IL-6 mediation of glucose homeostasis and insulin sensitivity during in-vivo influenza infection: a mice model experiment

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IL-6 mediation of glucose homeostasis and insulin sensitivity during in-vivo influenza infection: a mice model experiment

by

Hala Farouk Bastawros

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
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Iowa State University
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ABSTRACT

Infections are frequently associated with changes in glucose level, either hyperglycemia or hypoglycemia. The factors that influence glucose change and/or the mechanism(s) that result in altered glucose level during infection are not determined. Therefore, the purpose of this study was to investigate the role of infection-induced changes in IL-6 on blood glucose level. Blood glucose and insulin level were measured in Balb/c mice infected with influenza virus A/PR/8/34 at a dose of 50 ul containing 512 HAU or 1024 HAU (high dose). In addition, anti-IL-6 neutralizing antibody was used to determine the extent to which IL-6 may influence infection-associated changes in glucose or insulin. We found an increase in serum IL-6 at days 1, 2, 3, 5, and 6 post-infection and a significant decline in blood glucose at days 5 and 6 post-infection that was more pronounced with a higher dose of virus. Insulin level increased significantly on day 1 post-infection and then decreased on days 3, 4, and 5 post-infection when a higher dose of virus was used. With the administration of IL-6 neutralizing antibody, there was an attenuation of the decline in glucose level at day 5 post infection, suggesting a potential role for IL-6 in mediating blood glucose during infection. With respect to insulin, the mice treated with IL-6 neutralizing antibody had greater insulin levels than infected mice treated with irrelevant antibody at day 2 post-infection. However at day 5 post-infection, the decline in insulin was not prevented by IL-6 neutralizing antibody suggesting that IL-6 may mediate insulin response at only specific time points during infection. Taken together, the results demonstrate that insulin and glucose levels are altered by infection, and that IL-6 may play a role in mediating these responses. The actual mechanisms by which IL-6 is involved remain to be determined.
INTRODUCTION

Alteration in Blood Glucose Level

Multiple factors contribute to the regulation of blood glucose, however there are several conditions such as stress, infection, or myocardial infarction in which blood glucose levels may be temporarily altered. Chronic elevations of blood glucose occur in individuals with diabetes (a disease that affects 2–4% of the U.S. population) (Thomas et al., 1993). According to WHO, the diagnostic criteria of diabetes is characterized by fasting blood glucose level >140mg/dl. This high blood glucose level is due to either deficiency of the circulating insulin or increase in insulin resistance with the end result of inadequate action of insulin on different body tissues and elevation of blood glucose level. It is important to note that conditions in which blood glucose may be altered temporarily (i.e., infection) may further exacerbate the chronically elevated blood glucose levels in the diabetic. Other examples of temporary conditions that may cause acute hyperglycemia in diabetic or non diabetics include stroke, thermal injury, and acute infection (Thomas et al., 1993; Heismayr et al., 2006; Norhammar et al., 2002; Holm et al., 2004). The acute elevation in glucose level and hyperglycemia may be associated with severe and life threatening complications such as rapid mental deterioration, coma and death (Thomas et al., 1993). It has also been observed that mortality rates are greater in patients presenting with hyperglycemia upon admission to intensive care units (Whitcomb et al., 2005). For these reasons, a better understanding of the mechanism(s) by which certain conditions such as infection result in hyperglycemia is very important. With an understanding of the mechanisms, it may be possible to develop an appropriate intervention.
REVIEW OF LITERATURE

Acute Infection Promotes Changes in Blood Glucose Level

In addition to an increased incidence and susceptibility to infection in patients with high glucose levels (Segado et al., 1999), acute infections are associated with alteration in blood glucose level. For example, bacterial infections such as Cholera or tuberculosis may result in hyperglycemia. As infection is cleared, and/or after antibiotics are administered, the high glucose levels may return to normal (Ronan et al., 1997; Jaward et al., 1995). Viral infection is also correlated with hyperglycemia. For example, severe acute respiratory syndrome (SARS) has been associated with elevated fasting plasma glucose in both diabetics and non diabetic patients. Those with more severe disease as assessed by SaO2 measures, tended to show greater elevations in fasting plasma glucose (Yang et al., 2006). Studies in mice similarly show hyperglycemia during viral infection. For example, mice infected with coxsackieB virus developed hyperglycemia 8 weeks later. Reexposure to coxsackieB virus increased the frequency and onset of hyperglycemia in the infected mice (Horwitz et al., 2003). Also it has been demonstrated that insulin resistance occurs during infection and may persist for 1-3 months post-infection. One study evaluated insulin resistance during and following acute viral or bacterial infection in young adults (mean age 29). The degree of insulin resistance observed in these younger adults was equivalent to that predicted for an 84 year old normal person or an age-matched obese individual with a body mass index (BMI) = 37 kg/m² (Yki-Jarvinen et al., 1989). In addition, other studies showed that up to 40% of acute hyperglycemia and development of ketoacidosis in diabetic patients are precipitated by infection, particularly respiratory and urinary tract infections (Thomas et al., 1993; Segado et
al., 1999). Based on the important association between infections and elevation of blood glucose level, it has been suggested that continuous monitoring of blood glucose data in diabetic patients may have the potential to predict outbreaks of infectious diseases (Arsand et al., 2005).

Infection has also been reported to decrease blood glucose level leading to development of hypoglycemia, although it appears that the timing of blood glucose assessment in relation to stage of infection may be important. It has been shown that lipopolysaccaride (LPS) (a component of gram negative bacteria) decreased blood glucose level 4-6 hrs following intraperitoneal injection in BALB/c mice, and that decline was dependent on the dose of LPS (Oguri et al., 2002). It is possible that the type of the pathogen is important in determining whether hypoglycemia or hyperglycemia will occur. Moreover, the timing of blood glucose measurement in relation to pathogen challenge may play a critical role in determining the type of glucose alteration. For example, some evidence suggests that severe bacterial infection and sepsis may result in acute hyperglycemia due to increase in proinflammatory cytokines. The elevated proinflammatory cytokines may then cause insulin resistance, followed by a hyper-metabolic state with increased glucose consumption, glycogen depletion and gluconeogenesis inhibition eventually leading to the development of hypoglycemia (Yu et al., 2003; Andersen et al., 2004; Miller et al., 1980). In vibrio-cholera, hyperglycemia was noticed in the first few hours of diarrhea and that was corrected after electrolyte replacement. The hyperglycemia could be due to the elevation of diabetogenic hormones e.g. norepinephrine, glucagons and corticosteroid in response to stress produced by this type of infection (Ronan et al., 1997). The above are possible mechanisms that have been associated with alteration in glucose metabolism in certain types
of pathogens. Until the mechanism by which infection may alter blood glucose is elucidated, it will be difficult to predict which types of infection may alter glucose, and at what time point during the infection are blood glucose levels most likely to change.

**Infection and Chronic Elevation of Blood Sugar Level**

There is evidence from multiple studies to suggest that infections cause acute alteration in blood glucose level. In addition, certain viruses that may cause chronic disease may also predispose the individual to persistent elevation of blood glucose level, leading to the development of diabetes mellitus. Some evidence suggests that hepatitis C virus (HCV) could impair glucose tolerance and approximately 43.2% of HCV-infected patients became diabetic later in their lives (Kwon et al., 2005). Another study showed that diabetes occurs more frequently in HCV-seropositive than HCV-seronegative beta-thalassamia patients (Mowla et al., 2004). In addition to HCV virus, human immunodeficiency virus (HIV) infection is also associated with blood glucose elevation. It is found that HIV-patients treated with antiretroviral agent (HAART) are more likely to develop fat redistribution or lipodystrophy. These lipodystrophic-HIV infected patients exhibit glucose tolerance impairment and developed diabetes mellitus more often than non-lipodystrophic HIV- treated patients (Haugaard et al., 2005). Moreover, in renal transplant patients, asymptomatic cytomegalovirus (CMV) infection was associated with increased incidence of new-onset diabetes mellitus and impairment of insulin release (Hjelmesaeth et al., 2004). Case studies also exist in the literature documenting the development of Type 1 diabetes following a flu-like illness (Wasmuth et al., 2000). In general, numerous studies have shown an associated between prior virus infection and subsequent development of Type I diabetes as recently
reviewed (Jaeckel et al., 2002). In addition to chronic viral infections, chronic bacterial infections have also been associated with altered glucose. For example, helicobacter felis infection increased glycosylated hemoglobin HbA1c and mortality rate in streptozotocin-induced diabetic mice (Spinetti et al., 2005). Therefore, the results of numerous studies suggest that infection (acute or chronic) are situations in which blood glucose may be altered temporarily and potentially may lead to long-term alterations.

**Influenza Virus Infection in Mice or Humans and Blood Glucose**

The severity of influenza infection appears to be greater in diabetics. For example, the number of hospitalization of diabetic patients due to ketoacidosis and pneumonia was increased in epidemic from non-epidemics years ($P < 0.01$ and $P < 0.01$ respectively). The mortality rate among diabetic patients was increased, with the estimated relative risk of death in diabetic patients equal to 91.8 during epidemic years compared to 30.9 in non-epidemic years (Bouter et al., 1991; Diepersloot et al., 1990). The increased rates of morbidity and mortality among diabetic patients during influenza epidemic could be due to the effect of influenza virus-induced immune mediated effects on blood glucose, and/or may be due to hyperglycemia-associated changes of innate immune response. Some evidence in support of the latter possibility has been published, yet to our knowledge, it has not been determined how immune response to influenza infection may impact blood glucose. With respect to hyperglycemia-associated changes of innate immunity, it was found that hyperglycemia inhibits glucose 6 phosphate dehyrogenase G6PD and impairs phagocyte NADPH oxidase activity, leading to inhibition of the antimicrobial function of the polynuclear neutrophils (Moutschen et al., 2005; Engelich et al., 2001). Influenza infection may result in a secondary
bacterial infection, via compromised anti-bacterial innate defenses and may increase the likelihood of developing a secondary infection. Influenza virus infection has been shown to decrease neutrophil function either through interaction of viral hemagglutinin to sialylated neutrophil surface molecules or via acceleration of monocyte and neutrophil apoptosis (Engelich et al., 2001). The depression in monocyte and neutrophil function (due to hyperglycemia and/or via actions of the influenza virus) may increase susceptibility to bacterial infections, thereby resulting in elevated hospitalization rates and mortality in diabetic patients during influenza pandemic.

Also, in experimental studies, it has been found that diabetic mice are more susceptible to influenza virus infection than non-diabetic mice (Reading et al., 1998). In this study, viral load in lung tissues was higher in mice with elevated blood glucose levels at the time of infection. A 10-fold difference in virus yield was observed as early as 24 hours post-infection in diabetic mice compared to non diabetic mice. This enhanced susceptibility of influenza infection in diabetic mice was thought to be due in part to impairment of collectin-mediated host defense of the lung by glucose. Consequently, the neutralization of influenza virus particles by surfactant binding lectins was abolished. Therefore, evidence in humans and animal models suggests that elevated blood glucose could increase the incidence and severity of influenza infection.

Fewer studies were documented explaining the association between influenza virus and hypoglycemia. In experimental studies, intravenous inoculation of mice with influenza B/Lee virus impaired gluconeogenesis in the liver and led to the development of severe hypoglycemia. In this study blood glucose level felt down at 6 hrs and persists up to 30hrs later (Davis et al., 1993). Also, laboratory finding of influenza associated encephalitis
patients infected with influenza A and B virus revealed glucose level < 60mg/dl (Tsuneo et al., 2002). Taken together, evidence showed that influenza infection may be associated with either hyperglycemia or hypoglycemia. The alteration in glucose level may be depending on virus strain, severity of infection or could be related to timing of glucose measuring during infection.

Pathogenesis of Influenza Virus

Influenza virus is highly antigenic; it is a single strand RNA with an outer lipoprotein envelope. The envelope is covered by 2 different spikes, hemagglutinin and neuraminidase. Influenza virus is characterized by high molecular recombination of the genome RNA (antigenic shifts) that determines the type of hemagglutinin and neuraminidase proteins. A significant change in the type of hemagglutinin and neuraminidase protein may result in an in epidemic, and also may influence the pathogenesis of the disease (Enserink et al., 2004; Li et al., 2004). For example, H1N1 (Spanish flu) was the dominant strain during 1918 epidemic year. However H2N2 (Asian flu), H3N2 (Hong Kong flu), and H5N1 (avian flu) were the common type during the years 1957, 1968 and 1997 respectively and each of these major changes in hemagglutinin or neurominidase resulted in pandemics (Enserink et al., 2004; Li et al., 2004). Also, influenza virus antigenicity is dependent on minor gene mutation (antigenic drifts). Antigenic drift describes the minor changes that generally occur annually in either the hemagglutinin or the neuraminidase or in both. In contrast, major antigenic shift occurs infrequently and describes the appearance of viral strains with surface antigens that are only distantly related to those on previous strains. Antigenic shift may involve the hemagglutinin alone, or the neuraminidase as well. It is generally thought that minor
antigenic variations are due to mutations in hemagglutinin and/or neuraminidase genes whereas major antigenic shift may occur as a result of gene reassortment between a human and an animal strain. Major antigenic shift typically results in influenza epidemics or pandemics (Warren et al., 1994).

It has been suggested that the pathogenesis of influenza infection may be related to the associated inflammatory mediators that occur during infection. In vitro studies using alveolar macrophages demonstrated that influenza A virus infection resulted in increased IL-1β, IL-6, IL-8, and TNF-α (Seo et al., 2004). Recent studies with the influenza H5N1 virus which resulted in high mortality rates showed that gene transcription of the pro-inflammatory cytokine TNF-α in the infected human primary monocyte-derived macrophages was markedly elevated (Cheung et al., 2002). Also, human alveolar and bronchial epithelial cells infected with H5N1 virus produce higher level of IL-6 and interferon-β as compared to the H1N1 virus (Chan et al., 2005). Experiments using in vivo models have shown similar results. For example, alveolar macrophage isolated from pigs infected with H1N1 or H3N2 influenza virus produced high level of IL-6 and TNF-α cytokines (Seo et al., 2004). Also, in H1N1 infected mice, IL-6, TNF α and Type I IFNs in bronchoalveolar fluid (BAL) were elevated (Conn et al., 1995). Experiments using influenza virus NS gene reassortants in mice demonstrated that reassortants containing the H5N1 gene resulted in increased pulmonary concentrations of the inflammatory cytokines IL-1α, IL-1β, IL-6, and chemokine KC, similar to the findings obtained from humans who died from influenza H5N1 (Lipatov et al., 2005). The result from other studies suggests that high serum levels of IL-6, TNF-alpha and soluble TNF receptor 1 sTNFr1 observed during influenza infection was associated with encephalitis (Tsuneo et al., 2002). Taken together, there is evidence from in vitro models demonstrating
that influenza-infected cells may produce inflammatory cytokines, and findings from in vivo studies suggest that elevated pro-inflammatory cytokines contribute to severity of infection.

**Effect of IL-1α, IL-1β, and IL-2 on Blood Glucose**

Pro-inflammatory cytokines appear to be a critical factor in glucose homeostasis. Previous studies indicate that cytokines may act as hormones and alter blood glucose level. IL-1 has been identified as the major mediator of hypoglycemia during septic shock in a mice experiment (Oguri et al., 2002). The intra-peritoneal injection of low dose of IL-1 alpha and IL-1 beta (0.1ug/kg mice body weight) were found to be capable of the reducing blood glucose level (Oguri et al., 2002; Endo et al., 1991). This reduction in blood glucose level was due to increased tissue glucose uptake and inhibition of gluconeogenesis. Similarly, it was found that glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (the key enzymes in gluconeogenesis) were decreased in mice bearing IL-1-beta secreting tumor compared to mice injected with non secreting tumor cells (Metzger et al., 2004). In human studies, it has also been observed that alteration of blood glucose level could be induced by cytokines. Subcutaneous administration of IL-2 produced hypoglycemia (Harnish et al., 2005). Although direct administration of IL-1 and/or IL-2 appear to result in hypoglycemia, it is less clear whether infection-induced changes in IL-1α, IL-1β, or IL-2 contribute to altered blood glucose.

**Effect of IL-6 on Glucose Homeostasis and Insulin Sensitivity**

It has been shown that IL-6 may be produced by multiple cells including adipocytes, endothelial cells, hepatocytes, as well as cells of the immune system. In contrast to the
hypoglycemia observed after IL-1 or IL-2 injection, the subcutaneous injection of IL-6 increased blood glucose level in humans in a dose dependent manner (Harnish et al., 2005; Tsigos et al., 1997). Direct infusion of bacterial LPS in humans resulted in increased glucose (6-8%) and increased plasma insulin (19-38%) at 24 hours after infusion (Andersen et al., 2007). Plasma IL-6 and TNF showed greater than a 100 fold increase in concentration which reached a peak 2 hours after LPS infusion (but preceded the change in glucose and insulin). Also, LPS and TNF expression in adipocytes and monocytes peaked 2-4 hours after infusion. The findings from this study suggested that cytokines precede blood glucose and insulin changes in response to bacterial LPS.

IL-6 has been associated with insulin resistance. In humans, the hyperinsulinemic-euglycemic clamp was used to test insulin sensitivity, and it was demonstrated that plasma IL-6 levels were inversely correlated with insulin sensitivity (Heliovaara et al., 2005). Similarly in a mouse model, using the hyperinsulinemic-euglycemic clamp, it was observed that acute IL-6 administration decreased insulin-stimulated glucose uptake in muscle, which contributed significantly to the change in whole-body insulin resistance. Furthermore, these investigators demonstrated that this effect was associated with defects of the insulin-stimulated IRS-1-associated PI 3-kinase in muscle (Hypo-Jeong et al., 2004). In addition, in this same study, it was found that IL-6 administration increased tyrosine phosphorylation of STAT3 in skeletal muscle, and it is known that IL-6 binding results in recruitment of the signal transducing molecule STAT3 leading to the activation of suppressor of cytokine signaling (SOCS)-3. SOCS-3 has been shown to inhibit insulin signaling in hepatocytes and adipocytes (Emanuelli et al., 2000; Emanuelli et al., 2001). Other in vivo studies have demonstrated that adipose tissue IL-6 content is correlated with resistance to insulin
activation of glucose uptake (Bastard et al., 2002) and chronic exposure to IL-6 also causes hepatic insulin resistance in mice (Klover et al., 2003). Studies performed in vitro have shown that IL-6 inhibits insulin signaling and insulin action in isolated mice hepatocytes and in hepatoma cell lines via a decrease in tyrosine phosphorylation of insulin receptor 1 (IRS1) and protein kinase activation (Senn et al., 2002). Also, IL-6 impaired insulin signaling in 3T3-L1 subcutaneous adipose cells isolated from humans (Rotter et al., 2003). Therefore, a substantial number of studies have suggested that IL-6 may promote insulin resistance, although this final has not been entirely consistent. Other experimental studies in vivo demonstrated that acute infusion of IL-6 for 120 min with hyperinsulinaemic- euglycemic clamp in rats did not alter the effect of insulin on whole body glucose uptake (Sopasakis et al. 2004). Other researchers indicate that infusion of IL-6 in type 2 diabetic patients and aged matched control subjects does not impair or enhance glucose uptake and glucose level remain at the same level all the time (Peterson et al., 2004). In addition other investigators demonstrated that IL-6 may be associated with increase insulin sensitivity and enhancement in glucose uptake. For example, in vitro studies 3T3-L1 adipocytes treated with recombinant murine IL-6 for 5 hrs increased glucose transport by 20% (Stouthard et al., 1996). Also there was a high basal glucose level and impairment in response to the glucose tolerance test in IL-6 deficient mice (Wallennius et al., 2002). Also it has been shown that IL-6 increased glucose disposal during hyperinsulinemic-euglycemic clamp in healthy humans (Carey et al., 2006). Therefore, data with respect to the role of IL-6 role on insulin sensitivity is controversial. Figure 1 demonstrates a hypothesized role of IL-6 as a mediator of insulin sensitivity within insulin responsive cells (IR) (Carey et al., 2004). This figure demonstrates that IL-6 may act in a manner similar to insulin and result in phosphorylation of tyrosine residue of IRS-1.
IRS-1 phosphorylation will lead to PI3K activation and enhance insulin action. However in chronic elevation of IL-6, Akt phosphorylation can lead to phosphorylation of serine instead of tyrosine residue on IRS-1 via activation of the mammalian target of rapamycin (mTOR). This leads to impaired insulin signaling and increased insulin resistance. In addition, IL-6 may activate suppressors of cytokine signaling through Jak/Stat pathway to inhibit tyrosine phosphorylation of IRS-1. This also will inhibit insulin action. Therefore, IL-6 may increase or decrease insulin action and glucose uptake. However whether IL-6 is a main mediator of glucose homeostasis during infection is not clear.
HYPOTHESIS

We hypothesize that influenza-associated systemic increase of the pro-inflammatory cytokine IL-6, results in altered blood glucose and insulin (and/or tissue response to insulin). This hypothesis was tested by administering anti-IL-6 antibody prior to and during infection to determine whether the antibody blocks infection-induced changes in blood glucose and insulin levels.
MATERIAL AND METHODS

Animals

8-12 week-old Balb/c mice were obtained from the Jackson Laboratory. The animals were maintained in the animal facility at Iowa State University. Each mouse was housed in a separate cage at room temperature and kept on a 12 h dark-light cycle starting at 0600. Mice were fed the standard rodent chow and water (except at days in which fasting glucose was measured). The university animal care committee (COAC) approved all protocols used on animals.

Influenza Virus Infection and Collection of Blood for Assessment of Glucose, Insulin and IL-6

Mice were infected by intranasal inoculation of influenza virus under light anesthesia with CO₂. Different doses of the mouse adapted influenza virus (H1N1- A/PR/8/34) were tested in mice. The virus was grown in chicken embryo obtained at the Veterinary Diagnostic Laboratory at Iowa State University. In preliminary experiments several doses of virus were tested ranging from 25 μl of a virus with a titer equal to 12.5 hemagglutination units (HAU) up to a dose of 50 μl at a titer equal to 512 HAU. Based on the preliminary data, a dose of 50 μl at HAU=512 was the minimum dose in which an effect on blood glucose was observed. Subsequent experiments used 50 μl at either 512 HAU or 1024 HAU. Control mice were similarly anesthetized and inoculated with 50 μl of saline solution 0.9% NaCl.
In one set of experiments, blood glucose was measured prior to influenza infection, and post-infection at times points ranging from day 1 post-infection to day 6 post-infection. At the lower dose of virus test (50 μl of 512 HAU), 20 mice were infected with virus, and on days 1, 2, 3, 4, and 5 post-infection, 4 mice were euthanized (4 mice per day) and glucose was measured. In addition, glucose was measured in 4 non-infected mice as a control comparison for day to day variability in blood glucose. At a higher dose of virus, (50 μl of 1024 HAU), 8 mice were infected and blood glucose was measured at the following time points, 24 hr pre-infection, and days 1, 3, and 5 post-infection. A separate group of 8 mice were also infected and blood glucose was measured at the following time points, 24 hr pre-infection, and on days 2, 4, 6, post-infection. Again, a control group of non-infected mice was measured as a comparison for day to day variability in blood glucose. Body weight, food and water intake were measured in the same mice used to assess blood glucose at multiple time points post-infection in order to establish change over time in body weight and food/water intake.

A second set of experiments was used to assess insulin levels and serum IL-6. With a virus dose equal to 50 μl of 512 HAU, 20 mice were infected and 4 mice were euthanized at each of the following time points post-infection (day 1, 2, 3, 4, and 5). Non-infected mice (n=4) were used as a control comparison. At a higher dose of virus (50 μl of 1024 HAU), 30 mice were infected, and serum IL-6 and insulin were measured at the following time points post-infection (day 1, 2, 3, 4, 5, and 9). Two of the mice to be measured at day 9 post-infection died before day 9 and therefore at day 9, there were only 3 infected mice. Again, non-infected mice (n=5) were used as a control comparison.
**Sampling of Blood and Broncho-Alveolar Fluid for Cytokine and Hormone Assays**

Mice were euthanized by carbon dioxide before collection of broncho-alveolar fluid (BAL) and blood. Blood was drawn by cardiac puncture into a 1cc syringe with a 27 ½ gauge needle. Blood was stored at room temperature for 30 min. The blood was centrifuged at 2000rpm for 30 min. The serum was collected and stored at -20°C for until further analysis.

For BAL: Lungs were lavaged with 1ml sterile AIM-V media through a small catheter inserted into the trachea. Lungs were washed twice, and the broncho-alveolar fluid was collected and centrifuged for 15min at 1500rpm to remove cells and debris. The supernatant was stored at -20°C until assayed for IL-6.

**Determination of Blood Glucose**

In order to minimize stress that could produce an increase in blood glucose, mice were acclimated to the protocol for 7-10 days prior to actual glucose level measurement. Mice were placed in a 50 ml centrifuge tube with an opening hole at the cover, the right leg was shaved, and the saphenous vein was exposed. In order to obtain fasting blood glucose, food was taken away and mice were left to fast for 4-8 hrs with access only to water. The optimum level was detected after 6 of fasting and the blood glucose range between 80-120 mg/dL. Mice were divided into groups and fasted for 6-7 hrs. The blood taken from the saphenous vein was directly applied to a strip and glucose was determined with Accu-Check Advantage glucometer. Several experiments were done to determine differences between infected and non infected mice groups and also to detect the time course for changes in
glucose level. The time points and doses used were described above under “Influenza virus infection and collection of blood for assessment of glucose, insulin and IL-6”

**Measurement of Body Weight and Food and Water Intake**

Before infection mice were weighed on a top loading balance, and food and water in each cage were weighed on the same scale. Daily measurement was performed prior to infection and for five days post-infection. The total amount of food was subtracted from the total amount of food on the previous day to determine the actual amount of food consumed each day during infection. Water intake was assessed with the same method. Body weight, food intake and water intake were also measured in non-infected mice.

**Enzyme-Linked Immunoassay ELISA for IL-6 Cytokine Measurement**

IL-6 was measured by ELISA (BD Biosciences, San Diego, CA lot # MF 26406) with test sensitivity > 4pg/ml. For this ELISA kit, the intra-assay % CV = 6.4 and the inter-assay % CV = 6.5. The plate was coated with 100μl/well capture antibodies diluted at 1: 250 in a coating buffer 0.1 M sodium carbonate, pH 9.5 freshly prepared (8.40g NaHCO₃, 3.56g Na₂CO₃; q.s. to 1.0 L; pH to 9.5). The plate was sealed and kept overnight at 4°C. Wells were washed and blocked with 200 μl of assay diluent at room temperature for an hour. After several wash steps with PBS buffer, 100 μl of standard and samples were added to each well, and the plate was sealed and incubated for 2hrs. Several washes with PBS buffer were performed. Then, the working detector solution containing strepatavidin-HRP-conjugated and biotinylated anti-mouse IL-6 monoclonal antibody was prepared. 40 μl of strepatavidin-HRP-conjugated and 40 μl of the detection antibody were added to 10ml of assay diluent.
The fraction of the IL-6 bound to the enzyme conjugate was detected by reaction with 100μl/well of tetramethybenzidine and hydrogen peroxide solution. The colorimetric produced by the reaction was assessed by reading the optical density at 655nm using a BIO-RAD micro-plate reader.

**Measurement of Insulin Level**

Insulin level was measured by enzyme-linked immunosorbent assay kit (Mercodia Mouse Insulin ELISA, ALPCO diagnostics). The sensitivity of the assay was 0.10μg/l. For this ELISA kit, CV% within the assay = 2.2 and the CV % between the assay = 4.9. Twenty-five µl of samples and calibrators and 25 µl of peroxidase-conjugated anti-insulin antibodies were added into anti-insulin antibodies coated micro-titration wells. The plate was incubated on a shaker for 2 hrs at room temperature. Several washing steps were performed to remove unbound enzyme labeled anti-insulin antibodies. The bound conjugate was detected by reaction with tetramethylbenzidine at 200 µl/well. The reaction was stopped by adding sulfuric acid H₂SO₄ in the stop solution. The absorbance was read at 450 nm by spectrophotometer and compared with the calibrators.

**Treatment of Mice with anti-IL-6 Antibody**

In initial experiments, the dose of antibody tested was based on published literature. MP5-20F3 anti-IL-6 antibody was purchased from BD Pharmingen. Mice were injected through an intra-peritoneal (i.p.) route with 100 μg/100 μl of anti-IL-6 mAB. Control mice were similarly injected i.p. with 100 μl saline. Mice were injected with antibody at 4 hours
prior to infection. At days 24 and 48 hours post-infection serum was collected and IL-6 was measured. Prior administration of the anti-IL-6 antibody significantly blocked the infection associated increase in IL-6 at day 1 post-infection and tended to block to increase at day 2 post-infection (Fig. 2) (although serum IL-6 did not increase to the same extent at day 2 post-infection compared to day 1 post-infection based on our previous data). The dose of 100 μg was used in subsequent experiments.

**Administration of Anti-IL-6 Antibody in Vivo and Assessment of Blood Glucose Post-Infection**

Mice were injected through an intra-peritoneal (i.p.) route with 100 μg in a 100 μl volume of saline of anti-IL-6 mAb, clone MP5-20F3 (Anti-IL-6 antibody treatment group). Control mice were similarly injected i.p. with 100 μg rat Ig1 κ purchased also from BD Pharmingen (Control antibody treatment group). Injections were performed 4 hrs before infection and then daily for four days during infection. A non-infected mice group was similarly injected with 100μl saline at the corresponding times. In the first set of experiments five mice per treatment group were used, and blood glucose was measured before infection and again at day 3 (72 hrs) and day 5 (120 hrs) post infection. At the time of euthanization on day 5, insulin was also measured. In the second set of experiments, the same three treatment groups were used. On day 1 (24hr) post-infection, 5 mice per treatment group were euthanized and serum IL-6 and insulin were measured. On day 2 (48 hr) post-infection, again 5 mice per treatment group were euthanized and blood was collected for assessment of IL-6 and insulin.
Data Analysis

Statistical analysis of the data was performed using SPSS. In experiments in which IL-6, glucose, or insulin was measured once per mouse, a one way ANOVA was used to compare means from more than two groups. Post hoc tests were used with LSD as the post-hoc test to establish which treatment groups differed. In the experiments in which glucose was measured at more than one time point in the same mouse, a mixed ANOVA was used (treatment by time). If a significant treatment by time interaction was found, appropriate one-way ANOVA’s were performed to establish differences between groups at given time points.

Series of the Experiments

In initial experiments, different doses of virus were tested (ranging from 25 μl of 12.5 HAU/0.025 ml to 50 μl of 512 HAU/0.05ml) to determine the extent to which a different virus dose might affect IL-6, glucose, and insulin levels. At lower levels of virus, blood glucose and insulin responses were variable and did not show large fold changes. It was concluded that higher doses of virus should be tested to determine whether the higher doses would have a greater effect.

In the second set of experiments, two higher doses of virus (50 μl of 512 HAU/0.05 ml and 1024 HAU/0.05 ml) were used to determine the effect of infection on the pattern of changes on IL-6, glucose and insulin levels one week after infection. Body weight and food intake were also measured. Significant effects of infection on blood glucose, insulin, body weight and IL-6 were observed with the highest dose of virus. The next experiments sought to determine whether anti-IL-6 antibody administered in vivo could block the infection-
associated increase in IL-6. At the dose and time point tested, the IL-6 antibody did block the increase in IL-6. The last set of experiments were planned to evaluate the extent to which prior administration of anti-IL-6 antibody might mediate infected-associated changes in blood glucose, insulin, or body weight. Anti-IL-6 antibody or irrelevant antibody was administered prior to and/or during infection. Changes in blood glucose, insulin, IL-6, body weight, food and water intake were measured.
RESULTS

High Serum IL-6 Level with Infection

Mice were infected with 50μl of influenza A/PR/8/34 at a titer of 512 HAU, and IL-6 was measured at days 1-5 post infection (n=4 mice per day). A statistically significant increase in serum IL-6 was found at day 2 (p=.001), day 3 (p= 0.03) and day 5 (p=0.01) compared to the control group inoculated with 50μl saline (Fig. 3). At a higher dose of virus (50 μl of 1024 HAU), IL-6 was also found to be elevated significantly at day 1 (p= 0.03), day 2 (p<0.01), day 5 (p=0.01) and day 6 (p=0.03) post infection compared to day 0 (non-infected group) (Fig. 4).

Changes in Blood Glucose Level Post Infection

In the first set of experiments, 50 μl of A/PR/8/34 influenza virus at a titer of 512 HAU was administered and mice were euthanized day 1-5 post-infection. Four different mice were euthanized each day. The results of a one-way ANOVA showed that on day 5 post infection, the decrease in blood glucose compared to pre-infection was different than the day to day change in blood glucose of non-infected mice (p=0.052) (Fig. 5). However, days 1-4 were not significantly different. In the second set of experiments, a higher dose of virus was used (50 μl of 1024 HAU titer). The same mice were used to measure blood glucose on days 1, 3, and 5 post-infection (n=8) (Fig. 6), and a different set of mice were used to measure glucose on days 2, 4, and 6, post-infection (n=8) (Fig. 7). Change in blood glucose over time was compared to non infected mice (mixed ANOVA treatment X time). The results showed a significant treatment by time interaction for mice assessed on days 1, 3, 5
post infection (p=0.001) and for mice assessed days 2, 4, 6 (p=0.003) (Figs. 6 & 7). Upon post-test analysis (ANOVA comparison for each day), a significant decrease in blood glucose was found on days 2, 4, 5, and 6 post-infection, as compared to day to day variability in blood glucose of non-infected mice. In Table 1, the change in blood glucose during infection was compared between mice infected with the viral titer of 512 HAU with mice infected with the titer of 1024 HAU. With this day by day comparison of change in blood glucose, it was apparent that the higher dose of virus resulted in a greater decrease of blood glucose throughout the infection.

**Insulin Level in Response to Infection**

The same mice that were used to assess IL-6 in serum were also used to measure insulin at several time points post-infection. At the lower dose of virus (50 μl of 512 HAU), mice were euthanized day 1-5 post-infection (n=4 per day). No significant differences were found in insulin during infection (Fig. 8). In contrast, at the higher dose of virus (50 μl 1024 HAU), a significant increase in insulin was observed at day 1 post-infection p= 0.05. At day 3 and 4 there was a trend towards lower insulin levels than in non-infected, p=.06 for day 3, p=.08 for day 4 and on day 5, insulin was significantly lower than in non-infected mice at p=0.03 (Fig. 9). The table below (Table 2) compares the insulin response to the two doses of virus. It appears that the higher dose of virus affects insulin level to a greater extent.

**Body Weight, Water Intake, and Food Intake Changes with Infection**

Body weight and food intake were measured in the mice that were infected with 50 μl of the 1024 HAU titer A/PR/8/34 influenza virus. A significant main effect of treatment
A significant treatment by time interaction were observed. On days 2, 3, 4, and 5, body weight of infected mice was significantly lower than non-infected (Fig. 10). Food intake followed a similar pattern with a significant treatment effect (p<0.001) and a trend to a treatment by time interaction. Food intake of infected mice was significantly lower than non-infected mice at day 2, 3, 4, and 5 post-infection (Fig. 11). Water intake did not differ between non-infected and infected mice (data not shown)

Effect of Anti-IL-6 Ab Administration in Vivo on Infection-Associated Change in Blood Glucose

Blood glucose was measured pre-infection and then at day 3 and 5 post-infection in mice treated with one of the following treatments, anti-IL-6 antibody, anti rat IgG1 isotype immunoglobulin, or saline in non-infected mice. A significant main effect of treatment (p=0.001) and a treatment by time interaction (p=0.057) were found suggesting that the groups did not respond similarly over time. A follow-up one-way-ANOVA for glucose measured each day separately showed no difference between the groups at pre-infection. However at day 3 post-infection, a significant effect of treatment was observed (p<0.001) such that both infected groups had a significant decrease in blood glucose compared to non-infected mice, but the anti-IL-6 antibody treated mice were not different than the mice treated with irrelevant antibody. At day 5 post-infection when the drop in blood glucose was typically more pronounced, a significant effect of infection was found (p=0.01). However, the decrease in blood glucose was significantly different in mice receiving the irrelevant antibody when compared to non-infected (p=0.003), but NOT different between anti-IL-6
antibody-treated mice and non infected mice. This finding suggests that the administration of IL-6 antibody may attenuate the drop in blood glucose at day 5 post-infection (Fig. 12) (although it should be noted that this was not a consistent effect across all of the mice).

**Effect on Anti-IL-6 Ab Administration in Vivo on Infection-Associated Change in Insulin**

Five mice per group received the anti-IL-6 antibody treatment or the irrelevant antibody treatment whereas 22 mice per group served as non-infected controls (pooled experiments with all non-infected mice). At days, 1, 2, and 5 post-infection insulin was measured. At day 2 post-infection, insulin levels tended to decrease as previously observed (irrelevant antibody treated mice as compared to non-infected, although this did not meet statistical significant). However, the anti-IL-6 treated mice at day 2 post-infection did not demonstrate a decline in insulin and instead, their insulin level was significantly greater than infected mice treated with irrelevant antibody (p=0.04, Fig 13). At day 5 post-infection, the infected mice tended to show a reduction in insulin (p=0.057) as compared to non-infected mice. However, there was no difference between anti-IL-6 Ab-treated mice and irrelevant antibody-treated mice suggesting that IL-6 does not mediate the drop in insulin at day 5 post-infection.

**Effect on Anti-IL-6 Ab Administration in Vivo on Infection-Associated Change in Body Weight and Food/Water Intake**

Although infection was associated with a significant drop in body weight at days 2-5 post-infection (p<0.01) and food intake at days 1-4 post infection (p<0.015), there was no
difference between mice receiving IL-6 antibody as compared to irrelevant antibody suggesting that IL-6 does not mediate changes in infection-associated weight loss or food intake (Fig. 14, 15). Water intake did not change with infection and there were no differences between all three treatment groups with respect to water treatment (data not shown).
DISCUSSION

Overall Summary of Findings

The present study was designed to investigate the effect of infection-associated increases in serum IL-6 on glucose and insulin levels. The change in glucose and insulin taken together was used to predict insulin sensitivity (although insulin sensitivity at the tissue was not measured in this study). The results showed an elevation of serum IL-6 during infection and a decline in glucose level that reached statistical significance at day 5-6 post-infection. In contrast, insulin was significantly increased in response to infection on day 1 post-infection, but decreased on days 3, 4, and 5 post-infection. With administration of anti-IL-6 antibodies, the infection-associated decline in insulin as seen in previous experiments appeared to be prevented by administration of anti-IL-6 antibody only on day 2 post-infection. Also, the infection-associated decline in blood glucose at day 5 post-infection was attenuated in anti-IL-6 treated mice as compared to infected mice that received the irrelevant antibody. These findings support a role for IL-6 as a mediator of the infection-associated change in blood glucose and insulin levels. With infection there was a tendency to decrease insulin and glucose levels started at day 2 and that decrease became more pronounced at day 5 post-infection. These findings suggest that during infection there may be an increase in insulin sensitivity that predispose to the decline in glucose level at day 5. Whether infection-induced IL-6 release is the mediator in insulin sensitivity is not determined yet.
**Blood Glucose Changes in Association with Infection**

A change in blood glucose level is well documented during infection and therefore our results are consistent with other studies. However the direction of the infection-associated change in blood glucose (hyperglycemia vs. hypoglycemia) is variable. Patients infected with either bacterial or viral organisms may be hypoglycemic or hyperglycemic during infection (Ronan et al., 1997; Jaward et al., 1995, Yang et al., 2006, Andersen al., 2004; Miller et al., 1980)). It is not clear whether the type of infectious agent predicts hypo- or hyperglycemia, however the existing data with respect to this question are limited and it is not possible to draw a conclusion at this point.

Also the mechanism(s) responsible for the changes in blood glucose is not well understood. In an attempt to study the mechanism(s) that may lead to glucose changes with infection, we have studied influenza infection in mice. Evidence from other studies suggests that respiratory infection may precipitate the acute elevation of blood glucose in a diabetic person (Thomas et al., 1993), or could be associated with persistent glucose elevation after 3 months of the onset of infection (Wasmuth et al., 2000). Also, hypoglycemia is associated with influenza virus infection, and laboratory findings in influenza associated-encephalitis patients infected with either influenza A and B virus revealed glucose level < 60mg/dl (Tsuneo et al., 2002). Therefore, we have studied influenza infection in a mouse model to better understand how infection might affect glucose and insulin levels. Previous attempts to better understand the relationship between bacterial infection and glucose were performed by using LPS injection (s.c. or i.p.) in experimental mice (Oguri et al., 2002). This study showed a decrease in glucose level with LPS injection in a dose dependant manner. There was also another study that showed influenza B virus injection in mice led to inhibition of the
mitochondrial phase of gluconeogenesis and decreased glucose level (Davis et al., 1993). To our knowledge, the potential role of cytokines as mediators of infection-related changes in glucose and/or insulin has not been evaluated. Therefore, the experiments in our study were designed to test whether the cytokine IL-6 mediates changes in blood glucose and/or insulin during influenza infection.

Our results showed that mice infected with influenza A/PR/8/34 at a dose of 50 μl of 512 HAU showed a significant drop in blood glucose particularly at day 5, and that drop was more prominent with a greater dose of virus. Although there was a decline in food intake during infection that could potentially cause a decrease in blood glucose, the time course of decreased blood glucose did not correspond directly with food intake. Food intake is typically decreased on days 2-4 of infection, and begins to return to normal around day 5-6 post-infection. Therefore, there may be other mechanisms responsible for the decline in glucose.

**IL-6 Increased with Infection**

It is possible that inflammatory cytokines produced during infection may regulate glucose homeostasis. Findings from other studies have shown that IL-6 increased with influenza infection in both vitro and vivo experiments (Seo at al., 2004; Chan et al., 2005; Conn et al., 1995). The increase in serum IL-6 was correlated with severity of illness in human subjects (Tsuneo et al., 2002; Kaiser et al., 2001). Our results are consistent with these other studies; we have observed a significant elevation of serum IL-6 with both low and high doses of infection. However, with a higher infectious dose (50 μl of 1024 HAU) we did not observe a greater level in serum IL-6 level as compared to the lower infectious dose (512
HAU). This may be due to the fact that the standard curve used to determine the level of serum IL-6 with the lower infectious dose was not within the typical range that is usually observed using the ELISA kit from BD Biosciences. Therefore, it was not possible to directly compare absolute level of IL-6 between the two experiments using different doses of virus. However, in both experiments IL-6 was significantly increased on day 2 and day 5 post-infection. Also, our data showed that with the high virus dose the significant increase in IL-6 started earlier at day 1 post-infection as compared to day 2 with lower infectious dose.

**Correlation between IL-6 and Glucose / Insulin during Infection**

The data from our experiments indicated that the decline in glucose level was very prominent particularly at day 5-6 post-infection (at the same time in which serum IL-6 was elevated). However, although IL-6 was increased at days 1-2 post-infection, there was no change in glucose. That may suggest that early onset and increasing length of time in which circulating IL-6 is elevated is an important factor in predicting the impact of IL-6 on blood glucose during infection. Perhaps a longer duration of exposure to elevated IL-6 (i.e. several days rather than 24-48 hrs) is necessary before an effect on blood glucose is found.

It is possible that changes in insulin sensitivity result in altered blood glucose found at day 5-6 post-infection. In support of this possibility, IL-6 has been found to play a role in altering insulin sensitivity and subsequent changes in glucose. An in vitro study showed that 3T3-L1 adipocytes treated with recombinant murine IL-6 for 5 hrs increased glucose transport by 20% (Stouthard et al., 1996). Also it has been shown that IL-6 infusion enhances insulin stimulated glucose disposal in human during hyperinsulinemic-euglycemic clamp (Carey et al., 2006). Therefore, IL-6 may be associated with an increase in insulin sensitivity
during infection. The increase in insulin sensitivity and enhancement of glucose uptake may be responsible for the decrease in glucose level found in our data at day 5-6 post-infection. Our results may have supported this possibility, given that we found a significant increase in serum IL-6 and tendency to decrease insulin level over time during infection with a significant drop at day 5 post-infection. This decline in insulin level was also matched with a decrease in glucose and became statistically significant at day 5 and 6 post-infection. This suggested that IL-6 may influence blood glucose level during infection via enhancement of insulin sensitivity and glucose uptake. In our experiments when anti-IL-6 antibody was administered, at day 5 post-infection, there was no effect of the antibody on insulin level, but the glucose level increased in antibody-treated mice. With no effect of IL-6 on insulin, and blockade of IL-6 resulting in increased glucose, these findings suggested that acute increases in IL-6 might act to reduce blood glucose (or increase insulin sensitivity at day 5 post-infection). Given that serum IL-6 was increased for several days during infection and the significant decline in glucose and insulin was obvious later with infection, it is possible that prolonged exposure to IL-6 may be important to sensitize tissues to insulin and enhance glucose uptake which was predicted by decline in glucose level. Also important to consider is that the IL-6 antibody appeared to prevent the decline in insulin that occurred early during infection (day 2), but had no effect on glucose on day 3. This might suggest that early during infection (within hours), IL-6 acts to reduce insulin which is accompanied by a drop in blood glucose.

A possible mechanism by which IL-6 exerts its effect on the day5 decrease in blood glucose is through phosphorylation of tyrosine residue of IRS-1. IRS-1 phosphorylation will lead to PI3K activation as shown in Fig 1 (Carey et al 2004). Activation of PI3K can lead to
increase GlutT4 movements from the internal membrane vesicle to plasma membrane and increase glucose uptake resulting in a decline in blood glucose level.

**Other Factors that may Mediate Infection-Associated Decrease of Blood Glucose**

Other factors may be involved in the decrease of glucose level during infection. One study has shown a decrease in glucagon level during acute and convalescence periods in patient infected with either bacterial or viral pathogens (Sammalkorpi et al., 1989). The decrease in glucagon level may be responsible for the decrease in glucose level in our experiments. Also inhibition of gluconeogenesis could be a possible cause in decreasing glucose level during infection as well. It was found that influenza B injection in mice model led to inhibition of the mitochondrial phase of gluconeogenesis and the pyruvate carboxylase, the main key enzyme in gluconeogenesis was largely displaced into the cytosol of hepatocytes (Davis et al., 1993). Therefore, it is possible that IL-6 in combination with changes of other hormones that affect blood glucose level could be responsible for the hypoglycemic effect found in the infected mice. The results of our study suggested that IL-6 mediates the hypoglycemic response observed at day 5-6 post-infection. However, it is possible that IL-6 altered glucose by a direct effect on tissue insulin sensitivity as discussed earlier, or that IL-6 acted by altering other hormones that affect blood glucose.

Previous studies showed that both IL-1 alpha and IL-1 beta contribute to hypoglycemic effect in mice (Oguri et al., 2002; Endo et al., 1991). Therefore, it is also possible that IL-6 may also act on other cytokines and promote their release. IL-6 is known to induce acute phase protein including pro-inflammatory cytokine IL-1 (Janeway et al., 2001). IL-1 subsequently could alter glucose homeostasis. Therefore, other cytokines such
as IL-1 that are released in response to IL-6 may in fact be the cytokine that is directly responsible for alterations of blood insulin and glucose. It is also possible that multiple cytokines acting together are involved in mediating glucose and insulin. Further studies using knockout mice or antibodies against multiple cytokines would be required to establish whether multiple cytokines are involved.

**IL-6 and Insulin Resistance Early with Infection**

It was documented that direct injection of LPS in human led to immediate elevation in IL-6, insulin and glucose levels in the first 24 after injection (Andersen et al., 2007). That may indicate that there may be a state of increased insulin resistance due to IL-6 elevation with LPS injection. Our results in an infection model were somewhat consistent with the studies using LPS. Our data showed a higher infectious dose resulted in an early significant elevation of IL-6 and insulin level at day 1 post infection. This may suggest that there may be a state of increased insulin resistance early with infection (up to 24 hours post-infection). Although some of our findings at later time points during infection (day 5-6) suggest Based there is an increase in insulin sensitivity later with infection, it is possible that both insulin resistance and insulin sensitivity could occur at different time points during infection. The increase in insulin resistance may be occurring early with infection followed by gradual increase in insulin sensitivity. Perhaps a short-term exposure (hours) to IL-6 results in insulin resistance whereas prolonged exposure to IL-6 results in greater insulin sensitivity. At the cellular level, different signaling pathways might be influenced by short-term versus longer term exposure to IL-6. Another possibility is that different tissues are affected by different
lengths of time of exposure to IL-6. For example, there may be a partial increase in insulin resistance on hepatocytes early with infection followed by increase in glucose disposal and body uptake at the periphery (adipose and muscle). However, further experiments are required to determine which of these possibilities may be correct. In general, it was apparent that with either low or high virus dose there was a significant decline in glucose level that might be related to insulin sensitivity.
CONCLUSION

We have tested the role of IL-6 during infection on glucose and insulin via administration of IL-6 neutralizing antibodies. Based on the decrease in glucose and insulin levels and the potential increase in insulin sensitivity later (at day 5 post-infection), we hypothesized that IL-6 antibody treatment would block the increased insulin sensitivity resulting in either an increase in insulin level or attenuation in the decline of glucose level. The results showed that anti-IL6 antibodies treatment did attenuate the drop of glucose level associated with influenza infection in mice (at day 5 post-infection). Also, there were no effects of anti-IL-6 antibody treatment on body weight or food intake. Those results taken together suggest a potential role of IL-6 exists with respect to the infection-mediated decrease of glucose (day 5-6 p.i.) (unrelated to the consumed food amount). Anti-IL-6 antibody treatment did also appear to prevent an infection-associated decrease in insulin at day 2 post-infection compared to infected group with irrelevant antibodies. This may indicate that IL-6 have a role in insulin homeostasis with infection. Taken together, these findings suggest that IL-6 acts to mediate glucose and insulin level during infection. IL-6 may increase insulin sensitivity during infection. However we have to determine whether IL-6 has a direct effect on tissue insulin sensitivity and subsequent blood glucose level or whether IL-6 acts on other cytokines or hormones which in turn mediate blood glucose.
REFERENCES


## TABLES

Table 1: Changes in blood glucose (post-pre-infection).

*Units = mg/dl mean ± S.E.*

<table>
<thead>
<tr>
<th>Virus dose</th>
<th>Non infected</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>512 HAU</td>
<td>0.0 ± 9.1</td>
<td>5.0± 17.1</td>
<td>1.25 ± 9.9</td>
<td>3.0 ±5.8</td>
<td>12.5± 4.1</td>
<td>-30.2 ±10.4 <em>(p=0.052)</em></td>
<td></td>
</tr>
<tr>
<td>1024 HAU</td>
<td>9.0±7.2</td>
<td>5.0 ±9.3</td>
<td>-21.7 ± 2.0 <em>(p=0.005)</em></td>
<td>-3.7± 3.5 <em>(p=0.111)</em></td>
<td>-26.3± 6.3 <em>(p=0.013)</em></td>
<td>-29.5± 6.7 <em>(p=0.008)</em></td>
<td>-60.5± 7.2 <em>(p=0.001)</em></td>
</tr>
</tbody>
</table>

Table 2: Change in insulin level with infection.

<table>
<thead>
<tr>
<th>Virus dose</th>
<th>Non infected</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>512 HAU</td>
<td>0.82 ± 0.42</td>
<td>0.41 ± 0.29</td>
<td>0.52 ± 0.27</td>
<td>0.36 ± 0.18</td>
<td>0.52 ± 0.15</td>
<td>0.33 ± 0.21</td>
</tr>
<tr>
<td>1024 HAU</td>
<td>0.51 ± 0.18</td>
<td>2.10 ± 0.57 <em>(p=0.04)</em></td>
<td>1.18 ± 0.61</td>
<td>0.21 ± 0.18</td>
<td>0.30 ± 0.14</td>
<td>0.11 ± 0.08</td>
</tr>
</tbody>
</table>
Figure 1: Hypothesized role for IL-6 in the etiology of insulin sensitivity. IL-6 may phosphorylate IRS-1 on tyrosine residue and enhance insulin action and sensitivity. However, IL-6 may activate suppressors of cytokine signaling through Jak/Stat pathway to inhibit tyrosine phosphorylation of IRS-1. This will lead to impair insulin signaling and increase insulin resistance. In chronic elevation of IL-6 may phosphorylate Akt, which in turn lead to phosphorylation of serine instead of tyrosine residue on IRS-1 and inhibit insulin action, (Pictorial is redrawn from Diabetologia 2004).
Figure 2: Intra-peritoneal injection of 100 μg/100μl of anti-IL6 neutralizing antibodies in mice infected with influenza A virus at a dose of 50 μl at 1024 HAU/0.05 ml. Successfully blocking serum IL-6 at day 1 post-infection with tendency to decrease at day 2 in the serum compared to infected mice injected with 100 μl saline.
Figure 3: Infection with influenza A virus at titer of 512 HAU/0.05ml. Significant increase in serum IL-6 at day 2, 3 and 5 post-infection compared to non-infected
Figure 4: Infection with influenza A at titer of 1024 HAU/0.05ml. A statistically significant increase in serum IL-6 at days 1, 2, 5 and 6 post-infection compared to day 0 (non-infected) $p<0.05$. 
Figure 5: Infection with influenza A virus at titer of 512 HAU/0.05ml. A significant decline in glucose level was observed at day 5 post-infection compared to pre-infection level.
Glucose level with 1024 HAU/0.05ml
days 1,3 and 5 PI

Figure 6: Infection with influenza A virus at titer of 1024 HAU/0.05ml and blood glucose was measured at days 0, 1, 3 and 5 post-infection in the same mice. A significant decline in glucose level at day 5 post infection was found compared to pre-infection.
Figure 7: Infection with influenza A virus at titer of 1024 HAU/0.05ml, and blood glucose was measured at days 0, 2, 4 and 6 post-infection in the same mice group. A significant decline in glucose level was found at day 2, 4 and 6 post-infection compared to pre-infection.
Figure 8: Infection with influenza A virus at titer of 512 HAU/0.05ml. No significant changes in insulin level were found as compared to the non infected group.
Figure 9: Infection with influenza A virus at titer of 1024 HAU/0.05ml. A significant increase in insulin level at day 1 post infection was observed as compared to day 0 non infected group. There is a trend towards lower insulin levels at days 3 and 4 post-infection with a significant decrease of insulin level at day 5 post-infection p=0.03.
Figure 10: Infection with influenza A virus at titer of 1024 HAU/0.05ml. A significant weight loss at day 2, 3, 4, 5 and 6 post-infection was found as compared to non-infected mice.
Figure 11: Infection with influenza A virus at titer of 1024 HAU/0.05ml. The amount of food intake was calculated by subtracting the total amount of food present in the mouse cage from the total amount of food on the previous day. A significant reduction in food intake at day 2, 3, and 4 post-infection was found as compared to the non-infected group.
Figure 12: Intra-peritoneal injection of IL-antibody treatment in mice infected with influenza A virus at a dose of 50 μl at 1024 HAU/0.05 ml. At day 3, glucose level in IL-6 Ab and control Ab were significantly lower than non-infected, and at day 5 the IL-6 Ab attenuated the drop in blood glucose such that glucose level of control Ab-treated mice was significantly lower than non-infected mice.
Figure 13: Insulin level in mice treated with anti-IL-6 Ab, control Ab, or non-infected mice (infected with 50 μl of 1024 HAU/0.05 ml). At day 2, insulin level in IL-6 treated group was significantly greater than insulin level in infected group with control antibody $p=0.04$. 
Figure 14: Body weight changes in mice treated with Anti-IL-6 Ab, control Ab or non-infected (infected with 50 μl of 1024 HAU/0.05 ml). There were no significant differences in body weight loss between anti-IL-6 Ab-treated infected mice as compared to infected mice treated with irrelevant antibodies. However body weight of both infected groups was lower than non-infected at days 1,2,3,4, and 5 post-infection.
Figure 15: Food intake changes in antibodies treated groups. There was no difference in food intake between mice treated with anti-IL-6 Ab as compared to control Ab, however both infected groups of mice had significantly lower food intake on days 2-5 post-infection as compared to non-infected mice (infected with 50 μl of 1024 HAU/0.05 ml).
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