J Periodont Res 2007; 42; 31–38
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JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2006.00911.x

High glucose-boosted inflammatory responses to lipopolysaccharide are suppressed by statin

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Nareika A, Maldonado A, He L, Game BA, Slate EH, Sanders JJ, London SD, Lopes-Virella MF, Huang Y. High glucose-boosted inflammatory responses to lipopolysaccharide are suppressed by statin. J Periodont Res 2007; 42: 31–38.
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Background and Objective: It has been established that periodontal diseases are more prevalent and of greater severity in diabetic patients than in nondiabetic patients. Recent studies have underscored the role of monocytes and macrophages in periodontal tissue inflammation and destruction in diabetic patients. Although it has been shown that monocytes isolated from diabetic patients produce more inflammatory cytokines and that gingival crevicular fluid collected from diabetic patients contains higher levels of inflammatory cytokines than that obtained from nondiabetic patients, the underlying mechanisms are not well understood.

Material and Methods: U937 histiocytes cultured in medium containing either normal (5 mM) or high (25 mM) glucose were treated with 100 ng/ml of lipopolysaccharide for 24h. After the treatment, cytokines in the medium and cytokine mRNA in the cells were quantified using enzyme-linked immunosorbet assay and real-time polymerase chain reaction, respectively.

Results: In this study, we demonstrated that the pre-exposure of U937 histiocytes to high glucose concentrations markedly increased the lipopolysaccharide-induced secretion of pro-inflammatory cytokines and chemokines and the cellular inducible nitric oxide level compared with pre-exposure to normal glucose. Our data also showed that the increased secretion of cytokines was a result of increased mRNA expression. Furthermore, the effects of statin and peroxisome proliferators-activated receptor agonists on high glucose-enhanced secretion of cytokines were determined. The results showed that simvastatin, but not fenofibrate or pioglitazone, inhibited high glucose-enhanced cytokine release.

Conclusion: This study has shown that high glucose concentrations and lipopolysaccharide act synergistically to stimulate the secretion of inflammatory mediators, and that statin is capable of suppressing the high glucose-boosted proinflammatory response. This study therefore delineates a novel mechanism by which hyperglycemia enhances the inflammatory responses of macrophages and suggests that statin may be useful in the treatment of periodontal disease in diabetic patients.

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Key words: diabetes mellitus; glucose; lipopolysaccharide; periodontal diseases

Accepted for publication April 18, 2006

Clinical studies have established that periodontal diseases are more prevalent and of greater severity in patients with either type 1 or type 2 diabetes than in nondiabetic patients (1). Previous studies carried out to obtain a greater understanding of the pathogenesis of diabetes-associated periodontal disease have indicated that multiple mechanisms may be involved in the progression of the disease (1). Impaired host defense to bacterial pathogens, increased susceptibility to infection, decreased collagen production and advanced glycosylation end products are the factors believed to contribute to periodontal diseases in diabetic patients (1–3).

In recent years, the role of monocytes and macrophages in diabetic periodontal diseases has been highlighted, and several lines of evidence indicate that monocytes and macrophages are important players in periodontal tissue inflammation and destruction in diabetic patients (4,5). For example, it has been shown that monocytes isolated from diabetic patients produce a greater amount of tumor necrosis factor-α (TNF-α) and interleukin-1β *in vitro* than do nondiabetic controls (4). It was also reported that diabetic subjects with periodontal disease have significantly higher levels of both TNF-α and interleukin-1β in gingival crevicular fluid when compared with nondiabetic controls matched for periodontal disease severity (4). Furthermore, studies have shown that cytokines (such as TNF-α and interleukin-1β) released from monocytes and macrophages, stimulate matrix metalloproteinase (MMP) expression and secretion by polymorphonuclear leukocytes (5). Cytokines also stimulate macrophages and polymorphonuclear leukocytes to release reactive oxygen species (ROS) that are believed to be cytotoxic to fibroblasts in periodontal tissues and to contribute to periodontal tissue destruction (5).

Although it has been shown that monocytes from diabetic patients produce more cytokines, and that the gingival crevicular fluid from diabetic patients with periodontal disease contains higher levels of inflammatory cytokines than that from nondiabetic patients (4), it remains unclear how the cytokine release is increased in diabetic patients. In the present study, we demonstrated that a high glucose content markedly augmented lipopolysaccharide-induced inflammatory cytokine and inducible nitric oxide synthase (iNOS) expression in U937 macrophages, suggesting that hyperglycemia in diabetic patients may boost the inflammatory response that leads to increased cytokine release. Furthermore, as it has been shown that antidiabetic drugs, such as thiazolidinediones (TZDs), and antidyslipidemic drugs, such as fibrates and statins, have anti-inflammatory effects (6–8), we proposed that these drugs might inhibit the inflammatory responses of U937 cells to high glucose concentration and lipopolysaccharide. Our results showed that statin, but not TZD or fibrate, suppressed effectively the stimulatory effect of high glucose and lipopolysaccharide on the secretion of pro-inflammatory cytokines.

Material and methods

Cell culture

U937 histiocytes (9) (American Type Culture Collection, Manassas, VA) were cultured in a 5% CO_2 atmosphere in RPMI 1640 (Gibco, Invitrogen Corp., Carlsbad, CA, USA) containing 10% fetal calf serum (FCS), 1% MEM nonessential amino acid solution, 0.6 g/100 ml of HEPES, and 5 mM (normal glucose) or 25 mM (high glucose) of D-glucose. The medium was changed every 2–3 d. Histiocytes are also called resident macrophages, and the histiocytic origin of U937 cells was shown by its capacity for lysozyme production and strong esterase activity (9). U937 cells were treated with lipopolysaccharide (Sigma, St Louis, MO, USA) that was highly purified from *Escherichia coli* by phenol extraction and gel-filtration chromatography, and was cell culture tested.

Cell DNA assay

Cellular DNA was quantified with a CyQUANT cell proliferation assay kit, according to the procedures provided by the manufacturer (Molecular Probes, Eugene, OR, USA).

Enzyme-linked immunosorbent assay (ELISA)

TNF-α, interleukin-1β, interleukin-6, interleukin-8, macrophage inflammatory protein (MIP)-1α, MIP-1β and MMP-1 in cell-conditioned medium were quantified using sandwich ELISA kits according to the protocol provided by the manufacturer (R & D System, Minneapolis, MN, USA). Briefly, 50 μl of conditioned medium was added to each well of the plate provided by the kits and incubated for 1–2 h. After washing, anticytokine, chemokine or MMP-1 immunoglobulin conjugated with horseradish peroxidase was added to the wells and incubated for 1 h. After the incubation, wells were washed and protein was detected by adding color reagent A (hydrogen peroxide) and color reagent B (tetramethylbenzidine). The protein was quantified by reading the plate at 450 nm in a mocroplage reader within 30 min. Cytoplasmic proteins were extracted using a cytoplasmic-protein extraction kit (Pierce, Rockford, IL, USA), and 5 μg of protein for each sample was used to quantify the amount of iNOS by ELISA (R & D System).

Real-time polymerase chain reaction (PCR)

Total RNA was isolated from cells using the RNeasy minikit (Qiagen, Santa Clarita, CA, USA). First-strand complementary DNA (cDNA) was synthesized using the iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) in 20 μl of reaction mixture containing 0.25 μg of total RNA, 4 μl of 5× iScript reaction mixture and 1 μl of iScript reverse transcriptase. The complete reaction was then 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 1 : 10 with nuclease-free water and used for PCR amplification in the presence of the primers (Table 1). The Beacon Designer Software (PREMIER Biosoft

Table 1. The primer sequences for real-time polymerase chain reaction

Genes	5′ primer sequence	3′ primer sequence	AET
TNF-α	CCCCAGGGACCTCTCTCTAA	TTTGCTACAACATGGGCTACAG	52°C
Interleukin-1β	CTGTACGATCACTGAACTGC	CACCACTTGTTGCTCCATATC	54°C
Interleukin-6	AACAACCTGAACCTTCCAAAGATG	TCAAACTCCAAAAGACCAGTGATG	53°C
GAPDH	GAATTTGGCTACAGCAACAGGGTG	TCTCTTCCTCTTGTGCTCTTGCTG	52°C

AET, annealing/extension temperature; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNF-α, tumor necrosis factor-α.

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 carried out in duplicate using 25 μl of reaction mixture that contained 1.0 μl of reverse transcription mixture, 0.2 μM of both primers and 12.5 μl of iQ™ SYBR Green Supermix (Bio-Rad). The real-time PCR was performed using the i-Cycler™ 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 (95°C for 10 s) and annealing/extension (different temperatures for different primers, see Table 1, for 45 s). A melt-curve was then performed (55°C for 1 min and then the 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. Amplicon size and reaction specificity were confirmed by 2.5% agarose-gel electrophoresis. Data were analyzed using the ICYCLER IQ™ software. The average starting quantity (SQ) of fluorescence units was used for analysis. Quantification was calculated using the SQ of cytokine cDNA relative to that of GAPDH cDNA in the same sample.

Stimulation of cells with lipopolysaccharide

To stimulate the expression of pro-inflammatory cytokines, U937 cells pre-exposed to 5 mM (normal) glucose or 25 mM (high) glucose concentrations were treated with 100 ng/ml of lipopolysaccharide for 24 h. This concentration of lipopolysaccharide has been applied previously to stimulate the expression of pro-inflammatory cytokines (10,11) and also significantly stimulated MMP expression in U937 cells, as shown in our previous study (12).

Treatment of cells with statin, TZD and fibrate

In the studies to determine the effects of the cholesterol-lowering drug (statin), insulin-sensitizing drug (TZD) and antidyslipidemic drug (fibrate), on the stimulation of cytokine expression by high glucose and lipopolysaccharide, U937 cells pre-exposed to a normal or to a high concentration of glucose were treated with 100 ng/ml of lipopolysaccharide in the absence or presence of different doses (5, 10, 20, 30 μM) of simvastatin, pioglitazone or fenofibrate for 24 h. Previous studies have shown that these concentrations of drugs effectively inhibit gene expression and signal transduction (13–15). We applied these different concentrations in the present study to demonstrate the concentration-dependent effect of the drugs on cytokine expression.

Statistical analysis

Data were presented as mean ± standard deviation. Student's *t*-tests were performed to determine the statistical significance of cytokine expression among different experimental groups. A p value of < 0.05 was considered significant.

Results

High glucose concentration enhances lipopolysaccharide-induced inflammatory cytokine and chemokine secretion

To determine if pre-exposure to increased concentrations of glucose has any effect on lipopolysaccharide-induced secretion of inflammatory cytokines and chemokines, U937 histiocytes were cultured in medium containing either normal (5 mM) or high (25 mM) concentrations of glucose for at least 1 mo before treatment with lipopolysaccharide for 24 h. After the treatment, we quantified inflammatory cytokines TNF-α, interleukin-1β and interleukin-6, and the chemokines interleukin-8, MIP-1α and MIP-1β, as these cytokines and chemokines have been shown to be involved in periodontal disease (16–18). Results from ELISA showed that high glucose plus lipopolysaccharide markedly increased the secretion of inflammatory cytokines TNF-α, interleukin-1β and interleukin-6, and of chemokines interleukin-8, MIP-1α and MIP-1β, as compared with normal glucose plus lipopolysaccharide (Fig. 1A–F). Clearly, these results show a synergistic effect of high glucose concentration and lipopolysaccharide on the stimulation of cytokine and chemokine release.

High glucose concentration-enhanced cytokine and chemokine release in response to lipopolysaccharide is caused by an increased cellular mRNA level

It is known that the induction of cytokine and chemokine secretion from macrophages by lipopolysaccharide is controlled at the transcriptional level (19,20). As the above results show that high glucose concentration further increases lipopolysaccharide-induced secretion of cytokines and chemokines, it is important to determine if the increase in lipopolysaccharide-induced secretion of these inflammatory mediators by high glucose is caused by an

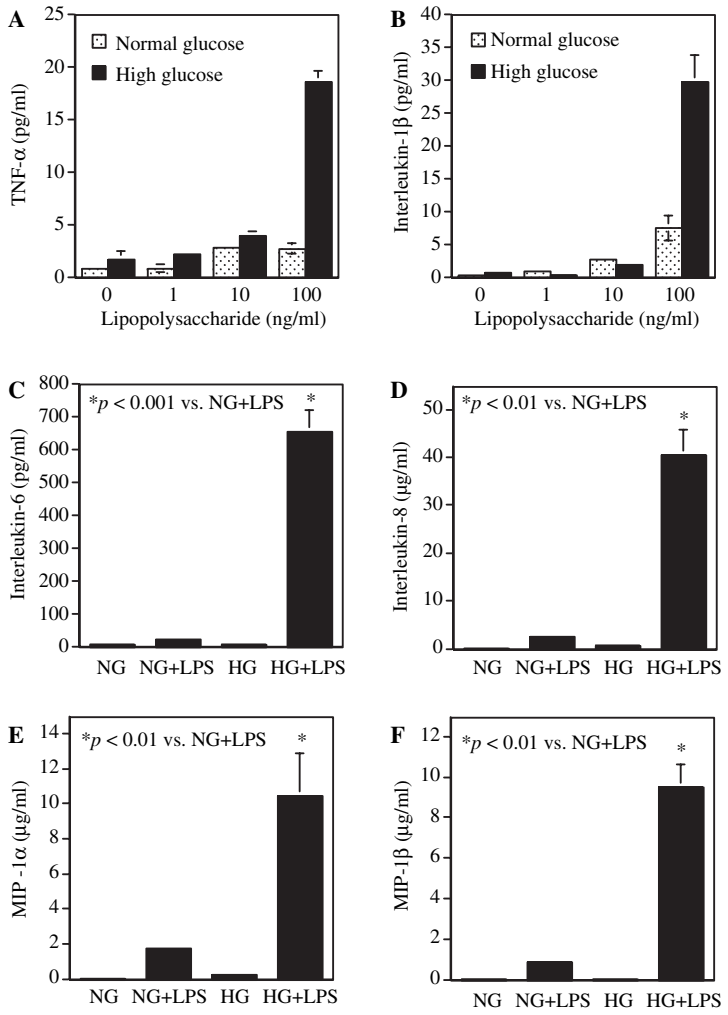

Fig. 1. High glucose concentration boosts lipopolysaccharide-induced inflammatory cytokine and chemokine secretion. (A,B) U937 cells pre-exposed to normal glucose (5 mM) or high glucose (25 mM) were treated with different concentrations of lipopolysaccharide for 24 h. After the treatment, conditioned medium was collected for quantification of tumor necrosis factor-α (TNF-α) (A) and interleukin-1β (B) using enzyme-linked immunosorbent assay (ELISA), as described in the Material and methods. (C–F) U937 cells pre-exposed to normal glucose (NG; 5 mM) or to high glucose (HG; 25 mM) were treated with 100 ng/ml of lipopolysaccharide (LPS) for 24 h. After the treatment, conditioned medium was collected for quantification of interleukin-6 (C), interleukin-8 (D), macrophage inflammatory protein-1α (MIP-1α) (E) and MIP-1β (F) using ELISA, as described in the Material and methods. The data (mean ± standard deviation) presented are the representative of two experiments, with similar results obtained from each.

increase in mRNA level. Thus, real-time PCR was conducted to quantify the amount of mRNA of the inflammatory mediators in cells exposed to normal or to high glucose concentration and lipopolysaccharide. The results showed that compared with normal glucose concentration, high glucose concentration significantly increased the lipopolysaccharide-induced cellular mRNA levels of TNF-α, interleukin-1β and interleukin-6 (Fig. 2A–D). Interestingly, although high glucose concentration alone had no effect on interleukin-1β and interleukin-6 mRNA expression, it amplified the lipopolysaccharide-stimulated interleukin-1β and interleukin-6 expression (Fig. 2B–D).

High glucose concentration and lipopolysaccharide also have a synergistic effect on the expression of iNOS

In addition to increased cytokine release, increased iNOS expression is also an important feature of macrophage activation (21). Thus, the effect of high glucose concentration on iNOS expression by U937 cells was determined. Results obtained by immunoassay (Fig. 3) showed that high glucose concentration alone did not increase iNOS expression compared with normal glucose concentration, and that lipopolysaccharide had no effect on iNOS expression in cells exposed to normal glucose. Interestingly, lipopolysaccharide markedly stimulated iNOS expression in cells exposed to high glucose, suggesting that high glucose concentration and lipopolysaccharide have a synergistic effect on iNOS expression in U937 histiocytes.

Simvastatin, but not pioglitazone or fenofibrate, inhibits high glucose concentration-enhanced inflammatory response to lipopolysaccharide

It has been shown that cholesterol-lowering drug statins, and the antidiabetic drug, TZD, known as peroxisome proliferator-activated receptor (PPAR) agonists, and the antidyslipidemic drug, fibrate, known as a PPARα agonist, have anti-inflammatory properties (7,8). Clinically, statins, TZDs and fibrates are used to treat hypercholesterolemia, hyperglycemia and hypertriglyceridemia, respectively, in diabetic patients. Therefore, we decided to examine the effect of these drugs on the high glucose-enhanced inflammatory response to lipopolysaccharide. The results showed that simvastatin inhibited high glucose-enhanced TNF-α, interleukin-1β and interleukin-6 secretion in a dose-dependent manner (Fig. 4A). It inhibited TNF-α, interleukin-1β, interleukin-6 secretion by 70%, 50% and 50%, respectively, at 5 μM, and by 83%, 71% and 80%, respectively, at 20 μM. Furthermore, as our previous study showed that high

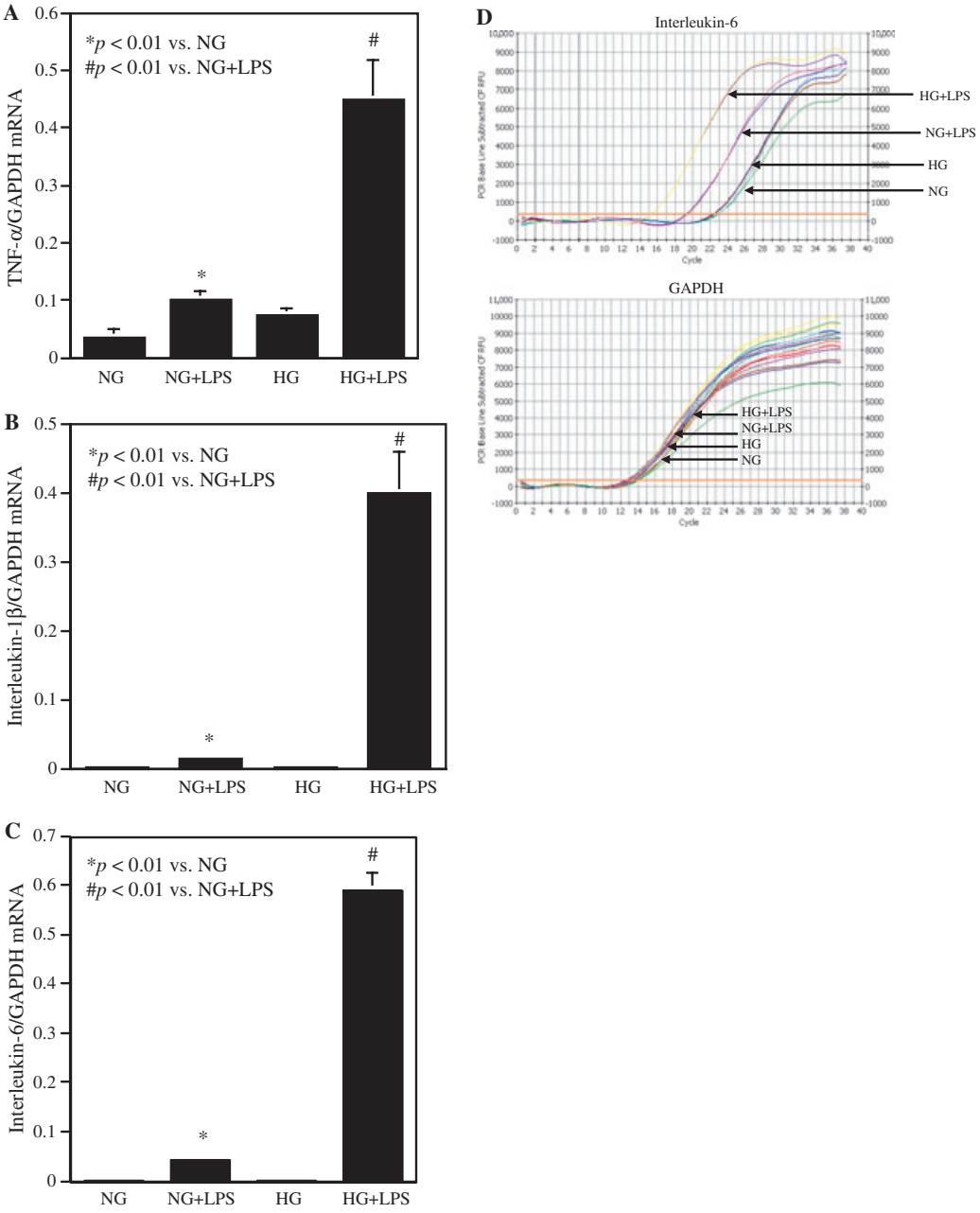

Fig. 2. High glucose concentration boosts lipopolysaccharide-induced cytokine mRNA expression. U937 cells pre-exposed to normal glucose (NG; 5 mM) or to high glucose (HG; 25 mM) were treated with 100 ng/ml of lipopolysaccharide (LPS) for 24 h. After the treatment, total RNA was isolated and real-time polymerase chain reaction was conducted to quantify tumor necrosis factor-α (TNF-α) (A), interleukin-1β (B) and interleukin-6 mRNA (C), as described in the Material and methods. Panel D shows the curves that represent the real-time amplification of interleukin-6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (control).

glucose enhanced lipopolysaccharide-induced MMP-1 expression in U937 cells (12), the effect of simvastatin on MMP-1 secretion was also determined. Interestingly, the results showed that 5 μM simvastatin suppressed 85% of glucose-enhanced MMP-1 secretion (Fig. 4A). These results indicate that simvastatin effectively inhibited high glucose-enhanced cytokine and MMP-1 expression.

The effect of PPARα and PPARγ agonists on high glucose-enhanced secretion of TNF-α, interleukin-1β and interleukin-6 was also examined. The results showed that fenofibrate, a PPARα agonist, increased high glucose-enhanced TNF-α secretion at 20 and 30 μM (Fig. 4B) but had no effect on interleukin-1β and interleukin-6 secretion (Fig. 4B). The PPARγ agonist, pioglitazone, increased high glucose-enhanced TNF-α, interleukin-1β and interleukin-6 secretion at 5 μM, but

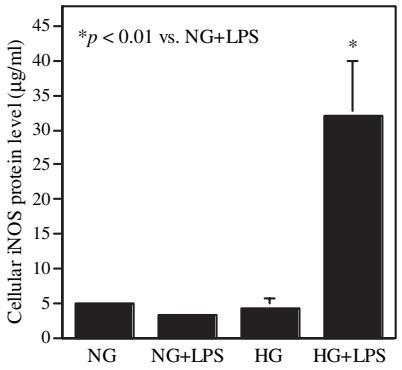

Fig. 3. The effect of high glucose and lipopolysaccharide content on inducible nitric oxide synthase (iNOS) production. U937 cells pre-exposed to normal glucose (NG; 5 mM) or to high glucose (HG; 25 mM) were treated with 100 ng/ml of lipopolysaccharide (LPS) for 24 h. After this treatment, the cells were lysed and the amount of inducible nitric oxide synthase (iNOS) in the cell lysate was quantified using enzyme-linked immunosorbent assay (ELISA), as described in the Material and methods. The data (mean ± standard deviation) presented are representative of two experiments, with similar results obtained on each occasion.

no further increase was observed at higher concentrations (Fig. 4C).

Discussion

In addition to atherosclerosis, nephropathy, retinopathy, neuropathy and peripheral vascular disease, periodontal disease has been recognized as another diabetic complication (22). The primary cause of periodontal disease is bacterial infection (23), which is unique among the diabetic complications. It has been shown that some periodontopathic bacteria exhibit a number of virulence factors, such as lipopolysaccharide, lipoteichoic acids, toxins, proteinases and short-chain fatty acids, which elicit inflammatory responses (24). Inflammatory mediators, such as cytokines and chemokines, released by activated monocytes and macrophages, play a central role in tissue inflammation and destruction in periodontal disease (5). Studies have shown that the expression of MMPs (the powerful proteinases that degrade collagen and other extracellular matrices in periodontal tissue) by macrophages and polymorphonuclear leukocytes are up-regulated by cytokines (5). Given the crucial role of bacterial virulence factors in periodontal disease, it is likely that the diabetes-associated pathogenic factors, such as hyperglycemia, interplay with bacterial virulence factors to promote the progression of periodontal disease in diabetes. In this study, we demonstrated that a high concentration of glucose (25 mM) markedly enhanced the lipopolysaccharide-induced expression of several pro-inflammatory cytokines and chemokines in U937 macrophages. Clearly, a high concentration of glucose together with lipopolysaccharide has a synergistic effect in up-regulating the expression of genes that are involved in inflammation and tissue destruction. Therefore, this study has delineated a novel mechanism potentially involved in the periodontal disease observed in diabetic patients.

Previous studies have shown that statins, which are potent cholesterol-lowering drugs, inhibit lipopolysaccharide-induced expression of pro-inflammatory genes, such as monocyte chemoattractant protein-1 (MCP)-1 (25), iNOS (26), intercellular adhesion molecule-1 (ICAM-1) (27) and interleukin-6 (28). The present study has further demonstrated that simvastatin is also capable of blocking the augmentation of lipopolysaccharide-stimulated pro-inflammatory cytokine production by a high concentration of glucose. Nevertheless, it is not clear at this point how simvastatin inhibits high glucose-enhanced inflammatory responses to lipopolysaccharide, although several mechanisms are likely

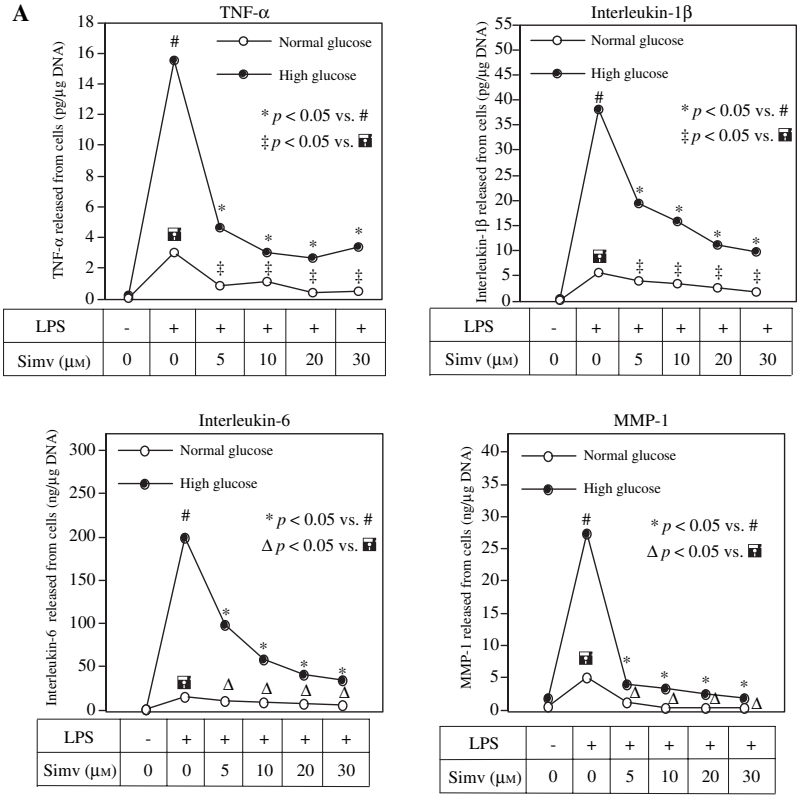

Fig. 4. The effect of simvastatin (A), fenofibrate (B) and pioglitazone (C) on the release of cytokines. U937 cells pre-exposed to normal glucose (NG; 5 mM) or to high glucose (HG; 25 mM) were treated with 100 ng/ml of lipopolysaccharide in the presence or absence of different doses of simvastatin, fenofibrate and pioglitazone for 24 h. After the treatment, conditioned medium was collected for quantification of tumor necrosis factor-α (TNF-α), interleukin-1β and interleukin-6 using enzyme-linked immunosorbent assay (ELISA), as described in the Material and methods. The experiments were run in duplicate and data are presented as the mean ± standard deviation.

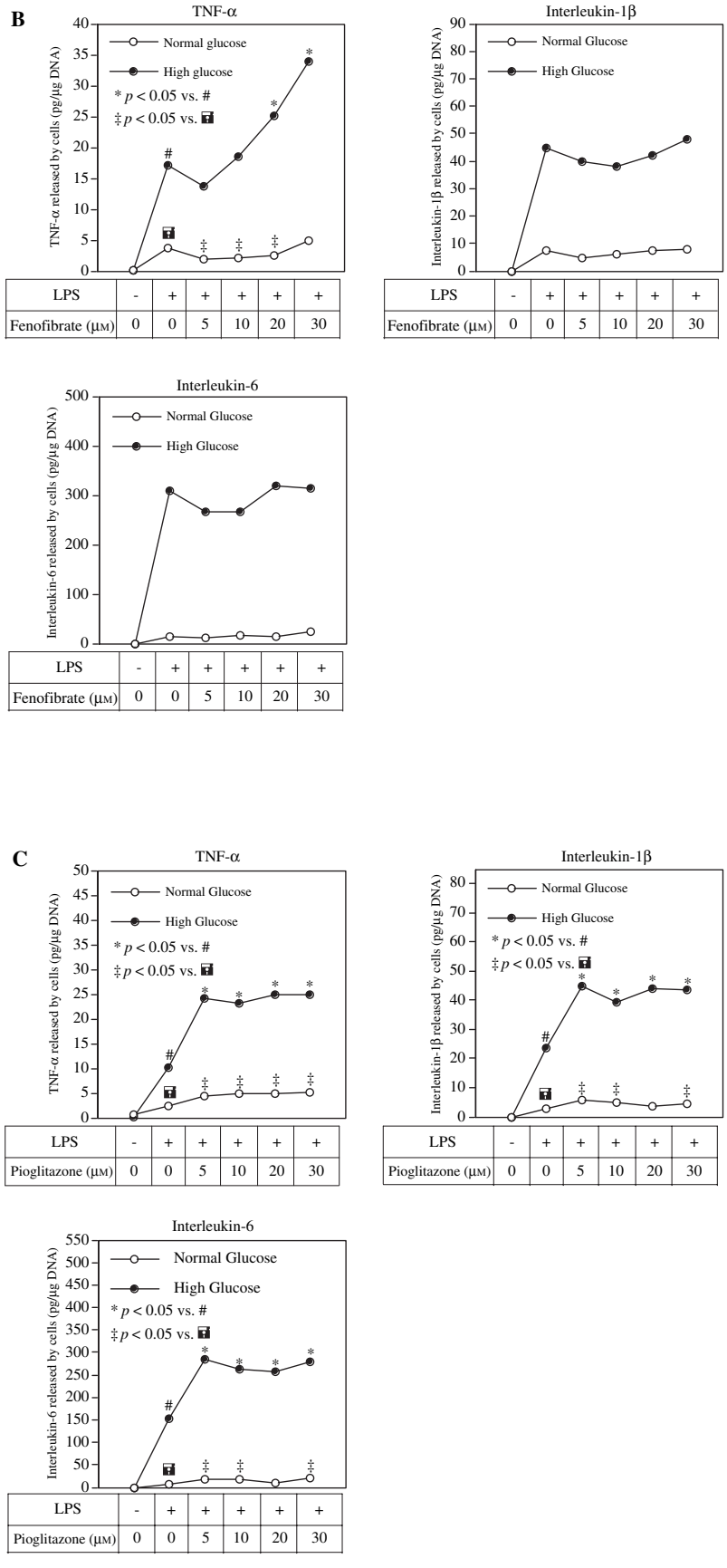

Fig. 4. (Continued)

to play a role, such as direct inhibition of signaling pathways involved in the expression of the genes, or indirect actions through interfering with cholesterol metabolism. It is obvious that more studies are necessary to explore the mechanism by which simvastatin inhibits the cytokine expression up-regulated by high glucose and lipopolysaccharide.

It has been shown that statins have an anti-inflammatory effect on diabetic patients. For example, Yamada *et al.* showed that atorvastatin not only improved hypercholesterolemia, but also reduced the level of C-reactive protein in patients with type 2 diabetes (29). Takebayashi *et al.* reported that low-dose atorvastatin (10 mg/d) significantly decreased the level of C-reactive protein and MCP-1 in patients with type 2 diabetes (6). Economides *et al.* also reported that atorvastatin improved endothelial function and decreased the levels of markers of endothelial activation and inflammation in patients with type 2 diabetes (30). Thus, our finding that simvastatin effectively inhibits the expression of pro-inflammatory cytokines by U937 macrophages in response to high glucose and lipopolysaccharide is consistent with these reports. Indeed, our study, and those of others, indicate that further clinical investigation is necessary to evaluate the potential use of statins in the treatment of periodontal disease in diabetic patients.

PPAR agonists are another group of drugs shown to inhibit inflammation. As these agonists are used to treat either diabetic dyslipidemia (fibrates) or hyperglycemia (TZDs) and are widely prescribed to type 2 diabetic patients, we determined whether they inhibited the high glucose-enhanced inflammatory response. In contrast to what was expected, they failed to exhibit an inhibitory effect. These results are not surprising because the previous reports on the effects of PPAR agonists on lipopolysaccharide-induced inflammation remain controversial (31,32). For example, Shu *et al.* showed that fenofibrate and rosiglitazone failed to modulate the lipopolysaccharide-induced secretion of interleukin-8 from THP-1 cells (31), while Morimoto

et al. showed that pioglitazone did not affect lipopolysaccharide-induced increase in plasma TNF-α levels in a diabetic mouse model (32). Furthermore, as our results showed that fenofibrate increased lipopolysaccharide-induced TNF-α secretion, and pioglitazone increased lipopolysaccharide-induced TNF-α, interleukin-1β and interleukin-6 secretion, it is necessary to explore the mechanisms involved in the stimulatory effect of fenofibrate and pioglitazone on lipopolysaccharide-induced gene expression.

In summary, the present study has shown that a high concentration (25 mM) of glucose markedly enhances lipopolysaccharide-induced expression of inflammatory cytokines and chemokines, but simvastatin largely blocked the enhanced expression. This study therefore uncovered a mechanism by which diabetes with poor glycemic control enhances periodontal disease and suggested that statins may be useful not only in dyslipidemic control but also in reducing periodontal inflammation and probably the progression of the disease.

Acknowledgement

This work was supported by a Merit Review Grant from the Research Service of the Department of Veterans Affairs and NIH grant DE16353 (to Y.H.).

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