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**INSULIN REGULATION OF GLUCOSE TRANSPORT IN SKELETAL MUSCLE:
INFLUENCE OF CAFFEINE, ADENOSINE AND EPINEPHRINE**

A Thesis

Presented to

The Faculty of Graduate Studies

of

The University of Guelph

by

FARAH S.L. THONG

In partial fulfillment of requirements

for the degree of

Doctor of Philosophy

June, 2002

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ABSTRACT

INSULIN REGULATION OF GLUCOSE TRANSPORT IN SKELETAL MUSCLE: INFLUENCE OF CAFFEINE, ADENOSINE AND EPINEPHRINE

Farah S.L. Thong
University of Guelph, 2002

Advisor:
Dr. Terry E. Graham

This thesis investigated the putative roles of A₁ adenosine receptor (AR) and caffeine (CAF, non-selective AR antagonist) in insulin regulation of skeletal muscle glucose transport. In Study 1, reduced skeletal muscle glucose uptake (~50%) was the major determinant of CAF impairment of insulin sensitivity during physiological hyperinsulinemia (100 µU/ml) in humans. CAF did not affect insulin stimulation of muscle IRTK, IRS-1 PI3K and PBK activities, but increased plasma epinephrine and muscle cAMP concentrations, possibly indicating that this might contribute to its insulin antagonistic effects. In Study 2, CAF administered in conjunction with propranolol (β-receptor blocker) abolished the CAF-induced impairment of glucose tolerance in humans. In contrast to previous reports in rat muscle, the insulin antagonistic effects of CAF are engendered by epinephrine and not AR antagonism in human skeletal muscle. In Study 3, the effects of adenosine on 3-*O*-methylglucose transport were characterized in isolated rat soleus muscle. Following adenosine removal (ADA and AOPCP), N⁶-cyclopentyladenosine (CPA, A₁ agonist) caused a dose-dependent increase (~15%) in submaximal insulin (0.1 mU/ml), but not basal (no insulin) or maximal (10 mU/ml) insulin stimulated glucose transport. 8-cyclopentyl-1,3-dipropylxanthine (CPX, A₁ antagonist) dose-dependently inhibited the maximal stimulatory effects of CPA on insulin (0.1 mU/ml)-mediated glucose transport. These effects were likely achieved by altering translocation and not intrinsic activity of GLUT4 because addition of CPA and CPX to muscles preincubated with 10 mU/ml insulin alone (30 min) did not alter glucose

transport. These findings suggest A_1 AR mediates adenosine-insulin interaction. Since the presence of A_{2A} and A_{2B} , but not A_1 AR has been conclusively demonstrated in skeletal muscle, the possibility exists that A_2 AR might mediate adenosine-insulin interaction. The relevance of these data to humans is unknown since species difference in adenosine-insulin interaction and a lack of effect of adenosine on insulin sensitivity and muscle glucose transport in humans have previously been reported. CPA and CPX have greater affinity for binding AR than interstitial adenosine and despite their use at pharmacological concentrations, only a minor change in glucose transport (~15%) was observed. Collectively, these findings raise uncertainty as to whether adenosine is an integral component of insulin regulation of skeletal muscle glucose transport.

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June 2002
Guelph, Ontario, Canada

Farah S.L. Thong

*Oh Fee, you're trying to live a life
That's completely free
You're racing with the wind
You're flirting with death
So have a cup of coffee
And catch your breath...
(Phish, Junta, 1998)*

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LIST OF ABBREVIATIONS

Abbreviation	Definition
ADA	Adenosine deaminase
AOPCP	α,β -methylene adenosine diphosphate
AR	Adenosine receptor
AUC	Area under the curve
CAF	Caffeine
cAMP	Cyclic AMP
CPA	<i>N</i> ⁶ -cyclopentyladenosine
CPX	8-cyclopentyl-1,3-dipropylxanthine
FFA	Free fatty acid
G6P	Glucose-6-phosphate
G _i	Inhibitory G-protein
GP	Glycogen phosphorylase
G _s	Stimulatory G-protein
GS	Glycogen synthase
GSK	Glycogen synthase kinase
IR	Insulin receptor
IRS	Insulin receptor substrate
IRTK	Insulin receptor tyrosine kinase
OGTT	Oral glucose tolerance test
PDE	Phosphodiesterase
PDH	Pyruvate dehydrogenase
PDK	3'-phosphoinositide-dependent kinases
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PL	Placebo
PLC	Phospholipase C
PR	Propranolol
T2DM	Type II diabetes mellitus

CHAPTER 1. INTRODUCTION

*Here was a first principle not formally recognized by scientific methodologists:
When you run into something interesting, drop everything else and study it.
Skinner, B.F.*

Glucose represents one of the major fuel sources in mammalian cells. Skeletal muscle by virtue of its mass (~ 40% of body mass) accounts for ~ 80% of glucose disposal under insulin-stimulated conditions and is a key factor affecting glycemic levels (31). One of the central effects of insulin is the maintenance of glucose homeostasis. This is accomplished by stimulation of glucose clearance by skeletal muscle and adipose tissue and inhibition of glucose production by liver. In addition to insulin, glucose uptake and utilization in skeletal muscle are also increased by other “insulinomimetic” stimuli (exercise/contraction and hypoxia) (131). Regulation of glucose transport, metabolism or its storage as glycogen by insulin and exercise is of critical importance to maintaining glucose homeostasis and can have significant implications for patients with insulin resistance. Diminished response to insulin, but not to exercise, leading to decreased glucose transport in skeletal muscle is a major contributing factor to insulin resistance associated with obesity and Type II diabetes mellitus (T2DM). Consequently, research has intensified to elucidate the molecular and regulatory machineries involved in insulin- and exercise-mediated glucose transport in an effort to understand and to procure therapies to overcome insulin resistance in these endemic pathophysiological conditions. While significant advances have been made in recent years in defining the molecular machineries involved in insulin regulation of glucose transport, much less is known about the underlying factors that may contribute to this complex regulatory mechanism.

Adenosine is a ubiquitous substance that is produced, released and metabolized by tissues that utilize ATP as an energy source. It serves a number of important physiological functions in the body including inhibition of adipose tissue lipolysis and stimulation of insulin-mediated glucose transport in fat and heart cells. However, in skeletal muscle, these findings are less reproducible and have produced controversy as to whether adenosine receptor activation is essential to insulin stimulation of glucose transport and whether such effects are stimulatory or inhibitory.

To date, 4 adenosine receptor populations have been described and cloned – A₁, A_{2A}, A_{2B} and A₃. Adenosine has a half-life of a few seconds and is rapidly metabolized. Thus, investigation of the endogenously produced adenosine is difficult and has limited usefulness as an agonist in experimental models. Pharmacological agents that either remove adenosine, selectively activate or inhibit a subpopulation of adenosine receptors have proven to be quite useful and are routinely used to delineate adenosine receptor function. By using these approaches, it has been shown that adenosine stimulation of glucose transport and inhibition of lipolysis in fat cells are mediated by the A₁ receptor. Activation of the A₁ receptor has also been proposed to mediate adenosine stimulation of glucose transport in contracting rodent muscle in the presence (34, 64, 173) and absence (64) of insulin. However, this latter conclusion may be erroneous because these results were obtained using the A₁ receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (CPX) at concentrations that exceeded its selectivity for this receptor population. Moreover, while the A₁ receptor is expressed in abundance in adipose tissue and in heart muscle, its presence in skeletal muscle has not been conclusively demonstrated. Thus, it remains unclear whether adenosine is indeed an essential component of insulin regulation of glucose transport in skeletal muscle and if its effects are mediated by activation of the A₁ receptor.

In the 1970s, methylxanthines (caffeine, theophylline) were reported to increase blood glucose levels, insulin secretion and impair glucose tolerance in humans (156) and in rodents (29, 144). There has been a recent resurgence in interests in the effects of caffeine on glucose management in healthy humans and its implications in insulin-resistant conditions. These studies have also found that caffeine raises blood glucose levels (19), impairs insulin sensitivity (58, 84) and glucose tolerance at rest (53, 145). However, the primary target tissue accountable for the caffeine-induced impairment of insulin action remains unknown. It has generally been accepted that caffeine at “physiologically-relevant” concentrations ($\sim 40 \mu\text{M}$), which is achievable by drinking 3-4 cups of coffee or by administering 5-6 mg/kg body mediates its physiological responses by adenosine receptor antagonism. Accordingly, it has been proposed that caffeine impairs glucose transport in rodent skeletal muscle (173) and reduces insulin sensitivity (58) and glucose tolerance in humans (53) by antagonism at the A_1 receptor. This conclusion is tenuous because it was also based on results obtained with CPX at concentrations that far exceeded its selectivity for the A_1 receptor. Moreover, the mechanism of caffeine’s actions are not limited to adenosine receptor antagonism. In particular, in intact animals including humans, caffeine administration results in a simultaneous rise in plasma epinephrine levels. Epinephrine is a potent counterregulator of insulin actions in peripheral tissues and could theoretically be responsible for caffeine impairment of insulin sensitivity and glucose tolerance in humans. In support of this hypothesis, several studies have shown that the hyperglycemic and peripheral insulin resistance effects of methylxanthines are eliminated in adrenalectomized (treated with cortisol) and adrenal demedullated rodents (29, 144). However, it is not known if the insulin antagonistic effects of caffeine in humans are indeed engendered by elevated epinephrine levels.

Therefore, the aims of this doctoral thesis were: (1) to identify the target tissue responsible for caffeine impairment of insulin sensitivity and to characterize the effects of caffeine on glucose uptake, glycogen synthase (GS) activity and several key signalling intermediates in human skeletal muscle, (2) to test the hypothesis that the caffeine-induced impairment of glucose tolerance in humans is secondary to epinephrine and not to peripheral adenosine receptor antagonism in healthy humans, and (3) to examine the effects of adenosine removal, selective A₁ receptor agonist and antagonist and caffeine on glucose transport in isolated rodent skeletal muscle in the absence (basal) and presence of submaximally and maximally-effective insulin concentrations.

CHAPTER 2. REVIEW OF LITERATURE

True science teaches us to doubt and in ignorance, to refrain.
Claude Bernard

(A portion of the literature review has been published in Can J Appl Physiol, 27: 152-179, 2002)

Glucose represents a major source of metabolic fuel for mammalian cells. Blood glucose levels are maintained tightly at ~ 5 mM by a coordinated regulation of the demand-supply loop where the brain and other peripheral tissues are the consumers and the liver is the main supplier (89). The maintenance of glucose homeostasis in response to changing physiological conditions is dependent on the balance between glucose uptake, utilization (glycolysis), storage (glycogen synthesis) and production (glycogenolysis/gluconeogenesis). These processes are tightly regulated by the interplay between insulin, epinephrine and glucagon and can be altered by insulinomimetic agents and by stimuli which increase cellular energy demand such as hypoxia, exercise and other challenges to the oxidative phosphorylation process.

At rest and in the postabsorptive or fasting state, the brain with an obligatory requirement for glucose is the main consumer. Approximately half of the body's basal glucose production is accounted for by cerebral glucose uptake. Since this glucose uptake is concentration-dependent, hypoglycemia can rapidly lead to cerebral dysfunction. Thus, defense mechanisms consisting of metabolic and hormonal regulation by the key counterregulators of insulin actions (glucagon and epinephrine) are in place to prevent hypoglycemia. Following a meal, the increase in insulin secretion promotes glucose disposal and storage as glycogen by liver, skeletal muscle and adipose tissue and inhibition of hepatic glucose production. These processes are vital in averting hyperglycemia. Conversely, in the fed to fasted state, rapid degradation of glycogen and glucose production by the liver is crucial to provision of energy source and in the prevention of hypoglycemia. Of these tissues, skeletal muscle by virtue of its mass (~ 40% of body mass) accounts for ~ 80% of whole body glucose disposal under insulin-stimulated conditions and is thus a key player in maintaining glucose homeostasis (31). Skeletal muscle is also a major site of glucose uptake and utilization during physiological conditions that increase energy demand such as during moderate to high intensity exercise and in response to hypoxia (131). Glucose entry into all cells is achieved by specific membrane-bound carrier molecules - the glucose transporter (GLUT) family which facilitate glucose diffusion into cells (131). The specific properties intrinsic to the glucose transporter isoform that is expressed in each tissue as well as the regulation of the activity or availability of these transporters are critical to respond to the diversity of demands for glucose (89). The maintenance of an efficient unidirectional influx of glucose is achieved by rapid removal of free glucose and phosphorylation into glucose-6-P (G6P) in the cytosol by hexokinase or glucokinase for its subsequent metabolism (131).

2.1.1 Acute Insulin Regulation of Glucose Transport

Glucose Transporters. Until recently, 5 transporter genes and their products have been identified (89). The GLUT isoforms differ in their expression among tissues, kinetic characteristics and substrate specificity. GLUT1 is ubiquitously expressed in mammalian tissues, including skeletal muscle, but is found in abundance in the brain and erythrocytes (89). GLUT1 is responsible for basal glucose transport. GLUT2 is found in the β -pancreatic and hepatic cells and functions in the coupling of blood glucose levels to insulin secretion (89). GLUT3, with the highest affinity for glucose is expressed predominantly in neurons in adult mammals (89). GLUT4 expression is limited to insulin-responsive tissues - skeletal muscle, adipose tissue and cardiac muscle (89). GLUT5, the high affinity fructose transporter is expressed predominantly in intestine and is also expressed in skeletal muscle (89). Several novel GLUT family members have recently been described (78). Of note is GLUT12 whose expression in adult humans is limited to skeletal muscle and fat, providing evidence for the existence of other insulin-regulatable glucose transporters in these tissues (141). However, the function and characteristics of most of these novel GLUT members are still not completely understood.

GLUT4 Translocation. Glucose transport into skeletal muscle cells is achieved by GLUT1 and GLUT4 transporters. GLUT1 is localized mostly at the plasma membrane in the basal state (106). GLUT4 transporters are mostly sequestered in its preferred intracellular locations under basal conditions, but exhibits constitutive recycling to the plasma membrane (38). Insulin and exercise stimulation of glucose transport is achieved by activating GLUT4 translocation to the plasma membrane and transverse tubules (T-tubules) and possibly by restraining transporter

internalization (35, 71). In 3T3-L1 adipocytes, insulin induces GLUT4 translocation to the plasma membrane caveolae (81).

Intracellular GLUT4 vesicular compartments. Most studies have shown that insulin and other “insulinomimetic agents” recruit GLUT4 from distinct intracellular vesicular pools in skeletal muscle (37, 70, 123). However, their location(s) has yet to be clearly defined. Similarly, muscle contraction also stimulates GLUT4 translocation from 2 distinct intracellular populations – a recycling endosomal pool that is selectively recruited to the plasma membrane and another pool of storage vesicles that are recruited to the T-tubules (and possibly plasma membrane) that may represent a specialized GLUT4 storage compartment (98). These findings suggest that the intracellular GLUT4 pools that are responsive to these physiological stimuli are functionally and anatomically distinct. However, the insulin and muscle contraction regulated membrane compartments have different sedimentation coefficients, but yet, the major protein composition of these compartments appear to be similar (24).

GLUT4 Trafficking. The excursion of GLUT4 vesicles to and from the intracellular compartments and cell surface and vesicle-membrane fusion are regulated by intricate mechanisms involving intracellular traffic and vesicular fusion machinery (47). Once the GLUT 4 vesicles are at the cell surface, successful docking and fusion to the cell membrane are critical in accomplishing the final step in the glucose transport process (47). Various *N*-ethylmaleimide-sensitive fusion attachment protein receptors (SNARE protein) complexes are required for vesicle-plasma membrane fusion. The current hypothesis for this model is that interaction of the vesicle-associated (v-) SNARE, VAMP2, found in GLUT4 vesicles with their cognate

counterpart, the target-associated (t-) SNAREs, syntaxin 4 and SNAP 23 in the plasma membrane is necessary to the docking and fusion of GLUT4 vesicles (47). The SNARE-associated accessory proteins (Munc 18c, synip, Rab4 and VAP-33) provide the ancillary machinery for regulating GLUT4 traffic (47) (Fig. 1). Successful docking and fusion of GLUT4 vesicles with the plasma membrane appear to be regulated by various physiological stimuli. For instance, isoproterenol pretreatment in adipocytes (44, 172) permitted GLUT4 vesicles to dock at the plasma membrane but maintained them in an occluded conformation rendering them inaccessible, thereby lowering glucose uptake. It has also been demonstrated that an intact microtubule cytoskeleton is essential to insulin-mediated GLUT4 translocation acting both as a barrier separating docked from stored synaptic vesicles and as a facilitator of exocytosis (87).

Is translocation sufficient to account for maximal insulin-stimulated glucose transport? Insulin stimulation of glucose transport follows saturation kinetics. The increase in glucose transport in response to insulin occurs via an increase in the maximum velocity ($V_{\max}$) without any considerable changes in the Michaelis constant, K_m , (half-maximal saturation constant) (72). Increase in $V_{\max}$ is achieved by increasing the number of functional GLUT4 proteins at the cell surface and/or increasing the transporter turnover number and a concomitant decrease in the intracellular membranes (122). All studies to date (notwithstanding the techniques used) agree that GLUT4 translocation is a major mechanism by which insulin stimulates glucose transport into skeletal muscle. However, it has remained difficult to prove that translocation alone is sufficient to account for the maximal increase in insulin stimulation of glucose uptake. In addition to translocation, it has been postulated that changes in the intrinsic activity of the GLUT4 at the cell surface is subjected to regulation (158).

2.1.2 Molecular Aspects of Insulin Signalling

Insulin receptor and insulin receptor tyrosine kinase. Insulin initiates its pleiotropic effects on metabolism and cellular growth by binding to specific cell-surface receptors. The insulin receptor (IR) is expressed in most mammalian tissues although the number of receptors varies among tissues (17). In plasma membranes of adipocytes, IR are found to be localized in caveolae which appears to be crucial for IR signalling mechanisms (61). The IR is a heterotetramer ($\alpha_2\beta_2$) covalently linked through disulfide bonds (17). Insulin binding in the extracellular α -subunit initiates autophosphorylation of Tyr residues in the β -subunits. A signal is transmitted through the plasma membrane resulting in activation of insulin receptor tyrosine kinase (IRTK) located in the intracellular domain of the β -subunits. The exact mechanism by which the extracellular insulin signal is communicated through the membrane resulting in activation of IRTK is still unclear. In the basal state, phosphorylation of the IR occurs on Ser and Thr residues. Following insulin stimulation, there is an increase in both Tyr and Ser/Thr phosphorylation (17). Ser/Thr phosphorylation sites of the IR serves as potential substrates for various protein kinases, including PKC (160), cAMP-dependent protein kinase A (PKA) (153), casein kinase II (17), and insulin (160). Ser/Thr phosphorylation of IR has been associated with a decrease in IRTK activity and subsequent loss of insulin sensitivity and it has been speculated that this provides a means of “turning off” insulin action (160).

Proximal Signalling Intermediates: IRS/PI3K Pathways. Following IR autophosphorylation, the IRTK domain is catalytically active and specifically phosphorylates a number of cellular substrates on Tyr residues. Various modular docking/scaffolding proteins that are recruited to its vicinity further recruit and activate numerous key proteins, thereby assembling signal

transduction complexes. The focal point in insulin signalling mediating most of its mitogenic and metabolic effects is the IRS protein, which acts as a docking site for various downstream proteins (Fig. 1) (17). Four different isoforms of the IRS proteins have been identified, of which the IRS-1 and IRS-2 isoforms are predominantly expressed in skeletal muscle (157). Following multisite Tyr phosphorylation, all 4 IRS proteins can recruit and bind to a multitude of signalling proteins. It is not clear to what extent the various IRS proteins produce reciprocal versus redundant pathways of insulin signalling.

A key proximal effector protein that interacts with IRS and is directly involved in the metabolic actions of insulin is the lipid kinase, phosphatidylinositol-3-kinase (PI3K) (17) (Fig. 1). PI3K is a heterodimer composed of a p85 α regulatory subunit and a p110 α catalytic subunit (17). Activation of PI3K is achieved by IRS-1/2 interaction with the p85 subunit (17). Additionally, the p85 subunit of PI3K has been shown to associate directly with the IR (17). Interaction of the p85 regulatory subunit of PI3K with Tyr phosphorylated IRS activates the catalytic p110 subunit and targets PI3K to its subcellular functional sites (17). Multiple adapter subunit isoforms of PI3K in human skeletal muscle are recruited by insulin, suggesting that the different isoforms of the regulatory subunits of PI3K may provide specificity in insulin signalling (17). Activation of PI3K leads to formation of phosphorylated inositol-containing lipids, including phosphatidylinositol-3,4,5-triphosphate (PIP₃). The PI3K lipid products and phosphorylation of 3'-phosphoinositide-dependent kinases (PDK1 and PDK2) recruit signalling proteins further downstream (171) (Fig. 1).

Figure 1. Schematic representation of the insulin signalling pathways central to the regulation of glucose transport and glycogen synthase activity in skeletal muscle

Solid and dashed arrows indicate activating and inhibitory inputs, respectively. Arrows (→) represent metabolic pathways. IRTK, insulin receptor tyrosine kinase; IRS, insulin receptor substrate; PI 3-kinase, phosphatidylinositol 3-kinase; PDK, phosphoinositide-dependent kinase; GRB2, SH2 domain containing adaptor molecular that links IR activation to the ras pathway; ras, small GTP-binding protein, SOS, son of sevenless; PKB, protein kinase B; PKC, protein kinase C; MAPK, mitogen-activated protein kinase, PPIG, glycogen-associated protein phosphatase 1; PK, phosphorylase kinase; GS, glycogen synthase, GP, glycogen phosphorylase; HK, hexokinase; LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase; G6P, glucose-6-phosphate, TCA, tricarboxylic acid cycle. Some of the proposed accessory proteins involved in GLUT4 trafficking include pantophysin, VAMP-2, Rab4 which are located on the GLUT4 vesicles; Munc18, synip are bound to syntaxin 4 in basal state; SNAP-23 and GLUT4 are associated with the actin cytoskeleton.

Downstream Targets. While it is clear that activation of IRS/PI3K is essential to insulin activation of glucose transport, the downstream signalling intermediates that link IRS/PI3K activation to translocation of GLUT4 transporters are less apparent. The search for these distal signalling intermediates has led to the identification of several potential candidates. Currently, substantial attention has been directed at protein kinase B (PKB; also known as Akt), protein

kinase C (PKC) and to a lesser extent, the stress-activated p38 mitogen-activated protein kinase (MAPK) (Fig. 1). PKB/Akt (Ser/Thr kinase) is currently thought to provide an integrated mechanism in the IRS/PI3K directed signalling system in insulin activation of translocation of GLUT4 transporters (21) (Fig. 1). The atypical PKC isoforms (λ and ζ) are also involved in glucose transport stimulation (6) (Fig. 1). p38 MAPK has recently been proposed to provide an additional regulatory step, i.e., activation of GLUT4 proteins at the cell surface in L6 cells (150). At present, much controversy remains as to the specific roles of these signalling intermediates in insulin regulation of glucose transport and clearly further studies will be necessary to resolve this issue. The search for more distal targets of either PKB or PKC λ/ζ leading to GLUT4 translocation is currently in progress. It is likely that PKB provides a bifurcation point in insulin signalling cascade since it has been implicated in many of the metabolic actions of insulin including glycogen (28) and protein synthesis (111).

Additional Pathways. It is likely that other distinct but complementary pathways in addition to those outlined above are activated in response to insulin and in the regulation of glucose transport. The existence of complementary pathways in insulin-targeted signalling is supported by evidence that homozygous IRS-1 knockout mice exhibited 50% reduction in insulin-stimulated glucose disposal (2). IRS-2 has also been confirmed to be an alternative substrate of the IR in the IRS-1 knockout mice (118). It remains to be ascertained whether IRS-3 and IRS-4 are also alternate substrates to IRS-1 (177). Similarly, while it is clear that activation of PI3K is essential for insulin stimulation of GLUT4 translocation to the cell membrane, it is not sufficient to stimulate glucose uptake in insulin sensitive cells (74). Moreover, observation of a lack of insulin resistance in p85 α knockout mice also provide evidence for the existence of other linear pathways to that of

PI3K activation in mediating insulin action (162). Several “insulinomimetic agents” including exercise/muscle contraction (181), hypoxia (181), osmotic shock (18) and GTP γ S (39, 139) stimulate GLUT4 translocation/glucose transport by PI3K-independent pathways. Moreover, numerous agents that activate the heterotrimeric G-protein, G α (G $_{\alpha q}$, G $_{\alpha 11}$ and G $_{\alpha 12}$) are effective stimulators of GLUT4 translocation and glucose uptake (73, 184) in 3T3-L1 adipocytes. Collectively, these findings suggest the existence of redundancy and cross-talk in the insulin signalling cascades and activation of these distinct, parallel pathways may provide an integrative mechanism in regulating metabolic processes.

Increased Insulin Sensitivity After Exercise. Following a bout of exercise, skeletal muscle glucose transport and glycogen synthase (GS) activity are markedly enhanced in an effort to replenish glycogen stores used during exercise (14, 133). The enhanced insulin sensitivity to glucose transport appears to be limited to previously exercised muscle and is mediated by locally-induced rather than by systemic factors (134). It has been shown that translocation of a greater number of GLUT4 transporters to the cell surface in rodents (66) and in humans during an insulin clamp (163) account for increased insulin sensitivity to glucose transport. However, it is not known if the intrinsic activity of GLUT4 at the cell surface is also enhanced.

How does exercise increase muscle insulin sensitivity? The persistent effects of prior exercise on insulin sensitivity appears to be a non-specific increase in the susceptibility of the glucose transport system that is not exclusive to insulin alone (14). In this regard, it has been proposed that enhanced insulin sensitivity to glucose transport is mediated by exercise amplification of a common step in the glucose transporter system in response to both exercise and insulin (14). It is

possible that prior contractile activity simply leaves the muscle in a state that optimizes replenishment of fuel source used during exercise (14). This can be accomplished by directly or indirectly leaving the GLUT4 transporter system in a more recruitable state and/or activating or priming the GS regulatory systems (14). Reversal of GS activation after exercise is suggested to be related to the rate of glycogen resynthesis (132). In support of this hypothesis, it has been found that glycogen content is more critical in determining the subcellular localization and activity of GS than insulin and exercise *per se* (112).

The molecular mechanism(s) that mediates post-exercise insulin sensitivity to glucose transport and GS activity remains to be elucidated. Several studies that have examined changes in insulin signalling following a single bout of exercise have found that neither insulin binding to its receptor (11, 164) nor insulin receptor-mediated signalling to the level of IRTK, IRS-1 associated PI 3-kinase and glycogen synthase kinase 3- α (GSK-3 α , a GS inhibitor) (179, 180) are mechanisms by which exercise increases insulin sensitivity in skeletal muscle. Whether enhanced PKB activity links insulin stimulation to glucose transport in the post-exercise period is still unclear (163, 179).

2.2 CARBOHYDRATE METABOLISM: INFLUENCE OF ADENOSINE AND METHYLXANTHINES

Adenosine is ubiquitously produced, released and metabolized by tissues that utilize ATP as an energy source and serves many important physiological functions. In adipose tissue and the heart, adenosine has consistently been found to potentiate insulin stimulation of glucose uptake. However, in skeletal muscle, the notion that adenosine has a functional role in insulin regulation

of glucose transport is controversial. In addition, studies that have examined adenosine interaction with insulin action have produced diametrically opposing results (i.e., no effect, stimulatory and inhibitory effects). The following section summarizes the findings published to date on the effects of adenosine removal, adenosine receptor agonists and antagonists on insulin and/or contraction stimulation of glucose transport and metabolism in various insulin-sensitive tissues. This section will also include findings from studies on the effects of caffeine on insulin actions because at “physiologically relevant” concentrations, the mechanism of action of caffeine is commonly attributed to adenosine receptor antagonism (49). A brief overview of adenosine metabolism, adenosine receptor function and the pharmacological agents commonly used to study adenosine receptor function will first be presented.

2.2.1 Adenosine Metabolism

Adenosine is a purine nucleoside and is produced by tissues that utilize ATP as an energy source. In skeletal muscle, it is proposed that muscle cell, vascular endothelial cells and/or potentially nerve cells contribute to the production of adenosine (127). Adenosine is produced continuously in the intra- and extracellular compartments. The transmethylation pathway catalyzed by S-adenosylhomocysteine (SAH) hydrolase contributes to intracellular adenosine formation in various tissues (127). However, it is not known if this pathway is of any significance to intracellular adenosine production in skeletal muscle since SAH hydrolase activity/level in mouse and rabbit tissue is low (167). The intracellularly produced adenosine can be transported to the extracellular space by bi-directional saturable transporter proteins (60).

Figure 2. The potential sources of adenosine in skeletal muscle

T, adenosine transporter; *PDE*, phosphodiesterase; *5'NT*, *5'* nucleotidase; *SAHH*, *S*-adenosylhomocysteine hydrolase; *ADA*, adenosine deaminase; *PDE*, phosphodiesterase; *RBC*, red blood cells. (Adapted from (195))

5'-nucleotidase (NT), which dephosphorylates AMP to adenosine or IMP to inosine is found in 3 specific locations (membrane-bound, cytosolic and ecto-) in various cell types, but their relative distribution appears to be heterogeneous among tissues and among species (Fig. 2) (187). They also differ in their kinetics and preferential affinities for their substrates (187). The membrane-bound NT prefers AMP as a substrate while the ecto-NT has a preference for IMP. There are 2 cytosolic forms of NT which may play different roles – cNT-I breaking down AMP to adenosine and cNT-II breaking down IMP to inosine intracellularly (Fig. 2) (187).

Adenosine formation is dependent on substrate availability and the prevailing energy status of the cell. When cellular energy demand increases, such as during contraction, the capacity for dephosphorylation of AMP by *5'*-NT is enhanced due predominantly to increased extracellular

ATP hydrolysis, thereby markedly increasing adenosine concentration (Fig. 2). In the interstitial space of human skeletal muscle, adenosine concentration estimated by use of microdialysis increases from $\sim 0.2 \mu\text{M}$ at rest to $\sim 1.5 \mu\text{M}$ during exercise (67). When adenosine concentration is high in the extracellular space, it is transported into the cell (105). In the intracellular space, adenosine can then be deaminated to inosine by adenosine deaminase (ADA) or phosphorylated to AMP by adenylate kinase (50).

2.2.2 Adenosine Receptors

Adenosine must bind to cell surface receptors in order to mediate its effects. Adenosine receptors belong to the superfamily of heterotrimeric G-protein coupled receptors (116). To date, four receptor subtypes have been cloned and described – denoted as A_1 , A_{2A} , A_{2B} , A_3 . Adenosine receptors are highly homologous across species and among tissues (116). They are widely distributed in mammalian tissues, but the relative distribution and the expression level of each receptor subtype differ between tissues and likely between species (116). The A_1 receptor is most highly expressed in the brain, adrenal gland and eye and are found in intermediate levels in adipose tissue, liver, heart and thyroid gland, but not in skeletal muscle. The A_{2A} receptor is found in high levels in spleen, thymus, brain striatum and blood platelets and is also expressed in rat skeletal muscle. The A_{2B} receptor is expressed in the colon, caecum, bladder at high levels, with lower levels found in adipose tissue, liver, adrenal gland and rat skeletal muscle. The A_3 receptor is expressed in brain, adrenal, liver and in heart in low levels (116).

Are A_1 receptors present in skeletal muscle? While functional roles have been ascribed to $A_1\text{AR}$ in skeletal muscle, its presence in this tissue is uncertain. The A_{2A} and A_{2B} , but not the A_1

receptors were identified on sarcolemmal membrane in giant vesicle preparations of rodent and human skeletal muscle by use of immunohistochemistry (104). Similarly, Northern blot analysis was unable to detect A₁ receptor mRNA in rodent (130) and in human (175) skeletal muscle. In contrast, preliminary findings using western blotting indicate the presence of both A₁ and A₂ receptor populations in human and rodent skeletal muscle (59). An mRNA transcript that is distinct from that found in tissues with high A₁ receptor expression has been detected in tissues with low A₁ receptor expression, including skeletal muscle (129). While the functional significance is not known, it does suggest that A₁ receptor are expressed, but perhaps in low abundance, in skeletal muscle. Since vesicle preparations are devoid of T-tubular membranes, it is possible that A₁ receptor may be present on T-tubular membranes and was undetected in the preparations used by Lynge and Hellsten (104). Moreover, in this study, small amounts of A₁ receptor were found in muscle homogenates, which the authors attributed to the presence of endothelial cells where A₁ receptor is known to be located. However, in muscle homogenates, muscle cells are present in far greater proportion than endothelial cells thus, it is possible that the A₁ receptor reflect those present in T-tubules as well as endothelial cells. Moreover, expression of the A₁ receptor may be too low to be detected and/or the antireceptor antisera used may be poorly characterized resulting in erroneous conclusion. Thus, radioligand binding may be a more preferable method to verify the presence or absence of this adenosine receptor population in skeletal muscle.

Pharmacological tools used to study adenosine receptor functions. The endogenous agonist adenosine is of limited use as a research tool for the investigation of adenosine receptor function because of its susceptibility to extensive metabolism by a host of enzymes (half life ~ 0.6-1.5 s)

(148). Consequently, pharmacological agents that remove adenosine (ADA) activate or inhibit a selective adenosine receptor population are commonly used to delineate adenosine receptor function (Table 1). The pharmacological agents commonly used to study A₁AR-mediated responses include *N*⁶-cyclopentyladenosine (CPA) and 1,3-dipropyl-8-cyclopentylxanthine (CPX). Methylxanthine derivatives, such as caffeine and theophylline are non-selective adenosine receptor antagonists, i.e., they are nearly equipotent at the A₁ and A₂ receptors (49). However, caffeine is more potent at blocking the effects mediated by the A_{2A} than the A₁ receptor (49). The A₃ receptor is insensitive to inhibition by xanthine derivatives. There is more information available on the distribution of the A₁ and A₂ receptors in part because of the relatively good pharmacological agents that are available. At present, research on the functional role of the A_{2B} and A₃ receptors has been impeded by the lack of selective agonists for these receptors.

Table 1. Examples of several common adenosine receptor agonists and antagonists

Agonists	Selectivity
<i>R</i> -phenylisopropyladenosine (R-PIA)	non-selective: A ₁ /A ₃
<i>N</i> ⁶ -Cyclopentyladenosine (CPA)	A ₁ (A ₁ < 0.5 μM)
2-chloro- <i>N</i> ⁶ -cyclopentyladenosine (CCPA)	A ₁
5'- <i>N</i> -ethylcarboxamidoadenosine (NECA)	non-selective: A ₁ /A _{2A} /A ₃
CGS21680	A _{2A}
Antagonists	
1,3-dipropyl-8-cyclopentylxanthine (CPX)	A ₁ (A ₁ < 0.3 μM)
Caffeine	non-selective: A ₁ ~ 29–40 μM, A ₂ ~ 48 μM
Theophylline	non-selective: A ₁ ~ 8.5 μM, A ₂ ~ 25 μM

Sources of information: (49, 165)

Caffeine: Mechanisms of Actions. Caffeine is best known as a stimulant of the CNS (49). In addition to its central effects, caffeine also affects peripheral tissues as evidenced by its ability to

increase circulating catecholamines, free fatty acid (FFA), insulin, and also stimulates contraction in skeletal muscle. The proposed biochemical mechanisms (direct) include (1) competitive inhibition of A_1 and A_2 receptors, (2) inhibition of phosphodiesterases (PDE) leading to an increase in cyclic AMP (cAMP), and (3) activation of ryanodine receptor resulting in Ca^{+2} mobilization from sarcoplasmic reticulum (49) (Fig. 3). At “physiologically relevant” concentrations, adenosine receptor antagonism is the accepted mechanism that mediates the effects of caffeine (especially central/behavioural). However, because epinephrine can also have similar impacts on peripheral tissues as caffeine (for example, stimulation of lipolysis and FFA mobilization), some of the effects of caffeine on peripheral tissues might be mediated by epinephrine stimulation of β -adrenoceptors (indirect).

Figure 3. Intracellular mechanisms of caffeine actions

(Adapted from (49)). The responses(central/behavioural) elicited by caffeine at “physiologically relevant” concentrations are generally due to adenosine receptor antagonism. Although caffeine is considered a non-selective adenosine receptor antagonist, it is more potent at blocking the effects mediated by the A_{2A} than the A_1 receptor. At high μM or low mM concentrations, caffeine’s effects are elicited by inhibiting phosphodiesterases or by increasing intracellular Ca^{+2} .

2.2.3. Carbohydrate Metabolism: Influence of Adenosine and Methylxanthines

The most consistent effects demonstrated with adenosine analogs are its antilipolytic actions (via A_1 receptor) in adipocytes (69, 170) and its vasodilatory actions (via A_{2A}) in skeletal muscle (127). The notion that adenosine plays a role in insulin regulation of carbohydrate metabolism in skeletal muscle is controversial. The published findings are incongruent with reports of diametrically opposing effects of adenosine on carbohydrate metabolism in skeletal muscle.

2.2.3.1. Whole Body Effects

Activation of A_1 receptors leads to the inhibition of lipolysis in fat cells of several species, including humans (69, 170). In rodents, acute (108) and chronic (8-day) (124) treatment with agents that activate A_1 receptor (CPA and GR79236) suppressed circulating FFA and glucose. In addition, 8-day treatment with GR79236 resulted in marked improvement in glucose tolerance in both normal and fructose-fed insulin-resistant rodents (124). It has been suggested that the glucose-lowering effects of adenosine agonists may be secondary to its antilipolytic abilities since decreases in plasma glucose in response to CPA and GR79236 lagged behind the fall in plasma FFA by ~ 1h (108).

2.2.3.2. Glucose Uptake: Isolated Adipocytes and Cardiac Muscle

In adipocytes isolated from rodents (79) and from humans (69), selective A_1 receptor antagonists or removal of adenosine by ADA reduced insulin-stimulated glucose transport. In rodent adipocytes, these pharmacological agents did not alter basal glucose transport (22) (Table 2). In cardiac muscle, adenosine receptor activation increases glucose uptake in response to insulin (96).

These data do provide some evidence for a functional role for adenosine in potentiating insulin stimulation of glucose transport in adipocytes and in cardiac muscle.

2.2.3.3 Insulin Sensitivity and Skeletal Muscle Glucose Uptake/Transport

Adenosine receptor agonism and antagonism. The notion that adenosine contributes to insulin regulation of glucose transport in resting muscle is controversial since basal adenosine concentration is relatively low. In support of this hypothesis, a recent study was unable to show any effects of dipyridamole (an adenosine reuptake inhibitor) on whole body glucose disposal in resting humans (84). However, during exercise adenosine concentration increases by ~10-fold in interstitial space of human skeletal muscle (127) and may contribute to the regulation of metabolic processes in this tissue. Indeed, both CPX (A_1 receptor antagonist) and caffeine inhibited glucose uptake to similar extents in contracting perfused rat hindquarter, but this was observed only in the presence of insulin (173) (Table 2). Since the effect of adenosine receptor antagonists on glucose transport was observed only when insulin and contraction were present simultaneously, this would imply that adenosine mediates the synergistic but not the independent actions of insulin and contraction in stimulating glucose transport. In agreement, CPX has been shown to decrease contraction-mediated glucose uptake in the presence of insulin in perfused rat hindlimb (34) and in isolated soleus strips (64) (Table 2). In contrast, CPX also inhibited glucose transport when either stimulus was present in isolation implying that the effects of adenosine are not limited to the synergistic interactions between insulin and exercise (64). In the presence of 1 μ M CPA, a selective A_1 receptor agonist, maximal glucose transport in response to insulin and/or contraction was not further increased (173) (Table 2). The authors concluded that endogenously produced adenosine is adequate in potentiating the maximal effects of insulin and/or contraction

on glucose transport in skeletal muscle. Reduction in GLUT4 proteins at the cell surface (assessed by the radiolabel bis-mannose photolabel, ATB-BMPA) accounted for the CPX-induced reduction in glucose transport (64).

Table 2. Summary of findings on adenosine receptor agonists, antagonists and methylxanthines on glucose uptake/transport and metabolism

Ref.	Experimental Model	Pharmacological Agents Used and Effects
97	Isolated rodent muscle	ADA: ↑ insulin sensitivity
93	Isolated soleus muscle from sedentary and exercised rodents	2-chloroadenosine: ↑ insulin sensitivity
173	Perfused rat hindlimb	CPA, CPX: No effect on insulin or contraction CPX: ↓ insulin + contraction
34	Perfused rat hindlimb	CPX: ↓ insulin + contraction
64	Isolated soleus and epitrochlearis muscle	ADA: ↓ insulin, contraction, insulin+contraction CPA: No effect on maximal insulin stimulation CPX: ↓ insulin, contraction, insulin+contraction
108	Normal rodents	Acute CPA and GR79236: ↓ plasma glucose
124	Normal rodents	8-day GR79236: improved glucose tolerance
84	Sedentary humans + insulin clamp	Dipyridamole: no effect on insulin sensitivity
125	Humans: Exercise (75% VO ₂ peak)	THEO: No effect on Ra, ↓ Rd of glucose [†]
51	Humans: Exercise (70% VO ₂ max)	CAF: ↑ arterial glucose concentration No effect on net leg glucose uptake [‡]
145	Sedentary humans + IVGTT	CAF: ↓ glucose tolerance
84	Sedentary humans + insulin clamp	CAF: ↓ whole body glucose disposal
53	Sedentary humans + OGTT	CAF: ↓ glucose tolerance
58	Sedentary humans + insulin clamp	CAF: ↓ whole body glucose disposal
144	Normal rats + glucose infusion Adrenalectomized rats + glucose infusion	THEO: ↑ HGP, ↑ insulin, induced insulin resistance THEO: ↑ insulin, but developed hypoglycemia
110	Overweight/insulin resistant humans + insulin clamp	Adenosine infusion: No effect on forearm glucose uptake
124	Fructose-fed insulin resistant rodents	8-day GR79236: ↓ plasma glucose (improved glucose tolerance)
182	Lean and obese Zucker rats + OGTT	Acute and 6 wk BWA1433: ↑ glucose tolerance
27	Lean and obese Zucker rats + insulin clamp	1 week BWA 1433: Obese rats: ↑ glucose disposal; ↑ glucose uptake Lean rats: ↓ glucose disposal, ↓ glucose uptake

*Glucose transport was assessed by either 2-deoxyglucose or 3-O-methylglucose uptake; [†]Glucose uptake was measured as Rd using stable isotope infusion method; [‡]Leg glucose uptake was measured by direct Fick method. The abbreviations used are: EDL, extensor digitorum longus; OGTT, oral glucose tolerance test; Ra, rate of appearance; Rd, rate of disappearance; ADA, adenosine deaminase; R-PIA, N⁶-phenylisopropyl-adenosine (A₁AR agonist); CPX, 8-cyclopentyl-1,3-dipropylxanthine (A₁AR antagonist); CPA, N⁶-cyclopentyladenosine (A₁AR agonist); GR79236, N-[(1S, trans)-2-hydroxycyclopentyl]adenosine (A₁AR agonist); CAF, caffeine (non-selective antagonist); THEO, theophylline (non-selective antagonist); BWA1433, 1,3-dipropyl-8-(p-acrylic) phenylxanthine (A₁AR and A_{2B}AR antagonist).

In contrast to the studies mentioned above, Challiss and colleagues in a series of publications, have provided striking evidence for an opposing action of adenosine on insulin stimulation of glucose transport in isolated rodent muscle (12, 41) (Table 2). Using various adenosine receptor antagonists, agonists or ADA, they have consistently demonstrated that adenosine inhibits insulin stimulation of glucose transport in soleus muscle isolated from sedentary rats (12, 16, 41) and opposes the enhanced insulin sensitivity of soleus muscle isolated from rats after a single bout of exercise (93) (Table 2).

Methylxanthines. In humans, theophylline infusion reduced the rate of glucose disappearance (Rd), which the authors attributed to reduction in glucose uptake by active skeletal muscle during exercise (125) (Table 2). In contrast, when caffeine was administered prior to exercise, arterial glucose concentration was increased, but net glucose uptake by exercising leg was unaffected (51) (Table 2). In humans, caffeine decreased insulin sensitivity (58, 84) and glucose tolerance at rest (53, 145) and 12-14h following a bout of moderate exercise (Thong, FSL, unpubl observations) (Table 2). Methylxanthines have also been shown to induce glucose intolerance/insulin resistance in normal rats under insulin-stimulated conditions (29, 144). However, in adrenalectomized (treated with cortisol) and adrenal demedullated rats, the hyperglycemic (156) and peripheral insulin resistant effects (29, 144) of methylxanthines were not observed. Furthermore, because of a lack of insulin counterregulatory actions by epinephrine to the theophylline-induced increase insulin secretion, these adrenalectomized rats developed lethal hypoglycemia (144). These data suggest that in intact animals, the changes in glucose homeostasis and peripheral insulin resistance induced by methylxanthines resulted from the simultaneous activation of the sympathetic nervous system. The insulin antagonistic effects of

methylxanthines have been ascribed to catecholamine stimulation of hepatic glucose production, increased insulin secretion and a secondary inhibition of insulin-dependent glucose clearance (29, 144, 156). The methylxanthine-induced increase in insulin has been attributed to a direct stimulation of insulin release, both *in vivo* and *in vitro* (144).

2.2.3.4 Glucose and Glycogen Metabolism: Liver, Adipose tissue and Skeletal muscle

Liver and adipose tissue. Adenosine has been suggested to modify glucose/glycogen metabolism in many cell types. In rat livers, perfusion of adenosine inhibited glycogen phosphorylase activity in isolated hepatocytes and transiently increased hepatic glucose output (13). This effect is attributed to activation of A₂ receptor (13). In adipose tissue, adenosine stimulated [U-¹⁴C]glucose incorporation into glycogen, as well as basal and insulin-induced [1-¹⁴C]glucose oxidation (149).

Skeletal muscle. In skeletal muscle, numerous studies have also found that adenosine influences glucose utilization pathways within skeletal muscle. However, these findings are equally divergent as those reported on glucose transport. In insulin-stimulated muscle, the ADA-induced increase in glucose uptake was directed towards enhanced glycolysis (97). In contrast, adenosine receptor agonism increased pyruvate and lactate release and glycogen synthesis in isolated rodent soleus muscle but not in human rectus abdominus muscle (175). These data suggest species differences might exist in interactions between adenosine and insulin. Activation of A₁ receptor had no effect on [¹⁴C]glucose incorporation into glycogen in muscle isolated either from sedentary rats (16) or from rats following a bout of exercise (93). In sedentary humans, caffeine ingestion reduced carbohydrate storage and increased oxidation (assessed by indirect calorimetry)

during a euglycemic-hyperinsulinemic clamp (58). In the study by Raguso et al. (125), observations of reduced Rd of glucose during theophylline infusion in exercising humans led to the conclusion that theophylline reduced glycogenolysis in skeletal muscle. However, neither net glucose uptake nor intramuscular glycogen was quantified in this study. Moreover, the finding of a methylxanthine-induced “glycogen-sparing” during exercise in humans is not consistently supported by studies in which intramuscular glycogen was quantified (51, 95). However, caffeine ingestion prior to exercise has been shown to reduce muscle glycogenolysis over the first 15 min of exercise (152). In contracting perfused rat hindlimb, in the presence of both insulin and isoproterenol (β -adrenergic receptor agonist), caffeine or the concomitant presence of both A_1 and A_{2A} receptor antagonists exerted an exaggerated stimulation of net glycogenolysis during contraction by decreasing the fractional activity of GS (174). However, because isoproterenol was present in all conditions, it cannot be excluded that inhibition of GS activity resulted from β -adrenergic receptor stimulation.

2.2.3.5. Cellular Insulin Resistance: Adipose Tissue and Skeletal Muscle

There are several physiological conditions under which adenosine sensitivity of adipocytes, in particular to its antilipolytic effects, is altered. Amongst this is in pathological conditions associated with insulin resistance such as diabetes and obesity (80). Adipocytes isolated from massively obese humans display reduced sensitivity to the antilipolytic effects of adenosine (80) that appeared to normalize with weight reduction (83). Similarly, while the antilipolytic action of A_1 receptor agonists is normal in adipocytes isolated from lean animals, this response was markedly attenuated in fat cells isolated from obese Zucker rats (170). Acute (55) and chronic (54) incubation of rat adipocytes with A_1 agonists resulted in downregulation of adenosine

receptor and insulin resistance. Downregulation of receptor number (80), decreased receptor responsiveness to receptor binding to agonist (115) and impaired receptor coupling to G-proteins which mediate adenosine receptor actions (55) have been proposed as potential causes of attenuated antilipolytic response to adenosine in adipocytes. Overexpression of the A₁ receptor in adipose tissue of mice had no effect on glucose or insulin concentrations or on body weight compared to their lean counterparts (36). Interestingly, when the animals were fed a high fat diet, both the control and transgenic animals became equally obese and yet, unlike the control mice, the transgenic animals did not develop insulin resistance or glucose intolerance (36). The authors suggested that overexpression of A₁ receptor in adipocytes imparted a protective mechanism against the high-fat-insulin resistance diet on these animals. Overexpression of the A₁ receptor in adipocytes decreased plasma FFA levels which remained significantly lower during high fat feeding (presumably from an enhanced antilipolytic effects in adipose tissue) and could be a mechanism that protected these mice against development of insulin resistance in response to a high fat diet.

In patients with T2DM, acute theophylline administration stimulated insulin secretion that was associated with a transient decrease in endogenous glucose production (119). In overweight patients, adenosine infusion into the forearm induced vasodilation. However, despite an increase in blood flow, no further increase in muscle glucose uptake was observed (110). The authors suggested that *in vitro*, the antilipolytic action of adenosine likely accounts for the larger glucose uptake observed (110). In this regard, the authors observed a small rise in RQ with adenosine infusion. However, there were no changes in either net FFA or glycerol balances. Thus it is

difficult to conclude that a Randle cycle effect could fully explain the adenosine-induced increase in muscle glucose uptake (110).

In fructose-fed insulin resistant rodents, chronic treatment (8 d) with GR79236 (A_1 AR agonist) markedly improved intravenous glucose tolerance and reduced serum triglycerides (124). However, in obese Zucker rats, 1 week treatment with an adenosine receptor antagonist (BWA1433) improved *in vivo* glucose tolerance (182), increased skeletal muscle glucose uptake (27) and abolished the decreased sensitivity of glycolysis to insulin (15) (Table 2). Interestingly, while BWA1433 decreased glucose uptake in adipose tissue of both lean and obese rats, it elicited diametrical responses in whole body glucose disposal that were attributed to an insulin-mediated glucose uptake in skeletal muscle in lean (decreased) and obese (increased) rodents. However, glucose uptake at baseline in the obese rats was ~50% lower than the lean rats. In response to BWA1433, glucose uptake in obese rats approached that of the lean rats. It is plausible that at least in obese Zucker rats, the sum total of the differences in lean body mass to fat ratio and/or possibly an improved sensitivity to the antilipolytic actions of A_1 receptor ultimately determined the overall response in whole body insulin sensitivity.

2.2.4 Signal Transduction Mechanisms

The cellular effects of A_{2A} and A_{2B} receptors are mediated via stimulatory G-proteins (G_s), while A_1 and A_3 receptor act via inhibitory G-proteins (G_i), coupled to adenylate cyclase. The subsequent increase or decrease in cAMP initiates a complex amplification cascade of phosphorylation reactions to generate a myriad of intracellular responses (Table 3). However, A_1

receptor in several mammalian cells can also stimulate numerous adenylate cyclase/cAMP-independent effector systems (Table 3).

Table 3. G-protein coupling of adenosine receptors

Adenosine receptor	G protein	Response to G-protein coupling
A ₁	G _{i1/23}	↓ cAMP (General, CHO cells)* ↑ IP ₃ /DAG (PLC) (General/CHO cells) ↑ K _{ATP} Channels (heart) [†] ↓ Ca ²⁺ (neurons) ↓ PLA ₂ (brown fat) ↑ PLD
A _{2A}	G _s , G _{olf} G _{15/16}	↑ cAMP (General) ↑ IP ₃ (COS cells)
A _{2B}	G _s G _{q/11}	↑ cAMP (General) ↑ IP ₃ /DAG (PLC) (General, HEK 293 cells)
A ₃	G _{i2/3} G _{q/11}	↓ cAMP (General, CHO cells) ↑ IP ₃ /DAG (PLC) (CHO cells)

Sources of information: (50). *Receptor transfected cell system; †May not be physiological. The abbreviations used are: cAMP, cyclic AMP; CHO cells: Chinese Hamster Ovary cells; IP₃, inositol triphosphate; DAG, diacylglycerol; PLC, phospholipase C; PLA₂, phospholipase A₂; PLD, phospholipase D.

Adenosine activates responses mediated by A₁ and A_{2A} receptors with higher potency than it activates responses mediated by the A_{2B} and A₃ receptors (148). Since adenosine concentration in the interstitial space constitutes the physiologically active adenosine that is available to activate adenosine receptor, mechanisms that regulate interstitial adenosine levels are critical in determining adenosine-mediated physiological responses (116). Circulating adenosine is predominantly sequestered by endothelial cells in skeletal muscle, thus the endothelium is a critical regulator of the interchange of adenosine between the plasma and the interstitial space (116). In addition to adenosine activation of distinct adenosine subtypes, its ability to mediate various physiological actions in tissues are also governed by mechanisms that regulate purine nucleoside and nucleotide availability. Thus, the release, reuptake, production and metabolism of ATP, AMP, ADP and adenosine are factors that contribute to the availability of adenosine to bind

to its receptors. It is common for a single cell to simultaneously express more than one receptor population with opposing actions at the cellular level. Thus, the response to local adenosine production is in part determined by the relative expression level of each receptor population (116). Moreover, the ability of activation of adenosine receptor to couple to a specific signal transduction/effector pathway may also require expression of a threshold amount (116).

Adenosine interaction with insulin action. Is it mediated by cAMP-dependent mechanisms? It is possible that adenosine modulation of glucose transport could be linked to cAMP-dependent processes. In this regard, cAMP analogues have been shown to counter the stimulatory actions of insulin on glucose transport (85). In adipocytes, phosphorylation of GLUT4 transporter proteins by cAMP-dependent protein kinases inhibited glucose transport (76). However, the role of cAMP-dependent processes in regulating glucose transport is debatable since numerous studies have found that GLUT4 transporter phosphorylation has minimal effects on glucose transport (121). Although the exact mechanism by which cAMP inhibits insulin stimulation of glucose transport is not known, it has been postulated to occur via inhibition of PKB activity by blocking the membrane localization of PDK1 (88) (Fig. 1) and impairment of the ability of insulin to enhance cell-surface receptor binding capacity (40). The long-term inhibitory effects of cAMP was suggested to occur by downregulating GLUT 4 gene expression (185). However, the inhibitory effects of cAMP on insulin-stimulated glucose transport are equivocal since cAMP has also been shown to paradoxically activate key insulin signalling intermediates known to participate in stimulation of glucose transport (46). Similarly, epinephrine which couples to G_s protein and commonly mediates its effects via cAMP-dependent mechanisms increased GLUT4 translocation, but inhibited glucose transport in rat skeletal muscle (10). However, the inhibitory

effects of epinephrine on insulin-stimulated glucose transport occurred without any noticeable change in cAMP levels (65). These findings suggest that both cAMP-dependent and independent mechanisms are likely involved in G-protein coupled receptor interaction with insulin-mediated regulation of glucose transport (82).

Other Pathways Mediating Adenosine-Insulin Interaction. At present, adenosine stimulation of glucose transport has been suggested to occur via cAMP-independent mechanisms (64, 173) and in adipocytes, it may involve PI3K and PKB (159). In insulin-stimulated adipocytes, adenosine potentiates glucose transport by promoting an accessible/functional form of GLUT 4 at the cell surface (44, 172). Similarly, in insulin-stimulated adipocytes, catecholamines in the absence of adenosine promoted an inaccessible/occluded form of GLUT4 at the cell surface, thereby inhibiting glucose transport (44, 172). However, there is also evidence in insulin-stimulated adipocytes that suggests adenosine potentiates glucose transport by possibly altering the intrinsic activity of glucose transporters (likely GLUT 4) at the plasma membrane (90). These studies provide evidence that at least in adipocytes, there exist several cAMP-independent levels of control of insulin-stimulated glucose transport – one which modulates the translocation process and another which affects the intrinsic activity or possibly the steric conformation of GLUT4 at the plasma membrane. It is presently not known if a similar mechanism of action by adenosine or epinephrine occurs in skeletal muscle in the regulation of insulin-stimulated glucose transport. To date, only 1 study has provided evidence for interaction between adenosine receptor activation and insulin signalling cascade in skeletal muscle. Chronic treatment with the adenosine receptor antagonist, BWA1433 increased IRTK activity and lowered protein tyrosine phosphatase (PTP) activity, the purported negative regulator of insulin signalling in skeletal muscle isolated from

obese, but not from lean Zucker rats (26). Prior to BWA1433 treatment, the obese Zucker rats had higher PTP activity which the authors attributed to higher adenosine receptor function. Thus, adenosine receptor antagonism may increase skeletal muscle insulin sensitivity, at least in obese Zucker rats by decreasing PTP activity (26).

CHAPTER 3. PROPOSED RESEARCH QUESTIONS

Research is to see what everybody else has seen and to think what nobody else has thought.
Albert Szent-Györgi

It is clear from the aforementioned findings that many aspects of adenosine and caffeine interaction with insulin stimulation of glucose uptake in skeletal muscle are either poorly understood or are lacking. In particular, findings on adenosine interaction with insulin action in skeletal muscle are less reproducible than those reported in adipose tissues and heart muscle and the conclusions regarding the effects of adenosine on insulin action are diametrically opposed. The notion that adenosine has a functional role in insulin stimulation of glucose transport in resting skeletal muscle is questionable because basal adenosine concentration is relatively low. Similarly, the idea that – since caffeine at “physiologically relevant” concentrations mediates its known effects (central/behavioural) by adenosine receptor antagonism, this must also be *the* mechanism that mediates its insulin antagonistic effects– is also dubious. Finally, that activation and inhibition of A₁ receptor respectively mediates adenosine and caffeine interaction with insulin stimulation of skeletal muscle glucose transport is also tenuous since this receptor population has yet to be identified in this tissue. In this chapter, a brief analysis/perspective of the findings summarized in the previous chapter and the rationale and proposed research questions for this Ph.D. dissertation will be presented (see Fig. 4).

Figure 4. Schematic model of the proposed research studies for the Ph.D. dissertation
Solid and dashed arrows indicate stimulatory and inhibitory actions, respectively. Details of each study are outlined in text

Study 1. In intact rodents and in humans, methylxanthines decreased glucose tolerance and whole body insulin sensitivity at rest and following a single bout of moderate exercise. The majority of these experiments were performed using hyperinsulinemic clamps and oral glucose tolerance tests. While these experimental approaches do provide qualitative data on the impact of methylxanthine on whole body glucose disposal, they do not identify the tissue that accounts for the caffeine-induced impairment of insulin actions. Although it is known that skeletal muscle accounts for the majority of glucose disposal under insulin-stimulated conditions, it is not known if reduction in muscle glucose uptake is responsible for reduction in insulin sensitivity caused by caffeine ingestion. In addition, the intracellular mechanism that might mediate the inhibitory actions of caffeine on insulin action has not been identified.

Proposed research questions: Is reduction in skeletal muscle glucose uptake responsible for caffeine-induced impairment in whole body insulin sensitivity in healthy men? If so, is increased intracellular cAMP and/or downregulation of key insulin signalling intermediates responsible for mediating the inhibitory effects of caffeine on insulin actions in human skeletal muscle? (Thong et al., Diabetes 51:583-590, 2002). This study was performed in collaboration with Dr. Erik Richter in Copenhagen, Denmark

Study 2. In order to understand how caffeine impairs insulin actions, it is important to elucidate its underlying mechanism of actions. In the majority of the human studies, caffeine is usually administered at 5-6 mg/kg doses, which would elicit plasma caffeine concentrations of ~ 30-45 μM . At these concentrations, caffeine approaches the K_i of adenosine receptors suggesting that it is indeed a competitor for binding to these receptors. Methylxanthines are non-selective adenosine receptor antagonists – i.e., nearly equipotent at A_1 ($K_i \sim 29\text{-}40 \mu\text{M}$) and A_2 ($K_i \sim 48 \mu\text{M}$) receptors). Based on the finding that 70 μM caffeine and 77 μM CPX (selective A_1 antagonist) elicited similar reductions in glucose transport in perfused contracting rat hindlimb exposed to submaximal insulin (173), it has generally been hypothesized that caffeine impairs whole body insulin sensitivity (58) and glucose tolerance (53) by antagonism at the A_1 receptor. This conclusion is inaccurate because CPX was used at concentrations that exceeded its selectivity for the A_1 receptor by 200-fold (173). Moreover, caffeine is more potent at blocking the effects mediated by the A_{2A} than the A_1 receptor (49). In addition, the mechanism that mediate the physiological responses to caffeine is not limited to adenosine receptor antagonism (Fig. 3). For example, caffeine stimulates Ca^{+2} release from the sarcoplasmic reticulum (161) and inhibits PDE (49). Methylxanthines at high μM or low mM concentrations are effective inhibitors

of PDE ($K_i \sim 91 \mu\text{M}$ for inhibition of PDE in rat fat cells (147)), thereby raising intracellular cAMP levels which could mediate the insulin antagonistic effects of caffeine. In particular, methylxanthine ingestion stimulates epinephrine release and results in a concomitant rise in plasma epinephrine (51, 57). Epinephrine has been shown to effectively inhibit insulin-stimulated glucose transport, glycogen synthesis (7, 10, 65, 94) and downregulate GS activity (99). Moreover, amelioration of the caffeine-induced responses (hyperglycemia and peripheral insulin resistance) in adrenalectomized rats (29, 144, 156) supports the hypothesis that the *in vivo* insulin antagonistic effects of caffeine is mediated by elevated epinephrine. The impact of caffeine on glucose tolerance and whole body insulin sensitivity in humans could theoretically be secondary to elevated epinephrine levels and not to antagonism of skeletal muscle adenosine receptors.

Proposed research question: Is the caffeine-induced impairment in glucose tolerance mediated by epinephrine activation of β -adrenergic receptors and not by peripheral adenosine receptor antagonism? (Thong and Graham, J. Appl. Physiol, 92:2347-52, 2002)

Study 3. Perspectives on adenosine and insulin action in skeletal muscle. While diametrically opposed findings on adenosine interaction with insulin action are usually reported between research groups, it is noteworthy that the results are consistent within a specific research group. The majority of these experiments have been conducted *in vitro*, using either the perfused rat hindlimb (34, 173) or incubated muscle models (12, 16, 41, 64, 93). One could speculate that the conflicting results could have arisen from differences in the experimental models used, but yet, even when the same experimental models were used (i.e., isolated soleus muscle), the results were equally conflicting. Presumably, one would expect qualitatively similar findings, not diametrically opposing results despite different experimental models used, i.e., perfused rat

hindlimb vs. isolated soleus muscle. While it is possible that some of the conflicting findings could be attributed to differences between species, experimental models, the use of different agonists or antagonists at varying concentrations, these may not adequately account for all the differences observed. For example, 1 μ M CPA (see Table 1 for K_i values) was shown to have either no effect (64) or inhibited (16) glucose transport in the presence of a submaximal insulin concentration (50-60 μ U/ml) in soleus strips isolated from male Wistar rats. Perhaps experimental methods that are consistent within but are incongruent between the research groups (e.g., variations in size of the soleus strips, incubation temperatures and whether the soleus strips were isolated from animals prior to or following euthanasia (9)) might account for some of the diverging findings reported. In the majority of these experiments, adenosine concentration has not been measured. Since all cells continuously produce adenosine, it may not have been adequately removed despite the presence of ADA and could conceivably activate adenosine receptor, thereby producing quantitatively misleading data. However, even if adenosine was measured in the experimental medium, it may not accurately reflect the local concentration present in proximity of the receptors. Therefore, the conclusions drawn from the findings in these studies may be somewhat limited.

In skeletal muscle, evaluation of adenosine interaction with insulin action has consistently been accomplished by using pharmacological agents that either activate or inhibit adenosine receptors and/or ADA which removes extracellular adenosine. The agonists and antagonists commonly used are either A_1 -selective (R-PIA, CPA, CPX, GR79236) or are non-selective for A_1 and A_2 receptors (methylxanthines, BWA1433). The receptor population that mediates adenosine interaction with insulin action is usually not addressed in the majority of these studies. However,

in 3 studies, observations that 77 μ M CPX inhibits glucose transport in rodent muscle led to the authors to conclude that adenosine stimulation of muscle glucose transport is coupled to activation of the A₁ receptor (34, 64, 173). As previously mentioned, CPX at 77 μ M concentration exceeds its selectivity for the A₁ receptor by ~ 200-fold (K_i for A₁ receptor < 1.5 nM) and may block the actions mediated by activation of the A₂ receptor instead. Moreover, the presence of the A₁ receptor has not been conclusively demonstrated in skeletal muscle (59, 104, 130, 175).

In skeletal muscle, it is also concluded that adenosine stimulates glucose transport by altering GLUT4 translocation (64). This conclusion was based on the finding that the CPX-induced reduction in glucose transport paralleled the reduction in GLUT4 proteins at the cell surface (assessed by using the radioactive bis-mannose photolabel ATB-BMPA). However, in insulin-stimulated adipocytes, augmentation of glucose transport by adenosine has been suggested to occur by altering the docking/fusion of GLUT4 proteins, rendering them more accessible to transport glucose (44, 172) and/or possibly by increasing the intrinsic activity of GLUT4 at the cell surface (90). ATB-BMPA is commonly used to detect and quantify levels of GLUT4 transporters at the cell surface since it interacts with the substrate-binding site. However, bis-mannose photolabels only bind to active transporters and cannot determine if the intrinsic activity of those GLUT4 molecules present at the cell surface is altered by either insulin or other insulinomimetic agents. Thus, at present, it is not known if adenosine receptor activation affects the intrinsic activity and/or the conformation of GLUT4 proteins at the cell surface in skeletal muscle.

Proposed research questions: Will CPA and CPX used at concentrations within the selectivity range for the A₁ receptor affect basal, submaximal and maximal insulin stimulated glucose transport in isolated rodent skeletal muscle? If so, are these changes related to alterations in the translocation or the "intrinsic activity" of GLUT4 transporters? Does caffeine inhibit insulin-stimulated glucose transport in isolated rat muscle?

This study was performed in collaboration with Dr. David J. Dyck, Dr. John C. Shryock and Dr. Arend Bonen.

CHAPTER 4. STUDY 1: Caffeine-induced impairment of insulin action but not insulin signalling in human skeletal muscle is reduced by exercise

Proposed research questions: Is reduction in skeletal muscle glucose uptake responsible for caffeine-induced impairment in whole body insulin sensitivity in healthy men? If so, is increased intracellular cAMP and/or downregulation of key insulin signalling intermediates responsible for mediating the inhibitory effects of caffeine on insulin actions in human skeletal muscle?

This study was performed in collaboration with Dr. Erik Richter in Copenhagen, Denmark (Thong et al., Diabetes 51:583-590, 2002).

It is hypothesized that:

1. Caffeine will decrease whole body glucose disposal and that insulin-stimulated glucose uptake in rested and previously exercised skeletal muscle will account for the majority of this decline,
2. Caffeine will decrease glycogen synthase (GS) activity and reduce intramuscular glycogen storage in rested and previously exercised skeletal muscle, and

3. The caffeine-induced reduction in insulin-stimulated glucose uptake and GS activity will be accompanied by increase in intracellular cAMP and alterations in the key insulin signalling intermediates (decreased IRTK, PI3K, PKB and increased GSK-3 α) in skeletal muscle.

4.2. RESEARCH DESIGN AND METHODS

4.2.1. Subjects

Seven healthy, moderately active men were recruited to participate in this study. The subjects' age, weight, height and BMI were 26 ± 2 years, 78 ± 4 kg, 180 ± 3 cm and 24 ± 1 kg/m², respectively. This study was approved by the Ethics Committee of Copenhagen and Frederiksberg County and informed consent was obtained from each subject. The subjects were accustomed to the one-legged knee extensor apparatus before determination of peak work capacity (VO_{2peak}) via an incremental knee extensor test. They were instructed to follow a mixed diet, to abstain from caffeine-containing products and alcohol and to avoid strenuous physical activity 2 d before the experiment. On the day of the experiment, the subjects ate a light breakfast 2h before arriving at the laboratory at 7am.

4.2.2. Experimental Protocol

On 2 separate occasions, separated by 10-14d, the subjects performed 60 min of repeated (1 kick/s) one-legged knee extensor exercise alternating every 5 min at a workload eliciting 75% and 100% the knee extensor VO_{2peak}. The selection of the exercising leg was randomized. Three subjects exercised the left, while four exercised the right legs. Following the exercise, the subjects rested in a supine position. Teflon catheters were then inserted below the inguinal ligament in the femoral artery and bilateral femoral veins. A thermistor (Baxter, CA) was

inserted through each femoral venous catheter and was advanced 6–8 cm proximal to the catheter tip. The thermistors were connected to an Edslab cardiac output computer (Baxter, CA), and a Gould TA 2000 recorder (Cleveland, OH) was used to record venous temperature for blood flow measurements. For glucose and insulin infusions, catheters were inserted into an antecubital and a forearm vein, respectively.

At 2h after exercise, 5 mg/kg body weight of either dextrose (placebo, PL) or caffeine in gelatin capsules was administered to the subject in a randomized, double-blind fashion. A one-step euglycemic-hyperinsulinemic clamp was initiated 3h postexercise by an intravenous bolus injection of insulin (Actrapid, Novo Nordisk, Denmark) over 1 min (9 mU/kg). This was followed by 100-min constant infusion of insulin at 1.5 mU/min/kg. Plasma glucose was infused at a variable rate to maintain euglycemia. In this study, plasma glucose concentration during the insulin infusion was maintained at $\sim 5.2 \pm 0.1$ mM and 5.3 ± 0.1 mM in the placebo and caffeine trials, respectively. To maintain plasma K^+ concentration during the insulin clamp, an oral dose of 30 mmol KCl (Kaleorid, Leo, Denmark) was administered to each subject 15 min prior to initiating the insulin clamp. Blood samples were drawn simultaneously from the femoral arterial and both femoral vein catheters at –60 and at 0 (1h post-pill ingestion), 10, 20, 30, 50, 75 and 100 min following the initiation of the insulin clamp. Blood flow was measured in both thighs using a modified thermodilution method as previously described (135). Pneumatic cuffs were placed below the knees and were inflated to 230 mmHg during blood sampling and blood flow measurements. Bilateral muscle biopsies were taken under local anaesthesia from the vastus lateralis at 0, 30 and 100 min of insulin infusion and were quickly frozen in liquid nitrogen. The 2 muscle biopsies were obtained within 30s at each time point. Pulmonary O_2 consumption

(VO₂) and RER were measured at 0, 60 and 90 min of insulin infusion by collecting expired air in Douglas bags over a 1 min interval (RER, pulmonary and leg VO₂ data are shown in Appendix A).

4.2.3. Analytical Procedures

Plasma and blood glucose and lactate were determined in duplicates using a dual-channel glucose-lactate analyzer (YSI-2700 Select, Yellow Springs Instruments, Yellow Springs, OH). Lactate data are reported in Appendix A. Plasma insulin was measured using a radioimmunoassay method (Pharmacia, Sweden). Plasma free fatty acid was determined by a nonesterified fatty acid kit (Wako Bioproducts, Richmond VA, USA). Plasma glycerol was measured by an enzymatic method (102). Plasma catecholamines were determined in 120 µl of 0.24M ethylene glycol-bis (β-aminoethyl ether)- *N,N,N',N'*-tetraacetic acid (EGTA) and reduced glutathione (GSH)-treated plasma by an HPLC method as described by Weiker (176). Plasma caffeine concentration was analyzed by an HPLC method as previously described (58). Blood gases, hemoglobin, hematocrit and O₂ saturation in blood were analyzed using an OSM III hexometer (Radiometer, Copenhagen, Denmark) (data not shown). The expired air volume collected in the Douglas bags was measured using a Tissot spirometer. O₂ and CO₂ contents were determined by paramagnetic (Servomex) and infrared (Beckman LB-2) systems, respectively.

Muscle biopsies were freeze-dried and dissected free of blood, fat and connective tissue before measuring muscle glycogen, GS activity, glucose, G6P and cyclic AMP (cAMP), but not for measuring the insulin signalling intermediates. Muscle glycogen concentration was measured by a modified acid hydrolysis method as previously described (1). Muscle glucose and G6P were

measured using a standard fluorometric method (102). Muscle GS activity was determined by a modified method as previously described (135). GS activity was determined in the presence of 0, 0.17 and 8 mM G6P. GS activities are expressed as either % FV (fractional velocity: 100 x activity in the presence of 0.17 mM G6P divided by the activity at 8 mM G6P) or as % I-form (% G6P-independent GS) (100 x activity in the absence of G6P divided by the activity at 8 mM G6P [saturated]). GS at 100 min in the placebo trial was determined for six subjects due to analytical difficulty with this sample in one subject. Muscle cAMP was measured using a radioimmunoassay kit (Amersham Pharmacia Biotech, Uppsala, Sweden).

For determination of IRTK activity and PKB Ser⁴⁷³ phosphorylation, approximately 10 mg of frozen muscles were processed as previously described (180). Solubilized protein concentrations were determined using a bicinchoninic acid (BCA) Protein Reagent kit (Pierce Chemical) using a microtiter plate protocol at 37°C for 30 min. The chemicals used were of analytical grade from Sigma Chemical, unless otherwise stated.

IRTK activity was measured as previously described (180). For determination of IRS-1 associated PI3K and GSK-3 α , muscle biopsies weighing approximately 20-30 mg were processed and quantified according to previously described methods (180). Phosphorylation of PKB on Ser⁴⁷³ was measured as an indication of *in vivo* PKB activity since these have been shown to be highly correlated (Wojtaszewski, J.F.P., unpublished observations). Briefly, for immunoblotting, aliquot of muscle lysates containing 50 mg of protein were separated by SDS-PAGE and transferred to PDVF membranes. The PDVF membranes were blocked with 1% BSA in Tris-buffered saline containing 10 mM Tris, 50 mM NaCl and 0.05% Tween-20 was

incubated with phospho-specific serine (S473) PKB antibody (New England Biolabs, Beverly, MA). Detection was made with an alkaline phosphatase conjugated anti-rabbit IgG (Zymed). Specific bands for PI 3K and PKB were quantified using a phosphorimager (Molecular Dynamics) and GSK-3 α was quantified using a Beckman scintillation counter.

4.2.4 Calculations and Statistical Analyses

The net exchanges of glucose, lactate, insulin and oxygen were calculated using the direct Fick method by multiplying the arteriovenous differences in concentration by blood flow. Total insulin-stimulated glucose uptake was calculated as the area under the curve (AUC) over 100 min using the trapezoidal method. Leg exchanges, glucose AUC, and blood flow data are expressed per kilogram of thigh muscle mass. Lean thigh volume was estimated using the method described by Jones and Pearson (77) where skin and subcutaneous adipose tissue volumes were subtracted from the total thigh volume. Total thigh muscle mass was calculated from the volume ratio (40:60) between the quadriceps femoris and the hamstring and adductor muscles (180). Whole body and leg glucose oxidation and storage were calculated using the equations by Frayn (48). For calculations of limb glucose oxidation and storage, it is assumed that whole body RER is equivalent to leg RQ. Activities of the insulin signalling intermediates are expressed in arbitrary units relative to an insulin-stimulated standard. One, two or three-way ANOVA with or without repeated measures were used to evaluate statistical differences (SAS, Cary, NC). A Tukey post-hoc comparison was made when statistical significance was found between observations. Statistical significance was accepted at $p < 0.05$. All data presented are the means $\pm$ SE.

4.3 RESULTS

Methylxanthine Concentrations. Arterial plasma caffeine concentration 1h (time 0 min) after caffeine ingestion was higher ($p<0.05$) compared to basal and to the placebo trial and remained higher ($p<0.05$) than placebo and basal throughout the insulin clamp (Table 4). Caffeine concentrations in the placebo trial were negligible.

Table 4. Arterial plasma methylxanthine concentrations (μM) following caffeine ingestion

		Time (min)							
		-60	0	10	20	30	50	75	100
Caffeine	PL	0.43 (0.15)	0.46 (0.18)	0.42 (0.18)	0.42 (0.19)	0.44 (0.19)	0.36 (0.16)	0.32 (0.14)	0.31 (0.13)
	CAF	0.57 (0.36)	32.0* [†] (1.95)	32.0* [†] (1.85)	30.7* [†] (1.74)	29.2* [†] (1.68)	27.7* [†] (1.61)	25.4* [†] (1.40)	23.7* [†] (1.61)
Theophylline	PL	0.19 (0.09)	0.14 (0.07)	0.09 (0.05)	0.07 (0.05)	0.06 (0.05)	0.06 (0.04)	0.06 (0.04)	0.06 (0.04)
	CAF	0.17 (0.12)	0.38 (0.07)	0.52* [†] (0.15)	0.58* [†] (0.18)	0.76* [†] (0.33)	0.91* [†] (0.44)	0.78* [†] (0.28)	0.87* [†] (0.35)
Theobromine	PL	3.64 (1.08)	3.20 (1.01)	3.05 (0.91)	2.99 (0.92)	3.05 (0.82)	2.96 (0.82)	2.73 (0.72)	2.59 (0.70)
	CAF	2.80 (1.07)	3.06 (1.03)	3.12 (0.94)	3.16 (0.99)	3.09 (0.96)	3.33 (1.02)	3.26 (0.91)	3.26 (0.89)
Paraxanthine	PL	1.08 (0.67)	1.21 (0.70)	1.02 (0.56)	0.99 (0.56)	0.97 (0.57)	1.00 (0.62)	1.01 (0.62)	0.97 (0.61)
	CAF	1.44 (1.31)	5.54* [†] (0.93)	6.51* [†] (1.04)	6.99* [†] (1.01)	7.41* [†] (1.20)	8.51* [†] (1.29)	9.26* [†] (1.26)	9.87* [†] (1.49)

Values are means $\pm$ SE ($n=7$). Numbers in brackets are SE. PL, placebo; CAF, caffeine. * $p<0.05$ vs. -60 min (within trial), [†] $p<0.05$ vs. PL

Whole body glucose disposal. Arterial plasma insulin increased ($p<0.05$) immediately following the bolus injection of insulin and reached a plateau by 20 min in both the placebo and caffeine.

Plasma insulin concentrations were similar between placebo (556 ± 40 pmol/L) and caffeine (510

± 45 pmol/L) trials during the insulin clamp. Following caffeine ingestion, whole body glucose disposal was decreased ($p < 0.05$) compared to placebo as indicated by a 30% reduction in glucose infusion rates needed to maintain euglycemia (Fig. 5).

Figure 5. Glucose infusion rates during 100-min euglycemic-hyperinsulinemic clamp. Values are means $\pm$ SE ($n=7$) PL, placebo; CAF, caffeine. * $p < 0.05$ vs. PL.

Leg glucose uptake. The arteriovenous differences for glucose are from 6 subjects due to technical difficulty with femoral artery catheterization in 1 subject. Thigh insulin clearance was similar in rested and exercised legs and was not affected by caffeine ingestion (see Appendix A). In both the placebo and caffeine trials, insulin-stimulated glucose uptake by the exercised leg was significantly higher compared to the rested leg (Fig. 6). Glucose uptake in the caffeine trial was decreased ($p < 0.05$) compared to the placebo trial in both rested and exercised legs during the 100-min insulin infusion (Fig 6). Total insulin-stimulated leg glucose uptake was decreased ($p < 0.05$) by 2.59 ± 1.36 mmol/kg/100min in the rested (55%) and by 4.10 ± 1.98 mmol/kg/100 min in the previously exercised (51%) legs following caffeine ingestion (Fig. 7).

Figure 6. Insulin-stimulated glucose uptake in PL and CAF during insulin infusion
Values are means $\pm$ SEM (n=6). PL, placebo, CAF, caffeine. Glucose uptake was higher ($p < 0.05$) in the exercised vs. rested legs in PL and CAF. CAF decreased ($p < 0.05$) glucose uptake in both rested and exercised legs from 10–100 min of insulin infusion vs. PL.

Figure 7. Total ($\text{AUC}_{0-100\text{min}}$) glucose uptake in rested and exercised thighs
Values are means $\pm$ SE (n=6). PL, placebo; CAF, caffeine. * $p < 0.05$ vs. rested leg, † $p < 0.05$ vs. PL.

Thigh blood flow increased with insulin infusion and was similar between the rested and exercised leg and between placebo and caffeine trials throughout the entire duration of the insulin clamp (Table 5). Thus, the significantly higher glucose uptake by the exercised leg was due to higher glucose extraction by the previously exercised muscle. Similarly, the significantly lower glucose uptake after caffeine ingestion was due to lower glucose extraction by both the rested and previously exercised leg.

Table 5. Blood flow in rested and exercised legs during insulin infusion

	Time (min)	PL Rest	PL Ex	CAF Rest	CAF Ex
Blood flow	0	29 ± 4	29 ± 3	33 ± 5	38 ± 4
(ml/kg thigh muscle/min)	10	38 ± 7*	49 ± 12*	51 ± 6*	51 ± 6*
	20	44 ± 7*	50 ± 13*	56 ± 6*	54 ± 6*
	30	47 ± 7*	51 ± 12*	56 ± 6*	55 ± 7*
	50	49 ± 8*	54 ± 11*	60 ± 8*	64 ± 9*
	75	53 ± 10*	54 ± 10*	61 ± 9*	66 ± 10*
	100	53 ± 10*	53 ± 8*	62 ± 9*	64 ± 10*

Values are means ± SE (n=6). PL, placebo; CAF, caffeine. *p<0.05 vs. time 0 min (within leg)

Arterial FFA, Glycerol and Catecholamines. Prior to caffeine ingestion, arterial plasma FFA and glycerol were similar to the placebo trial. However, 60 min following caffeine ingestion, FFA and glycerol were significantly higher compared to the placebo trial (Table 6). Insulin infusion suppressed FFA and glycerol ($p<0.05$) in both placebo and caffeine trials, but FFA and glycerol remained significantly higher in caffeine than placebo trial until 50 and 30 min of insulin infusion, respectively (Table 6). Plasma epinephrine and norepinephrine were significantly increased following caffeine ingestion (Table 6). Insulin infusion did not affect catecholamine concentrations.

Table 6. Arterial FFA, glycerol and catecholamine concentrations during insulin infusion

		Time (min)							
		-60	0	10	20	30	50	75	100
FFA (μ M)	PL	490 (112)	524 (75)	421* (72)	238* (36)	171* (22)	128* (9)	122* (11)	117* (10)
	CAF	484 (64)	839* [†] (132)	584* [†] (47)	315* [†] (24)	218* [†] (14)	175* [†] (16)	145* (13)	143* (16)
Glycerol (μ M)	PL	51.9 (4.2)	54.6 (7.8)	42.3* (5.1)	33.0* (3.4)	34.0* (3.8)	31.8* (5.3)	33.7* (6.3)	32.4* (5.5)
	CAF	51.7 (6.3)	74.8* [†] (8.2)	59.3* [†] (6.8)	53.6* [†] (8.9)	44.3* [†] (3.8)	42.7* [†] (2.6)	37.9* (2.9)	40.5* (2.4)
Epinephrine (nM)	PL	0.19 (0.02)	0.19 (0.02)	0.22 (0.02)	0.21 (0.02)	0.23 (0.01)	0.25 (0.02)	0.21 (0.02)	0.26 (0.05)
	CAF	0.19 (0.02)	0.39* [†] (0.03)	0.44* [†] (0.02)	0.49* [†] (0.04)	0.60* [†] (0.07)	0.49* [†] (0.06)	0.43* [†] (0.04)	0.40* [†] (0.04)
Norepinephrine (pM)	PL	0.55 (0.06)	0.55 (0.06)	0.52 (0.04)	0.54 (0.04)	0.55 (0.05)	0.53 (0.03)	0.51 (0.04)	0.54 (0.05)
	CAF	0.55 (0.06)	0.65* (0.04)	0.65* [†] (0.04)	0.69* [†] (0.06)	0.71* [†] (0.06)	0.70* [†] (0.06)	0.68* [†] (0.07)	0.68* [†] (0.06)

Values are means $\pm$ SE (n=7); Numbers in brackets are SE. PL, placebo; CAF, caffeine. * $p < 0.05$ vs. -60 min (within trial); [†] $p < 0.05$ vs. PL

Muscle GS activity. GS activity, expressed as either % fractional velocity (Fig. 8a) and % I-form (Fig. 8b) was significantly higher in the exercised compared to the rested leg in both the placebo and caffeine trials before and during the insulin clamp. In the placebo trial, insulin infusion increased GS activity at 30 min in rested and exercised legs compared to basal, and remained higher ($p < 0.05$) at 100 min compared to basal in both legs. In the caffeine trial, insulin increased GS activity at 30 min, with no further detectable increase observed after 30 min of insulin infusion in both rested and exercised legs. GS activity remained higher ($p < 0.05$) in the exercised compared to rest leg in caffeine trial. Caffeine ingestion resulted in a significant reduction in GS

activity in the rested and exercised legs at all time points. The caffeine-induced reduction in GS activity was observed prior to and throughout the insulin infusion compared to the placebo trial.

Figure 8. Skeletal muscle GS activity expressed as % fractional velocity (A) and % independent-form (B) during the insulin clamp
Values are means $\pm$ SE (n=7). * p <0.05 vs. rested leg, † p <0.05 vs. PL.

Muscle glycogen, G6P and cAMP. Three hours following exercise, muscle glycogen concentration was significantly lower in the exercised compared to the rested leg in both the placebo (45% lower) and caffeine (44% lower) trials (Table 7). Insulin significantly, but modestly, increased muscle glycogen at 100 min in the exercised, but not in the rested leg in the placebo trial. Glycogen concentration in the exercised leg remained lower ($p<0.05$) compared to the rested leg in both placebo and caffeine trials at all time points. Caffeine ingestion did not alter glycogen concentration (Table 7). Neither insulin infusion nor caffeine affected muscle G6P or glucose concentrations in rested or exercised legs (Table 7). Neither insulin nor prior exercise affected muscle cAMP concentrations, whereas caffeine ingestion resulted in significantly higher, but similar muscle cAMP levels in both the rested and exercised legs (Table 7).

Table 7. Skeletal muscle glycogen and cAMP concentrations in rested and exercised legs during insulin infusion

	Time (min)	PL Rest	PL Ex	CAF Rest	CAF Ex
Glycogen (mmol glucosyl units/kg dw)	0	496 ± 35	277 ± 39*	509 ± 27	286 ± 40*
	30	507 ± 33	286 ± 30*	492 ± 36	285 ± 27*
	100	487 ± 36	310 ± 30* [†]	496 ± 40	307 ± 29*
Glucose (mM/kg dw)	0	1.4 ± 0.2	1.9 ± 0.3	2.5 ± 0.5	2.6 ± 0.4
	30	1.7 ± 0.6	1.8 ± 0.2	2.5 ± 0.6	2.0 ± 0.3
	100	1.9 ± 0.3	1.3 ± 0.3	1.9 ± 0.4	1.6 ± 0.3
G6P (mM/kg dw)	0	0.3 ± 0.1	0.2 ± 0.1	0.7 ± 0.4	0.7 ± 0.4
	30	0.3 ± 0.1	0.2 ± 0.1	0.7 ± 0.3	0.4 ± 0.2
	100	0.3 ± 0.1	0.3 ± 0.2	0.8 ± 0.4	0.4 ± 0.2
cAMP (μmol/kg dw)	0	2.8 ± 0.5	2.2 ± 0.7	3.3 ± 0.6 [‡]	3.7 ± 0.4 [‡]
	30	2.3 ± 0.4	2.5 ± 0.7	3.4 ± 0.7 [‡]	3.8 ± 0.3 [‡]
	100	2.9 ± 0.6	3.1 ± 0.5	4.1 ± 0.3 [‡]	3.4 ± 0.5 [‡]

Values are means ± SE (n=7). Data are expressed per kg dry muscle weight (dw). PL, placebo; CAF, caffeine. * $p<0.05$ vs. Rest leg (within trial), [†] $p<0.05$ vs. 0 min, [‡] $p<0.05$ vs. PL.

Insulin signalling. Basal IRTK, IRS-1 associated PI3K and GSK-3 α activities and PKB phosphorylation on Ser⁴⁷³ were similar between the rested and exercised legs and between placebo and caffeine trials. Insulin induced a significant, but similar increase in IRTK activity (~3-fold) in both the rested and exercised legs in the placebo and caffeine trials (Fig. 9).

Figure 9. Skeletal muscle IRTK activity during the insulin clamp

Values are means $\pm$ SEM ($n=7$) and are expressed in arbitrary units normalized to the number of insulin receptors. PL, placebo, CAF, caffeine.

Similarly, insulin resulted in a comparable 3-fold ($p<0.05$) increase in PI3K activity at 30 min in both rested and exercised legs and between placebo and caffeine trials (Fig. 10). At 100 min of insulin infusion in placebo, but not in caffeine trial, insulin-stimulated PI3K activity decreased ($p<0.05$) in the exercised leg and was significantly lower compared to the rested leg (Fig. 10).

Insulin infusion resulted in a significant, but a similar 2.5-fold increase in PKB phosphorylation at 30 min in rested and exercised legs in placebo and caffeine trials (Fig. 11). No further increase in PKB activity was observed after 30 min of insulin infusion.

Figure 10. Skeletal muscle IRS-1 associated PI 3K activity during the insulin clamp
 Values are means $\pm$ SE ($n=7$) and are expressed in arbitrary units. PL, placebo, CAF, caffeine

Figure 11. Skeletal muscle PKB^{Ser-473} phosphorylation during the insulin clamp
 Values are means $\pm$ SE ($n=7$) and are expressed in arbitrary units. PL, placebo, CAF, caffeine

Insulin decreased ($p<0.05$) GSK-3 α activity similarly (~50%) in both the rested and exercised legs in placebo and in caffeine trials at 30 min of insulin infusion, and no further decreases were observed thereafter (Fig. 12). Caffeine ingestion did not alter the effects of insulin on IRTK, PI3K and GSK-3 α activities and PKB phosphorylation in either the rested or the exercised legs.

Figure 12. Skeletal muscle GSK-3 α activity during the insulin clamp
Values are means $\pm$ SE (n=7). PL, placebo, CAF, caffeine

Calculated whole body and leg glucose oxidation and storage. Basal RER values were similar between placebo and caffeine trials. Insulin significantly increased RER at 90 min compared to 0 min in the placebo trial, but not in the caffeine trial (Table 9, Appendix A). From whole body glucose disposal data, pulmonary VO_2 (Table 9, Appendix A) and RER data, it is estimated that caffeine resulted in a significant reduction in carbohydrate storage but not carbohydrate oxidation compared to placebo (Fig. 13). Similarly, caffeine ingestion had no effect on glucose oxidation in both the rested and exercised legs, but reduced ($p<0.05$) glucose storage in both the rested and the exercised legs (Figs. 14a and 14b).

Figure 13. Whole body glucose storage and oxidation during insulin infusion

Values are means $\pm$ SEM ($n=7$). PL, placebo; CAF, caffeine. * $p<0.05$ vs. PL.

Figures 14. Glucose storage and oxidation in rested (A) and exercised (B) legs during 100 min of insulin infusion

Values are means $\pm$ SEM ($n=6$). Data are expressed per kg thigh muscle mass. PL, placebo; CAF, caffeine. * $p<0.05$ vs. PL.

4.4 DISCUSSION

Ingestion of caffeine at a dose equivalent to drinking 3-4 cups of coffee decreased whole body glucose disposal by ~ 30% (Fig.5) , in agreement with previously reported findings (58, 84). Moreover, it was demonstrated for the first time that the caffeine-induced reduction in insulin-stimulated glucose uptake in human skeletal muscle is a major contributor to diminished whole body glucose disposal (regression analysis of absolute change in insulin-stimulated leg glucose uptake vs. glucose infusion rates: $r^2=0.93$, $p<0.05$). In the present study, caffeine decreased glucose uptake by approximately 55% in the rested leg and 51% in the exercised leg. Yet, after caffeine ingestion, glucose uptake by exercised muscle remained 40% higher ($p<0.05$) compared to the rested leg, suggesting that the enhanced insulin sensitivity to glucose uptake in the post-exercise period was not completely abolished by caffeine, but merely that the general level of glucose uptake was decreased by caffeine ingestion. Stated differently, these results show that exercise reduces the deleterious effects of caffeine and caffeine reduces the beneficial effects of exercise on muscle insulin action. Furthermore, following caffeine ingestion, the reduction in GS activity in the exercised leg was not different compared to the rested leg in the placebo trial. Yet, GS activity was significantly higher in the exercised compared to the rested leg following caffeine ingestion. These findings suggest that the stimulatory effects of prior contractile activity on GS was not completely overridden by caffeine. Since caffeine resulted in similar relative magnitudes of inhibition in glucose uptake and GS activity in both the rested and previously exercised legs, it likely inhibited glucose uptake and GS activity by a common mechanism in both rested and exercised muscle.

A potential mechanism by which caffeine exerted its inhibitory effects on glucose uptake and GS activity in skeletal muscle is by competitively blocking adenosine binding to its receptors. In the perfused contracting rat hindlimb in the presence of insulin, caffeine at 77 μM reduced glucose uptake comparable to that observed with a selective A_1 adenosine receptor antagonist, CPX (173), suggesting that caffeine exerted its inhibitory effect by selectively blocking the A_1 adenosine receptor. A dose of 5 mg/kg of caffeine in the present study elicited plasma caffeine concentration of $\sim 30 \mu\text{M}$ which approaches the K_i for A_1 and A_2 receptors suggesting that it is a competitor for binding to adenosine receptors (49). However, while it is possible that caffeine in this study mediated its inhibitory effects on glucose uptake by blocking adenosine binding to the A_1 receptor, the presence of this receptor in skeletal muscle remains uncertain (104).

After caffeine ingestion, plasma FFA was significantly higher compared to placebo (Table 6). Numerous studies have demonstrated by use of intralipid and heparin infusion that high circulating FFA inhibits glucose uptake in skeletal muscle during insulin stimulation (86, 140). However, in the present study, while plasma FFA was higher 1h after caffeine ingestion compared to placebo, insulin infusion effectively decreased plasma FFA to that observed in placebo after 50 min, and yet, glucose uptake remained significantly lower during 100 min of insulin infusion. In addition, caffeine ingestion did not alter pulmonary RER values (Table 9, Appendix A). Intramuscular G6P and glucose concentrations were not different between placebo and caffeine trials and between the rested and exercised legs during the insulin clamp. Therefore, it is unlikely that in the present study, the inhibitory action of caffeine on skeletal muscle glucose uptake resulted from either high circulating plasma FFA or from G6P inhibition of hexokinase.

In the present study, caffeine ingestion resulted in significantly higher plasma epinephrine and intramuscular cAMP concentration. The intracellular mechanism by which caffeine inhibited glucose uptake and GS activity is likely via epinephrine stimulation of cAMP-dependent processes. Epinephrine has been shown to inhibit insulin-stimulated glucose transport and glycogen synthesis in human (7, 94) and in rodent (20, 65) skeletal muscle. The recent finding that insulin sensitivity to glucose uptake is increased in $G_s\alpha$ -knockout mice provide further evidence that $G_s\alpha$ -coupled pathways, possibly including epinephrine stimulation of G_s protein, negatively regulate insulin signalling (184). Similarly, epinephrine is known to potently counteract insulin activation of GS (117). Thus, it is likely that the inhibitory actions of caffeine on glucose uptake and GS activity in the present study were secondary to epinephrine. However, the inhibitory effects of caffeine in this study were not mediated by alterations in the known components of the insulin signalling cascade since after caffeine ingestion, the responses of IRTK, PI3K, PKB and GSK-3 α were comparable to that observed in the placebo trial. Similarly, the caffeine-induced reduction in GS activity was not accompanied by alterations in insulin-stimulated GSK-3 α activity. The observation that downregulation of GS activity after caffeine ingestion occurred prior to insulin infusion would further imply that the mechanism involved in caffeine-induced inhibition of skeletal muscle GS activity must be distinct from those activated by insulin. Further, these data also suggest that the insulin antagonistic effects of epinephrine are not mediated by downregulating the insulin signalling intermediates measured in this study. The molecular mechanism involved in the epinephrine effect is not well characterized. One possibility is that cAMP activation of PKA leads to serine/threonine phosphorylation of the insulin receptor, or possibly of different isoforms of the signalling intermediates measured in this study or in separate pathways which might decrease the ability of these molecules to be activated

by insulin. Furthermore, downregulation of GS activity could be mediated by an epinephrine-induced decreased association of GS with the glycogen targeting subunits of protein phosphatase-1 leading to GS inactivation (99).

After an acute bout of exercise, whole body glucose disposal is enhanced and this is attributed mainly to enhanced insulin sensitivity to glucose transport and glycogen metabolism in skeletal muscle (131, 133). In accordance with a number of studies (135, 179, 180), it was shown that net glucose uptake was about 45% higher in the previously exercised leg, compared to the rested leg during physiological hyperinsulinemia 3h following one-legged knee extensor exercise. This was observed regardless of whether the subjects received placebo or caffeine, and regardless of the fact that insulin induced similar increases in IRTK, IRS-1 associated PI3K and PKB in the rested and exercised legs. A decrease in insulin-stimulated IRS-1 associated PI3K activity in the exercised leg by 100 min of insulin infusion resulting in significantly lower activity compared to the rested leg was actually observed in this study, which is in accordance with previous observations (180). Similarly, GSK-3 α activity was reduced by insulin to similar extents in the rested compared to exercised leg, despite a significantly higher GS activity in the previously exercised leg, consistent with previous reports (180). Nonetheless, the findings from the present study suggest that increased insulin sensitivity of glucose transport and GS activity in the post-exercise period results from activation of signalling intermediates that are either more distal of IRTK, IRS-1 PI3K, PKB and GSK-3 α or are independent of the PI3K pathway (179, 180).

Intramuscular glycogen concentration has been suggested to play a regulatory role in determining the capacity of skeletal muscle to increase glucose uptake (33, 136). Since skeletal muscle

glycogen concentration has been shown to be inversely correlated with glucose uptake, the lower glycogen stores in previously exercised muscle could account for the higher glucose uptake (33, 136). This would also provide an explanation for the observation of higher glucose uptake in the exercised leg with lowered glycogen content following caffeine ingestion. Thus, it is possible that the enhanced insulin sensitivity to glucose transport and GS activity is merely a general response to glycogen depletion following exercise.

Despite significant decrease in GS activity in response to caffeine, intramuscular glycogen concentration was not different compared to the placebo trial. However, whole body and leg glucose storage (estimated from pulmonary RER and VO_2 data) were significantly reduced. The potential amount of glycogen that could be synthesized was estimated using the AUC leg glucose uptake data over 100 min of insulin infusion (assuming the water content of muscle is 76%), assuming that all the glucose that entered the leg was taken up by the previously exercised quadriceps muscle, is directed towards glycogen synthesis. From these estimations, a maximal increase in glycogen store of approximately 54 mmol glucosyl unit/kg dwt in quadriceps muscle in the previously exercised leg in the placebo trial would be expected. Similarly, in the caffeine trial, it is estimated that a maximal increase would be 28 mmol glucosyl unit/kg quadriceps muscle of glycogen over 100 min of insulin infusion. In the rested leg, maximal increases of 20 mmol glucose/kg dwt thigh muscle and 9 mmol glucose/kg dwt thigh muscle of glycogen could be synthesized over 100 min insulin infusion in the placebo and caffeine trials, respectively. It is highly unlikely that these modest changes in intramuscular glycogen could have been easily detectable especially given an approximately 10% variation in the glycogen assay (1) and it is likely that some glucose taken up by the hamstring muscles could be oxidized. It is also possible

that the attendant inhibition of GS and activation of GP by epinephrine resulted in no differences in intramuscular glycogen content following caffeine ingestion. Although caffeine has been found to inhibit GP in rodent skeletal muscle (143), this was achieved at caffeine concentrations far higher than that observed in the present study.

In summary, caffeine ingestion reduced whole body insulin sensitivity in healthy humans. This can be attributed to an approximately 50% reduction in glucose uptake by both rested and exercised skeletal muscle during physiological hyperinsulinemia. Caffeine also reduced GS activity in both rested and exercised muscle. These inhibitory effects of caffeine were not accompanied by alterations in either IRTK, IRS-1 associated PI3K, and GSK-3 α activities or in PKB Ser⁴⁷³ phosphorylation. The inhibitory effects of caffeine effects may be mediated by signalling pathways that are either further downstream from these insulin signalling intermediates or may be independent of this insulin signalling cascade. These effects of caffeine on insulin sensitivity and muscle glucose uptake were reduced by exercise. Caffeine increased circulating levels of epinephrine and muscle concentrations of cAMP possibly indicating that this might contribute to the insulin antagonistic effects of caffeine.

CHAPTER 5. STUDY 2: Caffeine-induced impairment in glucose tolerance is abolished by β -adrenergic receptor blockade in humans

Proposed research question: Is the caffeine-induced increase in insulin response to an oral glucose load mediated by epinephrine activation of β -adrenergic receptors and not by peripheral adenosine receptor antagonism? (Thong and Graham, J. Appl. Physiol, 92:2347-52, 2002)

It is hypothesized that:

1. Caffeine will impair glucose tolerance during the oral glucose tolerance test (OGTT)
2. Impairment of glucose tolerance by caffeine will be ameliorated by β -adrenergic receptor blocker, propranolol.

5.2 RESEARCH DESIGN AND METHODS

5.2.1. Subjects

Seven healthy, active males (age: 24 ± 1 yr, weight: 76 ± 4 kg, BMI: 23 ± 1 kg $\cdot$ m⁻²) were recruited to participate in this study. This study was approved by the University of Guelph Human Ethics Committee and informed written consent was obtained from each subject prior to the experiment. They were instructed to follow a mixed diet containing no less than 150g of carbohydrates, to abstain from caffeine-containing products, alcohol and to avoid strenuous physical activity 2 d prior to the experiment. The subjects reported to the laboratory on 4 separate occasions following an overnight fast.

5.2.2. Experimental Protocol

The subjects rested for 15 min and a catheter was inserted into an antecubital vein for blood sampling and was kept patent with a normal saline drip. A resting blood sample, heart rate (HR) and blood pressure (BP) were obtained (-105 min) and another blood sample was obtained 15 min later (-90 min). Subsequently, the subject received either 5 mg/kg body weight dextrose (placebo, PL), 5 mg/kg caffeine (CAF), 80 mg propranolol (PR) or 5 mg/kg body weight caffeine and 80 mg propranolol (CAF+PR) in a randomized, double-blind fashion. The subjects remained at rest for 90 min. A blood sample, HR and BP were obtained (0 min) and a 120-min OGTT was initiated by ingestion of 75g glucose (Trutol). The amount of dextrose administered in PL is a

small percentage (<1%) of the glucose administered for the OGTT. Resting blood samples were obtained at 15, 30, 60, 90 and 120 min following the glucose drink. To provide measures of β -blockade, HR and BP were monitored throughout the OGTT and the subjects then performed cycling exercise for 2-min at each 100W, 150W and 200W workloads following the OGTT. HR and rating of perceived exertion (RPE) were obtained at the end of each workload.

5.2.3. Analytical Procedures

Approximately 7-10 ml of blood was collected in a sodium heparinized tube. 200 μ l of whole blood was transferred to an eppendorf tube and was treated with 1 ml of 0.6 M perchloric acid (PCA) for glucose, lactate and glycerol analyses. The remainder of the blood was treated with 120 μ l of 0.24 M EGTA/glutathione for determination of catecholamines. Following centrifugation, aliquots of supernatant from the PCA treated samples and plasma from EGTA/GSH treated samples were transferred to eppendorf tubes and were stored at -20°C and -80°C, respectively, until the time of analysis. Another 7-10 ml of blood was collected in non-heparinized tube for serum FFA, insulin and C-peptide analyses. Blood samples were allowed to clot at room temperature. Samples were separated by centrifugation and serum was transferred to eppendorf tubes and were stored at -20°C until the time of analysis.

All blood metabolites and hormone concentrations were determined as the average of duplicate determinations. To minimize the effects of assay variability, samples from each subject were analysed in the same assay. Blood glucose, lactate and glycerol were analyzed according to the methods by Bergmeyer (8), Gutmann and Wahlefeld (62) and Lowry and Passoneau (102), respectively. Plasma catecholamines were determined as described in Study I (176). Serum

FFA was measured using a NEFA kit (Wako Chemicals USA, Inc.). Radioimmunoassay kits were used to measure serum insulin (Coat-a-Count Insulin, Diagnostic Products Corporation, CA) and serum C-peptide (Human C-Peptide RIA Kit, Linco Research Inc). The minimal detectable limits were 7.2 pmol/L for insulin and 0.0331 nmol/L for C-peptide. The intra- and inter-assay coefficients of variation were approximately 4 and 7%, respectively.

5.2.4. Calculations and Statistical Analyses

The area under the curve (AUC) for glucose, insulin and C-peptide concentrations during the OGTT were calculated over 120 min using the trapezoidal method. Indices of whole body insulin sensitivity during the OGTT were calculated according to the following equation proposed by Matsuda and DeFronzo (107):

$$\frac{10,000}{\sqrt{(FPG \times FPI) \times (\bar{G} \times \bar{I})}}$$

FPG, fasting plasma glucose (mM)

FPI, fasting plasma insulin (pM)

$\bar{G}$, mean plasma glucose concentration during OGTT (mM)

$\bar{I}$, mean plasma insulin concentration during OGTT (pM)

This index of whole body insulin sensitivity in OGTT calculated by using this equation is highly correlated ($r=0.73$, $p<0.0001$) with the rate of whole-body glucose disposal during the euglycemic insulin clamp (107).

Statistical analyses were performed using a one- or two-way ANOVA, with and without repeated measures, as appropriate (SAS, Cary, NC). A Tukey post-hoc comparison was made when statistical significance was found between observations. Statistical significance was accepted at $p<0.05$. Data are reported as the means $\pm$ SE.

5.3. RESULTS

HR and BP data are presented in Appendix B. Basal HR and BP were similar among groups. HR was decreased ($p<0.05$) in PR and in CAF+PR compared to PL and CAF during the OGTT. CAF resulted in significantly higher systolic BP, but not diastolic BP, compared to PL, PR and CAF+PR during the OGTT. There were no differences in diastolic BP among groups. The exercise-induced increase in HR was approximately 50% less in PR and CAF+PR compared to PL and CAF and RPE was significantly greater in PR and CAF+PR compared to PL and CAF.

Blood lactate data are presented in Appendix B. Blood glucose was similar between all 4 trials at -105 min (Fig. 15). In response to an oral glucose load, blood glucose increased similarly ($p<0.05$) during the OGTT in all trials (Fig. 15).

Figure 15. Blood glucose prior to and during 120-min OGTT

Values are means $\pm$ SEM ($n=7$). Blood glucose increased ($p<0.05$) similarly in all 4 trials in response to 75 g glucose load at 15 min and throughout the OGTT.

Insulin (Fig. 16) and C-peptide (Fig. 17) were not different at basal between trials. In response to an oral glucose load, insulin (Fig. 16) and C-peptide (Fig. 17) concentrations were significantly higher at 15 min and remained higher ($p<0.05$) for the remainder of the OGTT compared to basal. The insulin (Fig. 16) and C-peptide (Fig. 17) responses to CAF were greater from 15 min to 60 min of OGTT compared to PL, PR and CAF+PR. Insulin and C-peptide were not different compared to PL, PR or CAF+PR for the remainder of the OGTT. No differences were observed in insulin or C-peptide response to an oral glucose load in PR and CAF+PR compared to PL. The area under the insulin (Fig. 16 insert) and C-peptide (Fig. 17 insert) curves were greater in CAF compared to PL, PR and CAF+PR. No differences were observed in $AUC_{(0-120\text{min})}$ for insulin and C-peptide between PL, PR and CAF+PR. The index of whole body insulin sensitivity was reduced ($p<0.05$) following CAF ingestion compared to PL, PR and CAF+PR. No differences were observed in whole body insulin sensitivity between PL, PR and CAF+PR (Fig. 18).

Figure 16. Serum insulin prior to and during 120-min OGTT

Insert: AUC (0-120 min) insulin. Values are means $\pm$ SEM ($n=7$). Insulin was increased ($p<0.05$) in response to an oral glucose load in all trials. * $p<0.05$ vs. PL, PR and CAF+PR.

Figure 17. Serum C-peptide prior to and during 120-min OGTT

Insert: AUC (0-120 min) C-peptide. Values are means $\pm$ SEM (n=7). C-peptide was increased ($p < 0.05$) in response to an oral glucose load in all trials. * $p < 0.05$ vs. PL, PR and CAF+PR.

Figure 18. Index of whole body insulin sensitivity

Values are means $\pm$ SE (n=7). * $p < 0.05$ vs PL, PR, CAF+PR. Whole body insulin sensitivity was calculated by using the equation by Matsuda and DeFronzo (107).

FFA (Fig. 19) and glycerol (Fig. 20) were similar at -105 and -90 min in all 4 trials. CAF ingestion resulted in significant increase in FFA and glycerol at 0, 15 and 30 min. FFA and

glycerol were lower ($p<0.05$) in PR at 0 and 15 min compared to PL and CAF+PR. FFA and glycerol were similar between PL, PR and CAF+PR for the remainder of the 120 min OGTT.

No differences were observed in FFA and glycerol at any time point between PL and CAF+PR.

Figure 19. Free fatty acid concentration prior to and during 120-min OGTT

Values are means $\pm$ SE ($n=7$). FFA concentration was decreased ($p<0.05$) in response to insulin in all trials. * $p<0.05$ vs. PL, PR and CAF+PR, † $p<0.05$ vs. PL, CAF, CAF+PR

Figure 20. Glycerol concentration prior to and during 120-min OGTT

Values are means $\pm$ SEM ($n=7$). Insulin decreased glycerol concentration in all trials. * $p<0.05$ vs. PL, PR and CAF+PR, † $p<0.05$ vs. PL, CAF, CAF+PR

Plasma epinephrine concentration was elevated ($p<0.05$) after caffeine ingestion (CAF and CAF+PR) compared to PL and PR (Table 8). There were no differences in plasma epinephrine between PL and PR and between CAF and CAF+PR. Plasma norepinephrine concentration was similar between trials (Table 8). There was no effect of an oral glucose load on catecholamine concentrations.

Table 8. Plasma catecholamine concentrations prior to and during 120-min OGTT

		Time (min)						
		-90	0	15	30	60	90	120
Epinephrine (nM)	PL	0.22 (0.01)	0.24 (0.01)	0.23 (0.02)	0.25 (0.02)	0.24 (0.03)	0.24 (0.03)	0.26 (0.01)
	CAF	0.25 (0.03)	0.49 (0.07)* [†]	0.50 (0.09)* [†]	0.54 (0.08)* [†]	0.46 (0.03)* [†]	0.42 (0.03)* [†]	0.38 (0.05)* [†]
	PR	0.21 (0.03)	0.32 (0.05)	0.33 (0.07)	0.28 (0.02)	0.25 (0.02)	0.29 (0.01)	0.22 (0.02)
	CAF+PR	0.25 (0.04)	0.49* [†] (0.09)	0.59* [†] (0.09)	0.41* [†] (0.07)	0.32 [†] (0.06)	0.33 [†] (0.07)	0.34* [†] (0.04)
Norepinephrine (pM)	PL	1.85 (0.31)	1.79 (0.17)	2.20 (0.19)	2.43 (0.34)	2.25 (0.09)	2.38 (0.42)	2.15 (0.33)
	CAF	1.61 (0.09)	2.21 (0.32)	2.69 (0.42)	2.67 (0.22)	2.36 (0.22)	2.27 (0.28)	2.04 (0.31)
	PR	1.71 (0.14)	2.09 (0.14)	2.36 (0.20)	2.62 (0.20)	2.74 (0.29)	2.40 (0.19)	2.17 (0.24)
	CAF+PR	1.86 (0.37)	2.67 (0.37)	2.41 (0.27)	2.30 (0.27)	2.50 (0.35)	2.64 (0.54)	2.43 (0.47)

Values are means ± SE (in brackets) (n=5). * $p<0.05$ vs. -90 min (within trial), [†] $p<0.05$ vs. PL and PR

5.4 DISCUSSION

In the present study, caffeine administered in doses that would elicit plasma caffeine concentration of approximately 25–45 μM (see Table 4 from Study 1) (51, 58) increased insulin

response by 42% and reduced whole body insulin sensitivity index by 25% during an OGTT. These findings are in accordance with findings from Study 1 and those previously reported (53, 58, 84). Based on previous finding from Study 1 that reduction in net glucose uptake in skeletal muscle was the major determinant of the caffeine-induced reduction in whole body glucose disposal, it is reasonable to speculate that the reduced glucose tolerance following caffeine ingestion in the present study resulted from decreased insulin-dependent glucose clearance in skeletal muscle.

It has been proposed that the mechanism by which caffeine impairs glucose uptake in *in vitro* rodent experimental models (155, 173) and insulin sensitivity and glucose tolerance in humans (53, 58) is adenosine receptor antagonism. However, caffeine also stimulates epinephrine release (Table 6, Study 1) (51, 58), which is a potent inhibitor of insulin actions in peripheral tissues. It is conceivable that the caffeine-induced impairment of insulin actions could be mediated by epinephrine and not by adenosine receptor antagonism. Moreover, while a functional role for adenosine has been shown in isolated adipocytes (54, 69, 79, 90), cardiac muscle (96) and in rodent skeletal muscle (12, 34, 41, 64, 97, 173, 175), its role in insulin regulation of glucose transport in humans is presently unclear since whole body insulin sensitivity (84) and glucose uptake in the forearm (110) were unaffected by infusion of dipyridamole (an adenosine reuptake inhibitor) and adenosine, respectively. Moreover, the presence the A₁ receptor in human skeletal muscle which is proposed to mediate adenosine interaction with insulin is uncertain (104).

To test the hypothesis that reduction in insulin action *in vivo* following caffeine ingestion is mediated by elevated epinephrine levels and not by adenosine receptor antagonism, caffeine was

administered in the presence and absence of a β -adrenergic receptor blocker, propranolol. Propranolol was used because the insulin antagonistic effects of epinephrine is reportedly mediated by epinephrine stimulation of β_2 -adrenergic receptor (92). The observation of marked reduction in heart rates during the OGTT and during exercise in the propranolol trials is consistent with reduction of sympathetic tone, suggesting that β -blockade was indeed achieved in this study. When caffeine was administered in combination with propranolol, insulin and C-peptide concentrations were comparable to that observed in placebo in response to an oral glucose load, in spite of similar epinephrine levels as when caffeine was administered alone. This finding suggests that the greater insulin response to an oral glucose load in the presence of caffeine was indeed secondary to elevated epinephrine levels. A large body of evidence indicates that epinephrine by activating β -adrenergic receptors selectively induces whole body insulin resistance by transiently increasing hepatic glucose production and by impairing glucose clearance by skeletal muscle (5, 7, 32, 75, 91). It has also been found that epinephrine enhances β -cell responsitivity to glucose during an intravenous glucose tolerance test (5). In the present study, the enhanced insulin response to a glucose load following caffeine ingestion is likely elicited to counter the opposing actions of epinephrine on insulin-stimulated glucose clearance by peripheral tissues and possibly, an increase in hepatic glucose output, in an effort to maintain glucose homeostasis. Thus, these data counter the notion that the insulin antagonistic effects of caffeine *in vivo* are mediated by adenosine receptor antagonism in skeletal muscle. Instead, findings from the present study and Study 1 suggest that the caffeine impairment of insulin sensitivity and glucose tolerance is coupled to increased epinephrine production and its subsequent inhibition of insulin-mediated glucose uptake in skeletal muscle. Although the effect of epinephrine on insulin response to a glucose load *per se* was not assessed in this study, the

notion that endogenous catecholamines are implicated in the insulin antagonistic effects of caffeine is supported by previous findings that the methylxanthine-induced hyperglycemia (144, 156) and peripheral insulin resistance (144) were eliminated in adrenalectomized rats and in control rats in the presence of propranolol (156). Although neither intramuscular glucose nor G6P was measured in this study, it was shown in Study 1 (Table 7) that the caffeine-induced impairment of leg glucose uptake was not accompanied by changes in muscle glucose or G6P. Thus, epinephrine likely inhibited glucose uptake by affecting glucose transport rather than by G6P inhibition of hexokinase in skeletal muscle.

It is possible that the higher insulin response to an OGTT following caffeine ingestion resulted from a decrease in insulin clearance rather than to an increase in insulin secretion, to oppose epinephrine-induced impairment of insulin action following caffeine ingestion. Similarly, normalization of the caffeine-induced insulin response in the presence of propranolol could be secondary to inhibition of insulin secretion or stimulation of insulin clearance by some non-specific effects of propranolol. However, it is found that propranolol, in the absence of caffeine, had no effect on either insulin or C-peptide concentrations nor did it alter blood glucose levels compared to placebo in response to an oral glucose challenge. This would suggest that the effects observed when caffeine and propranolol were present concomitantly resulted from β -adrenergic receptor blockade on peripheral tissues, rather than from any non-specific effects of propranolol. The observation of a lack of effect of propranolol on blood glucose or glucose tolerance in this study is concordant with those previously reported (113, 154). Similarly, C-peptide levels paralleled by that of insulin in response to caffeine in the presence and absence of propranolol.

Based on these observations, it can be concluded that the greater insulin response to caffeine in the present study reflects altered insulin secretion rather than altered clearance.

Caffeine ingestion resulted in higher circulating FFA, likely secondary to epinephrine stimulation of lipolysis or via antagonism of the A_1 receptor. The finding that the caffeine-induced rise in circulating FFA was abolished in the presence of propranolol suggests that stimulation of lipolysis following caffeine ingestion resulted from elevated epinephrine levels. These data are in agreement with those reported in humans (168). Numerous studies have demonstrated by use of intralipid and heparin infusion that high circulating FFA inhibits glucose uptake in skeletal muscle during insulin stimulation (86, 140). However, it is unlikely that the inhibitory actions of caffeine on glucose tolerance in our study resulted from high circulating FFA. This conclusion is supported by several findings. While circulating FFA was higher 90 min following caffeine ingestion and prior to the OGTT, the increase in insulin response was not observed until 15 min following the oral glucose load. Moreover, following caffeine ingestion, the increase in insulin concentration effectively decreased FFA to that observed in placebo by 30 min of the OGTT, and yet insulin levels remained significantly higher until 90 min of the OGTT. Similarly, insulin infusion either prevented (58) or decreased the caffeine-induced rise in plasma FFA to that observed in placebo (Study 1) and yet, whole body insulin sensitivity (58) and net glucose uptake in skeletal muscle (Study 1) remained significantly lower compared to placebo during the euglycemic-hyperinsulinemic clamp.

In summary, in healthy males caffeine increases insulin response to an oral glucose challenge. It is also shown that caffeine impairment of glucose tolerance was reversed in the presence of a β -

adrenergic receptor blocker, propranolol. These data suggest that while caffeine can exert its inhibitory effects on glucose uptake *in vitro* by adenosine receptor antagonism, the insulin antagonistic effects of caffeine *in vivo* are mediated by elevated epinephrine levels and a requisite stimulation of β -adrenergic receptors. The findings from this study certainly cannot discount the potential role for adenosine receptor interaction with insulin actions in humans. Rather, these findings suggest that caffeine may not be an appropriate pharmacological tool to assess adenosine modulation of insulin actions on carbohydrate metabolism *in vivo* in light of the confounding effects elicited by the concomitant presence of epinephrine, which has profound counterregulatory effects on insulin's diverse actions in peripheral tissues.

CHAPTER 6. STUDY 3. A₁ Adenosine Receptor and Caffeine Modulation of Insulin-stimulated 3-O-Methylglucose Transport in Isolated Rodent Soleus Muscle

Proposed research questions: Will CPA and CPX at concentrations within the selectivity range for the A₁ receptor affect basal, submaximal and maximal insulin stimulated glucose transport in isolated rodent skeletal muscle? If so, are these changes related to alterations in the translocation or the "intrinsic activity" of GLUT4 transporters? Does caffeine inhibit insulin-stimulated glucose transport in isolated rat muscle?

It is hypothesized that:

1. N⁶-cyclopentyladenosine (CPA, A₁-selective agonist) and/or 1,3-dipropyl-8-cyclopentylxanthine (CPX, A₁-selective antagonist) would have no effect on either basal (no insulin) or maximal (10 mU/ml) insulin-stimulated glucose uptake
2. Adenosine removal would decrease submaximal (0.1 mU/ml) insulin-stimulated glucose transport

3. Following adenosine removal,
  - a. CPA would increase submaximal insulin-stimulated glucose transport in a dose-dependent fashion
  - b. CPX would not affect submaximal-insulin stimulated glucose transport, but would decrease the CPA-enhanced insulin-stimulated glucose transport in a dose-dependent manner,
4. Caffeine (CAF) would have no effect on submaximal insulin-stimulated glucose transport, but would decrease CPA-enhanced glucose transport, AND
5. CPA and/or CPX would have no effect on the “intrinsic activity” of glucose transporters

6.2 RESEARCH DESIGN AND METHODS

6.2.1. Materials

3-*O*-methyl-D-[1-³H]glucose and D-[1-¹⁴C]mannitol were purchased from Amersham Pharmacia Biotech. Fatty acid free bovine serum albumin (BSA) and adenosine deaminase (ADA) were obtained from Roche Molecular Biochemical. All other chemicals used were purchased from Sigma Aldrich.

6.2.2. Animals and Muscle Preparation

Male Wistar rats (Charles River, PQ) weighing approximately 128 ± 21 g (n=82) were fed standard Purina chow and water *ad libitum* and were allowed to adjust to the environment for ~5-7 days prior to the experiment. The rats were kept on a reverse 12-12h light-dark cycle. On the day of the experiment, rats were anaesthetized with 0.1 ml/100 gbody weight of pentobarbital sodium. This study was approved by the University of Guelph Animal Care Committee.

The rat soleus muscle has been used extensively for *in vitro* experiments and was selected to characterize the effects of these pharmacological agents on glucose transport in this study. The soleus muscle comprises predominantly Type I or slow twitch oxidative muscle fibres. However, the intact soleus muscle is relatively thick, thereby creating potential problems with regards to diffusion of oxygen and substrates (9). Glucose uptake has been shown to be proportional to the weight of the soleus muscle up to ~ 25-30 mg (9). Therefore, in this study, the intact soleus muscle was split longitudinally into strips with average weights of ~18-25 mg.

6.2.3. Muscle Incubation

Immediately after dissection, muscles were incubated in 2 ml well-oxygenated (95% O₂, 5% CO₂) Krebs Henseleit buffer (KHB) containing 8 mM glucose, 32 mM mannitol and 0.1% BSA and were allowed to recover for 40 min. Muscles were blotted quickly on filter paper and were transferred to vials containing 2 ml of KHB containing 2 mM pyruvate, 38 mM mannitol, 0.1% BSA, without insulin (basal) and with either 0.1 mU/ml insulin (submaximal) or 10 mU/ml insulin (maximal) (Fig. 21a). To assess the effects of adenosine removal on submaximal insulin-stimulated glucose transport, 2 U/ml adenosine deaminase (ADA) and 50 µM of α,β -methylene adenosine diphosphate (AOPCP) were added to incubation vials. Stock solutions of CPA, CPX, CAF and AOPCP were prepared in dimethylsulfoxide (DMSO) and 10-20 µl was then added to the incubation medium to obtain the appropriate concentrations. The equivalent amount of DMSO was also added to the control experiments.

To assess the interaction between A₁ receptor and submaximal and maximal insulin-stimulation of glucose transport, CPA and CPX at concentrations ranging from 0.001 µM to 1.0 µM were

added to the incubation medium. The effects of CAF on submaximal insulin-glucose transport was assessed at 70 μ M concentration. Once the maximal stimulatory concentration of CPA on insulin-stimulated glucose transport was determined, the ability of CPX (at concentrations from 0.001 μ M to 1.0 μ M) to inhibit the stimulatory effects of CPA on basal, submaximal and maximal insulin stimulated glucose transport uptake was then assessed. The ability of 70 μ M CAF to inhibit CPA-induced glucose transport was also assessed. In this study, the maximally effective stimulatory effect of CPA on glucose transport was achieved at 0.1 μ M concentration. Since adenosine is continuously produced by most cells, 2 U/ml ADA and 50 μ M AOPCP were added to all the incubation vials that contained CPA, CPX and CAF to prevent any confounding effects from endogenously produced adenosine. In the experiments that contained both CPA and either CPX or CAF, muscles were incubated with CPX or CAF for 10 min and CPA was then added after this initial incubation period. The entire incubation period was for 30 min. The vials were continuously gased (95% O₂, 5% CO₂) and shaken at 30°C throughout the entire incubation (Fig. 21a).

To assess whether the CPA and CPX affected insulin-stimulated glucose transport by altering the intrinsic activity of GLUT 4 at the cell surface, the muscles were incubated in KHB containing 10 mU/ml insulin, 2 mM pyruvate, 38 mM mannitol and 0.1% BSA for 30 min to achieve maximal GLUT4 translocation (Fig. 21b). The muscles were then transferred to 2 ml of identical buffer for 10 min to remove extracellular glucose. Following this, muscles were transferred to 2 ml KHB containing 10 mU/ml insulin, 2 mM pyruvate, 38 mM mannitol, 0.1% BSA, 2 U/ml ADA, 50 μ M AOPCP and 1 of the following: 0.1 μ M CPA, 1.0 μ M CPX, and 0.1 μ M CPA + 1.0 μ M

CPX and were incubated for an additional 10 min. In the incubations that contained 0.1 μ M CPA + 1.0 μ M CPX, CPX was added for 5 min before CPA was added to the vials.

A. To examine the effects of AR agonist/antagonists on insulin-stimulated glucose transport

<u>PREINCUBATION</u>	<u>INCUBATION</u>	<u>WASH</u>	<u>TRANSPORT</u>
40 min 8 mM glucose 32 mM mannitol 0.1% BSA	30 min 2 mM pyruvate, 38 mM mannitol 0.1% BSA (1) No insulin (2) 0.1 mU/ml insulin (3) 10 mU/ml insulin +/- Drugs +/- 2 U/ml ADA and 50 μ M AOPCP	10 min Additions same as Incubation	10 min [³ H]3MG (8 mM) ¹⁴ C-mannitol (32 mM) 0.1% BSA Same as incubation

B. To examine the effects of AR agonist/antagonists on "intrinsic activity"

<u>PREINCUBATION</u>	<u>INCUBATION</u>	<u>WASH</u>	<u>DRUG</u>	<u>TRANSPORT</u>
40 min 8 mM glucose 32 mM mannitol 0.1% BSA	30 min 2 mM pyruvate 38 mM mannitol 0.1% BSA 10 mU/ml insulin	10 min Additions same as Incubation	10 min 2 mM pyruvate 38 mM mannitol 0.1% BSA 10 mU/ml insulin 2 U/ml ADA 50 μ M AOPCP Drugs	10 min [³ H]3MG (8 mM) ¹⁴ C-mannitol (32 mM) 0.1% BSA Same as Drug

Figure 21. Schematic representation of the experimental design

Drugs refer to 0.001–1.0 μ M CPA, 0.001–1.0 μ M CPX, 0.1 μ M CPA + 0.001–1.0 μ M CPX, 70 μ M CAF, 0.1 μ M CPA + 70 μ M CAF. For basal (no insulin conditions), the experiments consisted of 0.1 μ M CPA, 1.0 μ M CPX and 0.1 μ M + 1.0 μ M CPX only. In all experiments that contained Drugs, 2 U/ml ADA and 50 μ M AOPCP were present in the incubations.

6.2.4. Measurement of glucose transport activity

Glucose transport activity was measured using the non-metabolizable glucose analog, 3-methylglucose. Following the incubations, muscles were briefly blotted on filter paper and were transferred to vials with 2 ml of identical KHB and were incubated for 10 min to remove glucose from the extracellular space. The muscles were quickly blotted and transferred to vials containing 2 ml KHB with 8 mM 3-O-methyl-D-[1-³H]glucose (1.0 μ Ci/ml), 32 mM [¹⁴C]mannitol (0.1 μ Ci/ml) and the same additions as before (Fig. 21). The reaction was stopped after 10 min by quickly blotting the soleus strips and freeze-clamping in tongs cooled to the temperature of liquid N₂. The muscles were weighed and were then digested in 500 μ l 1N NaOH. The muscle extract was neutralized with 200 μ l 1N HCl in scintillation vials containing 5 ml of scintillation fluid

(Fisher Scientific) and were counted in a Beckman scintillation counter with channels preset for simultaneous ^3H and ^{14}C counting. The amount of each isotope present in the samples was determined and this information was used to calculate the extracellular space and the intracellular concentration of 3-methylglucose (109). Total muscle water was assumed to be ~ 80% of muscle weight.

6.2.5. Evaluation of *in vitro* incubation on viability of muscle preparation

In order to evaluate the effect of the incubation period on the viability of the soleus muscles, ATP, phosphocreatine (PCr) and creatine (Cr) were measured in muscles that had been freeze-clamped in tongs cooled to the temperature of liquid N_2 immediately after dissection (control) and in muscles incubated in 2 ml KHB containing 2 mM pyruvate, 38 mM mannitol, 0.1% BSA, 10 mU/ml insulin for 30, 60 and 120 min. The vials were continuously gased (95% O_2 , 5% CO_2) and shaken at 30°C throughout the entire incubation. Following the incubation, muscles were blotted dry and freeze-clamped. The muscles were freeze-dried and stored at -80°C until the time of analyses. Muscle phosphagen concentrations were measured using a spectrophotometric method by Bergmeyer (8).

6.2.6. Statistical Analyses

Statistical analyses were performed with one way ANOVA (SAS, Cary, NC). Post-hoc comparisons were made with a Scheffé test when statistical significance was detected. Statistical significance was accepted at $p < 0.05$. All data presented are the means $\pm$ SE.

6.3 RESULTS

6.3.1 Evaluation of *in vitro* incubation on muscle viability

In muscles incubated for a period of up to 120 min, intramuscular ATP, PCr, or Cr levels remained unaltered compared to soleus muscles that were immediately freeze-clamped following dissection (0 min) (Fig. 22). This clearly shows that the muscles maintained their energy stores and that tissue oxygenation was adequate during this incubation period.

Figure 22. Time course of changes in levels of ATP, PCr and Cr in soleus muscles

Muscles were freeze-clamped immediately after dissection (0 min) or after different incubation times in KHB containing 2 mM pyruvate, 38 mM mannitol, 0.1% BSA and 10 mU/ml insulin. Values are means $\pm$ SE ($n=4-5$ muscle at each time point per group).

6.3.2. Evaluation of linearity of 3-*O*-methylglucose uptake in soleus muscle

In all experiments, muscle glucose transport was evaluated by measurements of ^3H labelled 3-*O*-methylglucose uptake. 3-methylglucose is a glucose analogue that is not metabolized in the cell and therefore allows the study of transport process *per se* independent of glucose metabolism. As shown in Fig. 23, 3-methylglucose uptake remains linear for at least 15-20 min, but begins to

deviate from linearity thereafter, which has been estimated to occur when intracellular concentration increases to above ~ 20% of the extracellular concentrations (183). Therefore, in the present study, measurement of glucose transport at 10 min is appropriate as 3-methylglucose uptake remains linear up to this time.

Figure 23. Time course of measuring 3-O-methylglucose uptake in incubated soleus muscle in the absence (basal) and presence of 10 mU/ml insulin

The dotted line indicates the predicted intracellular 3-O-methylglucose concentration if the uptake had continued to be linear. Results are expressed per ml of intracellular water. Values are means $\pm$ SE for 5 soleus muscles in each group.

6.3.3 Effects of CPA and CPX on basal glucose transport

In the absence of insulin, basal 3-methylglucose transport was 0.63 ± 0.02 $\mu\text{mol/ml/hr}$ (Fig. 24).

Addition of CPA, CPX and 0.1 μM CPA with 1.0 μM CPX had no effect on basal 3-methylglucose uptake (Fig. 24).

Figure 24. Effects of CPA and CPX on basal 3-O-methylglucose transport

Values are means $\pm$ SE (n=6 muscles per group with average weight $\sim 25 \pm 2$ mg). All treatments except control contains 2 U/ml ADA and 50 μ M AOPCP.

6.3.4. Effects of adenosine removal, CPA, CPX and CAF on submaximal insulin-stimulated glucose transport

In the presence of 0.1 mU/ml insulin, 3-methylglucose transport was increased by ~ 2 -fold (1.44 ± 0.08 μ mol/ml/hr) (Fig. 25) compared to basal. The addition of ADA and AOPCP reduced ($p < 0.05$) insulin-stimulated 3-methylglucose transport by $\sim 15\%$ (Fig. 25). 3-methylglucose transport was increased ($p < 0.05$) in a dose-dependent manner by CPA compared to treatments wherein adenosine was removed by ADA and AOPCP, but not compared to insulin alone. The maximal stimulatory effects of CPA was achieved at 0.1 μ M concentration. The relative increases in 3-methylglucose uptake elicited by 0.01-1.0 μ M CPA were negligible (2%) and $\sim 15\%$ compared to insulin in the absence and presence of ADA and AOPCP, respectively.

Figure 25. Effect of adenosine removal and CPA (A_1 agonist) on submaximal insulin (0.1 mU/ml) stimulated 3-methylglucose uptake

Values are means $\pm$ SE ($n=6-11$ muscles per group with average weight of 21 ± 2 mg). All treatments contained 0.1 mU/ml insulin. * $p < 0.05$ vs. insulin, † $p < 0.05$ vs. insulin in the presence of 2 U/ml ADA and 50 μ M AOPCP.

CPX at all concentrations did not alter insulin-stimulated 3-methylglucose transport (Fig. 26).

The stimulatory effects of 0.1 μ M CPA on glucose transport in the presence of a submaximal insulin concentration was reduced ($p < 0.05$) by CPX at 0.01-1.0 μ M concentrations (Fig. 27).

The maximal reduction ($\sim 15\%$) in CPA-induced increase in glucose transport was observed at 0.01 μ M CPX. CAF at 70 μ M had no effect ($p=0.09$) on insulin- (0.1 mU/ml) or CPA-induced 3-methylglucose uptake in the presence of 0.1 mU/ml insulin (Fig. 28).

Figure 26. Effects of the A₁-selective antagonist CPX on insulin-stimulated 3-methylglucose uptake

Values are means $\pm$ SE ($n=7-11$ muscles per group with average weight of 21 ± 3 mg). All treatments contained 0.1 mU/ml insulin. * $p<0.05$ vs. insulin

Figure 27. Inhibitory effects of CPX on CPA-induced 3-methylglucose transport

Values are means $\pm$ SE ($n=7-11$ muscles per group with average weight of 21 ± 2 mg) Each point shows the inhibition of glucose transport by CPX stimulated by 0.1 μM CPA. All treatments contained 0.1 mU/ml insulin, 0.1 μM CPA, 2 U/ml ADA and 50 μM AOPCP. * $p<0.05$ vs. 0.1 μM CPA.

Figure 28. Effects of caffeine on 3-methylglucose transport in response to insulin and CPA
Values are means $\pm$ SE ($n=6-11$ muscles per group with average weight of 20 ± 1 mg). All treatments contained 0.1 mU/ml insulin. CAF had no effect on glucose transport in the presence or absence of 0.1 μ M CPA. * $p<0.05$ vs. insulin.

6.3.5. Effects of CPA and CPX on maximal insulin-stimulated glucose transport and intrinsic activity

In response to a maximal insulin stimulus (10 mU/ml), 3-methylglucose transport was increased by ~2-fold compared to submaximal insulin concentration. In the presence of maximal insulin concentration, neither CPA, CPX nor CPA + CPX altered 3-methylglucose transport (Fig. 29).

To assess the possibility that CPA and CPX might affect the intrinsic activity of GLUT4 at the cell surface, soleus muscles were pre-incubated for 30 min with maximal insulin concentration (10 mU/ml) in the absence of these pharmacological agents. Addition of these agents to these muscles (followed by an additional 10 min incubation) did not alter insulin stimulation of glucose transport (Fig. 30).

Figure 29. Effect of CPA and CPX on maximal insulin (10 mU/ml) stimulated 3-methylglucose transport

Values are means $\pm$ SE (n=5-14 muscles per group with average weight of 22 ± 2 mg).

Figure 30. Effects of CPA and CPX on the "intrinsic activity" of glucose transport

"Intrinsic activity" was assessed by addition of CPA and CPX, 2 U/ml ADA and 50 μM AOPCP after 30 min pre-incubation with 10 mU/ml insulin to maximally stimulate GLUT4 translocation. Values are means $\pm$ SE (n=5-14 muscles per group with average weight of 22 ± 2 mg).

6.4. DISCUSSION

Adenosine has consistently been shown to potentiate glucose transport in isolated adipocytes (69, 79, 90) and in cardiac muscle (96). However, in rodent skeletal muscle, investigation of adenosine modulation of glucose transport has produced controversy as to whether adenosine has no effect (173), stimulates (64) or inhibits (12, 41) glucose transport in response to insulin stimulation alone. By use of 77 μ M CPX, it has been concluded that activation of A₁ receptor mediates the synergistic interaction between insulin- and contraction-mediated glucose transport in rat hindlimb (173). However, at this concentration, CPX far exceeds its selectivity for the A₁ receptor (selectivity < 0.3 μ M) (165) and may activate responses mediated by inhibition of the A₂ receptors instead. In the present study, CPA and CPX were used at concentrations within the selectivity range for the A₁ receptor in order to assess whether the interactions between adenosine and insulin-stimulated glucose transport is indeed mediated by activation of A₁ receptor.

In agreement with previously reported findings (64, 173) basal glucose transport (Fig. 23) was unaffected by CPA in the absence and presence of CPX, suggesting that adenosine modulation of glucose transport is limited to its effects on GLUT4-, but not GLUT1-mediated glucose transport. However, adenosine removal by ADA and AOPCP was necessary in order to show a stimulatory effect of CPA on submaximal insulin-stimulated glucose transport. The observation that CPA-induced increase in glucose transport was similar to insulin alone (without adenosine removal), suggests that endogenously produced adenosine is maximally effective at potentiating glucose transport. This finding is in agreement with those previously reported (64, 173). The maximal effect of CPA on glucose transport was observed at 0.1 μ M (selectivity for A₁ receptor < 0.5 μ M (165)). When adenosine was first removed, addition of CPX to the incubation medium had no

effect on glucose transport. This is not surprising since antagonists merely occupy the receptor, but do not alter the receptor conformation or its interaction with downstream signalling mechanisms. However, the maximal glucose transport induced by 0.1 μM CPA was reduced by $\sim 15\%$ in the presence of 0.01 μM CPX (selectivity for A_1 receptor $< 0.3 \mu\text{M}$ (165)). Therefore, it can be concluded that adenosine interaction with insulin stimulation of glucose transport in isolated soleus rodent muscle is mediated by activation of the A_1 receptor. It is noteworthy that the quantitative reduction in glucose transport elicited by adenosine removal ($\sim 0.21 \mu\text{mol/ml/hr}$) is similar to the stimulatory effects elicited by CPA ($\sim 0.19 \mu\text{mol/ml/hr}$) which identifies that a fraction of insulin-mediated glucose transport is dependent on adenosine. However, the contribution of adenosine to insulin regulation of glucose transport is quite small (the maximal inhibitory/stimulatory effects are $\sim 15\%$).

The maximal inhibitory effect of CPX on CPA-induced glucose transport in response to 0.1 mU/ml insulin is much smaller ($\sim 15\%$) than the 50% reduction induced by 77 μM CPX reported by Han et al. (64). Since the concentration of CPX used in the latter study far exceeds the selectivity for the A_1 receptor, a greater reduction in glucose transport could be attributed to antagonism of the A_2 receptor. At present, only the A_{2A} and A_{2B} , but not the A_1 receptor, have been identified in rodent and human skeletal muscle (104, 130, 175). While these agents are purportedly selective for activating the A_1 receptor, they are not completely selective for a given adenosine receptor population *per se*. Thus, in the absence of the A_1 receptor in skeletal muscle, it has to be considered that the effects elicited by CPA and CPX on 3-*O*-methylglucose transport might be secondary to activation/inhibition of the A_2 receptor instead. In general, the effects of a receptor antagonist can usually be explained by its ability to reverse or prevent the actions of an

endogenous or exogenous agonist. However, CPX, like other xanthines, are effective inhibitors of PDE, albeit at much less effectiveness than binding to adenosine receptors. However, the IC_{50} reported for CPX inhibition of PDE is $\sim 10 \mu M$ (23) which is still 7-fold lower than the concentration used by Han et al. (64). Thus, it is possible that CPX inhibition of glucose transport in that study was mediated by either PDE inhibition or by a combination of PDE inhibition and adenosine receptor antagonism. A much smaller effect was observed in the present study possibly because at $0.1 \mu M$ concentration, the inhibition of CPA-induced stimulation of glucose transport by CPX is mediated solely by antagonism at the A_1 receptor and not by inhibition of PDE or vice-versa.

Insulin stimulation of glucose transport is achieved by increasing the number of GLUT4 transporters translocated to the cell surface and possibly, by enhancing the intrinsic activity of GLUT4 already present at the cell surface. Thus, the changes in glucose transport in response to these various agents could be achieved by affecting either or both of these processes. In insulin-stimulated adipocytes, it has been found that adenosine potentiates glucose transport by stimulating the intrinsic activity of glucose transporter (likely GLUT4) at the cell surface (90) and by promoting an unoccluded conformation of GLUT4 docked at the membrane rendering it accessible to bind to and transport glucose into the cell (44, 172). Conversely, Han et al. (64) by use of the bis-mannose photolabel, ATB-BMPA, demonstrated that reduction in GLUT4 translocation to the cell surface accounted for the ADA-induced inhibition of glucose transport in rodent skeletal muscle. However, ATM-BMPA cannot detect changes in the intrinsic activity of GLUT4 transporters at the cell surface (89). Thus, it is possible that adenosine receptor activation

could alter the intrinsic activity of GLUT4 proteins that are present at the cell surface in skeletal muscle.

In the present study, the possibility that adenosine could affect GLUT4 transporters already present at the cell surface (“intrinsic activity”) was investigated by incubating soleus muscle with 10 mU/ml of insulin in the absence of the pharmacological agents for 30 min to achieve maximal GLUT4 translocation to the cell surface. CPA in the presence and absence of CPX was then added to the incubation vials for an additional 10 min prior to measuring 3-*O*-methylglucose transport. When these agents were added to soleus muscle preincubated with 10 mU/ml insulin, no changes were observed in 3-*O*-methylglucose transport elicited by insulin. These data suggest that in rodent soleus muscle, adenosine does not alter the GLUT4 glucose transporters already present at the cell surface. Rather, the data suggest that adenosine modulation of glucose transport likely occurs at a step in translocation of the glucose transport proteins.

The intracellular signalling mechanism that may couple adenosine receptor interaction with insulin stimulation of glucose transport in skeletal muscle is presently unknown, but has been suggested to occur via both cAMP-dependent and cAMP-independent mechanisms in adipocytes (82, 90). The inability to observe any changes in cAMP concentrations despite a CPX-induced reduction in glucose transport in rodent skeletal muscle suggest that cAMP-independent processes are involved in adenosine receptor modulation of insulin stimulated glucose transport (103). In isolated adipocytes, adenosine has been shown to enhance insulin activation of PI3K and PKB (159). Activation of both A₁ and A_{2B} receptors upregulates phospholipase C (PLC) activity via coupling to G_i and G_{q/11}, respectively. Increased PLC activity and/or possibly its

resultant product (such as inositol phosphates) are required for GLUT4 translocation in adipocytes (169) and could represent a pathway by which adenosine enhances insulin stimulation of glucose transport in rodent skeletal muscle. Whether these signalling intermediates provide a link between adenosine receptor activation and insulin stimulation of glucose transport in skeletal muscle remains to be elucidated.

In the present study, caffeine tended to ($p=0.09$) decrease insulin-stimulated glucose transport in the presence and absence of CPA. This is a surprising finding in light of the fact that endogenously produced adenosine would have been adequately removed by the concomitant presence of ADA and AOPCP. However, methylxanthines, such as caffeine and theophylline are also non-specific PDE inhibitors ($K_i \sim 90 \mu\text{M}$ in rat fat cells) (146). Although hypothetical, since inhibition of PDE would result in a rise in intracellular cAMP, it is possible that caffeine at $70 \mu\text{M}$ concentration might have inhibited PDE and possibly via a cAMP-dependent mechanism resulted in the observed inhibitory effects. Nonetheless, the effects of caffeine on insulin-stimulated glucose transport in the present study were not statistically significant.

In summary, the findings in the present study demonstrate that adenosine has no effect on either basal or maximal insulin stimulated glucose transport in rat soleus muscle. Adenosine modulation of submaximal insulin-stimulated glucose transport is likely mediated by activation of the A_1 receptor. The interaction of adenosine and insulin stimulation of glucose transport is likely mediated by altering the translocation of GLUT4 transporters, possibly by interacting with a key insulin signalling pathway and not by affecting GLUT4 transporters already present at the cell surface. Caffeine had no effect on insulin-stimulated glucose transport in rat soleus muscle.

At present, the role of adenosine in insulin regulation of glucose transport in human skeletal muscle remains uncertain in light of previous findings of a lack of any effects of adenosine on insulin actions in human skeletal muscle (84, 110, 175). Furthermore, since the A₁ receptor has yet to be identified in skeletal muscle and findings that species differences might exist in adenosine receptor interaction with insulin action, the significance of these findings to human skeletal muscle is presently unknown. It has to be considered that adenosine enhancement of insulin stimulation of glucose transport is achieved by activating the A₂ receptors, which are present in skeletal muscle. At present, extrapolation of the findings from this study in rodent skeletal muscle to humans must be done with extreme caution.

CHAPTER 7. GENERAL DISCUSSION AND CONCLUSIONS

Success in scientific theory is won, not by rigid adherence to the rules of logic, but by bold speculation which dares even to break those rules if by that means new regions of interest may be opened up. Dingle, H. , Through Science to Philosophy

Unlike other insulin-sensitive tissues, the notion that adenosine has a regulatory role in insulin regulation of glucose transport in skeletal muscle is controversial. The results published to date are less reproducible than those reported in adipocytes and the conclusions derived from these data are diametrically opposed. Similarly, the idea that since caffeine at “physiologically relevant” concentrations mediate its known effects (central/behavioural) by adenosine receptor antagonism, this must also be *the* mechanism that mediates its insulin antagonistic effects, is also dubious. Finally, that activation and inhibition of A₁ receptor respectively mediate adenosine and caffeine interaction with insulin stimulation of skeletal muscle glucose transport is also tenuous since this receptor population has yet to be identified in this tissue. Caffeine ingestion results in a

simultaneous secretion of epinephrine, which is a potent counterregulator of insulin actions.

Thus, this doctoral thesis was undertaken to investigate the putative roles of adenosine receptor and caffeine in insulin regulation of glucose transport in human and in rodent skeletal muscle.

Caffeine inhibits insulin action by epinephrine and not peripheral adenosine receptor

antagonism in humans. In keeping with previously reported findings (53, 58, 84, 145), it was shown in Study 1 that caffeine ingestion reduces whole body insulin sensitivity in humans. The main finding of this study is that reduction in net leg glucose uptake was the major determinant of caffeine-induced reduction in insulin sensitivity in humans. It was also demonstrated that caffeine inhibits GS activity in human skeletal muscle. These actions of caffeine were unaccompanied by changes in the insulin signalling intermediates (IRTK, PI3K, PKB/Akt, GSK-3 α) which are thought to be central to insulin stimulation of glucose transport in skeletal muscle. The mechanism by which caffeine reduces insulin action is commonly attributed to adenosine receptor antagonism. Indeed, in rat skeletal muscle, it is proposed that caffeine inhibits glucose transport by antagonism at the A₁ receptor. However, it is more likely that the inhibitory effects of caffeine on glucose uptake and GS activity *in vivo* are secondary to increased epinephrine levels, rather than peripheral adenosine receptor antagonism. In Study 2, when caffeine was administered with a β -blocker, propranolol, in spite of comparable increases in epinephrine levels as when caffeine was administered alone, the insulin response to glucose loading was similar to that observed in placebo. Thus, the quantitative difference in glucose tolerance can be ascribed to the lack of catecholamine stimulation of β -adrenergic receptors. This finding counters the proposition that caffeine inhibits the stimulatory actions of insulin by peripheral adenosine receptor antagonism (skeletal muscle). The notion that endogenous catecholamines are essential

to caffeine opposition of insulin action *in vivo* is further supported by observations that the methylxanthine-induced hyperglycemia and peripheral insulin resistance were ameliorated in adrenalectomized and adrenal demedullated rats (144, 156).

In Study 2, the possibility exists that some of the inhibitory effects of caffeine may have occurred independently of epinephrine. However, this is unlikely because the mechanism of propranolol actions (i.e., β -adrenergic receptor antagonist) is well-established and the use of propranolol to assess the insulin antagonistic effects of epinephrine is a reliable method (113, 126, 168).

Moreover, it has been reported that the insulin antagonistic effects of epinephrine is mediated by β_2 -adrenergic receptor stimulation (92). Similarly, acute (113) and chronic (154) propranolol administration does not alter glucose or insulin levels nor does it affect glucose tolerance. In the studies that have reported epinephrine inhibits insulin actions in peripheral tissues, epinephrine concentrations were used at high physiological or pharmacological doses (5, 7, 32, 75, 91). However, the exclusion of epinephrine at lower physiological concentrations in these studies does preclude the possibility that epinephrine at these concentrations can inhibit insulin actions. In this regard, findings from Study 2 as well as those previously reported by Hamburg et al. (63) that epinephrine levels at ~ 0.4 - 0.5 nM can impair glucose tolerance suggest that small physiologic increments of epinephrine is adequate to elicit an inhibitory effect on insulin actions.

If caffeine indeed mediates its inhibitory actions on carbohydrate metabolism by adenosine receptor antagonism *in vivo*, one would expect caffeine to have a greater impact during exercise because adenosine concentration is ~ 10 -fold higher compared to resting concentrations (68). Yet, leg glucose uptake during exercise in humans was unaffected by caffeine ingestion (51).

However, one could argue that since insulin levels are low during exercise, the inhibitory actions of caffeine are limited to conditions when insulin levels are high. Indeed, as shown in Study 1, the inhibitory effects of caffeine on leg glucose uptake occurs only in the presence of high insulin levels (since glucose uptake was not different at 0 min compared to the placebo trial) (Fig. 5). This is in accordance with previous observations that caffeine reduced glucose disposal during a hyperinsulinemic euglycemic clamp in humans (58, 84) and during electrical stimulation of perfused muscle in the presence of 100 μ U/ml of insulin (173), but not during exercise in humans where plasma insulin is low (51). It appears that caffeine exerts its inhibitory effects on glucose uptake only when insulin is above normal basal concentrations.

Skeletal muscle is the major site of glucose disposal under insulin-stimulated conditions (31) and plays a vital role in the counterregulation of hypoglycemia (25). If the plasma glucose lowering effect of insulin is unopposed, hypoglycemia will inevitably ensue. Consequently, hypoglycemia can lead to cerebral dysfunction since cerebral tissues which use glucose almost exclusively as a substrate. To ensure adequate glucose delivery to cerebral tissues, the body has a dual defense mechanism against hypoglycemia – hormonal and metabolic (137). A principal objective of the interplay between insulin and the counterregulatory hormones (epinephrine and glucagon) is the maintenance of euglycemia. During 3h of hyperinsulinemic-hypoglycemic clamp in non-diabetic individuals, a 2-fold reduction in GS activity, 1.5-fold increase in GP activity, increase in plasma lactate, increase in glucose production (G6P can then be redirected towards glycolysis, resulting in increased production of 3-carbon compounds which may be used by liver for gluconeogenesis) and reduction in glucose uptake were observed in skeletal muscle (25). Under hyperinsulinemic-hypoglycemic conditions, stimulation of glucose oxidation was only 80% of

basal, compared to 2.5-fold increase under euglycemic conditions. These changes in skeletal muscle glucose kinetics and enzyme activities were associated with increases in plasma epinephrine (25). Thus, when the prevailing exigency is glycemic recovery, these coordinated changes in skeletal muscle are important mechanisms by which muscle can contribute to providing alternate fuels for noncerebral tissues and in turn redirect glucose towards vital organs that are more dependent on glucose supply for energy (25). The findings from Study 1 that caffeine ingestion (and a corresponding increase in epinephrine) reduced muscle glucose uptake and muscle GS activity in humans are similar to those observed under hyperinsulinemic-hypoglycemic conditions when both insulin and epinephrine levels are elevated (25).

A prominent effect of epinephrine is its inhibitory actions on insulin-mediated glucose transport in skeletal muscle. The effect of epinephrine on skeletal muscle carbohydrate metabolism is dependent on the magnitude of the stimulatory effect of insulin relative to the mass action effect of glucose. During exercise when insulin levels are low, but epinephrine levels are elevated, an increase in blood flow would increase glucose delivery to contracting muscle. Thus, glucose transport is unaffected despite the presence of high epinephrine levels during exercise (4). These observations and the findings that caffeine opposes insulin actions during an insulin clamp and during an OGTT in resting humans, but not during exercise, further supports the conclusion that the insulin antagonistic effects of caffeine in resting humans are engendered by a caffeine-induced elevation in epinephrine, and not by peripheral adenosine receptor antagonism. In insulin-stimulated adipocytes, the inhibitory effects of epinephrine appear to be contingent upon removal of adenosine (44, 79, 172). However, in skeletal muscle, epinephrine effectively inhibited insulin-stimulated glucose transport when adenosine was not removed (7, 10, 65, 94).

Thus, it is unlikely that the epinephrine-induced reduction in muscle glucose uptake observed in Study 1 was dependent on removal of adenosine action by caffeine antagonism at adenosine receptors. This conclusion is also supported by observations that caffeine had no effect on glucose uptake or whole body insulin sensitivity in adrenalectomized and adrenal demedullated rodents (144, 156).

The intracellular mechanisms that might mediate the insulin antagonistic effects of caffeine.

In Study 1, the inhibitory effects of caffeine were not mediated by alterations in the known components of the insulin signalling cascade since following caffeine ingestion, the responses of IRTK, PI3K, PKB and GSK-3 α were comparable to that observed in placebo. The intracellular mechanism by which caffeine inhibited glucose uptake and GS activity is likely mediated by epinephrine activation of β -adrenergic receptors and subsequent coupling to Gs-protein/cAMP-dependent processes. However, the molecular mechanism involved in the epinephrine effect is not well characterized. Epinephrine impairment of insulin stimulation of glucose uptake in skeletal muscle could be mediated by alterations in signalling intermediates that are either found in a separate pathway or are further downstream from PI3K/PKB pathway or possibly by altering the GLUT4 transporters at the cell surface, possibly mediated by both cAMP-dependent and independent mechanisms.

An additional site of regulation of glucose metabolism may be at the level of pyruvate dehydrogenase (PDH), a key enzyme governing the rate of glucose entry into the mitochondria and its subsequent oxidation in skeletal muscle. The net activity of PDH is governed by numerous stimulatory (insulin, pyruvate) and inhibitory (epinephrine, cAMP, Ca²⁺, acetyl CoA,

FFA) factors. Although PDHa activity was not measured in Study 1, it is possible that its activity might be downregulated in light of the presence of numerous inhibitory factors in this study, thereby leading to downregulation of glucose oxidation. Moreover, the stimulatory effects of insulin on PDHa activity can be overridden by increased FFA (86). Although calculated whole body and leg glucose oxidation were unaffected by caffeine ingestion in Study 1, indicating that PDHa activity was unaltered by caffeine. However, this conclusion is based on pulmonary RER might not be truly reflective of leg RQ. It would be interesting to learn whether caffeine does affect PDHa activity in skeletal muscle under insulin-stimulated conditions.

In resting rodent skeletal muscle, epinephrine at physiological concentrations has been found to stimulate triglyceride (TG) hydrolysis (in the absence of insulin) (120). However, no detectable increases in FFA oxidation was observed and the authors suggest the possibility that FFA released from the TG pool could be spread among other lipid pools (such as diacylglycerol, etc.) (120). Thus, it is possible that in Study 1, exposure of skeletal muscle to a caffeine-induced increase in epinephrine over a 4h period might have resulted in increased intramuscular TG hydrolysis. Consequently, elevated intramuscular diacylglycerol and/or possibly FFA could have impaired glucose transport. However, in Studies 1 and 2, the net effect of caffeine on intramuscular TG hydrolysis is likely dependent on the sum total lipolytic effect of epinephrine compared to the antilipolytic actions of insulin. The acute inhibitory effects on glucose uptake/glucose tolerance after caffeine ingestion are likely not associated with elevated FFA. However, because FFA in both Studies 1 and 2 remained higher following caffeine ingestion compared to placebo despite the ability of insulin to decrease FFA concentration, the possibility that reduction in muscle glucose uptake was secondary to FFA cannot be entirely excluded.

Does caffeine cause insulin resistance? Caffeine is a pervasive component of the human diet. The dose of caffeine administered to the subjects in the studies that have examined the impact of caffeine on insulin action is “typical” of human consumption – i.e., obtainable by consuming 3-4 cups of strong coffee. An important question that arises from these findings is whether they can be extrapolated to imply that chronic use of caffeinated products can induce insulin resistance or at least should be avoided by healthy humans, those predisposed to T2DM and individuals with T2DM. It is imperative to note that these studies are not without their limitations. In particular, the findings that acute caffeine ingestion reduces whole body glucose disposal, muscle glucose uptake and glucose tolerance were obtained under “artificially-simulated” laboratory settings. In these studies, caffeine was administered in its alkaloid form in a bolus that is equivalent to drinking 3-4 cups of coffee simultaneously, caffeine was administered for at least 1-2h before insulin infusion at high physiological concentrations or ingestion of 75 g of glucose, and the subjects were required to abstain from caffeine-containing products, alcohol and physical exercise for 48h before reporting to the laboratory. These are certainly not typical of normal human dietary habits and/or lifestyle practices. Moreover, only men were recruited to participate in these studies (with the exception of Keijzers et al. (84) who studied both men and women, but their results were not separated by gender).

The notion that caffeine is *not* coffee is certainly not a new one. The most convincing data that caffeine is *not* coffee is a report that ingestion of caffeine from coffee is ineffective in enhancing performance in humans (52). Moreover, a cup of coffee is extremely complex, in that in addition to caffeine, it supplies hundreds of other chemical compounds, all with potential pharmacological/physiological effects (151). Very little is known of these chemical

compounds and much less is known of their potential interactions with caffeine. Individuals also differ greatly in their “sensitivity” to caffeine. Caffeine “sensitivity” and its metabolism can be influenced by a number of physiological and environmental factors, including (but not limited to) the use of oral contraceptives, cigarette smoking, concurrent use of other drugs, exercise, age, pregnancy, obesity and foods commonly found in the human diet (brassica vegetables) (3). These findings suggest that the complexity of a cup of coffee and the capricious nature of the human diet and lifestyle practices could alter caffeine metabolism which in turn, could alter its effects on insulin actions.

Despite the overwhelming research on the potential adverse impact of caffeine/coffee consumption on human health, the medical literature is conflicted regarding the harmful effects of coffee and caffeine, and caffeine has not been implicated in any adverse health effects associated with coffee consumption (178). In fact, one study suggests that higher caffeine and coffee intake lowers the incidence of Parkinson’s disease (142). Chronic use of methylxanthine derivatives attenuates the humoral and hemodynamic effects associated with acute methylxanthine use (138). Development of tolerance to chronic caffeine consumption (generally associated with upregulation of adenosine receptors (186)) is often cited as the reason for the lack of adverse effects of chronic caffeine consumption on cardiovascular effects (178). If the impact of caffeine on insulin action is also subjected to tolerance, one might expect reversal of the caffeine impairment of insulin sensitivity with chronic use of caffeinated products. However, caffeine-induced impairment of insulin actions in intact animals is secondary to epinephrine and not to adenosine receptor antagonism. Chronic caffeine ingestion does not elevate plasma epinephrine levels in healthy humans (30), but downregulates β -adrenergic

receptors in rats making production of cAMP less favoured (56). Similarly, the ability of caffeine in coffee to raise plasma epinephrine levels is much smaller than that achieved with acute caffeine ingested in its alkaloid form (52). In accordance, the caffeine-induced increase in insulin response to an oral glucose load was also much smaller when caffeine was consumed in the form of coffee compared to its alkaloid form (Graham et al., unpublished observations). These findings suggest that long-term use of caffeinated products might not alter insulin sensitivity. In Study 1 and that by Keijzers et al. (84), insulin was infused at high physiological levels and how this would translate to normal physiological conditions is not known. It is also uncertain whether the reduction in insulin sensitivity by caffeine would be of similar magnitude at more physiological insulin levels or in patients with insulin resistance who already start at a lower insulin sensitivity. Prudence dictates that before concluding caffeine causes insulin resistance and before recommending the general public avoid caffeinated products with respect to carbohydrate management, it is necessary to first establish that chronic consumption of caffeinated products under conditions that reflect typical human dietary and lifestyle practices can significantly impair preservation of glucose homeostasis in healthy humans and in individuals with insulin resistance. Prudence also dictates that these research findings must be put into perspective in order to provide the general public with meaningful, accurate and understandable messages regarding the effects of caffeinated products on carbohydrate management and maintenance of metabolic homeostasis.

Adenosine interaction with insulin stimulation of glucose transport. Is it an A_1 receptor-mediated process? Findings from previous studies that have investigated the role of adenosine receptor in mediating insulin stimulation of glucose transport have produced controversy as to whether activation of adenosine receptor stimulates (64), inhibits (12, 41, 42, 93, 97) or has no

effect (34, 173) on this process in rodent skeletal muscle. In Study 3, the putative role of the A₁ receptor in mediating insulin stimulation of glucose transport was examined in isolated rodent soleus muscle by using pharmacological agents at concentrations within the selectivity range for the A₁ receptor. The findings from this study suggest that A₁ receptor activation does indeed contribute to the insulin regulation of glucose transport in rodent skeletal muscle. It appears that the effect of A₁ receptor activation on glucose transport is limited to its ability to alter submaximal, but not maximal or basal glucose transport. It was also found that in contrast to isolated adipocytes, these pharmacological agents did not alter glucose transport in muscles that were previously incubated with maximal insulin. This finding would suggest that activation of A₁ receptor does not affect GLUT4 transporters already present at the cell surface and likely stimulates glucose transport by altering a step in GLUT4 translocation.

A caveat must be added to the above discussion on A₁ receptor interaction with insulin action – inherent in the inferences that adenosine modulates insulin regulation of carbohydrate metabolism in skeletal muscle is the assumption that this receptor population is indeed expressed in skeletal muscle. At present, only A_{2A} and A_{2B}, but not A₁ receptors, have been identified in the rodent and human skeletal muscle (104, 130, 175). Accordingly, it cannot be excluded that the effects elicited by the pharmacological agents in the *in vitro* studies that have examined adenosine receptor interaction with insulin action, including Study 3 in this Ph.D. dissertation might be unrelated to activation and antagonism of the A₁ receptor in skeletal muscle.

If not A₁, how about A₂ receptor? Although the use of various agonists and antagonists have proven useful to study a specific functional significance of adenosine receptors, none of these

pharmacological agents are totally selective for a specific receptor population, which complicates their use as pharmacological agents. CPA is generally considered to have an 8-fold greater affinity/selectivity for the A₁ compared to A₂ receptor, but nonetheless, it is capable of activating the A₂ receptor. Thus, it is highly plausible that in the absence of A₁ receptor in skeletal muscle, CPA could activate A₂ (or A₃) receptor instead. In Study 3, A_{2A} receptor would likely not be the candidate in mediating CPA-induced changes in glucose transport because in general activation of A_{2A} receptor coupled to G_s protein stimulates adenylate cyclase would increase intracellular cAMP. The latter would be expected to inhibit and not stimulate insulin actions. However, both CPA and CPX have a good-to-reasonable affinity for A_{2B} receptor (CPX has a nM binding affinity for A_{2B}) (43). It is possible that CPA-induced increase in insulin-stimulated glucose transport in Study 3 could result from activation of A_{2B} receptor. It is interesting to note that with respect to intracellular pathways, A_{2B} receptor have as much in common with A₁ (PLC) or with A_{2A} (adenylate cyclase) receptors (43). It has been found that G_{q/11} mediated signalling participates in insulin stimulation of glucose transport (73, 184). Activation of A_{2B} receptor by coupling to G_{q/11} stimulates phospholipase C (PLC) activity (43), which appears to be necessary for insulin-stimulated glucose transport. It has also been postulated that the subsequent formation of its resultant products, inositol phosphates enhances GLUT4 association with plasma membrane (169). Furthermore, activation of A_{2B} receptor increases intracellular Ca²⁺ (a known factor involved in glucose transport), but this process appears to be independent of cAMP and PLC (43). Thus, in Study 3, CPA or CPX could conceivably activate or inhibit A_{2B} receptor-coupled to PLC and subsequently increase or inhibit insulin-stimulated glucose transport, respectively. Xanthines (including caffeine and CPX) are effective PDE inhibitors and PDE activation is a critical step for insulin to elicit its antilipolytic effects and glucose transport (103).

Thus, the possibility also exists that these pharmacological agents may have opposed insulin stimulation of PDE and could inhibit glucose transport by increasing intracellular cAMP levels, independently of activation/antagonism of adenosine receptor. Until the presence of A₁ receptor in skeletal muscle is confirmed, the conclusions that adenosine-insulin interaction is facilitated by this adenosine receptor subtype is premature. Future studies are certainly warranted to delineate whether activation of A_{2B} receptor alters insulin regulation of glucose transport.

Are the in vitro findings obtained using pharmacological agents relevant to the physiological milieu? With a half-life of only a few seconds, the sites of adenosine production and release, the relative number and spatial location of the adenosine receptors on cell membranes and the proximity of adenosine release relative to a specific receptor subtype to predominantly activate them are likely crucial factors in determining the ultimate physiological response. The magnitude and type of responses to adenosine can vary depending on the “physiologically active” adenosine concentration, which in turn, is governed by numerous events, including the release, reuptake, production and metabolism of adenine nucleotides and adenosine. Thus, the total production rate of adenosine is dependent on provision of substrates (ATP breakdown), the affinity of AMP deaminase and 5’NT AMP or IMP as a substrate (Fig. 2). Under well-oxygenated resting conditions, net ATP breakdown in muscle cells is minimal since energy demand is relatively low. Thus, the majority of adenosine production under basal conditions would occur via the transmethylation pathway which is catalyzed by SAH (Fig. 2). However, the significance of this pathway to adenosine production in skeletal muscle is questionable since SAH hydrolase activity/level in this tissue is low (167). Nonetheless, one would expect total adenosine production in resting muscle to be relatively low.

The response to local adenosine production is in part determined by the relative expression level of each adenosine receptor population (116). Moreover, the ability of activation of adenosine receptor to couple to a specific signal transduction/effector pathway may also require expression of a threshold amount (116). The conclusion that adenosine is a major factor in insulin regulation of skeletal muscle glucose transport is based upon the underlying assumption that basal interstitial adenosine concentration in skeletal muscle is adequate to activate adenosine receptors. One could argue that in Study 3, the concentration of CPA (0.1 μ M) that elicited an increase (~ 15%) in insulin-stimulated glucose transport is within the range of the interstitial adenosine concentration found in rat soleus and EDL muscles (~ 0.3 μ M (100)), suggesting that basal adenosine concentration must be adequate to exert an effect on insulin-stimulated glucose transport. Moreover, the lack of an effect of CPA on 3-methylglucose transport when adenosine was not removed would also indicate that endogenous adenosine is adequate to insulin stimulation of glucose transport (Study 3 and (64)). However, CPA has a higher affinity for binding to adenosine receptors than adenosine found in the interstitial space¹. One could also argue that basal adenosine concentrations, albeit low, can activate adenosine receptors in numerous tissues, so why not skeletal muscle? However, although adenosine at basal levels can cause some activation of receptors, this is limited to tissues with an abundant expression of adenosine receptors (50). Therefore, the question still remains as to whether adenosine level found under resting conditions in muscle is adequate to bind to its receptors and elicit an effect on insulin stimulation of glucose transport especially in a tissue where the A₁ receptor has yet to be identified and the A_{2A} and A_{2B} receptors are expressed in low/intermediate levels. To extrapolate this theory to caffeine, would one have expected caffeine to impair insulin actions in human

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¹ Shyroch, J., personal communication (18 April 2002)

skeletal muscle under resting conditions if this response was elicited by adenosine receptor antagonism?

One might be tempted to speculate that the adenosine-mediated responses might be different at rest compared to conditions when interstitial adenosine concentration in human skeletal muscle increases by ~10-fold, such as during exercise (68). The ability of adenosine to activate a specific receptor population and the ensuing physiological responses are concentration-dependent. When adenosine production is enhanced, activation of A₂ receptor may be greater than activation of A₁ receptor. A response that is associated with adenosine activation of A₂ receptor in skeletal muscle is an increase in blood flow (127). Thus, the possibility exists that the ability of adenosine to increase glucose transport under hypoxic or cellular energy imbalance (e.g., exercise) conditions, is limited to its ability to increase blood flow. Consequently, enhanced substrate delivery and an increase in skeletal muscle glucose uptake could result from a mass action effect. However, during contraction when the rate of ATP hydrolysis exceeds that of ADP phosphorylation, IMP has been shown to accumulate in rodent muscle (166). This finding suggests that the affinity of AMP deaminase for AMP far exceeds that of 5'NT for AMP as a substrate and that even under conditions when energy demand is high, adenosine production via 5'NT might still be relatively low. Thus, the extent to which adenosine might play a role in skeletal muscle glucose transport during exercise remains uncertain.

What is the significance of these in vitro findings to a whole body situation? The majority of the studies that have examined adenosine receptor activation on insulin action to date have been performed using *in vitro* experimental model. Thus, the relevance of these findings to an *in vivo*

whole body condition must be addressed. In adipose tissue, basal adenosine exerts a tonic inhibition of lipolysis (101). As such, in both isolated adipocytes and in intact animals, A₁ receptor agonists maximally inhibit lipolysis (69, 101). The magnitude of adenosine suppression of lipolysis in adipocytes is in the order of 75-92% (69, 101), in contrast to the relatively minor change in insulin sensitivity to glucose transport (~15% in rat skeletal muscle in Study 3 and ~30% in isolated adipocytes (69)). The notion that lipid oversupply can induce insulin resistance by impairing muscle glucose transport is supported by studies using an intralipid/heparin infusion protocol (86, 140). However, the intralipid/heparin infusion protocol is usually initiated 2-3h prior to insulin infusion, followed by quantification of insulin sensitivity or muscle glucose uptake. The findings in these studies suggest that the FFA-induced impairment of glucose transport requires a more chronic timeline (i.e., hours or days). Nonetheless, it is conceivable that the contribution of adenosine to insulin regulation of glucose transport is limited to its plasma FFA lowering effect. In this regard, the A₁ receptor agonists (GR79236 and R-PLA) reduced plasma FFA and glucose levels in normal rodents (108, 128) and enhanced insulin-mediated glucose uptake in adipocytes obtained from diabetic, but not normal rodents (128). The hypoglycemic effects in normal (108) and in diabetic rats (128) and enhanced adipocyte glucose uptake (in both normal and diabetic rats) (128) of these agents were associated with its ability to lower plasma FFA because these responses lagged behind by ~ 1h after reduction in plasma FFA. Thus, it is possible that the underlying mechanism by which adenosine influences acute glucose transport/metabolism in skeletal muscle (and possibly in insulin-resistant states) might be secondary to its potent antilipolytic function in adipose tissue and not directly to adenosine stimulation of insulin-mediated glucose transport in skeletal muscle *per se*.

The stimulatory effects of *R*-PIA on insulin-mediated glucose uptake in isolated adipocytes observed in adipocytes isolated from both normal or diabetic rats were modest (~15%). Moreover, the ability of *R*-PIA to lower plasma glucose was observed in diabetic, but not in normal rats (128). If an increase in glucose uptake was the major cause of decrease plasma glucose, one might have anticipated that these drugs would also lower plasma glucose in normal rats – which was not the case (128). Thus, it is possible that the effects of adenosine on insulin action/glucose metabolism might be limited to pathophysiological conditions associated with insulin resistance. In this regard, there is some evidence to indicate that there are dysfunction in adipocyte A₁ receptor reduced sensitivity to the antilipolytic effects of adenosine and/or its coupling to downstream G-protein signalling processes, in adipose tissue and liver in insulin-resistant states (e.g., obesity and diabetes) (80, 115). Furthermore, overexpression of the A₁ receptor in adipocytes which resulted in reduction in FFA in these mice prevented against the development of insulin resistance and glucose intolerance. (36). Since circulating FFA levels remained lower during high fat feeding compared to control animals during high fat feeding, this could possibly be a mechanism which counteracted against the insulin resistance inducing effects of the high-fat diet (36).

In the study by Reaven et al. (128), the effects of *R*-PIA (hypoglycemic and stimulation of adipocyte glucose uptake) were similar to those elicited by nicotinic acid (an antilipolytic agent). Nicotinic acid (or its analogue Acipimox) is routinely used to assess the impact of changes in circulating FFA on carbohydrate metabolism. These agents also reduce plasma FFA, which in turn results in improvement in glucose disposal (45, 114). This finding would also suggest that perhaps the ability of these drugs to alter plasma glucose levels or insulin action is simply due to

their shared antilipolytic function (128). Thus, to suggest that because *R*-PIA, CPA, GR79236 improved glucose disposal, adenosine is essential to insulin regulation of glucose uptake is perhaps as tenuous as suggesting that because Acipimox improved glucose disposal, nicotinic acid is essential to insulin stimulation of glucose transport. Given the complexity and the disparity in these findings, it is difficult at the present time to definitively conclude that adenosine *per se* indeed has a role in insulin action in either healthy or in insulin-resistant states.

In summary, it has been found that reduction in skeletal muscle glucose uptake is the major determinant of the caffeine-induced reduction in whole body insulin sensitivity in healthy, lean males. Caffeine also reduced glycogen synthase activity, but did not alter muscle glycogen levels. The inhibitory effects induced by caffeine are reduced by exercise. It has also been shown that the insulin antagonistic effects of caffeine *in vivo* are mediated by elevated epinephrine and not by peripheral adenosine receptor antagonism (in skeletal muscle) in humans. However, the caffeine-induced impairment in insulin action is not associated with alterations in any of the known components of the insulin signalling cascade (IRTK, PI3K, PKB, GSK3 α). The intracellular mechanism by which caffeine/epinephrine inhibits insulin action remains to be ascertained. Several possibilities include alterations in signalling intermediates that are either further downstream from IRTK/PI3K/PKB or are independent of this insulin signalling cascade, alteration of the steric conformation and/or the intrinsic activity of GLUT4 at the cell surface. These mechanisms of actions may include both cAMP-dependent (PKA) and independent mechanisms. In isolated rat soleus muscle, the A₁-selective agonist, CPA mildly stimulated (~15%) submaximal insulin-stimulated glucose transport, but had no effect on either basal or maximal insulin-stimulated glucose transport. The stimulatory effects of CPA are likely

associated with alterations in the GLUT4 translocation process, possibly by influencing a key insulin signalling pathway and not by affecting GLUT4 transporters at the cell surface. The supposition that adenosine modulates insulin stimulation of glucose transport in skeletal muscle is mediated by activation of A₁ receptor is based on the assumption that this receptor population is indeed expressed in muscle. However, the presence of A₁ receptor has not been conclusively demonstrated in rodent and human skeletal muscle. Thus, it has to be considered that the mechanism by which these pharmacological agents altered insulin-stimulated glucose transport in rodent muscle might be unrelated to activation of A₁ receptor. At present, the role of adenosine in insulin regulation of glucose transport in humans is also uncertain in light of previous reports of a lack of effect of adenosine on insulin sensitivity and forearm glucose uptake and that species differences exist in adenosine-insulin interaction. Thus, the relevance of the findings in rodent muscle to human muscle is uncertain and extrapolation of these findings to humans must be done with extreme caution.

Despite the valiant efforts made by numerous researchers, the putative roles of adenosine on insulin actions remain inconclusive and uncertain. Countless questions regarding adenosine and insulin interaction in skeletal muscle are still unanswered. For example, is adenosine under basal conditions adequate to activate receptors and to elicit an effect on insulin regulation of glucose transport in skeletal muscle – a tissue where A₁ receptor has yet to be found and has only an intermediate expression of the A₂ receptor? Perhaps a more important fundamental question is why would insulin-mediated glucose transport be regulated by adenosine whose production is dependent on ATP hydrolysis which is minimal under resting conditions? Are the *in vitro* findings obtained using pharmacological agents with greater affinity for binding adenosine

receptors than endogenous adenosine in isolated rat adipocytes and skeletal muscle exposed to supraphysiological insulin concentrations relevant to humans? Adenosine is a well-known antilipolytic and vasodilatory agent. That these physiological processes can profoundly alter glucose transport in skeletal muscle independently of adenosine activation of adenosine receptors in muscle also raises uncertainty of the significance/contribution of adenosine to insulin regulation of skeletal muscle glucose transport *per se*. Is the ability of adenosine to stimulate insulin regulation of glucose transport insofar as it is able to inhibit adipose tissue lipolysis acutely and/or increase blood flow/substrate delivery to skeletal muscle? Resolving these issues will be fundamental to determining whether the paradigm of insulin regulation of glucose transport should be extended to include adenosine as an integral component of this complex regulatory mechanism.

CHAPTER 8. LIMITATIONS

Invariably, the inability to control for every element of all experimental designs and to measure all possible metabolites/enzymes/hormones limits the conclusions that can be drawn from the findings generated from these studies. Thus, the aims of this chapter are to address some of the limitations of the experimental designs used in this dissertation and to propose other experimental approaches that may be more appropriate.

In Study 1, the effects of caffeine were examined 3h after a bout of one-legged knee extensor exercise. The purpose was to examine whether caffeine would alter carbohydrate metabolism specifically in skeletal muscle and whether caffeine would affect exercised muscle differently than rested muscle. Although exercise was confined to one leg, the possibility exists that there

may have been some whole-body effects induced by a bout of exercise on the rested skeletal muscle. Thus, a better study would have been to specifically test the subjects under resting and exercised conditions on 2 separate occasions, thereby eliminating any potential confounding effects of prior exercise on non-exercised muscle. In addition, quantification of ancillary enzymes (PDH and PKA activities) might provide a greater insight into the cellular mechanisms associated with caffeine and epinephrine inhibition of insulin actions.

In Study 2, an OGTT was used to assess the effects of caffeine on insulin action. However, because an OGTT is considered a “closed loop approach”, any changes in glucose will inevitably be paralleled by changes in insulin and vice-versa. Consequently, any potential effects of caffeine on the pancreas, thereby altering the insulin response independently of changes in glucose levels, was not controlled for. Thus, the possibility that caffeine directly or indirectly (epinephrine, FFA) stimulated insulin secretion from β -cells, rather than that the insulin response was secondary to epinephrine inhibition of insulin actions in peripheral tissues, cannot be excluded. Similarly, any direct effects of caffeine or epinephrine on the liver were also not restrained. It is difficult to assess whole body insulin sensitivity using an OGTT despite the use of the equation proposed by Matsuda and De Fronzo to obtain an index of whole body insulin sensitivity. However, these issues can be easily resolved by using a euglycemic-hyperinsulinemic clamp. In this study, the subjects were tested following an overnight fast, a condition that can have profound effects on various metabolic processes, hormone concentrations and enzyme activities in liver, adipose tissue and skeletal muscle. Perhaps a different approach involving the use of an insulin clamp in individuals in a postprandial state (like Study 1) might prevent some of the physiological and metabolic changes associated with fasting.

In Study 3, the effects of various A₁ agonists and antagonists were used to assess the putative roles of adenosine in insulin regulation of glucose transport. Ideally, definitive proof that A₁ receptors are indeed present in skeletal muscle should have preceded investigation of the effects of various pharmacological agents purported to selectively activate this adenosine receptor population. The soleus muscle was used to characterize the effects of these agents on glucose transport and the conclusions are therefore limited to slow twitch, oxidative fibres only. It might be useful and more informative to include other muscles such as the epitrochlearis which consists of fast-twitch Type II fibres. The ability of adenosine to alter the “intrinsic activity” of GLUT4 transporters was assessed in this study by adding these pharmacological agents to the incubation medium after an initial preincubation with insulin alone. This is an indirect means of assessing “intrinsic activity”. To draw any definitive conclusions regarding the effects of these pharmacological agents on GLUT4 at the cell surface assessment of GLUT4 transporters at the cell surface and their functional conformation will be necessary. These will undoubtedly allow a more definitive conclusion as to whether adenosine alters the intrinsic activity and/or the steric conformation of cell surface GLUT4 transporters. Inclusion of A₂ receptor agonists and antagonists in a similar study would elucidate whether adenosine modulation of insulin action involves this receptor population. In Study 3, the inhibitory effects of epinephrine on insulin stimulation of muscle glucose transport were not investigated because this has already been extensively studied and is well characterized. In addition, unlike adipocytes, the insulin antagonistic effects of epinephrine in skeletal muscle are not contingent upon the removal of adenosine, therefore, this was also precluded in Study 3.

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APPENDIX

A. Supplementary Data: Study 1

Table 9. RER and pulmonary VO₂ during 100-min of insulin infusion

	Time (min)	PL	CAF
RER	0	0.81 ± 0.01	0.81 ± 0.03
	60	0.87 ± 0.02*	0.84 ± 0.03
	90	0.89 ± 0.02*	0.83 ± 0.04
VO₂ (ml/kg/min)	0	3.7 ± 0.3	4.1 ± 0.1
	60	3.6 ± 0.2	4.0 ± 0.2
	90	3.7 ± 0.2	4.2 ± 0.2

Values are means ± SE (n=7). PL, placebo; CAF, caffeine. *p<0.05 vs. 0 min (within trial). Insulin infusion increased RER at 100 min vs. 0 min in PL, but not CAF. CAF ingestion did not alter RER or pulmonary VO₂.

Table 10: Oxygen uptake (ml/kg thigh muscle/min) in rested and exercised legs during 100 min of the euglycemic-hyperinsulinemic clamp

Time (min)	PL Rest	PL Ex	CAF Rest	CAF Ex
0	1.4 ± 0.2	1.6 ± 0.2	1.2 ± 0.2	1.3 ± 0.2
10	1.8 ± 0.4	1.7 ± 0.5	2.0 ± 0.3*	1.8 ± 0.2*
20	2.1 ± 0.4*	2.5 ± 0.6*	2.2 ± 0.3*	2.1 ± 0.2*
30	2.4 ± 0.6*	2.5 ± 0.6*	1.9 ± 0.1*	1.9 ± 0.2*
50	2.9 ± 0.8*	3.0 ± 0.9*	2.1 ± 0.3*	2.3 ± 0.2*
75	2.7 ± 0.5*	2.6 ± 0.5*	2.7 ± 0.2*	2.7 ± 0.2*
100	2.6 ± 0.6*	2.5 ± 0.3*	2.5 ± 0.4*	2.3 ± 0.4*

Values are means ± SE (n=7). PL, placebo; CAF, caffeine. *p<0.05 vs. time 0 min (within leg). Insulin infusion increased leg VO₂ in both legs in PL and CAF. CAF ingestion did not alter leg VO₂.

Figure 31. Arterial lactate during 100 min of insulin clamp

Values are means $\pm$ SE ($n=7$). Insulin infusion increased ($p<0.05$) arterial lactate in placebo (PL) and caffeine (CAF). Arterial lactate was increased ($p<0.05$) in CAF vs. PL from 10-100 min of insulin clamp.

Figure 32. Net lactate release from rested and exercised muscle during insulin infusion

Values are means $\pm$ SE ($n=6$) (calculated per kg thigh muscle). PL, placebo; CAF, caffeine. Lactate release is calculated from blood lactate concentrations ($[L]$, mmol) and blood flow rates (F , ml) according to the following equation: $(([L]_A - [L]_V) \cdot F)$. Negative numbers indicate release.

B. Supplementary Data: Study 2

Table 11. Heart rate and blood pressure before and during 120-min of OGTT

	TIME (min)					
	-105	0	30	60	90	120
	ORAL GLUCOSE TOLERANCE TEST					
	Heart Rate (beats/min)					
PL	49 ± 2	48 ± 2	50 ± 2	50 ± 2	51 ± 3	52 ± 1
CAF	51 ± 3	46 ± 2	49 ± 2	48 ± 2	48 ± 3	51 ± 3
PR	49 ± 2	41 ± 2 [†]	44 ± 2 [†]	41 ± 2 [†]	43 ± 2 [†]	42 ± 2 [†]
CAF+PR	48 ± 3	45 ± 3	43 ± 3 [†]	43 ± 2 [†]	42 ± 2 [†]	42 ± 2 [†]
	Blood Pressure (mm Hg)					
PL	111/67	115/64	114/67	114/66	112/65	109/70
CAF	118/71	121/74 [‡]	120/69 [‡]	121/71 [‡]	124/68 [‡]	125/70 [‡]
PR	117/71	110/68	109/64	105/59	103/60	102/62
CAF+PR	115/66	111/73	109/64	110/66	112/63	110/71

Values are means ± SE (n=7). PL, placebo; CAF, caffeine; PR, propranolol; CAF+PR, caffeine + propranolol. ^{*}*p* < 0.05 vs. PL, [†]*p* < 0.05 vs. CAF, [‡]*p* < 0.05 vs. PL, PR and CAF+PR

Figure 33. Blood lactate before and during 120 min OGTT

Values are means ± SE (n=7). Basal lactate was similar among trials. CAF increased lactate at 0-30 min of OGTT. ^{*}*p* < 0.05 vs. PL, CAF, CAF+PR.