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Tissue Factor and Toll Like Receptor (TLR)4 in Hyperglycemia-Hyperinsulinemia: Effects in Healthy Subjects, and Type 1 and Type 2 Diabetes Mellitus

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SUMMARY

Background—Diabetes mellitus (DM) patients have increased cardiovascular events. Blood tissue factor-procoagulant activity (TF-PCA), the initiating mechanism for blood coagulation, is elevated in DM. We have shown that hyperglycemia (HG), hyperinsulinemia (HI) and combined HG+HI (induced using 24 hr infusion clamps) increases TF-PCA in healthy and T2DM subjects, but not in T1DM subjects. The mechanisms for this are unknown. DM patients have elevated plasma lipopolysaccharide (LPS), a toll-like receptor (TLR) 4 ligand. We postulated that TLR4 plays a role in modulating TF levels.

Objectives and Methods—We studied the effect of HG+HI on TLR4 and TF-PCA *in vivo* during 24 hr HG+HI infusion clamps in healthy subjects, and T1DM and T2DM subjects, and *in vitro* in blood.

Results—*In vivo*, in healthy subjects, 24 hr HG + HI infusion increased TLR4 6-fold, which correlated with TF-PCA ($r = 0.91$, $p < 0.0001$). T2DM patients showed smaller increases in both. In T1DM subjects, TLR4 declined (50%, $p < 0.05$) and correlated with TF-PCA ($r = 0.55$; $p < 0.05$). *In vitro*, HG (200 mg/dl added glucose) and HI (1-100 nM added insulin) increased TF-PCA in healthy subjects (~2-fold, 2-4 hr). Insulin inhibited by ~30% LPS-induced increase in TF-PCA and high glucose reversed it. TLR4 levels paralleled TF-PCA ($r = 0.71$, $p < 0.0001$); HG and HI increased TLR4 and insulin inhibited LPS-induced TLR4 increase.

Conclusions—This is first evidence that even in healthy subjects, HG of short duration increases TLR4 and TF-PCA, key players in inflammation and thrombosis. TLR4-TF interplay is strikingly different in non-diabetic, T1DM and T2DM subjects.

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Disclosure of Conflict of Interest

The authors declare no conflict of interest.

Keywords

Diabetes mellitus; Hyperglycemia; Hyperinsulinemia; Tissue factor; Toll-like receptor-4

INTRODUCTION

Patients with diabetes mellitus (DM) have a 2–8-fold higher risk of cardiovascular morbidity and mortality compared with matched controls [1]. Patients with type 2 DM (T2DM) have hyperinsulinemia (HI) and hyperglycemia (HG), both independent risk factors for mortality. Hyperglycemia in hospitalized patients, with or without diabetes, is associated with increased risk for complications and mortality [2-5]. Numerous alterations have been described in patients with T2DM and type 1 diabetes mellitus (T1DM) in blood coagulation system and platelets [6-10], which indicate they are in a prothrombotic state.

Tissue factor (TF) is the primary physiological initiator of blood coagulation and thrombosis [8, 11, 12]. In addition to the TF present in the adventitia of blood vessels and in atherosclerotic plaques, which initiates blood coagulation when vessel walls are injured or plaques fissured [13], there is a circulating pool of TF in blood that is thrombogenic [8, 11, 14-16]. We and others have shown that patients with both T2DM and T1DM have elevated levels of whole-blood TF-PCA and are in a procoagulant state [17-19]. Our studies in healthy non-diabetic subjects using glucose and insulin infusion clamps showed that HG (~200 mg/dl), HI and combined HG+HI elevate whole blood TF-procoagulant activity (TF-PCA) by two, six and nine-fold, respectively [20]. This was associated with increased plasma factor VIII and thrombin-antithrombin complexes indicating there was thrombin generation *in vivo*. Further, we have reported that raising blood insulin levels, and especially raising blood glucose and insulin levels (HG +HI) together to levels frequently seen in diabetic patients, increased TF-PCA and thrombin generation in T2DM patients [18]. In striking contrast, in T1DM patients [19], HG+HI did not cause any increase in TF-PCA by 24 h, rather a decline was seen with the combination of HG and HI and with selective HG. These findings indicated that the mechanisms regulating the TFPCA and the effects of HG and HI in these three subject groups are distinct, and they are unknown.

Multiple studies highlight the link between TF, toll-like receptor (TLR)4, lipopolysaccharide (LPS) (a TLR4 ligand), and the role of TLR4 in regulating TF expression. Monocytes are a major source of TF in blood and LPS induces monocyte TF surface expression and procoagulant activity [12]. Serum LPS is elevated in both T1DM [21] and T2DM patients [22, 23], and the levels have correlated with insulin and triglyceride levels [23]. LPS stimulates monocytes and other cells via the TLR4, which is an evolutionarily preserved pattern-recognition receptor, expressed on several cell types, including monocytes and platelets [24-26]. Activation of the innate immune system via TLR is implicated in the pathogenesis of insulin resistance and inflammation in DM [27-29]. TLR4 and TLR2 expression is increased in insulin resistant target tissues, skeletal muscle and adipose tissue, of T2DM subjects [27]. Moreover, nutritional free fatty acids whose levels are increased in obesity and DM activate TLR4 signaling in macrophages and adipocytes providing a link between immunity and insulin resistance [29]. Of note, TLR4 gene Asp299Gly

polymorphism, which impairs inflammatory responses, is associated with a reduction in circulating C-reactive protein level and a decrease in the risk of angiographic coronary artery disease and clinical DM [30]. In vitro, TF production by LPS-stimulated monocytes is suppressed by insulin [31], similar to insulin's inhibition of platelet function [32], and monocytes from T2DM have impaired sensitivity to the inhibitory effects of insulin, resulting in enhanced TF production [31, 33].

To explore the potential role of TLR4 in regulating the effects of HG+HI on TF expression we studied TLR4 levels during HG+HI infusion clamps using samples from our studies in healthy nondiabetic subjects [20] and patients with T1DM and T2DM [18, 19]. In addition, we studied the effect *in vitro* of high glucose, high insulin and the combination on whole blood TF-PCA and TLR4 in healthy non-diabetic subjects in the presence and absence of LPS. Our studies document for the first time evidence that HG (~200 mg/dl) of relatively short duration (6 hr) in healthy subjects leads to a proinflammatory and prothrombotic state, with elevated TLR4 and TF-PCA. They provide new insights into changes in TLR4 and the relationship between TLR4 on TF-PCA, and on the strikingly differential effects in T1DM and T2DM patients.

MATERIALS AND METHODS

Materials

Insulin, D-glucose and LPS were obtained from Sigma Aldrich (St. Louis, MO). For the TF-PCA assay factor VIIa, factor X, pooled normal human plasma, phospholipids vesicles and Hemosil recombiplastin were obtained from American Diagnostica (Stamford, CT), Haematologic Technologies Inc. (Essex Junction, VT), George King Bio-Medical Inc. (Overland Park, KS), Avanti Polar Lipids (Alabaster, AL), and Instrumentation Laboratory Company (Lexington, MA) respectively. All the other reagents used were of analytical grade.

Methods

***In vivo* studies in healthy non-diabetic subjects and patients with T1DM and T2DM during hyperglycemic-hyperinsulinemic infusion clamps**—Whole blood TLR4 levels were measured in blood samples available from our previously published studies on TF-PCA using infusion clamps in healthy non-diabetic subjects [20], and patients with type T1DM [19] and T2DM [18]; the details regarding the subjects and the protocols have been described. The study protocols were approved by the Institutional Review Board of Temple University Hospital. Informed written consent was obtained from all subjects after explanation of the nature, purpose and potential risks.

Subjects

Healthy non-diabetic subjects—None of the subjects had a family history of DM or any other endocrine disorder and none were taking any medication. Basal TLR4 and TF-PCA were measured in 18 healthy subjects (mean age 37.3 ± 4.1 years; 10 males). Infusion clamps were performed on 9 subjects (6 males; mean age 37.6 ± 4.2 years); in addition to

basal samples, TLR4 was measured in 5 subjects at 6 hr and in 4 subjects at 24 hr, based on availability.

Patients with T1DM—Infusion clamps were performed in 6 subjects [19] (42.3 ± 6.8 years; 5 males; duration of DM 18 ± 8 years). Three subjects were studied over 6 hr; 3 were for 6 hr. In these subjects basal C-peptide levels were very low (0.25 ± 0.09 ng/ml) and did not respond to intravenous glucose. None had clinical signs of renal insufficiency and proliferative retinopathy. Six T1DM patients were on a basal/bolus insulin regimen consisting of insulin glargine in doses ranging from 15-70 units taken at night. Three patients took Novolog 70/30 mix (insulin aspartate-protamine/insulin aspartate) twice daily in doses ranging from 45-50 units. Only basal TLR4 and TF-PCA levels were measured in 11 T1DM patients (mean age 44.6 ± 5.1 ; 8 males).

Patients with T2DM—HG+HI infusion clamps were performed in 5 T2DM patients (mean age 49.2 ± 3.9 years, 4 males, duration of DM 7.5 ± 4 years) [18]. Only basal TLR4 and TF-PCA were measured in 17 T2DM patients (56.8 ± 3.4 years; 10 males).

Study Design: Combined elevation of glucose and insulin (High glucose/high insulin clamps)

Healthy, non diabetic subjects—The infusion clamps studies began at ~8AM after an overnight fast, as described [20]. The purpose of these studies was to examine effects of raising both blood glucose and insulin levels. A 20% glucose solution was infused intravenously at variable rates adjusted to maintain plasma glucose at ~200 mg/dl (~11 mmol/l). Serum insulin rose (~1000 pmol/L) in response to the HG [20]. Small blood samples (0.25 ml) were collected every 30-60 min initially and every 1-2 hr later for measurement of glucose concentrations. Subjects were fasting but were allowed to drink water ad lib. Plasma electrolytes were monitored every 6 hr, body weight every 12 hr and fluid balances every 6 hr. Potassium (20 mg) and magnesium (400 mg) were given PO every 12 hr. Blood samples were obtained at baseline and at 6 and 24 hr.

Type 1 diabetes mellitus patients—Study participants came to the CRC after an overnight fast. They had their last meal and insulin injection at ~8 PM. At ~8AM the next day, blood samples were collected and a 20% glucose solution was infused IV at variable rates, which were adjusted to maintain plasma glucose ~200-300 mg/dl (~11-16 mmol/l) [19]. Insulin was infused at a rate of 1 mU/kg min. The insulin levels were ~100 U/ml (~600 pmol/L) [19]. Blood glucose concentrations and electrolyte and fluid balances were monitored as described [19]. HG-HI clamps were continued for 6 h in 6 patients and for 24 h in 3 patients.

Type 2 Diabetes mellitus patients—A 20% glucose solution was infused intravenously at variable rates, adjusted to maintain plasma glucose at ~200 mg/dl (~11 mmol/l) [18]. Blood samples (0.25 ml) were collected every 30-60 min initially and every 1-2 h later for measurement of blood glucose. Serum insulin levels rose from 148 ± 49 pmol/L at 0 h to a mean of 390 ± 139 pmol/L over 24 h.

Effects of hyperglycemia and hyperinsulinemia in vitro on TF-PCA and TLR4

Blood was obtained from 5 healthy nondiabetic subjects (mean age 31.2 ± 6.1 years; 4 males) who were not on any medications. They were recruited from the staff at Temple University Health Sciences Center. All subjects provided an informed consent and the studies were approved by the Institutional Review Board of Temple University.

Whole blood (105 ml) was drawn into one-tenth volume of 3.8% sodium citrate by venipuncture from healthy, medication-free volunteers ($n=5$). Aliquots of blood (7 ml) were incubated at 37°C for 4 hr with added 200 mg/dL glucose alone, insulin alone (1 nM, 10 nM or 100 nM), or combinations of glucose and insulin, in the presence and absence of LPS (1 $\mu\text{g/ml}$) [31]. Aliquots (1.5 ml) of blood were centrifuged for 20 min at 1590 g to obtain cell-free plasma for glucose and insulin measurements. Aliquots (1 ml) of whole blood were frozen at baseline (0 hr). Remaining blood was incubated at 37°C and aliquots (1 ml - 1.5 ml) were removed at 2 and 4 hr for TF-PCA and TLR4 and for harvesting plasma. Samples were stored at -70°C until assayed.

Assays

Whole-blood tissue factor-procoagulant activity (TF-PCA) assay—This was measured as described previously [15, 20]. Blood lysates and cellular components were collected from aliquots of whole blood subjected to three cycles of freezing and thawing followed by centrifugation and washing, and they were finally suspended in HBSA buffer (10 mmol/l HEPES, 137 mmol/l NaCl, 5.38 mmol/l KCl, 5.55 mmol/l glucose, and 0.1% bovine serum albumin, pH 7.5) [15]. TF-PCA was measured in whole-blood cell lysates with a two-stage clotting assay using recombinant factor VIIa, factor X, and normal human plasma containing phospholipid vesicles [15]. This assay measures cell-bound and microparticle-associated TF in lysed whole blood. Recombinant human TF (Hemosil Recombiplastin) was used as a standard. The clotting times were measured using a Coagulometer (Amelung KC-4, Trinity Biotech). This assay has been validated by Key et al (15) and by us to show that it is specific for TF; the activity is entirely dependent on the addition of FVIIa and is inhibited ~95% by an anti-TF antibody. Moreover, the activity is highly inducible by LPS (Fig 5) (15), which further supports that TF is being measured. The TF-PCA levels during infusion clamps are from our published studies [18-20].

Other Assays—TLR4 levels were measured in the same samples as used for TF-PCA using an ELISA (USCN Life Science, Inc., Wuhan, China); the whole-blood lysates were solubilized by overnight incubation with 0.1% Triton X-100. Plasma glucose was measured with a glucose analyzer using the glucose oxidase method and serum insulin by RIA (Linco Research, St. Charles, MO).

Statistical analysis—Comparisons of the values obtained within each group at different time points were performed using the Student's *t* test. Statistical significance was defined as $p < 0.05$. All results are presented as means $\pm$ SE. The relationships between variables were assessed using Pearson correlation coefficients.

RESULTS

Basal TLR4 levels in non-diabetic and diabetic subjects

Basal TLR4 levels in T2DM patients (0.81 ± 0.08 ng/ml) were markedly decreased to ~35% of those in healthy subjects (2.23 ± 0.30 ng/ml, $p < 0.001$) (Fig 1A). The levels were lower than in T1DM subjects (3.47 ± 0.98 ng/ml, $p < 0.05$). The corresponding basal TF-PCA levels in the same subjects are shown in Fig 1B.

In vivo studies on TLR4 levels during hyperglycemia-hyperinsulinemia clamps

We measured TLR4 levels in samples obtained from our studies in healthy subjects and DM patients during combined HG + HI infusion clamps [18-20]. These studies showed marked differences in the TLR4 responses between the 3 groups. In non-diabetic healthy subjects there was a striking elevation in TLR4 with ~2-fold increase by 6 hr and 6-fold by 24 hr (Fig 2A). T2DM patients started with markedly lower basal levels as compared to non-diabetic and T1DM subjects and showed a 25% increase over basal levels. The levels in T2DM at baseline and 24 hr were 0.67 ± 0.05 ng/ml and 0.84 ± 0.07 ng/ml, respectively ($p < 0.05$) (Fig 2A). In contrast, in T1DM subjects, TLR4 declined from 3.33 ± 1.36 ng/ml at baseline to 2.24 ± 1.30 ng/ml ($p = 0.03$, $n = 6$) at 6 hr and to 1.18 ± 0.10 at 24 hr ($p = 0.02$, $n = 3$). Thus, there was a striking elevation in TLR4 in non-diabetic subjects, a decrease in T1DM subjects, and a small increase (25%) in T2DM patients.

The pattern of changes in TF-PCA (Fig 2B) and TLR4 (Fig 2A) following HG+HI clamps were identical both in non-diabetic subjects and in T1DM with increases over 24 hr in the former group and a decline in the latter. There was a good correlation between TLR4 and TF-PCA levels (T1DM, $r = 0.55$, $p = 0.03$; nondiabetic controls, $r = 0.91$, $p < 0.0001$) (Fig 3), even though TFPCA and TLR4 levels increased in nondiabetic subjects and declined in T1DM. In T2DM patients, TFPCA levels rose with HG+HI by ~30% over already elevated basal levels – this was associated with a 25% increase in TLR4 (Fig 2). The correlation between TLR4 and TF-PCA ($r = 0.43$, $p = \text{NS}$) was not significant.

We explored the relationship between TLR4 and insulin and glucose levels during HG+HI clamps (0, 6, 24 hr) in the 3 groups (Table 1). The relationship between TLR4 and insulin in these three groups was distinctly different. In healthy non-diabetic subjects TLR4 levels were positively correlated with insulin levels ($r = 0.60$, $p < 0.01$); in T1DM there was an inverse relationship ($r = -0.68$; $p = 0.01$, with exclusion of one subject with extremely high TLR4 levels at 0 and 6 hr). In T2DM there was no relationship observed ($r = 0.07$, $p = \text{NS}$). TLR4 levels were positively related to glucose levels in non-diabetic subjects ($r = 0.73$; $p < 0.001$). No relationship was noted in T1DM or T2DM subjects.

Effect *in vitro* of high glucose and high insulin on TF-PCA in healthy non-diabetic subjects

To better understand the effects of high glucose and high insulin on TF-PCA and TLR4 we performed studies *in vitro* in the presence and absence of LPS. The mean basal glucose and insulin levels prior to addition of exogenous glucose or insulin were $75 \text{ mg/dL} \pm 4.2$ (mean $\pm$ SE, $n = 3$) and $99 \pm 24.4 \text{ pmol/L}$, respectively. With added 200 mg/dL glucose, TF-PCA increased by ~2-fold (Fig 4A) at 2 hr and by ~2.5-fold by 4 hr (Fig 4B). Addition of insulin,

1, 10 or 100 nM increased TF-PCA by 2-fold at 2 hr and 2-4 fold by 4 hr. Thus, with added glucose or insulin, there was an increase in TF-PCA. The combination of high glucose (200 mg/dL) with insulin (1, 10 or 100 nM) increased TF-PCA at 2 hr by 2-3 fold, and by 3-4 fold at 4 hr. In general, the highest levels were with the combination of HG and HI. TF-PCA level with high glucose plus high insulin (100 nM) was significantly higher than with glucose alone at both 2 hr (69.6 ± 10.8 vs 45 ± 6.7 , $p=0.02$) and 4 hr (104.0 ± 18.5 vs 62 ± 10 , $p=0.01$). At 2 hr TF-PCA with the HG+HI combination was higher than with respective insulin concentration alone (Fig 4A). At 4 hr, it was significantly higher in combination with 10 nM insulin (Fig 4B).

Effect *in vitro* of high glucose and high insulin on TF-PCA in the presence of LPS

LPS (1 $\mu\text{g/ml}$) alone markedly increased the mean TF-PCA by 32 fold at 2 hr and by 25-fold at 4 hr (Figure 5C and D). With added glucose TF-PCA was higher than with LPS alone but the difference was not statistically significant. However, added insulin inhibited LPS-induced increase in TF-PCA levels. Insulin 10 nM inhibited the levels by ~35% ($p=0.04$) at 2 hr and 30% ($p=0.01$) by 4 hr. This was in contrast to that observed in the absence of LPS, where insulin by itself increased TF-PCA (Fig 4A). Most strikingly, with the combination of LPS, high glucose and high insulin (1 nM or 100 nM) there was a marked and further increase by ~3 fold in TF-PCA over the already high levels with LPS alone. These elevated TF-PCA levels were higher than with LPS plus glucose and LPS plus insulin (Fig 4C and D). Thus, glucose reversed the inhibitory effect of insulin and enhanced the levels even higher.

Effect *in vitro* of high glucose and high insulin on TLR4

TLR4 levels were measured at 4 hr. In general, the effects of glucose and insulin on TLR4 levels (Fig 5A) appeared to parallel the TF-PCA levels (Fig 4) and there was a strong correlation between TF-PCA and TLR4 ($r=0.71$, $p<0.0001$) (Fig 5B). In the absence of LPS, there was a ~4-5 fold increase in TLR4 with added high glucose and with added high insulin (1 nM) (Fig 5A). Combined high glucose plus high insulin increased TLR4 over basal, but not over that with added glucose or insulin alone. LPS caused a ~10- fold increase in TLR4 over basal (Fig 5A). In presence of LPS, added glucose did not further increase TLR4. However, insulin (1 and 100 nM) inhibited LPS-induced TLR4 by ~65%. The highest TLR4 levels were noted with the combination of LPS, high glucose and high insulin suggesting that high glucose overrides the inhibitory effect of insulin on LPS-stimulated TLR4.

Discussion

The combination of our *in vivo* studies following infusion of HG and HI in healthy non-diabetic subjects and DM patients and the *in vitro* studies provides novel insights into the inter-play between TF-PCA and TLR4. Our *in vivo* infusion studies provide the novel and important evidence that HG of a relatively short duration (6 hr), even in healthy non-diabetic subjects, induces a striking increase in TLR4 and TF-PCA, both key players in inflammation, thrombosis and atherosclerosis [24-26, 34]. Our previous studies have shown that the TF-PCA elevation induced by HG+HI is associated with a strong evidence of an *in*

vivo procoagulant state with elevation in plasma TAT complexes and factor VIII, increased expression of CD40 ligand on platelets and TF on monocytes, and increased circulating platelet-monocyte aggregates [20, 35]. High glucose *in vitro* has been shown to induce TLR expression in human monocytes [36]. Our previous studies [18-20] involving combined HG + HI infusion clamps showed striking differences between the 3 subject groups studied with respect to TF-PCA, and now we provide new evidence of a parallel effect on TLR4 (Fig 2). Nondiabetic healthy subjects showed marked increases in TF-PCA and TLR4 levels (Fig 2) with a good correlation between the two (Fig 3A). Previous studies have established that activation of monocyte TLR4 leads to downstream activation of NF- κ B and TF production [24-26]. Our findings, therefore, are consistent with an *in vivo* regulatory effect of TLR4 on TF-PCA expression and as the basis for downstream TF levels.

In vitro, both HG and HI individually increased TF-PCA in blood from healthy subjects with a further increase with combined HG+HI (Fig 4A, 4B). However, insulin inhibited by ~30% (Fig 4) LPS-induced marked increase in TF-PCA, which was in contrast to an increase with insulin alone (Fig 4A). More importantly, in the presence of LPS, the combination of HG + HI induced the highest TF-PCA levels (Figs 4C, 4D) indicating that HG overrides the insulin inhibition and increases levels further. The changes in TLR4 levels paralleled and strongly correlated with TF-PCA (Fig 4). HG and HI alone induced 4-5-fold increases over basal TLR4 levels. As with TF-PCA, insulin alone inhibited (~65%) LPS-induced marked increase in TLR4, and added HG reversed this inhibition and further increased TLR4 levels (Fig 4A). These parallel changes in TF-PCA and TLR4 are in line with the established role of TLR4 in regulating TF expression on activation by ligands including LPS [24-26]. Our findings are consistent with the paradigm that insulin inhibits TLR4 and TF-PCA while high glucose stimulates their expression, and that this is influenced by LPS levels, which are known to be increased in T1DM and T2DM [21-23].

HG-induced upregulation of TLR4 is also relevant in processes beyond recognition of pathogen associated molecular patterns (PAMPs), innate immunity and cytokine release [24]. TLR4 is activated by host-derived molecules and is implicated in pathogenetic effects. Free fatty acids activate TLR4 on macrophages, adipocytes and liver to induce pro-inflammatory cytokine expression and insulin resistance [29], and this is particularly relevant in DM. TLR4 downregulation is associated with a decrease in inflammation and clinical DM [30]. Hypercholesterolemia-induced prothrombotic state and monocyte TF expression is driven by engagement of TLR4/TLR6 complex by oxidized low-density lipoproteins [37]. Moreover, TLR4 is implicated in cardiac ischemia and failure [38]. There is an increase in TLR4 in myocardial tissue of diabetic mice as early as 3 days after appearance of hyperglycemia [39]. HG-induced myocardial apoptosis was prevented by silencing of TLR4 gene expression [39]. These studies reflect on the significance of HG-induced elevation of TLR4.

In our studies, the effects of combined HG + HI on TLR4 and TF-PCA are strikingly different between T1DM and T2DM (Fig 2 and 3). In T2DM patients, TLR4 levels started low and showed a small (~25%) increase by 24 hr, as also noted with TF-PCA (Fig 2). We postulate that the lack of a marked increase in TLR4 in T2DM patients (as was noted in healthy subjects) is due to an inhibitory effect of chronic HI. In contrast to nondiabetic and

T2DM subjects, T1DM subjects showed a decline in TLR4 at 6 and 24 hr, which was accompanied by a decrease in TF-PCA (Fig 2,3). Moreover, there was an inverse relationship between insulin and TLR4 levels (Table 1) consistent with an inhibitory effect of insulin.

We found that the basal TLR4 levels were distinctly different between the three groups. They were lower in T2DM patients than in T1DM and non-diabetic individuals (Fig 1). The two subjects with the highest TLR4 levels (>9.5 ng/ml) had T1DM suggesting that TLR4 levels may be elevated in T1DM patients, as noted previously in monocytes from T1DM patients [40]. High glucose levels are reported to increase monocytes TLR4 levels *in vitro* [36]. Because, in general, T1DM subjects have HG but not HI, the normal or possibly elevated TLR4 levels may reflect a lack of chronic inhibitory effect of HI. Insulin has been shown to decrease monocyte TLR4 mRNA levels in T2DM [41], and based on our *in vitro* studies (Fig 4C, 4D), we postulate that chronic HI present in T2DM decreases basal TLR4 levels. Low TLR4 levels have been observed in tissue from skin ulcers in T2DM patients [42]. In T2DM the basal TF-PCA was elevated (Fig 2) and this may be related to a stimulatory effect via pathways other than TLR4, such as through the advanced glycation end products (AGEs) and oxidized lipoproteins, which can stimulate TF-PCA via receptor for AGE (RAGE) and CD36, respectively [43, 44] (Fig 2).

TLR4 levels were measured in our studies in whole blood lysates, which includes membranes from blood cells, including monocytes and platelets, both of which express TLR4. Both TF-PCA and TLR4 are cell surface membrane proteins and were measured in the same membrane samples, which supports our conclusions regarding their expression and relationship. Moreover, we have previously shown that HG+HI associated increase in whole blood TF-PCA is accompanied by a parallel increase in TF surface expression by flow cytometry and in monocyte TF mRNA [20, 35].

Overall, our findings document a hitherto unrecognized proinflammatory and prothrombotic state, with elevated TLR4 and TF-PCA, induced by HG of a relatively short duration even in healthy subjects. Our studies are consistent with the paradigm that high glucose elevates and insulin inhibits TLR4 and TF-PCA with a potential effect of endogenous LPS, a TLR4 ligand. They document marked differences in the circulating levels and on the effect of HG and HI on TLR4 and TF-PCA in healthy and T1DM and T2DM subjects, and provide a cogent explanation for the strikingly different effects on TF-PCA we have observed [18-20] in these subjects.

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What is known on this topic

- Diabetes mellitus (DM) patients are recognized to be in a prothrombotic state with an increased incidence of cardiovascular events.
- Blood tissue factor-procoagulant activity (TF-PCA), the initiating mechanism for blood coagulation, is elevated in DM, along with elevated plasma lipopolysaccharide (LPS), a ligand for the toll-like receptor (TLR)4.

What this paper adds

- In healthy nondiabetic subjects, 24 hr of hyperglycemia – hyperinsulinemia induced by glucose infusion results in a 6-fold increase in TLR4, which correlates with TF-procoagulant activity.
- This is the first evidence that even in healthy subjects, hyperglycemia of short duration increases TLR4 and TF-PCA, key players in inflammation and thrombosis.
- TLR4-TF interplay is strikingly different in healthy non-diabetic subjects and patients with T1DM and T2DM.

Figure 1. Basal TLR4 (A) and TF-PCA (B) levels in healthy nondiabetic subjects, and patients with type 1 and type 2 diabetes mellitus

Figure 2. TLR4 (left panel) and TF-PCA (right panel) in whole blood samples during combined hyperglycemia and hyperinsulinemia clamps in healthy nondiabetic subjects, and patients with type 1 and type 2 diabetes mellitus

Shown are results in 9 nondiabetic subjects (squares), with levels at 6 hr in 5 subjects and at 24 hr in 4 subjects; in 6 T1DM subjects (circles) with levels in 3 subjects at 0, 6 and 24 hr and 6 subjects at 0 and 6 hr; and in 5 T2DM subjects (diamonds) with levels at 0, 6 and 24 hr in all subjects. TF-PCA results are from our previously published studies in non-diabetic subjects [20], T1DM [19] and T2DM [18]. * $p < 0.05$ compared to 0 hr; # $p < 0.05$ compared to 6 hr.

Figure 3. Correlation between TF-PCA and TLR4 levels in healthy non-diabetic subjects, and patients with T1DM

Shown are levels obtained before and during hyperglycemia/hyperinsulinemia infusion clamps in 9 healthy subjects and 5 T1DM patients and the Pearson correlation coefficients.

Figure 4. Effect of hyperglycemia and hyperinsulinemia on whole blood TF-PCA in the absence (A, B) and presence of lipopolysaccharide (LPS) (C, D) at 2 hr (A, C) and 4 hr (B, D)

Whole blood was incubated with added glucose (200 mg/dl), insulin (1, 10, or 100 nM), alone or in combination for up to 4 hr, in the presence or absence of added LPS (1 µg/ml).

Aliquots were removed at baseline, 2 and 4 hr for measurement of TF-PCA. Each experiment was performed using blood from 5 health subjects.

Figure 4A and 4B: * p < 0.05 compared to baseline (control), ** p < 0.05 compared to added glucose alone.

Figure 4C and 4D: * p < 0.05 compared to LPS alone; and ** p < 0.05 compared to LPS plus glucose.

Figure 5.

A. Effect of hyperglycemia and hyperinsulinemia on whole blood TLR4 in the absence and presence of LPS. Whole blood was incubated with added glucose (200 mg/dl), insulin (1 or 100 nM), alone or in combination for 4 hr. This was performed in the presence or absence of added LPS (1 μ g/ml). Aliquots were removed at baseline and 4 hr for measurement of TLR4. Each experiment was performed using blood from 5 health subjects. * $p < 0.05$ compared to control (basal).

Figure 5B. Correlation between TF-PCA and TLR4 levels. Shown are the TF-PCA and TLR4 levels at baseline and 4 hr in samples from experiments shown in Fig 5A, and the Pearson correlation coefficient.

Table 1

Relationship between TLR4 and insulin and between TLR4 and glucose levels in 9 non-diabetic subjects, 5 T2DM patients and 6 T1DM patients before and during HG+HI infusion clamps.

TLR4	vs Glucose r (p)	vs Insulin r (p)
Non-diabetic healthy subjects	0.73 (<0.001)	0.60 (<0.01)
Type 2 diabetes mellitus patients	0.22 (0.44)	0.07 (0.8)
Type 1 diabetes mellitus patients	−0.21 (0.44)	−0.68 (0.01)

Shown are the Pearson correlation coefficients and p values.