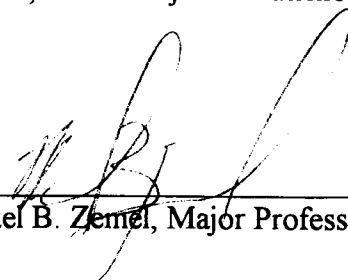


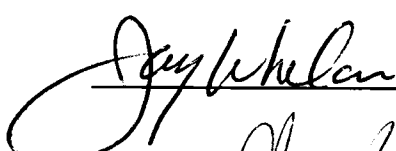
To the Graduate Council:

I am submitting herewith a thesis written by Dana Lynn Dibling entitled "Chromium Picolinate Antagonizes the Lipogenic and the Anti-Lipolytic Effects of Insulin." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.



Michael B. Zemel, Major Professor

We have read this thesis and
recommend its acceptance:





Nana Mustafa Muna

Accepted for the Council:



Associate Vice Chancellor and
Dean of the Graduate School

**Chromium Picolinate Antagonizes the Lipogenic and
the Anti-Lipolytic Effects of Insulin**

**A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville**

Dana Lynn Dibling
August 1998

Abstract

Recent studies indicate that chromium picolinate (CrPic) supplementation may improve body composition, as indicated by a selective reduction in body fat. Previous data from our laboratory has shown that CrPic increases the rate of vascular smooth muscle cell intracellular calcium ($[Ca^{2+}]_i$) recovery from agonist-induced loads, thereby decreasing $[Ca^{2+}]_i$. Although CrPic regulation of $[Ca^{2+}]_i$ has not been similarly investigated in adipocytes, we have found human fatty acid synthase (FAS) expression to be regulated, in part, by $[Ca^{2+}]_i$, and recent data indicates that lipolysis is suppressed by elevations in $[Ca^{2+}]_i$. Consequently, we have now investigated the potential role of CrPic in regulating FAS activity and lipolysis. Human adipocytes were maintained in primary culture with or without CrPic (1 μ M), insulin (10 nM), or both for 72 hours, and FAS activity, lipolysis and triglyceride content were measured. CrPic did not alter basal lipolysis and FAS activity. However, CrPic partially antagonized the inhibitory effect of insulin on glycerol release (26% increase, $p < 0.05$) and antagonized insulin stimulation of FAS activity (0.64 ± 0.11 vs 0.35 ± 0.05 nmol NADPH/min/mg protein in insulin and CrPic/insulin treated adipocytes, respectively $p = 0.04$). Since FAS is a key enzyme in *de novo* lipogenesis, this reflects a coordinated activation of lipolysis and inhibition of lipogenesis by CrPic. These effects were reflected in a 23% reduction in adipocyte triglyceride content over a 72 hour treatment period (3.99 ± 0.03 vs. 3.00 ± 0.04 mg triglyceride/mg protein, $p < 0.04$). Thus, CrPic coordinately

regulates lipolysis and lipogenesis, may thereby may inhibit insulin-mediated triglyceride storage.

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I. Introduction

Chromium (Cr) is an essential trace mineral necessary for potentiating insulin action, and for normal carbohydrate, lipid and protein metabolism (Mertz, 1975). Most functions of Cr are insulin dependent (Evans et al., 1973). Of the various forms of Cr, organic trivalent forms (Cr^{3+}) are most stable and are biologically active form (Swartz & Mertz, 1957). The mechanism whereby organic Cr is absorbed is uncertain, however, it is more complex than passive diffusion. Cr^{3+} binds with transferrin, organically complexed Cr may be transported via a different mechanism. Organic Cr is believed to be stored in a body pool of about 4-6 mg. Immediately upon absorption Cr may be released into the blood in response to the physiological stimulus insulin. Active Cr is then transported to tissue, where in the presence of insulin it potentiates insulin action (Mertz, 1975). The risk of toxicity for Cr in the trivalent state is "so low that there is a substantial margin of safety between the amounts normally consumed and those considered to have harmful effects", according to the 1989 National Research Council (National Research Council, 1989).

The estimated safe and adequate daily dietary intake of Cr is 50-200 micrograms a day for people over 7 years of age (National Research Council, 1989). Dietary sources of Cr include meats, whole grain products and spices (Kumpulainen et al., 1979, Jorhem and Sundstrom, 1993). Approximately

40% of women and 60% of men are consuming less than the 50-200 μg estimated safe and daily dietary intake (Kumpulainen et al., 1979). Other trace minerals, including zinc (Hahn and Evans, 1975) and iron (Moshtaghi et al., 1992) decrease the bioavailability of Cr. Final absorption of Cr is only about .5-3.0% of that ingested in fasted rats (Mertz, 1969, Sayato et al., 1980). These findings suggest that Cr supplementation may potentially be beneficial to those with consuming less than the recommended 50 μg . In the U.S. population 50 μg is adequate to prevent deficiency symptoms (National Research Council, 1989). However, Cr status in individuals is difficult to assess, due to low levels in tissues and inaccurate assessment techniques (as reviewed by Anderson, 1981).

In 1987, Gary Evans, patented a new Cr complex termed Chromium Picolinate (CrPic). CrPic structure is as follows (Figure 1) (U.S. Patent #33,988).

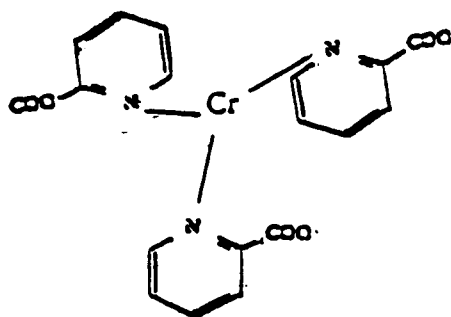


Figure 1. Chromium Picolinate Structure

This complex consists of one molecule of Cr and three molecules of picolinic acid. Picolinate (pyridine-2-carboxylic acid) is a metabolite of tryptophan produced in the kidneys (Evans, 1982). Picolinate (Pic) is a metal chelator and increases the bioavailability of zinc and chromium (Evans, 1982). In biological systems, picolinic acid may be a natural ligand for metals, increasing both absorption and transport (Evans, 1982). Consequently, this complex, CrPic, should increase the absorption and transport of Cr^{3+} (Evans, 1993).

Chromium picolinate supplementation has been shown to improve blood lipid levels, decreasing total cholesterol, low density lipoproteins, and apolipoprotein B (Press et al., 1990). Moreover, an increase in apolipoprotein A1, associated with high density lipoproteins, was found during CrPic supplementation (Press et al., 1990). In addition, studies consistently demonstrate that type II diabetics respond to CrPic supplementation with reductions in blood glucose levels (Evans, 1989), and one study has noted a reduction in triglycerides in type II diabetics when supplemented with 200 μg CrPic daily (Lee and Reasner, 1994). Further, the life-span of rats was increased significantly over controls when CrPic was included in the diet (Evans and Meyer, 1992). The reported effects of CrPic supplementation on body composition have been variable. However, evidence is accumulating that athletes and overweight individuals exhibit improved body composition in

response to daily supplementation of at least 200 micrograms CrPic, as a reduction in body fat and an increase in lean body mass has been demonstrated in these individuals (Evans et al., 1989, Hasten et al., 1992, Kaats et al., 1991). For example Evans (1989) in a double-blind study supplemented college football players with 200 µg CrPic or a placebo. After 42 days of exercise and CrPic supplementation lean body mass was increased by 2.6 kg and 3.4 kg of body fat was lost. In the control group only 1.8 kg were gained in lean body mass and 1 kg body fat was lost (Evans, 1989).

The mechanisms whereby CrPic improves body composition have not yet been elucidated. Body composition is regulated, in part, by insulin (Felig, 1975). Insulin increases glucose and amino acid uptake into muscle cells (Felig, 1975). Insulin resistance is a selective phenomenon, with some tissue exhibiting resistance to insulin while others remain responsive. For example, skeletal muscle cells become insulin resistant, while adipocytes continue to be responsive to insulin. This phenomenon causes increased insulin mediated triglyceride storage in adipocytes. Thus, selective insulin resistance may lead to the development of obesity and a disruption in normal energy balance (LaNoue and Martin, 1994). Chromium has been shown in some studies to increase insulin sensitivity in skeletal muscle cells (Evans and Meyer, 1992). Thus, it is possible that the resulting reduction in circulating insulin will reduce lipogenesis and improve body composition.

Alternatively, CrPic has also been demonstrated to increase the expression of Ca^{2+} -ATPase in vascular smooth muscle cells, resulting in reduced intracellular calcium ($[\text{Ca}^{2+}]_i$) (Moore et al., 1998). Obese patients exhibit increased adipocyte $[\text{Ca}^{2+}]_i$, (Draznin et al., 1988) and we have recently shown that intracellular calcium regulates, in part, the expression and activity of fatty acid synthase (FAS), the key regulatory enzyme in de novo lipogenesis (Xue et al., 1998). Accordingly if CrPic regulates adipocyte Ca^{2+} transport similar to its action on vascular smooth muscle cells (Moore et al., 1998), it may thereby decrease intracellular Ca^{2+} and consequently reduce FAS activity.

Accordingly, the objective of the present study to investigate the effects of CrPic on lipolysis and lipogenesis in the presence and absence of insulin, in human adipose tissue. It was theorized that CrPic would decrease insulin mediated triglyceride storage by attenuating lipogenesis and potentiating lipolysis.

II. Literature Review

Chromium the mineral

Chromium (Cr) was discovered in 1797 by a French chemist, Louis Vauguelin. It is found in nature as chromite or iron chromate (as reviewed by Anderson et al., 1981). In 1948, Chromium (Cr^{3+}) was identified as an essential trace mineral (as reviewed by Nielsen, 1994). Studies conducted in animals in the 1950s showed that Cr functions in maintaining glucose homeostasis, amino and fatty acid absorption, and protein synthesis (Jeejeebhoy et al., 1977). In biological systems Cr is active in the 3+ oxidation state. The ligand associated with Cr also has an influence on biological activity (Mertz, 1969). Schwarz and Mertz, in 1957, extracted a "glucose tolerance factor" (GTF) from porcine kidney (Schwarz and Mertz, 1957). This factor consists of one molecule Cr^{3+} and two molecules of nicotinic acid. GTF is an organic form that has been demonstrated to enhance the efficacy of insulin (Schwarz and Mertz, 1957).

Chromium is found in air, water, soil, and essentially all biological tissues that have been evaluated (as reviewed by Anderson, 1981). However, the concentration of Cr in tissues is very low, and it is therefore crucial to avoid contamination of specimens in laboratory settings (Vellion, 1989). Chromium is a hard, gray, brittle mineral with a melting point of $1903 \pm 10^\circ$

C. Chromium has an atomic number of 24 and an atomic weight of 51.996. Four stable isotopes have been identified, 50, 52, 53, and 54. Cr isotope 52 has the highest relative abundance, approximately 84% (as reviewed by Anderson et al., 1981). Chromium in the Cr^{6+} valence state is found in primarily industrial settings. Small amounts of Cr^{6+} are also found as dietary contaminants; however, it is reduced to Cr^{3+} in the stomach (Mertz, 1969). Cr^{6+} is a classified carcinogen in humans and animals. Although the exact mechanism of this effect is unknown, it appears to be linked to the reactive intermediates (Cr^{5+} and Cr^{4+}) formed (Lin et al., 1994). A radioactive form of Cr, with a half-life of 27.8 days, is available commercially and for use in radioactive tracer studies (as reviewed by Anderson, 1981).

Chromium the Nutrient

The value of dietary Cr in human nutrition has been extensively documented. Chromium is an essential trace mineral and a co-factor to facilitate insulin action, and functions in glucose, lipid, and amino acid metabolism (Mertz, 1975). The estimated safe and adequate daily dietary intake of Cr for people 7 years and older is .96-3.85 μM Cr/day (50-200 μg) (National Research Council, 1989). Estimates assessing the North American diet Cr composition are variable. Most investigators agree that consumption is less than 50 μg /day (Kumpulainen et al., 1979). Some investigators estimate that upwards of 40% of women and 60% of men are consuming less than the

recommended dietary intake (Kumpulainen et al., 1979). Cr status declines in the elderly possibly due to low Cr intake throughout life (Bunker et al., 1984). The risk of toxicity for Cr in the trivalent state is "so low that there is a substantial margin of safety between the amounts normally consumed and those considered to have harmful effects", according to the 1989 National Research Council (National Research Council, 1989). Even at several thousand times the Estimated Safe and Daily Dietary Intake Anderson et al. reported no toxicity symptoms (Anderson et al., 1997). However, exposure to hexavalent Cr, which is almost strictly man-made, does elicit toxic responses (Von Burg and Liu, 1993). Cr⁶⁺ toxicity symptoms include allergic dermatitis, skin and nasal lesions, and an increased risk of lung cancer. Cr⁶⁺ toxicity is rare, but occurs occasionally in stainless steel welders (Lin et al., 1994).

Cr is present in food in such low amounts that accurate analysis has been problematic. Neutron activation analysis may be used to determine Cr content in foods (as reviewed by Anderson, 1981). Good food sources of Cr are processed meats, whole grain products, and spices. Fruits and vegetables contain varying amounts of Cr, while meat, poultry, fish and dairy products are generally low in Cr (Kumpulainen et al., 1979, Jorhem and Sundstrom, 1993). Cr content may be determined in foods by using atomic absorption spectrometry. Biologically active Cr may be leached from stainless steel containers when the food products is acidic (Offenbacher and Pi-Sunyer,

1983). Diets high in refined foods and simple sugars are likely to be low in Cr (Kumpulainen et al., 1979). The low content of Cr in diets high in simple sugars is of particular concern, since sucrose stimulates the urinary excretion of Cr, further depleting Cr stores (Seaborn and Stoecker, 1989). A diet higher in starch slows the passage of food through the intestine more than diets higher in simple sugars. Cr travels very rapidly through the gastrointestinal tract, possibly with water. Starch, having a lower osmotic potential, draws less water into the intestinal lumen allowing for greater Cr retention (Seaborn and Stoecker, 1989). High-fat diets generally have lower Cr than isocaloric low-fat diets (Kumpulainen et al., 1979). Some chelating compounds can increase or decrease Cr availability. Pyridine-2-carboxylic acid (picolinic acid), a metabolite of tryptophan, will greatly enhance the bioavailability of Cr (U.S. Patent # 33,988). Chronic and acute exercise will increase urinary Cr losses. However, these losses are not problematic since exercise increases Cr retention as well (Rubin et al., 1998). Cr content also declines with age (Bunker et al., 1984).

Intestinal absorption of trivalent Cr is low, in fasted rats with a range of less than 0.5% to 3% (Mertz, 1969, Sayato et al, 1980). In human subjects an average of 0.69% is excreted in the urine after 72 hours. Absorption is difficult to measure and therefore this range is to be considered an estimate. The exact mechanism of Cr absorption in the intestine has not been identified, however, it is affected by dietary factors. Oxalate increases Cr absorption,

whereas, iron decreases Cr absorption. The effects of iron on Cr absorption have been studied in iron deficient mice (Lim et al., 1983).

Chronically low intakes of Cr will result in a Cr deficiency, which is most frequently manifested as impaired glucose tolerance, elevated blood glucose levels, hypercholesterolemia, and the development of aortic plaques (Evans, 1989) (Table 1). There is no clinical test available to test for Cr deficiency other than a reversal of deficiency upon Cr consumption (Jeejeebhoy et al., 1977). Chromium supplementation in the diet can help alleviate or entirely prevent these symptoms in deficient populations. For example, some patients receiving long term total parenteral nutrition (intravenous feedings), experienced glucose intolerance, which was relieved upon Cr supplementation (Jeejeebhoy et al., 1977). It is believed by some researchers that Cr forms a complex between insulin and insulin receptors facilitating insulin sensitivity (Evans and Bowman, 1992).

Chromium Picolinate

(CrPic) consists of one molecule of Cr and three molecules of picolinic acid. CrPic structure is as follows. (U.S. patent #33,988). Cr readily forms complexes with picolinic acid (Evans, 1982). Picolinic acid, a metabolite of the amino acid, tryptophan, is a metal chelator synthesized in human kidney

Table 1. Chromium deficiency symptoms

Impaired glucose tolerance
Elevated blood glucose levels
Hypercholesterolemia,
Development of aortic plaques
Elevated circulating insulin
Impaired growth
Peripheral neuropathy
Decreased fertility
Metabolic encephalopathy
Decreased longevity

(Adapted from Anderson,1981)

cells (Evans and Johnson, 1980) and in brewers yeast. Picolinic acid has also been found in intestinal cells and human breast milk (Evans and Johnson, 1980). Picolinic acid may be a naturally produced ligand to facilitate the utilization and/or transport of trace minerals (Offenbacher, et al., 1983). Picolinic acid combines with trace metals in the intestines and blood, this serves to facilitate the transport and metabolism of trace minerals (Evans, 1993). Complexes of nicotinic acid and Cr also increase the efficiency of Cr. Adequate levels of nicotinic acid, also synthesized from tryptophan increase the conversion of tryptophan to picolinic acid (Evans, 1993).

Cell membranes are virtually impermeable to Cr in the trivalent form. Organic complexes of Cr are more efficient in promoting insulin sensitivity in cultured cells (as reviewed by Mertz et al., 1993). One of such complexes is CrPic. In cultured cells, a three day incubation with CrPic enhanced insulin binding in smooth muscle cells (Evans and Bowman, 1992). Cellular uptake of glucose and leucine is increased by CrPic, whereas, other forms of Cr have not been demonstrated to have comparable effects. These effects are, however attributed to Cr and not picolinate. Other picolinic complexes, such as zinc-picolinate do not have comparable effects. Evans and Bowman (1992) compared the effects of zinc-picolinate and CrPic on insulin internalization and saw that zinc-picolinate was not effective in increasing insulin sensitivity (Evans and Bowman, 1992). Numerous in vitro and in vivo studies have investigated the physiological functions of CrPic. These studies have

investigated the effects of CrPic on various parameters in humans and animal models. The biological effects of CrPic as demonstrated by some studies are improvements in blood lipids, glucose regulation Type II diabetics (Evans, 1989), body composition (Bahadori et al., 1997, Kaats et al., 1991, Hasten et al., 1992), and in rats an increased lifespan (Evans et al., 1992).

Evans (1989) conducted a six week placebo-controlled crossover study of the effects of CrPic (200 µg/day) on serum lipids in hypercholesterolemic subjects. Total cholesterol, LDL cholesterol, and the associated lipoprotein B, were decreased, and HDL cholesterol, and the associated lipoprotein A1 were increased. Total cholesterol was decreased by 7% and LDL cholesterol was reduced by 10% after 42 days supplementation. In these patients, CrPic was found to be an effective agent for the treatment of hyperlipidemia (Press et al., 1990). According to the 7.4 year Lipid Research Clinics Coronary Primary Prevention trial an 8.5% reduction in cholesterol is associated with a 19% decrease in coronary artery disease (Newman et al., 1986). Moreover, this is not the only study to report an improvement in blood lipid levels. In Type II diabetics supplemented with 200 micrograms/day CrPic for two months, triglyceride levels were decreased by 17.4%, although there was no significant effect of CrPic on LDL or HDL cholesterol. Further research is needed to determine if the lipid lowering effects of CrPic would be sustained over long term periods (Lee et al., 1994). Other researchers do not support these

findings. Press et al. did not report a reduction in triglycerides in Type II diabetics when supplemented with CrPic (Press et al., 1990).

The role of CrPic and other Cr complexes has on glucose regulation has been extensively investigated. Brewer's yeast, which contains an organic form of Cr, has been well-documented to enhance insulin sensitivity in Type II diabetics (as reviewed in Anderson, 1981). After six weeks of 200 micrograms/day CrPic supplementation, 8 of 11 Type II diabetics showed an improvement in glucose regulation. Fasting blood glucose decreased by 24% and glycosylated hemoglobin was reduced by 19% (Evans, 1989). Other studies support these findings. In rats injected with Streptozotocin to induce a diabetic state no difference in blood glucose levels was seen in rats supplemented with CrPic as compared to controls (Morris et al, 1996). Inorganic forms of Cr, such as the salt, chromic chloride have not effected glucose regulation in Type II diabetics (McCarty, 1993).

Furthermore, much of the focus on CrPic research is on its' effects on body composition. In an Evans study (1989), after 42 days of supplementation (200 micrograms) subjects gained 2.6 kg lean body mass and lost 3.4 kg of body fat. (Evans, 1989). Hasten et al. also supplemented beginning weight lifting students with CrPic (200 µg/day). In males CrPic did not have significant effects on body circumference, skinfold thickness, or weight. It appeared from this study that CrPic has more effect in females than

in males. The females gained significantly more lean body weight, despite that skinfold thickness and circumferences were not different (Hasten et al., 1992). A recent study investigated the effects of 200 micrograms Cr-yeast or CrPic on weight loss and body composition during and after a very low calorie diet (Bahadori et al., 1997). Neither Cr-yeast nor CrPic reduced the loss of lean body mass during a low calorie diet. However, during the maintenance period CrPic did increase lean body mass over a long-term period (Bahadori et al., 1997). Other studies have seen no effects on body composition with 200 micrograms CrPic supplementation (Lukaski et al., 1996, Clancy et al., 1994). It has been suggested that CrPic supplemented may only have beneficial effects on body composition in those individuals or sub-groups consistently consuming less than the recommended 50 µg/day in Cr (Bahadori et al., 1997).

In a long term feeding study, rats supplemented with CrPic lived significantly longer than controls with an increase in median lifespan (Evans and Meyer, 1992). The median lifespan in the CrPic group was longer than the average maximum lifespan of control rats. This is the first report of inclusion of a nutrient to the diet increasing the lifespan of rats (Evans and Meyer, 1992). The rats maintained lower blood glucose levels during the study, this reduced blood glucose is indicative of improved insulin sensitivity (Evans and Meyer, 1992). Improved insulin sensitivity has benefits including reduced

cholesterol, improved body composition, and enhanced cellular immunity (McCarty, 1993) (Table 2).

Fatty Acid Synthase

Fatty acid synthase (FAS) is a multifunctional enzyme, which plays a central role in de novo lipogenesis (Wakil et al., 1983, Volpe and Vagelos, 1976). The enzyme complex is a polypeptide with seven active sites (Wakil et al., 1983) that is necessary for the endogenous production of fatty acids (Volpe and Vagelos, 1976). FAS catalyzes the synthesis of long chain fatty acids, such as, palmitate, from acetyl CoA, malonyl CoA, and NADPH (Wakil et al., 1983). This metabolic pathway provides fatty acids essential for the synthesis of cellular lipids, and for storage (Wakil et al., 1983). FAS is distributed in the liver, kidney, brain, lung, mammary gland, and adipose tissue in humans (Claycombe et al., 1998, Jayakumar et al., 1996, Semekovich, 95). An understanding of the regulation of this enzyme may be relevant in the treatment of several metabolic defects, such as obesity, hyperlipidemia, and diabetes. The liver and adipose FAS may be involved in the development metabolic disorders since human FAS activity is high in the liver and intra-abdominal fat (Claycombe et al., 1998, Semekovich et al., 1995). FAS is regulated by hormonal, nutritional, and developmental factors. FAS activity is not known to be regulated by allosteric effectors or covalent modification (Wakil et al., 1983). Most information about FAS is derived

Table 2. Physiological benefits associated with insulin sensitivity.

Improved glucose tolerance
Reduced LDL cholesterol, triglycerides, and free fatty acids
Improved body composition (Increased lean body mass, reduced body fat)
Enhanced cellular immunity
Decreased cancer risk
Reduced stress response

(Adapted from McCarty, 1993)

from animal studies, and considerably less is known about the human FAS gene (Jayakumar et al., 1996, Moustaid et al., 1996, Semenkovich et al., 1995). It is known that human tissue FAS activity is lower than rodent FAS activity. This finding is comparable to the lower activities of other human enzymes (pyruvate dehydrogenase, citrate lyase, and glucose-6-phosphate dehydrogenase) involved in glucose and fatty acid metabolism, when compared to other animals. These two findings indicate that human lipogenesis is repressed compared to other animals. (Zelewski and Swierczynski, 1990).

FAS is regulated hormonally and nutritionally (Sul et al., 1993). FAS is stimulated by a fat free, high carbohydrate diet (Laux and Schweizer, 1990). This nutritional effect is correlated with insulin stimulation, as insulin has been shown to increase human FAS activity three fold (Moustaid et al., 1996). In induced diabetic mice FAS mRNA does not increase upon carbohydrate feeding, levels of mRNA remain similar to those seen in fasted animals (Paulauskis and Sul, 1988). However, when insulin is injected into these animals a dramatic increase in FAS mRNA is observed (Laux and Schweizer, 1990). In diabetic induced mice a rapid insulin related reduction of FAS mRNA in response to carbohydrate intake is noted, whereas, a dramatic increase in FAS mRNA is observed upon insulin injections (Paulauskis and Sul, 1988). The same insulin response is seen in 3T3-L1 cells, an adipose cell line with mature insulin receptors. When 3T3-L1 cells are cultured in the

presence of insulin, FAS expression is upregulated (Paulauskis and Sul, 1988). Regulation of FAS by insulin is at the transcriptional level (Laux and Schweizer, 1990). A high carbohydrate, low fat diet is not the only nutritional factor that has been shown to affect FAS activity. If rats are fasted for 1-2 days a reduction in FAS synthesis is demonstrated. This is due to higher circulating concentrations of glucagon and repressed insulin levels. As glucagon increases, production of cAMP is increased thereby, activating lipolysis. Thyroid hormone (T₃), which causes increased fatty acid synthesis also decreases during fasting and is increased during feeding (Moustaid and Sul, 1991). The effects of cAMP and insulin are similar in rat liver and 3T3-L1 cells (Sul et al., 1993). A low fat diet increases FAS expression and consequently FAS activity (Laux and Schweizer, 1990, Moustaid et al., 1994).

It has recently been demonstrated that increasing levels of intracellular calcium stimulate FAS (Jones et al., 1996). Obese patients have increased intracellular calcium levels when compared to non-obese subjects (Draznin et al., 1988). Treatment of obese mice with a calcium channel antagonist, nifedipine, resulted in a reduction FAS activity and consequently a reduction of fat pad mass (Kim et al., 1996).

Active human FAS is characterized by two subunits arranged head-to-tail, generating two active catalytic sites (Wakil et al., 1993). Each polypeptide consists of seven active enzymes; beta-ketoacyl synthase, malonyl/acetyl

transferase, beta-hydroxyacyl, enoyl reductase, beta-ketoacyl reductase, acyl carrier protein (ACP), and thioesterase (Wakil et al., 1993). It is hypothesized that the multifunctional FAS evolved from the fusion of monofunctional enzymes (Amy et al., 1992). The combination of all the enzymes in the pathway into one functional unit permits greater efficiency and freedom from competing reactions (Amy et al., 1992).

The key to understanding the regulation of the FAS gene most likely lies in the DNA sequences flanking the transcribed portion of the gene (Amy et al., 1992). In the rat and the goose, these flanking regions have been identified. The gene is characterized by a 5' prime region of a TATA box between nucleotides -33 and -26, a positively acting inverted CCAAT box between nucleotides -98 and -92, and an Sp1 binding site between -241 and -236 (Okskouian et al. 1996). Insulin action is mediated by nucleotides -68 and -52 (Moustaid et al., 1994), and the negative action of cAMP is regulated by those nucleotides between -98 and -92 (Rangan et al., 1996).

Lipolysis

In humans, a number of complex pathways are involved in the conservation of fat store preservation, among these lipolysis plays a key role. One hypothesis is that obesity results, in part, from a reduction in the rate of lipolysis and/or fatty acid oxidation (Calles-Escandon and Driscoll, 1995).

Insulin has been demonstrated to inhibit adipocyte lipolysis (Arner and Engfeldt, 1987). Increases in plasma insulin ranging from 20-50 uU/mL will suppress plasma free fatty acid concentrations by 50% (Nurjhan et al., 1986). However, these studies used free fatty acid concentration as an index of lipolysis, whereas, glycerol release may be a more accurate indicator. In a similar study using glycerol release as the lipolytic index insulin suppressed glycerol release by 85%. (Nurjhan et al., 1986).

The mechanisms of insulin suppression of lipolysis are not fully defined (Arner and Engfeldt, 1987). It is well known that insulin inhibits cAMP production, leading to a reduction in protein kinase A activation. This leads to a reduction of active hormone sensitive lipase, thereby inhibiting lipolysis (Tebar et al., 1996). However, recent research has also proposed that phosphatidylinositol 3-kinase is involved in the anti-lipolytic effects of insulin.(Tebar et al., 1996). Also regional differences in the anti-lipolytic effects of insulin as abdominal adipocytes are more sensitive to this effect of insulin than are femoral or omental adipocytes (Cimmino et al., 1995). It has been suggested that insulin effects on intracellular calcium may potentiate the anti-lipolytic effect (Xue et al., 1998).

Nutritional factors, also play a role in determining the lipolytic rate. During fasting, biological adaptations take place and the action of insulin is altered. When healthy women are fasted for seven days, there is a 40%

increase in insulin sensitivity in subcutaneous fat cells and an enhancement of the anti-lipolytic effects of insulin is observed. This anti-lipolytic effect may serve as a protective mechanism to more efficiently conserve energy substrates (Arner and Engfeldt, 1987).

Recent data indicate that increased $[Ca^{2+}]_i$ inhibits lipolysis in a dose dependent manner (Tebar et al., 1996). Ca^{2+} channel blockers, such as nifedipine inhibit this effect indicating this effect is caused by increases in $[Ca^{2+}]_i$ (Xue et al., 1998). If $[Ca^{2+}]_i$ is increased only 1.2 fold, lipolysis is suppressed, indicating that lipolysis is very sensitive to increases in $[Ca^{2+}]_i$ (Tebar et al, 1996). An optimal range of Ca^{2+} is necessary for lipolysis to occur (Draznin, 1988).

The purpose of this study was to evaluate the effects of CrPic on lipolysis and fatty acid synthase. It has been previously demonstrated that CrPic improves body composition. Lipolysis plays a key role in the preservation of fat stores and fatty acid synthase is a key enzyme in *de novo* lipogenesis. It was hypothesized that CrPic would decrease insulin mediated triglyceride storage by stimulating lipolysis and inhibiting lipogenesis.

III. Materials and Methods

Human subjects. Human adipocytes were obtained from men and women between ages 28 and 48 years of age. Patients underwent required or elective abdominal surgery or liposuction. Exclusion criteria would include communicable diseases or a body mass index greater than 30. This protocol was approved by the University of Tennessee Institutional Review Board for Human Subjects.

Isolation and culture of human adipocytes. Adipose tissue was obtained from the abdominal region of patients and placed in sterile Hanks' medium from Gibco supplemented with penicillin (1 mM), streptomycin (100 mM), and gentamicin (50 mM). Adipocytes were washed with Hanks' medium several times, blood clots and visible connective tissue was removed. Adipose tissue was then finely chopped into small pieces using surgical scissors. Tissue was cultured in thin layers, so that all cells were able to obtain nutrients, minerals and hormones from the medium, in 250 mL Falcon tubes. Cells were kept in an atmosphere of 37°C, and 5% CO₂. Medium was gently removed with a pipet and changed to fresh medium after 24 hours. It was at this time cells were treated with insulin (10 nM), CrPic (1 µM), or CrPic and insulin (1 µM and 10 nM respectively) for 72 hours.

Fatty acid synthase activity. Fatty acid synthase (FAS) activity was measured spectrophotometrically. Cells were homogenized in 250 mM sucrose

buffer supplemented with 1 mM EDTA, 200 uM dithiothreitol, and 100 uM phenylmethylsulfonyl flouride (pH). The homogenate was centrifuged at 14,000 X g for 30 minutes. The supernatant was removed to be used for FAS activity assays. Acetyl CoA (12 mM) was added to each sample immediately prior to assays and malonyl CoA (1.17 μ M) was used to initiate the reaction. Fatty acid synthase activity was expressed in NADPH oxidized $\text{min}^{-1} \cdot \text{mg}$ protein⁻¹. Samples were corrected for protein using a modified Bradford method, as described later in this section under protein assay. Six samples were run for each treatment from one patient.

Lipolysis. Glycerol release was determined using the one-step enzymatic method as described by Boobis and Maughan (Boobis and Maughan, 1983). Media was removed from the adipocytes. Immediately following experimental incubation, 50 μ L alloquots were mixed with 200 uL hydrazine buffer consisting of 2-amino-2-methyl-1-propanol (0.1 M), hydrazine (0.2 M), EDTA (1 mmol/L) dissolved in distilled water and 4 uL NAD, and 1 uL glycerol dehydrogenase. Samples were adjusted to a pH of 9.9. The samples were mixed thouroughly by vortexing, and were incubated for 90 minutes. After the 90 minute incubation period the reaction was stopped by the addition of 1 mL, 20 mM sodium carbonate buffer with a pH of 10. The flouresence was measured with an excitation of 336 nm and an emission of 460 nm. Six samples were concurrently run for each treatment for each of 3 patients.

Protein Assay. This protocol is a modification of the Bradford method (Bradford, 1976). Human adipocytes, were harvested and homogenized in 250 mM sucrose buffer, supplemented with 1 mM phenylmethylsulfonyl flouride, 1 mM EDTA, 200 μ M dithiothreitol (pH 7.4). 50 μ L of each sample and 1.5 mL Coomassie Blue reagent were added to appropriately labeled test tubes. The absorbance was measured at 595 nM using water as the reference. A standard curve using bovin serum albumin was used to determine the concentration for the unknown sample (Bradford, 1976). Six samples were run for each treatment for each patient. Number of patients is dependent on number of patients used for respective assay. See lipolysis, fatty acid synthase, or triglycerides to determine n.

Triglycerides. Triglycerides were measured used a diagnostic kit from Sigma diagnostics (Sigma Chemical Company). Human adipocytes were harvested and placed in 250 mM sucrose buffer, supplemented with 1 mM EDTA, 100 μ M phenylmethylsulfonyl flouride (pH 7.4). A reagent blank, (10 μ L water) and a calibrator (10 μ L glycerol equivalent to 500 mg/dl) were prepared. 10 μ L of each sample were added to clean test tubes. 1.0 mL of triglyceride reagent (ATP, 2.0 mM, NAD 2.0 mM, Magnesium ions 3.0 mM, INT, 1.0 mM, glycerol kinase, 200 μ was added to each tube. Test tubes were incubated for 18 minutes at 28° C. The absorbance of the blank, calibrator, and test using

water as a reference, at 500 nM were recorded. Six samples were consecutively run for each patient.

Statistical Analysis. All data were evaluated via factorial analysis of variance using the General Linear Models method of SPSS® (SPSS® Chicago, Ill., 1998). This analysis treated patient source of fat as a fixed factor, replicates of each treatment within each patient sample as random factors, sample treatment as the independent variable and each outcome variable as a dependent variable.

IV. Results

To investigate the effects of CrPic on de novo lipogenesis, we measured the activity of adipose tissue FAS. Figure 1 shows the effects of CrPic on FAS activity. CrPic (1nM) had no effect on the rate of basal FAS activity. Insulin has been shown to increase FAS activity in a number of studies and our data are consistent with this observation ($p<0.04$). However, CrPic antagonized this lipogenic effect of insulin, reversing the insulin stimulation of FAS activity (Figure 2) ($p<0.05$).

To test the effects of CrPic on lipolysis, glycerol release was measured after 72 hours incubation. In the presence of insulin (1 nM) lipolysis was inhibited when compared to the basal rate ($p<0.04$). CrPic partially antagonized these effects (Figure 3). Since CrPic decreased FAS activity and increased the rate of lipolysis, adipocyte triglyceride level was examined. Indeed, a 23% reduction in adipocyte triglyceride content was seen ($p<0.05$), demonstrating attenuation of insulin mediated triglyceride storage (Figure 4).

Figure 2

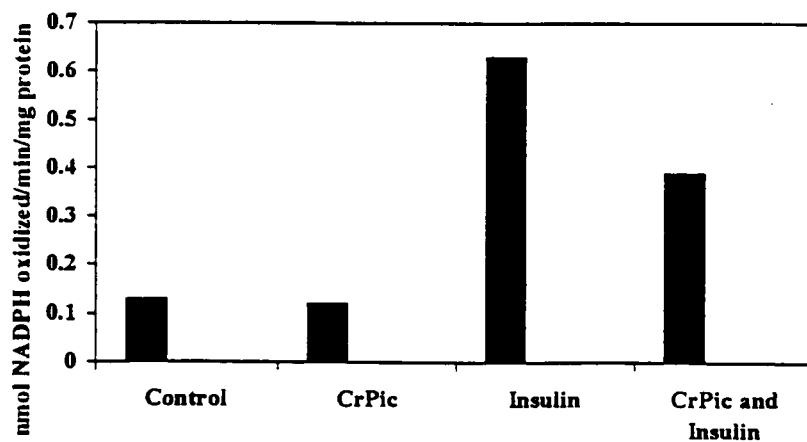


Figure 2. Effects of CrPic (1 μ M) and insulin (10 nM) on FAS in human adipocyte tissue pieces (n=1, average of 6 replicates). Human adipocytes tissue pieces were cultured in the presence or absence of CrPic for 72 hours and cells were harvested and FAS activity was measured as described in materials and methods. Data are expressed as nmol/NADPH oxidized/min/mg protein; (p<0.04).

Figure 3

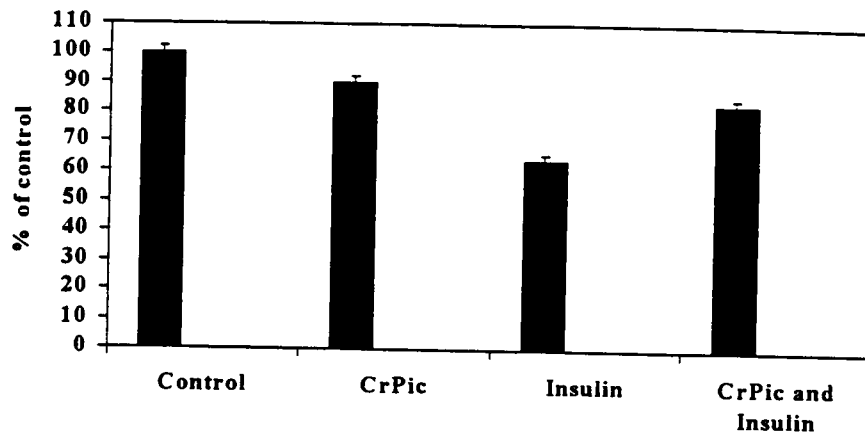


Figure 3. Effects of CrPic (1 μ M), insulin (10 nM) or CrPic (1 μ M) and insulin on lipolysis as measured by glycerol release. Human adipocyte tissue pieces were cultured in the presence or absence of CrPic (1 μ M) for 72 hours, cells were harvested and glycerol release was measured as described in materials and methods. Data are expressed as % control; control= 1.43 ± 0.22 μ M glycerol released/mg protein.

Figure 4

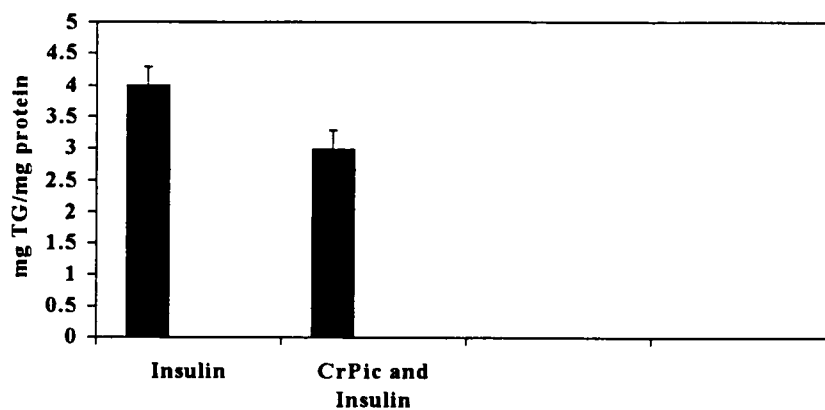


Figure 4. Effects of CrPic (1 μ M) or CrPic (1 μ M) and insulin (10 nM) on triglycerides in human adipocytes. Primary human adipocytes were cultured the absence or presence of CrPic for 72 hours. Cells were harvested and triglycerides were measured as described in materials and methods. Data are expressed as % control; control= 3.99 mg triglyceride/ mg protein.

V. Discussion

Data from this study demonstrate that CrPic coordinately regulates lipolysis and lipogenesis. The antilipolytic effect of insulin was antagonized by CrPic, and insulin stimulation of FAS was partially inhibited. This resulted in an approximate 20% reduction in triglyceride content in adipose cells.

Previous data from our laboratory demonstrate that CrPic modulates vascular smooth muscle cell Ca^{2+} has a corresponding effect when in the presence of insulin on Ca^{2+} recovery (Moore et al., 1998). Chromium potentiation of insulin has been well documented, as demonstrated by an improvement in glucose tolerance (as reviewed by Mertz, 1993). However, the recent report of Cr modulation of $[\text{Ca}^{2+}]_i$ is the first such report of Cr exerting an insulin-like action in the absence of insulin (Moore et al., 1998).

Further, recent studies demonstrated regulation of $[\text{Ca}^{2+}]_i$ modulates both lipolysis and lipogenesis. $[\text{Ca}^{2+}]_i$ plays a role in the metabolic disorders of obesity and diabetes and in human and mouse adipocytes, $[\text{Ca}^{2+}]_i$ plays a role in the regulation of *de novo* lipogenesis (Jones et al., 1996). Obese patients exhibit increased levels of $[\text{Ca}^{2+}]_i$ in adipocytes (Draznin et al., 1988). Increasing levels of intracellular calcium stimulate FAS activity, and calcium agonists, upregulate FAS activity (Xue et al., 1998). Treatment of obese mice with a calcium channel agonist, nifedipine, results in a reduction

of FAS activity and consequently a reduction in fat pad mass (Kim et al., 1996). Further, in vitro treatment of 3T3-L, adipocytes with a Ca^{2+} - channel blocker prevented Ca^{2+} mediated effects on FAS (Jones et al., 1996).

Recent data indicate that increased $[\text{Ca}^{2+}]_i$ inhibits lipolysis in a dose dependent manner (Tebar et al., 1996). Agents, such as the Agouti gene product, KCl, or AVP, stimulate increases in $[\text{Ca}^{2+}]_i$ and inhibit lipolysis. Ca^{2+} channel antagonists block this effect, further demonstrating the $[\text{Ca}^{2+}]_i$ dependence of lipolysis (Xue et al., 1998). Moreover, large increases in Ca^{2+} are not necessary to inhibit lipolysis. When $[\text{Ca}^{2+}]_i$ is increased only 1.2 fold by epidermal growth factor, lipolysis is suppressed, indicating that lipolysis is very sensitive to increases in $[\text{Ca}^{2+}]_i$ (Tebar et al, 1996). An optimal range of Ca^{2+} is necessary for lipolysis to occur (Draznin et al., 1988). Modulation of $[\text{Ca}^{2+}]_i$ provides one possible metabolic for the data in the present study.

Selective insulin resistance causes some tissue to become insulin resistant while other tissues remain responsive. For example, skeletal muscle cells in diabetic patient become insulin resistant, while adipocytes continue to be sensitive to insulin. This phenomenon causes increased insulin mediated triglyceride storage. Cr has been shown to increase insulin sensitivity in muscle (Evans and Bowman, 1992). The resulting reduction in circulating insulin will decrease lipogenesis and improve body composition.

Since adipocytes are likely to remain sensitive to insulin, the reduction in lipogenesis and the increase in lipolysis seen in the presence study is most likely, in part, via an insulin-dependent mechanism. However, further research is necessary to determine the mechanisms behind these data.

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