testosterone treatment, could counteract the cellular post-receptor defects in non-insulin-dependent diabetes mellitus. Thus, it may be argued that the combined metabolic defects as observed during the late stage of non-insulin-dependent diabetes mellitus can, in part, be compensated by testosterone and/or endurance training. Unravelling the exact mechanism of this effect of testosterone is a challenge for future investigations.

6.5 References

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7 Possible mechanisms of the testosterone induced changes in skeletal muscle carbohydrate and fatty acid metabolism

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Modulation of glycogen metabolism of rat skeletal muscle by endurance training and testosterone treatment

Eric van Breda, Hans A. Keizer, Peter Geurten, Gerrit van Kranenburg, Paul P.C.A. Menheere, Harm Kuipers, and Jan F.C. Glatz

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and

Modulation of fatty acid-binding protein content of rat heart and skeletal muscle by endurance training and testosterone treatment

Eric van Breda, Hans A. Keizer, Michaël M. Vork, Don A.M. Surtel, Yvonne, F. de Jong, Ger J. van der Vusse and Jan F.C. Glatz

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7.1 Introduction

In the preceding chapters we have described the effects of testosterone and/or endurance training on muscle energy metabolic properties in skeletal muscle of diabetic and non-diabetic female rats. We found that testosterone increases glycogenesis, decreases glycogenolysis and increases fatty acid handling in diabetic as well as non-diabetic rats. The mechanisms underlying these changes are incompletely understood. It can be hypothesized that on the cellular level these changes are caused by testosterone-induced alterations in fibre type composition (Holmäng *et al.*, 1990; van Breda *et al.*, 1993) of the skeletal muscles under investigation and on the subcellular level, by changes in total and translocatable GLUT4.

Unlike the effects of training, literature concerning the effects of testosterone-treatment on muscle fibre type distribution is very limited. Holmäng *et al.* (Holmäng *et al.*, 1990) reported a decreased number of oxidative (type I) fibres of the oxidative soleus muscle in sedentary testosterone treated rats and related this to androgen-induced insulin resistance of skeletal muscles. Interestingly, the decrease in the number of oxidative muscle fibres was accompanied by an increase in 2-deoxyglucose uptake. Thus, based upon these findings their results can not explain the increased glucose uptake rates in this type of muscle after testosterone treatment. Furthermore, it has been shown that human skeletal muscle is capable of aromatizing testosterone to estradiol-17 β (Longcope *et al.*, 1976; Longcope *et al.*, 1978; Matsumine *et al.*, 1986), it is possible that in rat muscle the aromatization product estradiol-17 β rather than testosterone induces these adaptations.

Testosterone can be converted to estradiol-17 β by the aromatase enzyme complex and since in different types of muscle fibres of different species testosterone as well as estradiol-17 β receptors have been identified (Michel and Baulieu, 1980; Dahlberg, 1982; Meyer and Rapp, 1985; Stumpf, 1990) testosterone-induced effects might be caused by either testosterone or estradiol-17 β or a combination of both. To date, however, it is unknown whether in rat aromatization exclusively takes place in the ovaries (Wouters *et al.*, 1989; Krekels *et al.*, 1990) or that rat skeletal muscle, like human skeletal muscle (Longcope *et al.*, 1976; Longcope *et al.*, 1978; Matsumine *et al.*, 1986), itself is capable of converting androgens into estrogens.

In order to shed more light upon the above mentioned findings we investigated whether: (1) testosterone either alone or in combination with endurance training causes by a shift in muscle fibre composition; (2) testosterone itself or its conversion product estradiol-17 β were responsible for the observed testosterone-induced effects and (3) rat skeletal muscle is capable of aromatizing testosterone into estradiol-17 β . For this purpose we investigated in oxidative and glycolytic muscles of non-diabetic female rats the effects of testosterone on the percentage of type I and type II fibres (trained rats only). Furthermore, the effects of a non-steroidal aromatase inhibitor, administered to both sedentary and trained testosterone treated rats and their controls, on the testosterone-induced effects on glycogen content and fatty acid-binding protein content (chapter 4 and 5). Finally, we investigated whether the muscles under investigation are capable of aromatizing testosterone into estradiol-17 β .

7.2 Materials & Methods

Animal treatment and experimental procedures

Animal treatment was exactly as described in chapter 4 and 5. To study the influence of testosterone and an aromatase inhibitor, during the last 2 weeks of the training period each group of sedentary (S) and trained rats (Tr) were randomly divided into four subgroups, i.e. control (SC and TrC), testosterone (ST and TrT), aromatase inhibitor (SCA and TrCA) and testosterone plus aromatase inhibitor (STA and TrTA) (Figure 7.1). To the rats of the SCA, STA, TrCA and TrTA groups, a new, highly specific, non-steroidal inhibitor, R76713 (gift from Janssen Research Foundation, Beerse, Belgium) was administered daily by gavage at a dose of 1 mg/kg body weight. This dosage has been shown to be very effective in suppressing the conversion of testosterone to estradiol-17 β for a period of 24 - 48 h (Wouters, 1989).

Muscle samples for muscle fibre distribution were taken from the trained control (TrC) and testosterone-treated groups (TrT) only. For this part of the study only these two groups were selected, since the influence of endurance training on fibre distribution is well known (cf. Holloszy and Coyle, 1984).

All analytical methods are described in detail in chapter 3.

Figure 7.1. Schematic representation of study design.

Table 7.1. Fatty acid-binding protein content in heart, *m. extensor digitorum longus* (EDL), and *m. soleus*

Subgroups	Heart ($\mu\text{g/g}$. wet weight)	EDL ($\mu\text{g/g}$. wet weight)	Soleus ($\mu\text{g/g}$. wet weight)
SC	497 $\pm$ 32	58 $\pm$ 12	255 $\pm$ 50
SCA	464 $\pm$ 75	n.d.	267 $\pm$ 49
ST	663 $\pm$ 58* ¹	48 $\pm$ 9	294 $\pm$ 18* ¹
STA	637 $\pm$ 82	46 $\pm$ 7	255 $\pm$ 24
TrC	639 $\pm$ 28* ²	50 $\pm$ 18	255 $\pm$ 21
TrCA	642 $\pm$ 28	n.d.	255 $\pm$ 37
TrT	759 $\pm$ 59* ³	55 $\pm$ 17	295 $\pm$ 15* ³
TrTA	729 $\pm$ 39	55 $\pm$ 11	248 $\pm$ 45

Values are means $\pm$ SD and are expressed as $\mu\text{g/g}$ wet weight. *¹ $P < 0.05$ SC versus ST;

*² $P < 0.05$ SC versus TrC; and *³ $P < 0.05$ TrC versus TrT. n.d. = not determined

Table 7.2. Cytochrome *c* oxidase activities in heart, *m. soleus* and *m. extensor digitorum longus*

Subgroups	Heart (U/g wet weight)	EDL (U/g wet weight)	Soleus (U/g wet weight)
SC	168 $\pm$ 23	14 $\pm$ 3	26 $\pm$ 5
SCA	n.d.	n.d.	n.d.
ST	165 $\pm$ 29	16 $\pm$ 2 * ¹	24 $\pm$ 4
STA	154 $\pm$ 36	17 $\pm$ 5	22 $\pm$ 6
TrC	276 $\pm$ 61 * ¹	12 $\pm$ 3	29 $\pm$ 5* ¹
TrCA	243 $\pm$ 32	12 $\pm$ 4	32 $\pm$ 4
TrT	315 $\pm$ 35 * ² * ⁴	15 $\pm$ 3	40 $\pm$ 9* ² * ⁴
TrTA	300 $\pm$ 42	11 $\pm$ 3 * ³	26 $\pm$ 2* ³

Values are means $\pm$ SD and are expressed as Units/g wet weight. SC = sedentary control; SCA = sedentary control plus inhibitor; ST = sedentary plus testosterone; STA = sedentary plus testosterone and inhibitor; TrC = trained control; TrCA = trained control plus inhibitor; TrT = trained plus testosterone and TrTA = trained plus testosterone and inhibitor. *¹ $P < 0.05$ versus SC; *² $P < 0.05$ versus TrC; *³ $P < 0.05$ versus TrT; *⁴ $P < 0.05$ versus ST. n.d. = not determined.

Table 7.3. Glycogen content in *m. extensor digitorum longus* (EDL) and *m. soleus*

Subgroups	EDL	Soleus
SC	117 ± 7	45 ± 16
SCA	n.d.	n.d.
ST	119 ± 9	51 ± 8
STA	118 ± 14	71 ± 16*1
TrC	123 ± 6	81 ± 18*2
TrCA	140 ± 13*4	89 ± 15
TrT	135 ± 13*3*5	82 ± 8*5
TrTA	138 ± 15	90 ± 15

Values are means ± SD and are expressed as $\mu\text{mol (g dry weight)}^{-1}$. *1 $P < 0.05$ ST versus STA; *2 $P < 0.05$ SC versus TrC; *3 $P < 0.05$ TrC versus TrT; *4 $P < 0.05$ TrC versus TrCA; *5 $P < 0.05$ ST versus TrT. n.d. = not determined.

7.3 Results

Plasma testosterone levels in the aromatase inhibitor treated control groups were mutually not different from the non-inhibitor treated control groups (0.9 ± 0.3 nmol/l, 0.9 ± 0.2 nmol/l, 0.8 ± 0.2 nmol/l and 0.9 ± 0.2 nmol/l for SC, SCA, TrC and TrCA, respectively). In the testosterone-treated animals plasma testosterone levels were significantly higher compared to the control groups (25.8 ± 3.9 nmol/l, 21.3 ± 2.2 nmol/l, 23.6 ± 4.1 nmol/l and 24.9 ± 3.2 nmol/l for ST, STA, TrT and TrTA, respectively; $P < 0.05$). No significant changes could be observed between plasma testosterone levels of the testosterone treated groups with or without the aromatase inhibitor.

Administration of the highly specific non-steroidal aromatase inhibitor R76713 resulted in barely detectable estradiol-17 β levels (Wouters *et al.*, 1989). Applying a modified radio-immunoassay (see chapter 3) we found levels below the detection limit of this assay (< 20 pmol/l). The inhibitor did not induce significant changes in muscle fatty acid-binding protein (FABP) content, cytochrome c oxidase activities (Tables 7.1 and 7.2), nor glycogen content in the soleus of the sham-plus-inhibitor treated group compared with the non-inhibitor sham-treated group (TrC versus TrCA) (Table 7.3). In the EDL, however, inhibitor treatment in the trained control group resulted in a significantly higher glycogen content compared with the non-inhibitor treated control group ($P < 0.05$; TrCA versus TrC) (Table 7.3).

Muscle fibre type composition

In both EDL and soleus muscles the combination of testosterone treatment and training, when compared with training alone, resulted in a significant transformation towards relatively more oxidative (type I) muscle fibres at the expense of glycolytic (type II) muscle fibres (TrC versus TrT; $P < 0.05$) (Table 7.4).

Response of FABP, cytochrome c oxidase, and glycogen content to training, testosterone treatment and aromatase inhibition

Fatty acid-binding protein (FABP) contents ($\mu\text{g/g}$ wet weight) in heart, soleus and extensor digitorum longus (EDL) are shown in Table 7.1 (for a more detailed description of part of the results see chapter 5). Inhibition of the androgen-to-estrogen-converting aromatase system in the heart did not change the testosterone-induced FABP increments in the sedentary (STA versus ST) or in the trained groups (TrTA versus TrT), suggesting a direct effect of testosterone.

In contrast to the effects of the aromatase inhibitor on cardiac FABP levels, are the results of the inhibitor on soleus muscle (Table 7.1). Inhibition of the aromatase system in both sedentary and trained rats counteracted the testosterone-induced effects completely (Table 7.1), indicating that the effect of testosterone is mediated by its conversion product estradiol-17 β . In the EDL muscle neither training nor testosterone treatment with or without the aromatase inhibitor had any significant effect on the muscular FABP content (Table 7.1).

The activities of the mitochondrial marker enzyme cytochrome c oxidase are depicted in Table 7.2. Inhibition of the aromatase system in both sedentary and trained animals significantly decreased cytochrome c oxidase in trained soleus and EDL, but not in heart.

Glycogen contents of EDL and soleus are shown in Table 7.3. Inhibition of the aromatase system in the sedentary EDL had no effect of the glycogen content (ST versus

Table 7.4. Relative muscle fibre composition (%)

Subgroups	EDL		Soleus	
	Type I	Type II	Type I	Type II
Testosterone (TrT)	6 $\pm$ 1*	94 $\pm$ 1*	84 $\pm$ 3*	16 $\pm$ 3*
Control (TrC)	3 $\pm$ 1	97 $\pm$ 1	78 $\pm$ 5	22 $\pm$ 5

Values are expressed as means $\pm$ SD for 8 animals * $P < 0.05$ versus control muscles.

STA). In the trained groups the inhibitor did not change the testosterone-induced glycogen increments (TrTA versus TrT), indicating a direct effect of testosterone (Table 7.3). In the soleus testosterone treatment had no effect on glycogen content in the sedentary and trained rats (ST versus SC and TrT versus TrC). Interestingly, inhibition of the aromatase enzyme in both sedentary as well as trained animals resulted in a significantly higher glycogen content (STA versus ST and TrTA versus TrT; $P < 0.05$).

Aromatase activity in rat skeletal muscle

From the experiments in vitro (see chapter 3) (each performed in duplicate) a K_m for aromatization of testosterone of $0.51 \mu\text{M}$ for soleus and $0.19 \mu\text{M}$ for EDL was calculated. Using the known 3H-distribution of [$1\beta, 2\beta\text{-}^3\text{H}$]testosterone (42% in 1β and 31.2% in 2β) a $V_{\max}$ of $5.1 \text{ pmol h}^{-1} (\text{g wet weight})^{-1}$ for soleus and $2.1 \text{ pmol h}^{-1} (\text{g wet weight})^{-1}$ for EDL could be calculated (Figure 7.2; Table 7.5).

7.4 Discussion

The aim of the present study was to gain more information on how testosterone and/or endurance training affect muscle energy metabolic parameters. The results from the preceding chapters demonstrate that the muscles examined, which show a wide variation in physiological and biochemical properties, respond differently to these interventions.

Effects of testosterone on muscle fibre type composition

The combination of endurance training and testosterone administration for 14 days was found to cause an increase in the relative number of oxidative fibres in both EDL and soleus muscles. These findings contrast sharply with those of Holmäng *et al.* (Holmäng *et al.*, 1990), who reported a decreased number of oxidative (type I) fibres of the soleus in

Table 7.5. Kinetic parameters of the skeletal muscle aromatase activity

	EDL	Soleus
$K_m (\mu\text{M})$	0.19	0.51
$V_{\max} (\text{pmol} \cdot \text{h}^{-1} \text{g}^{-1} \text{wet weight})$	2.1	5.1

Figure 7.2. Lineweaver-Burke plot using $[1\beta, 2\beta\text{-}^3\text{H}]$ testosterone as substrate of aromatase activity by homogenates of glycolytic (EDL) and oxidative (soleus) muscles of female rats.

sedentary rats and related this to androgen-induced insulin resistance of skeletal muscles. A possible explanation for these differences might be caused by (i) the different plasma concentrations of testosterone (ca. 5 nmol/l Holmång et al. (Holmång *et al.*, 1990) versus ca. 25 nmol/l the present study) or (ii) the combined effect of endurance training and testosterone administration (present study).

Response of aromatase inhibition on FABP content, cytochrome c oxidase activity and glycogen content

Steroid hormones act on heart and skeletal muscle by cytoplasmic and/or nuclear receptors. In heart as well as in skeletal muscle fibres of different species both testosterone and estradiol- 17β receptors have been identified (Michel, 1980; Dahlberg, 1982; Meyer and Rapp, 1985; Stumpf, 1990). The results of the present study suggest that both receptors are involved in the effects of testosterone on muscle FABP and glycogen content and cytochrome c oxidase activities. Evidence for this assumption is provided by

the results after inhibition of the aromatase enzyme system by the highly specific inhibitor R76713 (Wouters *et al.*, 1989). It has been shown that dose used in the present study was highly effective for suppressing the conversion of testosterone to estradiol-17 β (Wouters, 1989). We found that, upon administration of this inhibitor at this dose, the FABP content of soleus muscles of sedentary and trained animals and cytochrome c oxidase activity of trained animals returned to their baseline level. In contrast, we failed to observe an effect of the inhibitor on cardiac FABP content or cytochrome c oxidase activity. Therefore, it is conceivable that in the soleus estradiol-17 β and not testosterone is the active compound. Since in the heart the inhibitor was ineffective, testosterone is likely to be the active compound.

An explanation for the findings of the present study that in the soleus glycogen content is increased after inhibition of the aromatase enzyme system is not easy to provide. It indicates that testosterone is the active compound and that after inhibition more testosterone is available to induce its effect in this type of muscle. An interesting alternative hypothesis is that estradiol-17 β has an antagonistic effect on the testosterone-induced effects and that after inhibition of the conversion of testosterone into estradiol-17 β this antagonistic effects has disappeared

Although the androgen-to-estrogen-converting aromatase enzyme system has been reported to exist in other tissues besides the gonads and placenta (Longcope *et al.*, 1976; Longcope *et al.*, 1978; Matsumine *et al.*, 1986), we are not aware of studies showing a difference in activity of this P450-dependent enzyme system in each specific type of muscle. In the present study, however, we have shown that a difference in aromatase activity exists between oxidative (soleus) and glycolytic (EDL) muscles. The physiological meaning for these differences has to be elucidated.

Concluding remarks

The results of the present study demonstrate that the effects of testosterone on muscle metabolic parameters of female rats are different for glycolytic (m. extensor digitorum longus, EDL), oxidative (m. soleus, soleus) and heart muscles. The differences can be explained, in part, by a different aromatase activity in these muscles. The results of the present study indicate that both testosterone as well as estradiol-17 β are involved. The increase in the relative number of oxidative (type I) fibres in the trained and testosterone treated animals gives further evidence for the more pronounced metabolic effects in oxidative muscles in which aromatase activity was the highest. Interestingly, FABP, cytochrome c oxidase, and glycogen seem to be affected by different hormones. Whereas, FABP in soleus was most likely to be affected by the conversion product of testosterone, estradiol-17 β , glycogen content seems to be under the control of testosterone. Interestingly, in the heart both testosterone and estradiol-17 β seem to be involved, although more research is needed to elucidate this latter hypothesis.

Future research on the differences in aromatase activity among these muscles will help to unravel the exact mechanism of the effects of testosterone. Finally, it would be interesting to explore whether aromatase activity in skeletal muscle of diabetic rats is

different from non-diabetic rats and whether testosterone treatment in these diabetic animals results in a change in muscle fibre type distribution as well.

7.5 References

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8

General Discussion

8.1 Introductory remarks

Skeletal muscle energy metabolism is, among others, under control of the endocrine system. In contrast to the well known effects of insulin on carbohydrate and lipid metabolism, literature concerning the effects of sex-steroids, especially testosterone, on skeletal muscle energy metabolism is rather limited. Although it has been suggested that hyperandrogenism is associated with insulin resistance little is known about the nature of this putative relationship (Achard and Thiers, 1921; Stein and Leventhal, 1935; Landon, 1963; Holmäng *et al.*, 1990; Poretsky, 1991). Insulin resistance can be defined as a subnormal response to a given concentration of insulin. Since skeletal muscle is the dominant tissue for insulin mediated glucose uptake, insulin resistance at this level represents the impaired 'whole-body' glucose uptake as observed in non-insulin-dependent diabetes mellitus.

At the cellular level a reduced glucose uptake and/or glycogen synthesis appear to be the main defects of insulin resistance (see Figure 2.11)(Clauser, 1992). However, the effect of testosterone on skeletal muscle glucose uptake and glycogenesis is less clear. It has been reported that testosterone may reduce glucose uptake and glucose incorporation into muscle glycogen (Holmäng *et al.*, 1990), whereas other investigators found increased glucose uptake after testosterone treatment (Max and Toop, 1983).

Regular exercise forms a fundamental part of the treatment of non-insulin-dependent diabetes mellitus because of its ability to reduce insulin resistance and to increase 'whole-body' glucose uptake (Horton, 1988; Berger, 1992; Vanninen, 1992). However, our understanding of the intracellular mechanism(s) by which this improved insulin sensitivity is achieved remains inchoate.

Therefore, the studies described in the present thesis were designed to gain more insight into the effects of testosterone alone or in combination with endurance training on a set of muscle metabolic parameters that are modified during insulin resistance. The preceding chapters of this thesis have dealt with a number of aspects related to testosterone and training mediated effects on muscle metabolic properties. The goals of the study were essentially twofold:

- . to investigate the intracellular effects of testosterone on muscle carbohydrate and lipid metabolism in both sedentary and trained diabetic and non-diabetic rats.
- . to get more insight into the mechanisms by which testosterone acts on muscle carbohydrate and lipid metabolism.

The results showed that:

- testosterone treatment in control (non-diabetic) female rats does not lead to insulin resistance.
- testosterone administered to insulin resistant diabetic female rats improves glycogenesis rather than deteriorating it.

- testosterone induces a glycogen sparing effect in muscles of exercised endurance trained diabetic (m. extensor digitorum longus only) and non-diabetic (m. extensor digitorum longus and m. soleus) rats which effect probably is due to both increased glycogen synthesis and increased fatty acid metabolism.
- testosterone elicits different effects on different parameters of energy metabolism of glycolytic and oxidative muscle fibres.
- the effects of testosterone can, in part, be mediated by estradiol-17 β produced by aromatization of testosterone in heart and skeletal muscle.

The corollaries of these findings will be discussed in more detail below.

8.2 General discussion

Validation of the experimental models

To study the particular effects of hormones uncomplicated by side effects that might occur in vivo, a mice skeletal muscle preparation in vitro, commonly used by many investigators (Jones *et al.*, 1983; MacDonald *et al.*, 1990) for its assumed suitability for metabolic studies, was adopted initially. However, it turned out to be of limited value to investigate the specific aims of this thesis. Despite the fact that D-[3-³H]glucose incorporation into glycogen agreed well with the findings of other investigators using the same model (Le Marchand-Brustel *et al.*, 1978; Bonen *et al.*, 1981; Bonen *et al.*, 1984; Watson-Wright *et al.*, 1984; MacDonald *et al.*, 1990) and that pH, O₂ saturation, and osmolality remained stable during the incubation period, we could provide histochemical evidence that part of the incubated muscle had lost its viability. Large areas devoid of glycogen were found (chapter 3, Figure 3.2), suggesting an ischemic situation in the core of the muscle preparation. For these reasons it was decided to use a different approach namely the rat in vivo model. Despite the fact that this model has its drawbacks as well, e.g. the complicated competition of androgens and glucocorticoids for the same intracellular receptor (Mayer and Rosen, 1977; Danhaive and Rousseau, 1988), the model most likely resembles the true physiological situation of the intact organism.

Two in vivo approaches were used (i) a non-diabetic rat model and (ii) a diabetic rat model. According to the model of Portha *et al.* (Portha *et al.*, 1989), rats were rendered diabetic by injecting pups in the neonatal period at the day of birth with a high dose of streptozotocin. Although we did not actually measure glucose uptake, the diabetic rats showed an abnormal insulin response to an oral dose of glucose at 15 and 30 minutes. Furthermore, after 8-10 weeks the rats showed hyperglycemia, hypoinsulinemia and increased plasma levels of fatty acids. Furthermore, muscle parameters that are indicative of insulin resistance, (i.e. glycogen content, glycogen synthase activity) were depressed as well.

Based upon the above mentioned findings we assume that the diabetic rat model represents a valid model to study the effects of testosterone and/or endurance training on insulin resistance.

Effects of testosterone on muscle metabolism in sedentary rats

The findings of the present investigations do not support the hypothesis that hyperandrogenicity and insulin resistance, as determined by some selected parameters of glycogenesis, are related. Instead, testosterone treatment in diabetic (chapter 6) and non-diabetic (chapter 4) sedentary rats resulted in either an increased or unchanged rather than the anticipated decrease in muscle glycogen content. Indeed, comparison of glycogen contents in diabetic and non-diabetic rats revealed that in the diabetic rats glycogen content was increased by testosterone treatment in both the glycolytic and oxidative muscles in concert with an increased activity of the key enzyme in glycogenesis, glycogen synthase, whereas in non-diabetic rats no changes in glycogen content could be observed. These findings not only mean that testosterone is not a causative factor in the development of insulin resistance in non-diabetic rats but also provides evidence for an increased glycogenesis in diabetic (already insulin resistant) rats. Support for the results of the present study comes, in part, from the study of Holmång *et al.* (Holmång *et al.*, 1990) who showed an increased 2-deoxy-glucose uptake (not significant in EDL and soleus, but significant in oxidative gastrocnemius) in their female control rats after testosterone treatment. Further support for the results of the present study and those deduced from the study of Holmång *et al.* comes from other investigators who reported an increased glycogen content and/or glucose uptake in EDL and/or soleus muscles of testosterone treated male rats (Bergamini, 1974; Max and Toop, 1983; Guezennec *et al.*, 1984). However, the results of Bergamini (Bergamini, 1974) have to be interpreted with caution since they used the *in vitro* model shown to be of limited value for the study of muscle metabolism (chapter 3).

Interestingly, a closer examination of the results of Holmång *et al.* (Holmång *et al.*, 1990) reveals that 2-deoxy-glucose uptake and [^{14}C]glucose incorporation into glycogen of the EDL and soleus muscles of hyperandrogenetic ovariectomized non-diabetic female rats were reduced. Thus, the results of the present study and those of Holmång *et al.* (Holmång *et al.*, 1990) together could suggest that the facilitating action of testosterone probably requires the presence of intact ovaries. An explanation for this notion is not easy to provide, but it might be due to the conversion of testosterone to estradiol-17 β or a hormone or another agent of ovarian origin.

Effects of training and testosterone treatment on muscle metabolism

Another interesting observation in the present study was that endurance training increases glycogen content in concert with an increase in glycogen synthase activity in EDL and soleus muscles of diabetic female rats (chapter 6). These findings indicate a potential beneficial effect of endurance training on impaired glycogenesis (marker of

insulin resistance) as previously found in the diabetic state (Horton, 1988; Berger *et al.*, 1992; Vanninen *et al.*, 1992). The combined effect of testosterone and endurance training on glycogenesis was not different from that reached by each manipulation separately. These results raise the question about the physiological meaning of increased testosterone levels as found in insulin resistance. An interesting and challenging interpretation of the present findings is that the negative effect of insulin resistance on skeletal muscle is mitigated by testosterone.

Effects of training and testosterone treatment on muscle metabolism after a submaximal exercise bout

Following a submaximal acute exercise bout testosterone induces a glycogen sparing effect in muscles of exercised endurance trained diabetic (m. extensor digitorum longus only) and non-diabetic (m. extensor digitorum longus and m. soleus) rats. These findings could be explained by the increased ratio of glycogen synthase to glycogen phosphorylase activities. Only Guezennec *et al.* (Guezennec *et al.*, 1984) investigated this issue and failed to find such a glycogen sparing effect in muscles of castrated and testosterone treated male rats. It should be noted, however, that in their study muscle samples were obtained after 7 hours of running a protocol probably leading to complete glycogen depletion in both the control as well as in the testosterone treated rats.

An explanation that could explain the glycogen sparing effect of testosterone is that testosterone regulates glycogen metabolism during exercise through substrate competition. This means that an increased availability of one substrate (e.g. fatty acids) restrains the consumption (oxidation) of the other (e.g. glucose). This mechanism was originally demonstrated in rat heart and diaphragm muscles by Randle *et al.* (Randle *et al.*, 1963). The observation that fatty acid-binding protein content was increased in oxidative muscle of diabetic and non-diabetic rats upon testosterone treatment in concert with increases in marker enzymes for fatty acid oxidation (chapter 6) are in support of this hypothesis. Moreover, together with the findings of an increased glycogenesis and reduced glycogenolysis, the results of the present study support the concept that the fatty acid-glucose cycle of Randle (Randle *et al.*, 1963) is also operative in oxidative muscle of trained and testosterone treated diabetic rats. An attractive alternative hypothesis worth mentioning is the fact that testosterone could increase the efficiency of the oxidation of glycogen (i.e. a more complete oxidation to CO₂ rather than the incomplete breakdown to lactate). In this way less glycogen is needed to produce for the required ATP production.

Mechanism(s) by which testosterone exerts its effects

In order to understand the mechanisms by which testosterone exerts its effect we will first present a generalized model of its action. Testosterone most likely enters its target cell by simple diffusion. Subsequently testosterone binds to its specific receptor which is found in both the cytoplasmic and nuclear compartment of the cell. Once bound, conformational changes in the testosterone-receptor complex convert the complex from an inactive to an

Figure 8.1. Schematic and simplified overview of the cellular mechanisms by which steroids act on cells. After crossing the cell membrane steroids (S) bind to a specific receptor (R) in the cytoplasmic or nuclear region. Binding of the steroid results in conformational changes the receptor to an active conformation (SR). In the nucleus binding of the hormone-receptor complex at specific binding sites to the DNA usually result in gene activation, i.e. transcription to produce messenger RNA (mRNA). The mRNA is translated on cytoplasmic ribosomes to produce the new protein, which leads to physiological changes in cell function.

Adapted and modified from Williams Textbook of endocrinology (1992).

active structure. The active structure then binds with high affinity to miscellaneous nuclear binding sites where it usually results in gene activation. A schematic representation of this process is depicted in Figure 8.1.

Alternatively, testosterone can be aromatized to estradiol-17 β not only in the ovaries but also in skeletal muscle. Thus our findings of testosterone-induced changes in muscle metabolic properties (chapter 7) may be mediated by testosterone or estradiol-17 β . Indeed, we found that different muscle metabolic parameters are under control of either testosterone and/or estradiol-17 β (chapter 7). Inhibition of the aromatase enzyme complex by a highly specific non-steroidal aromatase inhibitor resulted in unchanged (EDL) or slightly increased (soleus) glycogen contents (chapter 7) indicating that estradiol-17 β is not involved. On the other hand, other muscle metabolic parameters, i.e. fatty acid-binding protein (FABP), were mediated by estradiol-17 β since they remained at the control value after inhibition with the aromatase inhibitor (chapter 7). The physiological meaning of the aromatase enzyme complex in skeletal muscle remains to be elucidated.

A last interesting finding that is noteworthy is that testosterone treatment at a patho-physiological dose (ca 5 nmol/l; chapter 6) resulted in a more pronounced effect in both sedentary and trained muscle fibres of diabetic rats than the higher pharmacological dose (ca. 25 nmol/l; chapter 4) in the non-diabetic animals. These findings could implicate that (i) muscles of diabetic rats are more sensitive to testosterone than muscles of non-diabetic rats or (ii) at higher doses of testosterone receptor down-regulation occurs, which is associated with a decline in testosterone action.

In conclusion, the results of the present study indicate that hyperandrogenecity does not lead to insulin resistance in intact non-diabetic rats nor does it aggravates already existing insulin resistance in the diabetic rats. Rather than the expected decrease in: (1) muscle glycogen content and glycogen synthase activity; (2) activity of marker enzymes for lipid oxidation and (3) a shift towards more glycolytic fibres, the combined effects of hyperandrogenecity and endurance training seem to be a stimulus for the incorporation into glycogen in concert with a concomitant decrease in glycolytic flux, and an increase in fatty-acid binding protein content and activity of marker enzymes for lipid oxidation in both diabetic and non-diabetic female rats. Furthermore, a relatively more pronounced increase in oxidative muscle fibre content was found after the combination of hyperandrogenecity and training as compared with training alone. We also found that testosterone had different effects in different muscles. These effects may be mediated by the androgen itself or by its conversion product estradiol 17- β .

8.3 Future directions

Although the results of the present thesis provide insight into the effects of testosterone and/or training on skeletal muscle energy metabolic pathways our knowledge of the mechanism(s) by which testosterone and/or training induce these changes is still incomplete.

Testosterone mediated effects may occur either through (i) a direct effect of

testosterone at the pre-transcriptional, transcriptional or post-transcriptional level, (ii) an indirect effect through its conversion to estradiol-17 β , (iii) modulation of membrane hormonal receptors (de Beer *et al.*, 1982; de Beer *et al.*, 1983; Duval *et al.*, 1983) or (iv) interference with the secretion of or binding competition for the same receptor of other hormones at the central level (hypothalamic-pituitary axis).

For instance, more research has to be conducted on the intriguing findings of the comparable effects of testosterone or endurance training on the intracellular metabolic pathways in skeletal muscles of diabetic and non-diabetic rats. For example, more knowledge has to be gathered at the level at which (pre- or posttranscriptional) the training-induced changes in glycogenetic activity, glucose uptake or fatty acid-binding protein increase are controlled. It would also be worthwhile to investigate the effects of testosterone and/or endurance training on the mitochondria's own synthetic machinery.

Although from the results of chapter 7 it can be concluded that testosterone can be aromatized to estradiol-17 β in skeletal muscle, more information about the physiological implication of this finding is necessary. Is estradiol-17 β involved in the regulation of testosterone-induced effects? What is the contribution of skeletal muscle to circulating estradiol-17 β levels? Finally, since it has been shown that androgens and glucocorticoids can compete for the same intracellular receptor (Mayer and Rosen, 1977; Danhaive and Rousseau, 1988), the questions may be posed whether or not estradiol-17 β can compete with testosterone for the same receptor, whether testosterone mediates its effect via increased growth hormone secretion, which hormone plays an important role in muscle metabolism (Koppeschaar and Keizer, personal communication) or whether testosterone changes the modulation of the number, and probably also the intrinsic activity, of membrane receptors. The potential effect of testosterone on for instance the insulin receptor (Duval *et al.*, 1983) may have major implications for the insulin-regulatable glucose uptake. Not only the synthesis of GLUT4 could thus be changed through the classic concept of steroid action, but also the translocation of these proteins in response to an intrinsic signal from the insulin receptor can be modified by steroid hormones. In a pilot study, recently performed in cooperation with the laboratory of A. Bonen (University of Waterloo, Canada), muscle from non-insulin-dependent diabetic rats appear to have an unchanged amount of glucose transporter isoform type 4 (GLUT4). These findings suggest that in line with the recent findings of Kahn and coworkers (Kahn, 1992) transporter intrinsic activity (not number) is altered in non-insulin-dependent diabetes mellitus. Interestingly, we observed a small, non-significant increase in the total number of GLUT4 after the combined effect of training and testosterone treatment which needs further investigation.

Molecular biological and electron-microscopic techniques are elegant and powerful tools available and in use at our department to help to unravel these above mentioned premises. For instance, the translocation of GLUT4 from internal stores to the sarcolemma can be investigated by immunohistochemical (electron-microscopic) techniques. Furthermore, the expression of GLUT4 protein (GLUT4 mRNA) under physiological and/or pathological conditions can be investigated with molecular biological tools. Finally, the use of labelled substrates (2 deoxyglucose, [^{14}C]glucose, and/or 3-O-methylglucose, [^{14}C]palmitate) in combination with analysis of the appropriate enzymes of energy metabolic pathways

(present study) could be of importance to unravel some of the hormone-induced effects on skeletal muscle energy metabolism. Especially the combined use of these techniques could help to shed more light on the effects of testosterone treatment on substrate competition (Randle cycle).

8.4 References

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S ummary

Summary

Skeletal muscle represents 35-40% of the total body mass and it therefore plays an important role in whole body energy metabolism. Despite the large number of studies dealing with muscle carbohydrate and lipid metabolism, aspects regarding the control of both metabolic pathways remain poorly understood. Especially the role of sex-steroids on muscle carbohydrate and lipid metabolism has not received a great deal of scientific attention. Therefore, the aim of the present thesis was to study the effect of testosterone on skeletal muscle energy metabolic parameters in diabetic and non-diabetic endurance trained female rats. The goals of the study were twofold:

- to investigate the effects of testosterone administration on selected parameters of muscle carbohydrate and lipid metabolism in both sedentary and trained, diabetic and non-diabetic rats.
- to get more insight into the mechanisms by which testosterone acts on muscle carbohydrate and lipid metabolism.

After a brief introduction (**chapter 1**), a general overview of muscle energy metabolism and its endocrine control is described in **chapter 2**.

In **chapter 3** two models were evaluated for their suitability for metabolic studies. Firstly, an isolated mouse skeletal muscle model *in vitro* was studied, in which dissected muscles are incubated in a buffer at 37 °C. This model makes it possible to study particular effects of hormones, uncomplicated by side effects that might occur *in vivo*. Despite the fact, however, that pH, O₂ saturation, and osmolality of the buffer remained stable, histochemical studies of the muscles showed large cores devoid of glycogen, suggesting that the incubated muscles had exhausted their endogenous energy stores during incubation. Further evaluation of the core showed that cells in this region lost their normal shape and positioning with respect to each other. From this study we concluded that caution should be exercised when interpreting the results of studies with intact isolated muscle, incubated at 37 °C. For these reasons we switched to the rat model "*in vivo*". Although we are aware of the drawbacks of this model, i.e. the complicated interaction of hormones with each other on distinct cellular processes, this model represents the true physiological situation of the animal. In order to achieve controlled and fairly constant plasma testosterone levels new drug dosing techniques (i.e. osmotic minipumps, depot drugs preparations and silastic tubes) are available. One major omission in current literature is that only steroid concentrations at the final experimental day are given without taken into consideration possible day-to-day fluctuations in testosterone delivery. In chapter 3 we provided evidence that the use of silastic tubes filled with crystalline testosterone offer a practical and economic alternative for medium-term (14 days) steady state delivery of exogenous testosterone as compared with osmotic minipumps and injections of depot preparations.

In **chapter 4** the effects of testosterone and endurance training, either alone or in combination, on glycogen content and the activities of glycogen synthase, glycogen

phosphorylase, and fructose-6-phosphate kinase were studied in extensor digitorum longus (EDL) and soleus muscles of intact adult female rats. We found that in the soleus, but not in the EDL, glycogen content was increased by training alone which could be explained, at least partly, by a decreased activity of active glycogen phosphorylase and an increased activity of active glycogen synthase.

In both muscles glycogen content of testosterone treated sedentary rats was not changed. Interestingly, in the EDL, but not in the soleus, the glycogen content increased significantly by an increased activity of active glycogen synthase in trained animals chronically treated with testosterone. Finally, we found that in the EDL and soleus of acute exercised testosterone treated animals a significant sparing of glycogen was observed compared with the non-testosterone treated animals. This glycogen-sparing effect could be explained, in part, by an increased ratio of active glycogen synthase to active glycogen phosphorylase in the testosterone treated groups.

Chapter 5 describes the effects of training and/or testosterone treatment on fatty acid-binding protein (FABP) content, a cytoplasmic protein most likely involved in the intracellular translocation of fatty acids, and cytochrome c oxidase activity in heart, extensor digitorum longus (EDL) and soleus muscles of intact adult female rats. For sake of comparison, samples of heart muscle were included in this study. In the EDL no changes were found upon testosterone treatment alone or in combination with endurance training whereas in the heart FABP content was increased after training, testosterone treatment or both manipulations, and in soleus FABP increased only after testosterone treatment. Among the three muscles studied the FABP content was found to be related to the cytochrome c oxidase activity in a non-linear way. The results of this study indicate that the FABP contents and mitochondrial activities of heart and skeletal muscle are differently affected by training and/or testosterone treatment.

In **chapter 6** the results of a study performed in streptozotocin-induced diabetic female rats are described. It was found that upon testosterone administration up to a pathophysiological concentration (ca. 5 nmol/l), glycogen content was increased in EDL and soleus of diabetic sedentary and diabetic trained rats in concert with an increased glycogen synthase activity. In EDL, but not in the soleus, of the testosterone treated diabetic trained subgroup we found less depletion of glycogen upon acute exercise than in the untreated group. This effect of testosterone could be explained, in part, by an increased glycogen synthase activity together with a relatively smaller increase in glycogen phosphorylase activity.

We concluded that mild hyperandrogenism (plasma testosterone 4-5 nmol/l), acts on energy metabolic parameters of diabetic animals by different mechanisms in glycolytic and oxidative muscle fibres, and that hyperandrogenism in combination with endurance training can help to compensate for diabetes-induced metabolic defects of muscle energy metabolism.

Since it has been shown that testosterone treatment differently affects effects on energy metabolic parameters of glycolytic and oxidative muscle fibres we decided to investigate in **chapter 7** whether the effects of testosterone either alone or in combination with endurance training are caused by: (1) testosterone itself or by its conversion to estradiol-17 β ; and/or (2) a shift in muscle fibre composition.

The results showed that the combination of endurance training and testosterone treatment increased the relative number of oxidative muscle fibres (type I) in both EDL and soleus muscle. Furthermore, we showed that the differences between glycolytic and oxidative muscles can be explained, in part, by a different aromatase activity in the muscles examined. After inhibition of the aromatase enzyme complex FABP content, cytochrome c oxidase activity and glycogen content of EDL and soleus muscles are affected by either testosterone or its conversion product estradiol-17 β .

From the results of the various studies described in this thesis we conclude that:

- testosterone treatment in control (non-diabetic) does not lead to insulin resistance, as the skeletal muscle glycogen content and glycogen synthase activity are not decreased.
- testosterone administered to insulin resistant diabetic female rats improves glycogenesis in skeletal muscles rather than deteriorating it.
- testosterone induces a glycogen sparing effect in exercised endurance trained diabetic and non-diabetic rats which probably is due to both increased glycogen synthesis and increased fatty acid metabolism.
- testosterone elicits different effects on energy metabolic properties of glycolytic and oxidative muscle fibres.
- the effects of testosterone are mediated, at least partly, by estradiol-17 β produced by aromatization of testosterone in heart and skeletal muscle.

S amenvatting

Samenvatting

Ondanks het gegeven dat ons lichaam voor 35-40% uit skeletspieren bestaat, wordt vaak voorbij gegaan aan de belangrijke rol die de skeletspieren vervullen bij de koolhydraat- en vethomeostase. Met name de controle van zowel het koolhydraat- als het vet-metabolisme in de skeletspieren door hormonen en met name de controle door geslachtshormonen is nog nauwelijks onderzocht.

Het onderzoek dat in dit proefschrift beschreven is, had betrekking op de effecten van het geslachtshormoon testosteron op het spiermetabolisme van vrouwelijke ratten. Dit is van belang daar bij vrouwen, die ten gevolge van bepaalde afwijkingen aan de eierstokken, te veel testosteron produceren meer afwijkingen in met name het koolhydraat-metabolisme worden gevonden. Dit suggereert een mogelijke relatie tussen de plasma testosteronspiegel en het ontstaan van diabetes mellitus. Om deze reden hebben we gekeken naar het effect van testosteron op de opslag van glucose in de vorm van glycogeen (glycogenese) in rust. Verder hebben we gekeken of regelmatige lichamelijke inspanning (training), al dan niet na testosteron behandeling, de glycogeenopslag deed veranderen en of dit mogelijk te maken had met een veranderd vetmetabolisme. Daar de opslag van glucose en het verbruik van vetzuren bij patiënten met diabetes mellitus eveneens verstoord is hebben we onderzocht of een verhoogd testosteron gehalte in het bloed bij diabeet gemaakte ratten de opslag van glucose in glycogeen deed veranderen. Tenslotte hebben we onderzocht of regelmatige inspanning bij deze diabeet ratten een mogelijk gunstig effect heeft op de opslag en/of afbraak van glycogeen en/of vetzuren in de spier.

De belangrijkste doelstellingen van dit proefschrift waren:

- te onderzoeken of testosteron een effect had op de glucose- en vetstofwisseling van de spier bij vrouwelijke diabeet en niet-diabeet ratten.
- het ontrafelen van het mechanisme welke ten grondslag ligt aan de effecten van testosteron op de spiercel.

Na een korte inleiding waarin de doelstellingen van dit proefschrift worden beschreven (**hoofdstuk 1**) wordt in **hoofdstuk 2** een algemeen overzicht gegeven over het energiemetabolisme en de rol van het hormonale systeem daarop.

Doordat er vele hormonen tegelijk in het bloed aanwezig zijn en bekend is dat zij elkaars werking kunnen beïnvloeden hebben we in **hoofdstuk 3** een bestaand model geëvalueerd waarmee de invloed van hormonen op de skeletspier kan worden onderzocht zonder dat er verstoringen optreden van andere hormonen. Dit model, het “*in vitro*” model, is een veel gebruikt model. Hierbij wordt een spier in een waterige oplossing van 37°C gehangen, waarin zuurstof en voedingsstoffen voor de spier aanwezig waren. Verder bevatte deze oplossing alleen die hormonen die we wilden onderzoeken. Nadere bestudering wees echter uit dat dit model ongeschikt was voor dit type van onderzoek. Na verloop van tijd (30 minuten) bleek dat er in het midden van de spier een gebied ontstond waarin de

spiercellen hun normale vorm bleken te hebben verloren, vermoedelijk door een gebrek aan zuurstof. Dit verschijnsel zou uiteraard de resultaten van het experiment negatief kunnen beïnvloeden. We zijn toen overgeschakeld naar het "hele rat" (*in vivo*) model. Een van de problemen, die we met dit model moesten trachten op te lossen, was de toediening van testosteron aan de rat. In ons onderzoek was het noodzakelijk om gedurende 14 dagen een vrijwel constante testosteron spiegel in het bloed te krijgen en te behouden. Hoewel er op dit moment vele toedieningsmethoden zijn is de goede werking van deze vaak dure methoden niet onomstotelijk bewezen. Daarom hebben we verschillende toedieningsmethoden met elkaar vergeleken. Het bleek dat siliconen slangetjes (binnendiameter 1.47 mm) een beter en tevens goedkoper alternatief zijn voor de toediening van testosteron aan ratten dan andere methoden zoals b.v. het osmotische minipompje.

Door gebruik te maken van de methode om testosteron toe te dienen met siliconen slangetjes, hebben we in **hoofdstuk 4** onderzocht of training, al dan niet in met testosteron behandelde ratten, invloed had op de opslag van glucose in glycogeen. Uit de resultaten bleek dat in een tweetal verschillende onderbeenspieren, t.w. een spier die voornamelijk geschikt is voor krachtige bewegingen, de lange tenen strekker (*extensor digitorum longus*, EDL) en in een spier die voornamelijk gebruikt wordt bij langdurige en daardoor minder krachtige bewegingen, de soleus: (1) training de opslag van glucose in glycogeen in de soleus maar niet in de EDL verhoogde; (2) bij getrainde ratten testosteron het gehalte van glycogeen in de EDL maar niet in de soleus verhoogde en (3) in de met testosteron behandelde getrainde dieren minder glycogeen werd afgebroken tijdens inspanning.

In **hoofdstuk 5** hebben we onderzocht of de in hoofdstuk 4 genoemde effecten van training, met of zonder testosteron, invloed had op het gehalte van een klein eiwit dat zich in de spieren bevindt. Dit vetzuurbindend eiwit (*fatty acid-binding protein*, FABP) speelt mogelijk een belangrijke rol bij het transport van vetzuren in de spiercel. Daar er weinig bekend is over het gehalte FABP in de skeletspieren hebben we ter controle eveneens het FABP gehalte van hartspiermonsters bepaald. Verder hebben we onderzocht of deze interventies invloed hadden op de activiteit van een oxidatief enzym uit de mitochondriën (*cytochrome c oxidase*). Uit de resultaten bleek dat noch training noch testosteron noch een combinatie van beide het gehalte van het FABP in de EDL deed verhogen. In het hart en in de soleus echter bleek de hoeveelheid FABP bij zowel ongetrainde als getrainde ratten onder invloed van testosteron te stijgen. Tenslotte bleek dat, gemeten aan de hand van de activiteit van het enzyme *cytochrome c oxidase*, er een relatie bestaat tussen het FABP gehalte van de spier en de oxidatieve capaciteit van die spier.

Hoofdstuk 6 beschrijft de effecten van testosteron, al dan niet in combinatie met training, op het energiemetabolisme in de EDL en soleus bij farmacologisch (*streptozotocine*) geïnduceerde diabetes mellitus bij vrouwelijke ratten. We vonden dat: (1) bij diabete ratten het glycogeen gehalte van de spieren was verlaagd; (2) training een verhoging van het glycogeen gehalte in beide spieren bewerkstelligde; (3) testosteron behandeling eveneens het glycogeen gehalte van beide spieren verhoogde; (3) in diabete ratten het FABP sterk verhoogd was en dat noch training noch testosteron noch een combinatie van beide enige invloed had op het FABP gehalte; en (4) na acute inspanning, dit in tegenstelling met de studie in niet diabete ratten (**hoofdstuk 4**), alleen in de EDL een glycogeen sparend effect van testosteron optrad.

effect van testosteron optrad.

Uit de resultaten van deze studie mogen we concluderen dat verhoging van de plasma testosteron spiegel tot 4 à 5 nmol per liter, dat wil zeggen concentraties die zich in het (patho)fysiologisch gebied bevinden een invloed heeft op zowel het koolhydraat- als de vetstofwisseling bij vrouwelijke diabeten ratten en dat deze effecten verschillend zijn in de verschillende spieren. Tenslotte concluderen we dat training een gunstig effect heeft op de defecten die optreden in de spierstofwisseling van diabeten vrouwelijke ratten.

De in de hoofdstukken 4 t/m 6 beschreven effecten van testosteron op de spierstofwisseling hoeven niet per sé aan de werking van testosteron zelf worden toegeschreven. Testosteron kan namelijk in bijvoorbeeld de eierstokken omgezet (gearomatiseerd) worden tot het vrouwelijke geslachtshormoon oestradiol. Daarom hebben we in **hoofdstuk 7** onderzocht of de effecten van testosteron alleen of in combinatie met training werden veroorzaakt door testosteron zelf of door de omzetting in oestradiol. De resultaten laten zien dat het verhinderen van de omzetting van testosteron naar oestradiol door de omzetting op farmacologische wijze te blokkeren met een “aromatase remmer”, de effecten van testosteron deels teniet doet. Een laatste interessante bevinding was het feit dat na behandeling met testosteron de spier die voornamelijk geschikt is voor krachtige bewegingen, de EDL, eigenschappen gaat vertonen die lijken op eigenschappen die meer voorkomen in de spier die voornamelijk gebruik wordt voor langdurige activiteit, de soleus.

We kunnen de belangrijkste conclusies uit dit proefschrift als volgt samenvatten:

- testosteron heeft een significante invloed op de energiestofwisseling van de spier.
- training heeft een gunstig effect op de defecten in de spierstofwisseling van diabeten ratten.
- de effecten van testosteron zijn verschillend in de verschillende spieren.
- de effecten van testosteron kunnen veroorzaakt worden door de omzetting ervan in oestradiol.

Dit alles wijst erop, dat hyperandrogenisme (teveel testosteron in het bloed) bij vrouwen waarschijnlijk niet primair verantwoordelijk is voor het ontstaan van diabetes mellitus.

C

urriculum vitae

Curriculum vitae

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