High glucose enhances lipopolysaccharide-stimulated CD14 expression in U937 mononuclear cells by increasing nuclear factor κ B and AP-1 activities

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Abstract

We have demonstrated recently that high glucose augments lipopolysaccharide (LPS)-stimulated matrix metalloproteinase (MMP) and cytokine expression by U937 mononuclear cells and human monocyte-derived macrophages. Since CD14 is a receptor for LPS, one potential underlying mechanism is that high glucose enhances CD14 expression. In the present study, we determined the effect of high glucose on CD14 expression by U937 mononuclear cells. After being chronically exposed to normal or high glucose for 2 weeks or longer, cells were treated with LPS for 24 h. Real-time PCR showed that although high glucose by itself did not increase CD14 expression significantly, it augmented LPS-stimulated CD14 expression by 15-fold. Immunoassay showed a marked enhancement of both membrane-associated and soluble CD14 protein levels by

high glucose. Further investigations using transcription factor activity assays and gel shift assays revealed that high glucose augmented LPS-stimulated CD14 expression by enhancing transcription factor nuclear factor κ B (NF κ B) and activator protein-1 (AP-1) activities. Finally, studies using anti-CD14 neutralizing antibody showed that CD14 expression is essential for the enhancement of LPS-stimulated MMP-1 expression by high glucose. Taken together, this study has demonstrated a robust augmentation by high glucose of LPS-stimulated CD14 expression through AP-1 and NF κ B transcriptional activity enhancement, elucidating a new mechanism by which hyperglycemia boosts LPS-elicited gene expression involved in inflammation and tissue destruction.

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Introduction

The incidence of periodontal and vascular diseases in diabetic patients is significantly higher than that in non-diabetic population, and inflammation plays an essential role in the progression of the diseases (Kannel & McGee 1979, Pyorala *et al.* 1987, Position paper 1999). In the investigation of the underlying mechanisms, hyperglycemia, a major metabolic disorder in both type 1 and type 2 diabetes, has been shown to upregulate gene expression involved in inflammation (Kannel & McGee 1979, Pyorala *et al.* 1987, Position paper 1999). Dandona *et al.* have shown in their patient studies that increased glucose concentration following a glucose load leads to an increase in the expression by mononuclear cells of tumor necrosis factor- α (TNF α), tissue factor, and matrix metalloproteinase (MMP; Mohanty *et al.* 2000, Aljada *et al.* 2004, 2006), which are known to be involved in inflammation (Matrisian 1992, Libby 1995). Increased glucose concentration also leads to an increased generation of reactive oxygen species by polymorphonuclear leucocytes (Mohanty *et al.* 2000, Aljada

et al. 2004, 2006). Furthermore, it was demonstrated that increased glucose level was associated with an enhanced transcriptional activities of AP-1, nuclear factor κ B (NF κ B), and early growth response 1, which play an important role in the transcriptional activation of genes involved in inflammation.

In addition to the effect of high glucose on gene expression as described above, our recent studies have demonstrated that chronic pre-exposure of mononuclear cells to high glucose augments lipopolysaccharide (LPS)-stimulated MMP and proinflammatory cytokine expression (Maldonado *et al.* 2004, Nareika *et al.* 2005, 2006). However, the mechanisms involved in the synergistic upregulation of the genes by high glucose and LPS have not been fully understood. The first step for the engagement of LPS to mononuclear cells is its binding to surface CD14, a LPS receptor, and the level of CD14 surface expression is a crucial factor to determine the extent of cellular engagement by LPS (Wong *et al.* 2000). Based on the clinical studies showing that CD14 expression by circulating mononuclear cells in patients with diabetes is higher than that in non-diabetic patients (Patino *et al.* 2000,

Fogelstrand *et al.* 2004), we postulated that high glucose may enhance CD14 expression and thus augments the engagement of cells by LPS, leading to an increased gene expression.

CD14 is a 55 kDa protein anchored to the cell membrane via glycosyl-phosphatidylinositol and a constituent of a multiligand pattern recognition receptor complex that plays an essential role in the innate immune responses (Pugin *et al.* 1994, Viriyakosol & Kirkland 1996, Antal-Szalmas 2000). CD14 binds to LPS/LPS-binding protein (LBP) complexes and then interacts with toll-like receptor (TLR)-4 and its adaptor protein, myeloid differentiation (MD)-2, triggering signaling activation and subsequent transcriptional activation of gene expression involved in inflammation (Antal-Szalmas 2000). In addition to LPS, CD14 also binds to other bacterial products such as teichoic acid and peptidoglycans (Pugin *et al.* 1994, Antal-Szalmas 2000), human heat shock protein (Kol *et al.* 2000), ceramide, and anionic phospholipids (Schmitz & Orso 2002). Aside from the membrane-bound CD14 (mCD14), CD14 was also found in circulating soluble form (sCD14; Amar *et al.* 2003). It has been shown that sCD14 is an acute phase protein (Bas *et al.* 2004). Clinical studies have shown that plasma level of sCD14 is elevated in a number of inflammatory diseases such as rheumatoid arthritis (Yu *et al.* 1998), systemic lupus erythematosus (Nockher *et al.* 1994), atopic dermatitis (Wuthrich *et al.* 1992), liver disease (Oesterreicher *et al.* 1995), Kawasaki disease (Takeshita *et al.* 2000), and atherosclerosis (Amar *et al.* 2003). Membrane-bound CD14 expression by mononuclear cells as determined by flow cytometry was found to be higher in diabetic patients than that in non-diabetic patients (Patino *et al.* 2000, Fogelstrand *et al.* 2004). Obviously, all these data indicate that CD14 plays a critical role in inflammation-associated diseases.

In this study, the effect of high glucose on CD14 expression by U937 mononuclear cells was investigated and the underlying mechanism was also explored. We found that high glucose markedly enhanced LPS-stimulated CD14 expression in U937 mononuclear cells by increasing NF κ B and AP-1 activities. This finding unveils a mechanism that may be involved in the synergistic stimulation of gene expression by high glucose and LPS.

Materials and Methods

Cell culture

U937 histiocytes (Sundstrom & Nilsson 1976) were purchased from American Type Culture Collection (Manassas, VA, USA) and have been used in our previous studies to show the augmentation of LPS-stimulated MMP and cytokine expression by high glucose (Maldonado *et al.* 2004, Nareika *et al.* 2005, 2006). The cells were cultured in a 5% CO₂ atmosphere in RPMI 1640 medium (GIBCO, Invitrogen Corp.) containing 10% fetal calf serum, 1% MEM non-essential amino acid solution, 0.6 g/100 ml HEPES, and 5 mM (normal glucose) or 25 mM (high glucose) D-glucose. The medium was changed every 2–3 days. After being

incubated with medium containing normal or high glucose for more than 2 weeks, U937 cells were treated with 100 ng/ml LPS (Sigma) that were highly purified from *Escherichia coli* by phenol extraction and gel filtration chromatography and was cell culture tested by the manufacturer. Our previous time course study has shown that pre-exposure of U937 cells to high glucose for 2 weeks or longer led to a significant increase in gene expression in response to LPS (Maldonado *et al.* 2004).

Membrane, cytosol, and nuclear protein extractions

The ProteoExtract kit (Calbiochem, San Diego, CA, USA) was used to isolate membrane and cytosol proteins from U937 cells. Briefly, cells were washed with ice-cold washing buffer and then incubated with the extraction buffer I at 4 °C under gentle agitation. After the incubation, cell lysate was centrifuged at 16 000 *g* for 15 min. The supernatant containing cytosol proteins was collected and the precipitate was incubated with the extraction buffer II at 4 °C for 30 min under gentle agitation. After the incubation, the samples were centrifuged at 16 000 *g* for 15 min and the supernatant containing membrane proteins was collected. Nuclear protein was extracted using NE-PER nuclear and cytoplasmic extraction reagents from Pierce (Rockford, IL, USA). The concentration of protein in cytosol, membrane, and nuclear fractions was determined using a protein assay kit (Bio-Rad).

ELISA

CD14 protein in cytosol and membrane fractions and in conditioned medium was quantified using sandwich ELISA kits according to the protocol provided by the manufacturer (R&D System, Minneapolis, MN, USA). MMP-1 level in conditioned medium was also determined using a sandwich ELISA kit (R&D System).

Real-time PCR

Total RNA was isolated from cells using the RNeasy minikit (Qiagen). First-strand cDNA was synthesized with the iScript cDNA synthesis kit (Bio-Rad) using 20 μ l reaction mixture containing 0.25 μ g total RNA, 4 μ l 5 $\times$ iScript reaction mixture, and 1 μ l iScript reverse transcriptase. The complete reaction was cycled for 5 min at 25 °C, 30 min at 42 °C, and 5 min at 85 °C using a PTC-200 DNA Engine (MJ Research, Waltham, MA, USA). The reverse transcription reaction mixture was then diluted in the ratio 1:10 with nuclease-free water and used for PCR amplification of CD14 and TLR4 in the presence of the primers (CD14 primers: 5' sequence, CCGCTGCCTCTGGAAG; 3' sequence, GGCGAGTGTGCTTGCG. TLR4 primers: 5' sequence, GTCCTCAGTGTGCTTGAG; 3' sequence, ATCCTGGCTTGAGTAGATAAC). The Beacon Designer Software (PREMIER Biosoft International, Palo Alto, CA, USA) was used for primer designing. Primers were synthesized by Integrated DNA Technologies Inc. (Coralville,

IA, USA). Real-time PCR was performed in duplicate using 25 µl reaction mixture that contained 1.0 µl RT mixture, 0.2 µM of both primers, and 12.5 µl iQ SYBR Green Supermix (Bio-Rad) and run in the iCycler real-time detection system (Bio-Rad) with a two-step method. The hot-start enzyme was activated (95 °C for 3 min) and cDNA was then amplified for 40 cycles consisting of denaturation at 95 °C for 10 s and annealing/extension at 53 °C for 45 s. A melt curve was then performed (55 °C for 1 min and then temperature was increased by 0.5 °C every 10 s) to detect the formation of primer-derived trimers and dimers. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control was amplified with the primers (5' sequence: GCCTTCCGTGTTCTACC; 3' sequence: GCCTGCTT-CACCACCTTC). Amplicon size and reaction specificity were confirmed by 2.5% agarose gel electrophoresis. Data were analyzed using the iCycler iQ software (Biorad Laboratories, Hercules, CA, USA). The average SQ (starting quantity) of fluorescence units was used for analysis. Quantification was calculated using the SQ of CD14 cDNA relative to that of GAPDH cDNA in the same sample.

Electrophoretic mobility shift assay (EMSA)

U937 cells cultured in normal or high glucose-containing medium were treated with 1 µg/ml LPS for 1, 2, 4, and 6 h. After the treatment, cells were harvested and nuclear protein was extracted as described above. Ten micrograms of nuclear proteins were used for the electrophoretic mobility shift assay to determine NFκB DNA and AP-1 binding activities. DNA-protein binding reactions were performed at room temperature for 20 min in a buffer containing 10 mM Trizma base (pH 7.9), 50 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 1 µg poly(dI-dC), 5% (v/v) glycerol, and ~0.3 pmole of NFκB or AP-1 oligonucleotide (Promega) labeled with DIG-ddUTP using terminal deoxynucleotidyl transferase (Roche Molecular Biochemicals). Protein-DNA complexes were resolved from protein-free DNA in 5% polyacrylamide gels at room temperature in 50 mM Tris (pH 8.3), 0.38 M glycine, 2 mM EDTA, and electroblotted onto positively charged nylon membranes. The chemiluminescence detection of digoxigenin (DIG)-labeled probes was conducted by following the instruction provided by the Roche Molecular Biochemicals.

Transcription factor activity assay

Two micrograms of nuclear protein of each sample were applied to the assay for NFκB and AP-1 activities using the TransAM kits produced by Active Motif (Carlsbad, CA, USA) according to the protocol provided by the manufacturer. These kits contain a 96-stripwell plate to which the consensus-binding site oligonucleotides were immobilized. Activated nuclear extract is added to each well and the transcription factor of interest binds specifically to bound oligonucleotides. TransAM assays are up to 100 times more sensitive than gel mobility shift assay and detect transcription factors with specific antibodies.

Treatment of cells with the inhibitors of signaling pathways and curcumin

In the studies to determine the effects of the specific inhibitors of MAP kinase (MAPK) and NFκB pathways on the stimulation of CD14 expression by high glucose and LPS, U937 cells pre-exposed to normal or high glucose were treated with 100 ng/ml of LPS in the absence or presence of 10 µM PD98059 (extracellular signal-regulated kinase (ERK) pathway inhibitor), SP60025 (c-Jun N-terminal kinase or JNK pathway inhibitor), SB203580 (p38 MAPK pathway inhibitor), 1 µM Bay11-7085 (NFκB pathway inhibitor), or gelastol (NFκB pathway inhibitor). These concentrations of the inhibitors have been applied in the previous study to block LPS-stimulated gene expression (Nareika *et al.* 2005). To determine the inhibitory effect of curcumin on CD14 expression, U937 cells were treated with 100 ng/ml LPS in the presence or absence of different concentrations of curcumin for 18 h. Previous studies have shown that the concentrations of 10–30 µM curcumin are effective in the inhibition of gene expression (Dickinson *et al.* 2003, Aggarwal *et al.* 2005, Nareika *et al.* 2005). After the treatment, conditioned medium was collected for sCD14 assay using ELISA as described above.

Blocking studies on MMP-1 secretion using anti-CD14 neutralizing antibody

U937 cells exposed to high glucose were treated with 100 ng/ml LPS in the presence or absence of 5 or 10 µM sheep anti-CD14 neutralizing antibody (R&D Systems) for 24 h. After the treatment, MMP-1 in the conditioned medium was quantified using ELISA. A control sheep anti-human IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used to determine the non-specific inhibition of MMP-1 secretion by IgG.

Statistical analysis

Data were presented as mean ± s.d. Student's *t*-tests were performed to determine the statistically significant differences of gene expression among different experimental groups. A value of *P* < 0.05 was considered significant.

Results

Augmentation of LPS-stimulated CD14 expression by high glucose

Results from quantitative real-time PCR showed that 100 ng/ml LPS stimulated CD14 mRNA expression in normal and high glucose-exposed U937 cells by 6-fold (0.14 ± 0.05 vs 0.022 ± 0.01) and 42-fold (2.09 ± 0.28 vs 0.05 ± 0.026) respectively when compared with cells treated without LPS (Fig. 1A, C and D). Although high glucose itself did not stimulate CD14 expression significantly, it led to a 15-fold augmentation of LPS-stimulated CD14 mRNA expression as compared with normal glucose (2.09 ± 0.28 vs 0.14 ± 0.05 ; Fig. 1A). Since

Figure 1 (A) Augmentation of LPS-stimulated CD14 mRNA expression in U937 cells by high glucose. U937 cells cultured in normal or high glucose-containing medium were treated with 100 ng/ml LPS for 24 h. After the treatment, total RNA was isolated from the cells and used for real-time PCR to quantify CD14 mRNA expression as described in Materials and Methods. The ratio of CD14 versus GAPDH mRNA was calculated and presented. Data presented are the representative of three duplicate experiments with similar results. (B) The effect of high glucose on TLR4 mRNA expression by U937 cells. The RNA isolated from the above experiments was also subjected to real-time PCR to quantify the cellular TLR4 mRNA level. (C and D) Real-time PCR amplification curves for CD14 (C) and GAPDH mRNA (D). Duplicate samples were run in each experiment. HG, high glucose; NG, normal glucose; C_t , cycles for threshold.

TLR4, another receptor for LPS, plays an important role in LPS signaling, the effect of high glucose on TLR4 expression was also investigated. Results showed that both high glucose and LPS had no stimulatory effect on TLR4 expression (Fig. 1B).

Since CD14 protein is present in the plasma membrane and cytoplasm and also shed into culture medium, CD14 localized in the membrane (mCD14), cytoplasm (cCD14) fraction, and cell-conditioned medium (sCD14) was quantified by ELISA. Results showed that LPS increased mCD14 protein level in cells exposed to normal glucose (0.16 ± 0.004 vs 0.002 ± 0.0002 pg/ μ g protein) or high glucose (7.20 ± 0.41 vs 0.05 ± 0.0003 pg/ μ g protein; Fig. 2A). The exposure to high glucose led to a 45-fold increase in mCD14 protein level in response to LPS when compared with the exposure to normal glucose. Similarly, high glucose also remarkably increased CD14 in the cytoplasm (Fig. 2A) and the conditioned medium (Fig. 2B). Figure 2C showed that glucose augmented LPS-stimulated sCD14 in a concentration-dependent fashion.

To exclude the possible involvement of increased molarity of high glucose in the upregulation of gene expression, cells were

treated with 25 mM mannitol instead of glucose and sCD14 was determined after the treatment. Results showed that the amount of sCD14 in response to 25 mM mannitol was similar to that in response to 10 mM glucose (187 ± 18 vs 182 ± 17 pg/ml) and only accounted for 28% of sCD14 released by cells in response to 25 mM glucose (187 ± 18 vs 660 ± 82 pg/ml, $P < 0.001$). Thus, the augmentation of CD14 expression by high glucose is not due to the increased molarity.

NF κ B and MAPK pathways are involved in the upregulation of CD14 expression by LPS

Since it is known that LPS stimulates gene expression in mononuclear cells through NF κ B and MAPK pathways, we confirmed the involvement of NF κ B and MAPK in the stimulation of CD14 expression by LPS. In this experiment, sCD14 was determined and served as a surrogate for CD14 expression since our above data showed that the increase in sCD14 protein (Fig. 2B) was similar to that in CD14 mRNA (Fig. 1A) in response to LPS. Results (Fig. 3A and B) showed

Figure 2 Augmentation of LPS-stimulated CD14 protein expression in membrane and cytosol fractions and in conditioned medium by high glucose. (A) U937 cells cultured in normal or high glucose-containing medium were treated with 100 ng/ml LPS for 24 h. After the treatment, cytosol and membrane proteins were extracted and used for quantification of CD14 protein using ELISA as described in Materials and Methods. (B) The soluble CD14 (sCD14) in conditioned medium was also quantified using ELISA. The CD14 protein level was normalized to cellular total protein. HG, high glucose; NG, normal glucose. (C) Concentration-dependent upregulation of sCD14 expression by glucose. U937 cells were cultured in medium containing different concentrations of glucose as indicated for 2 days and then treated with or without 100 ng/ml LPS for 24 h. After the treatment, sCD14 in the conditioned medium was determined as described above.

that in the absence of LPS stimulation, PD98059, SP60025, and SB203580 (specific inhibitors of ERK, JNK, and p38 MAPK pathways respectively) had no effect on the basal level of sCD14 in cells exposed to either normal or high glucose, while Bay11-7085 and gelastol (specific inhibitors for NFκB cascade) increased sCD14 by about twofold. In the presence of LPS stimulation, sCD14 levels were increased by 12-fold (113 vs 9 pg/μg DNA) and 63-fold (506 vs 8 pg/μg DNA) in normal and high glucose-exposed cells respectively. PD98059, SP60025, SB203580, Bay11-7085, and gelastol significantly inhibited LPS-stimulated sCD14 expression in normal glucose-exposed cells by 45–82% (Fig. 3A and

Table 1) and in high glucose-exposed cells by 67–97% (Fig. 3B and Table 1). These results demonstrated that both NFκB and MAPK (ERK, JNK, p38) pathways were involved in the upregulation of CD14 by LPS.

High glucose enhances LPS-stimulated NFκB and AP-1 DNA-binding activity

We determined whether high glucose augments LPS-stimulated CD14 expression by increasing LPS-triggered NFκB and AP-1 transcriptional activities using a quantitative assay system in which NFκB or AP-1 oligonucleotides are immobilized on a plate, and

Figure 3 Inhibition of sCD14 production by specific inhibitors of MAPK and NFκB pathways. U937 cells cultured in (A) normal glucose- or (B) high glucose-containing medium were treated with 100 ng/ml LPS in the presence or absence of 10 μM PD98059 (ERK pathway inhibitor), SP60025 (JNK pathway inhibitor), SB203580 (p38 MAPK pathway inhibitor), 1 μM Bay11-7085 (NFκB pathway inhibitor), or gelastol (NFκB pathway inhibitor) for 24 h. After the treatment, sCD14 in the conditioned medium was quantified using ELISA as described in Materials and Methods. The amount of sCD14 was normalized to the amount of cellular DNA. sCD14 level in each sample was presented as percent of sCD14 from cells treated with LPS in the absence of the inhibitors. Ctr, control; PD, PD98059; SP, SP60025; SB, SB203580; Bay, Bay11-7085; Gela, gelastol.

the bound NFκB or AP-1 is then detected by immunoassay with specific antibodies. This assay, therefore, is not only similar to gel mobility shift assay regarding DNA–protein interaction but also quantitative. Results showed that the DNA-binding activity of NFκB subunit p50, but not p65, was increased by high glucose (Fig. 4A and B). The DNA-binding activities of c-Fos and c-Jun, two major subunits of AP-1, were also determined. Results showed that while c-Fos and c-Jun activities had no statistical difference between cells exposed to normal or high glucose alone, the activities were significantly increased in high glucose-exposed cells after treatment with LPS for 4 h when compared with those in normal glucose-exposed cells (Fig. 4C and D). After LPS treatment for 6 h, the c-Fos activity continued to increase (Fig. 4C), while the c-Jun activity declined (Fig. 4D). Results from EMSA showed that, in the absence of LPS, the NFκB DNA-binding activity in cells exposed to high glucose was significantly higher than that in cells exposed to normal glucose (Fig. 5A and B). In the presence of LPS, the NFκB DNA-binding

activity assayed at the different times (1–6 h) was also higher in cells exposed to high glucose than that in cells exposed to normal glucose (Fig. 5A and B). Similarly, in the absence of LPS, the AP-1 DNA-binding activity was higher in cells exposed to high glucose than that in cells exposed to normal glucose (Fig. 5C and D). In the presence of LPS, the AP-1 DNA-binding activity at 1 and 2 h was also higher in high glucose-exposed cells than that in normal glucose-exposed cells. At 4 h, the AP-1 DNA-binding activity was similar between these two groups of cells (Fig. 5C and D). These results indicate that high glucose augments LPS-stimulated CD14 expression by enhancing the activities of transcription factors NFκB and AP-1.

Inhibition of CD14 expression by curcumin

Curcumin, also called diferuloylmethane, is a major pigment of the spice turmeric and a potent inhibitor of NFκB and AP-1

Table 1 Inhibition of sCD14 by inhibitors of MAP kinase and nuclear factor κB (NFκB) pathways (percentage of the control). Data shown are mean ± s.d.

	PD98059	SP60025	SB203580	Bay11-7085	Gelastol
Inhibitors					
Targeted pathway	ERK pathway	JNK pathway	P38 pathway	NFκB pathway	NFκB pathway
Low glucose					
Without LPS	NC	NC	NC	Slightly increased	Slightly increased
With LPS	45 ± 4%	52 ± 3%	77 ± 2%	76 ± 1%	82 ± 3%
High glucose					
Without LPS	NC	NC	NC	Slightly increased	Slightly increased
With LPS	67 ± 4%	67 ± 3%	89 ± 1%	96 ± 1%	97 ± 1%

NC, no change.

Figure 4 Enhancement of LPS-stimulated activities of NFκB subunit p50 and AP-1 subunits c-Fos and c-Jun by high glucose. U937 cells cultured in normal or high glucose-containing medium were treated with 100 ng/ml LPS for 1, 2, 4, 6, and 8 h. After the treatment, nuclear protein was extracted from cells and used for assays of p50 (A), p65 (B), c-Fos (C), and c-Jun (D) DNA-binding activities as described in Materials and Methods. The data were presented as fold increase in the DNA-binding activity when compared with that in cells cultured with normal glucose in the absence of LPS.

transcription activities (Dickson *et al.* 2003, Aggarwal *et al.* 2005). To further determine whether NFκB and MAPK pathways are involved in the upregulation of CD14 by LPS and high glucose, U937 cells were treated with LPS in the presence or absence of different concentrations of curcumin. After the treatment, sCD14 in the conditioned medium was quantified by ELISA. Results showed that curcumin inhibited sCD14 production in a concentration-dependent manner and 20 μM curcumin inhibited LPS-stimulated sCD14 production from both normal and high glucose-exposed cells by 80% (Fig. 6).

CD14 is essential for high glucose-augmented MMP-1 expression

Our recent study demonstrated that high glucose augments LPS-stimulated MMP-1 expression by U937 cells. Since CD14 is the receptor for LPS and increased CD14 expression could

enhance the interaction of cells with LPS, leading to upregulation of LPS-stimulated gene expression, we hypothesized that the enhancement of CD14 expression by high glucose may play an important role in the augmentation of LPS-stimulated MMP-1 expression. To test this hypothesis, we used anti-CD14 neutralizing antibody to block the interaction between CD14 and U937 cells. Results showed that the neutralization by 5 μg/ml anti-CD14 antibody inhibited the augmentation by high glucose of LPS-stimulated MMP-1 secretion by 81% (71.9 vs 387.1 ng/ml for secreted MMP-1 in the presence and absence of the neutralizing antibodies respectively; Fig. 7). Although the control IgG also partially inhibited MMP-1 secretion, 5 μg/ml of it inhibited MMP-1 secretion only by 36% (247.7 vs 387.1 ng/ml for secreted MMP-1 in the presence and absence of the control antibodies respectively) that is significantly less than the degree of inhibition by anti-CD14 antibody.

Figure 5 High glucose increases NFκB (A) and AP-1 (C) DNA-binding activities. U937 cells cultured in normal or high glucose-containing medium were treated with 100 ng/ml LPS for 0, 1, 2, 4, and 6 h. After the treatment, nuclear proteins were extracted from the cells and subjected to EMSA as described in Materials and Methods. Densitometric scanning was performed to quantify the intensity of the shifted NFκB (B) or AP-1 bands (D). The data presented are the representative of two experiments.

Discussion

CD14 is an initial receptor for LPS and plays an essential role in the pathogen recognition by the innate immune system (Bas *et al.* 2004). LPS binds to LBP and mCD14 to form a trimolecular complex that has a high affinity to TLR4, a LPS signaling receptor. The interaction between the complex and TLR4 triggers a strong inflammatory response through NFκB and MAPK pathways in mononuclear cells (Pugin *et al.* 1993). Besides mCD14, it has been shown that soluble CD14 mediates the LPS-stimulated activation of non-CD14-expressing cells such as late passage endothelial cells, epithelial, and smooth muscle cells (Haziot *et al.* 1993, Loppnow *et al.* 1995, Guha & Mackman 2001, Bas *et al.* 2004). Furthermore, studies have shown that macrophages isolated from CD14 knockout mice are unable to produce TNFα, IL-1β, and interferon-inducible protein-10 in response to LPS and these mice have an early death in pneumococcal infection (Vogel *et al.* 1998). Clearly, all these studies have well documented an essential role of CD14 in immunity and inflammation.

We reported in this study that high glucose incites a robust augmentation of LPS-stimulated CD14 expression by U937 mononuclear cells. It is interesting to note that high glucose did not have a significant effect and LPS had a 6-fold stimulation on CD14 mRNA expression, but the combination of high glucose and LPS led to a 95-fold stimulation on CD14 mRNA expression (2.09 ± 0.28 vs 0.022 ± 0.01). Similar robust augmentation by high glucose was consistently observed at the levels of membrane-associated CD14 and soluble CD14 in conditioned medium. Obviously, there is a strong synergy between high glucose and LPS on CD14 expression. Thus, this study has established a good cell model to further investigate the synergistic interaction between high glucose and LPS on CD14 expression.

In the following studies to explore the underlying synergistic mechanism, we found that high glucose enhances LPS-stimulated CD14 expression in U937 cells by increasing NFκB and AP-1 activities. Our EMSA study presented in Fig. 5 showed that exposure to high glucose increased LPS-stimulated NFκB DNA-binding activity at 1, 2, 4, and 6 h after the treatment. High glucose also increased LPS-stimulated AP-1 DNA-binding activity at 1 and 2 h after the treatment. Since it is known that transcription factors NFκB and AP-1 are essential for LPS-initiated inflammatory responses (Pugin *et al.* 1993), this finding suggests that hyperglycemia may boost inflammation in response to LPS. As illustrated in Fig. 8, our study suggests that mononuclear cells when exposed to hyperglycemia have an enhanced CD14 expression in response to LPS. The increased CD14 surface expression facilitates more engagement between mononuclear cells and LPS, which in turn further increases CD14 production under the condition of hyperglycemia. The enhanced LPS signaling leads to increased NFκB and AP-1 transcriptional activities and hence the expression of proinflammatory cytokines and MMPs.

Figure 6 Inhibition of sCD14 production by curcumin. U937 cells cultured in normal or high glucose-containing medium were treated with 100 ng/ml LPS in the absence or presence of curcumin (5–30 μM) for 24 h. After the treatment, conditioned medium was collected for quantification of sCD14 using an ELISA kit as described in Materials and Methods. The data were presented as percentage of the maximum from cells cultured in high glucose-containing medium and treated with LPS in the absence of curcumin.

Our results showed that high glucose and LPS stimulated the expression of CD14, but not TLR4 (Fig. 1), indicating that between LPS receptors CD14 and TLR4, high glucose and LPS specifically upregulate CD14 in U937 cells. Furthermore, our

Figure 7 Inhibition of LPS-stimulated MMP-1 secretion from U937 cells by anti-CD14 neutralizing antibody. U937 cells cultured in normal or high glucose-containing medium were treated with 100 ng/ml LPS in the absence or presence of 5 and 10 μg neutralizing anti-CD14 or control antibodies for 24 h. After the treatment, conditioned medium was collected for quantification of MMP-1 using ELISA as described in Materials and Methods. The data presented were the representative of two experiments.

results showed that the baseline level of CD14 expression was much lower than that of TLR4 expression when compared with the housekeeping gene GAPDH. However, after stimulation with high glucose and LPS, CD14 level was dramatically increased and became similar to TLR4 (about twofold of GAPDH mRNA; Fig. 1). Thus, it appears that CD14, but not TLR4, is responsible for the regulation of interaction between LPS and U937 cells by high glucose and LPS. It is expected that the robust increase in CD14 expression would lead to a marked increase in the formation of LPS–CD14–LBP complexes that interact with TLR4 and trigger TLR4-mediated signaling activation and gene expression.

We have reported previously that high glucose augmented LPS-stimulated MMP-1 expression by U937 histiocytes (Maldonado *et al.* 2004). However, the underlying mechanisms have not been well understood. In this study, we determine whether CD14 expression is essential for the augmentation of LPS-stimulated MMP-1 expression. We performed a neutralization study in which anti-CD14 antibodies were added to the high glucose-exposed cells when they were treated with LPS. It is expected that the binding of the neutralizing anti-CD14 to mCD14 or sCD14 would prevent the interaction of LPS to mCD14 or sCD14 and thus reduces the LPS-stimulated gene expression. Indeed, our results demonstrate that the addition of anti-CD14 antibodies significantly attenuates LPS-stimulated MMP-1 secretion from high glucose-treated cells (Fig. 7), confirming an important role of CD14 in MMP-1 expression in response to high glucose and LPS. In this experiment, we also observed that the control antibody for anti-CD14 partially inhibited MMP-1 secretion. While the cause remains unknown, it is possible that the partial inhibition may be caused by the close localization of CD14 and Fcγ-receptor II/III (CD32/16) on the cell surface. Hisaka *et al.* (1999) showed that the binding of anti-CD14 MAB to CD14 was inhibited by pretreatment of monocytes with anti-Fcγ-receptor II/III monoclonal antibodies. They postulated that the close localization of CD14 and Fcγ-receptor II/III led to the non-specific blocking of CD14 binding by anti-Fcγ-receptor II/III antibody. Since the control IgG antibodies used in the present study have potential to bind to Fcγ-receptor II/III through the Fc portion, it is possible that they can partially interfere with the binding of LPS to CD14.

It is known that diabetic patients have increased incidence and severity of infectious diseases such as those in the periodontal tissue (Bell *et al.* 2000, Grossi 2001) and urinary tract (Ooi *et al.* 2004) in which infection with the Gram-negative bacteria is the primary cause. For patients who have poor glycemic control, mononuclear cells may be pre-exposed to hyperglycemia before interacting with LPS. Thus, hyperglycemia may boost LPS-initiated inflammation by enhancing the expression of CD14, cytokines, MMPs, and other molecules involved in the inflammatory process. Our studies have shown that in addition to MMP-1, high glucose also augments LPS-stimulated expression of MMP-7,

Figure 8 The diagram showing the augmentation of LPS-stimulated CD14 expression and resulting CD14-mediated inflammatory responses by hyperglycemia.

MMP-8, MMP-9, as well as proinflammatory cytokines such as TNF α , IL-1 β , and IL-6 (Maldonado *et al.* 2004, Nareika *et al.* 2006). Therefore, the synergy between high glucose and LPS for CD14, cytokine, and MMP expression is likely a potential factor causing the increased risk and severity of periodontal disease and urinary tract infection in diabetic patients.

Human monocytes have both CD14 positive (CD+) and CD14 negative (CD14-) cells (Urbich *et al.* 2003). Given the different baseline levels of CD14 expression, it is possible that these two cell populations may respond to high glucose and LPS differently. Similar to U937 cells, CD14- cells have undetectable level of CD14 expression which may be induced by high glucose and LPS. In contrast, since the basal expression of CD14 is high in CD14+ cells, the effect of high glucose and LPS on CD14 expression in these cells may be less remarkable. Further experiments are necessary to investigate the effect of high glucose and LPS on CD14 expression by human CD14- and CD14+ monocytes.

In summary, our present study has demonstrated that high glucose markedly enhances LPS-stimulated CD14 expression in U937 mononuclear cells by enhancing NFκB and AP-1 transcriptional activities. Since CD14 plays a critical role in the pathogen recognition, these findings have revealed a molecular mechanism involved in diabetes-promoted inflammatory diseases.

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