Differential effects of triglycerides on in vitro glucose clearance by sheep aortic endothelial and arterial smooth muscle cells

Daniela Vavra

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DIFFERENTIAL EFFECTS OF TRIGLYCERIDES ON IN VITRO GLUCOSE CLEARANCE BY SHEEP AORTIC ENDOThelial AND ARTERIAL SMOOTH MUSCLE CELLS

By

Daniela Vavra


Presented in partial fulfillment of the requirements for the

Degree of Master of Science

Department of Pharmaceutical Sciences

School of Pharmacy and Allied Health Sciences

The University of Montana

1999

Approved by:

Chairman, Board of Examiners

Dean of Graduate School

Date
Diabetes is a serious endocrine and metabolic disease that affects many organs in the body. This disorder is characterized by hyperglycemia, and increased plasma levels of lipids and amino acids. There are many in vivo studies evaluating the interdependence of hyperglycemia and hypertriglyceridemia, however the relationship of these two factors in in vitro models is poorly understood.

Studies to evaluate the effects of hypertriglyceridemia on hyperglycemia in an in vitro model using two different cell types (sheep aortic endothelial cells and sheep arterial smooth muscle cells) were performed in the absence and presence of triglycerides (45 mg/dl, 200 mg/dl). The disappearance of glucose from medium containing two different initial glucose concentrations (70 mg/dl, 300 mg/dl) was measured in the absence or presence of insulin.

High lipid levels caused abnormalities in insulin-dependent glucose clearance in both cell types, but the responses of the cells were distinctive. Conditions mimicking hyperglycemia and hyperlipidemia induced a response from endothelial cells that resembled insulin resistance. These conditions also caused a loss of adherence by smooth muscle cells. The patterns of glucose clearance in the presence of 45 mg/dl triglycerides were very similar for both cell types, except in hyperglycemic conditions. The physiologic levels of lipids enhanced glucose clearance in both cell types.
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1.0 INTRODUCTION

Diabetes is a serious endocrine and metabolic disease that affects many organs in the body. The pathology includes a variety of clinical symptoms including premature mortality, blindness and renal disease. Diabetes is characterized by hyperglycemia as well as by increased plasma levels of lipids and amino acids. Most patients with diabetes have a predisposition to atherosclerosis and neuropathic disorders (88).

The number of diabetic patients in the U.S.A. increased greatly between 1980 and 1994. In 1994 Diabetes Mellitus was the 7th leading cause of death with 8 million new cases diagnosed (17). The 1997 Surveillance Report from Centers for Disease Control and Prevention indicated that the incidence of Type II Diabetes Mellitus was higher among African Americans, Asians and Native Americans when compared with whites (17).

There are two major types of Diabetes Mellitus; Insulin Dependent Diabetes Mellitus, or type I (IDDM) and Non-insulin Dependent Diabetes Mellitus also called type II (NIDDM). The division into the two major types of Diabetes Mellitus was first observed in the 1930s. Himsworth performed experimentations in which he observed blood glucose responses after the administration of insulin (7). Upon these studies, he suggested that there were two different types of Diabetes Mellitus. Patients in one group were insulin sensitive (Type I Diabetes Mellitus), and in the other group insulin insensitive (Type II Diabetes Mellitus).

Type I Diabetes Mellitus shows rapid onset usually in young individuals, but may occur at any age. Anti-islet-cell antibodies are present at the onset, leading to injury and
destruction of pancreatic cells. Endogenous insulin is usually minimal or absent. This type of diabetes presents commonly with ketoacidosis (88).

Type II shows insidious onset and is frequently seen in normal or obese individuals 35 years and older, but it may occur at any age (reviewed in ref. 88). Anti-islet-cell antibodies are not observed in this type of diabetes. There may be adequate amounts of insulin with delayed secretion, inadequate release of insulin, or adequate insulin in the presence of insulin resistance. Ketosis is seen mainly during infection or stress. When insulin is produced, but the body is refractory to its action (insulin insensitivity), several congenital syndromes may be present including acanthosis nigricans, hyperlipidemia, growth abnormalities, ovarian hyperandrogenism, ovarian cysts, elevated androgen levels, and neonatal leprechaunism. Many patients who suffer from these congenital syndromes do not have elevated plasma glucose levels, but usually show mildly impaired glucose tolerance. Glucose tolerance is determined by a glucose tolerance test, which measures blood glucose levels at intervals after ingestion of a known amount of glucose. The test result is considered normal if fasting plasma glucose is less than 115 mg/dl, and plasma glucose following 2hrs post ingestion is less than 140 mg/dl. The glucose intolerance is a pathological state in which fasting plasma glucose is less than 140 mg/dl and the 30, 60 or 90 min plasma concentration following a glucose tolerance test exceeds 200 mg/dl. Glucose intolerance is often seen in Type II Diabetes Mellitus. Hyperglycemia, or excess sugar in the bloodstream in Diabetes Mellitus leads to glycosylation of proteins, dehydration causing hyperosmolality (more than 325 mOsm/l), and end-organ damage (retinopathy, nephropathy). High plasma glucose causes osmotic shifts, and intracellular formation of other sugars (sorbitol) which
result in swelling of the lens of the eye, blurred vision, and formation of cataracts, as well as nerve damage. Proteins such as hemoglobin, albumin and collagen become glycosylated to a greater extent. This may contribute to the long-term tissue damage in the retina, kidneys, nerves, and cardiovascular system (7,27). In order to prevent damage from hyperglycemia, glucose homeostasis is very important and is accomplished through insulin–stimulated glucose uptake. Factors other than hyperglycemia that are relevant to Type II Diabetes Mellitus are hyperinsulinemia and hyperlipidemia (69,84,92,101).

Insulin resistance and abnormal triglyceride levels (12,96) are often found in patients with impaired glucose tolerance and Type II Diabetes (77,78,87). In addition, increased plasma insulin levels are found in patients with elevated plasma triglyceride levels (39,75). In the presence of high insulin levels, the liver increases production of triglycerides and very low density lipoproteins (VLDL) (93) leading to obesity and enhanced risk of atherosclerosis.

1.1 Insulin

Insulin is a peptide hormone of molecular weight 6,000 produced by the cleavage of proinsulin before being secreted by beta pancreatic cells. It consists of two amino acid chains, A and B, containing 21 and 30 amino acids respectively, which are linked by disulfide bonds (7).

The hormone, insulin, is secreted in response to changed levels of glucose, amino acids, or gut hormones. This hormone stimulates the uptake of glucose and, at the same time, inhibits mobilization of endogenous substrates, such as glycogen. When glucose decreases, insulin secretion will cease and mobilization of endogenously stored
carbohydrates will occur. There is a sigmoidal relationship between glucose and insulin (7)(Fig. 1). Normal fasting plasma glucose levels are 80-115 mg/dl. Insulin is not secreted when levels are below 50 mg/dl. On the other hand, the maximum insulin response is seen when glucose levels are about 300 mg/dl (7). Insulin is released when plasma glucose increases 10 mg/dl or more, but soon returns to baseline. If plasma glucose levels continue to increase, insulin release will be in its second phase, characterized by a slower rise of insulin and a plateau. In normal individuals the second phase can be seen for several hours (7).

1.2 Insulin regulation

Stimulatory factors for insulin secretion other than glucose include free fatty acids, amino acids (arginine, lysine, leucine and alanine), gastrin, cholecystokinin, secretin, intestinal peptide, and gastrin-releasing peptide. When glucose is given orally, the insulin response will be greater than if glucose is given intravenously. This is due to release of these gastrointestinal hormones (7). Sympathetic nerve activation, infection, surgery and stress inhibit insulin secretion through an alpha-adrenergic mechanism (8).

There are several glucose regulatory hormones that counteract the action of insulin (Reviewed in Ref. 7). These include glucagon, epinephrine, cortisol and growth hormone. Glucagon increases hepatic gluconeogenesis, and decreases hepatic storage of carbohydrates. Epinephrine, through its beta-adrenergic action, is known to cause glucose intolerance. It stimulates glycogenolysis, gluconeogenesis and proteolysis. Increased lipolysis leads to elevated free fatty acids. Ketogenesis is stimulated, whereas peripheral ketoacid use is inhibited. Other counter regulatory hormones include cortisol,
maintaining all the important enzymes in gluconeogenesis, and growth hormone, responsible for increased usage of free fatty acids, and causing insulin resistance as well.

The understanding of insulin regulation by hormones and other factors is an important concept. The complexity of the regulatory network requires that in vitro studies be carefully planned. Part of the goal of this project was to develop an in vitro model of glucose clearance using only insulin. The complex effects of other hormones on glucose regulation were eliminated to simplify the situation. However, the effects of other hormones are important and need to be evaluated in the future.

1.3 Insulin receptors

Insulin receptors are found on many cells and their number varies with different types of cells (reviewed in ref. 88). Insulin receptors consist of two α and β subunits, which are linked through disulfide bonds. Alpha subunits are extracellular and serve as the binding site for insulin, while the β subunits form transmembrane segments associated with integral protein tyrosine kinase activity (127). Insulin binding to the α subunit of the receptor causes conformational changes responsible for receptor aggregation and partial activation of the tyrosine kinase (32,98,125,126). Upon partial activation, the tyrosine kinase autophosphorylates by a trans-mechanism, stimulating phosphorylation of the β subunit at three key tyrosines (amino acids 1146, 1150 and 1151) (7) causing fully active tyrosine kinase activity. The activated tyrosine kinase, in turn, phosphorylates different intracellular proteins that initiate the signal transduction cascade. The mechanism of transmembrane signal transmission is not fully known. The hormone receptor complex is then internalized by endocytosis leading to hormone
degradation and receptor storage and recycling back to the plasma membrane. Hereditary defects in insulin receptors can cause diminished or decreased tyrosine kinase activity resulting in diabetic symptoms (7). Activation of insulin receptors and initiation of the signal transduction cascade results in glucose transport which is the rate-limiting step for the utilization of glucose (31,69,129).

1.4 Glucose transport

Glucose is transported across the plasma membrane via gradient driven facilitated diffusion mediated by glucose transport proteins. When the gradient reaches zero, facilitated diffusion of glucose or other substances stops (18).

There are six closely related genes encoding transporter proteins (GLUT 1-6) and one pseudogene (GLUT 7) shown in Table 1 (5,15,42,65). All six glucose transporters have sequence similarity in 12 hydrophobic transmembrane regions (36,57,68). Mutations in these transmembrane regions of genes encoding for glucose transport function can have negative effects on binding or transport. For example, mutations in N-linked glycosylation sites diminish the function of the GLUT 1 glucose transporter (2-4,30,36,48,57,68,106).

Each isoform of glucose transporter has different kinetic properties and kinetic studies are difficult, because of the mixture of different isoforms within a given tissue. The $K_m$ differs with respect to different cell types (44). GLUT 4 transports glucose faster and it has a higher affinity for glucose than GLUT 1. The $K_m$ for the GLUT 4 was found to be close to normal physiologic levels for glucose (2 – 10mM) and was lower than the $K_m$ for GLUT 1 (22). The $K_m$ for GLUT 3 is $1.4+/- 0.06$mM (89). The GLUT 5 and
GLUT 2 isoforms play a major role in fructose transport. GLUT 1, 2, and 3 are mannose transporters and xylose is also transported by isoform GLUT 3 (14,44,94).

The GLUT 2 isoform is found mainly in the plasma membrane of hepatocytes and in pancreatic β-cells, where it senses glucose concentrations resulting in alterations in insulin secretion. GLUT 7 is found in microsomes of hepatocytes involved in glucose export to the cytosol following gluconeogenesis (15,119,123,124).

GLUT 1 and GLUT 3, with intermediate $K_m$ constants, are mainly found in the blood brain barrier and neuronal tissue, providing a constant flow of glucose (43,81,83,104). GLUT 5 was also present, but the reason for its presence is not known (86,105).

The most important transporters for the laboratory experiments using muscle cells and endothelial cells, are the GLUT 4 and GLUT 1 isoforms, the main transporters in muscle and endothelial cells respectively (19,54,131,116) (see Table 1). When there is no insulin present, GLUT 4 is found intracellularly in myocytes and adipocytes. In the presence of insulin, there is translocation of GLUT 4 to the plasma membrane, which leads to rapid removal of glucose from the blood (41,49,70). This translocation is not observed for GLUT 1 and GLUT 5 isoforms (13,105).

It has been suggested that alterations in the number of GLUT4 transporters may be responsible for insulin resistance (2,45,64,66). Plasma membrane levels of GLUT 4 were found to be lower in type II diabetics accompanied by increased cAMP, and decreased GLUT 4 mRNA synthesis (63,113).

Translocation of glucose transporters from an intracellular pool to the plasma membrane (25,79) was studied using phorbol esters (55), which are known to stimulate
protein kinase C (37,121,122). It was found that phorbols also enhance the translocation of glucose transporters. Administration of protein kinase C inhibitors decreased glucose transporter translocation. This suggested that protein kinase C played a role in the translocation of glucose transporters (37,55,58,91,121,130). The exact sequence of the glucose transporter translocation and effects of protein kinase C are not known (79,90,102). Hormone signaling, exercise, and contraction can cause GLUT 4 translocation in muscle; however, the exact place where GLUT 4 is translocated in muscle cells is unknown (10,54,131). Circulating fatty acids are thought to influence translocation of GLUT 4 in adipocytes (102).

Previous research indicated that there was a delay between the translocation of GLUT 4 glucose transporter and the actual glucose transport, suggesting that transport was a regulated process (19,128). First the transporter had to be partially inserted into the cellular plasma membrane and then activation occurred, resulting in glucose transport. The activation process allowed the proper conformational change needed for glucose binding (41,94).

Once the correct position of the transporter is accomplished, its intrinsic activity can be modulated. The mechanism of this modulation is not known. The intrinsic activity can be influenced by a variety of substances such as: phlorizin, adenosine, isoproterenol, epinephrine and agents acting through G–protein coupled receptors (38,62,67,76). Glucose transporter recycling is also a regulated process that is presently not very well understood (107).
Fig. 1 In vivo relationship between plasma glucose and insulin secretion is sigmoidal. Diabetes 23:763, 1974 (7).
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<td>Erythrocytes, blood – brain barrier, placenta, kidney, perineurial sheath of muscle, lower levels in adipocytes</td>
<td>Largely a basal transporter; important in many tissues. Asymmetric glucose transport kinetics</td>
</tr>
<tr>
<td>GLUT 2</td>
<td>Liver, small intestine, kidney, pancreatic β - cells</td>
<td>High Km transporter. Therefore at physiological glucose levels will transport glucose at rate proportional to concentration of glucose</td>
</tr>
<tr>
<td>GLUT 3</td>
<td>Neurons, placenta. Lower levels in liver heart, small intestine</td>
<td>Basal glucose transporter found in tissues with constant need for glucose as a metabolic fuel</td>
</tr>
<tr>
<td>GLUT 4</td>
<td>Skeletal muscle, heart, brown and white fat</td>
<td>Low K_m, sequestered intracellularly in the absence of insulin. Translocated from intracellular pool to plasma membrane in response to insulin</td>
</tr>
<tr>
<td>GLUT 5</td>
<td>Small intestine. Lower levels in sperm, muscle, fat, brain and kidney</td>
<td>Basal transporter. Has a high affinity for fructose</td>
</tr>
<tr>
<td>GLUT 6</td>
<td>Pseudogene – not translated into protein</td>
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<tr>
<td>GLUT 7</td>
<td>Liver microsomes</td>
<td>Functions in intracellular transport of glucose</td>
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**Table 1.** Glucose transporters isoforms, their tissue distribution and characteristics (88).
A great deal of research has been done to explore the roles and regulation of insulin, insulin receptors, and glucose transporters in maintaining plasma glucose levels (2,5,10,25,45,70). However, it has become clear that many other factors affect glucose homeostasis. Hyperlipidemia, often seen with Type II diabetes, is receiving considerable attention because of its possible role in the development of cardiovascular diseases associated with diabetes (9, 75, 88).

1.5 Hyperlipidemia

Blood with increased amounts of lipids (hyperlipidemia) is an important symptom of Diabetes Mellitus. Hyperlipidemia may result from a defect in the gene controlling low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) receptors, or an inability to metabolize LDL and VLDL (88) or from enhanced rates of adipose tissue lipolysis.

A number of in vivo and in vitro studies have been performed to investigate the role of hyperlipidemia and insulin resistance in Diabetes Mellitus. Plasma of patients with Type II Diabetes Mellitus contained high levels of triglycerides, and decreased levels of high-density lipoprotein (HDL) and apolipoprotein A (75). Type II patients accumulated intermediate density lipoproteins and small dense LDL in the presence of hypertriglyceridemia, which showed impaired binding to LDL receptors (9, 53). High plasma triglyceride concentrations have also been associated with hyperinsulinemia, increased plasma levels of VLDL, and abnormal glucose tolerance (16).

Hyperlipidemia may also occur from excess free fatty acids accompanied by stimulation of gluconeogenesis, insulin resistance, glucose intolerance, or increased
lipolysis and may interfere with normal cellular metabolic function and signal transduction (85,88). Hypertriglyceridemia is associated with elevated glucose levels, and impaired cellular expression of the insulin gene (120). Elevated lipids also decrease the number of cellular glucose transporters (45,63).

### 1.6 Muscle cells

There are three types of muscle cells: skeletal muscle responsible for skeletal motion, cardiac muscle cells found in heart and large vessels, and smooth muscle cells present in blood vessel walls and elastic organs such as the uterus and bladder (117). Muscle is one of the most important organs in maintaining glucose homeostasis. Glucose uptake by muscle cells is regulated by insulin (88), with glucose transport as the rate-limiting step (31,70,129).

Although insulin normally stimulates glucose transport in muscle cells, in insulin resistance glucose levels remain high despite the presence of insulin. In this case, more and more insulin is secreted, leading to hyperinsulinemia. In the presence of high levels of insulin, studies have shown that glucose transport by muscle cells was depressed (24,59) and showed significant lipid uptake (114) with increased glucose conversion to lipids (84). It was found that lipoprotein lipase activity in muscle cells was inversely correlated with plasma insulin concentration (23). The rate of glucose utilization, insulin sensitivity, and maximum insulin responsiveness in muscle diminished with congenital generalized lipodystrophy (120), or aging (40), and was increased by the influence of exercise due to increased extraction and delivery of glucose to the muscle cells (50).
1.7  **Endothelial cells**

Endothelial cells play important roles in the formation of a smooth antithrombogenic surface in blood vessels, coagulation, thrombolysis, antigen presentation (28,95), and passage of nutrients, solutes, and hormones from blood to the extravascular space (21). Since dysfunction of vascular endothelium is associated with Diabetes Mellitus (20), several studies exploring lipoprotein degradation and insulin stimulated glucose uptake in endothelial cells have been performed.

Human endothelial cells grown in cell culture in the presence of hyperglycemic conditions showed formation of glycosylated LDL and inhibition of its degradation as compared to normal (nonglycosylated) LDL. The decreased recognition of these LDL molecules by the endothelial lining of blood vessels may play a role in abnormal tissue physiology (80). High concentrations of glucose added to the cell culture media reduced the number of endothelial cells by 78% compared to controls and the presence of insulin decreased glucose uptake of endothelial cells, possibly due to changes in osmolality (1). Decreased glucose uptake was also observed in diabetic endothelial cells (101).

1.8  **Significance**

Glucose uptake by cells has been extensively studied. Mechanisms of glucose transport, insulin signaling, and some aspects of signal transduction of insulin are known. However, a direct comparison of glucose clearance by different cells using an *in vitro* assay system under physiological and pathological conditions has not yet been performed. Such a study could provide important clues to our understanding of pathologies associated with Type II Diabetes Mellitus, such as atherosclerosis and insulin
resistance. Therefore, it was important to design an *in vitro* system which could accurately measure changes in glucose clearance in the presence of lipids.

In order to compare the action of insulin on different cell types *in vitro*, during hyperglycemia and hyperlipidemia, it was important to normalize the assay system in terms of the number of cells and culture conditions. The normalization of the assay system must also be considered when parameters are changed by increasing amounts of lipids to assure that only true functional differences between the different cells studied will be observed.

### 1.9 Hypothesis

When looking from a historic point of view, a basal level of plasma glucose was probably necessary for providing energy to cells in performing strenuous life sustaining work. On the other hand, the lifestyle of modern man has completely changed. Food is readily available, and less exercise is common. Thus, in general, less energy is required for daily activities, and very often more energy enters the system (body) than is expended. The result is an accumulation of metabolic fuels, which are stored mostly as fat. Excess stored body fat leads to obesity associated with insulin resistance (108). Because insulin resistance is associated with altered glucose clearance and hyperlipidemia, it is essential to explore the effects of hyperlipidemia on insulin-dependent glucose clearance. Endothelial cells can be likely affected by both hyperglycemia and hyperlipidemia since they are in direct contact with the plasma (1,20). In addition, alteration of arterial smooth muscle cells has been shown to be associated with atherosclerosis in Type II diabetes (9, 84). **The hypothesis to be tested is that**
high lipid levels can cause abnormalities in insulin dependent glucose clearance in aortic endothelial cells (EC) and arterial smooth muscle cells (SMC). We further hypothesize that the glucose clearance in the presence of high lipid levels will decrease from that seen without lipids in both aortic endothelial cells and arterial smooth muscle cells, and that there will be a significant difference between the cell types with respect to glucose handling. It is expected that the glucose clearance by endothelial cells and arterial smooth muscle cells under normal physiologic levels of lipids will remain the same as the glucose clearance in the absence of lipids.

1.10 Specific aims

1. To establish an in vitro experimental model to enable detection of insulin dependent glucose clearance in aortic endothelial cells and arterial smooth muscle cells.

Questions to be answered include:

a) Can glucose clearance be measured in standard cell culture media (DMEM)?

b) Does bovine insulin cause measurable increases in insulin-dependent glucose clearance in non-bovine cell types?

c) Does glucose starvation enhance insulin-dependent glucose clearance in vitro?

d) Are the triglyceride concentrations used for the experiments toxic to cells?
2. To examine glucose clearance under normal glycemic and hyperglycemic conditions in glucose-starved (and non-starved) EC and SMC.

Questions to be answered include:

a) What is the optimal glucose-starvation time for measurement of insulin-dependent glucose clearance in these two cell types?

b) Does the addition of insulin cause increased glucose clearance under both normal glycemic and hyperglycemic conditions?

c) Do SMC and EC respond differently to normal glycemic and hyperglycemic conditions?

3. To examine the effect of two widely different concentrations of triglycerides on glucose clearance by both cell types.

Questions to be answered include:

a) Do normal physiologic triglyceride levels (45 mg/dl) affect insulin-dependent glucose clearance for cells under normal and hyperglycemic conditions?

b) Do high triglyceride levels (200 mg/dl) affect insulin-dependent glucose clearance for cells under normal and hyperglycemic conditions?
1.11 Rationale for the experimental design

Endothelial cells and smooth muscle cells from sheep were chosen because of their availability and the acceptability of the sheep as a large animal model for many cardiovascular experiments.

Endothelial cells were chosen because of their homeostatic role at the blood interface, and their potential influence on lipid accumulation, and atherosclerosis in blood vessels (88), and because of the evidence of endothelial dysfunction in diabetes (20). Smooth muscle cells were selected because, like the tissue from which they originate, they require an adequate blood supply for their function. These cells also play an important role in glucose homeostasis and insulin action (26).

In order to compare the effect of hyperglycemia and high triglycerides on two different cell types, culturing conditions for both cell types must be normalized. For this purpose the cell number in each culture was determined so that all measurements could be expressed on a per cell basis. All experiments were also conducted soon after cells became confluent using normal medium containing fetal bovine serum. The experiments began with the starvation of cells in glucose- and serum-free medium. All subsequent glucose or triglycerides supplements were added to the glucose- and serum-free medium, so that the concentrations of both added supplements were well defined.

The success of this study depended heavily on the ability to measure changes in the glucose clearance from the medium. The detection of glucose clearance from the medium was the method of choice because the issue of intracellular breakdown of glucose during the experimental period can be avoided. The Sigma GAGO (glucose)
assay kit was chosen because the glucose oxidase/peroxidase assay system was simpler, more accurate and sensitive than an assay employing a straight chemical detection method such as the Nelson procedure (82).

It is possible that a high level of triglycerides could be toxic to cells, and this could invalidate any negative effects observed. For this purpose, lactate dehydrogenase assay (LDH) and Trypan Blue staining were used to assess cell viability prior to the termination of the experiments.

In Specific Aim 2, glucose-starvation was used to mimic clinical glucose-tolerance testing. A series of experiments were designed to determine the optimum starvation period to maximize glucose clearance by SMC and EC under normal glycemic and hyperglycemic conditions. The longest starvation period to be tested was cut to 6 hrs because some cells, such as the adipocytes, cannot tolerate or survive beyond this period (personal communication, V.R. Grund). Because of this, any data obtained from SMC and EC starved for longer periods would not be comparable with results from other cells in future studies. The normal physiological level of insulin (60 mg/dl) was chosen in these experiments. Likewise, normal glycemic (70 mg/dl) and hyperglycemic (300 mg/dl) levels of glucose (for both humans and sheep) were chosen for their clinical relevancy.

For Specific Aim 3, normal physiological (45 mg/dl) and elevated (200 mg/dl) concentrations of triglycerides were added to the system described in Specific Aim 2. Although the level (200 mg/dl) is considered moderately high, patients with such a level of triglycerides may be at the beginning stage of developing cardiovascular disease (88). Furthermore, preliminary experiments indicated that higher levels of triglycerides (in
combination with other fatty acids and lipids) were toxic to cells \textit{in vitro}. Triglycerides were used because they are associated with hyperinsulinemia, abnormal glucose tolerance, as well as high levels of LDL, VLDL, and decreased levels of HDL. Cell culture conditions again were normalized. Selected and counted cells were grown in the presence of triglycerides for 1, 7 and 14 days. Glucose clearance per cell from the culture media containing hyperglycemic and normal glycemic initial concentrations of glucose was determined. Sterile Intralipid 20% Fat Emulsion from Baxter Healthcare Corporation (Deerfield, IL) was used as a source of triglycerides, which are present in this emulsion at 200 mg/ml in a mixture with fatty acids such as linoleic (44-62%), oleic (19-30%), palmitic (7-14%), linolenic (4-11%), and stearic (1.4-5.5%). Other ingredients are Soybean Oil (20%), Egg Yolk Phospholipids (1.2%), Glycerin (2.25%), and water.
2.0 MATERIALS AND METHODS

2.1 Materials and reagents

Dulbecco's Modified Eagle's Medium (DMEM), containing gentamicin and fungizone was obtained from GIBCO BRL (Grand Island, NY), and supplemented with 10% fetal bovine serum (FBS) from Hyclone (Logan, UT). Another GIBCO product, RPMI 1640 Glucose-free medium was already prepared by the manufacturer and ready to be used for tissue cultures. Bovine insulin (GIBCO) was selected for the availability, cost and a high reported cross-reactivity with cells of other species. M199 medium also from GIBCO BRL was supplemented with 20% FBS, fungizone and gentamicin from the same company. Glucose assay kit GAGO-20 containing oxidase-peroxidase reagent and β-D-glucose were obtained from SIGMA (St. Louis, MO) and used according to the manufacturer's recommendations. Intralipid 20% fat emulsion was received from Baxter Healthcare Corporation (Deerfield, IL). The absorbance values were obtained using a Microplate reader UNISKAN II (McLean, VA). Pyruvate, NADH, and Lactate dehydrogenase (LDH) enzyme were obtained from SIGMA (St. LOUIS, MO). The absorbance readings for the LDH analysis were performed on a spectrophotometer (Thermomax Microplate Reader) at the University of Montana, Department of Pharmaceutical Sciences.
2.2 Methods

2.2.1 Endothelial cell isolation:

EC were harvested from the descending aorta. The ends of the aorta were kept in 70% ethanol for 5 min to prevent growth of cells other than endothelial, and to make sure that our further measurements of glucose clearance would not be positively or negatively influenced by a mixture of different cells. The outside surface of the sheep aorta was sterilized with 70% ethanol. The EC were captured following trypsinization (1% trypsin) and flushed through with medium [M199 (GIBCO) with 20% FBS, fungizone and gentamicin combination]. Endothelial cell growth factor was used to promote cell growth. EC were maintained in M199 medium and incubated at 37°C in a humidified environment containing 5% CO₂.

2.2.2 Smooth muscle cell isolation:

Sections of sheep artery were obtained and the outer tissue removed with forceps. The pieces were rinsed with FBS free DMEM, and sterilized with 70% ethanol. The margin was trimmed off and the remainder was cut into approximately 1 mm pieces. The pieces were placed in a 50 ml centrifuge tube with 6 ml of collagenase (0.1%) in PBS and incubated for 1 hr at 37°C. Following the incubation, the supernatant was removed and 10 ml of DMEM media with 10% FBS was added. The mixture was centrifuged at 1600 RPM for 5 min. Following centrifugation, the supernatant was removed and DMEM media with 10% FBS was added to the total volume of 20 ml, which was split into two plates. Cells were grown at 37°C in a humidified atmosphere containing 5% CO₂.
2.2.3 Modified glucose assay for microtiter plate using GAGO-20 Glucose Assay Kit:

Stock glucose standards stored at 4°C were made using directions in Table 1.

Table 1. Glucose Standards

<table>
<thead>
<tr>
<th>Tube</th>
<th>Volume of Glucose Free Media in μl</th>
<th>Volume of Glucose Standard Solution (1mg/ml) in μl</th>
<th>Concentration of Glucose in mg/ml</th>
</tr>
</thead>
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<tr>
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<td>Standard #4</td>
<td>920</td>
<td>80</td>
<td>0.08</td>
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</tbody>
</table>

Standards and samples vortexed to homogeneity, were added in the volume of 50 μl into microtiter wells and diluted to be in the range of the standard curve 0.02-0.08 mg/ml. Glucose-peroxidase assay reagent (100 μl) was pipeted into wells every 30 sec. The enzymatic reaction was stopped by the addition of 100 μl of 12N H₂SO₄, to the total volume of 250 μl, following the incubation for 30 min at 37°C in a dry incubator with 5% CO₂. Following this addition, samples were again kept in the same incubator under the same conditions for another 30 min to assure uniform color distribution in the wells. Absorbances were then determined with a microplate reader at 492 nm. Data were used to create a calibration curve where the X axis contained concentrations in mg/ml and the Y axis represented optical density. Regression analysis was used on every linear standard curve to determine the equation for the best fit line from which the sample concentrations were obtained.
2.2.4 Baseline glucose clearance:

Smooth muscle or endothelial cells were starved for 0, 2, 4 and 6 hrs in glucose free RPMI 1640 or fed by DMEM media with 10% FBS, and were rinsed twice prior to analysis with fresh glucose free media. Following the rinse, 10 ml of the same fresh media was added into each plate. When studying normal glycemic levels of glucose, both plates received 70 μl of glucose from 100 mg/ml stock, giving a final concentration of 70 mg/dl. This would actually correspond to normal fasting plasma glucose levels. For hyperglycemic levels, plates received 300 μl of glucose (100 mg/ml), giving a final concentration of 300 mg/dl. Samples were pipetted in duplicate from each plate in the volume of 130 μl. This sample served to measure glucose clearance at 0 min. Following this first sampling, one of the two plates received 300 μl of bovine insulin (20 mg/ml from stock). The normal physiologic concentration of insulin is 60-150 mg/dl. After the addition of insulin, samples of 130 μl were obtained in 5, 10, 15, 20, 40, 60, 90, 120 min and 24 hrs from both plates in duplicate. These samples were diluted, treated, assayed, and concentrations were obtained by the modified glucose assay protocol for the microplate reader. Sample concentrations were graphed as a function of time for each cell type.

Modifications in later experiments were made so that experiments could be done in 24-well tissue culture plates (Falcon).

2.2.5 Cell counting:

Medium used to feed EC and SMC was removed from plates. Plates were rinsed with FBS free DMEM. Cells were trypsinized and DMEM medium with 5% FBS was
added to the plates. Trypsinized cells in the mixture with DMEM medium were placed in centrifuge tubes and centrifuged at 2,400 RPM (Beckman, GPR Centrifuge). Following the centrifugation, supernatant was removed and 3ml of DMEM media free of FBS was added. The cell mixture was vortexed and 10μl was added to a hemocytometer. The total number of cells was obtained by counting under the microscope. Viability was determined by Trypan blue exclusion.

2.2.6 Lactate dehydrogenase analysis:

An LDH protocol, modified by the University of Montana, Department of Pharmaceutical Sciences, was used to measure the LDH reaction. This reaction follows conversion of glucose to pyruvate, and serves to regenerate NAD⁺ from NADH. KH₂PO₄ (13.6 g) was dissolved in 1L nanopure water to generate a 0.1M solution (pH 7.2) and 100 μl was mixed with 123 mg of pyruvate. This pyruvate assay solution was stored at 4°C. LDH enzyme (2 μl) was diluted in 4,000 μl 0.1 M KH₂PO₄. An NADH solution was prepared just prior to assay to prevent formation of potent enzyme inhibitors, which can form in frozen solution or damp powder. NADH (13 mg) was dissolved in 10 ml 0.1 M KH₂PO₄.

Pyruvate solution (50 μl) was added to all wells except the top left well, which was left as a blank. Standards were mixed in the following order and added to wells (Table 2):
Table 2. LDH Standard Curve

<table>
<thead>
<tr>
<th>Units</th>
<th>LDH (µl)</th>
<th>KH₂PO₄ (µl)</th>
</tr>
</thead>
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<tr>
<td>0</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>0.002</td>
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<tr>
<td>0.005</td>
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</tr>
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</tr>
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<td>30</td>
</tr>
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<td>30</td>
<td>20</td>
</tr>
<tr>
<td>0.04</td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td>0.05</td>
<td>50</td>
<td>0</td>
</tr>
</tbody>
</table>

In the next step 50 µl KH₂PO₄ was added to create a total volume of 150 µl. All samples were pipetted into additional wells in the volume of 100 µl. The total volume of samples and pyruvate was 150 µl. Parameters on the plate reader were set up and 50 µl NADH was rapidly added to all wells. Absorbance values at 492 nm (Spectrophotometer Thermomax Microplate Reader) were obtained immediately following the addition of NADH. For a positive control value, the cells from each sample were lysed in Triton-X 100, and cell lysates were assayed for LDH as above. Values for LDH in u were compared to this total cellular LDH and expressed as a percent of positive control.

2.2.7 Glucose clearance in the presence of triglycerides:

SMC and EC were kept in the presence of normal physiologic concentrations of triglycerides (45 mg/dl) or above normal triglyceride concentrations (200 mg/dl) for 1, 7 and 14 days. Intralipid 20% fat emulsion was used as a source of triglycerides present in this lipid emulsion in the concentration 200 mg/ml. Other major components in this lipid emulsion were fatty acids such as linoleic (44-62%), oleic (10-30%), palmitic (7-14%), linolenic (4-11%) and stearic (1.4-5.5%). The sterile lipid emulsion was stored at 4°C.
Studies were performed to demonstrate a consistent baseline glucose clearance in each cell type to which these obtained values were compared. When experiments were modified for 6 and 24-well plates, differences in volume and cell number were taken into account. Glucose clearance was measured for each cell type with 70 mg/dl and 300 mg/dl glucose exposure, with or without the presence of insulin, after 1, 7 and 14 days of incubation with the lipid emulsion as described in the modified glucose assay. Dilutions for the initial glucose concentrations of 70 mg/dl (1:10) and 300 mg/dl (1:50) were made to fit the calibration curve between 0.02-0.08 mg/ml.
3.0 STATISTICAL ANALYSIS

Several comparisons were made on the glucose clearance data obtained in this study. The two major effects analyzed statistically were, a) a statistically significant increase in glucose clearance when insulin was added to the culture, and b) differences in glucose clearance between the responses by the two cell types. Some experiments were performed with duplicate aliquots from single samples, which provided information regarding the reproducibility of the assay. Subsequently, triplicate (or more) samples were included in the statistical analyses where shown, using multiple concentrations as the variable treatments. Statistical variance (P) among treatment sets was determined by the One Way ANOVA test using Minitab Statistic Program. Statistically significant results were defined as p < 0.05.
4.0 RESULTS

4.1 To establish an in vitro experimental model to enable detection of insulin-dependent glucose clearance in aortic endothelial and arterial smooth muscle cells.

The initial experiments were conducted to measure glucose clearance by rabbit skeletal muscle cells and human fibroblasts in basic cell culture media. The amount of glucose present in the standard (DMEM) media (450 mg/dl) was too high, and therefore any change in glucose clearance by cells could not be accurately measured. Therefore, glucose-free medium with specific amounts of glucose added was used for subsequent measurements, and glucose concentrations in the media were measured at 0, 20, 60, 120, 240, 360 min, and 24 hr. Using the combination of this culture system and the GAGO glucose assay method, an approximately 10% insulin-dependent glucose clearance was observed after 24 hrs when an initial hyperglycemic level of glucose (300 mg/dl) was present (data not shown).

A significantly higher reduction of glucose from supplemented glucose-free medium was observed when insulin was used as compared to the baseline glucose clearance in the absence of insulin (data not shown, see later experiments). This confirmed that the culture conditions and the assay method were adequate to measure bovine insulin-dependent glucose clearance by both cell types.

Preliminary results also showed that glucose starvation for 4 hrs significantly increased the difference (30-60%) in glucose clearance seen with and without insulin by rabbit skeletal muscle cells, sheep corneal endothelial cells, and human fibroblasts.
All subsequent experiments were done using EC and smooth muscle cells SMC from sheep arteries. Cells were characterized by staining, using α-actin stain to identify SMC and von Willebrand Factor to identify EC. Cells were grown to confluence and photographed at 40x magnification for morphological appearance (Fig. 1 and 2).

Fig.1. SMC from sheep at 40X magnification.
A commercial preparation of triglycerides was used in this study. In order to ensure that this triglyceride preparation was not toxic to cells, LDH in the tissue culture media was measured as an indicator of cell death. LDH concentrations in the media of EC cultures remained below 10% of total cellular LDH for all concentrations of triglycerides used, with and without insulin (Fig. 3A). Similar results were obtained for SMC (Fig. 3B). Trypan blue staining also showed viability of cells remained above 90% throughout subsequent experiments.
Fig. 3A. LDH analysis for EC. Values represents [LDH] averaged percentages of positive control (total cellular LDH) for EC obtained over 14 days from tissue culture containing 1 mg/dl, 10 mg/dl or 100 mg/dl triglycerides. Error = s.d., n = 14.

Fig 3B. LDH analysis for SMC. Values represents [LDH] averaged percentages of positive control (total cellular LDH) for SMC obtained over 14 days from tissue culture containing 1 mg/dl, 10 mg/dl or 100 mg/dl triglycerides. Error = s.d., n = 14.
4.2 To examine glucose clearance under normal glycemic and hyperglycemic conditions in glucose-starved (and non-starved) EC and SMC (Specific Aim 2).

The effect of baseline insulin treatment under selected glucose starvation times (0, 2, 4, and 6 hr) was established to make sure that the period selected provided efficient insulin-dependent glucose clearance by EC and SMC. Another reason to evaluate the basal insulin treatment effect was the possibility that insulin resistance would be observed during our experiments in the presence of elevated triglycerides.

Insulin treatment caused increases in glucose clearance under most conditions. However, at the higher glucose concentration, insulin had little effect at 0 and 2 hr starvation for either cell type (Fig. 4A and B). The insulin treatment caused a significant increase in glucose clearance, and the glucose clearance/cell in the presence of insulin had the largest value for EC with an initial glucose concentration of 300 mg/dl following the 4 hr starvation time (Fig. 4A). The insulin treatment for SMC with an initial glucose concentration of 300 mg/dl was significant for a 4 hr starvation period (Fig. 4B). Therefore, the 4 hr glucose starvation period was used for subsequent experiments.
Figure 4A. Effect of glucose starvation on EC. EC were starved in glucose-free medium for the indicated times. The total glucose clearance/cell for 70 mg/dl and 300 mg/dl initial glucose concentration with and without insulin was measured. Values are averages of duplicate aliquots of single samples with error = range, except at the 4hr points where n=4, error bar = s.d., and * = p<0.05
Figure 4B. Effect of glucose-starvation on SMC. SMC were starved for indicated times. The total glucose clearance/cell for 70 mg/dl and 300 mg/dl initial glucose concentration with and without insulin was measured. Values represent averages of duplicate aliquots of single samples with error = range, except at the 4 hr points where n=4, error bar = s.d., and * = p<0.05.

The difference in glucose clearance between EC and SMC following glucose starvation was evaluated at a normal glycemic glucose concentration (70 mg/dl) (Fig 5A) and hyperglycemic glucose concentration (300 mg/dl) (Fig. 5B). Under most conditions EC took up slightly more glucose than SMC. The patterns of glucose clearance over the various starvation times varied between the cells, with glucose starvation time having a more dramatic effect on the SMC. This suggests that glucose clearance is regulated differently in the two cell types. Increasing the glucose concentration led to increased clearance with or without insulin in EC and SMC, but this increase was more pronounced for the SMC so that differences between the cell types diminished at the higher glucose concentration. For example, at 70 mg/dl glucose, at the 4 hr glucose starvation time point
the difference between the two cell types was statistically significant with and without insulin (Fig 5A), but there was no significant difference between the cells at 300 mg/dl.

**Figure 5.A.** EC and SMC were glucose starved for indicated times. The total glucose clearance/cell for 70 mg/dl initial glucose concentration with or without insulin was performed. Values represent averages of duplicate aliquots of single samples with error = range, except at 4 hr points where n=4, error bar = s.d., and * = p<0.05.
Figure 5.B. EC and SMC were glucose starved for indicated times. The total glucose clearance/cell for 300 mg/dl initial glucose concentration with or without insulin was measured. Values represent averages of duplicate aliquots of single samples with error = range, except at 4 hr points were n = 4, error bar = s.d., and * = p<0.05.

4.3 To examine the effect of two widely different concentrations of triglycerides on glucose clearance by both cell types (Specific Aim 3).

The last set of experiments evaluated glucose clearance by EC and SMC in the presence of normal physiologic plasma triglyceride concentrations (45 mg/dl), or slightly elevated plasma triglyceride concentrations (200 mg/dl). Graphical representations of the results are shown in Figures 6-9. The obtained measurements were also compared with appropriate baselines (glucose clearance in the absence of lipids) which have been shown to be consistent through repeated experiments. Calculated (Table 1-2), and graphical representations of differences from baselines were made, and can be found in Figure 10-17.
In the presence of normal physiologic concentrations of TGC (45 mg/dl) and glucose (70 mg/dl), the insulin treatment caused a significant increase in glucose clearance by both cell types. Interestingly, the glucose clearance by EC and arterial smooth muscle cells calculated per cell, in the absence or presence of insulin increased as compared with baselines after 1 day in TGC (Fig. 6, 10, 11). The glucose clearance further increased following the 7th day of incubation with TGC. After 14 days, the glucose clearance by both types of cells dropped, and reached values closer to baselines (Fig. 10 and 11). EC and SMC responded very similarly. This opposes the hypothesis that the glucose clearance by both cell types would remain the same as the baseline (Fig. 6A-B).

Similar results were seen for EC even when glucose was elevated to hyperglycemic levels (Fig. 7A). There was an increase in glucose clearance by EC during the 14 days of experimentation as compared with baseline in the presence or absence of insulin (Fig. 12, 13). However, in contrast to EC, the glucose clearance by SMC was not enhanced above baselines by the triglycerides at this concentration of glucose (Fig. 12).

With higher concentrations of triglycerides (200 mg/dl), and normal glycemic plasma glucose concentration 70 mg/dl, insulin treatment caused a significant increase in glucose clearance by EC on the 1st and 14th day of the experiments, while by SMC this significant increase in glucose clearance was seen on the 1st and 7th day of the experiments (Fig. 8A-B). In the absence of insulin, the glucose clearance by EC dropped during the 14 days of experimentation from well above the baseline to below it (Fig. 14). The same trend of decrease was seen in SMC during the 14 days of the experiment, but unlike the EC, there was not an initial increase above the baseline (Fig. 14, 15).
In the absence of insulin, the glucose clearance by both SMC and EC dropped below the baseline (Fig. 16). However, SMC remained sensitive to insulin whereas glucose clearance by EC was no longer significantly enhanced by the addition of insulin (Fig. 9A-B). Therefore, in these conditions both cells behaved similarly in the presence or absence of insulin except for the loss of sensitivity to insulin by EC (Fig. 16 and 17). These conditions also induced morphological changes in the SMC, including rounding up and loss of adherence, that were not seen in the absence of lipids (Fig. 1 and 18). These morphological changes were not observed in EC.
Fig. 6A. Effect of normal physiologic concentrations of triglycerides (45 mg/dl) on glucose clearance by EC at normal glycemic concentration (70 mg/dl). The averaged baseline uptake in the absence of insulin was 7.14 pg/dl/cell, and in the presence of insulin 12.88 pg/dl/cell. Error = s.d., n=4, * = p<0.05

Fig. 6B. Effect of normal physiologic concentrations of triglycerides (45 mg/dl) on glucose clearance by SMC at normal glycemic concentration (70 mg/dl). The averaged baseline uptake in the absence of insulin was 4.02 pg/dl/cell, and in the presence of insulin 9.65 pg/dl/cell. Error = s.d., n=4, * = p<0.05
Fig. 7A. Effect of normal physiologic concentrations of triglycerides (45 mg/dl) on glucose clearance by EC at hyperglycemic concentration (300 mg/dl). The averaged baseline uptake in the absence of insulin was 20.16 pg/dl/cell, and in the presence of insulin 31.18 pg/dl/cell. Error = s.d., n=4. * = p<0.05

Fig. 7B. Effect of normal physiologic concentrations of triglycerides (45 mg/dl) on glucose clearance by SMC at hyperglycemic concentration (300 mg/dl). The baseline uptake in the absence of insulin was 18.93 pg/dl/cell, and in the presence of insulin 22.83 pg/dl/cell. Error = s.d., n=4, * = p<0.05
Fig. 8A. Effect of elevated concentrations of triglycerides (200 mg/dl) on glucose clearance by EC at normal glycemic glucose concentration (70 mg/dl). The averaged baseline uptake in the absence of insulin was 7.14 pg/dl/cell, and in the presence of insulin 12.88 pg/dl/cell. Error = s.d., n=4, * = p<0.05

Fig. 8B. Effect of elevated concentrations of triglycerides (200 mg/dl) on glucose clearance by SMC at normal glycemic glucose concentration (70 mg/dl). The averaged baseline uptake in the absence of insulin was 4.02 pg/dl/cell, and in the presence of insulin 9.65 pg/dl/cell. Error = s.d., n=4, * = p<0.05
**Fig. 9A.** Effect of elevated concentrations of triglycerides (200 mg/dl) on glucose clearance by EC at hyperglycemic glucose concentration 300 mg/dl. The averaged baseline uptake in the absence of insulin was 20.16 pg/dl/cell, and in the presence of insulin 31.18 pg/dl/cell. Error = s.d., n=4, *= p<0.05

**Fig. 9B.** Effect of elevated concentrations of triglycerides (200 mg/dl) on glucose clearance by SMC at hyperglycemic glucose concentration (300 mg/dl). The averaged baseline uptake in the absence of insulin was 18.93 pg/dl/cell, and in the presence of insulin 22.83 pg/dl/cell. Error = s.d., n=4, *= p<0.05
Table 1. Averaged values (n=4) of glucose clearance by EC and SMC taken as a differences from appropriate average baselines for both cell types following incubation in 45 mg/dl TGC, initial glucose concentration 70 mg/dl and 300 mg/dl, absence and presence of insulin.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Initial Glucose Conc.</th>
<th>Insulin (Y/N)</th>
<th>Glucose clearance after 1&lt;sup&gt;st&lt;/sup&gt; day in TGC (pg/dl/cell)</th>
<th>Glucose clearance after 7&lt;sup&gt;th&lt;/sup&gt; day in TGC (pg/dl/cell)</th>
<th>Glucose clearance after 14&lt;sup&gt;th&lt;/sup&gt; day in TGC (pg/dl/cell)</th>
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</thead>
<tbody>
<tr>
<td>EC</td>
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<tr>
<td>SMC</td>
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<td>-8.83</td>
<td>21.17</td>
<td>4.67</td>
</tr>
</tbody>
</table>

Table 2. Averaged values (n=4) of glucose clearance by EC and SMC taken as a differences from appropriate average baselines for both cell types following incubation in 200 mg/dl TGC, initial glucose concentration 70 mg/dl and 300 mg/dl, absence and presence of insulin.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Initial Glucose Conc.</th>
<th>Insulin (Y/N)</th>
<th>Glucose clearance after 1&lt;sup&gt;st&lt;/sup&gt; day in TGC (pg/dl/cell)</th>
<th>Glucose clearance after 7&lt;sup&gt;th&lt;/sup&gt; day in TGC (pg/dl/cell)</th>
<th>Glucose clearance after 14&lt;sup&gt;th&lt;/sup&gt; day in TGC (pg/dl/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC</td>
<td>70 mg/dl</td>
<td>N</td>
<td>7.86</td>
<td>2.86</td>
<td>-4.64</td>
</tr>
<tr>
<td>SMC</td>
<td>70 mg/dl</td>
<td>N</td>
<td>-0.35</td>
<td>-2.22</td>
<td>-2.23</td>
</tr>
<tr>
<td>EC</td>
<td>300 mg/dl</td>
<td>N</td>
<td>21.84</td>
<td>11.84</td>
<td>-3.16</td>
</tr>
<tr>
<td>SMC</td>
<td>300 mg/dl</td>
<td>N</td>
<td>-0.93</td>
<td>-3.93</td>
<td>-15.93</td>
</tr>
<tr>
<td>EC</td>
<td>70 mg/dl</td>
<td>Y</td>
<td>27.12</td>
<td>0.12</td>
<td>10.12</td>
</tr>
<tr>
<td>SMC</td>
<td>70 mg/dl</td>
<td>Y</td>
<td>4.35</td>
<td>-2.65</td>
<td>-7.85</td>
</tr>
<tr>
<td>EC</td>
<td>300 mg/dl</td>
<td>Y</td>
<td>21.82</td>
<td>0.82</td>
<td>0</td>
</tr>
<tr>
<td>SMC</td>
<td>300 mg/dl</td>
<td>Y</td>
<td>1.17</td>
<td>14.17</td>
<td>-18.83</td>
</tr>
</tbody>
</table>
SAEC and SASMC, with 45mg/dl TGC, 70mg/dl initial glucose concentration and no insulin

![Graph showing glucose clearances by EC and SMC](image)

**Fig. 10** Glucose clearances by EC and SMC taken as differences from appropriate baselines obtained for both cells following 4hr starvation and no triglycerides present. * = p<0.05, n=4, s.d. shown in figures 6-9. The maximum change from the baseline is 5.8 fold (EC) and 3.2 fold (SMC), obtained by dividing highest or lowest value by the baseline.

SAEC and SASMC with 45mg/dl TGC, 70mg/dl initial glucose concentration, and insulin

![Graph showing glucose clearances by EC and SMC](image)

**Fig. 11** Glucose clearances by EC and SMC taken as differences from appropriate baselines obtained for both cells following 4hr starvation and no triglycerides present. * = p<0.05, n=4, s.d. shown in figures 6-9. The maximum change from the baseline is 4.5 fold (EC) and 2.3 fold (SMC).
SAEC and SASMC, with 45mg/dl TGC, 300mg/dl initial glucose concentration, and no insulin

Fig. 12 Glucose clearances by EC and SMC taken as differences from appropriate baselines obtained for both cells following 4hr starvation and no triglycerides present. * = p<0.05, n=4, s.d. shown in figures 6-9. The maximum change from the baseline is 2.57 fold (EC) and 0.92 fold (SMC).

SAEC and SASMC with 45mg/dl TGC, 300mg/dl initial glucose concentration, and insulin

Fig. 13 Glucose clearances by EC and SMC taken as differences from appropriate baselines obtained for both cells following 4hr starvation and no triglycerides present. * = p<0.05, n=4, s.d. shown in figures 6-9. The maximum change from the baseline is 2.73 fold (EC) and 1.86 fold (SMC).
Fig. 14 Glucose clearances by EC and SMC taken as differences from appropriate baselines obtained for both cells following 4hr starvation and no triglycerides present. * = p<0.05, n=4, s.d. shown in figures 6-9. The maximum change from the baseline is 2.1 fold (EC) and 0.37 fold (SMC).

Fig. 15 Glucose clearances by EC and SMC taken as differences from appropriate baselines obtained for both cells following 4hr starvation and no triglycerides present. * = p<0.05, n=4, s.d. shown in figures 6-9. The maximum change from the baseline is 3.26 fold (EC) and 0.19 fold (SMC).
SAEC and SASMC with 200mg/dl TGC, 300mg/dl initial glucose concentration, and no insulin

**Fig. 16** Glucose clearances by EC and SMC taken as differences from appropriate baselines obtained for both cells following 4hr starvation and no triglycerides present. * = p<0.05, n=4, s.d. shown in figures 6-9. The maximum change from the baseline is 2.1 fold (EC) and 0.13 fold (SMC).

SAEC and SASMC with 200mg/dl TGC, 300mg/dl initial glucose concentration and insulin

**Fig. 17** Glucose clearances by EC and SMC taken as differences from appropriate baselines obtained for both cells following 4hr starvation and no triglycerides present. * = p<0.05, n=4, s.d. shown in figures 6-9. The maximum change from the baseline is 1.82 fold (EC) and 0.13 fold (SMC).
Fig. 18. SMC from sheep following 5 day incubation in 200 mg/dl triglycerides, 40X magnification.
5.0 DISCUSSION

Diabetes is a serious endocrine and metabolic disease presenting with elevated glucose levels (hyperglycemia), and increased plasma levels of amino acids and lipids. Other important factors presently in the center of diabetic research are hyperinsulinemia and hypertriglyceridemia (9,16,53,97). There are many in vivo studies evaluating the relationships between hyperglycemia, hypertriglyceridemia and hyperinsulinemia. However the roles of these factors in in vitro models still needs to be explored.

In the present study, the effects of hypertriglyceridemia in an in vitro model using EC and SMC were evaluated. Two cell types were selected because the preliminary studies of glucose clearance from the medium by distinct cell types suggested that they have apparent differences in glucose utilization and metabolism in vitro. This corresponds to published information on the highly variable kinetics of specific glucose transporters expressed on different cell types (2,3,14,86). The main transporters in muscle and endothelial cells are GLUT 4 and GLUT 1, respectively (19,54,116,131). GLUT 4 transports glucose faster and it has a higher affinity for glucose than GLUT 1 (22,89).

Two initial glucose concentrations were selected. One was near normal (fasting) glycemic levels in sheep as well as in human (70 mg/dl), and the other was a hyperglycemic concentration (300 mg/dl). Higher concentrations were not used because the preliminary studies showed that the sensitivity of detecting the clearance of these high glucose concentrations diminished (Data not shown).
Triglycerides were also added in two different concentrations. One was a normal plasma triglyceride concentration (45 mg/dl), and the other was a slightly elevated plasma triglyceride concentration (200 mg/dl). Higher triglyceride concentrations were not selected because preliminary studies suggested that concentrations higher than 200 mg/dl were toxic to cells.

Preliminary studies also suggested that glucose starvation resulted in differences between insulin-dependent and non-insulin-dependent glucose clearance by different cells. With the different glucose starvation times selected (2, 4, and 6 hr), a statistically significant optimal period was not obtained. Instead, the 4-hr starvation time was chosen for subsequent experiments based on trends seen at high concentrations of glucose where starvation appeared to enhance the effect of insulin. The starvation time was intended to mimic clinical glucose tolerance tests, with the idea of maximizing intracellular glucose depletion and minimizing glucose transporter expression at the cell surface. In this way, glucose clearance upon addition of glucose would be driven by the glucose gradient, and the effect of insulin would be seen as additional glucose transporters were expressed on the cell surfaces in response to insulin binding.

Glucose clearance by EC (expressed on a per cell basis throughout this study) was greater than by SMC suggesting that these cell types may have different glucose requirements. This could be perhaps due to the earlier described differences in GLUT 1 and GLUT 4 transporters and their distinct affinity for glucose (22,89). Differences between the cell types diminished at the higher glucose concentration, and this may simply be a reflection of the dynamics of the particular glucose transporters expressed on these cells. However, it does suggest that the two cell types responded differently to the
increase. Insulin caused significant increases in glucose clearance in both cell types at both normal glycemic and hyperglycemic glucose concentrations.

The next set of experiments addressed the question of how the glucose clearance by EC and SMC is influenced by triglycerides. In the presence of a normal glycemic glucose concentration, addition of triglycerides at a physiologic level led to an increase in non-insulin dependent glucose clearance by both cell types. This increase was further affected by insulin (Fig. 6A-B). This enhancement effect of triglycerides could be due to an adjustment of the cells to the environment or to some other function of the lipids that affected glucose clearance. The lipids used in the study contained mixture of fatty acids. It was observed in vivo that addition of fatty acids lead to increased glucose utilization (132,133). However, at a high concentration of glucose, the normal level of triglycerides did not increase the non-insulin-dependent glucose clearance by SMC above the baseline level (Fig. 12). Similarly, after 1 day of incubation in a physiologic concentration of triglycerides, the increase of insulin-dependent glucose clearance by SMC was lost in the presence of hyperglycemic level of glucose (Fig 7B). SMC therefore appeared to be more sensitive to the increase in glucose concentration. This phenomenon is probably due to the presence of GLUT 4 transporters in smooth muscle cells that is responsible for higher affinity for glucose than GLUT 1 transporter (22,89).

When an elevated triglyceride level (200 mg/dl) was used in combination with a normal glycemic level of glucose, the non-insulin-dependent glucose clearance was no longer enhanced in either cell type (Fig. 8A and 8B). Also, the insulin effect was no longer significant for EC at day 7 (Fig.8A). This is consistent with what is observed in hypertriglyceridemia related insulin resistance in vivo (8,12,16,51,120), in which high
levels of triglycerides reduce cellular responses to insulin. Under the same conditions, although the insulin-dependent glucose clearance by SMC increased significantly on day 1 and 7, the glucose clearance dropped dramatically by day 14 (Fig. 8B).

The most intriguing results were obtained when an elevated concentration of triglycerides was used in combination with hyperglycemic plasma glucose concentration (Fig. 9A and B). Without insulin, both cell types showed the same pattern of progressively decreasing glucose clearance over the 14 days. Therefore, the main difference between the cell types under these conditions occurred in the presence of insulin, where SMC showed more insulin sensitivity than EC for the first 7 days. Data for day 14 was inconclusive for comparison between the cell types due to a loss of adherence by the SMC. The patterns of sensitivity differed between the cell types depending on triglyceride and glucose concentrations, but the EC clearly showed insulin insensitivity when both glucose and triglycerides were high. The important issue in Type II Diabetes Mellitus is the relationship between hyperglycemia, hypertriglyceridemia and insulin resistance. It has not been conclusively shown which of these three factors lead to the other two. The current study suggests that high triglyceride levels lead to insulin resistance in endothelial cells perhaps due to dysfunction of vascular endothelium that has been observed in diabetic patients (20). Interestingly, both concentrations of lipids enhanced overall glucose clearance above that seen without lipids only in the endothelial cells, whereas in SMC, this effect of lipids was only seen at the lower concentrations of both lipids and glucose.

The possibility exists that decreased glucose clearance by SMC was due to toxicity of triglycerides. It is important not to overlook the fact that the triglycerides that
were used in our model were in combination with fatty acids and other ingredients. These ingredients in the lipid emulsion could be responsible for cell toxicity; however, this does not seem to be the case since viability studies using Trypan blue suggested that the cells remained 90-95% viable. For future studies, it would be ideal if triglycerides without other ingredients could be used. There is, however, a difficulty in emulsifying triglycerides. Perhaps a liposome delivery system should be considered. Another possibility is to use plasma from patients with high triglyceride levels, which might require the use of human cells instead of sheep cells. Another explanation for some of the observed decrease in glucose clearance might have been a loss of adherence by SMC. Since they had to be nutritionally supplemented, changing of media depleted the number of cells and it was difficult to get accurate counts. Nevertheless, the loss of adherence by SMC suggests that the lipids strongly affected these cells, although not in the same way as the EC since they remained sensitive to insulin.

In in vivo systems EC cover the smooth muscle cells in vessels. These cells are exposed to constantly changing concentrations of chemicals, nutrients, and other important elements in the plasma. EC perhaps need to be able to adjust to rapidly changing insulin and glucose concentrations in the environment. On the other hand, SMC could be naïve to similar conditions. In in vitro experiments, our observations of SMC becoming intolerant may be due to the fact that these cells are no longer protected and perhaps they cannot adjust rapidly to the changes. Although the SMC were affected by high levels of lipids in terms of adherence, the EC's showed more insulin insensitivity. This insensitivity seen in EC may be a protective mechanism from changing environmental conditions that is lacking in SMC.
The \textit{in vitro} model used to measure glucose clearance did not evaluate long term effects of lipids. Perhaps long term presence of abnormally high triglyceride levels would cause dysfunction of EC, and this would increase the possibility of contact of naïve SMC with the chemically changing environment. Such an insult could perhaps lead to disease and rupturing of vessels. This is commonly seen in diabetic patients. Triglyceride concentration of 500 mg/dl could cause irreversible changes in EC and SMC already after a short time, and lead to a similar result of vessel damage.

It would be ideal to perform assays in the future using higher insulin concentrations. If these phenomena occurred or were alleviated in the presence of higher than normal plasma insulin concentration, it would suggest clues to insulin resistance in these conditions. This would give us a better understanding of the correlation between hyperlipidemia, and hyperglyceridemia and their effect on cardiovascular complications in patients with NIDDM (9,75,77). It is important to realize that this system was very simplified. It deliberately eliminated the effects of other factors including hormones (growth hormone, glucagon, cortisol,...) and other important components influencing glucose clearance \textit{in vivo} as well as influencing regulation of insulin secretion and resistance. These eliminated players should be considered for similar evaluation in the future for a better understanding of pathological basis of diabetes.

Complex models evaluating the effects of insulin, other hormones, enzymes, such as lipoprotein lipase, or combinations of these should be examined in the future. In order to evaluate the possibility of insulin resistance in the presence of triglycerides, different concentrations of insulin should be used. Much work needs to be done to understand the role of triglycerides on the function of glucose transporters corresponding to each cell.
type. More research must also be done to evaluate the significance of lipids in signaling pathways that may influence the action of insulin.

Future studies using Syndrome X animals in our model should be considered. The Syndrome X is presented with genetic hyperlipidemia, angina-like chest pain with a normal coronary arteriogram and positive exercise test. The individuals with Syndrome X are predisposed to Diabetes Mellitus and suffer from hypertension as well (9,26). In order to explore these complex interactions, cell types other than smooth muscle cells and endothelial cells should be examined. Good candidates would be hepatocytes, fibroblasts and adipocytes.

Careful interpretation of such \textit{in vitro} data is important to understand the normal and Type II diabetic \textit{in vivo} systems. Future development of a human model instead of the sheep model should be considered because of the difficulties of interpretation of results between the two species.
In conclusion, high triglyceride levels caused abnormalities in insulin-dependent glucose clearance in sheep aortic endothelial and arterial smooth muscle cells. Conditions mimicking hyperglycemia and hyperlipidemia induced a response from EC that resembled insulin resistance. These conditions also caused a loss of adherence by SMC that may have masked other significant effects. The patterns of glucose clearance in the presence of 45 mg/dl triglycerides were very similar for both cell types except when hyperglycemic glucose concentrations were used. As hypothesized, high lipid levels reduced overall glucose clearance compared to clearances seen at physiologic lipid levels. Interestingly, physiologic levels of lipids caused an enhancement of glucose clearance in both cell types that was not predicted in our stated hypothesis.
7.0 REFERENCES


a balanced overview. Diabetes Care 15:318-368.


