DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS
104

REGULATION OF ACTIVE AND PASSIVE MOLECULAR TRANSPORT IN THE TESTIS

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Dissertation is accepted for the commencement of the degree of Doctor of Medical Sciences on January 12, 2005 by the Council of the Faculty of Medicine, University of Tartu, Estonia

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Commencement: March 4, 2005

Publication of this dissertation is granted by the University of Tartu

ISSN 1024–395X
ISBN 9949–11–016–5 (trükis)
ISBN 9949–11–017–3 (PDF)

Tartu Ülikooli Kirjastus
www.tyk.ut.ee
Tellimus nr. 39

To Kristiina and Artur

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I **Kokk K**, Veräjänkorva E, Laato M, Wu XK, Tapfer H, Pöllänen P. Expression of the insulin receptor substrates-1-3 (IRS-1-3), GLUT-1-4, SIRP1α, PI3-kinase and PKB kinase genes at the protein level in the human testis. Accepted for publication in Anatomical Science International 01.09.2004.

II **Kokk K**, Veräjänkorva E, Wu XK, Tapfer H, Põldoja E, Pöllänen P. Immunohistochemical detection of glucose transporters class I subfamily in the mouse, rat and human testis. Medicina 2004; 40(2):156–60.

III **Kokk K**, Veräjänkorva E, Pöllänen P, Tapfer H. Insuliini retseptori substraatide 1–3 (IRS 1–3) ja glükoosi transporterite (GLUT1-4) esinemine valgulisel tasandil inimese munandis. Eesti Arst 2002; 7: 394–398.

IV Pöllänen P, Cooper TG, **Kokk K**, Saari T, Setchell BP. Microvascular permeability to the $F(ab')_2$ fragment of IgG in the male rat reproductive tract at puberty. J. Reprod. Immunol. 1997; 32: 221–240.

V **Kokk K**, Veräjänkorva E, Wu XK, Tapfer H, Põldoja, E., Pöllänen P. Expression of the insulin signaling transmitters and glucose transporters at the protein level in the rat testis. Submitted to Biology of Reproduction 26.11.2004.

ABBREVIATIONS

ANOVA	analysis of variance
BFGF	basic fibroblast growth factor
BSA	bovine serum albumin
cAMP	adenosine 3′,5′-cyclic monophosphate
Cdc	cyclin-dependent kinase
cGMP	guanosine 3′,5′-cyclic monophosphate
DAB	3,3′-diaminobenzidin
DABCO	1.4-diazabicyclooctane
EBA	endothelial barrier antigen
EC	endothelial cell
EGF	epidermal growth factor
ES	early spermatocytes
EDTA	ethylenediamine tetraacetic acid
FSH	follicle-stimulating hormone
FITC	fluorescein isothiocyanate-conjugated
$[^{125}I]F(ab')_2$-IgG	^{125}I-iodinated $F(ab'_2)$ fragment of Immunoglobulin G
GLUT	glucose transporter
GnRH	gonadotropin-releasing hormone
hCG	human chorionic gonadotropin
HMIT	H(+)/myo-inositol transporter
IF	interferon
IGF-I	insulin-like growth factor I
IL	interleukin
IR	insulin receptor
IRS	insulin receptor substrate
|K| value	the slope of the line of $\ln (1-V_t/V_{eq})$ against time
KD	kiloDalton
LDH	lactate dehydrogenase
LH	luteinizing hormone
LIF	leukemia inhibitory factor
M	macrophage-like interstitial cell
MAP kinase	mitogen-activated protein kinase
mIFN-gamma	murine interferon-gamma
mIL-1alpha	murine interleukin-1alpha
mTNF-alpha	murine tumor necrosis factor-alpha
PBS	phosphate buffered saline
PC	peritubular cell
PE	microvascular permeability
PI 3-kinase	phosphatidylinositol kinase
PKB	protein kinase B

RA	retinoic acid
SC	Sertoli cell
SDS	sodium dodecyl sulfate
SGLT	sodium-dependent glucose transporter
SIRP1α	signal regulatory protein 1α
TBS	Tris-buffered saline
TF	transferrin
Tris	Tris (hydromethyl)-aminomethane
VEGF	vascular endothelial cell growth factor
V_{eq}	maximum volume of distribution

1. INTRODUCTION

Male infertility and non-barrier contraception remain intractable problems. In addition, a rising incidence of testicular cancer is a cause for concern (Shabbir and Morgan, 2004; Safe, 2004). Approaches to each of these problems will require a basic understanding of the cellular and molecular biology of the testis. Answers to all of these problems may be hidden in the complex cellular and molecular interactions that take place in the testis and, in particular, in the seminiferous epithelium (Griswold, 1995).

Insulin plays a pivotal role in the development of the testis (Zhou and Bondy, 1993). The insulin receptor tyrosine kinase family is required for the appearance of male gonads and thus for male sexual differentiation (Nef *et al*., 2003). Insulin itself initiates a wide variety of growth and metabolic effects by binding to the insulin receptor and by activating a tyrosine kinase. Insulin signal transduction takes place through receptor-mediated tyrosine phosphorylation of Insulin Receptor Substrate (IRS) proteins (Sun *et al*., 1991; Sun *et al*., 1995).

Spermatogenesis – a complex process regulated e.g. by FSH and testosterone (Heller *et al*., 1950; Kalra and Prasad, 1967) is disturbed in diabetes (Sainio-Pöllänen *et al*., 1997), causing infertility. Factors involved in the development of infertility in males with insulin-dependent diabetes mellitus are, however, still poorly characterized (Sainio-Pöllänen *et al*., 1997). In the present study, insulin signal transduction in the testis is characterized to see, how glucose uptake into the testicular cells may be regulated.

IRS-s are also substrates for the insulin-like growth factor I (IGF-1) receptor and for tyrosine kinases associated with the receptors for growth hormone and certain interleukins and interferons (White, 1998) known to be present in macrophages (Kmicikiewicz and Bilinska,1997; Verajankorva *et al*., 2001) and Leydig cells (Lin *et al*., 1986), but no data has existed this far on the regulation of testicular macrophages or Leydig cells through the IRSs. As the testicular macrophages or Leydig cells may play a role in the creation of the immune privilege in the testis (von Euler, 2002), it is important to know how the testicular macrophages and Leydig cells are regulated by cytokines using IRSs in their signal transduction.

Glucose uptake and metabolism are essential for the proliferation and survival of cells. Glucose uptake into cells is usually carried out through glucose transporters, which are integral membrane proteins (Girard *et al*., 1992; Joost and Thorens, 2001; Wood and Trayhum, 2003), and thus concretely control the access of glucose through a barrier formed by the lipid double-layer plasma membrane. The number of distinct glucose transporter gene products, together with the presence of several different transporters in certain tissues and cells indicates that glucose delivery into cells is a process of considerable complexity (Wood and Trayhum, 2003). Germ cells do not use glucose in their

energy metabolism but lactate produced by Sertoli cells (Jutte *et al*., 1981). Lactate production in Sertoli cells has been shown to be predominantly under the control of the endocrine system including FSH (Jutte *et al*., 1983; Oonk *et al*., 1989; Esposito *et al*., 1991), insulin (Oonk *et al*., 1989; Esposito *et al*., 1991) and insulin-like growth factor I (IGF-I) (Oonk *et al.*, 1989; Borland *et al*., 1984). The importance of lactate for normal spermatogenesis was recently highlighted in a report showing that spermatogenesis in adult cryptorchid rat testis is improved by intratesticular infusion of lactate (Courtens and Plöen, 1999).

The presence of circulating molecules like e.g. glucose and immunoglobulin G, in the testis or inside the testicular cells, is not self-clear. Their access to the various compartments of the testis or into the cells is tightly controlled by active and passive mechanisms. The most important factors of the control of movements of fluids and substances into, out of and inside the testis, and into the cells include: the function of the testicular endothelial cells, the flow of the interstitial extracellular fluid, the tubular barrier-forming inter-Sertoli cell junctional complexes (Russell, 1977; Russell, 1980; de Kretser *et al*., 1981; Russell and Peterson, 1985), and the transporter molecules in the plasma membranes of the testicular cells. A fuller understanding of the ways in which substances move around in the testis, particularly how they cross the endothelial cell layer or penetrate into the tubules and get access to the intracellular environment, will be necessary for a better knowledge about testicular function (Setchell, 1986). In the past years, rapid advances in the tools available in cell and molecular biology have led to the excavation of a large number of growth factors, enzymes, transport proteins, and receptors involved in the regulation of these factors in the testis.

In the present thesis, the regulation of active and passive molecular transport in the testis is studied for explanation the control of substance transport through the testicular endothelial barrier and the control of substance transport through the plasma membrane barrier of testicular cells.

2. REVIEW OF THE LITERATURE

2.1. Structure and function of the testis

2.1.1. Testicular cells

The testis is divided into avascular seminiferous tubules and the vascular interstitial tissue.

The germinal epithelium of seminiferous tubules contains two distinct categories of cells, the actively differentiating and proliferating germ cells, and the Sertoli cells which act as supporting elements and nourish the germ cells. Sertoli cells are the somatic cells of the testis that are essential for the testis formation and spermatogenesis. Human Sertoli cells produce and/or store e.g. transferrin (TF) and IGF-1 (Forti *et al.,* 1989). Where two Sertoli cells border on each other, the contiguous surfaces show complex occluding junctional specializations (Russell, 1977; Russell, 1980). These sites of membrane apposition and fusion constitute the morphological basis of the blood-testis barrier in the seminiferous tubules (Fawcett *et al*., 1970; Dym *et al*., 1970). Sertoli cells secrete luminal fluid, including androgen-binding protein (French and Ritzen., 1973). They are stimulated by testosterone and follicle-stimulating hormone (FSH) to secrete chemical messengers that stimulate sperm production and maturation (Kalra and Prasad, 1967). Sertoli cells facilitate the progression of germ cells to spermatozoa via direct contact and by controlling the microenvironment within the seminiferous tubules (Roosen-Runge, 1969). The regulation of spermatogenesis by FSH and testosterone occurs by the action of these hormones on the Sertoli cells (Setchell, 2004). While the action of testosterone is necessary for spermatogenesis, the action of FSH minimally serves to promote spermatogenic output by increasing the number of Sertoli cells (Tapanainen *et al*., 1997; Griswold, 1998).

The wall of seminiferous tubules is surrounded by the basal lamina and peritubular (myoid) cells (Pöllänen *et al*., 1985). The myoid layer also contains mononuclear leucocytes (Mori *et al*., 1980) and mesenchymal peritubular cells (Mather and Phillips, 1984).

In the seminiferous epithelium there are several cell types of spermatogenesis. The undifferentiated germ cells, which are termed spermatogonia are found only in the basal compartment, between the tight junctions of the Sertoli cells and the basement membrane of the seminiferous tubule (Griswold, 1995). After several mitotic cycles, the spermatogonia give rise to primary spermatocytes. Each of the latter crosses a tight junction, enlarges, and divides into two secondary spermatocytes, which divide into spermatids, which differentiate into spermatozoa (Vander *et al*., 1990).

The interstitial tissue of the human testis contains collagenous fibers, blood and lymph vessels, nerves and several cell types, namely Leydig cells, macrophages, fibroblasts, mast cells and lymphocytes. The interstitial tissue of the rodent testis is a large lymph sinusoid in which there are lymph endothelial cells lining the sinusoids and the blood vessels surrounded by pericytes, Leydig cells and macrophages are floating in the lymph (Kuopio and Pelliniemi, 1989).

2.1.2. Function of the testis

The testis is a double gland: it contains both an exocrine and an endocrine part. The exocrine function consists of the production of spermatozoa. The endocrine function includes the production of androgenic hormones responsible for the maintance of spermatogenesis and the appearance of secondary sex characteristics and the libido. The principal endocrine product of the testis is testosterone, produced by Leydig cells, which constitute a peculiar type of an endocrine gland in that they do not develop from an epithelial surface, as do most glands, but from the mesenchymal stroma of the testis. Testosterone is essential for spermatogenesis (acts via Sertoli cells), induces differentiation of male accessory reproductive organs and maintains their function, induces male secondary sex characteristics and stimulates protein anabolism and bone growth. Testosterone also decreases gonadotropin-releasing hormone (GnRH) secretion via action on the hypothalamus and therefore decreases luteinizing hormone (LH) and FSH secretion (Vander *et al*., 1990).

Various alterations in germ cell proliferation/differentiation, survival and energy metabolism are potentially involved in hypospermatogenesis leading to male infertility. Energy metabolism in the testis exhibits some specificity in that lactate is the central energy metabolite used by germ cells. This metabolite is produced by somatic Sertoli cells, transported and used by germ cells in the context of an active cooperation under the control of the endocrine system and local cytokines (Boussouar and Benahmed, 2004).

2.2. Insulin signaling and the role of the IRS-s family in insulin signaling

Insulin Receptor Substrate (IRS) proteins are key mediators in insulin signaling from the insulin receptor (Myers and White, 1996). Insulin signaling takes place through receptor-mediated tyrosine phosphorylation of IRS proteins (White, 1997). Activation of these SH_2 domain proteins initiates signaling cascades (White, 1998), leading to the activation of multiple downstream effectors which ultimately transmit the insulin signal to a branching series of intracellular pathways that regulate cell differentiation, growth, survival and metabolism (Fig.1), (Sesti *et al*., 2001).

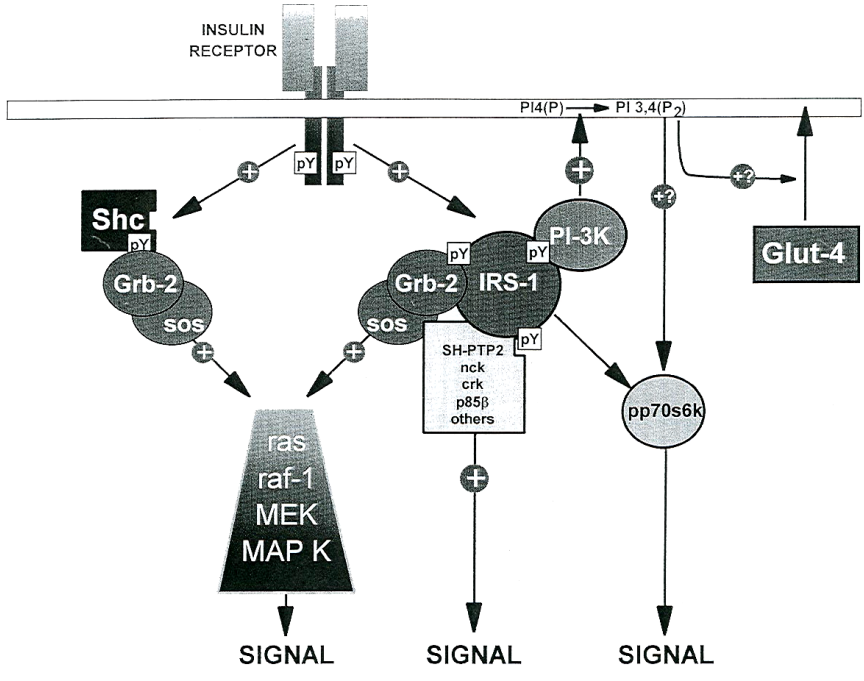

Figure1. Schematic diagram of the insulin receptor signaling mechanism (White, 1997).

Four members (IRS-1, IRS-2, IRS-3, IRS-4) of this family have been identified, which differ in their subcellular distribution, binding to the insulin receptor and interaction with SH2 domain proteins (Sun et al., 1991; Sun et al., 1995; Lavan *et al*., 1997 a; Lavan *et al*., 1997 b). IRS-1 is found in most tissues, including the insulin-responsive ones that contribute to glucose homeostasis such as muscle, adipose tissue and liver (Sun *et al*., 1995). In addition, differential IRS tissue- and developmental-specific expression patterns may contribute to specificity in their signal potential (Giovannone *et al.*, 2000). All these related molecules play a very different role in vivo – both IRS-1 and IRS-2 are important for insulin action and glucose homeostasis in vivo, whereas IRS-3 and IRS-4 appear to play a redundant role in the IRS signaling system (Tsuruzoe *et al*., 2001). IRS-1 is a cytosolic protein with a predicted molecular weight of 131,000 (Sun *et al*., 1991). IRS-1 contains over 30 potential serine/threonine phosphorylation sites with homologies to caseine kinase II, protein kinase C, mitogen-activated protein (MAP) kinases, cyclin-dependent kinase cdc2 and adenosine 3′, 5′-cyclic monophosphate (cAMP)- and guanosine 3′, 5′-cyclic monophosphate (cGMP)-dependent protein kinase consensus phosphorylation sites (Myers and White, 1993). IRS-1 and IRS-2 molecules have been considered plausible candidate genes involved in the pathogenesis of

Type 2 diabetes (Sesti *et al*., 2001). Several polymorphisms in the IRS genes have been identified, but only the Gly –> Arg72 substitution of IRS-1 seems to have a pathogenic role in the development of Type 2 diabetes (Sesti, 2000). IRS-1 and IRS-2 are not functionally interchangeable in tissues responsible for glucose production (liver), glucose uptake (skeletal muscle and adipose tissue), and insulin production (pancreatic ß cells). In fact, IRS-1 appears to have its major role in skeletal muscle whereas IRS-2 appears to regulate hepatic insulin action as well as pancreatic ß cell development and survival (Sesti *et al*., 2001).

IRS-3 and IRS-4 may act as negative regulators of the IGF-1 signaling pathway by suppressing the function of other IRS proteins at several steps (Tsuruzoe *et al*., 2001).

2.3. Regulation of glucose metabolism in somatic cells

A family of glucose transporters (GLUT) mediates the transport of glucose across the plasma membrane using facilitated diffusion. Two families of glucose transporters have been identified: the facilitated-diffusion glucose transporter family (GLUT family), and the Na(+)-dependent glucose transporter (SGLT) family (Scheepers *et al*., 2004). Proper localization of glucose transporters and gap junctions is a prerequisite for successful transepithelial transport of sugars (Takata, 1996). Active transport accumulates glucose in specific cells, whereas facilitative transport equilibrates blood glucose and intracellular glucose inside all mammalian cells (Girard *et al*., 1992). Glucose, fructose and dehydroascorbic acid enter mammalian cells via facilitated diffusion (Takata, 1996), a process regulated by different glucose transporter isoforms at the plasma membrane (Girard *et al*., 1992). Thirteen members of the family of facilitative sugar transporters (GLUT1-12 and the myo-inositol transporter HMIT) are now recognized (Wood and Trayhum, 2003). These various transporters exhibit different substrate specificities, kinetic properties and tissue expression profiles (Wood and Trayhum, 2003). On the basis of sequence similarities and characteristic elements, the extended GLUT family can be divided into three subfamilies, namely class I (GLUT1-4), class II (GLUT5, 7, 9, 11), and class III (GLUT6, 8, 10, 12 and HMIT) (Joost and Thorens, 2001).

GLUT3 has a high affinity for glucose (Joost and Thorens, 2001). Malignant cells have been shown to utilize more glucose than normal cells in vitro and in vivo and GLUT3 mRNA levels have been found to be elevated in human cancers, indicating that GLUT3 protein may play a role in glucose uptake by cancer cells. In human lung carcinomas, GLUT1 expression is seen in most cases, and a few cases of lung carcinoma show a positive staining for GLUT3 and GLUT4 (Ito *et al*., 1999). In normal tissues GLUT3 has been detected in placenta (Younes *et al*., 1997). GLUT3 is also present in the human adult and fetal myocardium (Grover-McKay *et al*., 1999) and in the rat lens

(Merriman-Smith *et al*., 1999). GLUT1, 3 and 4 were reported in abundance in several brain regions and GLUT3 seems to uphold its suggested role in synaptic energy provision and neurotransmitters synthesis (Choeiri *et al*., 2002). The transport of glucose across the mammalian blood-brain barrier is mediated by the GLUT1, which is concentrated in the endothelial cells of the cerebral microvessels (Simpson *et al.,* 2001). To accommodate the high glucose flux, platelets express extremely high concentrations of the most active glucose transporter isoform, GLUT3 (Sorbara *et al*., 1997).

After penetrating the plasma membrane by mediated transport, glucose is metabolized mainly by glycolysis in red blood cells (Cainelli *et al*., 1974). The end product of glycolysis is lactic acid, which is released from the cells back into the extracellular fluid, the blood plasma. The diabetic disorders appear to be associated with quantitative alterations of erythrocyte acidic phosphatase activity and other enzymes that depend on the glycolytic rate reductase (Marques *et al*., 2000). Erythrocyte acid phosphatase may have a role in the modulation of glycolytic rates through the control of insulin receptor phosphorylation (Marques *et al*., 2000).

The brain, like red blood cells, takes up glucose by mediated transport in an insulin-independent manner. Muscle and heart cells, as well as adipose tissue readily utilize glucose, and transport of glucose into them is dependent on the presence of insulin in the blood (Delvin, 1992). Muscle and heart cells are capable of synthesizing significant quantities of glycogen. In contrast to the other tissues, the liver is unique in that it also has the capacity to convert three-carbon precursors such as lactate, pyruvate, and alanine, into glucose by the process of gluconeogenesis. The glucose produced can then be used to meet the need for glucose of the other cells of the body (Delvin,1992).

2.4. Regulation of glucose metabolism in germ cells

Glucose metabolism in germ cells is in accordance with their specific position in the seminiferous epithelium. Mitosis of spermatogonia to yield primary spermatocytes takes place entirely in the basal compartment, outside the blood-testis barrier forming inter-Sertoli cell junctional complexes and thus accessible to those circulating substances, which can gain access to the extracellular fluid of the testicular interstitial tissue. The primary spermatocytes then move through the tight junctions of the Sertoli cells, which open in front of them to give entry into the luminal compartment (Russell, 1980). In this luminal compartment, the meiotic divisions of spermatogenesis occur, and the spermatids are remodelled into spermatozoa while contained in recesses formed by invaginations of Sertoli cell plasma membranes. When sperm formation is completed, the Sertoli cell cytoplasm around the sperm retracts, and the sperm is released into the lumen to be bathed by the luminal fluid. Sertoli cells serve

as the route by which nutrients reach developing germ cells (Vander *et al*., 1990).

Studies on the gulcose metabolism of germ cells have shown that Sertoli cells actively metabolize glucose but the majority of it is converted to lactate and is not oxidized via the citric acid cycle (Robinson and Fritz, 1981; Grootegoed *et al*., 1986). Post-meiotic germ cells, which are nutritied by Sertoli cells, are unable to use glucose for their energy metabolism and they prefer lactate as their energy source (Jutte *et al.,* 1981; Mita and Hall, 1982). Glucose transport into the cell and the lactate dehydrogenase (LDH) isoenzyme system (Plagemann *et al*., 1960; Pesce *et al.,* 1961) participate in the regulation of lactate production. Glucose transport through the plasma membrane and LDH A mRNA levels are regulated in a distinct manner by FSH (Riera *et al*., 2001), interleukin 1α (IL1α) (Nehar *et al*., 1998), tumor necrosis factor-α (TNF-α) (Nehar *et al*., 1997) and epidermal growth factor (EGF) (Boussouar and Benahmed, 1999) as well as basic fibroblast growth factor (bFGF) – the factors that modify Sertoli cell lactate production.

A novel member of GLUT family, GLUT8, has recently been detected in the testis as well as in blastocysts, brain, muscle and adipocytes (Joost and Thorens, 2001). GLUT8 is expressed predominantly in the testis, in smaller amounts in heart and kidney, and in negligible amounts in liver and spleen (Chen *et al*., 2003). Furthermore, GLUT8 mRNA was found to be highly expressed in crude interstitial cells, Leydig cells and testicular and epididymal germ cells. GLUT8 mRNA in Leydig cells was positively regulated by hCG and IGF-I and down-regulated by cytokines, murine interleukin-1alpha (mIL-1alpha), murine tumor necrosis factor-alpha (mTNF-alpha) and murine interferon-gamma (mIFN-gamma). These results indicate that human chorionic gonadotropin (hCG), growth factors and cytokines may directly or indirectly affect Leydig cell steroidogenesis by modulating the GLUT8 expression (Chen *et al*., 2003).

Based on homology with GLUT1-5 a cDNA for a novel glucose transporter GLUTX1 has also been isolated. The corresponding mRNA was detected in testis as well as in several other organs (Ibberson *et al*., 2000).

GLUT3 and GLUT5 hexose transporters were the main hexose transporters in dog spermatozoa (Rigau *et al*., 2002); however, other possible SGLT-family-related hexose transporters were also localized. These data indicate that in concentrations from 1 mmol l(–1) to 10 mmol l(–1), fructose has a stronger effect than glucose on the hexose metabolism of dog spermatozoa. These differences appear to be related to variations in the sensitivity of hexokinase activity. Moreover, the differential hexose metabolism induced by the two sugars had distinct effects on the function of dog spermatozoa, as revealed by the diverse patterns of tyrosine phosphorylation (Rigau *et al*., 2002).

GLUT14, a novel member of the glucose transporter family has been identified and cloned. GLUT14 (SLC2A14) maps to chromosome 12p13.3 (17.1M), about 10 Mb upstream of GLUT3, with which it shares remarkable

sequence similarities (Wu and Freeze, 2002). The mRNA level of GLUT14 in the testis is about four times higher than that of GLUT3 (Wu and Freeze, 2002). The multiple duplications of GLUT genes suggest that the GLUT family probably emerged by gene duplications and mutations during evolution in different lineages (Wu and Freeze, 2002).

2.5. Regulation of endothelial cell function and microvascular permeability in the testis

According to the literature many substances are transported to the testis by receptor-mediated processes (Bustamante *et al.,* 1982; Setchell *et al.,* 1984; Banks and Kastin, 1992; Banks *et al*., 1993; Bukovsky *et al*., 1993; Ghinea *et al*., 1994) or fluid-phase transcytosis (Weihe *et al*., 1979).

Testicular endothelial cells express specific receptors for several peptide and (glyco)protein hormones that may transport hormones across the cell to be delivered to the interstitial fluid and tissue target cells. These include the following: FSH, LH.

FSH and LH transcytosis across the testicular endothelial barrier is receptor-mediated and involves luminal uptake via coated pits/vesicles (Ghinea *et al.*, 2001), sorting at the level of early luminal endosomes, and transcellular transport through transcytotic tubulo-vesicular organelles (Misrahi *et al*., 1996). Similar receptor-mediated pathways are likely to be involved in the physiological functioning of a number of other proteins and peptide hormones that must translocate specifically from blood to the target cells (Vu Hai *et al*., 2004).

The endothelial barrier antigen (EBA) has been described as a 'barrier protein' and is used as a marker for the competent blood-brain barrier (Sternberger and Sternberger, 1987). EBA was expressed by the endothelial cells in most microvessels of the testis, and in a few vessels of the epididymis, seminal vesicle, prostate gland, vas deferens and bladder-neck region (Gharbiel *et al*., 2002). Furthermore, EBA was strongly and consistently detected in epithelial cells of the rete testis and dorsolateral prostate gland, and in a few epithelial cells of the ventral prostate gland, the seminal vesicle and the coagulating gland (Gharbiel *et al*, 2002). However, Sertoli cells, which are the main site of the blood-testis barrier, were negative for EBA (Gharbiel *et al*., 2002) in spite of their demonstrated ability to exclude small electron-dense particles from the luminal compartment of the seminiferous epithelium (Dym and Fawcett, 1970; Aoki *et al*., 1975). In conclusion, EBA may have a wider role in rat tissues than has been previously appreciated (Gharbiel *et al*., 2002).

The vascular endothelial growth factor (VEGF)/VEGF receptor system plays a central regulatory role in physiological and pathological angiogenesis (Breier, 2000). Testicular vascular endothelial cell growth factor secretion is

increased by hormonal stimulation of Leydig cells and that VEGF, through effects mediated via VEGF-R2, regulates endothelial cell proliferation in the rat testis. VEGF does not appear to regulate testicular blood flow and it is not involved in inducing the hCG-induced inflammation-like response in the testicular microvasculature. The permeability-increasing effect of VEGF is low in the testis under basal conditions but is apparently up-regulated by hCG treatment (Rudolfsson *et al*., 2004).

2.6. Regulation of testicular cells by IRS-dependent cytokines

It has been found that several cytokines, e.g. IL-2 (Johnston *et al*., 1996), IL-4 (Johnston *et al*.,1996), IL-7 (Johnston *et al*., 1996), IL-9 (Sun *et al*., 1995), IL-13 (Sun *et al*., 1995), IL-15 (Johnston *et al*., 1996), interferon-γ (Platanias *et al*., 1996), IGF-I (He *et al*., 1996) or the leukemia inhibitory factor (Argetsinger *et al*., 1996) use IRS-1 or IRS-2 in their signal transduction.

Several cytokines are potent modulators of steroid release from the testis. Testicular cytokines and growth factors (such as IL-1, IL-6, TNF, IFN-gamma, LIF and SCF) were shown to affect both the germ cell proliferation and the Leydig and Sertoli cells functions and secretion. Cytokines and growth factors are produced by immune cells and in the interstitial and seminiferous tubular compartments by various testicular cells, including Sertoli, Leydig, peritubular cells, spermatogonia, differentiated spermatogonia and even spermatozoa. Corresponding cytokine and growth factor receptors were demonstrated on some of the testicular cells. These cytokines also control the secretion of the gonadotropins and testosterone in the testis. Under pathological conditions the levels of pro-inflammatory cytokines are increased and negatively affected spermatogenesis. Thus, the expression levels and the mechanisms involved in the regulation of testicular paracrine/autocrine factors should be considered in future therapeutic strategies for male infertility (Huleihel and Lunenfeld, 2004).

IL-6, IL-2, and TNF-alpha acting in a stage specific manner have acute and/or chronic influences on the release of TF from Sertoli cells (Boockfor and Schwarz, 1991). These findings, when viewed in light of reports of the presence of these factors in the testis, suggest strongly that cytokines or cytokine-like substances, by modulating the release of Sertoli cell substances, may play an important role in testis function.

On the basis of literature, IL-2, is a potent inhibitor of steroidogenesis. IL-2 may play a paracrine role in modulating Leydig cell function (Guo *et al*., 1990). Maximal testosterone production in response to hCG was reduced 40% in the presence of IL-2, IL-2 significantly inhibited the conversion of 20-OH-cholesterol, 22-OH-cholesterol, pregnenolone, progesterone, 17 alpha-hydroxypregnenolone, and 17 alpha-hydroxyprogesterone to testosterone but did not

alter the conversion of dehydroepiandrosterone and androstenedione to testosterone (Guo *et al*., 1990).

Leukemia inhibitory factor (LIF) is a highly pleiotropic cytokine (Hilton, 1992; Gearing, 1993) known to control the proliferation and survival of stem cells (Smith *et al*., 1988) including primordial germ cells and gonocytes (Matsui *et al*., 1991). LIF was found to be produced by peritubular cells and, to a much lesser extent, by the other testicular somatic cell types. No LIF was detected in meiotic and postmeiotic germ cell-conditioned medium, and only low levels of LIF were detected in spermatogonia-conditioned medium. Large amounts of bioactive LIF were measured in testicular lymph (Piquet-Pellorce *et al*., 2000). Given the proliferative effect of LIF on immature germ cells, Piquet-Pellorce *et al.* (2000) suggest that peritubular LIF plays an important role in the regulation of testicular function.

One function of FSH and leukemia inhibitory factor in Sertoli cells is to increase the metabolism of retinol to the biologically active metabolite retinoic acid (RA) and to retinyl esters (Guo *et al.,* 2001).

IGF-1 seems to lower the levels of germ cell apoptosis, which may be important for protecting the testes from torsion/detorsion injury (Ozkurkcugil *et al*., 2004).

3. AIMS OF THE STUDY

The aims of the present study are:

I to investigate the control of substance transport through the testicular endothelial barrier using the permeability of the testicular microvessels to [^{125}I]F(ab')$_2$-IgG in the rat as the model

II to investigate the control of substance transport through the plasma membrane barrier of testicular cells using the insulin receptor substrate and glucose transporter systems in the human, mouse and rat as the model

To reach the aims of the present thesis, the following hypotheses are tested:

1) insulin can regulate transport of glucose across the plasma membrane through the insulin receptor substrate pathway in the testicular cells
2) insulin receptor substrate-mediated signal transduction to regulate glucose transport across the plasma membrane does not occur in the germ cells using lactate in their energy metabolism
3) transport of glucose across the plasma membrane occurs through one or several of the glucose transporters 1–4 in the testicular cells
4) transport of the F(ab)$_2$ fragment of IgG to the testicular interstitial tissue and the luminal compartment of the seminiferous tubules occurs
5) transport of the F(ab)$_2$ fragment of IgG to the interstitial tissue and the luminal compartment of the seminiferous tubules changes with age

4. MATERIAL AND METHODS

4.1. Animals (Papers II, IV and V)

Wistar rats (n=12; paper II and V) and BALB/c male mice (n=12; paper II) were used as donors of testis tissue. The testes were removed and frozen immediately in liquid nitrogen and stored in –70°C until use.
In paper IV, Sprague-Dawley rats were used as experimental animals. They were at 20, 30, 45 and 60 days of age (n=60).
All the animals had free access to food and water and they were maintained in a normal dark/light cycle. The rats and mice were supplied by the Experimental Animal Centre of the University of Turku.

4.2. Human testis tissue (I, III)

In papers I and III, human testis tissue was obtained from three patients undergoing orchiectomy either due to prostatic cancer (n=2) or hydrocele (n=1) in 1998–1999. The patients were being treated at the Turku University Central Hospital. Permission for tissue donations and to use organs for research purposes were granted by the Hospitals and University´s joint Ethical board. The testes were frozen immediately in liquid nitrogen and kept at –70°C until use.

4.3. Reagents

Mouse monoclonal anti-IRS-1 IgM antibody, mouse monoclonal anti-IRS-3 IgM antibody (Transduction Laboratories, Lexington, KY), rabbit polyclonal anti-IRS-2 IgG antibody (Upstate Biotehnology, Lake Placid, NY), goat polyclonal anti-GLUT1, -GLUT2, -GLUT3 and -GLUT4 antibodies were used as the primary antibodies in papers I, II, III, V. Goat polyclonal anti-PKB and -SIRP1α IgG (Santa Cruz Biotechnology) and rabbit polyclonal anti-PI 3-kinase IgG antibodies (Upstate Biotechnology, Lake Placid NY) were used as primary antibodies in papers I, III, V.

4.4. Immunohistochemistry (I, II, III, V)

The avidin-biotin-peroxidase method was used for the expression of IRS-1 and IRS-2 and the indirect immunofluorescence method for the expression of IRS-3, GLUT1-4, SIRP1α, PI 3-kinase and PKB genes.

4.4.1. Preparation of frozen tissue sections

Frozen sections of 6 μm in thickness were cut in a cryostat. They were dried on slides in the air at room temperature and fixed in cold (–20°C) acetone for 5 min. The sections were stored at –20°C.

4.4.2. Indirect immunofluorescence (I, II, III, V)

The sections were briefly soaked in phosphate-buffered saline (PBS; pH 7.4). The non-specific binding sites were blocked by incubating the sections for 15 minutes either in 5 % normal rabbit serum in PBS (for GLUT1-4, PKB, SIRP1α) or 5 % normal swine serum in PBS (for IRS-3 and PI 3-kinase). Thereafter, the sections were washed for 3x2 minutes in PBS and incubated for 60 minutes with the primary antibody at room temperature. All the primary antibodies were diluted 1:100 in 1% bovine serum albumin (BSA; Sigma) in PBS. After washing for 3x5 minutes in PBS, the sections were incubated for 30 min with the secondary antibody diluted 1:50 either in 5% normal human serum (paper I, III), 5% normal rat serum (paper V) or 5% normal mouse serum (paper IV) in PBS. Either FITC-conjugated rabbit-anti-goat Ig for GLUT1-4, PKB and SIRP1α or FITC-conjugated swine-anti-rabbit Ig for IRS-3 and PI 3-kinase were used as secondary antibodies. The sections were washed for 3x5 minutes in PBS and mounted in 1,4-diazabicyclooctane (DABCO, Sigma, St. Louis, MO). The sections were examined and photographed in ultraviolet-microscope.

4.4.3. Avidin-biotin-peroxidase method (I, II, III, V)

The sections were soaked in TBS (pH 7.4) for 3x10 minutes. Thereafter the sections were dehydrated in an increasing alcohol series (70%, 96%, absolute alcohol, methanol). Endogenous peroxidase blockade (0.3% H_2O_2 in methanol) was carried out for 30 minutes. The sections were then rehydrated in a decreasing alcohol series (methanol, absolute alcohol, 96%, 70%). After washing for 3x5 minutes in TBS, the non-specific binding sites were blocked by incubating the sections either in 2% normal horse serum (for IRS-1) or 2% normal goat serum (for IRS-2) for an hour. The sections were then incubated with the

primary antibody for 60 minutes at room temperature. The primary antibody was diluted 1:100 in 1% bovine serum albumine (BSA) in TBS. The sections were washed for 3x5 minutes in TBS and incubated with the secondary antibody: either with peroxidase-conjugated horse-anti-mouse IgG (for IRS-1; Research Diagnostics Inc) or goat-anti-rabbit Ig (for IRS-2; Research Diagnostics Inc) for 30 minutes. The biotinylated secondary antibody was diluted 1:50 in TBS containing 1% BSA. After washing for 3x5 minutes in TBS the sections were incubated with the VECTASTAIN ABC Reagent® (reagent A 90 µl and 10 ml TBS+ reagent B 90 µl, Vector Laboratories, Inc., Burlingame, CA) for 30 minutes. The sections were washed for 3x5 minutes in TBS and followed by a demonstration of peroxidase activity, using 3,3`–diaminobenzidine (DAB; Sigma, cat No. D-5905) as a substrate. The sections were examined and photographed in a microscope.

4.5. Western blot analysis (I, III, V)

Human testicular tissue (paper I, III) or rat testicular tissue (paper V) were used for immunoblotting. Tissue homogenates were prepared in ice-cold suspension buffer (0.1M NaCl; 0.01M Tris, pH 7.6; 0.001M EDTA, pH 8.0) supplemented with 1 µg/ml phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO, USA, cat No. P-7626) and soybean trypsin inhibitor (Sigma, cat no. T-9003) to avoid proteolysis. The tissue (1 g) was placed in 3 ml of suspension buffer and homogenized. After incubation the lysate was centrifuged at 10000g for 30 min. The salts were removed from the supernatant in a Sephadex G-25® column (PD-10, Pharmacia Biotech, Uppsala, Sweden). The eluate was then freeze-dried. The protein was diluted to 1 µg/ml in 2x Laemmli solution (1% SDS, 10% glycerol, 0.01% bromophenol blue and 2% 2-mercaptoethanol

[Fluka, cat no. 63690] in 50 mM Tris buffer, pH 6.8). The samples were boiled for 5 minutes.

Denaturated 12% SDS-polyacrylamide mini-gels were prepared and 30–40 µl samples were loaded into the wells. Low-molecular-weight markers (Pharmacia, cat no. 17-0446-01) were run in parallel to the samples. Gels were run with a 150 mA current and after electrophoresis proteins were transferred to the nitro-cellulose filter (Amersham International plc., Buckinghamshire, U.K.) for 60 minutes using a 400 mA current. The nitro-cellulose filter was stained with Ponceau S and each separate line was cut off. The strips were blocked with saline containing 2% bovine serum albumin (BSA) and 0.2% sodium azide and then incubated overnight at 4°C with immunosera diluted to 1:50 with PBS containing 2% BSA and 0.2% Triton X-100 (Acros Organics, NY, USA, cat no. 21568-0010). After incubation the strips were washed three times with PBS and then incubated for 1 h in 1:500 dilution with either horseradish peroxidase-conjugated rabbit-anti-mouse Ig or goat-anti-rabbit Ig in 0.3 ml PBS containing

2% BSA and 0.2% Triton X-100 per strip. The strips were washed three times with PBS and then allowed to react with 0.6 mg/ml diaminobenzidine and 0.03% H_2O_2 in 0.05M Tris (pH 7.6) for 10 minutes. Reactions were stopped with PBS containing 0.2% sodium azide and the strips were dried before photographing.

4.6. Determination of volumes of distribution (IV)

The rats were weighed, anaesthetised with pentobarbitone sodium (Mebunat, Orion, Finland) and ^{125}I-iodinated F(ab')2 fragment of sheep IgG (specific activity 10 µCi/µg, Amersham, Braunschweig, Germany, 0.1 µCi in 0.1 ml 0.9 % NaCl/100 g body weight) was injected into the right jugular vein through a 25 G needle. At stated times (3, 75, 150, 300 and 1200 min) after injection, the testes and epididymides were removed, a blood sample was collected from the posterior vena cava and the sera separated. The epididymides were divided into caput, corpus and cauda regions and weighed. The testes were decapsulated and the capsule (with the testicular artery and veins) and the parenchyma were weighed separately. Radioactivity in the segments of epididymides, testis parenchyma, testicular capsules and sera were measured in a gamma-counter (Wallac, Turku, Finland).

The volumes of distribution were calculated from the formula: volume of distribution in µl/g = cpm per gram tissue/cpm per µl serum. Although the blood-testis and blood-epididymis barriers prevent the access of whole immunoglobulins to the lumen of rat seminiferous (Johnson and Setchell, 1968; Koskimies et al., 1971; Johnson, 1972; Pöllänen and Setchell, 1989) and epididymal (Wong et al., 1983; Yeung et al., 1991) tubules in normal conditions, the possibility of the access of ^{125}I-F(ab')2 to the luminal compartment of the testis and epididymis could not be excluded. Therefore, the distribution volumes were calculated per gram of the whole tissue.

To compare the methods of calculation of permeabilities described by Amtorp (1980) and by Setchell (1990), the volumes of distribution were also calculated using mean cpm per µl serum between time t = 0 and time t = b. The function f (x) for the disappearance of ^{125}I-bound protein from the serum was first calculated using StatworksR program. Then an integral function $\int f(x)\,dx$ for the function f (x) was determined and the mean serum cpm concentration between time a = 0 and time b = t was calculated by subtracting the value of the integral function $\int f(x)\,dx$ at time b from the the value of the integral function $\int f(x)\,dx$ at time a and by dividing the obtained difference by the difference in time between time points a and b.

The functions f(x) for the disappearance of the ^{125}I-F(ab')2 fragment of IgG from serum were f(x)20 = –472.48log (x) + 1966.8, f(x)30 = –670.24 log (x) + 2402.9, f(x)45 = –793.70 log (x) + 2934.7 and f(x)60 = –784.28 log (x) + 2915.0.

The integral functions of the functions $f(x)$ 20–60 were: $\int_a^b f(x)\,dx$ 20 , = –472.48 log10e . b (ln | b | – 1) + 1966.8 b, $\int_a^b f(x)\,dx$ 30 , = –670.24 log10e . b (ln | b | – 1) + 2402.9 b, $\int_a^b f(x)\,dx$ 45, = –793.70 log10e . b (ln | b | – 1) + 2934.7 b, $\int_a^b f(x)\,dx$ 60 , = –784.28 log10e . b (ln | b | – 1) + 2915.0 b, when a = 0.

4.7. Estimation of microvascular permeabilities

Microvascular permeabilities (PE) were calculated in a similar way to Pöllänen and Cooper (1995) but with the improved formula:

1) $PE = 0.5\ r\ |K|\ Veq\ (1–H)/(c\ V3min)$,

where r = the standardized radius of the exchange vessels in the model, K = the slope of the line of ln (1–Vt/Veq) against time (fitted by the least squares method), Veq = the maximum volume of distribution, H = the haematocrit, c = the proportion of the exchange vessels of the whole circulation and V3min = the volume of distribution at t = 3 min. The serum spaces in the organs were corrected to blood space using the haematocrit (blood volume = serum volume/1–H, H = 35.3 %, Cremer and Seville, 1983).

4.8. Data presentation and statistical analysis

In paper IV, differences in percentages of initial serum radioactivity between the $F(ab')_2$ fragment of IgG and whole IgG were compared using Student's t-test. Differences in the distribution volumes per gram whole tissue were analysed statistically in all the age groups together using analysis of variance (ANOVA) and Tukey's test. The equalities of the regression lines in the organs at the various ages and in the age groups in the various organs were tested using the F-test and the differences of regression lines from zero were tested using Student's t-test as described by Sachs (1984). The parallelism of pairs of regression lines was analysed using Student's t-test with Bonferroni correction (Sachs, 1984).

5. RESULTS

5.1. Expression of insulin receptor substrates in the human testis (I, III)

Positive immunoreaction to IRS-1 was found in the human testis in the basal part of Sertoli cells, peritubular myoid cells and macrophage-like interstitial cells (Table 1). IRS-1 positive cells were not observed in the luminal part of Sertoli cells or spermatocytes or spermatids. No spermatogenic cycle stage-specificity was evident in tissue sections. No positive cells were present in negative controls.

IRS-1 was not detected in the Leydig cells or testicular blood vessels using immunohistochemistry. Positive immunoreaction for IRS-2 or IRS-3 was not found in the human testis.

In Western blotting, altogether 5 IRS-1 antigens were recognized in the testis extracts by the sera. These were of the following molecular ratios (kD): 87.5, 84, 58, 49 and 46.

Altogether 3 IRS-2 antigens were recognized in western blotting in the testis extracts by the antisera. These were of the following molecular ratios (kD): 59, 53 and 50.5.

IRS-3 was not detected by immunoblotting in the human testis.

5.2. Expression of glucose transporters in the human testis (I, II, III)

Positive immunoreaction to GLUT3 was found in Sertoli cells, peritubular myoid cells, macrophage-like interstitial cells, testicular endothelial cells and early spermatocytes (Table 1). GLUT3 positive cells were not found in the luminal part of Sertoli cells, spermatids or Leydig cells. No positive cells were present in negative controls.

Positive immunoreaction to GLUT1, GLUT2 and GLUT4 was not found in the human testis using immunohistochemistry.

In Western blotting, one GLUT3 antigen of the molecular ratio 72 kD was recognized in the testis extract by the antisera.

GLUT1, GLUT2 and GLUT4 proved negative in immunoblotting.

5.3. Expression of PI 3-kinase, PKB and SIRP1α genes in the human testis (I)

PI 3-kinase, PKB and SIRP1α proteins were not found in the human testis using immunohistochemistry or Western blotting.

Table 1. Expression of the IRS-1-3, GLUT1-4, PI 3-kinase, PKB and SIRP1α in the different types of cells in three different human testes by immunohistochemistry.

	Sertoli cells basal-part	Peri-tubular myoid cells	Sperma-tids	Early spermato-cytes	Macro-phage-like interstitial cells	Testicular endothelial cells	Leydig cells
IRS-1	+*	+*	–	–	+*	–	–
IRS-2	–	–	–	–	–	–	–
IRS-3	–	–	–	–	–	–	–
GLUT1	–	–	–	–	–	–	–
GLUT2	–	–	–	–	–	–	–
GLUT3	+#	+#	–	+#	+#	+#	–
GLUT4	–	–	–	–	–	–	–
PI 3-kinase	–	–	–	–	–	–	–
PKB	–	–	–	–	–	–	–
SIRP1α	–	–	–	–	–	–	–

*$p < 0.05$ vs. IRS-2 and IRS-3, χ^2-test
#$p < 0.05$ vs. GLUT1, GLUT2 and GLUT4, χ^2-test

5.4. Expression of insulin receptor substrates in the rat testis (V)

Positive immunoreaction to IRS-1 and IRS-2 was found in the rat testis in Sertoli cells, peritubular myoid cells, testicular endothelial cells, early spermatocytes and macrophage-like interstitial cells. IRS-2 was also detected in the Leydig cells (Table 2).

No spermatogenic cycle stage-specifity was evident in tissue sections. IRS-1 or IRS-2 was not detected in the spermatids and IRS-1 was not detected in the Leydig cells. No positive cells were present in negative controls.

Positive immunoreaction to IRS-3 was not found in the rat testis.

IRS-1 and IRS-2 antigens could be demonstrated in the rat testis using immunoblotting. IRS-1 was recognized at 40 kD and IRS-2 at 41 kD (molecular ratio).

5.5. Expression of glucose transporters in the rat testis (V)

Positive immunoreaction to GLUT1 was found in the peritubular myoid cells, macrophage-like interstitial cells, testicular endothelial cells and spermatocytes (Table 2). GLUT1 was not found in Sertoli cells, spermatids or Leydig cells.

Positive immunoreaction to GLUT2 and GLUT3 was detected in Sertoli cells, peritubular myoid cells, spermatocytes, spermatids, testicular endothelial cells and macrophage- like interstitial cells (Table 2). GLUT2 and GLUT3 were not expressed in Leydig cells. No positive cells were present in negative controls.

Positive immunoreaction to GLUT4 was not found in the rat testis.

In Western blotting, GLUT1, GLUT2, GLUT3 and GLUT4 antigens were present in the rat testis. These were of the following molecular ratios (kD): GLUT1-32, 67; GLUT2-26, 55; GLUT3-33, 42; GLUT4-34.

5.6. Expression of PI 3-kinase, PKB and SIRP1α in the rat testis (V)

Positive immunoreaction to SIRP1α was found in Sertoli cells, germ cells, peritubular myoid cells, testicular endothelial cells and macrophage- like interstitial cells (Table 2). No positive cells were present in negative controls.
PI 3-kinase and PKB genes were not detected in the rat testis.

In Western blotting, SIRP1α and PKB antigens were positive and PI 3-kinase antigen proved negative. SIRP1α was present at molecular ratios of 33 kD and 60 kD and PKB at 58 kD.

Table 2. Expression of IRS-1-3, GLU1-4, PI 3-kinase, PKB and SIRP1α in the rat testis using immunohistochemistry.

	Sertoli cells	Peri-tubular myoid cells	Sperma-tids	Early spermato-cytes	Macro-phage-like interstitial cells	Testicular endothelial cells	Leydig cells
IRS-1	+*	+*	–	+*	+*	+*	–
IRS-2	+*	+*	–	+*	+*	+*	+*
IRS-3	–	–	–	–	–	–	–
GLUT1	–	$+^{+}$	–	$+^{+}$	$+^{+}$	$+^{+}$	–
GLUT2	$+^{+}$	$+^{+}$	$+^{+}$	$+^{+}$	$+^{+}$	$+^{+}$	–
GLUT3	$+^{+}$	$+^{+}$	$+^{+}$	$+^{+}$	$+^{+}$	$+^{+}$	–
GLUT4	–	–	–	–	–	–	–
PI 3-kinase	–	–	–	–	–	–	–
PKB	–	–	–	–	–	–	–
SIRP 1α	+	+	+	+	+	+	–

*$p < 0.05$ vs. IRS-3, χ^2-test
$^{+}p < 0.05$ vs. GLUT4, χ^2-test

5.7. Expression of glucose transporters in the mouse testis (II)

Positive immunoreaction for GLUT2 was detected in the mouse testis in Sertoli cells, peritubular myoid cells, spermatocytes, spermatids, testicular endothelial cells and macrophage-like interstitial cells (Table 3). GLUT2 was not expressed by Leydig cells in the mouse testis.

Very intensive positive immunoreaction for GLUT3 was present in the mouse testis. GLUT3 was found in the basal part of Sertoli cells, peritubular myoid cells, macrophage-like interstitial cells, testicular endothelial cells and early spermatocytes. GLUT3 was not found in the luminal part of Sertoli cells, spermatids or Leydig cells.

GLUT1 and GLUT4 were not detected in the mouse testis.

Table 3. Expression of the GLUT1-4 in the different types of cells in different mice testes by immunohistochemistry.

	Sertoli cells basal part	Peri-tubular myoid cells	Sperma-tids	Early sper-mato-cytes	Macro-phage-like interstitial cells	Testicular endothelial cells	Leydig cells
GLUT1	–	–	–	–	–	–	–
GLUT2	+*	+*	+*	+*	+*	+*	–
GLUT3	+*	+*	–	+*	+*	+*	–
GLUT4	–	–	–	–	–	–	–

*$p < 0.05$ vs. GLUT1 and GLUT4, χ^2-test

5.8. Pharmacokinetics of the [^{125}I]F(ab')$_2$-IgG and the ^{125}I-whole IgG

The body and the organ weights of the rats used in the paper V increased steadily and significantly with age.
In the 20-day-old rats the ^{125}I-F(ab')2-IgG disappeared from serum as fast as the 125I-whole IgG. In the 30-day-old rats, there was a significant difference in the percentages of initial serum radioactivity between the F(ab')2 fragment of IgG and the whole IgG at 150 ($p < 0.05$) and 1200 ($p < 0.05$) minutes, but not before 150 minutes or at 300 minutes. At the age of 45 and 60 days there was a significant difference at 150, 300 and 1200 minutes ($p < 0.05$). The proportion of cpm bound to protein was more than 70 % in 30-, 45- and 60-day-old rats at 20 h after injection, but in the 20-day-old rats it remained above 90 % at all time points.

5.9. Distribution volumes of the [^{125}I]F(ab')$_2$-IgG

The distribution volumes increased steadily with time. Maximal distribution volumes (V_{eq}) were reached at 1200 min in all the tissues in each age group, except in the 45-day-old testicular capsule, where the maximal volume of distribution was reached at 300 min.

5.9.1. Differences between age groups

The 3 min spaces (μl/g whole tissue), reflecting the plasma volume in the tissue, decreased in the testis and caput between 30 and 45 days of age ($p < 0.01$, not shown). No significant changes were observed in the other tissues.

The V_{eq} in the testis of 45-day-old rats was significantly lower ($p < 0.05$) than that in the other age groups. In the testicular capsule, there were no significant differences in V_{eq} between the age groups. In the caput, the V_{eq} in the 60-day-old rats was significantly smaller than those in the 20- ($p < 0.01$) and 30-day-old ($p < 0.01$) rats, demonstrating that the Veq decreased with age in the caput. Also in the corpus the Veq decreased with age, since the Veq in the 20-day-old rats was significantly larger than that in the 60-day-old ($p < 0.01$) rats. In the cauda, the Veq in the 60-day-old rats was significantly smaller than that in the 30- ($p < 0.01$) and 45-day-old ($p < 0.05$) rats (not shown).

5.9.2. Differences between tissues

The Veq in the testicular capsule was significantly higher ($p < 0.05$) than those in the testis and caput at 20 days of age (not shown). At 30 days of age, the Veq in the testis was significantly smaller than those in the caput and cauda, and the Veq in the testicular capsule was significantly higher than in all the other studied tissues ($p < 0.05$, not shown). At 45 days of age, the Veq in the testis was significantly smaller and that in the testicular capsule larger than those in all the other studied tissues ($p < 0.05$, not shown). The Veq in the corpus was significantly smaller than that in the cauda ($p < 0.05$, not shown).

5.9.3. Differences between the $F(ab')_2$ fragment and whole IgG

Comparison of V_{eq} for the $F(ab')_2$ fragment and whole IgG showed that the V_{eq} for the $F(ab')_2$ fragment was significantly larger than that for the whole IgG in the testis of 30-, 45- and 60-day-old rats ($p < 0.001$), in the caput of the 30- ($p < 0.005$) and 45-day-old ($p < 0.01$) rats, in the corpus of 45-day-old rats ($p < 0.01$) and in the cauda of 30- ($p < 0.005$) and 45-day-old ($p < 0.05$) rats, suggesting better penetration of the $F(ab')_2$ fragment than that of whole IgG to the lumen in these tissues at these ages.

5.10. The regression curves for penetration of ^{125}I-F(ab')2-IgG into the testis and the various segments of the epididymis

The |K| values, reflecting the speed at which the maximum volume of distribution was reached, were not equal ($p < 0.05$) in any of the age groups or any of the organs and all the |K| values, except those for the 20-day-old corpus and cauda, were significantly different from zero ($p < 0.05$).

5.10.1. Differences between age groups

The |K| value in the testis at 20 days of age was significantly lower than that at 30 ($p < 0.005$), 45 ($p < 0.005$) and 60 days ($p < 0.001$) of age, demonstrating an increase between 20 and 30 days of age. In the testicular capsule, there were no significant differences between the |K| values. In the caput, the speed at which the equilibrium was reached increased between 45 and 60 days of age, as the |K| value at 60 days of age was significantly ($p < 0.001$) higher than those at 20, 30 and 45 days of age. In the corpus, the equilibrium was reached the faster the older the animals, since the |K| value at 20 days of age was significantly smaller than those at 45-day-old ($p < 0.01$) and 60-day-old ($p < 0.001$) rats. In the cauda, the |K| value at 45 days of age was significantly ($p < 0.001$) smaller than that at 60 days of age, but a clear age-dependent trend was not observed.

5.10.2. Differences between tissues

The |K| values in the various organs at 20, 30, 45 and 60 days of age were next compared. At 20 days of age, the |K| in the testicular capsule was significantly higher than those in the testis ($p < 0.01$), caput ($p < 0.005$) and corpus ($p < 0.005$). At 30 days of age, the |K| in the testis was significantly higher than those in the caput ($p < 0.005$), corpus ($p < 0.01$) and cauda ($p < 0.005$) and that in the testicular capsule was significantly higher than those in the caput ($p < 0.005$) and cauda ($p < 0.005$). At 45 days of age, equilibrium was reached faster in the testis, testicular capsule and corpus than in the caput ($p < 0.005$, 0.05 and 0.001, respectively) and cauda ($p < 0.005$ for all). The speed at which equilibrium was reached was also significantly higher in the testis than in the corpus ($p < 0.05$). In the 60-days-old rats, the |K| value for the cauda was significantly smaller than those in the other tissues ($p < 0.005$ for all) and the |K| value for the testicular capsule was significantly smaller than those for the testis ($p < 0.005$) and corpus ($p < 0.05$). The |K| value for the testis was also significantly higher than that for the caput ($p < 0.01$).

5.10.3. Differences between the F(ab')2 fragment and whole IgG

Comparison of the |K| values for the F(ab')2 fragment and whole IgG showed that there were no statistically significant differences in the speed at which equilibrium was reached in the testis. In the testicular capsule, the F(ab')2 fragment reached equilibrium faster than whole IgG at 60 days of age ($p < 0.001$). In the caput and corpus, the |K| value for the F(ab')2 fragment was significantly greater than that for the whole IgG at 60 days of age ($p < 0.05$ and 0.01, respectively), but smaller at 45 days of age ($p < 0.001$ and 0.05, respectively). Also in the cauda, the F(ab')2 fragment equilibrated more slowly than whole IgG at 45 days of age ($p < 0.001$).

5.11. Microvascular permeabilities to ^{125}I-F(ab')2 fragment of IgG in the testis and epididymis

Assuming that r, c and H were constant, the estimated permeabilities (PE) for the F(ab')2 fragment increased steadily from 20 to 60 days of age in the testis and caput. In the corpus, the PE's increased more abruptly between 30 and 45 days of age. In the testicular capsule and cauda epididymidis, the PE's remained at the same level during this developmental period.

The PE's in the testis were higher than those in the various segments of epididymis at 30, 45 and 60 days of age. The PE's in the testicular capsule were higher than those in the various segments of epididymis at 30 and 45 days of age and higher than that in the cauda at 60 days of age. The PE's in the caput and corpus were higher than that in the cauda at 60 days of age.

Comparison of PE's for the F(ab')2 fragment and whole IgG showed that the F(ab')2 fragment of IgG penetrated through the microvascular endothelium faster ($\geq$ 2 times) than whole IgG in the 20, 45 and 60-day-old testis, 20- and 60-day-old testicular capsule, 20-, 30- and 60-day-old caput and 20-day-old corpus.

Comparison of the methods of Amtorp (1980) and Setchell (1990) showed that the difference in the PE values obtained using these two methods was greatest in the 20-days-old rats and smallest in the 60-days-old rats.

6. DISCUSSION

6.1 Expression of the IRS-1 and IRS-2 in the human testis

The present finding of immunologically detectable IRS-1 in Sertoli cells, peritubular myoid cells and macrophage-like interstitial cells demonstrates that IRS-1-mediated signal transduction is active in these cells. Thus, the use of insulin in the regulation of glucose uptake by peritubular myoid and interstitial cells is possible if the insulin receptor is expressed in these cell types. Thus, the hypothesis 1 (see introduction) would seem to be true in the human testis in what comes to the Sertoli cells, peritubular myoid cells and macrophage-like interstitial cells. As well as insulin action, the effect of various cytokines may also be possible, as several cytokines use IRS-1 in their signal transduction. However, a precondition for these interpretations is the expression of the insulin receptor, the corresponding cytokine receptors and the other components of the IRS-mediated signal transduction pathway in the cells in question.

The absence of IRS-1 and IRS-2 from the germ cells in the luminal compartment is in accordance with the use of lactate in the energy metabolism of these cells. This finding supports the hypothesis 2, but does not finally prove it, because other insulin receptor substrates not yet known may exist. The absence of IRS-1 and IRS-2 from the germ cells further supports the dependence of germ cells on Sertoli cells in their energy metabolism.

Furthermore, the lack of IRS-1 and -2 from the germ cells indicates that the cytokines, e.g. IL-2 (Johnston *et al*., 1995), IL-4 (Johnston *et al*., 1995), IL-7 (Johnston *et al*., 1995), IL-9 (Sun *et al*., 1995), IL-13 (Sun *et al*., 1995), IL-15 (Johnston *et al*., 1995), interferon-γ (Platanias *et al*., 1996) or the leukemia inhibitory factor (Argetsinger *et al*., 1996), using IRS-1 or IRS-2 in their signal transduction can not have a full effect on these cells, even if their receptors were expressed by them. As IRS-2 interacts also with the insulin-like growth factor I receptors (He *et al*., 1996), it is evident that the lack of IRS-2 from the germ cells will also modulate the effects of IGF I on these cells.

The high expression of IRS-1 by the human peritubular cells is well in accordance with the high energy expenditure of these contractile cells using glucose in their energy metabolism and thus dependent on the insulin action. However, it is not excluded that the high IRS-1 signal from these cells indicates cytokine effects on these cells in the intra-testicular paracrine regulatory network.

6.2 Expression of the IRS-1 and IRS-2 in the rat testis

The present study clearly shows that IRS-1 and IRS-2 genes are expressed at the protein level in the rat testis. Thus, the hypothesis 1 (see aims of the study) would seem to be true also in the rat testis in what comes to the Sertoli cells, peritubular myoid cells, early spermatocytes, macrophage-like interstitial cells, testicular endothelial cells and Leydig cells, with the same preconditions as in the human testis (see above).

IRS-3, whose overexpression causes a decrease in association of IRS-1 and IRS-2 with the PI 3-kinase (Tsuruzoe *et al*., 2001) and modulates the biological responses to insulin, was not detected in the rat testis, suggesting that the differences in signal transduction between the IRS-1- and -2-mediated pathways and the IRS-3-mediated pathway may be functionally significant in the testicular environment.

As in the human testis, the high expression of both IRS-1 and IRS-2 by the peritubular cells is well in accordance with the high energy expenditure of these contractile cells. Thus, insulin would seem to regulate glucose transport across the plasma membrane of several testicular cell types in the rat, if only the insulin receptor is expressed by them.

As well as insulin action the effect of various cytokines may also be possible, but again, the precondition for this is the expression of the corresponding cytokine receptors.

The absence of IRS-1 and IRS-2 from the rat germ cells in the luminal compartment of seminiferous tubules would support hypothesis 2 (see aims of the study), but the observation of both IRS-1 and -2 in the early spermatocytes clearly speaks against it. Thus, it seems that the early spermatocytes may behave differently from the later germ cells in this respect.

IRS-1 and IRS-2 exhibit differences in their capacity to interact with various downstream signaling elements containing SH_2 domain (White, 1997). They may regulate their unique signaling pathways, but the expression of both IRS-1 and IRS-2 in Sertoli cells, peritubular myoid cells, blood vessels and interstitial cells supports binding to the insulin receptor and PI 3-kinase and insulin/IGF 1 signaling in these cell types.

6.3 Expression of the GLUT1-4 in the human, rat and mouse testis

The present study demonstrated that GLUT 3 was the prominent glucose transporter type in the human Sertoli cells, peritubular myoid cells, macrophage-like interstitial cells, testicular endothelial cells and early spermatocytes. The other glucose transporter types were not present at all. Thus, hypothesis 3 (see aims of the study) would seem to be true. However, transport of glucose

across the plasma membrane may occur also through those glucose transporter types that were not studied in the present investigation.

That GLUT3 was the only glucose transporter present suggests that in cases of diabetes-associated disturbances of the structure of the seminiferous epithelium (Sainio-Pöllanen *et al*., 1997), GLUT3 could possibly be involved. In addition, it is very interesting that the early spermatocytes expressed GLUT3 at the protein level in all the studied species, as generally it is thought that these cell types do not use glucose in their metabolism but lactate produced by Sertoli cells. However, the early spermatocytes expressed also IRS-1 and -2 in the rat testis, suggesting that the components to control glucose transport across the plasma membrane are at least in part present in these cells.

GLUT3 has a high affinity for glucose (Joost and Thorens, 2001). 86% of testicular, 16% of ovarian, 25% of gastric and 27% of non-small cell lung carcinomas were positive for GLUT3 (Younes *et al*., 1997), suggesting that also those tumor cells with their origin in the testis seem to use GLUT3 in their glucose transport.

In surgically induced unilateral abdominal cryptorchidism the GLUT3 expression was reduced by 85–95% compared with contralateral scrotal testis in rats (Fraooqui *et al*., 1997). This suggests that the degenerative changes in abdominal testis (impaired and incomplete spermatogenesis and lack of spermatozoa in the lumen of seminiferous tubules, see Sainio-Pöllänen *et al*., 1991) could be associated with decreased GLUT3 mediated glucose transport in seminiferous tubules and spermatogonia.

It has been found that human, rat and bull spermatozoa express the hexose transporter isoforms GLUT1-5 (Angulo *et al*., 1998), but the other testicular cell types have not been investigated yet. GLUT1, GLUT2, GLUT3, GLUT5 and low levels of the GLUT4 isoform showed a typical subcellular localization in the head and the tail of ejaculated spermatozoa (Angulo *et al*., 1998, Burant *et al*., 1992). The present study demonstrated that GLUT3 was the prominent glucose transporter type in the mouse, rat and human Sertoli cells, peritubular myoid cells, macrophage-like interstitial cells, testicular endothelial cells and early spermatocytes, but not in the mature spermatozoa. This discrepancy needs to be studied further, but it is possible that the expression of glucose transporters in the spermatozoa starts during the post-testicular maturation of spermatozoa, e.g. when the spermatozoa gain their ability to move in the epididymis.

From the present results, it can be judged that it is possible that the early spermatocytes are ready to increase transport of glucose into their cytoplasm, although the GLUT3 proteins were present only in the cytoplasmic vesicles. The functional significance of this finding needs to be studied.

The presence of GLUT1 in the rat testis and GLUT2 in the mouse and rat testis implies that it may not be possible to use the rodent models to investigate the role of different GLUT isoforms on glucose metabolism in human testis.

The members of the extended GLUT family exhibit a surprisingly diverse substrate specificity, and the definition of sequence elements determining this

substrate specificity will require a full functional characterization of all members (Joost & Thorens, 2001).

It has been recently observed that in recent onset type-1 diabetic patients autoantibodies against GLUT2 are present (Pehuet-Figoni et al., 2000). This raises questions about how the consequences of diabetes mellitus may occur. In the human testis, however, GLUT3 was the only glucose transporter present, although in the rat and the mouse testis GLUT2 was present in many cell types. Thus, these autoantibodies against GLUT2 are probably not responsible for the changes in fertility of the diabetic males.

6.4 Physiological significance of the microvascular permeability to the $F(ab')_2$ fragment of IgG in the male rat reproductive tract

The present results suggest that the permeabilities of the testicular microvessels and the epithelial barrier to the $F(ab')_2$ fragment of IgG are higher than to whole IgG. This suggestion is based on the estimates of microvascular permeability to the $F(ab')_2$ fragment of IgG, as observed in the present study (paper IV), and those of whole IgG (Pöllänen *et al*., 1995). As the inter-Sertoli cell tight junctions exclude molecules of the size of the $F(ab')_2$ fragment from the luminal compartment (Setchell 1979), the $F(ab')_2$ fragment of IgG probably gains access to the luminal fluid through the rete testis better than whole IgG (Johnson and Setchell, 1968; Maddocks and Sharpe, 1989; Jones *et al.,* 1996). This would also suggest that there was no active Fc-mediated transport of IgG through the testicular endothelium, as it was suggested earlier (Ahlström et al., 1996) or if there was, it was not very significant in relation to passive diffusion of the $F(ab')_2$ fragment. The hypothesis 4 (see introduction) would thus seem to be true. As there were changes in the transport of the $F(ab')_2$ fragment of IgG to the testis with age, hypothesis 5 is true. However, the changes with age were not similar as with whole IgG, suggesting changes in the control of substance transport through the testicular microvascular endothelium at puberty.

That the $F(ab')_2$ fragment of IgG seems to gain access to the rete testis may make passive immunization against sperm using sperm-specific $F(ab')_2$ or Fab' fragments possible. Although the use of exogenous $F(ab')_2$ fragments might cause problems in regard to immunization against them, this is not likely if recombinant $F(ab')_2$ fragments of the same species are used (Sawai *et al*., 1995). Thus, specific impairment of sperm-zona interaction or sperm motility might be possible by passive immunization of the male with sperm-specific $F(ab')_2$ or Fab' fragments of IgG. In the experimental animal work blockage of the functions of the sperm plasma membrane proteins by specific $F(ab')_2$ fragments may be possible as well.

7. CONCLUSIONS

1. The present study clearly shows that IRS-1 and IRS-2 are expressed at the protein level in the human, rat and mouse testis. IRS-3, the overexpression of which causes a decrease in association of IRS-1 and IRS-2 with the PI 3-kinase, and which can modulate the biological responses to insulin, was not detected in the rat testis.
2. The present finding of immunologically detectable IRS-1 and IRS-2 in Sertoli cells demonstrates that IRS-1 or IRS-2-mediated signal transduction may be active in Sertoli cells.
3. As well as insulin action the effects of various cytokines on the testicular cells may also be possible through IRS-1 and -2, as several cytokines, i.e. IL-2, IL-4, IL-7, IL-9, IL-13, IL-15, interferon-γ, IGF-I and the leukemia inhibitory factor use IRS-1 or IRS-2 in their signal transduction. However, a precondition for this is the expression of the corresponding cytokine receptors.
4. The absence of IRS-1 and IRS-2 from the germ cells in the luminal compartment of seminiferous tubules is in accordance with the use of lactate in the energy metabolism of these cells. It further supports the dependence of germ cells on Sertoli cells in their energy metabolism.
5. The high expression of IRS-1 by the peritubular cells is well in accordance with the high energy expenditure of these contractile cells using glucose in their energy metabolism and thus dependent on the insulin action.
6. Glucose uptake in different testicular cells was mediated by GLUT1, GLUT2 and GLUT3. GLUT3 was the prominent type of glucose transporters in the human, rat and mouse testis.
7. The $F(ab')_2$ fragment of IgG is transported to the lumen of the male reproductive tract in both the testis and the epididymis.

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SUMMARY IN ESTONIAN

Sissejuhatus

Spermatogenees on keerukas protsess, mis on reguleeritud erinevate hormoonide, peamiselt FSH ja testosterooni poolt. Diabeedi korral on spermatogenees häiritud, kutsudes esile infertiilsust. Vähe on teada aga selle tekkepõhjuste kohta. Samuti on murettekitav munandi vähi esinemissageduse tõus. Uurimistöös on vaadeldud insuliini signaali ülekannet munandi erinevates rakuliikides, et selgitada, kuidas on glükoosi metabolism munandis reguleeritud.

Insuliini retseptori substraadid (IRS) on olulised mediaatorid insuliini, kasvufaktorite ja mõnede tsütokiinide (IL-2, IL-4, IL-7, IL-9, IL-13, IL-15, IGF-1 ja interferoon γ) bioloogilises tegevuses. Nende käes on signaaliülekande võtmepositsioon insuliini retseptorilt rakku. Praeguseks ajaks on teada 4 IRS-perekonna liiget: IRS1-4. Neist tähtsaim insuliini retseptori tegevuse mõjutaja on IRS-1. IRS-2 on peamine alternatiivne mediaator.

Glükoos ja fruktoos sisenevad imetaja rakkudesse kergendatud difusiooni teel ning seda protsessi reguleerivad rakumembraanil suhtelise koespetsiifilisusega glükoosi transporterid GLUT 1-4. Neist afiinseim glükoosi suhtes on GLUT3.

Uurimistöö eesmärgid

1) uurida ainete transpordi regulatsiooni läbi testise-endoteliaalbarjääri kasutades mudelina roti munandi kapillaaride läbitavust [^{125}I]F(ab')$_2$-IgG suhtes
2) uurida ainete transpordi regulatsiooni läbi munandi rakkude rakumembraani kasutades mudelina insuliini retseptori substraatide ja glükoosi transporterite süsteemi inimese, hiire ja roti munandil

Käesolevas uurimistöös on uuritud aktiivse ja passiivse molekulaarse transpordi regulatsiooni munandis, et leida kinnitust järgmistele hüpoteesidele:

1) insuliin reguleerib glükoosi transporti läbi rakumembraani munandi rakkudes kasutades mediaatoritena insuliini retseptori subatraate
2) insuliini retseptori substraatide poolt vahendatud signaali ülekanne glükoosi transpordiks läbi rakumembraani ei toimu neis sugurakkudes, mis kasutavad ainevahetuses laktaati
3) glükoosi transpordiks läbi rakumembraani kasutavad munandi rakud ühte või mitut alljärgnevatest glükoosi transporteritest — GLUT1, GLUT2, GLUT3 või GLUT4.
4) IgG fragment F(ab)$_2$ transport munandi sidekoesse ja vääniliste seemnetorukeste valendikku on võimalik

5) IgG fragment $F(ab)_2$ transport munandi sidekoesse ja vääniliste seemnetorukeste valendikku muutub seoses vanusega.

Uurimismaterjalid ja uurimismeetodid

Eksperimendis insuliini retseptori substraatide ja glükoosi transporterite süsteemi transpordi regulatsiooni uuringus läbi rakumembraani on kasutatud 3 patsiendi munandeid, mis on eemaldatud eesnäärme vähi operatsioonil, 12 Wistar roti ja 12 BALB/c hiire munandeid. Munandid on kiiresti külmutatud vedelas lämmastikus ja hoitud kasutamiseni temperatuuril –70°C. Valguliste ülekandjate määramiseks on kasutatud immunohistokeemilist ja geelelektroforeesmeetodit (*Western blotting*). 6 μm paksused külmutuslõigud munandist on kuivatatud toatemperatuuril, fikseeritud –20°C atsetoonis 5 min ja säilitatud temperatuuril –20°C. IRS-1 ja IRS-2 kindlakstegemiseks on kasutatud *avidin-biotin* immunoperoksüdaasmeetodit, ülejäänud mediaatorite määramiseks immunofluorestsentsmeetodit.

Eksperimendis munandi kapillaaride läbitavuse määramiseks [^{125}I] $F(ab')_2$-IgG suhtes on kasutatud 20, 30, 45 ja 60-päevaseid Sprague-Dawley rotte (n=60). Määrati radioaktiivse isotoobiga märgistatud $F(ab')_2$ ja kogu IgG läbitavuse kiirust munandi ning munandimanuse pea, keha ja sabaosa kapillaarides.

Uurimuse tulemused

Positiivne immunoreaktsioon IRS-1-le leiti inimese, roti ja hiire munandis Sertoli rakkudes, peritubulaarsetes lihasrakkudes, sidekoerakkudes ja rotil ning hiirel ka munandi veresoonte seinas. IRS-2 inimese munandis ei leitud, rotil ja hiirel aga ilmnes positiivne immunoreaktsioon IRS-2-le samades rakuliikides nagu IRS-1. IRS-3 ühegi isendi munandis ei leitud. Glükoosi transporteritest oli kõige intensiivsem immunoreaktsioon GLUT3-le, mida leiti inimese, roti ja hiire munandi kõikides rakkudes, v.a. Leydigi rakud. GLUT1-positiivseid rakke leiti roti munandis ja GLUT2- positiivseid rakke roti ja hiire munandis. GLUT4-positiivseid rakke munandis ei leitud. PI 3-kinaasi ja PKBd munandis ei leitud. SIRP1α leidus aga roti ja hiire munandi erinevates rakuliikides. *Western blotting* uuring kinnitas kõikide immunohistokeemiliselt leitud valguliste ülekandjate esinemist inimese, roti ja hiire munandis.

IgG fragment $F(ab')_2$ jõudis nii munandi kui munandimanuse torukeste süsteemi läbides endoteliaalbarjääri. Munandis oli $F(ab')_2$ fragmendi maksimaalne mikrovaskulaarne läbitavus oluliselt kõrgem kui kogu IgG-l, sõltuvalt katseloomade vanusest isegi rohkem kui 2 korda kõrgem.

Järeldused

1. IRS-1 ja IRS-2, mis tegutsevad mediaatoritena insuliini signaali ülekandes, omavad mõju munandi erinevatele rakuliikidele. IRS-3, mis kutsub esile languse IRS-1 ja IRS-2 seostumisel PI3-kinaasiga ja võib seega moduleerida bioloogilist vastust insuliinile, munandi rakkudes ei leidunud.
2. Immunoloogiliselt määratud IRS-1 ja IRS-2 leidumine Sertoli rakkudes kinnitab, et IRS-1 ja IRS-2 poolt vahendatud signaali ülekanne on võimalik Sertoli rakkudes.
3. IRS-1 ja IRS-2 mitteleidumine vääniliste seemnetorukeste valendikupoolsetes sugurakkudes on kooskõlas laktaadi tarbimisega nende rakkude energiaainevahetuses. See kinnitab ka varajaste spermatosoidide sõltuvust Sertoli rakkudest.
4. Glükoosi omastamine munandi rakkude poolt on vahendatud GLUT1, GLUT2 või GLUT3 poolt. GLUT3 on enim levinud glükoosi transporterite tüüp munandi rakkudes.
5. Nii nagu insuliinil, nii ka erinevatel tsütokiinidel, mis kasutavad IRS-1 ja IRS-2 signaali ülekandjatena rakku, võib olla toime munandi erinevatele rakuliikidele, nt. IL-2, IL-4, IL-7, IL-9, IL-13, IL-15, interferoon γ, IGF-I. Selle eeltingimuseks on vastavate tsütokiinide retseptorite olemasolu neis rakkudes.
6. IgG fragment $F(ab')_2$ jõudis nii munandi kui munandimanuse torukeste süsteemi läbides endoteliaalbarjääri.

ACKNOWLEDGEMENTS

This study was carried out in the Department of Anatomy of the Institute of Biomedicine of the University of Turku and in the Department of Anatomy of the Institute of Anatomy of the University of Tartu, during the years 1999–2004. I would like to express my sincere gratitude to Professor **Kalervo Väänänen,** Head of the Department of Anatomy, the University of Turku and Professor **Risto Santti** for the opportunity to work in the Department of Anatomy, the University of Turku.

I express my deepest gratitude to my inspiring, encouraging and always optimistic supervisors, Associate Professor **Pasi Pöllänen**, the Department of Anatomy, the University of Turku and Associate Professor **Helle Tapfer**, the Department of Anatomy, the University of Tartu.

It was a great honour for me to be included in a team with long-time experience in the study of the function of testicular endothelial cells, and work together with Professor **Trevor Cooper** from the Institute of Reproductive Medicine, Westfalian Wilhelms-University and Professor **Brian Setchell** from the Department of Animal Sciences, University of Adelaide.

All members of our research group from the University of Turku have helped and contributed to this thesis: **Jari Sundström**, **Xiao-Ke Wu**, **Saara Sainio-Pöllänen**, **Laura Ylinen**, **Mika Martikainen**, **Kirsi-Marja Sallinen**, **Tamara Teros**, **Antti Liukas** and **Hanna Antola**. My special gratitude goes to **Esko Veräjänkorva** who was so patient and sedate and spent a great deal of time on introducing me to immunohistochemical methods.

I am grateful to **Iris Dunder** and **Soili Huhta** for their friendly and understanding support during the periods of my residency at the Department of Anatomy, the University of Turku.

My heartiest thanks go to Professor **Andres Arend**, Head of the Institute of Anatomy, the University of Tartu and to all my colleagues especially to Associate Professor **Aade Liigant** and my roommates **Eleonora Rajavee** and **Elle Põldoja** who have encouraged and supported me in many ways. I am thankful to my first anatomy teachers **Arne Lepp** and **Ela Lepp** for making this subject so interesting and attractive.

I thank my fellow doctoral students of our institute **Piret Hussar** and **Marina Aunapuu** for their encouragement and companionship throughout these years.

Also our secretaries **Liina Pärnsalu** and **Kaia Tohu** are acknowledged for their practical assistance.

I express my special and sincere gratitude to our Laboratory Engineer **Agu Raudheiding**, a friendly and helpful person, who was always available for technical help.

I am grateful to **OY Asko Foto** for good-quality photos and patience to make them over and over several times.

My special and sincere gratitude goes to my best friends **Laidi**, **Monika**, **Külli**, **Anu**, **Lilian**, **Kaja** and **Rain** who know me better than myself, for their good sense of humour and for supporting me during my whole life, including the period of preparation for this thesis.

I am thankful to my **Mother** and **Father** and other relatives who have always trusted and belived in me.

I express my warmest gratitude to my beloved children **Kristiina** and **Artur** and their father **Andres** for their love and support and being self-dependent during my completion of the thesis and long period of absence fron home.

And last but not least, I am thankful to my beloved **Enn** for his trust, love and care.

PUBLICATIONS

Kokk K, Veräjänkorva E, Laato M, Wu XK, Tapfer H, Pöllänen P. Expression of the insulin receptor substrates-1-3 (IRS-1-3), GLUT-1-4, SIRP1α, PI3 kinase and PKB kinase genes at the protein level in the human testis. Accepted for publication in Anatomical Science International 01.09.2004.

Kokk K, Veräjänkorva E, Wu XK, Tapfer H, Põldoja E, Pöllänen P. Immuno-histochemical detection of glucose transporters class I subfamily in the mouse, rat and human testis. Medicina 2004; 40(2):156–60.

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Kokk K, Veräjänkorva E, Wu XK, Tapfer H, Põldoja, E., Pöllänen P. Expression of the insulin signaling transmitters and glucose transporters at the protein level in the rat testis. Submitted to Biology of Reproduction 26.11.2004.

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Teadustöö

Uurimisvaldkond:	aktiivse ja passiivse transpordi regulatsioon mehe reproduktiivse trakti organites 7 teaduslikku publikatsiooni, 10 konverentsi teesid.

Expression of the Insulin Receptor Substrates 1–3 (IRS-1-3), GLUT 1-4, SIRP1α, PI 3-kinase and PKB at the protein level in the human testis

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A short title: Insulin signaling in the human testis

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ABSTRACT

Insulin receptor substrates (IRS) mediate biological actions of insulin, growth factors and cytokines. It takes place through receptor-mediated tyrosine phosphorylation of IRS proteins. The aim of present study is to demonstrate the distribution of Insulin Receptor Substrates 1–3 (IRS 1–3), glucose transporters class I subfamily (GLUT 1–4), signal regulatory protein 1α (SIRP1α), protein kinase B (PKB) and phosphatidylinositol kinase (PI 3-kinase) in the human testis to see if signal transduction mediated by these proteins is active in testicular cells. In the present study, expression of IRS 1–3, GLUT 1–4, SIRP1α, PI 3-kinase and PKB were studied in the human testis at the protein level using immunohistochemistry and Western blotting. Positive immuno-reaction for IRS-1 was found in the human testis in the peritubular myoid cells and macrophage-like interstitial cells. Positive immunoreaction for GLUT-3 was found in the human testis in the Sertoli cells, peritubular myoid cells, early spermatocytes, macrophage-like interstitial cells and cells in the small vessels walls. IRS-1, IRS-2 and GLUT-3 proteins were also demonstrated by Western

blotting in the human testis. Expression of IRS-3, GLUT1, GLUT2, GLUT4, SIRP1α, PI 3-kinase and PKB was not detected in the human testis. The present results suggest that proteins like insulin and certain cytokines using IRS-1, IRS-2 and GLUT-3 in their signal transduction can have effects on the different cell types of the testis in the human.

Key words: IRS, insulin signaling, glucose transporters, testis

1. INTRODUCTION

Insulin plays a pivotal role in the forming of the testis. The insulin receptor tyrosine kinase family is required for the appearance of male gonads and thus for male sexual differentiation (Nef et al.2003).

The insulin receptor substrates (IRS) play a key role in signal transduction from the insulin receptor. They are major intracellular targets of phosphorylation by the activated insulin receptor tyrosine kinase. They are also substrates for the insulin-like growth factor I receptor and for tyrosine kinases associated with the receptors for growth hormone and some interleukins and interferons (White 1998).

Recently the roles of IRS-1 and IRS-2 in insulin signaling have been extensively investigated. IRS-1 is a major effector of insulin receptor action. IRS-2 is the major alternative substrate of the insulin receptor. However, less is known about the role of IRS-3 in insulin signal transduction (White 1998). IRS-3 is a new member of the IRS family, cloned in 1997 (Lavant et al. 1997). Four members of the IRS family (IRS-1, -2, -3 and -4) are known for now (Liu et al. 1999).

The IRS-1 is a cytosolic protein with a predicted molecular weight of 131,000. IRS-1 contains over 30 potential serine/threonine phosphorylation sites with homologies to caseine kinase II, protein kinase C, the mitogen-activated protein (MAP) kinases, cyclin-dependent kinase cdc2 and adenosine 3′, 5′-cyclic monophosphate (cAMP)- and guanosine 3′, 5′-cyclic mono-phosphate (cGMP)-dependent protein kinase consensus phosphorylation sites (Myers & White 1993). The IRS-1 is found in most tissues, including the insulin-responsive ones that contribute to glucose homeostasis such as muscle, adipose tissue and liver (Sun et al. 1991). IRS-1 plays a role in mediating insulin-stimulated glucose transport in physiologically relevant insulin target tissue such as rat adipose cell (Quon et al. 1994).

IRS-1 and IRS-2 are required for normal growth and glucose homeostasis in mice. IRS-3 is not essential for normal growth, glucose homeostasis and glucose transport in adipocytes. In its absence no significant compensatory augmentation of insulin signaling through IRS-1/2 is evident (Liu et al. 1999).

IRS-3 may reconstitue some, but not all, of the signals required for insulin action in brown adipocytes. IRS-1 has a unique role for IRS-1 in triggering insulin action in brown adipocytes (Arribas et al. 2003).

Glucose and fructose enter mammalian cells via facilitated diffusion, a process regulated by five glucose transporter isoforms (GLUT 1–5) at the plasma membrane. GLUT 3 has a high affinity for glucose. It´s mRNA levels have been found to be elevated in human cancer cells. GLUT 3 has a limited expression in normal and malignant human tissues and may be an attractive target for monoclonal therapy or imaging of testicular germ cell tumor (Younes et al. 1997). GLUT 3 is present in human adult and fetal myocardium (Grover-McKay et al. 1999).

The PKB has recently been a focus of intense research. It appears that PKB lies in the crossroads of multiple cellular pathways and acts as a transducer of many functions initiated by growth factor receptors that activate PI 3-kinase. The activity of protein kinase B (Akt/PKB) is particularly important in mediating several metabolic actions of insulin and cell survival (Kandel & Hay 1999). Phosphorylation of IRS-1 by PKB positively regulates IRS-1 function. PKB phosphorylates IRS-1 and acts as feedback control regulator that turns off insulin signals by inducting the dissociation of IRS protein from insulin receptors (IR) (Paz et al. 1999).

The aim of present study is to demonstrate the distribution of Insulin Receptor Substrates (IRS 1–3), glucose transporters (GLUT 1–4), SIRP1α, PKB and PI 3-kinase in the human testis to see if signal transduction mediated by these proteins is active in testicular cells.

2. MATERIALS AND METHODS

2.1. Tissues

Human testis tissue was obtained from three patients undergoing orchiectomy either due to prostatic cancer (n=2) or hydrocele (n=1) in 1998–1999. The patients were being treated at the Turku University Central Hospital. Permission for tissue donations and to use organs for research purposes was granted by the Hospitals and University´s joint Ethical board. The protocol for the research project has been approved by a suitably constituted Ethics Committee of the University of Turku and it conforms to the provisions of the Declaration of Helsinki in 1995 (as revised in Edinburgh 2000).

The testes were frozen immediately in liquid nitrogen and kept at –70°C until use.

2.2. Immunohistochemistry

Sections of 6 µm in thickness were cut in a cryostat, air-dried briefly at room temperature, fixed in cold acetone (–20°C) for 5 min and stored at –20°C.
The avidin-biotin-peroxidase method was used for the expression of IRS-1 and IRS-2 and the indirect immunofluorescence method for the expression of IRS-3, GLUT 1–4, SIRP1α, PI 3-kinase and PKB.

2.2.1. Antibodies

Mouse monoclonal anti-IRS-1 IgM antibody, mouse monoclonal anti-IRS-3 IgM antibody (Transduction Laboratories, Lexington, KY), rabbit polyclonal anti-IRS-2 IgG antibody (Upstate Biotehnology, Lake Placid, NY), goat polyclonal anti-GLUT 1, -GLUT 2, -GLUT 3, -GLUT 4, -PKB and -SIRP1α IgG (Santa Cruz Biotechnology) and rabbit polyclonal anti-PI 3-kinase IgG antibodies (Upstate Biotechnology, Lake Placid NY) were used as primary antibodies.

2.2.2. Avidin-biotin-peroxidase method

After washing for 3x2 min in Tris buffered saline (TBS) (pH 7.4), the sections were dehydrated in an increasing alcohol series. Endogenous peroxidase blockade (0.3% H_2O_2 in methanol) was carried out for 30 min. The sections were then rehydrated in a decreasing alcohol series. After washing for 3x5 min in TBS, non-specific binding sites were blocked by incubating the sections either in 2% normal horse serum (for IRS-1) or 2% normal goat serum (for IRS-2) for an hour. The sections were then incubated with the primary antibody for 60 min at room temperature. The primary antibody was diluted 1:100 in 1% bovine serum albumine (BSA) in TBS. The sections were washed for 3x5 min in TBS and incubated either with horse-anti-mouse IgG (for IRS-1; Research Diagnostics Inc) or goat-anti-rabbit IgG (for IRS-2; Research Diagnostics Inc) for 30 min. The biotinylated secondary antibody was diluted 1:50 in TBS containing 1% BSA. After washing for 3x5 min in TBS the sections were incubated with the ABC reagent (reagent A 90 µl and 10 ml TBS+ reagent B 90 µl, Vector Laboratories, Inc., Burlingame, CA) for 30 min. The sections were washed for 3x5 min in TBS and followed by a demonstration of peroxidase activity, using 3,3′-diaminobenzidine (DAB, Sigma, cat no. D-5905) as a substrate.

2.2.3. Indirect immunofluorescence method

The sections were briefly soaked in phosphate buffered saline (PBS) (pH 7.4). Non-specific binding was blocked by 15 min incubation either in 5 % normal rabbit serum (for GLUT 1–4, PKB, SIRP1α) or 5 % normal swine serum (for IRS-3 and PI 3-kinase) in PBS. Thereafter, the sections were washed for 3x2 min in PBS and incubated for 60 min with the primary antibody at room temperature. The primary antibody was diluted 1:100 in 1% BSA in PBS. After

washing for 3x5 min in PBS, the sections were incubated for 30 min either with FITC-conjugated rabbit-anti-goat Ig (for GLUT 1–4, PKB and SIRP1α) or FITC-conjugated swine-anti-rabbit Ig (for IRS-3 and PI 3-kinase). The secondary antibody was diluted 1:50 in 5% normal human serum in PBS. The sections were washed for 3x5 min in PBS and mounted in 1,4-diaza-bicyclooctane (DABCO, Sigma, St. Louis, MO).

2.3. Western blotting

Human testicular tissue was used for immunoblotting. Tissue homogenates were prepared in ice-cold suspension buffer (0.1M NaCl; 0.01M Tris, pH 7.6; 0.001M EDTA, pH 8.0) supplemented with 1 μg/ml phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO, USA, cat no. P-7626) and soybean trypsin inhibitor (Sigma, cat no. T-9003) to avoid proteolysis. The tissue (1 g) was placed in 3 ml of suspension buffer and homogenized. After incubation the lysate was centrifuged at 10000g for 30 min. The salts were removed from the supernatant in Sephadex G-25® column (PD-10, Pharmacia Biotech, Uppsala, Sweden). The eluate was then freeze-dried. The protein was diluted to 1 μg/ml in 2x Laemmli solution (1% SDS, 10% glycerol, 0.01% bromophenol blue and 2% 2-mercaptoethanol [Fluka, cat no. 63690] in 50 mM Tris buffer, pH 6.8). The samples were boiled for 5 min.

Denaturated 12% SDS polyacrylamide mini-gels were prepared and 30–40 μl samples were loaded into the wells. Low-molecular-weight markers (Pharmacia, cat no. 17-0446-01) were run in parallel to the samples. Gels were run with a 150 mA current and after electrophoresis proteins were transferred to the nitro-cellulose filter (Amersham International plc., Buckinghamshire, U.K.) for 60 min using a 400 mA current. The nitro-cellulose filter was stained with Ponceau S and each separate line was cut off. The strips were blocked with saline containing 2% BSA and 0.2% sodium azide and then incubated overnight at 4°C with primary antibodies diluted to 1:50 with PBS containing 2% BSA and 0.2% Triton X-100 (Acros Organics, NY, USA, cat no. 21568–0010). After incubation the strips were washed three times with PBS and then incubated for 1 h in 1:500 dilution with horseradish peroxidase-conjugated rabbit-anti-mouse Ig or goat-anti-rabbit Ig in 0.3 ml PBS containing 2% BSA and 0.2% Triton X-100 per strip. The strips were washed three times with PBS and then allowed to react with 0.6 mg/ml diaminobenzidine and 0.03% H_2O_2 in 0.05M Tris (pH 7.6) for 10 min. Reactions was stopped with PBS containing 0.2% sodium azide and the strips were dried before photographing.

3. RESULTS

3.1. Immunohistochemistry

Positive immunoreaction for IRS-1 was found in the human testis in peritubular myoid cells and macrophage-like interstitial cells (Fig.1, Table 1). IRS-1 positive cells were not observed in Sertoli cells or spermatocytes or spermatids. IRS-1 was not detected in the Leydig cells or testicular blood vessels. No spermatogenic cycle stage-specifity was evident in tissue sections. No positive cells were present in negative controls.

Positive immunoreaction for GLUT-3 was found in Sertoli cells, peritubular myoid cells, macrophage-like interstitial cells, early spermatocytes and cells in the small vessels walls (Fig.2A,B, Table 1). GLUT-3 positive cells were not found in the luminal part of Sertoli cells, spermatids or Leydig cells. No positive cells were present in negative controls.

Positive immunoreaction for IRS-2, IRS-3, GLUT 1, GLUT 2, GLUT 4, PI 3-kinase, PKB and SIRP1α was not found in the human testis.

3.2. Western blotting

Altogether 4 IRS-1 antigens were recognized in the testis extracts by the sera of the following molecular ratios (kD): 84, 58, 50.5 and 46 (Fig.3).

Altogether 3 IRS-2 antigens were recognized in the testis extracts by the sera of the following molecular ratios (kD): 59, 53 and 50.5 (Fig.3).

One GLUT 3 antigen was recognized in the testis extract by the sera of the 72 kD molecular ratio (Fig.3).

IRS-3, GLUT 1, GLUT 2, GLUT 4, PI 3-kinase, PKB and SIRP1α proved negative in immunoblotting.

4. DISCUSSION

The study clearly shows that IRS-1 and IRS-2 are present in the human testis. IRS-1 expression was demonstrated both by immunohistochemistry and Western blotting in the testis. IRS-2 could be demonstrated only by Western blotting.

The present finding of immunologically detectable IRS-1 in peritubular myoid cells and macrophage-like interstitial cells demonstrates that IRS-1-mediated signal transduction is active in these cells. Thus use of insulin in the regulation of glucose uptake by peritubular myoid and interstitial cells is possible if the insulin receptor is expressed in these cell types. As well as insulin action also the effect of various cytokines may be possible, as several cytokines use IRS-1 in their signal transduction. However, a precondition for this is expression of the corresponding cytokine receptors.

The absence of IRS-1 and IRS-2 from the germ cells in the luminal compartment is in accordance with the use of lactate in the energy metabolism of these cells. It further supports the dependence of germ cells on Sertoli cells in their energy metabolism. Furthermore, it indicates that the cytokines, i.e. IL-2 (Johnston et al. 1995), IL-4 (Johnston et al. 1995), IL-7 (Johnston et al. 1995), IL-9 (Sun et al. 1995), IL-13 (Sun et al. 1995), IL-15 (Johnston et al. 1995), interferon-γ (Platanias et al. 1996) or the leukemia inhibitory factor (Argetsinger et al. 1996), using IRS-1 or IRS-2 in their signal transduction can not have a full effect on these cells, even if their receptors were expressed by these cells. As IRS-2 interacts also with the insulin-like growth factor I receptors (He et al. 1996), it is evident that the lack of IRS-2 from the germ cells will also modulate the effects of IGF I on these cells.

The high expression of IRS-1 by the peritubular cells is well in accordance with the high energy expenditure of these contractile cells using glucose in their energy metabolism and thus dependent on the insulin action. However, it is not excluded that the high IRS-1 signal from these cells indicates cytokine effects on these cells in the intra-testicular paracrine regulatory network.

In conclusion our results suggest that proteins like insulin and certain cytokines using IRS-1 in their signal transduction can have effects on the peritubular myoid cells and interstitial cells, if the corresponding receptors are expressed in these cells and that the effects of IRS-1 or IRS-2 dependent cytokines and insulin on germ cells is negligible or strongly modulated by the absence of IRS-1 and IRS-2 from these cells.

The present study demonstrated that GLUT 3 was the prominent glucose transporter type in the human Sertoli cells, peritubular myoid cells, macrophage-like interstitial cells, testicular endothelial cells and early spermatocytes. The other glucose transporter types were not present at all. This finding suggests that in case of diabetes-associated disturbances of the structure of the seminiferous epithelium (Sainio-Pollanen et al. 1997), GLUT 3 could be involved. In addition, it is very interesting that the early spermatocytes express GLUT 3 at the protein level, as generally it is thought that these cell types do not use glucose in their metabolism but lactate produced by Sertoli cells.

ACKNOWLEDGMENTS

This study was supported by the Centre for International Mobility (CIMO) of the State of Finland, the PhD Student Grant Program of the University of Tartu (DARAN 1549), A. and Dr. E. Traks Foundation stipend of the USA and the Exchange Program of the Universities of Tartu and Turku. Prof. Risto Santti is kindly acknowledged for the possibility to take part to the routine of anatomy in the Department of Anatomy of the University of Turku.

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TABLES AND FIGURES

Table1. Expression of the IRS-1-3, GLUT 1-4, PI 3-kinase, PKB and SIRP1α in the different types of cells in three different human testes by immunohistochemistry.

	Sertoli cells	Peritubular myoid cells	Germ cells	Testicular interstitial cells	Testicular blood vessels	Leydig cells
IRS-1	0/3	3/3*	0/3	3/3*	0/3	0/3
IRS-2	0/3	0/3	0/3	0/3	0/3	0/3
IRS-3	0/3	0/3	0/3	0/3	0/3	0/3
GLUT-1	0/3	0/3	0/3	0/3	0/3	0/3
GLUT-2	0/3	0/3	0/3	0/3	0/3	0/3
GLUT-3	3/3**	3/3**	3/3**	3/3**	3/3**	0/3
GLUT-4	0/3	0/3	0/3	0/3	0/3	0/3
PI 3-kinase	0/3	0/3	0/3	0/3	0/3	0/3
PKB	0/3	0/3	0/3	0/3	0/3	0/3
SIRP1α	0/3	0/3	0/3	0/3	0/3	0/3

*$p < 0.05$ vs. IRS-2 and IRS-3, χ^2-test
**$p < 0.05$ vs. GLUT 1, GLUT 2 and GLUT 4, χ^2-test

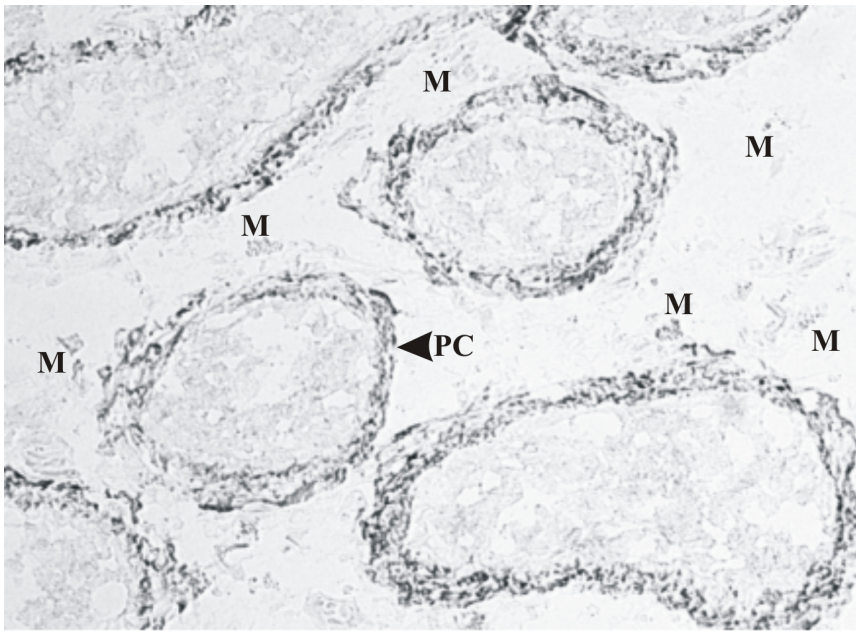

Fig. 1. Expression of the IRS-1 gene at the protein level in the human testis. Note the presence of IRS-1 in the peritubular myoid cell layers (PC) and in the macrophage-like interstitial cells (M). X 900, indirect immunohistochemistry on a frozen section.

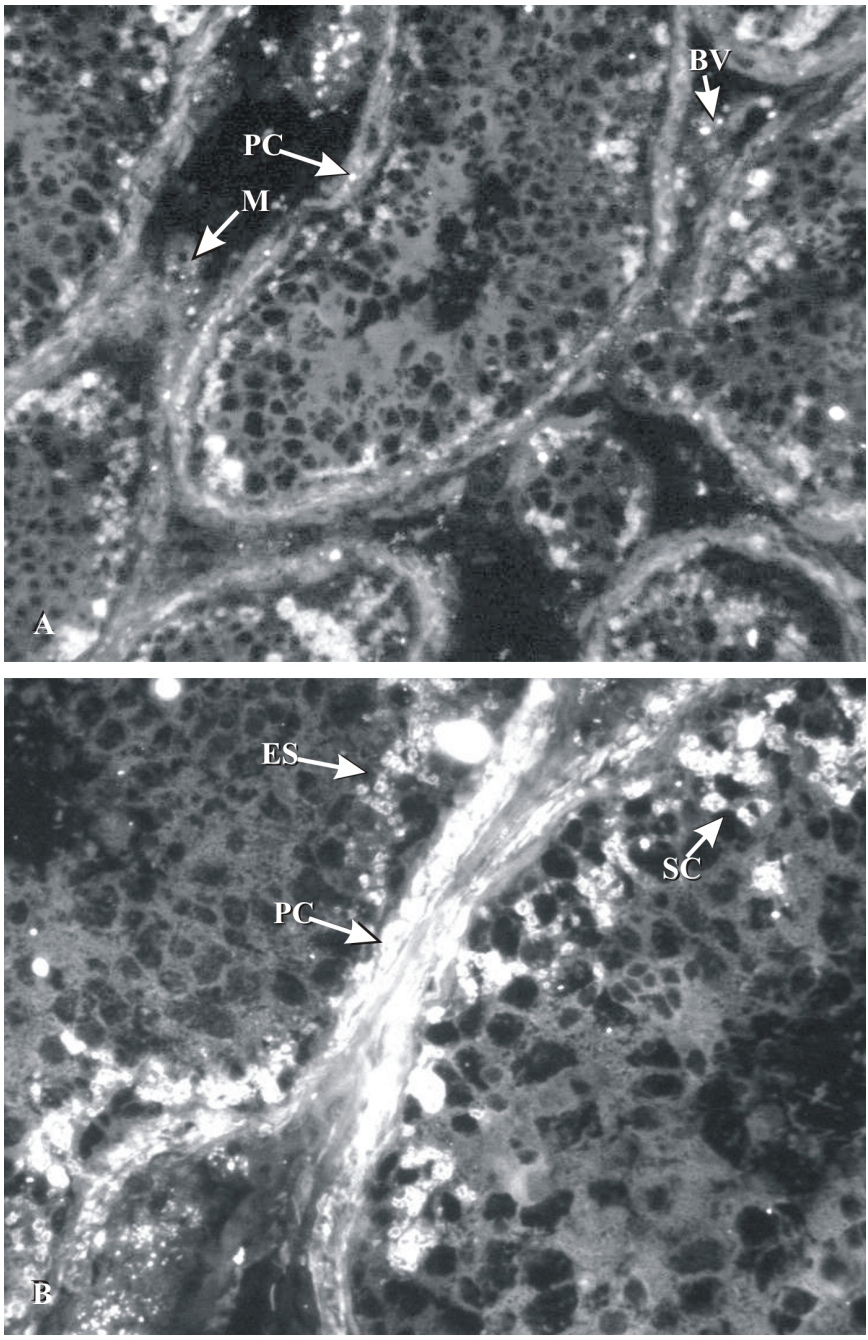

Fig. 2. Expression of the GLUT 3 gene at the protein level in the human testis. Note the presence of GLUT 3 in the peritubular myoid cells (PC), Sertoli cells (SC), macrophage-like interstitial cells (M), early spermatocytes (ES) and testicular blood vessels (BV). A X 2300, B X 3600, indirect immunohistochemistry on a frozen section.

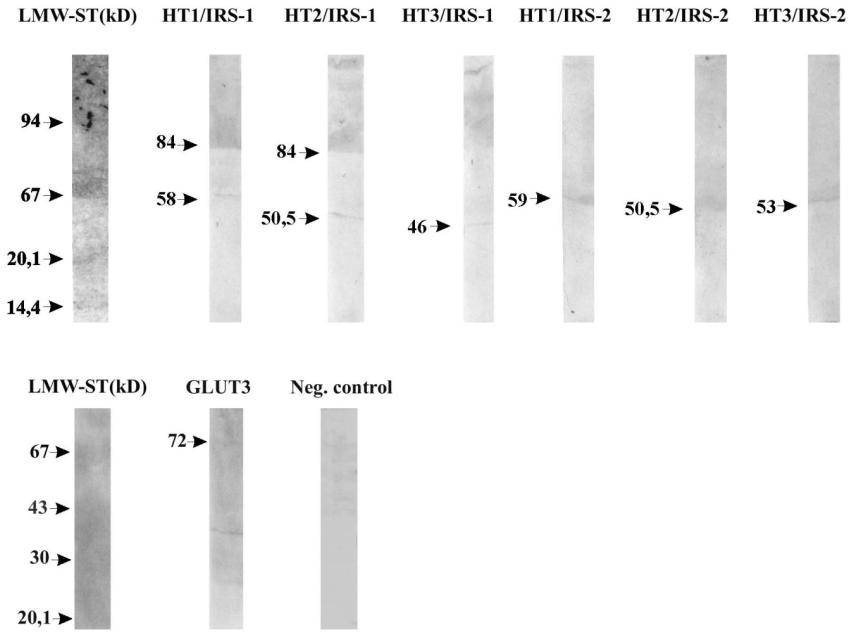

Fig. 3. Western blot of human testis for IRS-1 and IRS-2 (in three human testes HT1-3) and GLUT 3.

Expression of insulin signaling transmitters and glucose transporters at the protein level in the rat testis

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SUMMARY

Insulin Receptor Substrate (IRS) proteins are key mediators in insulin signaling from the insulin receptor. It takes place through receptor-mediated tyrosine phosphorylation of IRS proteins. The aim of the present study is to demonstrate the distribution of IRS 1–3, glucose transporters 1–4 (GLUT 1–4), signal regulatory protein (SIRP) 1α, PKB and PI 3-kinase in the rat testis to see if signal transduction mediated by these proteins is active in testicular cells. Wistar rats were used as donors of testis tissue. Expression of these genes was studied at the protein level by using immunohistochemistry and Western blotting. IRS-1, IRS-2, GLUT1, GLUT2, GLUT3 and SIRP1α were strongly expressed in the Sertoli cells (except GLUT1), early spermatocytes, peritubular myoid cells, macrophage-like interstitial cells and testicular endothelial cells in all the testes investigated by immunohistochemistry. IRS-2 was also expressed in the Leydig cells. Immunoblotting experiments demonstrated the presence of about 26–67 kD reactive with anti- IRS-1, IRS-2, GLUT1, GLUT2, GLUT3,

PKB and SIRP1α. The present results suggest that proteins like insulin and certain cytokines using IRS-1, IRS-2, GLUT1, GLUT2, GLUT3, PKB and SIRP1α in their signal transduction can have effects on the different types of testicular cells in the rat.

1. INTRODUCTION

Insulin initiates a wide variety of growth and metabolic effects by binding to the insulin receptor and activating tyrosine kinase. It takes place through receptor-mediated tyrosine phosphorylation of Insulin Receptor Substrate (IRS) proteins. IRS-s are also substrates for the insulin-like growth factor I (IGF-1) receptor and for tyrosine kinases associated with the receptors for growth hormone and some interleukins and interferons (White, 1998). Activation of these SH_2 domain proteins initiates signaling cascades, leading to the activation of multiple downstream effectors that ultimately transmit the insulin signal to a branching series of intracellular pathways that regulate cell differentiation, growth, survival and metabolism (Sesti *et al*., 2001).

Four members (IRS-1, IRS-2, IRS-3, IRS-4) of this family have been identified, which differ in their subcellular distribution, binding to the insulin receptor and interaction with SH2 domain proteins. The IRS-1 is found in most tissues, including the insulin-responsive ones that contribute to glucose homeostasis such as muscle, adipose tissue and liver (Sun *et al*., 1995). In addition, differential IRS tissue- and developmental- specific expression patterns may contribute to specificity in their signal potential (Giovannone *et al.*, 2001). All these related molecules play a very different role in vivo – both IRS-1 and IRS-2 are important for insulin action and glucose homeostasis in vivo, whereas IRS-3 and IRS-4 appear to play a redundant role in the IRS signaling system. IRS-1 and IRS-2 molecules have been considered plausible candidate genes involved in the pathogenesis of Type 2 diabetes. Several polymorphisms in the IRS genes have been identified, but only the Gly –> Arg72 substitution of IRS-1 seems to have a pathogenic role in the development of Type 2 diabetes (Sesti, 2000). IRS-1 and IRS-2 are not functionally interchangeable in tissues responsible for glucose production (liver), glucose uptake (skeletal muscle and adipose tissue), and insulin production (pancreatic ß cells). In fact, IRS-1 appears to have its major role in skeletal muscle whereas IRS-2 appears to regulate hepatic insulin action as well as pancreatic ß cell development and survival (Sesti *et al*., 1998).

IRS-3 and IRS-4 may act as negative regulators of the IGF-1 signaling pathway by suppressing the function of other IRS proteins at several steps (Tsuruzoe *et al*., 2001).

PKB lies in the crossroads of multiple cellular pathways and acts as a transducer of many functions initiated by growth factor receptors that activate

PI 3-kinase. (Kandel & Hay, 1999). PKB phosphorylates IRS-1 and acts as a feedback control regulator that turns off insulin signals by inducting the dissociation of IRS protein from IR (insulin receptor) (Paz *et al*., 1999).

A family of glucose transporters (GLUT) mediates the cellular uptake of glucose at the plasma membrane by facilitated diffusion. We investigated the presence of isoforms GLUT 1–4 of class I subfamilies in different types of cells in the rat testis.

The aim of present study is to demonstrate the distribution of Insulin Receptor Substrates 1–3 (IRS 1–3), glucose transporters 1–4 (GLUT 1–4), SIRP1α, PKB and PI 3- kinase in the rat testis to see if signal transduction mediated by these proteins is active in testicular cells.

2. MATERIALS AND METHODS

2.1. Experimental animals and tissues

Adult male Wistar rats (n=12, body weight 180–220g) were used as donors of normal testicular tissue. The animals had free access to food and water and they were maintained in a normal dark/light cycle. Permissions for the experiments and to use organs from the animals after sacrifice with CO_2 were granted by the local animal authorities. The testes were removed and frozen immediately in liquid nitrogen. Sections of 6 μm in thickness were cut in a cryostat, dried on slides in the air, fixed in cold (–20°C) acetone for 5 min and stored at –20°C.

2.2. Immunohistochemistry

The avidin-biotin immunoperoxidase method was used for the expression of IRS-1 and IRS-2 and indirect immunofluorescence method for the expression of IRS-3 GLUT 1–4, SIRP1α, PI 3-kinase and PKB genes.

2.2.1. Avidin-biotin peroxidase method

The sections were washed in TBS (pH 7.4) for 3x2 min and dehydrated in an increasing alcohol series. Endogenous peroxidase blockade (0.3% H_2O_2) was carried out for 30 min and the sections were rehydrated in a decreasing alcohol series. After three washings in TBS, nonspecific binding sites were blocked by incubating the sections either in 2% normal horse serum (for IRS-1) or 2% normal goat serum (for IRS-2) for 1 hour. The sections were then incubated with the primary antibody for 60 min at room temperature. Mouse monoclonal anti-IRS-1 IgM antibody (Transduction Laboratories, Lexington, KY) and rabbit polyclonal anti-IRS-2 IgG antibody (Upstate Biotehnology, Lake Placid, NY) were used as primary antibodies diluted 1:100 in 1% bovine serum

albumine (BSA) in TBS. The sections were washed for 3x5 min in TBS and incubated with horse-anti-mouse IgG (for IRS-1 and IRS-3; Research Diagnostics Inc) or goat-anti-rabbit IgG (for IRS-2; Research Diagnostics Inc) for 30 min. The biotinylated secondary antibody was diluted 1:50 in TBS containing 1% BSA. After 3x5 min washing in TBS, the sections were incubated with the ABC reagent for 30 min (reagent A 90 μl and 10ml TBS+ reagent B 90 μl, Vector Laboratories, Inc., Burlingame, CA). The sections were washed 3x5 min in TBS and followed by a demonstration of peroxidase activity, using 3,3′-diaminobenzidin (DAB; Sigma, cat no. D-5905) as a substrate.

2.2.2. Indirect immunofluorescence method

After washing for 3x2 min in phosphate-buffered saline (PBS), non-specific binding sites were blocked by incubating the sections for 15 min with 5% normal rabbit serum in PBS (for GLUT 1–4, PKB, SIRP1α) or 5% normal swine serum in PBS (for IRS-3 and PI 3-kinase). Thereafter, the sections were washed for 3x5 min in PBS and incubated for 60 min with a primary antibody at room temperature. Mouse monoclonal anti-IRS-3 IgM antibody (Transduction Laboratories, Lexington, KY), goat polyclonal anti-GLUT 1, GLUT 2, GLUT 3, GLUT 4, PKB kinase and SIRP1α IgG (Santa Cruz Biotechnology) and rabbit polyclonal anti-PI 3-kinase IgG antibodies (Upstate Biotechnology, Lake Placid NY) were used as primary antibodies, all in a dilution of 1:100 in 1% BSA in PBS. After 3x5 min washing in PBS, the sections were incubated for 30 min with a secondary antibody diluted 1:50 in 5% normal rat serum in PBS. Either FITC-conjugated rabbit-anti-goat Ig for GLUT 1–4, PKB and SIRP1α or FITC-conjugated swine-anti-rabbit Ig for IRS-3 and PI 3-kinase were used as secondary antibodies. The sections were washed for 3x5 min in PBS and mounted in 1,4-diazabicyclooctane (DABCO, Sigma, St. Louis, MO).

2.3. Western blotting

Rat testicular tissue was used for immunoblotting. Tissue homogenates were prepared in ice-cold suspension buffer (0.1M NaCl; 0.01M Tris, pH 7.6; 0.001M EDTA, pH 8.0) supplemented with 1 μg/ml aprotinin and soybean trypsin inhibitor to avoid proteolysis. The tissue (1 g) was placed in 3 ml of suspension buffer and homogenized. After incubation the lysate was centrifuged at 10000x for 30 min. The salts were removed from the supernatant in the Sephadex G-25 column. The eluate was then freeze-dried. The protein was diluted to 1 μg/ml in 2 x Laemmli solutions (1% SDS, 10% glycerol, 0.01% bromophenol blue and 2% -mercaptoethanol in 50 mmol l^{-1} TRIS buffer, pH 6.8). The samples were boiled for 5 min.

Denaturated 12% SDS polyacrylamide mini-gels were prepared and 10–20 µl samples were loaded into the wells. Low-molecular-weight markers were run parallel to the samples. Gels were run with a 150 mA current and after electrophoresis proteins were transferred to the nitro-cellulose filter for 60 min using a 400 mA current. The nitro-cellulose filter was stained with Ponceau S and each separate line was cut off. The strips were blocked with saline containing 2% BSA and 0.2% sodium azide and then incubated overnight at 4°C with immunosera diluted to 1:500 with PBS containing 0.1% BSA. After incubation the strips were washed three times with PBS and then incubated for 1 h in 1:50 dilution of horseradish peroxidase-conjugated rabbit-anti-mouse Ig (for IRS-1 and IRS-3) or goat anti-rabbit Ig (for IRS-2) in 0.3 ml PBS containing 0.1% BSA per strip. Strips were washed three times with PBS and then allowed to react with 0.6 mg/ml diaminobenzidine and 0.03% H_2O_2 in 0.05M Tris (pH 7.6) for 10 min. Reactions was stopped with PBS containing 0.2% sodium azide and the strips were blotted dry before photographing.

3. RESULTS

3.1. Expression of IRS-1, IRS-2 and IRS-3 genes at the protein level in the rat testis

Immunohistochemistry

Positive immunoreaction for IRS-1 and IRS-2 was found in the rat testis in Sertoli cells, peritubular myoid cells, testicular endothelial cells, early spermatocytes and macrophage-like interstitial cells. IRS-2 was also detected in the Leydig cells. (Fig.1A, B, C, Table 1).

No spermatogenic-cycle-stage specifity was evident in tissue sections. IRS-1 or IRS-2 was not detected in germ cells in the luminal compartment of the seminiferous tubule. No positive cells were present in negative controls.

Positive immunoreaction for IRS-3 was not found in the rat testis.

Western blotting

IRS-1 and IRS-2 antigens were present in the rat testis investigated in immunoblotting. IRS-1 was recognized of the 63 kD and IRS-2 of the 41 kD molecular ratio (Fig. 3).

3.2. Expression of GLUT 1, GLUT 2, GLUT 3 and GLUT 4 genes at the protein level in the rat testis

Immunohistochemistry

Positive immunoreaction for GLUT 1 was found in peritubular myoid cells, macrophage-like interstitial cells, testicular endothelial cells and early spermatocytes (Fig.2A, Table 1). GLUT 1 was not found in Sertoli cells or Leydig cells.

Positive immunoreaction for GLUT 2 and GLUT 3 was detected in Sertoli cells, peritubular myoid cells, spermatocytes, spermatides, testicular endothelial cells and macrophage- like interstitial cells (Fig.2B,C, Table1). GLUT 2 and GLUT 3 were not expressed in Leydig cells. No positive cells were present in negative controls.

Positive immunoreaction for GLUT 4 was not found in the rat testis.

Western blotting

GLUT 1, GLUT 2 and GLUT 3 antigens were present in the rat testis of the following molecular ratios (kD): GLUT 1- 32, 67; GLUT 2- 26, 55; GLUT 3- 33, 42 (Fig. 3). GLUT 4 antigen proved negative in immunoblotting.

3.3. Expression of PI 3-kinase, PKB and SIRP1α genes at the protein level in the rat testis.

Immunohistochemistry

Positive immunoreaction for SIRP1α was found in Sertoli cells, germ cells, peritubular myoid cells, testicular endothelial cells and macrophage-like interstitial cells (Fig. 2D, Table 1). No positive cells were present in negative controls.

PI 3-kinase and PKB genes were not detected in the rat testis.

Western blotting

SIRP1α and PKB antigens were positive and PI 3-kinase antigen proved negative in immunoblotting. SIRP1α was present of the 33 kD and 60 kD and PKB of the 58 kD molecular ratio.

4. DISCUSSION

The present study clearly shows that IRS-1 and IRS-2 genes are expressed at the protein level in the rat testis. IRS-2 expressions were demonstrated by immunohistochemistry and Western blotting analyses, IRS-1 was demonstrated by immunocytochemistry. IRS-3, whose overexpression causes a decrease in association of IRS-1 and IRS-2 with PI 3-kinase (Tsuruzoe *et al*., 2001) and can modulate the biological responses to insulin, was not detected in the rat testis.

The high expression of IRS-1 and IRS-2 by peritubular cells is well in accordance with the high energy expenditure of these contractile cells using glucose in their energy metabolism and thus dependent on insulin action.

The present finding of immunologically detectable IRS-1 and IRS-2 in Sertoli cells demonstrates that IRS-1 or IRS-2-mediated signal transduction is active in Sertoli cells. Thus the use of insulin in the regulation of glucose uptake by Sertoli cells is possible if the insulin receptor is expressed in these cell types. As well as insulin action the effect of various cytokines may also be possible, as several cytokines, i.e. IL-2 (Johnston *et al*., 1996), IL-4 (Johnston *et al*.,1996), IL-7 (Johnston *et al*., 1996), IL-9 (Sun *et al*., 1995), IL-13 (Sun *et al*., 1995), IL-15 (Johnston *et al*., 1996), interferon-γ (Platanias *et al*., 1996), IGF-I (He *et al*., 1996) or the leukemia inhibitory factor (Argetsinger *et al*., 1996) use IRS-1 or IRS-2 in their signal transduction. However, a precondition for this is the expression of the corresponding cytokine receptors.

The absence of IRS-1 and IRS-2 from the germ cells in the luminal compartment of seminiferous tubules is in accordance with the use of lactate in the energy metabolism of these cells. It further supports the dependence of germ cells on Sertoli cells in their energy metabolism, spermatides are closely applied to the surface of Sertoli cells where they commonly lie in deep recesses. Furthermore, it indicates that the cytokines, using IRS-1 or IRS-2 in their signal transduction, also can not have a full effect on these cells, even if their receptors were expressed by these cells.

IRS-1 and IRS-2 exhibit differences in their capacity to interact with various downstream signaling elements containing SH_2 domain (White, 1997). They may regulate the unique signaling pathways, but the expression of both IRS-1 and IRS-2 in Sertoli cells, peritubular myoid cells, blood vessels and interstitial cells supports binding to the insulin receptor and PI 3-kinase and insulin/IGF 1 signaling in these cell types.

This study clearly demonstrated that GLUT 1, GLUT 2 and GLUT 3 were glucose transporter types that mediate cellular uptake of glucose in Sertoli cells (except GLUT 1), peritubular myoid cells, interstitial cells, early spermatocytes and testicular blood vessels. GLUT 4 was not present in the rat testis. This finding suggests that in cases of diabetes-associated disturbances of the structure of the seminiferous epithelium (Sainio-Pollanen *et al*., 1997), GLUT 1, GLUT 2 and GLUT 3 are involved. In addition, it is very interesting that the early spermatocytes express GLUT 1, GLUT 2 and GLUT 3 at the protein level,

as generally it is thought that these cells do not use glucose in their metabolism but use lactate produced by Sertoli cells instead. From the present results it can be judged that it is possible that the early spermatocytes are ready to increase the transport of glucose into their cytoplasm, although GLUT 1, GLUT 2 and GLUT 3 proteins were present only in the cytoplasmic vesicles. The functional significance of this finding needs to be studied yet.

5. ACKNOWLEDGMENTS

This study was supported by the Centre for International Mobility (CIMO) of the Republic of Finland, the PhD Student Grant Programme (DARAN 1549) of the University of Tartu, A. and Dr. E. Traks Foundation scholarship of the USA and the Exchange Programme of the Universities of Tartu and Turku.

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TABLES AND FIGURES

Table1. Expression of the IRS1-3, GLUT1-4, PI 3-kinase, PKB and SIRP1α genes in the different types of cells in different rat testes by immunohistochemistry.

	Sertoli cells	Peri-tubular myoid cells	Sperma-tids	Early spermato-cytes	Macro-phage-like interstitial cells	Testicular endothelial cells	Leydig cells
IRS-1	+*	+*	–	–	+*	+*	–
IRS-2	+*	+*	–	–	+*	+*	+*
IRS-3	–	–	–	–	–	–	–
GLUT 1	–	+*	–	+*	+*	+*	–
GLUT 2	+*	+*	+*	+*	+*	+*	–
GLUT 3	+*	+*	+*	+*	+*	+*	–
GLUT 4	–	–	–	–	–	–	–
PI 3-kinase	–	–	–	–	–	–	–
PKB	–	–	–	–	–	–	–
SIRP 1 α	+*	+*	+*	+*	+*	+*	–

*$p < 0.05$ vs. IRS-3, GLUT 4, PI 3-kinase and PKB, χ^2-test

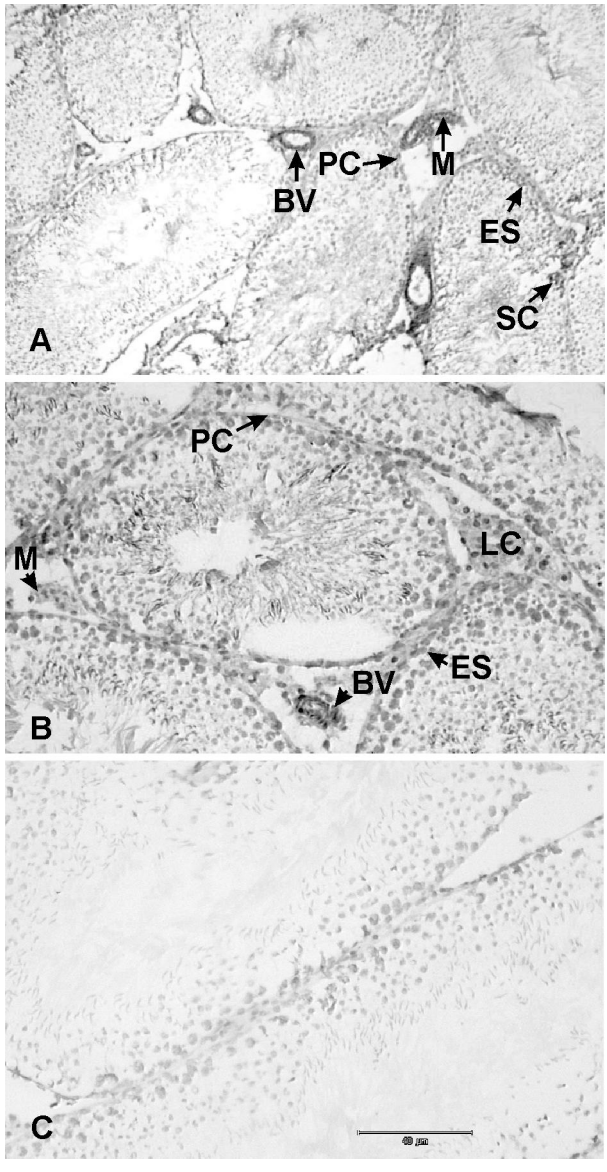

Fig. 1. Expression of IRS-1 (A) and IRS-2 (B) genes at the protein level in the rat testis and the control (C). Note the presence of IRS-1 and IRS-2 in the peritubular myoid cells (PC), Sertoli cells (SC), blood vessels (BV), early spermatocytes (ES), Leydig cells (LC) and macrophage-like interstitial cells (M). Magnification A x 2300, B x 3600, indirect immunocytochemistry (avidin-biotin-peroxidase) on a frozen section.

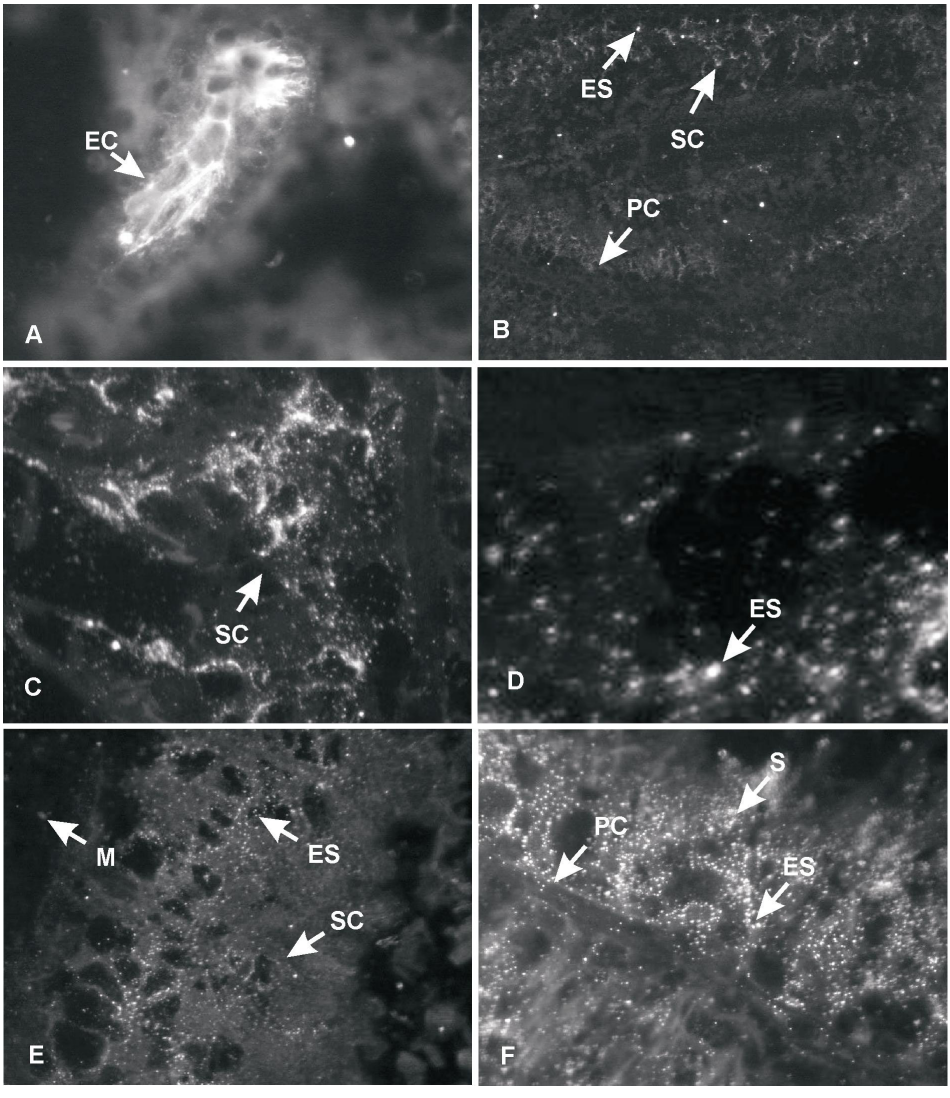

Fig. 2. Expression of the GLUT 1 in the testicular endothelial cells (A), GLUT 2 (B, C, D), GLUT 3 (E) and SIRP1α (F) at the protein level in the rat testis. Note the presence of glucose transporters in the early spermatocytes (ES), Sertoli cells (SC), peritubular myoid cells (PC), macrophage-like interstitial cells (M) and spermatids (S). Magnification A, C, D x 3600; B, E, F x 1900, indirect immunocytochemistry (immunofluorescence) on a frozen section.

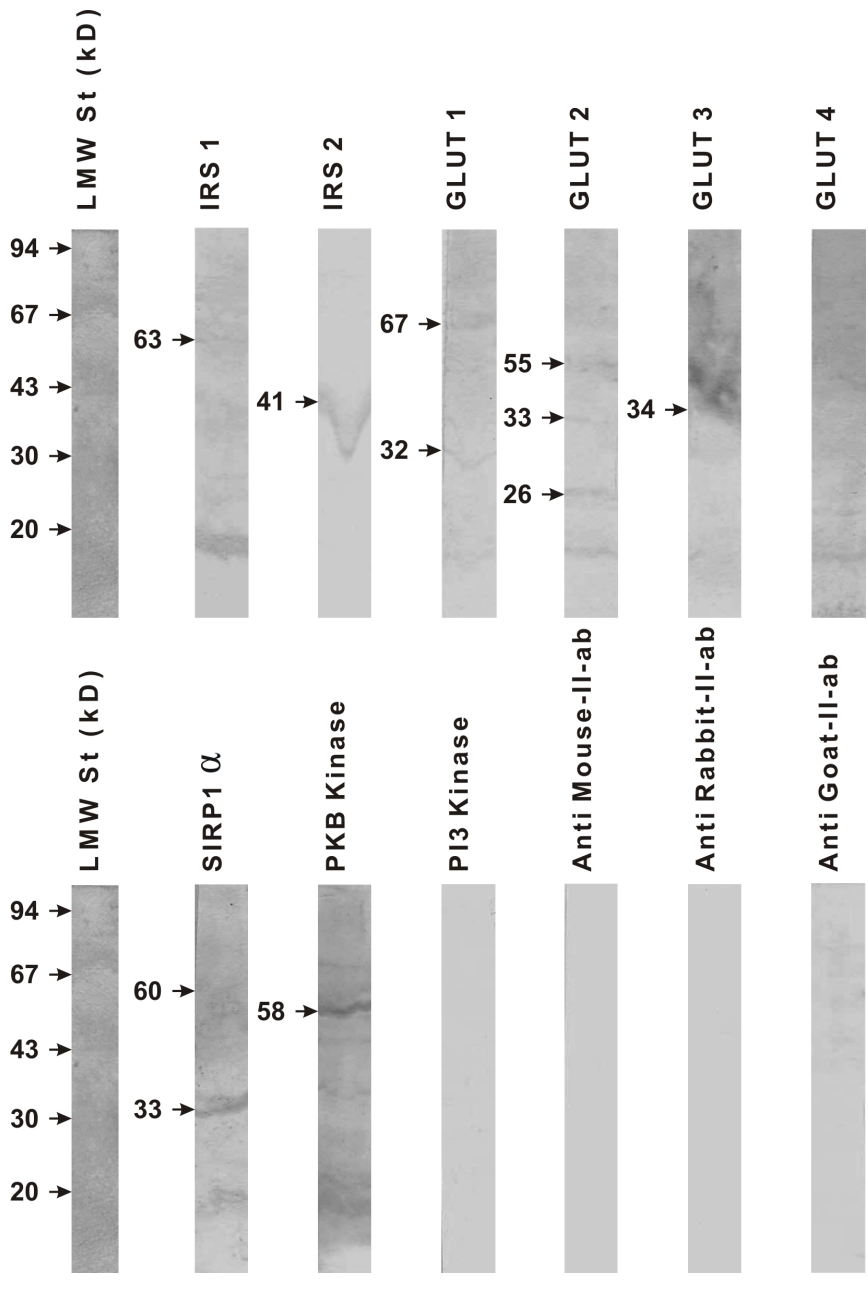

Fig. 3. Western blot analyses of the rat testis for IRS-1, IRS-2, GLUT 1, GLUT 2, GLUT 3, GLUT 4, SIRP1α, PKB and PI 3-kinase.