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Regulation of the Expression of Adiponectin, Resistin and GLUT4 in Omental Adipose Tissue of Baboon

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Regulation of the Expression of Adiponectin, Resistin and GLUT4 in Omental Adipose Tissue of Baboon

by

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The purpose of this research was to identify the chromosomal regions influencing the mRNA expression of the hormones resistin and adiponectin, and the glucose transporter 4 (GLUT4) in omental adipose tissue of baboons. These baboon genes were cloned using a two-step reverse transcription- polymerase chain reaction (RT-PCR) technique. Real- time, quantitative RT-PCR assays were developed and standardized for measurement of mRNA levels of each gene. Total RNA was isolated from 460 samples of adipose tissue from adult pedigreed baboons, and used for the quantification of adiponectin, resistin and GLUT4 mRNA. Quantitative genetic analyses were conducted using the mRNA abundance of each gene as a quantitative phenotype applying the variance decomposition approach. Heritabilities were calculated for resistin ($h^2 = 0.23$, $p = 0.005$), adiponectin ($h^2 = 0.23$, $p = 0.001$) and GLUT4 ($h^2 = 0.24$, $p = 0.001$). Genome

scan analyses were conducted to locate the chromosomal regions influencing the expression of the studied genes. The identified regions and corresponding LOD scores are: 19p13 (LOD score = 3.8) for resistin, 6q13 (LOD score =1.6) for adiponectin mRNA and same location (LOD score =1.0) for adiponectin protein, and 10q24-26 (LOD score = 1.4) for GLUT4 mRNA. A parallel study in 120 baboons indicated a relationship between body weight and indicators for insulin sensitivity, and an association between adiponectin levels and insulin resistance (HOMA-IR index) in baboons. No correlation between the analyzed phenotypes and resistin expression in monocytes was found. The relationships between mRNA expression in adipose tissue of resistin, adiponectin and GLUT4 and circulating levels of selected cytokines (TNFα, IL-6 and IL-1β) and phenotypes associated to insulin resistance were investigated in a sub-sample of unrelated baboons (n=40). Resistin expression in adipose tissue was related to insulin sensitivity, adiponectin mRNA was inversely associated with cytokines in plasma, and GLUT4 abundance and the HOMA-IR index were correlated. Collectively, these results revealed novel findings on the genetic component of the endocrine function of adipose tissue, and confirmed the value of the baboon as a model for the genetic study of obesity-related conditions.

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Chapter 1: Review of Literature

Obesity is a major risk factor for development of chronic diseases such as type 2 diabetes and cardiovascular disease. These diseases share a number of metabolic abnormalities such as insulin resistance, and adipose tissue plays a key role in the development of this particular condition through various mechanisms. One mechanism linking adipose tissue and insulin resistance is related to the endocrine function of this tissue (Saltiel, 2001; Trayhurn and Beatie, 2001). Adiponectin (Maeda et al., 2001; Hotta et al., 2001) and resistin (Steppan et al. 2001) are hormones expressed by adipose tissue that have been associated with insulin resistance. Another mechanism linking adipose tissue with insulin sensitivity involves the expression of glucose transporter 4 (GLUT4) (Charron et al., 1999; Ducleazu et al. 2002). Low levels of GLUT4 mRNA in adipose tissue are associated with insulin resistance in liver and skeletal muscle (Carvalho et al., 2001 and Garvey et al. 1991). Finally, the plasma levels of three cytokines that are associated with chronic inflammation and believed to regulate the expression of adiponectin, resistin and GLUT4 will be determined. These cytokines include tumor necrosis factor-α (TNF-α), interleukin -6 (IL-6) and interleukin 1β.

Specific aims

1) To identify the positional candidate genes that influence the expression of adiponectin, resistin and GLUT4 in omental adipose tissue of baboons.

Hypothesis: There will be a significant influence of genes on the expression of adiponectin, resistin and GLUT4 mRNA levels.

2) To conduct univariate and bivariate quantitative analyses to estimate the heritabilities and the degree of pleiotropy of mRNA levels of adiponectin, resistin and GLUT4 in adipose tissue and other phenotypes such as body weight, glucose, insulin and C reactive protein (CRP) levels.

Hypothesis: There will be significant evidence of additive genetic contribution in mRNA levels of adiponectin, resistin and GLUT4 in adipose tissue and other insulin resistance-related phenotypes in baboons.

3) To identify chromosomal regions that influence the expression of mRNA levels of adiponectin, resistin and GLUT4 in omental adipose tissue of baboons.

Hypothesis: There will be a significant evidence of shared genetic effects among mRNA levels of adiponectin, resistin and GLUT4 and between the mRNA levels of these genes and phenotypes such as weight and glucose levels.

4) To analyze the association between the expression of resistin in monocytes and insulin resistance-related phenotypes in baboons.

Hypothesis: There will be an association between the resistin mRNA expression in monocytes and phenotypes related to insulin resistance in baboons.

5) To investigate the relationship between the expression of adiponectin, resistin and GLUT4 in omental adipose tissue and circulating levels of TNF-α, IL-6 and IL-1β in baboons.

Hypothesis: There will be a significant relationship between the expression in adipose tissue of adiponectin, resistin and GLUT4 and selected pro-inflammatory cytokines insulin in baboons.

The overall goal of this project is to study genetic influences on the expression of adiponectin, resistin and GLUT4 in omental adipose tissue and its relationship with circulating levels of cytokines associated with chronic inflammation. The variance decomposition approach will be utilized to explore the association of the expression of these genes with insulin resistance-related phenotypes in baboons.

Introduction

Obesity is a global epidemic that poses one of the greatest threats to human health. In 1991 the reported rate in the United States was 1 out of 8 persons; in 1998, the prevalence of obesity escalated to 1 out of 5 (Mokdad et al., 1999; Dove, 2000).

The recent increase is alarming as it is a major risk factor for a number of chronic diseases such as type 2 diabetes, hypertension and cardiovascular disease (Dove, 2000; Carter et al., 1996; Serrano-Rios, 1998)**.** This research focused primarily on the relationship between obesity and insulin sensitivity. Since human obesity is a complex

condition with a large genetic component, we explored factors that affect genetic regulation in adipose tissue.

Diverse studies have demonstrated a relationship between adiposity and insulin resistance, which is an early defect in developing type 2 diabetes (Saltiel, 2001; Taylor, 1999), characterized by slow glucose clearance and hyperinsulinemia (De Fronzo et al., 1992). Findings from research groups suggest that adipose tissue amount and physiological activity are profoundly involved with insulin resistance (Garvey et al., 1993). Arner (2000) proposed that the main tissue for the study of obesity and insulin resistance should be adipose tissue. This tissue has a complex endocrine activity that is represented by the production and release of peptides, such as leptin, tumor necrosis factor alpha (TNF-α), adipsin, interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin 1β (IL-1β), plasminogen 1 (PAI-1), adiponectin and resistin (Saltiel, 2001; Trayhurn and Beatie, 2001 and Bruun et al., 2003).

The first three aims of this study determined the genetic contribution to the expression of adiponectin, resistin and GLUT4 in omental adipose tissue of baboons. Aims 4 and 5 explored the relationship between the expression of these genes and phenotypes associated with chronic inflammation and insulin resistance.

In recent years, adipose tissue was acknowledged as a highly active endocrine organ, with a profound effect on health (Arner, 2000; Saltiel, 2001). Adipose tissue has been classified as either subcutaneous or omental (visceral or central) (Lebovitz, 2003; Atzmon et al., 2002). These forms of adipose tissue differ according to location in the

body, as well as biological function. Studies suggest that the subcutaneous adipose tissue is more abundant, but less metabolically active, than omental (Lebovitz, 2003). Comparison between these two types indicate that the subcutaneous form expresses less β3-adrenergic receptors, phosphoenolpyruvate carboxykinase (PEPCK), PPARγ, hormone sensitive lipase, fatty acid transporter, angiotensinogen, resistin and adiponectin (Acrp 30), as well as other genes related to glucose homeostasis and insulin action, than does the visceral (Atzmon et al., 2002). Other studies have suggested that omental fat releases more products such as free fatty acids (Hellmer et al., 1992) and resistin (Mc Ternan, 2002a and Fain et al., 2003). Collectively, these observations support a deleterious effect of central or omental body fat on health.

Adiponectin

The hormone adiponectin is a 30 kD protein produced exclusively by white and brown adipose tissue (Hotta et al., 2001; Yamauchi et al., 2001) and has a three-dimensional structure that is similar to TNFα (Arita et al., 1999). The expression of adiponectin mRNA and protein is reduced in obese mice and humans, and its deficiency is associated with obesity-related insulin resistance, type 2 diabetes and cardiovascular disease (Yang et al., 2001). The expression of adiponectin correlates well with the insulin sensitivity state, and conditions such as restriction of caloric intake increase the expression of this protein and enhance glucose tolerance (Yang et al., 2001). Adiponectin also increases the expression of genes involved in fatty acid utilization such as CD36, acyl CoA oxidase and UCP2 (Saltiel, 2001; Yamauchi et al., 2001). A study by Fruebis et al. (2000) suggests that the insulin sensitizing effects are secondary to the fat burning, energy utilization and weight loss induced by adiponectin.

Results in humans report that following weight loss, adiponectin mRNA levels in adipose tissue and circulating protein significantly increase, with concomitant improvement of insulin sensitivity (Esposito et al., 2003). Adiponectin also appears to have an anti-inflammatory effect on the endothelial tissue, and it is believed to protect this tissue from damage induced by cytokines (Shimabukuro et al., 2003). Recent studies by Bruun et al. (2003) and Engeli et al. (2003) indicate that adiponectin expression is related to adipose derived cytokines such as TNF-α, IL-6, interleukin 8 (IL-8) and circulating levels of the C reactive protein (CRP). Findings from these human studies indicate that pro-inflammatory cytokines reduce the expression of adiponectin in adipose tissue, and that this mechanism may contribute to the insulin resistance

associated with sub-clinical inflammatory conditions (Bruun et al., 2003, Engeli et al., 2003).

A genome scan analysis by Comuzzie et al. (2000) identified a significant LOD score on region chromosome 3p27, using traits associated with the metabolic syndrome as quantitative phenotypes in humans. This region harbors the adiponectin gene, which is considered to be the major candidate influencing the expression of the metabolic syndrome. Another genome-wide scan was conducted using plasma levels of adiponectin as a phenotype in humans. This study reveals four chromosomal regions influencing the expression of this hormone on chromosomes 5 (LOD = 4.1), 14 (LOD = 3.2), 10 (LOD = 1.9) and 17 (LOD = 1.5). Some of these quantitative trait loci (QTL) have been reported in a prior study in association with obesity and the metabolic syndrome (Kissebah et al., 2001).

Resistin

Resistin is a newly discovered hormone secreted by adipose tissue whose biologic effect on insulin sensitivity was first described in mice (Steppan et al., 2001). This hormone is highly expressed in white adipose tissue in mice and secreted to the plasma where it travels throughout the body to exert its effect (Steppan et al., 2001; Yanakoulia et al., 2003). It is found in the serum of normal mice and its concentrations are sensitive to fasting and re-feeding conditions (Steppan et al., 2001; Nogueiras et al., 2003a). In mice, elevation of resistin levels leads to insulin resistance and glucose intolerance, symptoms of type 2 diabetes (Steppan et al., 2001). Administration of recombinant resistin to animals resembled these symptoms, i.e., impaired glucose tolerance without

changing insulin levels. This negative effect was reversed by blocking its action with anti-resistin IgG. In addition, administration of an anti-diabetic drug, rosiglitazone, lowers blood levels of resistin and improves glucose tolerance. These findings suggest that resistin is antagonistic to insulin, but at the level of peripheral resistance, not secretion. However, a receptor for resistin has not been identified to date.

Resistin has been associated with decreased insulin sensitivity in tissues, and some studies have shown that it is down-regulated by activation of peroxisome proliferator activated receptor γ (PPARγ) (Steppan et al., 2001; Patel et al., 2003 and Moore et al. 2001). Steppan et al. (2001) found a significant decrease of resistin mRNA after treatment of db/db (inactive leptin receptor) mice with PPARγ agonists, substances that are used as anti-diabetic drugs. Moore et al. (2001) confirmed these findings in the same strain with a reduction of 72% of resistin mRNA in white adipose tissue after treatment with two different PPARγ agonists (BRL-35135 or thiazolidinediones). In cultured human monocyte-derived macrophages Patel et al. (2003) reported decreased resistin expression after treatment with thizolinediones (TZD), a PPARγ ligand. These observations agree with findings from Savage et al. (2001), who reported that obese subjects had higher expression of resistin mRNA in adipose tissue as compared with lean controls. In another study by Holcom et al. (2002), a total lack of resistin was observed in subjects with a dominant-negative mutation of PPARγ.

However, the relationship between PPARγ activation and resistin expression remains controversial as Way et al. (2001) did not find any meaningful association between resistin expression and obesity or PPARγ activation in genetically obese mice. Also Levy

et al. (2002) reported that the activation of PPARγ **increased** the transcription of resistin in adipose tissue. Thus, further research is needed to resolve this dilemma.

The association between resistin expression and obesity is also unclear. A study from Nagaev and Smith (2001) analyzed the expression of resistin in 42 human subjects. These included six type 2 diabetic individuals, 18 insulin-resistant and 18 insulin-sensitive controls. Samples of abdominal subcutaneous adipose tissue obtained by biopsy and the adipocytes were isolated and cultured for resistin analysis. No relationships were observed between the expression of resistin in human adipocytes and obesity or insulin resistance. Subsequent observations in human studies found resistin expression in adipose tissue, with higher production in omental and abdominal tissue than in peripheral subcutaneous fat (McTernan et al., 2002a). In a study by Fain et al. (2003) explants of human omental and subcutaneous adipose tissue were analyzed for resistin expression and release. Their study reported that adipocytes have a negligible production of this hormone, as compared to the other non-fat cells in human adipose tissue. Interestingly, resistin expression is found in tissues other than fat in humans, including mononuclear blood cells (Nagaev and Smith, 2001), lung and bone marrow (Patel et al., 2003). A recent study by Nogueiras et al. (2003b) found expression of this hormone in adrenal gland, stomach, small and large intestines, and muscle in rats.

Two human studies (Yannakoulia et al., 2003; Zhang et al., 2003) show that circulating levels of the resistin protein are linked to insulin resistant-related phenotypes in humans. Yannakoulia et al. (2003) reported a positive significant association of resistin in plasma with body fat, and a negative correlation with the waist-to-hip ratio in

both sexes. Higher resistin values were found in females as compared to males. A study by Zhang et al. (2003) observes that resistin levels were related positively to the area under the curve of glucose following a standard glucose tolerance test. Therefore, these findings support a possible connection between circulating levels of resistin and phenotypes associated with the metabolic syndrome in humans.

Investigations on the relationship between genetic polymorphisms of resistin and traits related with the metabolic syndrome have reported other positive findings. Some single nucleotide polymorphisms (SNPs) in this gene seem to have an effect on body mass index (BMI) (Engert et al., 2002), insulin sensitivity, mRNA levels in adipose tissue and fatty liver (Smith et al., 2003). These results suggest that variations in the genetic structure of resistin are related to obesity and insulin-resistance related phenotypes.

Collectively, the available information on the biological activity of resistin indicates a role for this hormone in insulin resistance. Further research is needed to characterize the relationship between the endocrine function of adipose tissue and insulin resistance.

GLUT4

A third factor that is included in this research is GLUT4. Glucose is cleared from the bloodstream by a family of facilitative transporters (Garvey et al., 1991; Watson and Pessin, 2001; Garvey et al., 1994) and five isoforms of these transporters have been described (Watson and Pessin, 2001). Insulin resistance is characterized by decreased glucose transport and metabolism in muscle and adipocytes (Garvey et al., 1992).

GLUT4 is the major insulin-responsive transporter that is predominantly restricted to skeletal muscle, heart and adipose tissue (Charron et al., 1999).

The glucose transporter GLUT4 mediates insulin-stimulated glucose uptake in adipocytes and muscle by moving the GLUT4 protein from intracellular storage sites to the plasma membrane and increasing its expression (Garvey et al., 1992; Watson and Pessin, 2001). Abel et al. (2001) developed a transgenic model with adipose-selective reduction of GLUT4 expression. Although GLUT4 expression in muscle was preserved, these mice developed insulin resistance in muscle and liver. In humans other studies have found low expression of GLUT4 mRNA in adipose tissue of diabetic and obese subjects and this feature is recognized as a major cause of insulin resistance (Garvey et al., 1994; Charron et al., 1999 and Ducluzeau et al., 2002).

The mechanism of insulin resistance seems to be tissue-specific. In insulin resistant states such as obesity and type 2 diabetes, GLUT4 expression is decreased in adipose tissue but preserved in skeletal muscle (Ducluzeau et al., 2002). Minokoshi et al. (2003) developed an animal model of ablation of GLUT4 transporter in adipose tissue (Adipose-G4), and compared the metabolic profile of these animals with mice with suppression of GLUT4 in skeletal muscle (Muscle-G4). Results of this investigation reveled that both groups had similar insulin levels and abnormal glucose tolerance, although the glucose uptake in muscle is significantly higher than in adipose tissue. No effect was observed on resistin, adiponectin or TNFα levels in plasma. However, this adipose-specific defect in GLUT4 expression could contribute to the pathogenesis of insulin resistance in

obesity and type 2 diabetes. In addition, elective suppression of GLUT4 expression in adipose tissue resulted in insulin resistance in muscle, supporting the concept of a possible "cross-talk" between tissues for glucose homeostasis regulation. Thus, the first aim of this investigation is to identify the positional candidate genes that influence the expression of adiponectin, resistin and GLUT4 in omental adipose tissue of baboons.

Quantitative genetic analyses

In this project, the expression of adiponectin, resistin and GLUT4 was studied in omental adipose tissue. The mRNA abundance of each of these genes was used as a phenotype for quantitative genetic studies using variance components linkage approach (Comuzzie et al., 2001). Results of these analyses established values for heritability and identified quantitative trait loci (QTL) influencing the expression of these genes in adipose tissue. These analyses allowed the identification of pleiotropic or gene-gene interactions among the selected genes, and decomposition of the sources of variation into their environmental and genetic components.

The variance component linkage method is based on classical quantitative genetic principles, in which phenotype is assumed to be influenced by both genetic and environmental components (Comuzzie et al., 2001; North et al., 2003). The genetic factors can include specific loci at defined chromosomal locations. Evidence of genes is obtained from correlations among different classes of relatives. A matrix provides the a priori structure for these correlations among individuals in a family, which contain the coefficients of relationship for all pairs of individuals. These are the prior probabilities

that two individuals share a particular allele identical-by-descent (IBD) (Comuzzie et al., 2001; Almasy and Blangero, 1998).

Variance components linkage analyses uses the average IBD probability across the genome and the location-specific IBD probabilities estimated from the genetic markers typed for each individual. All the information regarding linkage is a function of the estimated IBD matrices calculated for each genetic marker. A method for accurate and fast calculations for multipoint IBD calculations in large pedigrees has been developed and IBD sharing at any specific point on the chromosome is predicted using a linear function of observed IBD probabilities at genetic markers at known locations. For hypothesis testing, the observed covariances among individuals are compared to expected covariances obtained under the linkage model including locus-specific and genomic factors. If the variance component for a specific chromosomal location is significantly greater than zero, there is evidence for a locus influencing the phenotype in that location (Comuzzie et al., 2001; Almasy and Blangero, 1998). The concept of the method is summarized in the following equation:

$$Ù = Ó Ï_¡ ó^2_{qi} + 2Ö ó^2_a + I ó^2_e$$

Where:

Ù: covariance

Ï ¡: IBD estimated matrix in marker X

$ó^2_{qi}$: additive genetic variance $QTL_¡$

Ö: kinship matrix

$ó^2_a$: additive genetic residual variance

I: identity matrix

$\acute{o}^2_e$: environmental matrix

The aim 2 in this project is to conduct univariate and bivariate quantitative analyses to estimate the heritabilities and the degree of pleiotropy of mRNA levels of adiponectin, resistin and GLUT4 in adipose tissue and other phenotypes such as body weight, glucose, insulin and C reactive protein (CRP) levels.

Genomic screening involves a complete search of all chromosomes for genes influencing quantitative and qualitative traits. Genotypic data for 330 STR markers typed in 500 baboons will be used for a genome scan. The phenotypes will be those traits which had significant ($p<0.05$) additive genetic contributions to the phenotypic variance in initial univariate statistical genetic analyses. A maximum likelihood variance decomposition approach will be then employed to detect, measure and localize the effects of quantitative trait loci (QTLs) (Falconer, 1989). The variance components approach is designed to fully exploit all of the genetic linkage information in pedigrees of arbitrary size and complexity and has been demonstrated to be effective in analyses of data from the baboon pedigrees (Almasy and Blangero, 1998). Thus, aim 3 in the present study is to identify chromosomal regions that influence the expression of mRNA levels of adiponectin, resistin and GLUT4 in omental adipose tissue of baboon.

Cytokines

Finally, the fourth factor analyzed in this project was the circulating levels of three cytokines associated with chronic inflammation and insulin resistance: TNFα, IL-6 and IL-1β. These cytokines were identified initially as messenger molecules in the immune system (Festa et al., 2000). Recent investigations report that these proteins are synthesized and released from adipose tissue (Trayhurn and Beatie, 2001; Bruun et al., 2003). The circulating levels of these pro-inflammatory proteins appear to be in association with total body fat and inversely related with insulin sensitivity in independent studies (Saltiel et al., 2001a; Bruun et al., 2003; Zhang et al., 2001).

TNF-α is believed to block the action of insulin (Hotamisligil, 1999) and to exert catabolic effects in adipose cells. This cytokine may function in a feedback pathway to regulate the number and lipid content of adipocytes (Saltiel, 2001a).

IL-6 is produced by subcutaneous adipose tissue in large amounts, and its secretion is correlated with BMI (Mohamed-Ali et al., 1997). A study on the effect of IL-6 on insulin-responsiveness identified the presence of IL-6 receptors in adipose tissue and suggested an autocrine/paracrine action of this cytokine on adipocytes *in vivo*. In addition, there was a possible participation of IL-6 in the insulin-resistant state observed in human obesity (Bastard et al., 2002).

The IL-1β cytokine is expressed in subcutaneous preadipocytes and adipocytes (Zhang et al., 2001). Previous studies report that IL-1β is regulated by TNFα, and

synergizes the metabolic effects of this cytokine. Il-1β may regulate metabolic pathways in the adipocyte by the stimulation of lipolysis and inhibition of lipogenesis (Memon et al., 1998).

Another phenotype of chronic inflammation included in this study was C reactive protein (CRP). The CRP is known to be produced in the liver; and recently its expression was detected in adipose tissue (Yang et al., 2002). This protein is considered a marker for acute and chronic inflammation. Recently, CRP has been identified also as a reliable marker for subclinical inflammation in humans. Circulating levels of this protein are predictive of coronary disease and other metabolic abnormalities (Festa et al., 2002). Interestingly, the hormones resistin and adiponectin have been associated with inflammatory process (Stumvoll and Haring, 2002; Holcomb et al., 2000, Bruun et al., 2003) and are considered "adipokines" altogether with TNFα and IL-6. It is believed that pro-inflammatory cytokines may influence the production of resistin and adiponectin (Engeli et al., 2003, Kern et al., 2003, Bruun et al., 2003), and contribute to the regulation of insulin resistance. However further research is required to clarify these observations.

Finally, the aim 4 in this research is to analyze the association between the expression of resistin in monocytes and insulin resistance-related phenotypes in baboons, and aim 5 proposes to investigate the relationship between the expression of adiponectin, resistin and GLUT4 in omental adipose tissue and circulating levels of TNFα, IL-6 and IL-1β in baboons.

The baboon as a nonhuman primate model for the study of obesity-related conditions

The baboon is the animal model used for this research because a 10% of adults develop spontaneous obesity and type 2 diabetes (Comuzzie et al., 2003; Stokes, 1986). The baboon is closer evolutionarily to humans than other species used in the study of obesity, and this relationship is expressed by the high genetic similarity between humans and this primate model (Comuzzie et al., 2003). A significant homology between the two species has been found in comparison of chromosomal staining patterns (Dutrillaux et al. 1978) Subsequent investigations have demonstrated that a significant group (approximately 300) of short tandem repeats markers (STR) from the human genome have the capacity to amply segments from baboon DNA (Rogers et al., 1995). In addition, the analyses of the DNA sequences of genes such as lipoprotein lipase (LPL) (Cole and Hixon, 1995) and leptin (Cole et al., 2003) have shown a high degree of identity between humans and baboons. Thus, the baboon is an excellent nonhuman primate model for the study of the genetic component in obesity-related conditions.

In summary, the overall goals of this project are to identify novel genes influencing the expression of adiponectin, resistin and GLUT4 in baboon omental adipose tissue and to explore the association of pro-inflammatory cytokines on the expression of these genes and their relationships with obesity and insulin resistance.

Chapter 2: Genome Wide Scan of Resistin mRNA Expression in Omental Adipose Tissue of Baboons

The hormone resistin was recently discovered in adipose tissue of mice. Functional tests suggest a role for resistin in the regulation of insulin sensitivity. However, human studies have reported controversial results on the metabolic function of this hormone. A 1 g omental adipose tissue biopsy was obtained from 404 adult baboons. Resistin mRNA expression was assayed by real time, quantitative RT-PCR, and univariate and bivariate quantitative genetic analyses were performed, via the variance decomposition approach. A genome scan analysis was conducted using resistin mRNA abundance in omental adipose tissue as a quantitative phenotype. A significant heritability of $h^2 = 0.23$ ($p = 0.003$) was found for resistin mRNA abundance in omental adipose tissue. A genome scan detected a quantitative trait locus (QTL) for resistin expression with a LOD score of 3.84, in the region between markers D19S431 and D19S714, corresponding to human chromosome 19 p13. This chromosomal region contains genes related to insulin resistance phenotypes, such as resistin, insulin receptor, angiopoietin like-4 protein and LDL-receptor. Variation among animals in resistin mRNA expression has a significant genetic component, and a gene or genes on chromosome 19p13 may regulate resistin mRNA levels in baboon omental adipose tissue.

Introduction

Resistin is a recently discovered hormone secreted by white and brown adipose tissue (Viengchareun et al, 2002; Steppan et al. 2001), monocytes, bone marrow and other tissues (Patel et al., 2003). The first study on resistin by Steppan et al. suggested a possible metabolic effect on insulin sensitivity in murine models. In mice, elevation of resistin levels leads to insulin resistance and glucose intolerance, symptoms of type 2 diabetes (Steppan et al., 2001). Administration of recombinant resistin to animals mimics these symptoms, i.e., impaired glucose tolerance without changing insulin levels. This negative effect was reversed by blocking resistin action with an anti-resistin protein. In addition, administration of rosiglitazone, an anti-diabetic agent, lowered blood levels of resistin and improved glucose tolerance. These findings suggested that resistin impairs peripheral insulin sensitivity.

Further studies in mice have reported contradictory findings on the role of this hormone in the regulation of insulin sensitivity. Moore et al. (2001) confirmed the initial findings of Steppan in the same strain of mice. A decrease of 72% in resistin mRNA levels in white adipose tissue was observed after treatment with two different PPARγ agonists (BRL-35135 or thiazolidinediones). In contrast, Way et al. (2001) reported low mRNA levels of resistin in adipose tissue of several different models of genetically obese mice, as compared with lean counterparts.

Subsequent human studies by Janke et al. (Janke et al., 2002) and Nagaev et al. (Nagaev and Smith, 2001) did not find any relationship between resistin mRNA levels in adipocytes and insulin resistance, type 2 diabetes or obesity. However, Zhang et al. (2000) observed associations between circulating levels of resistin protein and the calculated area under the curve of glucose (AUCG) during a 2 h glucose tolerance test, as well as percentage of body fat and the insulin sensitivity index. Also, Yannakoulia et al. (2003) reported that circulating resistin protein was related positively to body mass, and negatively to waist-to-hip ratio in healthy individuals. Finally, Fain et al. (2003) found that the release of resistin protein from human explants of omentum was 250% greater than from subcutaneous adipose tissue. Also, the isolated adipocytes had a negligible resistin production, as compared with non-fat cells present in adipose tissue. Collectively, these studies suggest that resistin is expressed in adipose tissue by cells other than adipocytes, and may have a role in regulation of insulin sensitivity.

Other studies by McTernan et al. (2002 a; 2002b) found higher levels of resistin protein and mRNA in visceral and subcutaneous abdominal adipose tissue, as compared to thigh and breast. Resistin expression was detected in isolated human pre-adipocytes and adipocytes, suggesting a possible link between central adiposity and insulin resistance-related conditions. However, the role of resistin in insulin sensitivity in humans is unclear and further research is needed.

The expression of proteins involved in the inflammatory response is increased in obesity states, implying that the observed metabolic abnormalities may be associated with chronic inflammation (Festa et al., 2000). One marker of inflammation, the C

reactive protein (CRP), is produced in the liver and adipose tissue, and contributes to regulation of insulin sensitivity (Ford, 2003). This protein is associated with adipokines, and is considered a robust marker for diabetes and cardiovascular disease (Yudkin et al., 1999; Rifai and Ridker, 2002).

Resistin is another protein believed to be involved in the inflammatory response. This protein was initially found in allergic pulmonary inflammation in mice. Stumvoll and Haring proposed that resistin could be involved in subclinical inflammatory processes (Stumvoll and Haring, 2002).

The human resistin gene is located on human chromosome 19p13.3. Several polymorphisms of this gene have been identified in humans, and associated with traits related to obesity and insulin sensitivity (Wang et al., 2002; Cao and Hegele, 2001). The complex nature of obesity and its related disorders indicates that the single candidate gene approach may be not the most appropriate for the study of these conditions (Comuzzie, 2002). Thus, the genome scan approach has been applied to investigate the genetic component in obesity in a variety of populations (Comuzzie, 2002; Clement et al, 2002; Chagnon et al., 2003). This method allows the analysis of large numbers of polymorphisms across the entire genome, with no a priori assumptions of the influence of particular genes on a phenotype (Comuzzie, 2002). Some quantitative trait loci (QTLs) influencing obesity and insulin resistance-related phenotypes have been detected consistently by this approach (Clement et al., 2002). In this project, a genome scan will be conducted to identify the QTLs influencing the expression of resistin mRNA in omental adipose tissue of baboons.

Animals and Methods

Animals

The data for the present study were obtained from 404 randomly-bred, pedigreed, adult baboons from the colony at the Sotuhwest Foundation for Biomedical Research (SFBR) in San Antonio, TX. The majority of the animals were olive baboons (P. h. anubis), with a smaller proportion of yellow baboons (P. h. cynocephalus), and crosses between those two subspecies. Animals are gang-housed and share the same low-fat diet (Harlan Teklad 15% Monkey Diet, 8715).

Sampling and Phenotype Collection

For sample collection, baboons were sedated with ketamine after overnight fasting. Body weight was measured on a calibrated electronic balance (GSC, Chicago, IL) and body length was measured between head and feet, with the animals lying on their back. Body mass index (BMI) was calculated as weight in kg divided by the squared length expressed in meters. Blood samples were collected from the antecubital vein as 4 mL in sodium fluoride tubes for glucose analysis, and 7 mL in EDTA tubes for insulin and cytokines measurement. The plasma was separated by centrifugation at 2000 x g, aliquoted and frozen for further analyses. A 1 g biopsy of omental fat was obtained as described elsewhere (Cole et al., 2003). Glucose analyses were performed in an Analox spectrophotometer (Analox Instruments, Lunenburg, MA) and insulin was measured by RIA using the kit by Linco Research, Inc (St Louis, MO). The C-reactive protein was

analyzed by radioimmunoassay (Linco Research Inc.). Parameters with variations >5% were reanalyzed.

Samples of omental adipose tissue were analyzed for triglyceride content, cell size and cell number per gram of tissue as described elsewhere (Lewis et al., 1986). The remaining sample was frozen immediately and stored at –80^{o} C.

Total RNA was isolated from adipose tissue with TRI REAGENT (Molecular Research Center, Inc, Gaithersburg, MD). RNA integrity was verified by staining with ethdium bromide on a 1.2% agarose gel. The isolated RNA samples were treated with RQ1 DNAse (Promega, Madison, WI) for 15 minutes at 37^{o} C for digestion of traces of DNA.

The cloning of a partial baboon cDNA for baboon resistin and the development of a quantitative, real time RT-PCR assay were performed as described by Tejero et al (2003).

The sequences of forward and reverse primers for the real time, quantitative RT-PCR were:

5' TCCTCCTGCCTGTCCTGG 3' and 5' CGCCCTCCTGAATCTTCTCAT 3', respectively. The sequence for the resistin probe was:

5'TCTAGCCAGACCCTGTGCTCCATGG 3'.

Ribosomal 18S RNA (rRNA) was used as an internal control and measured by the Universal 18S system from Ambion (Austin, TX). The primers to competimers ratio was 4:6. The probe for 18S was the r RNA Ambiprobe from Applied Biosystems.

A sample of 50 ng of total RNA was used per assay. RT-PCR conditions were 48^{o} C at 50 min for reverse transcription, and 40 cycles of 60^{o} C for 1 minute, followed by 90^{o} C for 15 seconds. Data were obtained as Ct values (the number of cycles at which logarithmic plots of PCR product accumulation cross a specific threshold line), according to the manufacturer's specifications. Resistin expression was corrected for measurement error, and calculated as resistin Ct divided by the 18S rRNA Ct, and corrected by the 18S rRNA Ct mean in any given run. Inter and intra-assay coefficients of variation for resistin expression were 6 and 4%, and for the 18S rRNA, 5 and 7%, respectively.

Genotyping

The DNA was isolated from leukocytes using a phenol-chloroform method as described by Rogers et al. (1995). Published human PCR primers were used to amplify homologous microsaellite loci from baboon genomic DNA. The PCR products were analyzed on electrophoresis gels on ABI 373 and ABI 377 Automated sequencers (ABI Biosystems, Foster City, CA), using fluorescently labeled primers and Genescan and Genotyper software (Applied Biosystems). The genotyping procedure included 330 short tandem repeat polymorphisms (STRs) and yielded a map density of 7.2 cM (Rogers et al., 2000). The initial analysis validated the relationships between animals. The PEDSYS software was use for the management and preparation of baboon genotypes and pedigrees.

Statistical Genetic Method

Quantitative genetic analyses were conducted on 404 pedigreed baboons using the maximum likelihood-based, variance decomposition approach that is implemented in the computer package SOLAR (Southwest Foundation for Biomedical Research, San Antonio, TX) (Almasy and Blangero, 1998; Hopper and Mathews, 1982). The variance of the phenotype (resistin mRNA abundance), represented as σ^2_P, was partitioned into the additive genetic (σ^2_G) and environmental components (σ^2_E). It is possible to calculate heritability because these components are additive, $\sigma^2_P = \sigma^2_G + \sigma^2_E$. The heritability of each trait is the proportion of the phenotypic variance attributable to additive genetic effects, as $h^2 = \sigma^2_G/\sigma^2_P$. The p-values for the heritability estimates are obtained by likelihood ratio tests, where the likelihood of a model is estimated and compared with the likelihood of a model in which the heritability is constrained to zero. Two times the difference in the natural logarithmic likelihood is distributed asymptotically as a 1/2:1/2 mixture of a χ^2 variable with one degree of freedom and a point mass at zero (Hopper and Mathews, 1982). Heritability of resistin mRNA was calculated using covariates such as sex, age, age-by-sex interaction, age^2, age^2-by-sex interaction, body weight, adipose cell volume and number, and adipose triglyceride content. Bivariate analyses were conducted to identify genetic correlations among the traits. The phenotypic correlation between two traits can be expressed in terms of the underlying genetic and environmental correlations, correcting for the use of related individuals, by use of the equation:

$$\rho_P = \rho_G \sqrt{h_1^2}\ \sqrt{h_2^2} + \rho_E \sqrt{(1-h_1^2)}\ \sqrt{(1-h_2^2)}$$

In this equation, $h^2_{\ 1}$ and $h^2_{\ 2}$ correspond to the heritability values of each of the traits.

Evidence for pleiotropic effects (same genes influencing several phenotypes) was analyzed between the resistin mRNA levels, body weight, BMI, cytokines, glucose and insulin concentrations in fasting plasma.

The use of the variance decomposition approach requires the estimation of the identical by descent (IBD) matrix. A pair-wise maximum likelihood –based procedure was used to estimate IBD probabilities. An extension of the technique described by Fulker et al. was applied to perform the multipoint analysis and the QTL mapping (Hopper and Mathews, 1982).

Results

The baboons in this sample were comprised of 11 classes of relative pairs (Table 1), and included 277 females and 127 males. The mean and SD for the expression of resistin mRNA in adipose tissue was 26.1 $\pm$ 3.2 Ct, with no difference according to sex (Table 2). Males had higher body weights and lower insulin levels than females. No differences were observed between males and females in BMI or levels of plasma glucose or CRP.

Univariate quantitative analysis found a significant additive heritability of 23% ($p = 0.003$) for resistin mRNA levels in adipose tissue. Significant heritability values were observed for the other traits in baboons (Table 2). A highly significant genetic correlation was observed between resistin mRNA levels and CRP ($\rho = 0.97$, $p = 0.02$). No significant genetic correlation was observed between resistin expression and the other traits in baboons.

The genome scan detected a maximum LOD score of 3.84 for resistin mRNA levels in the region between markers D19S431 and D19S714, at 17.6 to 34.7 cM, corresponding to human chromosome 19 p13.3 (www.research/marshfieldclinic.org). A secondary signal was observed on chromosome 17 (LOD score 1.65) at marker ATAG010. Results of the string plot are shown in Figure 1.

Discussion

The present study used resistin mRNA expression levels in omental adipose tissue of baboons as a novel phenotype for genetic analyses. The genome scan identified a QTL (LOD score 3.84) in the location that corresponds to human chromosome 19p13.3. The resistin gene is located in this region (Stumvoll and Haring, 2002), as well as other genes associated with the phenotypes of the metabolic syndrome. This study replicates other genome scans that have identified QTLs in this region for hypertension, blood pressure and BMI. Using hypertension as a phenotype in a French Canadian population, Rice et al. (2000) reported a LOD score of 3.1 in this region. A smaller LOD score of 1.74 was found by Cooper et al. (2002) in a linkage study at the D19S714 marker for systolic blood pressure in Nigerians.

Yang et al. (2002) reported the mRNA expression profile of human visceral adipose tissue using microarray analysis. The region on chromosome 19 was found to encode a large number of the genes expressed in this tissue. These genes are related to processes implicated in the metabolic syndrome as described below, including the resistin gene (RSTN), the insulin receptor substrate (INRS), angiopoietin-like 4 molecule (PGA) and low density lipoprotein receptor (LDL-R).

Results from the present study imply that regulatory elements in the resistin gene may exhibit an influence on its own transcription rate. The resistin gene is polymorphic in humans and genetic variation has been associated with obesity and insulin sensitivity phenotypes (Wang et al., 2002; Cao and Hegele, 2001). Studies on the association of resistin polymorphisms in humans have identified single nucleotide polymorphisms (SNPs) that appear to be associated with a BMI >30 kg/m^2 in subjects from the Quebec City area. However, this observation was not replicated in a population from Scandinavia. No association between genetic variants in the 5' flanking region of the resistin gene and type 2 diabetes was found in US Caucasian subjects. Yet a synergistic effect did exist between different sequences and obesity and risk of type 2 diabetes (Engert et al., 2002). Another study by Ma et al. (2002) identified a SNP in the resistin promoter region that is associated with abnormalities in insulin sensitivity in US Caucasians. Three other resistin SNPs in noncoding regions also showed a relationship with insulin sensitivity index that interacted with BMI. A recent study by Smith et al. (2003) reported that the G/G genotype of the –180 C-G SNP in the promoter region of the human resistin gene had higher mRNA abundance in cultured adipocytes and *in vivo*

abdominal subcutaneous fat. These findings support our observations, suggesting that polymorphisms in regulatory elements in the resistin gene regulate variations in mRNA expression levels. In addition, the study by Smith et al. (2003) found a relationship of resistin mRNA abundance in human subcutaneous adipose tissue with insulin resistance and hepatic fat, and proposed a possible role for resistin in the development of the metabolic syndrome.

The second candidate gene in the region of our QTL that could influence resistin mRNA levels is the insulin receptor (INSR). This gene encodes a key protein in the insulin response cascade. The insulin receptor binds insulin, and has a tyrosine-protein kinase activity. Mutations in INSR have been observed in insulin resistance syndromes, and a polymorphism in intron 9 of the INSR gene was associated with hypertension (Zee et al., 1994). As described above, resistin has been associated with insulin sensitivity in humans (Yannakoulia et al., 2003; Smith et al., 2003) and mice (Steppan et al., 2001), but its relationship with the insulin receptor gene is unexplored.

The third candidate gene in the 19p13 chromosomal region is the novel angiopoietin-like 4 protein (PGAR). This gene encodes a protein expressed in adipose tissue. Expression of PGAR protein is induced by PPAR γ activation, which is known to elicit insulin sensitizing (Yoon, 2000). PGAR expression is elevated in genetic models of obesity. Studies on this protein suggest a role as an intercellular or inter-tissue signaling molecule, but its function has not yet been defined.

Finally, the gene for the LDL receptor (LDL-R) is located approximately 3 Kb from the resistin gene. This receptor has a critical role in the uptake of triglycerides from LDL, and a mutation in this gene has been associated with obesity in normotensive (Griffiths et al., 1995) and hypertensive subjects (Zee et al., 1995). Interestingly, a recent study by Jove et al. (2003) reported a negative correlation between resistin mRNA levels in omental fat and cholesterol levels after treatment with fenofibrate. This study indicated a possible link between cholesterol and resistin expression, however the mechanism remains to be studied.

It is interesting that phenotypes related to genes in the 19p13.3 region, such as insulin resistance, high blood pressure, dyslipidemia and elevated BMI, are components of the metabolic syndrome (Groop, 2000). The clustering of these genes suggests that they may segregate together (Yang, 2002). Further studies on the significance of the genetic proximity of these genes are needed to elucidate the possible combined effect that genetic variation in this region has on phenotypes associated with the metabolic syndrome.

The characteristics of baboons presented in table 2 confirm findings in previous studies from our laboratory (Tejero et al., 2003). A sexual dimorphism is present in adult baboons, reflected by the difference in body weight between male and female animals. However, no difference in resistin mRNA expression was observed according to sex. This finding is in contrast to Nogueiras et al. (Nogueiras et al., 2003) who reported higher levels of resistin mRNA in adipose tissue in male rats, as compared to females, using the Northern blot technique.

Genetic correlation is a direct measurement of pleiotropy, and indicates that a common set of genes regulate a set of traits (Comuzzie et al., 1996). In the present study, a significant genetic correlation exists between resistin mRNA levels and circulating levels of CRP, an inflammatory protein elevated in obesity. Circulating levels of the CRP marker are of prognostic value for development of chronic conditions (Rifai and Ridker, 2002). Since resistin was initially discovered in a murine model of lung allergic response, Stumvull and Haring (2002) proposed that this hormone might induce insulin resistance under subclinical inflammatory states. Findings in our study suggest that a common set of genes may regulate circulating levels of CRP and resistin expression.

In summary, our findings indicate that a region on chromosome 19 harbors a gene or a group of genes that influence the expression of resistin mRNA. The genetic correlation between resistin mRNA and CRP levels indicates that a common set of genes are influencing the expression of these phenotypes, and further research is needed to elucidate the relationship of these traits. Fine mapping studies are needed to identify the genetic variation responsible for the QTL for resistin expression in omental adipose tissue.

Table 1

Distribution of Relative Pairs Among the 404 Baboons

RELATIONSHIP	*N*
1st cousin	9
Avuncular	56
Grandparent-grandchild	4
1 st cousins	9
Half avuncular	460
Half 1st cousins	15
Half siblings & 1st cousins	4
Half siblings & half 1st cousins	38
Half-siblings & half avuncular	9
Parent-offspring	116
Siblings	232
Half siblings	2692
Total	3676

Table 2

Characteristics (Mean ± SD) of Male and Female Baboons

TRAIT	MALE *(N = 127)*	FEMALE *(N = 277)*	HERITABILITY ± SE	*P**
Weight (kg)	31.5 ± 4.7	19.6 ± 4.1	0.79 ± 0.09	0.001
BMI (kg/m^2) [a]	26.3 ± 3.7	22.8 ± 4.3	0.46 ± 0.13	0.001
Resistin mRNA (Ct)	26.5 ± 3.3	25.8 ± 3.1	0.23 ± 0.11	0.003
Glucose (mmol/l)	5.1 ± 0.8	5.4 ± 0.8	0.19 ± 0.11	0.01
Insulin (pmol/l)	115.2 ± 92.9	199.8 ± 92.9	0.46 ± 0.20	0.005
CRP (pmol/mL) [b]	256.4 ± 66.7	303.1 ± 63.7	0.20 ± 0.17	0.07

**P* = *P* values corresponding to estimates of heritability

[a] BMI = Body mass index

[b] CRP = C reactive protein

Table 3

Genetic (r_G) and Environmental (r_E) Correlations Between Resistin mRNA Abundance and Metabolic Traits in Baboons

TRAIT	ρ_G	*P*	ρ_E	*P*
Weight	0.10	0.70	0.04	0.75
BMI [a]	-0.04	0.88	0.13	0.35
Glucose	-0.04	0.91	0.08	0.47
Insulin	-0.56	0.11	0.22	0.28
CRP [b]	0.97	0.02	0.09	0.52
HOMA-IR [c]	-0.48	0.18	0.20	0.34

[a] BMI = body mass index

[b] CRP = C reactive protein

[c] HOMA = Homeostasis model for insulin resistance (Matthews et al. 1985)

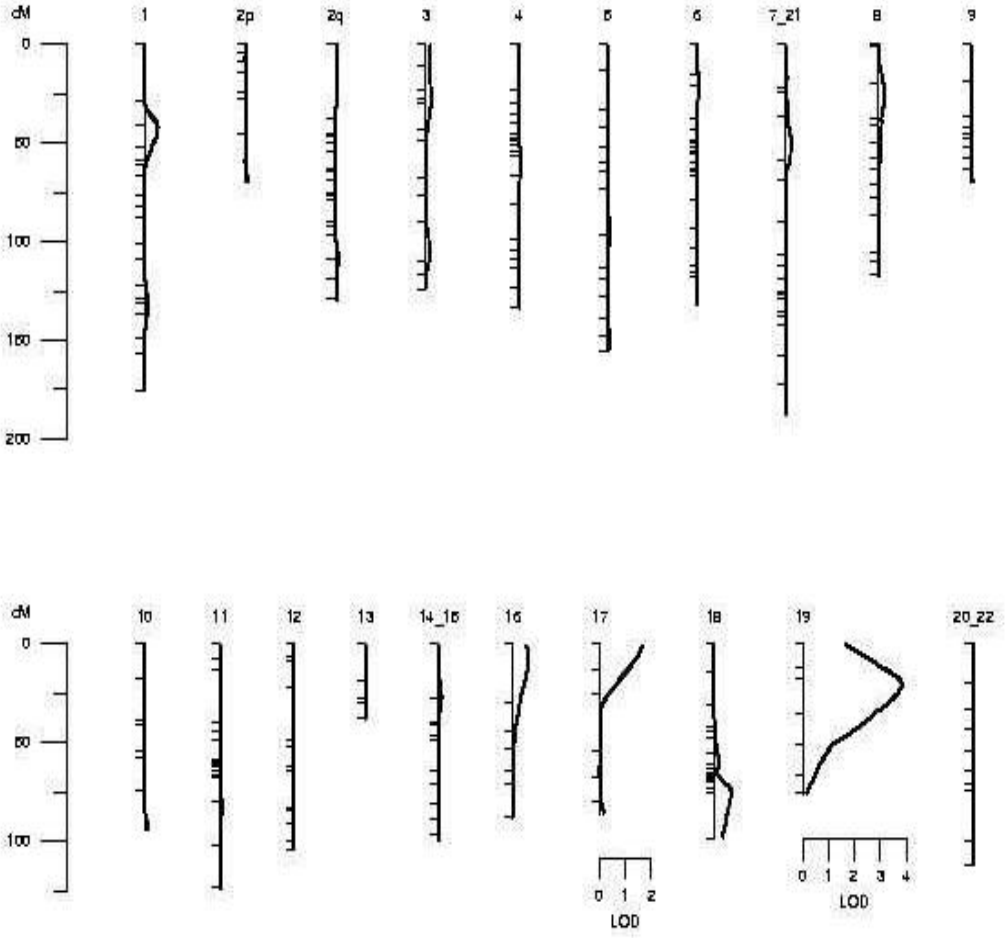

Figure 1 String plot for the genome scan for resistin mRNA levels in omental adipose tissue of baboons. The *y* axis corresponds to distance along the chromosome in cM, and the *x* axis indicates the LOD score value on a given region. A significant LOD score was found on chromosome 19p.

Chapter 3: Genetics of GLUT4 mRNA expression in omental adipose tissue of baboons

GLUT4 is an insulin-responsive glucose transporter expressed in adipose tissue. A decrease of the mRNA abundance of GLUT4 in adipose tissue has been observed in conditions of insulin resistance. This study proposes to use GLUT4 mRNA abundance as a novel phenotype for quantitative genetic analysis. A blood sample and a biopsy of omental adipose tissue were collected from 418 adult, pedigreed baboons. Biopsies were analyzed for triglyceride content, cell volume and number per weight of tissue, and total RNA was isolated. GLUT4 mRNA abundance in adipose tissue was measured by quantitative, real-time RT-PCR. Insulin and glucose were determined in fasting plasma by standard methods. Univariate and bivariate quantitative genetic analyses were conducted using GLUT4 mRNA, insulin and glucose as quantitative traits. GLUT4 mRNA expression in omental adipose tissue was heritable, with $h^2 = 0.23$, $p = 0.001$. A bivariate quantitative analysis revealed a significant genetic correlation between GLUT4 mRNA abundance and body weight ($\rho = 0.63$, $p = 0.007$), BMI ($\rho = 0.59$, $p = 0.02$) and with insulin ($\rho = 0.72$, $p = 0.04$). A genome scan was conducted, and detected a signal on chromosome 10q26 (LOD score = 1.4), a region previously associated with BMI. Thus, GLUT4 mRNA abundance in omental adipose tissue has a significant genetic component. These results suggest that adipose GLUT4 mRNA abundance, plasma insulin levels and body weight may be regulated by common genes.

Introduction

Glucose is cleared from the bloodstream by a family of facilitative transporters (Buse et al., 1992; Ezaki, 1999) that is composed of five isoforms (Watson and Pessin, 2001). Glucose transporter 4 (GLUT4), the major insulin-responsive transporter, is restricted predominantly to skeletal muscle and adipose tissue, and plays an important role in whole body glucose homeostasis (Ezaki, 1999; Watson and Pessin, 2001). Stimulation of glucose uptake by insulin involves translocation of GLUT4 from its intracellular storage sites to the plasma membrane (Garvey et al., 1992).

Nutritional conditions such as fasting, high fat feeding and obesity lead to a decrease in the level of GLUT4 mRNA in adipose tissue (Ezaki, 1999). Changes in GLUT4 gene expression are observed in physiologic states of altered glucose homeostasis. GLUT4 mRNA abundance varies in a tissue-specific manner and turns over at a more rapid rate in adipose tissue than in skeletal muscle (Garvey et al., 19992; Charron et al. 1999). Charron et al. (1999) reported that changes in the steady state of GLUT4 mRNA are reflective of the rate of mRNA transcription.

Diabetic and obese subjects have reduced levels of GLUT4 mRNA in adipose tissue (Garvey et al., 1991, Garvey et al., 1991). Ducluzeau et al. (2001) compared the change in GLUT4 mRNA abundance in human adipose tissue of type 2 diabetic and obese patients after an insulin infusion. The GLUT4 mRNA levels did not change in the type 2 diabetic subjects, but did increase in nondiabetic obese and control patients. These findings confirm that low expression of GLUT4 mRNA is associated with insulin

resistance and suggest decreased glucose transport and metabolism in muscle and adipocytes (Ezaki, 1999). In insulin resistant states such as obesity and type 2 diabetes, GLUT4 expression is diminished in adipose tissue but preserved in skeletal muscle (Garvey et al., 1992). In genetically modified mice with adipose-selective reduction of GLUT4 (G4A-/-), the muscle and liver exhibited insulin resistance (Abel et al., 2001). Conversely, the upregulation of GLUT4 mRNA in adipose tissue via treatment with an anti-diabetic agent, troglitazone, was related to an improvement of insulin resistance in type 2 diabetic rats (Furuta et al., 2002). Thus, the increase of the expression of this glucose transporter might contribute to enhanced insulin sensitivity. Collectively, these studies suggest that expression of the GLUT4 gene is a clinically relevant target to study insulin-resistant disease states (Charron et al., 1999).

Observations of the baboon colony at the Southwest Foundation for Biomedical Research (SFBR) have shown that baboons develop spontaneous obesity, insulin resistance and type 2 diabetes (Comuzzie et al, 2003; Stokes,1986), even though all animals share the same diet and living conditions. Thus, the potentially confounding environmental factors impacting phenotypic expression are minimized. At the genetic level, studies on chromosomal structure support a high degree of similarity between humans and baboons. For example, Rogers et al. (2000) found > 400 polymorphic loci that amplify from baboon DNA using human sequence-based PCR primers. Based on these markers, a 10cM linkage map was developed for the baboon, making it possible to search for genes involved in complex diseases. Findings from our laboratory confirm a high degree of identity between human and baboon DNA sequences for lipoprotein lipase (LPL) (Cole and Hixon, 1995), resistin (Tejero et al., 2003), leptin (Cole et al.,

2003) and adiponectin (Unpublished data). These homologies suggest that the baboon is an excellent model for the genetic study of obesity and related conditions. The present study will analyze the genetic component of the regulation of the expression of GLUT4 mRNA in omental adipose tissue, and proposes its abundance as a novel phenotype for quantitative genetic studies.

Animals and Methods

Animals

The data for this study were obtained from 460 randomly-bred, 134 male and 326 female pedigreed baboons chosen without regard to any pre-existing clinical condition. The majority of the animals are olive baboons (P. h. anubis), with a smaller proportion of yellow baboons (P. h. cynocephalus), and crosses between those two subspecies.

Sampling and Phenotype Collection

Baboons were sedated with ketamine under fasting conditions. Body weight was measured on an electronic balance (GSC, Chicago, IL) and body length was measured between head and feet, with the animals lying on their back. Body mass index was calculated as weight in kg divided by the squared length in meters. Blood samples were collected from the antecubital vein as 4 mL in sodium fluoride tubes for glucose analysis, and 7 mL in EDTA tubes for insulin measurement. The plasma was separated by centrifugation at 2000 x g and frozen at –80^{o} C for further analyses. A biopsy of omental fat was obtained as described elsewhere (Lewis et al., 1986).

Insulin was measured by radioimmunoassay (Linco Research, Inc., St Charles, MO) and glucose, in an Analox spectrophotometer (Analox Instruments, Lunenburg, MA Parameters with variations > 5% were re-analyzed. The homeostasis model for estimation of insulin resistance (HOMA-IR) was calculated as described by Matthews et al. (1985).

Samples of omental adipose tissue were analyzed for triglyceride content, cell size and number per gram of tissue as described by Lewis et al. (1986). The remaining sample was frozen immediately using liquid nitrogen and stored at –80^{o} C. Total RNA was isolated from adipose tissue with TRI REAGENT (Molecular Research Center, Inc, Gaithesburg, MD). All RNA samples were analyzed at 260, 230 and 280 nm UV for quality control and quantity calculations. RNA integrity was verified by staining with ethidium bromide on a 1.2% agarose gel. All samples were treated with RQ1 DNAse (Promega, Madison, WI) for 15 minutes at 37^{o} C for digestion of traces of DNA.

The cloning of a partial baboon cDNA for GLUT4 was performed by reverse transcription, followed by polymerase chain reaction (RT-PCR) using the THERMOSCRIPT ^{TM} System kit from Gibco BRL Life Technologies (Gaithersburg, MD). Primers for cloning were designed using published human sequences (GeneBank gi 13632785). A fragment of 250 bp was amplified from the region corresponding to human exons 4A and 4B, which is highly specific for GLUT4 (Chiuh et al., 1994). Clones were sequenced using Big Dye Terminator system (Applied Biosystems, Forest City, CA) on a 377 Sequencer. The baboon DNA sequence was 95% identical to the human sequence.

The baboon GLUT4 cDNA clone was used for the design of primers and probe for the Taq Man quantitative, RT-PCR system (Applied Biosystems, Foster City, CA) using the Primer Express Software V. 1 (Applied Biosystems). Sequences of forward and reverse primers and probe were:
5' TGG CCA GGC CCA TGA G 3' and 5' ATG GAA GGA AAA GGG CTA TGC 3', respectively, and for the GLUT4 probe: 5' CCC AGC ACT GCC AGG ACA TTG TTG 3'.

An assay to determine 18s ribosomal mRNA (18s rRNA) was used to estimate measurement error. The master mix contained primers and competimers from the 18S Universal System from Ambion (Austin,TX) in a 4 to 6 ratio. The probe for the18S rRNA was rRNA Ambiprobe, from Applied Biosystems.

Inter assay coefficients of variation for GLUT4 mRNA and 18S rRNA were 4.7% and 6%, and the intra-assay coefficients were and 4.0 and 5.2%, respectively. All samples were analyzed in duplicate, and average of the two measures was used for analyses. 50 ng of total RNA was used per 25 μl reaction. Conditions for RT-PCR were: 50 minutes at 48^{o} C for reverse transcription, followed by 40 cycles of 95^{o} C for 15 seconds and 60^{o} C for 1 minute for the PCR amplification. The GLUT4 mRNA abundance data are expressed in Ct, which is the number of PCR cycles at which logarithmic plots of product accumulation cross a specific threshold. The expression values were calculated as GLUT4 Ct divided by 18S rRNA and multiplied by the average 18S rRNA Ct in a given run.

Genotyping

A sample of 8 ml of blood was drawn from all subjects for separation of leukocytes. The isolated cells were used for DNA extraction using a phenol-chloroform method (Rogers et al., 1995). Published human PCR primers were used to amplify homologous short tandem repeats (STR) loci from baboon genomic DNA. The PCR products were run on electrophoresis gels on ABI 373 and ABI 377 automated sequencers, using fluorescently labeled primers and Genescan and Genotyper software (Applied Biosystems). Data was generated on 330 STRs. This technique yielded a map density of 7.2 cM. Initial analysis included validation of the reported relationships between individuals. The management and preparation of baboon genotypes and pedigrees was accomplished by analysis in PEDSYS routines (Dyke, 1994).

Statistical Genetic Methods

Analyses included the univariate and bivariate additive genetic analyses, performed on 418 related baboons. Quantitative genetic analyses were conducted in the pedigreed animals using the maximum likelihood-based, variance decomposition approach that is implemented in the computer package SOLAR (Southwest Foundation for Biomedical Research, San Antonio, TX) (Almasy and Blangero, 1998). The variance of the trait, (GLUT4 mRNA abundance), represented as σ^2_P, was partitioned into the additive genetic (σ^2_G) and environmental components (σ^2_E). The heritability of each trait is the proportion of the phenotypic variance attributable to additive genetic effects, as $h^2 = \sigma^2_G/\sigma^2_P$. The p-values for the heritability estimates are obtained by likelihood ratio tests, where the likelihood of a model is estimated and compared with the likelihood of a model

in which the heritability is constrained to zero. Two times the difference in the natural logarithmic likelihood is distributed asymptotically as a ½ ½ mixture of a χ^2 variable with one degree of freedom and a point mass at zero (Self and Liang, 1987). Heritability of GLUT4 mRNA expression was calculated using covariates such as sex, age, age-by-sex interaction, age^2, age^2-by-sex interaction, body weight, adipose cell volume and number, and adipose triglyceride content. Bivariate analyses were conducted to identify genetic correlations among the studied variables. The phenotypic correlation between two traits can be expressed in terms of the underlying genetic and environmental correlations, correcting for the use of related individuals, by use of the equation:

$$\rho_P = \rho_G \sqrt{h_1^2} \sqrt{h_2^2} + \rho_E \sqrt{(1-h_1^2)} \sqrt{(1-h_2^2)}$$

In this equation, h^2_1 and h^2_2 correspond to the heritability values of the pair of traits.

Evidence of pleiotropic effects (same genes influencing several phenotypes) was analyzed between the GLUT4 mRNA abundance, body weight, BMI, glucose and insulin levels in plasma. Descriptive values of the traits were calculated using the Pedysis software. A genome scan was conducted using SOLAR software with GLUT4 mRNA level in omental adipose tissue as a quantitative phenotype (Hopper and Mathews, 1982).

Results

The 418 baboons included in the analyses were comprised of 11 classes of relative pairs (Table 4)**.** The mean ages of the female and male animals were 15.8 $\pm$ 4.8, and 12.6 $\pm$ 3.9 years**,** respectively. The mean and SD for the expression of GLUT4 mRNA in adipose tissue was 34.4 $\pm$ 3.1 Ct. Differences were not observed according to sex. The general characteristics of the traits examined in this study are listed in Table 5. As illustrated, males had higher weights than females. GLUT4 expression, age, plasma glucose and insulin plasma levels did not differ significantly by sex.

Univariate quantitative analyses found a significant additive heritability of 23% for GLUT4 mRNA abundance in omental adipose tissue, using triglyceride content per gram of fat as covariate ($p = 0.005$). In addition, body weight, BMI, plasma glucose and insulin levels and the HOMA-IR index had significant heritabilities, as shown in Table 5. The bivariate quantitative analyses (Table 6) revealed significant genetic correlations between GLUT4 mRNA levels and body weight, BMI and fasting insulin levels. Weight and BMI had significant genetic correlations with insulin, with $\rho = 0.43$, $p = 0.05$ and $\rho = 0.58$, $p = 0.03$, respectively. No significant relationships were observed between GLUT4 expression and glucose levels and the HOMA-IR index. Environmental correlations were not significant among the traits in the present study.

Results from a preliminary genome scan are presented in Figure 2 as a string plot. A suggestive LOD score of 1.4 was found with marker D10S1230, corresponding to the

10q26 region on human chromosome 10 at 142.78 cM

(www.research/marshfieldclinic.org)

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Discussion

Results from the present study confirm that GLUT4 mRNA abundance is a quantitative trait associated with insulin resistance (Garvery et al., 1992; Ducluzeau et al. 2001). This phenotype had a significant genetic component and was genetically related with other insulin resistance-related phenotypes such as body weight, BMI and plasma insulin.

Univariate genetic analysis determined a heritability value of 23% for GLUT4 mRNA abundance. This genetic contribution to the variation of this phenotype is not surprising, since quantitative genetic studies have consistently found a significant heritability for other insulin resistance-related traits in humans. These include obesity-related traits (Pausova et al., 2001), insulin level (Panhuysen et al., 2003) and secretion (Lethovirta et al., 2000), indirect insulin resistance indexes (Cai et al., 2003), 2 h postpandrial insulin levels (Mitchell et al., 1996), and clinical diagnosis of type 2 diabetes (Hanis et al., 1996). As found in humans and in the results of a previous study from our laboratory, plasma levels of insulin and glucose, body weight, BMI and the HOMA-IR index, are significantly heritable in baboons (Cai et al., 2003).

The magnitude of the genetic correlation between a pair of traits represents the degree of pleiotropy (Comuzzie et al., 1996). Studies in humans have found significant genetic correlations between BMI and other indicators of adiposity and insulin levels in fasting conditions in Mexican Americans ($\rho = 0.52$, $p < 0.001$) (Comuzzie et al., 1996; North et al., 2003). In the present study the significant genetic correlations found among GLUT4 mRNA in omental adipose tissue and body weight, BMI and fasting insulin levels suggest that a common set of genes influence the expression of these phenotypes. This is a novel insight into the association between increasing levels of adiposity and conditions such as insulin resistance and type 2 diabetes.

No significant environmental correlations among variables were found in baboons, as opposed to findings in human studies (Comuzzie et al.,1996). This lack of association is presumably due to the homogenous environment in this animal model.

The 10q24-26 chromosomal region showing suggestive linkage (LOD = 1.4) to GLUT4 mRNA levels in baboons harbors the genes encoding the adrenergic receptors α2a (ADRA2) and β1 (ADRB1) (located between 137.6 to 141.8 cM on chromosome 10 in humans). The hormones epinephrine and norepinephrine are agonists for these G-protein coupled receptors. ADRA2 is involved in inhibition of mobilization of fatty acids in adipose tissue (Carpene et al., 1998). Garenc et al. (2002) found a relationship between the C1291G polymorphism in the promoter region of the ADRA2 receptor and the trunk-to-extremities skinfold ratio (TER) in blacks. This polymorphism was related to a higher TER in men, and the opposite observation was reported in women. Rosmond et al. (2002) reported a low cortisol response to dexamethasone and elevated glucose

levels in subjects with this polymorphism. A study by Ukkola et al. (2001) found that subjects homozygous for the ADRA2 DraI 6.3/6.3 genotype had smaller increases in glucose and insulin levels in plasma during the oral glucose tolerance test (OGTT). These studies indicate a possible role for ADRA2 in insulin sensitivity. As opposed to the ADRA2, ADRB1 in adipose tissue induces lipolysis and energy expenditure (Carpene et al., 1998). A study by Dionne et al. (2002) reported that a polymorphism in the amino acid sequence of ADRB1 (Gly389Arg) was associated with obesity in humans.

In summary, results from this study indicate that the variation of GLUT4 mRNA expression in omental adipose tissue has a significant genetic component. A common set of genes seems to influence GLUT4 mRNA abundance, weight, BMI and fasting insulin. These associations support the complex nature of the genetic components in obesity and insulin resistance- related conditions.

Table 4

Distribution of Relative Pairs Among the 418 Baboons

RELATIONSHIP	*N*
1st cousin	7
Avuncular	59
Grandparent-grandchild	2
Half 1st cousins	11
Half avuncular	562
Half siblings & 1st cousins	4
Half siblings & 1st cousins	35
Half-siblings & half avuncular	9
Parent-offspring	112
Siblings	247
Half siblings	2731
Total	3809

Table 5

Characteristics (Mean ± SD) of Male and Female Baboons

TRAIT	MALE *(N = 128)*	FEMALE *(N =280)*	HERITABILITY ± SE	*P*[*]
Weight (kg)	31.9 ± 5.1	19.8 ± 4.1	0.79 ± 0.09	0.001
BMI (kg/m^2) [a]	26.6 ± 3.8	22.9 ± 4.5	0.46 ± 0.09	0.001
GLUT4 mRNA (Ct)	34.8 ± 3.2	34.5 ± 3.2	0.23 ± 0.10	0.001
Glucose (mmol/l)	5.0 ± 0.7	5.3 ± 0.7	0.19 ± 0.11	0.01
Insulin (µUl/l)	29.7 ±17.8	51.3 ± 26.1	0.46 ± 0.20	0.005

[a] BMI = Body mass index

[*] *P* values correspond to the estimated heritability.

Table 6

Genetic (r_G) and Environmental (r_E) Correlations Between GLUT4 mRNA Abundance and Insulin Resistance-Related Traits in Baboons

TRAIT	ρ_G	*P*	ρ_E	*P*
Weight	0.63	0.007	-0.24	0.06
BMI [a]	0.59	0.02	-0.21	0.10
Insulin	0.71	0.04	-0.68	0.11
Glucosa	0.04	0.91	0.02	0.83
HOMA-IR [b]	-0.25	0.48	-0.02	0.93

[a] BMI = body mass index

[b] HOMA-IR = homeostasis model for insulin resistance (Matthews et al., 1985).

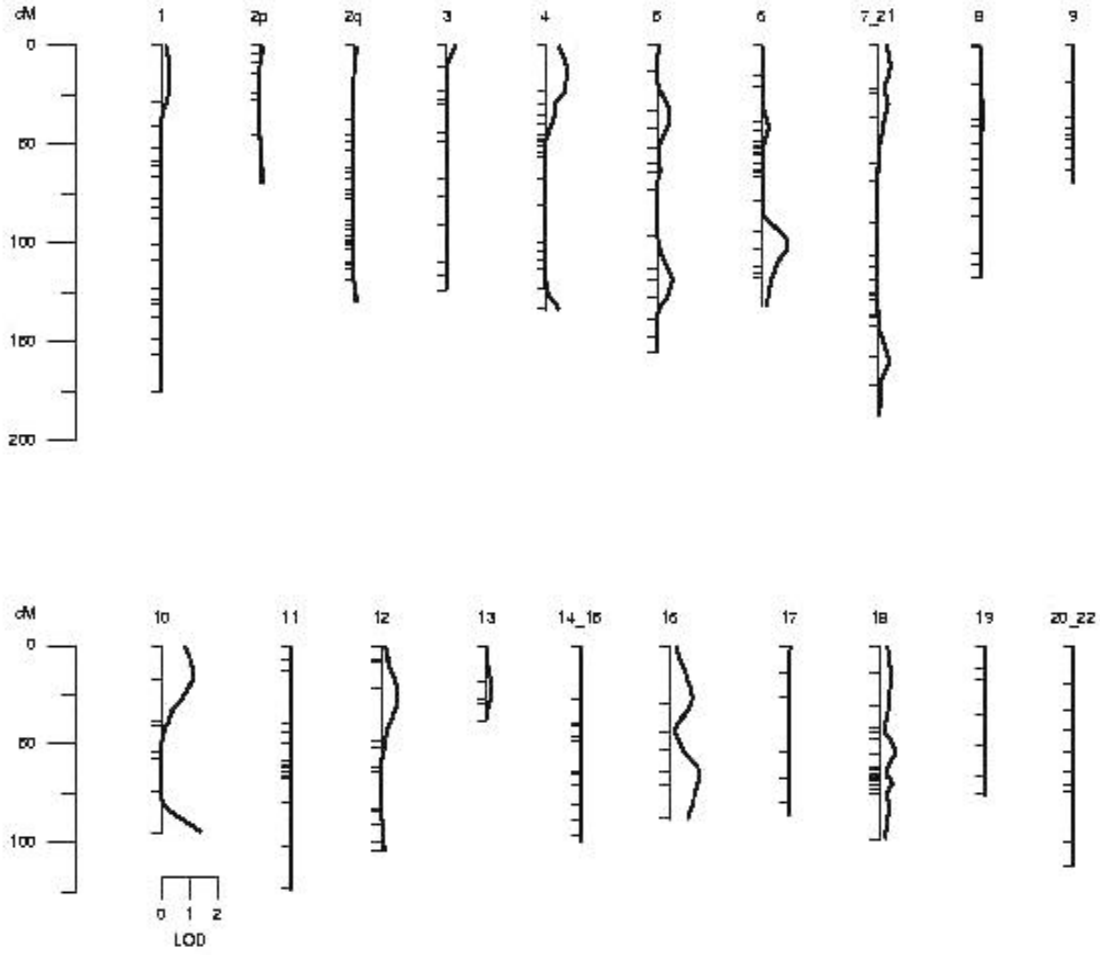

Figure 2 Sting plot for the genome scan for GLUT4 mRNA in omental adipose tissue. The *y* axis corresponds to distance along the chromosome in cM, and the *x* axis indicates the LOD score value on a given region.

Chapter 4: Plasma Adiponectin, but not Resistin mRNA Expression in Monocytes is Associated with Insulin Resistance-Related Phenotypes in Baboons

Resistin has been proposed as a possible link between obesity and insulin resistance, but findings in humans and animal models have produced controversial data on this association. This paper analyzed the potential relationship between resistin expression in monocytes and insulin resistance in baboons.

108 adult baboons (84 female and 24 male) were studied. Weight was measured and a blood sample was collected in fasting conditions for plasma and monocyte isolation. Fasting glucose, insulin and C-peptide levels in plasma were measured by standard methods. Insulin resistance was calculated by HOMA and Quicki index. Data were clustered by weight terciles for statistical analysis. Resistin mRNA abundance in monocytes was measured by real time RT-PCR.

According to the HOMA index, 32% female and 12.5% male baboons had elevated insulin resistance. As observed in humans, the insulin resistance-related phenotypes are related with weight and with C-peptide levels. No significant association between resistin expression in monocytes and the insulin resistance-related phenotypes was found in baboons. These findings support the baboon as an animal model for the study of obesity and insulin resistance, and suggest no association between resistin expression in monocytes and insulin resistance-related phenotypes.

Introduction

The adipose tissue-derived hormones adiponectin and resistin have been proposed as possible links between adiposity and insulin resistance (Stumvull and Häring, 2002; Yamauchi et al.,2001). It is believed that adiponectin improves insulin resistance, while resistin antagonizes insulin effects (Stumvull and Haring, 2002).

Adiponectin is an adipose tissue-derived hormone that is abundant in the plasma and affects insulin resistance (Yamauchi et al., 2001; Weyer et al., 2001). Low levels of plasma adiponectin are associated with obesity and type 2 diabetes in humans (Weyer et al., 2001) and rhesus monkeys (Hotta et al., 2001), and with coronary heart disease (Diez and Iglesias, 2003) and risk factors for the metabolic syndrome in humans (Stumvull and Haring, 2002; Yang et al., 2001). Yang et al. (2001) and Esposito et al. (2003) observed a significant increase of adiponectin levels after weight reduction in humans, with concomitant improvement of insulin resistance. Collectively, these studies indicate that circulating levels of adiponectin influence insulin sensitivity.

Resistin belongs to the resistin-like molecules (RELM) family of proteins. These proteins are detected in the inflammatory response to allergic pulmonary inflammation (Holcom et al.,2000). The human form of murine resistin has been mapped to chromosome 19 and is produced by white (Steppan et al., 2001; Sumvull and Haring, 2002; Holcom et al., 2000, Wang et al., 2002) and brown adipose tissue (Viengchareun et al., 2002), bone marrow, lung and monocytes (Patel et al., 2003; Nagaev and Smith, 2002).

Initial observations from Steppan et al. (2001) reported that circulating levels of resistin protein and mRNA in adipose tissue were increased in obese mice. Administration of intraperitoneal recombinant resistin protein to mice diminished glucose tolerance and raised plasma insulin levels. In addition, blocking with specific murine antibodies to inactivate resistin improved insulin resistance. These observations implied that resistin reduced insulin sensitivity and was a possible link between adipose tissue and insulin resistance. In 2002 Mc Ternan et al. (2002a) reported detectable levels of resistin mRNA in human adipose tissue. Higher resistin expression was found in both abdominal subcutaneous and omental depots, as compared to those in the thigh (Mc Ternan et al., 2002b). These higher values validate the proposed association between omental body fat abundance and insulin resistance. Also, Patel et al. (2003) detected significant amounts of resistin in human macrophages and other tissues. They concluded that thiazolidinediones (TZD), a PPARγ activator, reduced resistin expression at the RNA and protein level in these cells. Thus, these two studies support conclusions from the original paper by Steppan that resistin antagonized insulin action.

In contrast, Way et al. (2001) reported that treatment with TZD enhanced resistin expression and that obese rodents had lower resistin mRNA levels in adipose tissue as compared with lean pairs. Similar results were described in a human study by Nagaev and Smith (2001). Resistin expression in subcutaneous adipose tissue was not related with insulin resistance, as measured by euglycemic clamp in insulin-sensitive, insulin-resistant or type 2 diabetic patients. Although little or no resistin expression in human adipose tissue was found, the expression in monocytes was measurable. A possible

connection between resistin expression in these cells and insulin resistance was not explored. In 2002 Janke et al. (2002) analyzed the association between resistin mRNA abundance in cultured human subcutaneous preadipocytes and adipocytes, and insulin resistance via the homeostasis model assessment (HOMA-R) using fasting glucose and insulin levels. No association was found between resistin expression and weight, or insulin sensitivity. Thus, the conflicting reports on the biological role of resistin in insulin sensitivity are puzzling.

The baboon is an excellent animal model for the study of insulin resistance-related phenotypes because there is a close correspondence to humans in the interaction of genes involved in obesity and type 2 diabetes (Rogers et al., 2000). Approximately 10% of a captive colony of adult baboons developed spontaneous obesity and 4% become hyperglycemic or diabetic, even though they were housed under similar conditions and ate the same diet (Comuzzie AG, unpublished observations). Recently, variations in plasma adiponectin (Comuzzie AG and Carey K, unpublished observations) and resistin (Comuzzie AG and Lazar M, unpublished observations) across different baboons were detected in plasma using a human antibody. These observations confirm the presence of circulating levels of these hormones in baboons, and a high structural similarity with the human proteins. The purpose of this study is to analyze the association of plasma adiponectin levels, resistin mRNA expression in monocytes and insulin resistance-related phenotypes in adult baboons.

Animals and Methods

Animals

The 108 baboons consisted of 24 male and 84 non-pregnant or lactating females from the pedigreed colony at the SFBR. This colony was founded in San Antonio, TX with 400 feral animals (360 females and 40 males) from a mixture of two species, *P. h. anubis* and *P. h. cynocephalus*. All animals are gang-housed and fed *ad libitum* on a standard chow diet (Harlan Tecklad 15% Monkey Diet, 8715).

Sampling and Phenotype Collection

All samples were collected after an overnight fast (12 h), with the animals under sedation with ketamine. Weight was measured on a calibrated electronic scale (GSE. Chicago, IL). A 20 ml sample of blood was drawn from the antecubital vein and divided as follows: 8 ml sample was collected in CPT tubes containing a density gradient polymer gel and sodium citrate (Becton Dickinson) for monocyte isolation, 4 ml in sodium fluoride tubes for glucose analysis, and 7 ml in EDTA tubes for analysis of adiponectin, insulin and C-peptide. All samples were centrifuged for 10 minutes at 2000 X g. The resultant plasma from the sodium fluoride and EDTA tubes was collected, and frozen at –80^{o} C for subsequent analysis.

A 1 g omental adipose tissue biopsy was collected from a healthy male baboon, while under sedation with ketamine. This sample was frozen for further RNA isolation with Trizol Reagent (Molecular Research Center, Inc. Gaithersburg, MD). A 445 bp fragment

of baboon resistin was cloned from this RNA sample by a two-step RT-PCR method using the THERMOSCRIPT TM RT-PCR System (Gibco BRL, Life Technologies, Inc, Gaithersburg, MD). Primers for cloning were designed based on the human sequence (GenBank Accession No. AF352730). The amplified product was cloned using the CloneAmp pAMP1 kit (Gibco BRL, Life Technologies), according to their protocol. The cloned baboon resistin cDNA fragment was sequenced on an ABI 377 automated DNA sequencer using the Big Dye Terminator kit (Applied Biosystems, Foster City, CA). This fragment was 95% identical to the human mRNA sequence.

Monocytes were collected by centrifugation in CPT tubes (Becton Dickinson, Franklin Lakes, NJ), washed twice with 10 ml of sterile PBS and frozen at -80^{o} C for subsequent RNA extraction. RNA was isolated using a 4 PCR RNAquous system from Ambion (Austin, TX). RNA integrity was verified on 1% agarose gel using ethidium bromide stain. The RNA yield and purity were analyzed by UV spectrophotometry. Resistin expression in monocytes was measured by real-time, quantitative RT-PCR (Taq Man, Applied Biosystems, Foster City, CA). The primers and probe sequences were designed with the Primer Express Software Version 1 (Applied Biosystems, Foster City, CA) using the baboon resistin clone. The sequences of forward and reverse primers were: 5' TCCTCCTGCCTGTCCTGG 3' and 5' CGCCCTCCTGAATCTTCTCAT 3', respectively. The sequence for the resistin probe was: 5'TCTAGCCAGACCCTGTGCTCCATGG 3'.

Ribosomal 18S RNA (rRNA) was used as an internal control and measured by the Universal 18S system from Ambion (Austin, TX). The master mix primers to

competimers ratio was 4:6. The probe for 18S was r RNA Ambiprobe from Applied Biosystems.

A sample of 50 ng of total RNA was used per assay. RT-PCR conditions were 48^{o} C at 50 min for reverse transcription, and 40 cycles of 60^{o} C for 1 minute, followed by 90^{o} C for 15 seconds. Data were obtained as Ct values (the number of cycles at which logarithmic plots of PCR product accumulation cross a specific threshold line), according to the manufacturer's specifications. Resistin expression was corrected for sample-to-sample measurement error, and calculated as resistin Ct divided by the 18S rRNA Ct, and corrected by the 18S rRNA Ct mean in any given run. Inter and intra-assay coefficients of variation for resistin expression were 6 and 4% and for 18S rRNA, 5 and 7%, respectively.

Glucose was analyzed by the glucose oxidase method on an Analox spectrophotometer (Analox Instruments, Lunenburg, MA). Adiponectin levels were measured by radioimmunoassay (Linco Research, Inc, St Charles, MO). Insulin and C-peptide were analyzed by quimioluminiscence in a Luminex100 using the Endocrine Multiplex Immunoassay (Linco Research, Inc.). Parameters with variations greater than 5% were reanalyzed. All samples were analyzed in duplicate and compared with standard curves. The HOMA was calculated as indicated by Matthews et al. (1985).

Statistical Analysis

Descriptive values and analytical tests were calculated via SPSS. Comparisons between male and female baboons were performed by t tests for independent samples.

Pearson's correlations among variables were calculated. Baboons were classified in tertiles by weight, and the resulting groups were compared, using one-way ANOVA and LSD as post hoc tests.

Results

In this study the female to male ratio was 3 to 1, which represents the sex ratio at the baboon colony at the SFBR. The weight values for baboons ranged from 13 to 32 kg for females, and 26 to 40 kg for males. Three females and one male were hyperglycemic, as indicated by glucose fasting levels > 125 mg/dL. The HOMA-IR index for insulin resistance ranged from 0.1 to 12.1 in females, and 0.4 to 4.3 in males. Values greater than 3.0 are considered as elevated insulin resistance (Rodriguez-Moran and Guerrero-Romero, 2003). In this sample, 26 females (32%) and 3 males (12%) had elevated insulin resistance, as classified by the HOMA-IR index.

Table 7 compares the analyzed traits between female and male baboons. Females had lower body weights and were older than the males. Fasting glucose and insulin levels, as well as the glucose/insulin index, did not differ according to sex. Higher concentrations of C-peptide and the HOMA-IR index values were observed in females as compared to males, indicating higher insulin resistance. Adiponectin levels and resistin mRNA expression in monocytes were not significantly different according to sex.

Table 8 shows the analyzed phenotypic mean values within each weight tertile in female baboons. Insulin, C-peptide levels and HOMA-IR index values increased with weight. The reduction in adiponectin plasma levels with weight increments was not

significant (p=0.18), however, the log-transformed values of this hormone were correlated significantly to plasma insulin levels ($r = -0.3$, $p < 0.05$), the HOMA-IR index ($r = - 0.3$, $p < 0.05$) and the glucose/insulin ratio ($r = 0.34$, $p < 0.05$) in female baboons. The glucose/insulin ratio varied across tertiles, but only values in the highest tertile were significantly lower than the other groups. Data on male baboons are presented in Table 9. Males showed a similar trend, with increasing insulin and C-peptide circulating levels according to weight increments, but differences among tertiles were not significant, presumably due to the smaller sample size. Levels of adiponectin decreased with higher body weight, with no significant difference as observed in females. The glucose/insulin ratio declined significantly as body weight increased. Resistin mRNA abundance in monocytes was not associated with tertiles of body weight of any of the studied phenotypes in either sex group.

Discussion

Some human studies have reported higher adiponectin circulating levels in females than in males (Arita et al., 1999). In this study adiponectin levels were not different between sex groups, and circulating levels in baboons were within the range observed in humans (Yang et al., 2001; Esposito et al., 2003; Arita et al., 1999). Adiponectin was not associated with weight, but was related to plasma insulin levels, HOMA-IR and glucose/insulin indexes in female baboons. Similar findings have been observed in a human study by Matsubara et al. (2003) who found an inverse association between adiponectin levels, and indicators of insulin resistance in Japanese nondiabetic women. Plasma log-transformed adiponectin levels were significantly correlated with insulin levels ($r = -0.3$, $p<0.01$) and the HOMA-IR index ($r = -0.3$, $p<0.01$).

Since its discovery in 2001, the biological role of resistin has been under extensive investigation. Most of the published work has focused on resistin production in adipose tissue, and there are few studies on resistin expression in monocytes and its potential contribution to insulin resistance. Recently Patel et al. (2003) reported a down-regulating effect of PPARγ activators on resistin expression in cultured monocyte-derived macrophages in vitro. These findings support initial observations from Steppan et al. (2001) in mice and suggest a possible contribution of monocyte resistin to insulin sensitivity. However, in our study resistin mRNA expression in monocytes was not related to any indicator of insulin resistance. Although there are significant differences by sex in insulin resistance-related phenotypes, resistin expression in monocytes was the same in female and male baboons.

In this study a profound sexual dimorphism in size existed between female and male baboons, with females having a wider range in weight and age. Although female baboons were older than males, both groups are considered as adults, with females at childbearing stage. Previously Comuzzie et al. (2003) reported that percentage of body fat and weight are significantly correlated in baboons ($r = 0.76$, $p = 2 \times 10^{-5}$). The levels of insulin and C-peptide in baboons in the present study are close to those reported for humans (Humphriss et al., 1997). The increase in these parameters of insulin resistance with escalating body weights is supported by the parallel increase in C-peptide levels and the HOMA-IR index, as illustrated in Figure 3. The effect of weight on insulin resistance-related phenotypes was more pronounced in the female baboons, with significant differences across the weight tertiles. The male baboons showed a similar

trend, but the smaller sample size and narrow weight interval in the male sample presumably precluded significance. A previous study on body composition of the baboon found that weights of 20 kg in females, and 38 kg in males, are associated with approximately 20% body fat (Comuzzie et al., 2003). In this study, 35 females and one male exceeded this reference weight. It should be noted that standards for overweight and obesity have not been established in baboons.

In summary, levels of plasma adiponectin levels were associated significantly with insulin and the HOMA index. Body weight was associated with variations in insulin resistance, as indicated by levels of fasting insulin and C-peptide levels, and the HOMA-IR index. Resistin mRNA was expressed in significant amounts in monocytes in baboons, but it was not related to any parameter measured in this study. The observations above support the value of the baboon as a model for the study of obesity-related conditions.

Table 7

Characteristics (Mean ± SD) of Female and Male Baboons

TRAIT	FEMALE *(N = 84)*	MALE *(N = 24)*	*P*
Weight (Kg)	19.3 ± 4.14	31.6 ± 3.5	0.001
Age (years)	18.2 ± 4.9	14.9 ± 3.2	0.001
Glucose (mmol/L)	5.4 ± 0.8	5.1 ± 0.6	NS
Insulin (μU/dL)	11.4 ± 9.7	6.5 ± 4.6	NS
C-peptide (pg/dL)	518.9 ± 292.0	287.0 ± 124.1	0.001
HOMA-IR [a]	2.8 ± 2.7	1.4 ± 1.1	0.01
Glucose/insulin ratio	17.8 ± 17.1	24.0 ± 15.1	NS
Resistin mRNA (Ct)	24.3 ± 3.6	23.4 ± 3.7	NS
Adiponectin (μg/mL)	6.3 ± 1.7	6.9 ± 2.3	NS

[a] HOMA-IR = Homeostasis model for insulin resistance (Matthews et al., 1985)

Table 8

Characteristics (Mean ± SD) of Female Baboons According to Weight Tertiles

TRAIT	TERTILE 1 <16.8 kg *(N =28)*	TERTILE 2 16.8 - 20.7 kg *(N = 28)*	TERTILE 3 >20.7 kg *(N = 28)*	*P*
Weight (kg)	15.0 ± 1.2	18.8 ± 1.1*	24.2 ± 2.6**	0.001
Age (years)	18.7 ± 5.2	17.2 ± 4.9	18.8 ± 4.6	NS
Glucose (mmol/L)	5.2 ± 0.7	5.3 ± 0.8	5.6 ± 0.9	NS
Insulin (μU/dL)	7.2 ± 5.9	10.6 ± 10.4	16.2 ± 10.3**	0.002
C-peptide (pg/dL)	413.9 ± 237.7	491.2 ± 289.2	656.5 ± 301.9*	0.001
HOMA-IR [a]	1.7 ± 1.6	2.6 ± 2.7	4.2 ± 3.0	0.003
Glucose/insulin ratio	20.2 ± 13.3	23.3 ± 22.6	9.7 ± 9.9*	0.007
Resistin mRNA (Ct)	24.2 ± 3.6	24.1 ± 3.4	24.8 ± 4.0	NS
Adiponectin (μg/dL)	6.8 ± 1.6	6.1 ± 1.4	6.1 ± 2.0	NS

*p < 0.05 vs Tertile 1 (LSD after ANOVA) .

** p < 0.05 vs Tertiles1 and 2 (LSD after ANOVA).

[a] HOMA-IR= Homeostasis model for insulin resistance (Matthews et al. 1985)

Table 9

Characteristics (Mean ± SD) of Male Baboons According to Weight Tertiles

TRAIT	TERTILE 1 <29.8 kg *(N = 8)*	TERTILE 2 29.8- 32.5 *(N = 8)*	TERTILE 3 >32.5 kg *(N = 8)*	*P*
Weight (kg)	27.8 ±1.3	31.4 ± 0.8*	35.6 ± 2.3**	0.001
Age (years)	15.8 ± 2.8	14.0 ± 2.8	15.0 ± 3.9	NS
Glucose (mmol/L)	4.9 ± 0.4	5.2 ± 0.79	5.2 ± 0.4	NS
Insulin (µU/dL)	3.4 ± 2.4	6.7 ± 3.0	8.0 ± 5.9	NS
C-peptide (pg/dL)	262.6 ± 89.7	284.0 ± 149.3	314.2 ± 144.8	NS
HOMA-IR [a] index	0.72 ± 0.5	1.6 ± 0.9	1.9 ± 1.5	NS
Glucose/insulin ratio	36.2 ± 17.3	16.1 ± 7.0*	19.2 ± 12.0*	0.008
Resistin mRNA (Ct)	21.7 ± 2.1	23.7 ± 3.0	25.1 ± 4.9	NS
Adiponectin (µg/dL)	70.9 ± 8.1	66.0 ± 29.4	69.6 ± 33.7	NS

*p < 0.05 vs Tertile 1 (LSD after ANOVA).

** p < 0.05 vs Tertiles1 and 2 (LSD after ANOVA).

[a] HOMA-IR = Homeostasis model for insulin resistance (Matthews et al., 1985)

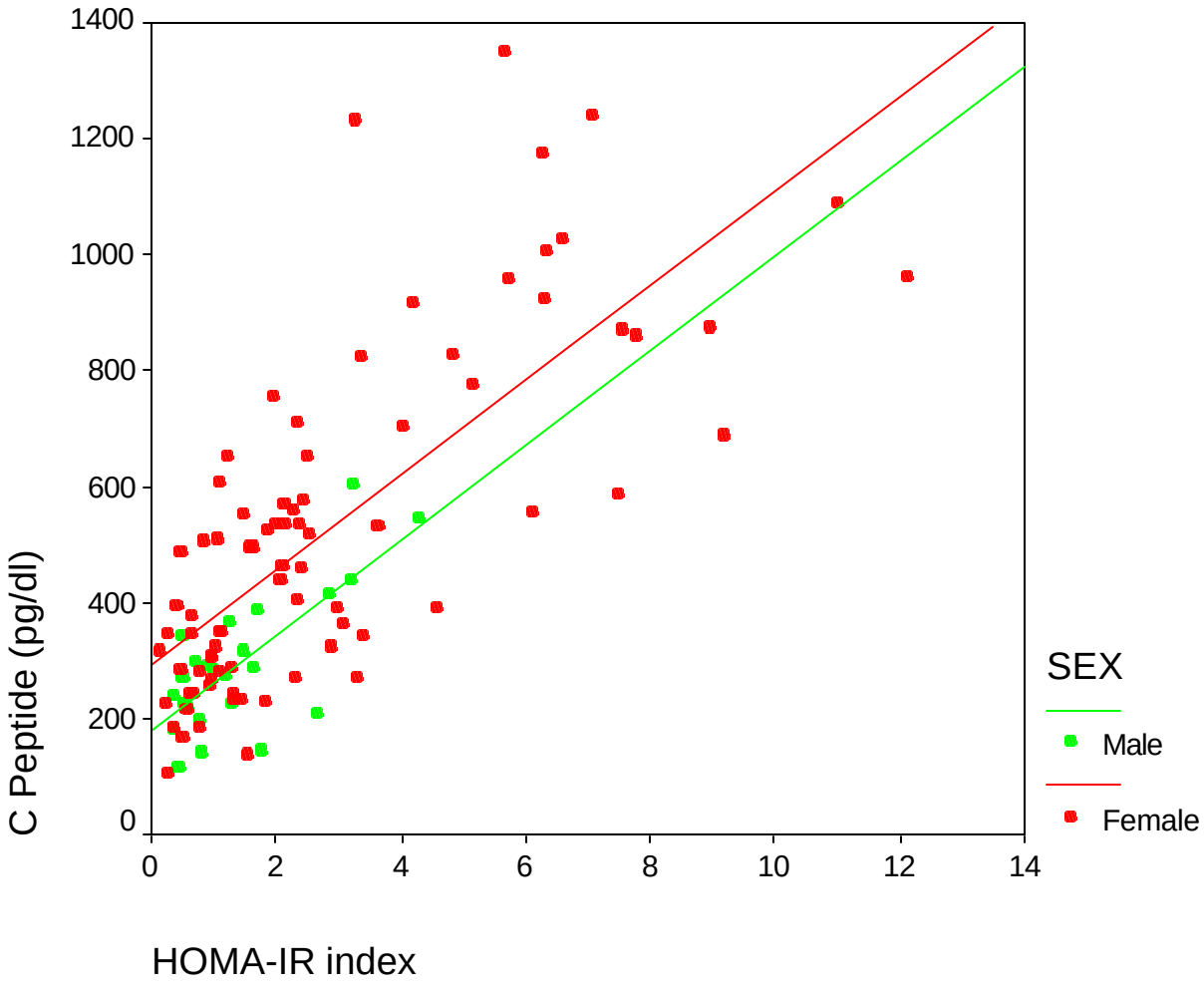

Figure 3 Association of C-peptide plasma levels and the homeostasis model assessment (HOMA-IR) index in baboons. Significant correlations were observed for females ($r = 0.74$, $p < 0.001$) and males ($r = 0.73$, $p < 0.001$), respectively.

Chapter 5: The Baboon as a Model for the Study of Chronic Inflammation and Insulin Resistance-Related Phenotypes

Adipose tissue produces a number of proteins that are believed to be involved in regulation of insulin sensitivity and the development of chronic conditions related to obesity. The baboon was used as an animal model for the study of pro-inflammatory cytokines and hormones expressed in adipose tissue. A 1 g adipose tissue biopsy and a blood sample were collected from 40 unrelated adult baboons. Plasma was separated for analyses of glucose, insulin, adiponectin, IL-6, IL-1β and TNFα. Total RNA was isolated from fat biopsies, and mRNA for adiponectin, resistin and GLUT4 were measured using real time, quantitative RT-PCR. Significant associations were observed between the expression of adiponectin mRNA and plasma IL-6 ($r = 0.38$, $p = 0.02$), with IL-1β ($r = 0.47$, $p < 0.05$) and with TNF-α ($r = 0.45$, $p < 0.05$); resistin mRNA and the HOMA-IR ($r = -0.51$, $p < 0.05$) and plasma insulin ($r = -0.45$, $p < 0.05$); and GLUT4 mRNA and glucose levels ($r = 0.47$, $p < 0.05$). These results confirmed observations in humans that levels of pro-inflammatory cytokines are associated with insulin sensitivity-related phenotypes in adipose tissue. This study supports the baboon as a model for the study of inflammation-related phenotypes in obesity and comorbidities.

Introduction

Adipose tissue is believed to contribute to the regulation of insulin sensitivity through several mechanisms, including the production and release of bioactive proteins (Trayhurn and Beatie, 2001; Saltiel, 2001). This paper will discuss several of these cytokines [interleukin 6 (IL-6), interleukin 1β (IL-1 β (Zhang et al., 2001), tumor necrosis factor α (TNF-α)] and hormones (adiponectin and resistin) (Steppan et al., 2001; Weyer et al., 2001; Stumvoll and Haring, 2002). The pro-inflammatory cytokines were selected as they are believed to be a key link between adipose tissue and the risk for metabolic syndrome, and cardiovascular disease (Trayhurn and Beatie, 2001; Saltiel, 2001; Sethi and Hotamisligil, 1999; Bastard et al., 2002). Recently, the chronic effect of subclinical inflammatory conditions has been proposed to contribute to the metabolic abnormalities found in obesity (Festa et al., 2000).

The cytokines IL-6 and TNF-α are produced and released from the adipose tissue, and seem to promote inflammation and reduce peripheral insulin resistance (Sethi and Hotamisligil, 1999; Bastard et al., 2002). TNFα is believed to induce insulin resistance on the adipocyte (Sethi and Hotamisligil, 1999). Bastard et al. (2002) reported that the IL-6 content in adipose tissue was correlated with insulin action *in vivo*, during hyperinsulinemic normoglycemic clamp, and *in vitro* by measuring glucose transport in adipocytes from subjects with and without diabetes.

These adipose-derived cytokines are believed to have a regulatory effect on the expression of the hormones adiponectin (Bruun et al., 2003), resistin (Fasshauer et al.,

2001) and the insulin responsive glucose transporter 4 (GLUT4) (Stephens and Pekala, 1999).

The cytokine IL-1β appeared to promote apoptosis, and is known to play a role in the host defense against infections. In 2001, production of this cytokine was found in subcutaneous adipose tissue, but its function was unclear (Zhang et al., 2001). TNFα induced the release of IL-1β through the activation of a cleavage enzyme (Tornberry et al., 1992). The IL-1β cytokine then regulated the expression of enzymes in the lipid metabolism (Memon et al., 1998). The present is the first report of an association of this cytokine on the expression of adiponectin, resistin or GLUT4 in omental adipose tissue.

The hormone adiponectin appears to be regulated by pro-inflammatory cytokines and is involved in the regulation of insulin sensitivity and is thought to play a role in preventing the development of atherosclerosis in humans and animal models (Engeli et al., 2003; Matsuda et al., 2002). Low levels of this hormone have been related to type 2 diabetes and cardiovascular disease (Hotta et al., 2000). Another adipose tissue hormone, resistin, was first discovered in the inflammatory response to allergy in mice (Holcom et al., 2000). Subsequent studies in humans (Janke et al., 2002), rodents (Steppan et al., 2001) and baboons (Tejero et al., 2003) have explored the possible contribution of resistin to the regulation of insulin sensitivity. However the role of this protein in inflammation and insulin resistance remains unclear (Gomez-Ambrosi and Fruhbeck, 2001).

The baboon is used as an animal model for inflammation in this study given the significant similarities with humans at both the physiologic and genetic level exists (Tejero et al., 2003; Dutrillux et al., 1978; Rogers et al., 1995). Genetic studies have shown a high homology between the two species in chromosome band staining (Dutrillux et al., 1978) in genetic markers (Rogers et al., 2000). In 2000, Rogers et al. (2000) developed a linkage map for the baboon using human short tandem repeats markers. In addition, findings from our laboratory indicate a high degree of identity between human and baboon sequences for genes expressed in adipose tissue (Tejero et al., 2003, Cole and Hixon, 1995). Thus, it is believed that the baboon can be a model for chronic inflammation, similar to its use as a model for obesity (Comuzzie et al., 2003), insulin resistance (Tejero et al., 2003) and type 2 diabetes (Stokes, 1986).

In this study, we analyzed the association of adipose-derived pro-inflammatory cytokines, insulin sensitivity phenotypes and the expression of adiponectin, resistin and GLUT4 mRNA in omental adipose tissue of baboons. Given the similarities between humans and this nonhuman primate, we proposed this animal as a model for the study of chronic inflammation and insulin sensitivity.

Animals and Methods

Animals

A sample of unrelated adult baboons (*P. h. anubis* and *P.h. cynocephalus*) (n= 40) from the pedigreed colony at the Southwest Foundation for Biomedical Research at San Antonio, TX (SFBR) was selected for the present study. The animals are olive and

yellow baboons, and a mixture between the two sub-species. Baboons were gang-housed under the same living conditions and shared the same standard low fat diet (Harlan Taklad15% Monkey, 8715).

Sampling and Phenotype Collection

All phenotypes were collected during the annual health assessment of the baboons. Body weight was determined on a calibrated electronic scale (GSE, Chicago, IL); body length was measured with a metric tape with the animals on their back. Bioimpedance was measured on a multi-frequency Xitron (Xitron Technologies, San Diego, CA). A 1 g biopsy of omental adipose tissue was obtained from baboons under sedation with ketamine, as described by Cole et al. (2003) after an overnight fast. Biopsies were frozen in liquid nitrogen for further isolation of total RNA. Blood samples were collected by venopuncture as follows: 8 mL of blood in EDTA tubes for measurement of hormones and cytokines, and a 3 ml in a sodium fluoride tube for glucose analysis. Glucose was assayed in an Analox spectrophotometer (Analox Intruments, Lunenburg, MA); insulin and adiponectin were analyzed by radioummunoassay (RIA) (Linco Research, St Louis, MO). Cytokines (IL-6, TNF-α and IL-1β) were assayed by chemioluminiscence via the Cytokine multiplex (Linco Research). All parameters were measured in duplicate and variations >5% were reanalyzed. The homeostasis model assessment index for insulin resistance (HOMA-IR index) was calculated according to the method of Matthews et al. (1985).

Total RNA was isolated from the adipose tissue samples using TRI-REAGENT (Molecular Research Center, Inc, Cincinnati, OH, USA). All RNA samples were digested with RQ1 DNAse (Promega, Madison, WI) for 30 min at 37^{o} C to eliminate traces of DNA, and checked for integrity in a 1% agarose gel stained with ethidium bromide.

Fragments of cDNA were cloned for baboon GLUT4, adiponectin and resistin via a two step reverse transcription-polymerase chain reaction (RT-PCR), using total RNA from omental adipose tissue. Details on the baboon clones are described elsewhere (Tejero et al., 2003). The clones were sequenced on an ABI 377 (Applied Biosystems, Forest City, CA), with the Big Dye Terminator sequencing kit (Applied Biosystems). Sequences were used for the development of real time, quantitative RT-PCR. Primers and probes were designed with the Primer Express software V.1 (Applied Biosystems). Primers and probe sequences for adiponectin, resistin and GLUT4 were:

For adiponectin: forward: 5' GCTCTTCACCTATGACCAGTACCA 3' and reverse: 5' TCCAGATGCAGGAGCACAGA 3'. The sequence for the adiponectin probe was: 5' TAAACGTGGACCAGGCCTCCGG 3'.

For resistin, forward: 5' TCCTCCTGCCTGTCCTGG 3' and reverse: 5' CGCCCTCCTGAATCTTCTCAT 3', the sequence for the resistin probe was: 5' TCTAGCCAGACCCTGTGCTCCATGG 3'.

For GLUT4 forward: 5' TGG CCA GGC CCA TGA G 3' and reverse 5' ATG GAA GGA AAA GGG CTA TGC 3', and for the GLUT4 probe: 5' CCC AGC ACT GCC AGG ACA TTG TTG 3'.

Ribosomal18S RNA was used as the endogenous control, using the primers and competimers from the Universal 18S system (Ambion, Austin, TX) in a 4:6 ratio and the rRNA Ambiprobe (Applied Biosystems). The inter and intra-assay variation coefficients for each real-time, quantitative RT-PCR were 3% and 3.7% for adiponectin, 6% and 4% for resistin, 4% and 5.1% for GLUT4 and 5% and 7% for 18S rRNA, respectively.

A sample of 50 ng of total RNA was used for every assay. All samples were analyzed in duplicate. Conditions for the real time, quantitative RT-PCR were 50 minutes at 48^{o} C for reverse transcription, followed by 40 cycles of 1 minute at 60^{o} C and 15 seconds at 90^{o} C.

Data were obtained in Ct values (the number of cycles at which logarithmic plots of PCR product accumulation cross a specific threshold lines), according to the manufacturer's specifications. The mRNA abundance of the analyzed genes was corrected for measurement error, and calculated as gene Ct divided by the 18S rRNA Ct, and corrected by the 18S rRNA Ct mean in any given run. The resulting Ct number has an inverse correlation with the number of mRNA copies present in the analyzed sample, with higher Ct values in those samples with lower content of the assayed mRNA form. Thus, positive correlation coefficients indicate an inverse association between the

mRNA levels of a given gene and a phenotype, and negative correlation coefficients imply a positive association between the two variables.

Statistical Methods

Data were analyzed using the SPSS V.9 (Chicago, IL) software. Descriptive data, student's t test for independent samples and Pearson's correlation coefficients were calculated for the studied parameters.

Results

As observed in Table 10, female baboons were older and had lower weights and BMI than males. The % of body fat in females (4.7 to 36.6%) was higher, but not significantly so, than males (6.2 to 24.3%). Table 11 contains the descriptive information on the phenotypes analyzed in plasma and Table 12 in adipose tissue. No differences were observed according to sex.

Table 13 contains information on the relationships among the circulating parameters for insulin sensitivity and the expression in the adipose tissue. The adiponectin protein was not related to its mRNA concentration in omental adipose tissue, or any other mRNA form assayed in this study. Circulating levels of TNF-α and IL-1β were significantly associated with the expression of adiponectin mRNA. No significant correlation was observed between adiponectin mRNA and parameters of insulin sensitivity. Resistin mRNA expression showed relationships with plasma insulin, and the

HOMA-IR index, but not the circulating cytokines. Levels of GLUT4 mRNA were related to glucose levels.

Significant associations were observed within the cytokines, including IL-6 and TNF-α ($r = 0.56$, $p < 0.001$) (Figure 4), IL-6 and IL-1β ($r = 0.61$, $p < 0.001$) (Figure 5) and TNFα and IL-1β ($r = 0.88$, $p < 0.001$) (Figure 6). Cytokine levels were not related to the concentrations of insulin, glucose or HOMA-IR in this study. No relationship was observed for the mRNA expression levels of adiponectin, resistin and GLUT4 in baboon adipose tissue.

Discussion

Collectively, these findings suggest a similar relationship between chronic inflammation and phenotypes related to insulin sensitivity in humans and baboons. As observed in humans, cytokines are negatively related to adiponectin mRNA levels (Bruun et al., 2003). Resistin expression in adipose tissue was linked to insulin sensitivity (Smith et al., 2003). GLUT4 mRNA abundance was associated with glucose levels, as previously reported in rhesus monkeys (Hotta et al., 1999). These observations closely resemble human studies that measured the expression of the genes in subcutaneous adipose tissue (Bruun et al., 2003; Ducluzeau et al., 2001; Smith et al., 2003). The present is the first report of these associations in omental adipose tissue in baboons, as well as the relationship between adiponectin mRNA expression and plasma concentrations of IL1-β.

In baboons, the adiponectin mRNA levels were associated with circulating TNFα, IL-6 and IL-1β. These results imply that higher levels of these cytokines in plasma are related to lower adiponectin expression in omental adipose tissue. An investigation by Bruun et al. (2003) supports our findings. They observed an inhibitory effect of IL-6 and TNFα on adiponectin mRNA levels in cultured fragments of human subcutaneous adipose tissue. A previous investigation in cultured adipocytes by other group confirms these results (Fasshauer et al., 2002). In contrast, an in vivo study by Kern et al. (2003) did not find any relationship between adiponectin expression and IL-6 levels in plasma in humans.

The association between adiponectin mRNA in adipose tissue and IL-1β in plasma in this study might be explained by the influence of TNFα on both phenotypes. Studies by Bruun et al. (2003) reported that TNF-α decreased adiponectin expression in human adipose tissue. Also Tornberry et al. (1992) indicated that this cytokine up-regulated the production of IL-1β by the activation of the IL-1β-converting enzyme. A possible inhibitory effect of IL-1β on adiponectin mRNA levels remains to be elucidated.

The correlation between resistin mRNA and the HOMA-IR in our study supports initial findings from Steppan et al. (2001), who suggested that this hormone regulated insulin sensitivity in peripheral tissues. Yet Janke et al. (2002) and Nagaev and Smith (2001) did not find any relationship between resistin mRNA in adipocytes and type 2 diabetes, or the HOMA index. However, subsequent studies reported that non-fat cells in human adipose tissue produce and release of resistin (Fain et al., 2003). The disparity in these

observations may be due to the different tissue type measured. Smith et al. (2003) recently found an inverse

association of resistin in subcutaneous abdominal adipose tissue and the HOMA-IR index in humans. This result confirms our observations in baboons in the present study.

The protein concentrations of resistin in the circulation have been linked to insulin resistance-related phenotypes (Yannakoulia et al., 2003; Zhang et al., 2002). The association between resistin and insulin in our study suggest that elevated resistin mRNA is found with high insulin levels under fasting conditions. However, Shojima et al. (2002) found the opposite in cultured 3T3-L1 adipocytes. This investigation also reported that TNFα down-regulated resistin mRNA in cultured cells. Our study did not show any association of these cytokines (TNFα, IL-6 and IL-1β) with resistin mRNA. Reasons for these contrasting observations might be due the dissimilar resistin expression pattern between adipocytes and adipose tissue (Fain et al., 2003) as well as the variations inherent between in vitro and in vivo studies.

The coupling of values for GLUT4 mRNA in adipose tissue and glucose levels in plasma has been previously observed in studies by Hotta et al. (1999) in rhesus monkeys. However, our study did not confirm that GLUT4 mRNA abundance in baboons was related to insulin resistance, body weight or BMI, as reported in humans (Ducleauzu et al., 2001) and Garvey et al. (1992).

In vitro studies by Hotamisligil et al. (1994) and Ruan et al. (2002) suggest a suppressive effect of TNFα on the expression of GLUT4 in adipocytes. Yet our in vivo study found no such association between GLUT4 and any cytokine.

In our study, no difference was observed in expression levels of any of the analyzed genes between the sexes. Neither, adiponectin protein and mRNA were significantly different by sex. This is contrary to several human studies, which reported that the adiponectin protein is higher in women than in men. A study by Kern et al. (2003) reported higher mRNA levels of adiponectin in subcutaneous adipose tissue in women than men using Northern blot, which may not be as sensitive as PCR, also the use of subcutaneous rather than omental adipose fat may account for the difference.

Nogueiras et al. (2003a) reported higher resistin expression in male rats than in females analyzed by Northern blot and real time , quantitative RT-PCR (2003b). In our study, no difference in resistin expression was detected between male and female baboons in omental adipose tissue, however the analytical method, the type of adipose tissue and the species were different between the two studies.

Adiponectin and resistin mRNA levels were not related in the present investigation. Fain et al. (2003) reported that expression and release of these hormones were not related to each other in human adipose tissue, contrary to findings in mice (Makimura et al., 2002).

Zhang et al. (2001) suggested that TNFα induced the production of IL-1β in subcutaneous adipose tissue. IL-1β is known to play a key role in the immune response, and induce lipolysis and inhibit lipogenesis in adipocytes (Memon et al., 1998). No information on the possible role of this cytokine on the metabolic abnormalities observed in obesity has been reported yet. We did not observe any relationship between this cytokine and indicators of insulin sensitivity in baboons.

In summary, these results imply a substantial interaction between plasma levels of selected cytokines and insulin sensitivity-related phenotypes. Most of the mechanisms behind the observed associations remain to be studied. Our findings sustain previous observations in baboons, and show the value of this nonhuman primate as a model for the study of the relationship between chronic inflammation and insulin resistance.

Table 10

Characteristics (Means ± SD) of Age and Body Composition in Baboons

TRAIT	MALE (*N* = 12)	FEMALE (*N* = 28)	*P*
Age (years)	16.7 ± 3.6	24.5 ± 4.7	0.001
Weight (kg)	32.1 ± 3.9	19.8 ± 4.9	0.001
BMI (kg/m^2) [a]	27.5 ± 4.9	22.4 ± 4.1	0.002
Body fat (%)	16.7± 3.6	20.4 ± 7.9	NS

[a] BMI = Body mass index

Table 11

Phenotypes Analyzed in Plasma in Baboons

TRAIT	MALE		FEMALE		RANGE
	Mean ± SD	Range	Mean ± SD	Range	IN HUMANS
Glucose (mmol/l)	5.5 ±1.1	4.5 - 8.1	5.3 ± 0.8	4.0 – 7.1	4.9 - 5.3
Insulin (μU/dL)	35.5 ± 20.0	4.0 – 61.6	33.4 ± 20.0	3.7 – 73.5	0.5 - 50
HOMA-IR [a]	8.9 ± 5.7	0.8 -19.3	8.0 ± 5.1	0.7 -18.6	7.1 – 26.9
Adiponect (μg/L)	5.9 ± 3.3	1.6 –13.3	5.3 ± 2.2	1.9 – 9.6	1.0 – 20.0
TNFα (pg/mL) [b]	11.8 ± 7.3	2.8 – 28.5	10.8± 9.0	0.7 – 32.3	4.6 – 4.8
IL-1β(pg/mL) [c]	6.9 ±7.1	2.6 –19.5	5.7 ± 4.6	0.7 – 15.2	0.5 – 20.0
IL-6 (pg/mL) [d]	7.2 ± 4.8	2.5 – 15.6	8.5 ± 7.5	0.5 – 28.9	3.0 – 80

[a] HOMA-IR = Homeostasis model for insulin resistance (Matthews, 1985)

[b] TNFα = tumor necrosis factor α

[c] IL-1β =interleukin -1β

[d] IL-6 = interleukin-6 *$P < 0.05$

Table 12

Levels of mRNA in Baboon Adipose Tissue

TRAIT	MEAN ± SD	RANGE
Adiponectin (Ct)	20.6 ± 3.0	15.8 – 29.6
Resistin (Ct)	26.3 ± 3.1	20.3 – 33.5
GLUT4 (Ct)	35.7 ± 3.1	29.3 – 45.4

Table 13

Correlation Coefficients for Plasma Levels of Adipokines, Insulin Sensitivity-Related Phenotypes and Levels of Adiponectin, Resistin and GLUT4 mRNA in Adipose Tissue

	PLASMA ADIPOKINES			INSULIN SENSITIVITY RELATED PHENOTYPES			
TRAIT RNA (Ct)	Adiponectin Protein	TNFα [a]	IL-1β [b]	IL-6 [c]	Insulin	Glucose	HOMA-IR [d]
Adiponectin	0.09	0.45*	0.47*	0.38*	-0.05	0.06	0.17
Resistin	0.17	0.06	-0.11	0.13	-0.45*	-0.17	-0.51*
GLUT4	0.06	-0.02	-0.04	-0.13	-0.23	0.45*	-0.08

[a] TNFα = tumor necrosis factor α

[b] IL-1β =interleukin 1β

[c] IL-6 = interleukin 6

[d] HOMA-IR = Homeostasis model for insulin resistance (Matthews et al., 1985)

*$P < 0.05$

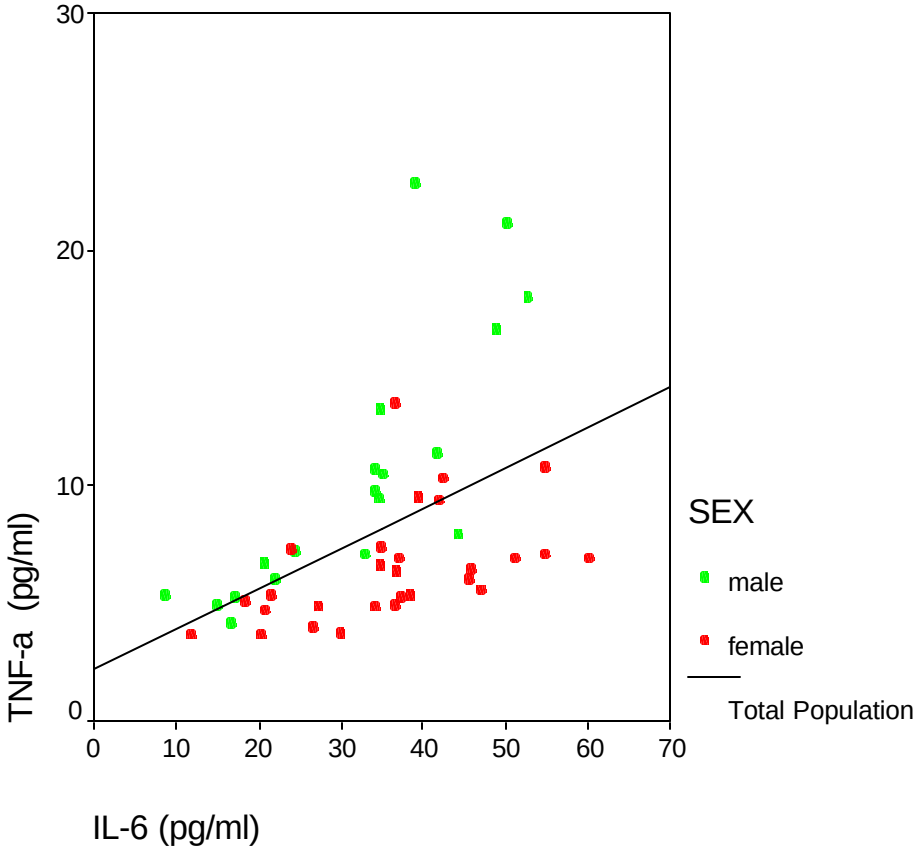

Figure 4 Correlation between TNFα and IL-6 in plasma in baboons (r = 0.56, p = 0.001) .

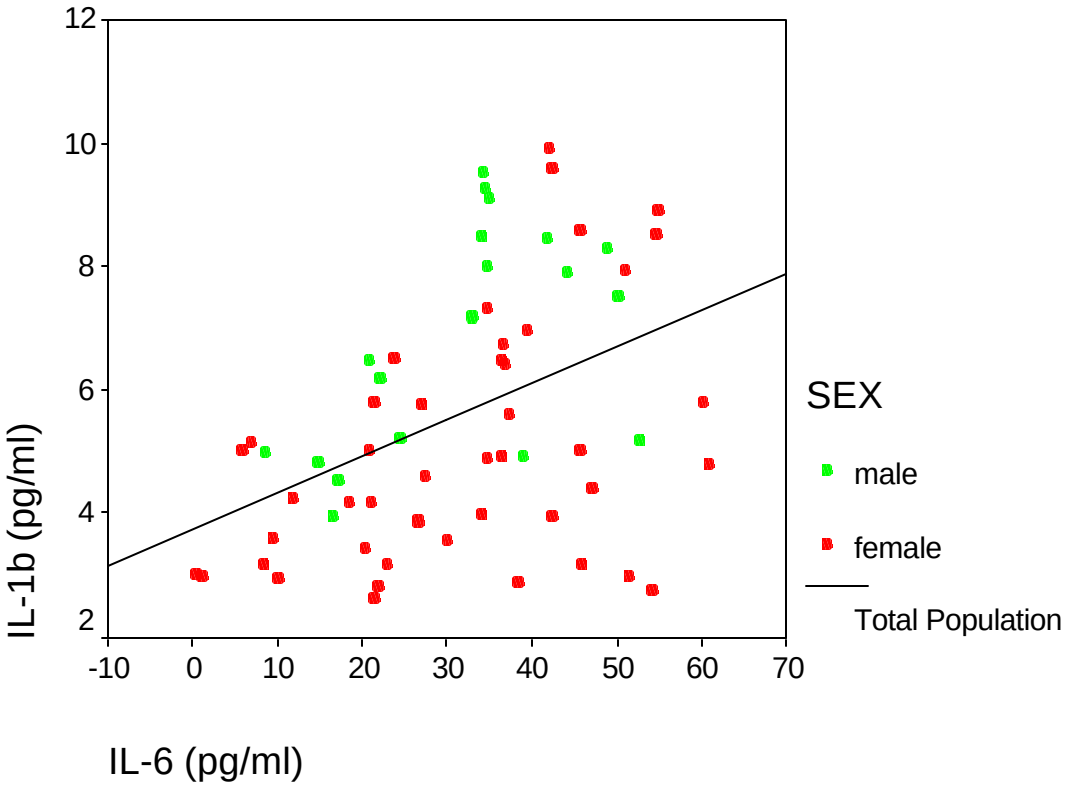

Figure 5 Correlation between IL-6 and IL-1β in plasma in baboons (r = 0.60, p = 0.001).

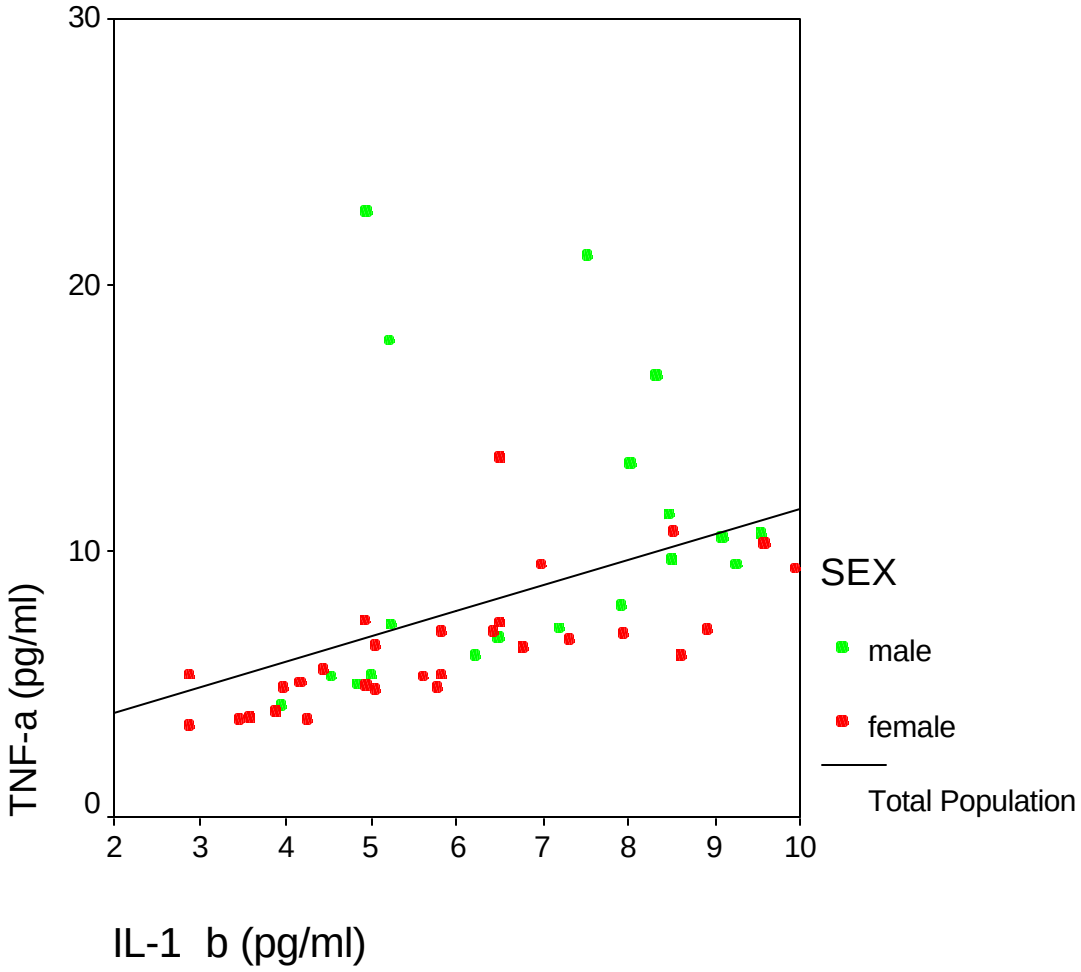

Figure 6 Correlation between IL-1β and TNFα in plasma in baboons (r = 0.8, p = 0.001).

Chapter 6: Conclusions and Recommendations

Results from this project suggest a high genetic and physiologic similarity between the baboon and human, and support the value of this nonhuman primate as a model for chronic diseases related to obesity. This dissertation shows that the sequences for the glucose transporter 4 (GLUT4) and hormones resistin were 95% identical to the human sequence, and adiponectin was 96%. Results from chapters 2 and 5 in the present investigation document that the circulating levels of glucose, insulin, C-peptide, adiponectin, TNFα, IL-6, IL-1β and the HOMA-IR index closely resemble human studies. Observations on the association of these phenotypes and the mRNA expression of GLUT4, adiponectin and resistin in omental adipose tissue also confirm findings in human studies. This dissertation is the first analysis of insulin sensitivity-related phenotypes in baboons. Also is the first time that mRNA abundance in adipose tissue of baboons is measured using real time, quantitative RT-PCR for genetic analysis and genome-wide scan.

A major challenge in the present study was the utilization of mRNA levels as a quantitative phenotype in genetic analyses. For this purpose, reliable quantitative methods for measurement of the mRNA abundance of the studied genes were applied. The development of the real time, quantitative reverse transcription- polymerase chain reaction (RT-PCR) method for the measurement of GLUT4, resistin, adiponectin and the

18S ribosomal RNA required the cloning of baboon cDNA fragments, sequencing, validation and standardization of the assays. The results of the real time, quantitative RT-PCR were highly reproducible, with low inter and intra-assay coefficients of variation for all the analyzed mRNA. Initially, we used the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an endogenous standard for the RNA quantification. However, it was necessary to switch to 18S ribosomal RNA because of an unexpected change in the sequence of the commercial kit. The 18S Universal system from Ambion (Austin, TX) contains primers and competimers to adjust the efficiency of the PCR reaction. A series of assays were conducted to find the specific ratio between primers and competimers, and match the reaction efficiency observed for target genes. The slope of the standard curve of the RT-PCR reaction is reflective of the efficiency. For this study slopes were standardized between –3.2 to –3.6 for all RT-PCR assays. Standard curves for the target genes were developed using specific mRNA produced by in vitro transcription using the clones as DNA templates.

The present study found that the mRNA abundance of GLUT4 ($h^2 = 0.23$, $p = 0.001$), resistin ($h^2 = 0.23$, $p = 0.001$) and adiponectin ($h^2 = 0.21$, $p = 0.005$) in omental adipose tissue of baboons is heritable, and it is possible to use these traits as quantitative phenotypes in genetic analyses. These results demonstrated a significant genetic component in the variation of the mRNA abundance of these genes in omental adipose tissue of baboons.

The bivariate genetic analyses detected substantial correlations for GLUT4 mRNA, fasting insulin levels, weight and body mass index, (BMI). These observations are a

novel finding for obesity and insulin resistance, suggesting that these traits are regulated by a common set of genes. The correlations indicate that higher weight, BMI and insulin levels are associated with lower GLUT4 mRNA abundance, and that BMI and insulin are genetically correlated in baboons and in humans (Comuzzie et al., 1996). It is interesting that the environmental correlations were not significant, presumably due to the homogenous living conditions within the baboon colony. The obtained LOD score of the preliminary genome scan for GLUT4 was 1.4 on chromosome 10p26. The positional candidates in this region are the adrenergic receptor $\alpha 2$ and $\beta 1$ (ADRA2 and ADRB1 respectively), which have been previously linked to obesity-related phenotypes.

The resistin mRNA levels were genetically correlated to the concentrations of C-reactive protein in the present study. The genome scan detected a highly significant LOD score (3.84) in the region corresponding to the human chromosome 19p13. Positional candidates in this QTL include the resistin gene, and other candidates that are linked to the metabolic syndrome. Results from the GLUT4 and resistin quantitative analyses and genome scan were analyzed in detail in the chapters 2 and 3. It is recommended to increase the sample size of GLUT4 mRNA (n = 750) to improve the LOD score and to identify the chromosomal regions regulating the expression of this glucose transporter.

For the resistin study, the next step would be the fine mapping of the identified region. The strongest candidate in this location is the resistin gene itself. This gene should be sequenced in full in order to find polymorphisms associated with the mRNA abundance. The other candidate genes in the detected region may be sequenced as

well, in order to find an association with the resistin mRNA abundance. It is also recommended to measure the resistin protein in plasma and conduct the genetic analyses to identify the chromosomal regions influencing this phenotype. Hopefully, this will be done in a future study, given that a reliable assay for this protein is available. However, the antibody must be tested first in baboon plasma before the analysis of a large number of samples.

The mRNA levels of adiponectin in baboon adipose tissue were significantly heritable ($h^2 = 0.23$). The bivariate analyses identified significant genetic correlations with the TNFα soluble receptor 2 (TNFSR2) ($r = 1.0$, $p = 0.02$) and with CRP ($r = 1.0$, $p = 0.02$). Given the large standard error of the heritability of the TNFSR2 ($h^2 = 0.27$, SE = 0.19) and CRP ($h^2 = 0.2$, SE = 0.17), it was not possible to calculate a reliable genetic correlation coefficient. The genome scan of the mRNA abundance of adiponectin identified a LOD score of 1.6 in the region corresponding to human chromosome 6q13 (marker D6S402). The adiponectin protein was used as a quantitative phenotype as well. The multipoint (genome scan) analysis identified a LOD score of 1.0 in the region corresponding to human chromosome 6p13 (D6S1048), which is located beside D6S402. This region has been reported before in association with insulin resistance. A study by Arya et al. (2002) reported a LOD score of 4.9 in this region using adiposity-insulin as a phenotype. It is interesting as well that both adiponectin phenotypes showed a signal in the same region. These findings were not reported as a publication because the LOD score were not as high as recommended for a linkage study (LOD = 3) and the genetic correlations do not have a standard error of the mean. It is recommended to increase the sample size (n = 750) for the adiponectin mRNA levels and plasma protein,

C-reactive protein and other mediators for inflammatory response. A larger sample may allow the identification of genetic correlations among traits associated with insulin resistance, inflammation and diabetes and to detect significant LOD scores in the genome scan using both, uni or bivariate analyses.

The genetic correlations between C-reactive protein with resistin, and with adiponectin deserve a thorough analysis. These three genes are expressed in adipose tissue, C-reactive protein is a robust marker for inflammatory conditions, linked to cardiovascular disease and diabetes (Festa et al., 2000), and resistin seems to be associated with inflammation (Steppan et al., 2002). In the other hand, the hormone adiponectin is believed to have an anti-inflammatory effect in peripheral tissues. The expression of these three adipose-derived proteins is probably regulated by common sets of genes. However, the analysis of a larger number of animals is needed to clarify these associations.

Collectively, the results from this investigation produced novel information on the genetics of the endocrine function of adipose tissue, and its participation in insulin sensitivity. This study characterized some phenotypes for insulin resistance in baboons, and contributed to the validation of this model.

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