Original Article

Sepsis-Induced Inflammation Is Exacerbated in an Animal Model of Type 2 Diabetes

Asha Jacob, Marissa L. Steinberg, Juntao Yang, Weifeng Dong, Youxin Ji, and Ping Wang

Department of Surgery, North Shore University Hospital and Long Island Jewish Medical Center and The Feinstein Institute for Medical Research, Manhasset, NY 11030, USA

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Abstract: Hyperglycemia is common in critically ill patients and pronounced hyperglycemia may lead to complications which include severe infections, polyneuropathy, multiple organ failure and death in such patients. Sustained hyperglycemia is generally observed in patients with Type 2 diabetes. To explore sepsis-induced inflammation in Type 2 diabetes, polymicrobial sepsis was induced by cecal ligation and puncture (CLP) in the Goto-Kakizaki (GK) rat, a spontaneous animal model of Type 2 diabetes. The Wistar-Kyoto (WKY) rats, non-diabetic inbred rats, were used as controls for the experiment. Blood glucose levels were measured at basal, 2 hr and 20 hr after CLP. At 20 hr after CLP, blood and tissue samples were collected. Plasma levels of lactate, IL-6, IL-10 and endotoxins were measured. Total RNA from liver tissues were extracted and subjected to reverse transcription-polymerase chain reaction using rat specific IL-6 primers. GK rats exhibited significantly elevated basal glucose levels compared to WKY rats. Glucose levels in septic GK rats were significantly elevated compared to WKY rats at all time points studied. While both WKY and GK rats showed significant increases in IL-6 at 20 hr after CLP, the GK rats exhibited an average 2.68-fold increase than that of WKY rats. At 20 hr after CLP, hepatic IL-6 gene expression in GK rats was 1.77-fold greater than that of WKY rats. Although, both WKY and GK rats showed significant increases in plasma lactate levels at 20 hr after CLP, the GK rats exhibited an average increase of 1.69-fold, from the already elevated basal levels, than that of WKY rats. Since the lactate levels in GK sham groups were slightly higher than that of WKY sham, the relative changes in the fold induction by CLP between strains were similar. Both WKY and GK rats showed significantly elevated endotoxin levels at 20 hr after CLP, but no statistical differences were observed between the two groups. These studies suggest that sepsis-induced inflammation is exacerbated in an animal model of Type 2 diabetes.

Key Words: Type 2 diabetes, sepsis, GK rats, IL-6, IL-10, rodent model of type 2 diabetes, cecal ligation and puncture

Introduction

Sepsis, septic shock and multiple organ dysfunction continue to be the most common causes of death in the intensive care units. The incidence of severe sepsis in the United States is estimated to be 750,000 cases per year and continues to increase at a rate of 1.5% annually. Despite the advancement of therapies, mortality remains as high as 40% in patients with severe sepsis and increases to 70% with septic shock [1]. It is also well established that individuals with diabetes are at a higher risk for a variety of bacterial infections such as cystitis, cellulitis and postoperative wound infections [2,3]. However, the reason for this increase in susceptibility is not clear.

Hyperglycemia plays a significant role in the development of postoperative infections [4-7]. Hyperglycemia is common in critically ill patients [8,9] and pronounced hyperglycemia may lead to complications which include severe infections, polyneuropathy, multiple organ failure and death in such patients. Surgical stress results in an initial, transient hyperglycemia presumably due in part to insulin resistance brought by an elevation in counter-regulatory hormones epinephrine, norepinephrine, glucagon, growth hormone and cortisol. The metabolic effects of these
hormones result in an increase in insulin resistance causing increased hepatic glucose production and a decrease in peripheral glucose utilization [10,11]. However, sustained hyperglycemia is generally observed in patients with Type 2 diabetes. In a recent study, it has been suggested that diabetes and hyperglycemia are strong and independent risk factors for hospitalization as a result of infection [12]. The production and/or release of cytokines such as TNF-α, IL-6 and IL-10 have been shown to play a significant role in the development of hyperglycemia. In vivo studies in animals have shown that hyperglycemia induced by glucose infusion for only 3 hr can significantly diminish immune function and induce a state of hepatic oxidative stress due to cytokine activation [13,14]. Furthermore, it has been shown that hyperglycemia due to stress is associated with increased cytokine production and adverse clinical outcome following sepsis [15]. However, the effect of sustained hyperglycemia and insulin resistance, as observed in Type 2 diabetes, on the response of pro-inflammatory cytokines to sepsis is not clearly understood.

Type 2 diabetes is considered to be associated with insulin resistance and a relative defect in insulin secretion. The insulin resistance in peripheral tissues decreases glucose utilization and increases blood glucose levels. The decrease in glucose uptake and the changes in glucose production contribute to the pathogenesis of diabetes. The Goto-Kakizaki (GK) rat is a spontaneous model of Type 2 diabetes, which is widely used in the analysis of the mechanism of Type 2 diabetes [16-20]. These rats are generated by selective breeding, repeated over many generations, with glucose intolerant non-diabetic Wistar rats [16]. Between 3 and 4 weeks of age, GK rats exhibit mild hyperglycemia and hyperinsulinemia. The pathogenesis of the diabetes in the GK rats includes insulin resistance [21,22], impaired insulin secretion [21], abnormal glucose metabolism [23] and an impaired development of pancreatic islet cells [24]. In contrast to other models of Type 2 diabetes, GK rats do not become obese and do not develop hyperlipidemia [25]. By about 24 months of age, these rats exhibit structural changes such as glomerular hypertrophy and thickening of glomerular basement membrane which are characteristics of early stage of human diabetic nephropathy [26,27]. Therefore, the GK rat is a suitable animal model to study the effect of sustained hyperglycemia and insulin resistance on the response of pro-inflammatory cytokines to sepsis.

To understand the pathophysiology of sepsis, much research has been directed toward animal models. One such model is the cecal ligation and puncture (CLP) [28-30] and this model mimics the features of sepsis in humans. In this model, animals exhibit an initial hyperglycemia at 2 hr after induction of sepsis. At 6 hr, these rats become euglycemic and by 24 hr they display a mild hypoglycemia [31]. However, it is unknown how already elevated glucose levels, as observed in Type 2 diabetes, influence these phases of sepsis. In addition to the changes in glucose levels, the pathophysiology of sepsis is characterized by two distinct mechanisms involving extrinsic and intrinsic mediators [32]. The extrinsic mediators include endotoxin, viruses, fungi or protein particles from gram-positive bacteria. Endotoxin or LPS activate the macrophages, endothelial cells and complement lead to the release of various pro-inflammatory mediators, tumor necrosis factor (TNF), interleukin-1 (IL-1), interleukin-6 (IL-6), high-mobility group box-1 protein (HMGB-1), platelet activating factor (PAF), nitric oxide (NO), complements, and eicosanoids [33,34]. These pro-inflammatory cytokines and non-cytokines released by the host immune cells are the intrinsic mediators of sepsis. Among these, IL-6 is the main inducer of the acute phase proteins [35] which are produced predominately by the liver in response to inflammation or tissue injury [36]. In the present study, we sought to explore the effect of sustained hyperglycemia, as observed in Type 2 diabetes, on the response to sepsis induced inflammation. Our study suggests that in the GK rat, a spontaneous animal model of Type 2 diabetes, sepsis-induced inflammation is exacerbated.

Materials and Methods

Animal model of sepsis

Male Wistar Kyoto (WKY) and Goto Kakizaki (GK) rats (275-325 g) at 11 weeks of age were purchased from Charles River Lab and Taconic Labs, respectively. They were housed in a temperature controlled room on a 12-hr light/dark cycle and fed a standard Purina rat chow diet for one week prior to experiment.
Before the induction of sepsis, rats were fasted overnight but allowed water ad libitum. Rats were anesthetized with isoflurane inhalation, and the abdomen and groin were shaved and washed with 10% povidone iodine. Cecal ligation and puncture (CLP) was performed as described previously [37]. Sham operated animals (controls) underwent the same procedure with the exception that the cecum was neither ligated nor punctured. All animals were resuscitated with 3 ml/100 g of body weight (BW) normal saline subcutaneously, immediately after surgery. At 20 hr post-surgery (i.e. late sepsis), animals were re-anesthetized with isoflurane inhalation, blood and tissue samples were collected for further analysis. All experiments were performed in accordance with the National Institutes of Health Guidelines for the use of experimental animals. This project was approved by the Institutional Animal Care and Use Committee of The Feinstein Institute for Medical Research.

Measurement of blood glucose levels

Blood samples were taken from the tail vein at different time points under isoflurane anesthesia and measured on a commercially available glucose meter (Freestyle, Therasense, Alameda, CA) [38].

Determination of plasma levels of lactate, IL-6 and IL-10

Plasma concentrations of lactate were determined using commercial assay kits (Pointe Scientific, Lincoln Park, MI). Plasma IL-6 and IL-10 were quantified using enzyme-linked immunosorbent assay kit specific for rat IL-6 or IL-10 (BD Biosciences Pharmagen, San Diego, CA). The assays were carried out according to the manufacturer’s instructions.

RNA extraction and determination of IL-6 mRNA expression

Total RNA was extracted from liver samples by TRI-Reagent (Sigma). Briefly, 100 mg tissues were homogenized in 1 ml TRI-Reagent and the homogenate was separated into aqueous and organic phases by 1-bromo-3-chloro-propane (BCP) addition and centrifugation. RNA was precipitated from the aqueous phase by addition of isopropanol and centrifugation. The RNA pellet was washed with 75% ethanol and resuspended in 100 μl DEPC-treated water. RNA concentration and purity were determined by measuring the absorbance at 260 and 280 nm. Then 4 μg of RNA was reverse transcribed in a 20 μl reaction volume containing 50 mM KCl, 10 mM Tris-HCl, 5 mM MgCl2, 1mM dNTP, 20 units of RNase inhibitor, 2.5 mM oligo d(T)16 primer, and 50 units of reverse transcriptase. The mixture was incubated at 42°C for 1 hr, followed by 95°C for 5 min. Then, 5 μl of cDNA was amplified by polymerase chain reaction with 0.2 μM each of 3’ and 5’ primers in a 25 μl reaction volume for 28 cycles with denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec, and extension at 72°C for 7 min. The primers are as follows: 5’-CCA GTT GCC TTC TTG GGA CTG ATG-3’ (forward) and 5’-ATT TTC TGA CCA CAG TGA GGA ATG-3’ (reverse) for rat IL-6 (NM 012589) and 5’-TGA AGG TCG GTG TCA ACG GAT TTG GC-3’ (forward) and 5’-CAT GTA GGC CAT GAG TGC CAC CAC-3’ (reverse) for rat glyceraldehyde 3-phosphate dehydrogenase (G3PDH; M17701). The amplicons were electrophoresed on a 1.6% agarose gel containing 0.22 μg/ml ethidium bromide. The gel was then examined and band intensities were measured using a Bio Rad image system and normalized against G3PDH.

Figure 1: Changes in blood glucose in septic WKY and GK rats. Polymicrobial sepsis was induced by CLP in WKY and GK rats. Blood glucose levels were measured at different time points by a commercially available glucose meter. Basal glucose levels were significantly increased in GK rats as compared to WKY rats. At 2 hr and 20 hr after CLP, glucose levels were significantly higher in GK rats as compared to corresponding time points in WKY rats. The data are presented as mean ± SE and compared by one-way ANOVA and Student-Newman-Keuls method. *P<0.05 versus WKY rats; #P<0.05 versus respective basal levels.
Determination of plasma endotoxin levels

Plasma concentrations of endotoxin were determined according to the manufacturer’s instructions, using the microplate method of the Chromogenic Limulus Amebocyte Lysate (LAL) Test (QCL-1000, Cambrex, Walkersville, MD). Plasma samples were diluted 1:10 in LAL Reagent Water and heated to 70°C for 10 min. The microplate was pre-equilibrated to 37°C and 50 μl of each diluted sample or standard was dispensed into the appropriate microplate well. The control wells contained 50 μl of LAL Reagent Water. Subsequently, 50 μl of LAL was added to each well in timed intervals beginning with the first well. After 10 minutes, 100 μl of substrate solution was added to each well in the same order. Six minutes later, the reaction was terminated by adding 50 μl of stop reagent to each well. The absorbance at 405-410 nm was measured using a microplate reader (μQuant MQX200, BioTek Instruments, Inc.). The concentrations of endotoxin were calculated using a standard curve.

Statistical analysis

All data are expressed as mean ± SE (n=4-6/group) and compared with one-way ANOVA and Student-Newman-Keuls test. Differences in values were considered significant if P<0.05.

Results

Changes in plasma glucose in sepsis

Immediately before CLP, blood glucose levels were measured in both WKY and GK rats (Figure 1). GK rats exhibited significantly elevated basal glucose levels of 138 ± 12.2 mg/dL while WKY rats produced 85.0 ± 7.4 mg/dL (P=0.002). Glucose levels increased by 18%, in WKY rats, at 2 hr after CLP (104 ± 9.4 mg/dL) but no statistical significance was evident. The levels then decreased to 70 ± 5.3 mg/dL at 20 hr after CLP, which is slightly lower than the basal value, but not statistically significant (P=0.075). Interestingly, in GK rats at 2 hr after CLP, glucose levels increased to 231± 15.7 mg/dL, from the already elevated basal level (P<0.001), and at 20 hr after CLP, the level was lowered to 143.7 ± 5.4 mg/dL, which is slightly higher than the basal level, but not statistically significant. Furthermore, glucose levels in GK rats were significantly elevated compared to WKY rats at all time points studied (P<0.001).

Changes in plasma lactate and IL-6 and IL-10

The plasma lactate, IL-6 and IL-10 levels were measured in WKY and GK rats in sham (control) and 20 hr after CLP (Figures 2-4). The basal levels of plasma lactate in GK rats (1.65 ± 0.07 mmol/L) were slightly higher than that of WKY rats (1.05 ± 0.05 mmol/L) but no statistical significance was evident (Figure 2). While both WKY and GK septic rats showed a significant increase in plasma lactate levels (1.81 ± 0.09 mmol/L, and 3.06 ± 0.40 mmol/L, respectively), the GK rats exhibited an average increase of 1.69-fold than that of WKY rats (P<0.05). Since the lactate levels in GK sham groups were slightly higher than that of WKY sham, only a 13% increase in lactate levels were observed in the GK septic rats. Thus, even though there was a significant increase in lactate levels between the strains, the relative changes in the fold induction by CLP were similar. Plasma IL-6 levels were extremely low in control samples of both groups (Figure 3). These levels were significantly elevated in both groups of septic rats (216 ± 9.44 pg/ml and 578 ± 137 pg/ml, respectively). Thus, the plasma IL-6 levels in septic GK rats were 2.68-fold greater than that of WKY rats (P<0.001). Since there were undetectable levels of IL-6 in both WKY and
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GK sham rats, we were unable to determine the relative changes in the fold induction by CLP between strains. Furthermore, plasma IL-10 (Figure 4) was similar in WKY and GK sham rats (53 ± 4.56 pg/ml and 59 ± 5.13 pg/ml, respectively). The levels increased to 3.0 and 4.0 fold in WKY and GK septic rats (162 ± 14.32 pg/ml and 245 ± 21.08 pg/ml, respectively). Thus, the plasma IL-10 in septic GK rats were 1.52 fold greater than that of WKY rats (P<0.001).

Changes in hepatic IL-6 gene expression

Total RNA from WKY and GK rats in control groups and 20 hr after CLP was extracted from the liver and subjected to RT-PCR using rat specific IL-6 and G3PDH primers (Figure 5). Liver IL-6 mRNA levels were extremely low in control samples of both WKY and GK rats. Following sepsis, IL-6 levels were greatly increased in both groups. Furthermore, IL-6 levels in septic GK rats were 1.77 fold greater than that of WKY rats (0.797 ± 0.05 vs. 0.450 ± 0.09, P<0.001). G3PDH was used as an internal control and the levels of IL-6 were normalized against G3PDH expression. Since there were undetectable levels of liver IL-6 mRNA in both WKY and GK sham rats, the relative changes in the fold induction by CLP within the strains were not determined.

Discussion

The purpose of this study was to understand the role of sustained hyperglycemia on the response of pro-inflammatory mediators in sepsis. To explore this, we induced CLP in a rat model of Type 2 diabetes, the GK rats. First, we examined the changes in blood glucose levels following CLP. Polymicrobial sepsis by CLP in normal Sprague-Dawley rats is characterized by an initial transient hyperglycemia by 2 hr followed by sustained hypoglycemia by 20 hr [31]. This early hyperglycemic phase is presumably due to the increase in hepatic glycogenolysis and gluconeogenesis. The increased production of glucose exceeds the rate of glucose disposal causing the hyperglycemia. Under severe septic conditions, the rate of glucose use exceeds the rate of glucose production resulting in hypoglycemia [39,40]. Furthermore, hyperglycemia has been shown...
Figure 5: Changes in IL-6 gene expression in septic WKY and GK rats. Sepsis was induced by CLP and liver tissues were collected at 20 hr after CLP. RNA was extracted and analyzed by RT-PCR using primers specific for rat IL-6 cDNA. G3PDH was used as an internal control. PCR products were electrophoresed on 1.6% agrose gel and visualized by ethidium bromide staining. A. Photograph of a representative agarose gel. B. Densitometric analyzes of the ratio between IL-6 and G3PDH mRNA expression (calculated as arbitrary densitometric values obtained from IL-6 gene expression divided by G3PDH values in corresponding lanes). The data are presented as mean ± SE and compared by one-way ANOVA and Student-Newman-Keuls method. *P<0.05 versus WKY rats; #P<0.05 versus respective controls.

to cause hepatic insulin resistance following trauma-hemorrhage [41].

In this study, we have shown similar phases of glucose levels in WKY rats. Interestingly, even though the glucose levels were significantly increased in both groups at 2 hr after CLP as expected, these levels were increased to 67% in GK rats while WKY rats exhibited only an 18% increase. Additionally, while glucose levels decreased to below basal levels in WKY rats at 20 hr after CLP, these levels were still slightly higher than basal levels in the GK rats. This study also showed that plasma IL-6 and liver IL-6 mRNA expression were significantly increased in GK rats at 20 hr after CLP.

Similarly, plasma IL-10 was also significantly increased in septic GK rats. These data suggest that pronounced hyperglycemia during sepsis exacerbates inflammatory responses in GK rats.

The CLP model of sepsis which exhibits an initial hyperglycemia followed by euglycemia and subsequent hypoglycemia at 24 hr has been described in Sprague-Dawley (SD) rats [31]. The control rats used in this study are Wistar rats, which are the non-diabetic counterparts of the GK rats. To our knowledge, we are the first to report the glucose changes in these rats following a septic challenge. Even though the Wistar rats exhibited similar
phases of glucose levels as to SD rats, the changes were not as pronounced. Thus, it is conceivable that factors other than hyperglycemia play important roles in sepsis-induced inflammation and tissue injury. Nevertheless, the GK rats exhibited higher glucose levels at all time points studied which suggest that pronounced hyperglycemia as observed in the GK rats could stimulate inflammatory factors.

Significant increases in plasma IL-6 have shown to predict mortality in this CLP model of sepsis [42]. Elevated levels of IL-6 have also been observed in patients who became moribund [43], those with abdominal sepsis [44] or septic patients with renal failure [45]. Increased IL-6 in serum has also been associated with insulin resistance and IL-6 contributes to hyperglycemia through glucose release from hepatic glycogen stores [46,47]. In addition, the hepatic IL-6 gene expression was also significantly increased in GK rats at 20 hr after CLP. Other reports have shown high correlation between IL-6 expression in mice with CLP induced sepsis and survival, which mimics clinical patterns of sepsis [48,49]. Using the IL-6 deficient mice, Fattori et al. [50] demonstrated IL-6 as an essential mediator of the inflammatory response to localized inflammation. Thus, our study further confirmed that IL-6 is an important factor causing a poor outcome in septic GK rats.

To determine whether alterations in plasma endotoxin are responsible for the observed increase in inflammatory responses in septic GK rats, we also measured plasma endotoxin in both septic GK and WKY rats. Our results showed that the plasma endotoxin were not different between the two groups either at control state or at 20 hr after CLP. This suggests that the increase in IL-6 in septic GK rats is not directly due to any substantial increase in endotoxin levels. Our data is in agreement with previous observations that immune dysfunction in CLP induced sepsis is not caused by endotoxin alone but could also be due to the necrotic tissue and other microbial components [51-54].

To our knowledge, this is the first report which examined the inflammatory responses following sepsis in this rat model of Type 2 diabetes, the GK rats. We chose this model because at 3-4 weeks of age, these rats exhibit mild hyperglycemia and hyperinsulinemia. These rats also show insulin resistance, impaired insulin secretion, and abnormal glucose metabolism which are characteristics similar to humans with Type 2 diabetes. In contrast to other rodent models of Type 2 diabetes, these rats do not become obese and do not develop hyperlipidemia, the two confounding factors that influence Type 2 diabetes [25]. Therefore, the observed increase in sepsis-induced inflammatory response is indeed due to the diabetic phenotype of the GK rats rather than other factors such as obesity or the metabolic syndrome.

The mechanisms responsible for the enhanced inflammatory response in diabetic animals have not been elucidated. Endotoxemia has been used as a model of sepsis in obese rodent models of Type 2 diabetes such as the leptin deficient (ob/ob) and leptin receptor deficient (db/db) mice [55-58]. These studies showed that endotoxin induced mortality and organ injuries were greater in the ob/ob compared to the db/db mice. These studies suggest the ob/ob mice are more susceptible to endotoxin injury presumably due to the lack of leptin in these mice [55]. Altered hepatic macrophage function in obesity sensitizes
hepatocytes to endotoxin has also been implicated [56]. Another study showed equivocal metabolic response to endotoxemia in Zucker diabetic fatty rats (fa/fa) in comparison to its lean littermates (fa/+)[59]. A recent study demonstrated that diabetes exacerbates inflammatory response in cardiovascular tissues of endotoxemia induced db/db mice [60]. Another study showed that diabetes prolongs the inflammatory response to a bacterial stimulus through cytokine dysregulation [61]. Nevertheless, all prior data on endotoxemia in rodent models of Type 2 diabetes indeed had obesity as a confounding factor.

In the current study, we have demonstrated that sepsis-induced inflammatory responses are exacerbated in a non-obese rat model of Type 2 diabetes, the GK rats, suggesting that the observed increase in inflammatory response is indeed due to the diabetic phenotype. Further studies are warranted to understand the mechanism responsible for this enhanced inflammatory response in Type 2 diabetes.

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Address correspondence to: Ping Wang, MD, Division of Surgical Research, The Feinstein Institute for Medical Research, 350 Community Drive, Manhasset, NY 11030, Fax: (516) 562-1022, E-mail: pwang@nshs.edu.

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