The Acute Modulation of Toll-Like Receptors by Insulin

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**Background:** Low dose insulin infusion has been shown to exert a prompt and powerful anti-inflammatory effect. Toll-like receptors (TLRs) are major determinants of the inflammatory response to viral and bacterial pathogens.

**Hypothesis:** We have now hypothesized that low dose insulin infusion in obese type 2 diabetic patients (T2DM) suppresses TLRs expression.

**Methods:** Ten T2DM were infused with a low dose of insulin (2 Units/hr) and dextrose to maintain normoglycemia for 4 hours while another 14 T2DM were infused with either dextrose or saline for 4 hours and served as controls. Blood samples were collected before and at 2, 4 and 6 hours TLRs expression was determined in MNC.

**Results:** Insulin infusion significantly suppressed TLR1, 2, 4, 7 and 9 mRNA expression in MNC within 2 hours of the infusion with a maximum fall at 4 hours by 24±9%, 21±5%, 30±8%, 28±5% and 27±10%, (P<0.05, for all) respectively, below the baseline. TLR2 protein was suppressed by 19±7% (P< 0.05) below the baseline at 4 hours. The DNA binding of PU.1, a major transcription factor regulating many TLR genes was concomitantly suppressed by 24±10% (P<0.05) by 4 hours in the MNC. There was no change in TLRs expression or DNA binding by PU.1 following dextrose or saline infusion in the control groups.

**Conclusion:** Insulin suppresses the expression of several TLRs, at the transcriptional level possibly through its suppressive effect on PU.1.
We have shown previously that insulin exerts a prompt and powerful anti-inflammatory effect, including the suppression of Nuclear Factor-κB (NFκB) binding, reactive oxygen species (ROS) generation and p47phox expression by mononuclear cells (MNC) (1;2) in the human. Insulin has also been shown to reduce the plasma concentrations of many pro-inflammatory mediators including C-Reactive protein (CRP) and serum amyloid A (SAA) within 24 hours in patients with acute myocardial infarction (AMI) and in patients undergoing coronary artery bypass surgery (3;4).

Toll like receptors (TLR) are a variety of pathogen pattern recognition receptors (PRR) which recognize bacterial and viral products and other pathogens (5). TLR4 recognizes endotoxin or lipopolysaccharide (LPS) and may be a mediator of endotoxin shock(6). TLR4 has also been shown to play an important role in the pathogenesis of atherosclerosis (7;8), diet-induced obesity and the related insulin resistance (9;10). TLR2 (in a heterodimeric association with TLR1 or TLR6) recognizes certain lipopeptides, peptidoglycans and other lipid moieties derived from Gram positive bacteria (5). Recently, TLR2 has been shown to mediate and aggravate myocardial tissue injury in ischemia-reperfusion based experimental animal models and that a deletion of TLR2 is associated with a reduction in the size of the experimental myocardial infarct (11). TLR2 deletion also results in the preservation of post-ischemic coronary endothelial function and the prevention of abnormal ventricular remodeling (12;13).

TLR7 and TLR9 recognize single stranded viral RNA (ssRNA) and microbial DNA respectively. TLR9 also modulates adaptive immune responses including autoimmunity against DNA and chromatin, e.g., systemic lupus erythematosus (SLE)(14). A role for TLR9 and TLR2 has also been shown in the pathogenesis of experimental T1DM, specifically related to the autoimmune inflammation in the pancreatic islet (15;16).

Transcription of many TLRs is dependent upon myeloid specific transcription factors including PU.1. PU.1 is a member of the ets gene family and is a master switch in the regulation of an array of genes involved in myeloid cells activation and differentiation including TLR2, 4 and 9 (17;18). PU.1 binds to purine rich regions of the TLRs gene promoter to activate their transcription.

Recent data in the human has demonstrated that TLR2 and TLR4 expression and plasma LPS concentration are increased in patients with type 2 diabetes and that LPS concentration is related to plasma insulin concentration and insulin resistance (19). However, the possibility that insulin or macronutrient intake may modulate TLR expression has not been investigated.

In view of the recent data on TLR involvement in insulin resistance and atherosclerosis and our previous work on the anti-inflammatory effect of insulin, it is important that the potential effects of insulin on the TLRs be investigated.

METHODS

Subjects: Ten obese patients with type 2 diabetes (T2DM), 5 females and 5 males, were recruited for the insulin infusion study (Age: 47.9±8.9 years; BMI: 39.2±6.5 kg/m² and HbA1c: 7.0±0.8%). The subjects were well -controlled diabetics with average HbA1c 7±0.81. They were on stable oral anti-diabetic medications. All patients were on metformin (1-2g/day) and 6 patients were on sulfonylureas (glyburide or glipizide 5-10mg/day). None of the subjects was on insulin or thiazolidenedione therapy or taking any antioxidant or nonsteroidal anti-inflammatory drugs (NSAIDs). After an overnight fast, subjects
were Infused with Insulin (2 unit/hour) with 5% glucose and 20 mEq of potassium chloride for 4 hours followed by 2 hrs of observation and wash out. The glucose infusion rate was titrated to maintain blood glucose at a target level of 80-130 mg/dL. Blood glucose levels were measured every 15 minutes. None of the patients had any hypoglycemic symptoms. Another 2 groups of T2DM patients, were infused with either 5% glucose (8 patients, 4 females and 4 males, age: 45.8±7.6 years; BMI 38.6±7.2 kg/m² and HbA1c: 7.3±0.9%) or normal saline (6 patients, 4 females and 2 males, age: 41.5±8.2 years, BMI 36.9±6.7 kg/m², HbA1c of 7.5±1.1%) alone at a rate of 100 ml/hour for 4 hours and served as controls. Blood samples were collected at baseline, 2, 4 and 6hrs. The protocol was approved by the Human Research Committee of the State University of New York at Buffalo. An informed consent was signed by all subjects.

**MNC isolation:** Blood samples were collected in Na-EDTA and carefully layered on Lympholyte medium (Cedarlane Laboratories, Hornby, ON). Samples were centrifuged and two bands separate out at the top of the RBC pellet. The MNC band was harvested and washed twice with Hank's balanced salt solution (HBSS). This method provides yields greater than 95% MNC preparation.

**Quantification of TLRs (1, 2, 4, 7 and 9) mRNA in MNC by RT-PCR:** Total RNA was isolated using commercially available RNAqueous®-4PCR Kit (Ambion, Austin, TX). Real Time RT-PCR was performed using Cepheid Smart Cycler (Sunnyvale, CA), Sybergreen Master mix (Qiagen, CA) and gene specific primers for TLRs (1,2,4,7 and 9) (Life Technologies, MD). All values were normalized to the expression of a group of housekeeping genes including actin, ubiquitin C and cyclophilin A.

**PU.1 DNA binding activity:** Nuclear PU.1 DNA binding activity was measured by electromobility shift assay (EMSA). Nuclear extract was prepared from MNC and by high salt extraction as described before (1). PU.1 assay was performed using specific binding site oligonucleotide corresponding to the PU.1 binding sites on TLR4 promoter (18): sense: `CGCTTTCACTTCTCTTCACCCTT` and anti-sense: `AAGGGTGAGAGGAAGTGAAAGCG`. The specificity of the band was confirmed by supershifting the band with specific antibody against PU.1 (Santa Cruz Biotechnology, CA) and by competition with cold oligonucleotides. PU.1 DNA binding was adjusted to Oct-1 DNA binding.

**Western blotting:** MNC total cell lysates were prepared as electrophoresis and immunoblotting were carried as described before (1). Monoclonal antibodies against TLR2, TLR4 (Abcam, Cambridge, MA) and actin (Santa Cruz Biotechnology, CA) were used and all values were corrected for loading to actin.

**Plasma measurements:** Glucose concentrations were measured in plasma by YSI 2300 STAT Plus glucose analyzer (Yellow Springs, Ohio). ELISA was used to measure plasma concentrations of insulin (Diagnostic Systems Laboratories Inc., Webster, TX), MCP-1 and s-ICAM-1 (R&D Systems, MN).

**Statistical analysis.** Statistical analysis was conducted using SigmaStat software (SPSS Inc., Chicago, IL). All data are represented as mean ± S.E. Statistical Analysis from baselines was carried out using Holm-Sidak one-way repeated measures analysis of variance (RMANOVA). Dunnett’s two-factor RMANOVA method was used for multiple comparisons between different groups.

**RESULTS**

Insulin and glucose concentrations during infusions: Plasma insulin concentration increased by 166% over the
baseline (from 20.9±10.9 μU/ml to 50.5±22.4 μU/ml, \(P<0.001\)) during the insulin infusion while it fell slightly in the dextrose groups from 27.6±5.6 μU/ml to 22.9±6.5 μU/ml at 4 hrs (NS) and in the normal saline group from 20.6±5.5 μU/ml to 17.9±4.7 μU/ml at 4 hrs (NS). The mean blood glucose concentrations changed from 122±15mg/dL at baseline to 111±10mg/dL at 4 hrs following insulin infusion which was not significantly different from that observed in the control groups (133±40mg/dL at baseline to 125±29mg/dL at 4 hours in the dextrose group and from 135±13mg/dL at baseline to 109±13mg/dL at 4 hours in the saline group).

**Suppressive effect of Insulin on TLR mRNA and protein expression in DM patients.** Insulin infusion suppressed the mRNA expression of TLR1, 2, 4, 7 and 9 in MNC within 2 hours with a maximum fall at 4 hours by 24±9%, 21±5%, 30±8%, 28±5% and 27±10% respectively below the baseline (\(P<0.05\) for all Figure.1). The expression of mRNA for all five TLRs reverted to baseline 2 hours after the end of the infusion. There was no significant change in mRNA expression of TLR6 and TLR8 (data not shown). TLR2 protein was suppressed by 19±7% below the baseline at 4 hrs following insulin infusion (\(P<0.05\)) when compared to baseline and to controls (Figure 2) while TLR4 protein levels were reduced by only 8±5% (NS). There was no significant change in TLR expression in the control group. We were unable to detect the other TLRs proteins possibly due to the lower expression levels of these genes in the MNC and the relatively low sensitivity of the western blotting technique.

MyD88 and CD14 are essential proteins involved in TLR signal transduction and LPS binding, therefore their expression following insulin infusion was also investigated. MyD88 and CD14 expression was not significantly altered following insulin infusion (data not shown).

**Effect of insulin infusion on PU.1.** The binding of PU.1 to a specific and necessary motif (PU-12) on the TLR4 promoter in MNC was suppressed by 24±10% at 4 hrs following insulin infusion (\(P<0.05\)) compared to baseline and controls (Figure 3). This suppression did not revert to baseline at 6 hrs.

**Effect of insulin infusion on Pro-inflammatory Mediators.** Following insulin infusion, plasma concentrations of MCP-1 fell significantly at 2 hrs and continued to fall at 4 hrs by 15±4% below the baseline (from 270±43 to 228±32ng/ml, \(P=0.026\), RMANOVA). Plasma concentrations of sICAM-1 were also suppressed significantly by 4 hrs following insulin infusion by 10±3% below the baseline (from 301±41 to 263±24ng/ml, \(P=0.017\), RMANOVA). The fall in MCP-1 and sICAM-1 concentrations was also significant (\(P<0.05\)) when compared to control group by 2-way RMANOVA.

**DISCUSSION**

Our data clearly show for the first time a potent and rapid suppressive effect of a low dose of insulin infusion on the expression of TLR1, 2, 4, 7 and 9 by 20-30%, evident at 2 hours, continuing till 4 hours and reverting back to baseline 2 hours after the cessation of infusion. This low dose infusion of insulin has previously been shown to exert a rapid and profound anti-inflammatory effect within 2h as reflected in the suppression of NF B binding and an increase in I B expression. The fall in TLR2 mRNA was also associated with a reduction in the expression of TLR2 protein. The absence of a clear suppression of TLR4 protein level may be due to the relatively short period of infusion. This issue should be addressed in future by longer periods of infusion. Our data also demonstrate clearly for the first time that insulin rapidly suppresses the DNA binding of PU.1 to a specific sequence of TLR4 gene promoter. PU.1 is a key transcription factor in the
regulation of TLR transcription and thus its suppression is reflected in the suppression of many of TLRs to a similar extent. The pattern of fall in TLRs mRNA expression was similar to that observed with other pro-inflammatory mediators like MCP-1 and sICAM-1 which fell at 2 hrs, remained low for the duration of the infusion and returned to the baseline at 6hrs, 2 hrs after the cessation of the insulin infusion. This is also consistent with our previous data on the suppressive effect of insulin on other indices of inflammation. Thus, there is a remarkable consistency in the pharmacodynamics of the various aspects of the anti-inflammatory effects of insulin.

Recent work has shown that TLR4, and possibly TLR2, might mediate diet induced obesity and insulin resistance and might, therefore, be involved in the pathogenesis of type 2 diabetes mellitus. It has been shown that TLR4−/− mice are protected from high fat diet induced insulin resistance (9;10). Also, RNA interference-mediated inhibition of TLR2 expression in muscle cells inhibited palmitate-induced insulin resistance (20). TLR2 and TLR4 expression is increased in adipose tissue of type 2 diabetics (19) and in the MNC of type 1 diabetics (21). The expression of TLR-4 in the MNC in type 1 diabetics is related significantly to HbA1c and the incubation of MNC with glucose increases TLR4 expression (21). LPS concentration is also higher in type 2 diabetics and related to plasma insulin concentration and insulin resistance (19). These facts support a role of the TLR pathway in the pathogenesis of type 1 and type 2 diabetes and the pathogenesis of diabetic complications. This is consistent with the observations that inflammatory mediators interfere with insulin signaling and might play a significant role in the development of insulin resistance. Thus, the suppression of TLR4 and TLR2 expression following a low dose insulin infusion points towards a potential suppressive role for insulin on insulin resistance and atherosclerosis. Furthermore, since TLR9 is also involved in the pathogenesis of auto-immune mechanisms related to type 1 diabetes in experimental animals and TLR2 is involved in beta cell death, the suppression of TLR9 and TLR2 may potentially be relevant to the prevention of type 1 diabetes (15;16).

PU.1 is the transcription factor which binds to a purine rich region of the TLR genes promoters in order to activate the transcription of TLRs (17;18). Binding and activation would result in increased synthesis of TLRs at the transcriptional level. The fact that there is a suppression of PU.1 in parallel with the reduction in mRNA of TLR1, TLR2, TLR4, TLR7 and TLR9 within 2 hours of starting insulin infusion, suggest strongly that the suppression of transcription of TLRs by insulin is prompt and is probably mediated by the suppression of this key transcription factor. This observation expands our understanding of the anti-inflammatory effect of insulin.

Since TLR4 mediates the inflammatory response to endotoxin, it is possible that insulin may potentially reduce the inflammatory response to endotoxin by reducing the receptor population binding to endotoxin provided we can demonstrate a reduction in TLR4 protein expression with longer infusions in future. This effect would be in addition to the more direct anti-inflammatory effect of its own through the suppression of NF-κB and Egr-1, two major pro-inflammatory transcription factors. Such an additional effect may be of considerable importance since inflammation triggers an increase in TLR expression and thus provides a positive feedback for inflammation. This positive feedback which may lead to a more protracted and intense inflammation would potentially be prevented by insulin. A direct anti-inflammatory effect of insulin and its additional ability to suppress TLR expression provide it a profoundly potent combination in
combating inflammatory processes. The suppression of TLR2 expression at mRNA and protein level by insulin is relevant to Gram positive bacterial infections. These actions of insulin may have contributed to the beneficial effects of insulin observed by Van den Berghe in patients in critical care (22). It is also relevant that a similar low dose of insulin infusion causes a reduction of 40% in plasma CRP and SAA concentrations within 24 hours in acutely ill patients with AMI (3) and in patients undergoing coronary artery bypass surgery (4). Whether a significant part of this suppression of CRP and SAA and the cardioprotection observed in acute myocardial infarction receives a contribution from a reduction in TLR2 expression needs to be carefully assessed in future.

Recent work has shown that in atherosclerosis, the expression of TLR1, 2 and 4 in the arterial intima is increased especially in areas with inflammatory infiltration (23). The increase in TLR2 and TLR4 expression is associated with an increase in intranuclear NFκB. Several TLR4 ligands, such as oxidized LDL, HSP-60, HSP-70 and peptidoglycan are found in atherosclerotic plaques. They may activate NFκB and cause a release of cytokines and matrix metalloproteinases. This indicates that insulin action on TLRs expression might play a role in atherosclerosis suppression and potential prevention of plaque rupture.

Active pharmaceutical research aiming to reduce TLR4 expression to prevent the pro-inflammatory action of LPS is currently being undertaken. This includes an attempt to generate antibodies against TLR4. The rapid suppression of TLR2 and TLR4 expression by insulin implies that insulin can be used clinically in endotoxemia and Gram negative (TLR4) and Gram positive (TLR2) infections to limit their inflammatory effects. Furthermore, the involvement of TLR2 in ischemia–reperfusion injury (11) and that of TLR4 in atherogenesis further justifies the use of insulin in acute and chronic atherosclerotic syndromes. In this context the ability of insulin to suppress CRP is also relevant since CRP mediates injury during ischemia–reperfusion of the heart and synthetic small molecules (phosphotidyl choline derivatives) which bind to and block CRP action reduce the size of myocardial injury (24).

In conclusion, insulin suppresses TLR expression and the activity of the transcription factor PU.1. The suppressive effect of insulin on TLRs also has important potential implications in the treatment of inflammatory conditions including endotoxemia, other infections and acute coronary syndromes in which TLR2 related mechanisms are involved. The suppressive effect of insulin on TLR4 is also important in understanding the relationship of inflammation to obesity and insulin resistance and atherosclerosis.
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Figure 1: TLRs mRNA expression by RT-PCR following insulin or dextrose infusion in T2DM subjects. (A) TLR1 (B) TLR2, (C) TLR4, (D) TLR7 and (E) TLR9 mRNA expression in MNC of T2DM subjects following 2 Units/hour insulin infusion compared to dextrose and saline infusion controls. *= P<0.05, RMANOVA compared to baseline and # P<0.05, 2-way RMANOVA compared to control groups.

Figure 2: TLR2 protein expression by western blotting following insulin, dextrose or saline infusion in T2DM. A) Representative TLR2 immunoblot in total cell lysate from MNC of DM subjects following 2unit/hour insulin infusion compared to dextrose and saline infusion controls. B) TLR2 protein densitometry. *= P<0.05, RMANOVA compared to baseline and # P<0.05, 2-way RMANOVA compared to control groups.

Figure 3: PU.1 DNA binding activity to specific oligonucleotide on the TLR4 promoter following insulin dextrose and saline infusions in T2DM subjects by EMSA. A) Representative EMSA gel for PU.1 binding activity to PU-12 motif in TLR4 promoter in nuclear extracts from MNC of T2DM subjects following 2unit/hour insulin infusion. SS: Supershift of PU.1-PU-12 complex from 0hr sample by a specific PU.1 antibody B) Densitometry of PU.1 binding activity on TLR4 promoter. *= P<0.05, RMANOVA compared to baseline and # P<0.05, 2-way RMANOVA compared to control groups.
Figure 3

3A

3B

% Change in PU.1 DNA Binding Activity to TLR4 Promoter

- Insulin
- Dextrose
- Saline

Hours

0 2 4 6