

Elevated Glucose and Diabetes promote Interleukin 12 Cytokine Gene
Expression in Mouse Macrophages

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ABSTRACT

Inflammation is emerging as an important mechanism for micro and macrovascular complication of diabetes. The macrophage plays a key role in the chronic inflammatory response in part by generating particular cytokines. Interleukin (IL)-1 β , IL-6, IL12, IL-18, TNF- α and interferon (IFN)- γ are produced primarily in macrophages and have been associated with accelerated atherosclerosis and altered vascular wall function. In this study, we evaluated the effect and mechanism of high glucose (HG) on gene expression of these cytokines in mouse peritoneal macrophages (MPM). HG led to a 2-fold increase in the mRNA expression of these cytokines with IL-12 showing the highest activation (5.4-fold) in a time- (3-12h) and dose-dependent (10, 17.5, and 25 mmol/L) manner. The effects were specific to HG since mannitol and 3-O-methyl-glucose had no effect on cytokine mRNA expression. HG also increased IL-12 protein accumulation from MPM.

We also explored the role of induced and spontaneous diabetes on inflammatory cytokine expression in MPM. Increases in expression in MPM of multiple inflammatory cytokines including a 20-fold increase in IL-12 mRNA was observed in streptozotocin-induced type 1 diabetic mice as well as in type 2 diabetic db/db mice, suggesting that cytokine gene expression is increased by hyperglycemia *in vivo*. We next explored potential mechanisms of HG-induced increases in IL-12 mRNA. HG increased the activity of protein kinase C (PKC), p38 mitogen activated protein kinases (p38), c-Jun terminal kinase (JNK), and I κ B kinase (IKK) in MPM. Furthermore, inhibitors of these signaling pathways significantly reduced HG-induced IL-12 mRNA expression in MPM. These results provide evidence for a potentially important mechanism linking elevated glucose and diabetes to inflammation.

Abbreviations: IL, interleukin; TNF- α , Tumor necrosis factor- α ; MPM, mouse peritoneal macrophage; RT-PCR, reverse-transcription-polymer chain reaction; HG, high glucose; NG, normal glucose. STZ, streptozotocin; PKC, protein kinase C; p38, p38 MAPK, JNK, c-jun-N-terminal kinase; NF- κ B, nuclear factor-kappa B.

INTRODUCTION

The macrophage plays a central role in chronic inflammation in diabetes and atherosclerosis. Macrophages are a major source of pro-inflammatory cytokines including tumor necrosis factor (TNF)- α , (1) interleukin (IL)-1 β (2), IL-6 (3), IL-12, and IL-18. TNF- α , IL-1 β (1, 2), and IL-6, are important cytokines involved in chemotaxis cell adhesion. Evidence shows that IL-12 is an important cytokine that can program T cells to develop into Th1 cells (4, 5). IL-12 and Th1 cell infiltration has emerged as an important pathway for autoimmune diabetes as well as chronic inflammation associated with atherosclerosis (5, 6). Reduction of IL-12 is associated with reduced atherosclerosis in the mouse (7, 8).

Macrophages of alloxan diabetic mice with moderate diabetes (300mg/dl) produced significantly more IL-6 and TNF- α (9). Recent data have also shown that high glucose (HG) treatment activates monocytes and induces an increase in gene expression of TNF- α , IL-1 β , and monocyte chemoattractant protein (MCP)-1 in human THP-1 monocyte like cells (10). However, it is not clear whether HG affects cytokine expression in primary tissue macrophages. Furthermore, how HG affects cytokine gene expression in tissue macrophages has not been evaluated.

The present study is designed to examine the effect and mechanism of high glucose on mRNA expression of cytokines in mouse peritoneal macrophages (MPM) from C57BL6 mice. We find that HG treatment time- and dose- dependently leads to a more than 2-fold increase in mRNA expression of multiple cytokines with a marked increase in IL-12 gene expression. These effects are specific to HG since mannitol and 3-O-methyl-glucose had no effect. We also studied the in vivo relevance of these

findings. The mRNA expression of these cytokines are also markedly increased in MPM from streptozotocin (STZ)-induced diabetic C57BL6 mice as well as from type 2 diabetic db/db mice. This is the first study showing that HG can increase cytokine gene expression and protein production in primed macrophages. These results provide a new explanation for the inflammatory effects of HG.

MATERIALS and METHODS:

Male C57BL6/J (8-10 weeks old) and male db/db (8-10 weeks old) mice were obtained from Jackson Laboratory (Bar Harbor, Maine). Thioglycollate, Streptozotocin, GF-109203X, and Calphostin C were from Sigma (St. Louis, MO). SB-202190 from Biosource International (Camarillo CA), JNK inhibitor 1 and JNK inhibitor 1 control peptide were from Alexis Biochemicals (San Diego, CA), NF- κ B inhibitor and NF- κ B inhibitor control peptide were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA), RNeasy Mini Kit was from Qiagen (Valencia, CA). Culture media, Dulbecco's modified Eagle's medium (DMEM) from ATCC, RPMI-1640, and Hanks' Balanced Salt Solution (HBSS), were from Invitrogen (Carlsbad, CA) All animal studies were approved by our Institutional Animal Safety Committee.

MPM Isolation and Culture:

Mice were injected intra-peritoneal (IP) with 2 ml 4% thioglycollate solution. 3 days later, the ascites of these mice was collected and cells were spun down, re-suspended in RPMI-1640 medium containing 10% FBS and 100 units/ml of penicillin/streptomycin (P/S), seeded and cultured on 100 mm plates for 3 hours in 5% CO₂ incubator at 37 °C. Non-attached cells were carefully washed away by washing cells 4 times with HBSS. The attached MPM were re-suspended in the same medium as

above. MPM number was counted, seeded again on 60-mm culture dishes and incubated overnight. The attached cells comprised >95% MPM as reflected by specific macrophage antibody staining.

MPM treatment.

The MPM were serum depleted from typical RPMI 1640 medium containing 10 mmol/L D-glucose (NG) supplemented with 10% heat-inactive FBS and P/S for 24 hours and were then cultured in varied glucose concentration for certain periods of time. In some experiments cells were pre-treated with one of the following inhibitors: Calphostin C, GF-109203X, SB-202190, JNK MAPK inhibitor, NFκB inhibitor for 30 min. The cells were then incubated in basal or elevated glucose for 3 h in the presence of inhibitors.

Preparation of Streptozotocin-Induced Diabetic Mice

A single STZ injection (freshly dissolved in saline) at dose of 280mg/kg body weight was injected IP into C57BL6 mice. Blood glucose was measured with an Accu-Chek Advantage meter and was expressed as mg/dl. To avoid the effect of streptozotocin itself on cytokine expression, macrophages were isolated at least 10 or more days after STZ injection. All animal protocols were approved by the Institutional Animal Safety Committee of the University of Virginia.

RNA Extraction and Real-Time PCR

Total RNA was extracted from cells using the RNeasy[®] kit (Qiagen Inc. Valencia, CA), with deoxyribonuclease I treatment. The RNA was processed to cDNA synthesis using the SuperScript[™] II reverse transcriptase and oligo d(T) (Invitrogen Carlsbad, CA) according to the manufacturer's protocol. The sequences of forward and reverse

primers for target cytokine genes and house keeping gene GAPDH were selected based on published sequence data from the NCBI database and previous study (11).

Primers were synthesized from Integrated DNA technologies Inc (Coralville, IA). For quantitation, a double-stranded DNA dye, SYBR Green I (Molecular Probes Inc, Eugene, OR) was used along with AmpliTaq Gold and 0.1 μ M of each primer. All reactions were performed in triplicate in iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratory, Hercules, CA). The real-time PCR was carried out in two steps, Step 1: 94 °C for 10 min, one cycle. Step 2: 94 °C for 15 sec, followed by 60 °C for 60 sec, 45 cycles. GAPDH was used as an endogenous reference to correct for differences in the amount of total RNA added to the reaction and to compensate for different levels of inhibition during reverse transcription of RNA and during PCR. Data are calculated using the $2^{-\Delta\Delta CT}$ method (12) and are presented as fold induction of transcripts for target genes normalized to GAPDH in cells treated with HG or inhibitors.

Total RNA of macrophages from db/db and control db/+ mice was extracted with Trizol Invitrogen according to manufacturer's instructions. 1 μ g of total RNA was used for reverse transcription using the GeneAmp RNA PCR kit from Applied Biosystems. 18S primers were used as internal control. The software used for quantitation of PCR data was Quantity one (BioRad).

Cytokine Protein Release Measurement Using ELISA Assay:

Cells were seeded on 24-well plates with each well containing 1×10^6 cells of MPM in 1 ml medium. After serum depletion for 24 hours, cells were then treated either with HG (25 mmol/L D-glucose) or NG (10 mmol/L D-glucose). Conditioned medium was collected in a sequential fashion (e.g. 0-16, 16-24, 24-36, and 36-60 h) with a change of

1 ml fresh medium containing HG or NG at each time point. The collected culture-conditioned medium was centrifuged to separate floating cells in the medium and the supernatant was stored at -80°C . IL-12 protein levels were measured using a specific mouse IL-12p40 immunoassay kit (R & D System, Minneapolis, MN).

Kinase Activity Measurement using Western Blot with Specific Phospho-Antibodies:

50 μg HG-treated protein extracts were resolved on 10% SDS-polyacrylamide gel. Electrophoresis was run under constant voltage conditions. The proteins on the gel were transferred onto Immobilon-P polyvinylidene difluoride (PVDF) membrane from Bio-Rad (Hercules, CA) which was incubated consecutively with specific phospho-antibody and anti-rabbit or anti-mouse Ig conjugated with horseradish peroxidase and the protein bands were detected using ECL detection reagents from Amersham Biosciences Co (Piscataway NJ). The membrane was then stripped using Restore Western Blot Stripping Buffer from Pierce Co (Rockford, IL). and the membranes were incubated with non-phospho-antibody or β -actin and detected using ECL detection reagents. The result presented on figures was a representative of a minimum of 3 experiments using 3 different batches of primary cultured macrophages treated with HG for different times.

Data Analysis:

The results are expressed as mean \pm SEM from combined experiments as noted in each legend. For experiments running at one time period, the control and experimental samples are analyzed using the student's t test. These comparisons are based on a minimum of 3 experiments in triplicate per treatment. For multiple time periods of conditions, ANOVA with Duncan's or Dunnett's test was used.

RESULTS:

HG Increases Cytokine Expression:

Macrophages were incubated with RPMI-1640 medium containing 10% inactive FBS for 24 hours and then treated with NG or HG for the indicated amount of time. mRNA expression was measured with real-time RT-PCR in whole cell RNA extracts. The Fig 1A shows that HG dose-dependently (10, 17.5, and 25 mmol/L) increases the mRNA expression of multiple cytokines including IL-1 β , IL-6, IL-12, IL-18 and TNF- α more than 2-fold over that in NG-treated macrophages. Of these cytokine genes, IL-12 mRNA expression was increased the most. 17.5 mmol/L D-glucose induced a 3.5-fold increase while 25 mmol/L D-glucose induced a 5.5-fold increase in IL-12 gene expression compared to that under NG conditions ($p < 0.01$). HG also increases Interferon γ (IFN- γ) about 2 fold (data not shown). Fig 1B shows the time course of HG-induced mRNA expression of these cytokine genes, which occurred as early as 3 hours and some reached a peak at 3 h and remained elevated for 6 hours after HG treatment. The HG-induced increase in mRNA expression declined at 12 hours of treatment. However, the value remained elevated compared to that under the NG conditions (Fig 1B). At 24 hours of HG treatment, the HG-induced mRNA expression of most cytokine genes returned to the value seen under NG conditions (data not shown). To examine whether high osmotic pressure plays a role in HG-induced increase in cytokine gene expression, IL-12 was taken as an example. Macrophages were treated with 10 mmol/L D-glucose + 15 mmol/L of mannitol or 3-O-methyl-glucose (3OMG) for 3 hours and IL-12 gene expression was measured. Fig 1C shows that supplementation with 15 mmol/L of mannitol or 3OMG did not change the IL-12 gene expression compared to that under NG conditions.

We next examined whether the HG could alter IL-12 protein levels. MPM were treated either with NG or HG. Conditioned medium was collected in a sequential fashion at interval of 0-16, 16-24, 24-36, and 36-60 hours with changes of 1 ml fresh medium containing HG at each time interval. Released IL-12p40 protein was examined using a specific mouse IL-12p40 immunoassay kit. Figure 1D demonstrates that HG significantly induces IL-12 secretion at the interval of 0-16 hours. We did not see increases at other intervals (data not shown). Therefore, HG treatment significantly increases both IL-12 mRNA and protein expression.

Enhanced Cytokine Gene Expression in Macrophages from STZ-Induced Diabetic Mice and Diabetic db/db Mice:

To test the in vivo effects of diabetes on cytokine expression, macrophages were isolated from either saline injected control mice or STZ-injected mice. The baseline blood glucose of the mice before getting STZ was 140, 152, 141, and 161 mg/dl (mean = 148.5 ± 5.0). Thioglycollate was injected on day 8 and macrophages were isolated on day 11. The blood glucose of the STZ-induced diabetic mice at the time of euthanasia was 405, 444, 468, and 474 mg/dl (447.7 ± 15.7 , $p = 0.0002$ vs control mice). Macrophages from saline injected mice were taken as controls. Thioglycollate injection did not change the value of blood glucose in C57BL6 mice with 141.1 ± 5.6 mg/dl before injection and 154.5 ± 6.4 mg/dl after injection ($n = 10$, $p = 0.135$). Fig 2A shows that hyperglycemia significantly increases mRNA expression of the cytokine genes of IL-1 β , IL-6, IL-12, IL-18, and TNF- α in MPM from STZ-induced diabetic mice compared to that in macrophages from non-diabetic control mice. Noteworthy, a marked, nearly 20-fold and 9-fold increase in IL-12 and IL-1 β mRNA expression could

be seen in MPM from STZ-induced diabetic mice. Macrophages from spontaneous type 2 diabetic db/db (8-10 week-old male) mice were also used for examining the effects of hyperglycemia on cytokine gene expression. In these experiments, macrophages from 8-9 weeks old male heterozygotes from same mice colony (db/+) were taken as controls. The blood glucose of the db/+ and db/db mice before euthanized was 160 ± 24 and 548 ± 63 mg/dl ($p < 0.01$ vs db/+), respectively. Fig 2B shows that IL-1 β expression was increased almost 2-fold ($p < 0.0001$), IL-6 expression was increased about 17-fold ($p < 0.0001$), IL-12 expression was increased about 9-fold ($p < 0.0001$), and TNF- α was increased about 1.7-fold ($p = 0.0011$) in macrophages from db/db mice compared with that in macrophages from control db/+ mice. These data indicate that hyperglycemia *in vivo* is a potent stimulus for mRNA expression of inflammatory cytokines in macrophages.

Role of PKC in HG-Induced IL-12 mRNA Expression:

PKC is clearly one of the mechanisms associated with HG-induced complications. We first examined whether HG increased PKC activity in primary cultured macrophages. PKC activation was examined using western blot with a specific anti-phosphor-PKC (pan) (γ Thr514) antibody and specific anti-phosphorylated MARCKS antibody. Fig 3A shows a time dependent increase in PKC activity, illustrating that γ Thr514 phosphorylated PKC was increased at 5-10 minutes and a peak at 30 minutes of HG treatment. The density of phosphorylated MARCKS bands had a similar pattern (Fig 3B) showing an increase at 5-10 minutes and a peak at 30 minutes of HG treatment. We then tested whether 2 structurally distinct PKC inhibitors, GF-109203X or Calphostin C attenuated HG effects on IL-12 gene expression. After serum depletion,

macrophages were pretreated with fresh RPMI-1640 medium containing either GF-109203X (100 nmol/L), Calphostin C (100 nmol/L) or vehicle control DMSO for 30 min. MPM were then treated with HG or NG for 3 hours. Fig 3C shows that GF-109203X or Calphostin C completely inhibited HG-induced IL-12 gene expression, suggesting that PKC activation is important in HG effects on IL-12 expression. GF-109203X or Calphostin C alone at concentrations studied had no significant effect on IL-12 gene expression under NG condition.

Role of P38 MAPK in HG-Induced IL-12 Expression

Fig 4A shows a time course of p38 MAPK activity detected by western blot using a specific anti-phospho-p38 antibody. HG-induced p38 activity occurred as early as the 5 minute-treatment period and reached a peak at 10 minutes. β -actin on the stripped membrane was detected with anti- β -actin antibody, which showed equal protein loading among different samples. We also tested the effect of the relatively specific p38 MAPK inhibitor, SB 202190. After serum depletion, macrophages were pretreated with fresh RPMI-1640 medium containing either 2 μ mol/L SB 202190 or DMSO vehicle for 30 min. MPM were then treated with HG or NG for 3 hours. Fig 4B shows that even though SB 202190 alone had a slight effect to increase basal IL-12 gene expression in NG, SB 202190 almost completely inhibited HG-induced IL-12 gene expression. These results suggest that p38 MAPK is also important for HG action.

Role of JNK in HG-Induced IL-12 Gene Expression:

cJun amino terminal kinase (JNK) has been implicated in the cascade of glucose-induced complications. Fig 5A shows that HG activates JNK in cultured macrophages. To explore whether the JNK pathway is involved in HG action on IL-12, we tested the

effect of the specific JNK inhibitor, JNKI-1, a peptide which competitively blocks the interaction between JNK and c-Jun. After depletion, macrophages were pretreated with fresh RPMI-1640 medium containing 10 $\mu\text{mol/L}$ JNKI-1 or 10 $\mu\text{mol/L}$ control peptide for 30 min, MPM were then treated with HG or NG for 3 hours. Fig 5B shows that JNKI-1 significantly inhibited HG-induced IL-12 gene expression while the JNKI control peptide had no effect. Neither the JNKI nor the JNKI control peptide had an effect on IL-12 mRNA expression under NG conditions. These data suggest that the JNK pathway is involved in HG-induced IL-12 mRNA expression.

HG activates IKK and an NF κ B Inhibitor prevents HG-induced IL-12 Gene Expression:

NF κ B is also central to glucose-induced complications and oxidative stress. Since the IKK complex appears to be critical for NF κ B activation, IKK was used as an indicator of NF κ B activation. Fig 6A shows that HG time-dependently activated IKK activity. To explore the role of the NF κ B pathway in our model, we tested whether an NF κ B inhibitor could attenuate HG effects on IL-12 gene expression. After serum depletion, macrophages were pretreated with fresh RPMI-1640 medium containing 75 $\mu\text{g/ml}$ of a specific NF κ B inhibitor or 75 $\mu\text{g/ml}$ control NF κ B inhibitor (inactive control for NF κ B inhibitor) for 30 min. MPM were then treated with HG or NG for 3 hours. Fig 6B shows that the active NF κ B inhibitor completely blocked HG-induced IL-12 gene expression while the NF κ B inhibitor control had no effect. Neither the NF κ B inhibitor nor the NF κ B control compound altered IL-12 mRNA expression under NG conditions. These data suggest that the NF κ B pathway is implicated HG action on IL-12 mRNA expression.

DISCUSSION

In the present study, we investigated the effect of high glucose on the mRNA and protein expression of inflammatory cytokines. This study demonstrates that high glucose induces a significant increase in the mRNA expression of several important inflammatory cytokines including IL-1 β , IL-6, IL-12, and TNF- α . Taking IL-12 as an example, we have demonstrated that the HG-induced increase of mRNA expression was not elicited by high osmotic pressure since 25 mmol/L concentration of D-glucose + mannitol or of D-glucose + 3-O-MG did not change the mRNA expression of IL-12. We also demonstrate that high glucose induced an increase in IL-12 protein secretion from macrophages. This is the first observation showing that high glucose activates mRNA expression of multiple cytokines in primary cultured MPM. We further demonstrate that hyperglycemia increases the mRNA expression of multiple cytokine genes in macrophages isolated from STZ-induced diabetic mice as well as type 2 diabetic db/db mice, showing that the stimulatory effects of hyperglycemia on cytokine gene expression exists in vivo and suggesting that glucose independent of autoimmunity can activate these important cytokines.

Activation of IL-12 and downstream transcription factors have been linked to atherosclerosis in mouse models and in humans (6-8). Therefore, IL-12 was taken as an example to further investigate the regulatory mechanism of HG stimulation on cytokine mRNA expression.

It has been well established that increasing extra-cellular glucose concentration from 5.6 mmole/L to 22.2 mmol/L appears sufficient to accomplish an activation of diacylglycerol (DAG) and protein kinase C (PKC) (13). DAG-PKC activation has been considered as an independent biochemical pathway involved in the pathogenesis of

diabetic complications and is involved in the regulation of various cell functions including cytokine actions (14). There was a report showing an involvement of PKC in high glucose-induced monocyte chemotactic peptide-1 (MCP-1) in human peritoneal mesothelial cells (15). Interestingly, (MCP-1) is one of the target genes produced by downstream transcription factor activation by IL-12. We first demonstrated that HG activated PKC activity by detecting an increase in the amount of phospho-PKC (pan) at γ Thr514 using specific anti-phospho-PKC (pan) γ Thr51 antibody. It can be seen that HG time-dependently increases the density of phospho-PKC (pan). MARCKs protein is a widely accepted indicator of PKC activation. The level of phosphorylated MARCKS is generally believed to reflect PKC activation. Our data clearly illustrated that HG increases the density of phosphorylated MARCKS protein and the increase of the density of phosphor-MARCK protein has a same time pattern with that of the density changes of phospho-PKC (pan) protein, strongly indicating that HG activates PKC activity in primary cultured macrophages. To investigate the possible involvement of PKC in HG-induced increases in IL-12 mRNA expression, two specific and structurally distinct PKC inhibitors, GF 109230X and Calphostin C were used. Both inhibitors completely blocked HG-induced IL-12 mRNA expression, strongly suggesting a role of DAG-PKC activation in HG-induced IL-12 mRNA expression. The PKC family includes at least eleven isoforms. There are reports showing that macrophages from various tissues express multiple types of PKC isoforms including α , β 1 and 2, γ , ϵ , η , and ζ . However, it has been hypothesized that macrophage expression/function of PKC isoforms is tissue specific (alveolar vs peritoneal) (16). Further studies will be necessary

to elucidate the types of PKC isoforms that MPM express and which isoform(s) of PKC mediate HG-induced IL-12 mRNA expression in murine peritoneal macrophages.

There is increasing evidence demonstrating a critical role of mitogen-activated protein kinase activation in the inflammatory response of macrophages (17). Evidence also shows that mitogen-activated protein kinase phosphatase-1 (MKP), which dephosphorylates and inactivates these MAP kinases plays an important role in cytokine production induced by agonists. Stimulation of primary macrophages isolated from MKP-1-deficient mice by LPS resulted in a prolonged p38 MAPK phosphorylation and more robust and rapid TNF α production compared to wild type macrophages (18). In the present study, we have not only demonstrated that HG activated p38 activity in macrophages, which is consistent with other reports, but also, a p38 MAPK inhibitor, SB202190, significantly reduced HG-induced IL-12 mRNA expression, suggesting an involvement of the p38 MAPK pathway. Our data is consistent with the data in a report showing that the inhibition of the phosphorylation of p38 MAPK is concomitant with a down regulation of IL-12p40 production (19).

c-Jun N-terminal kinase (JNK) is an inflammation-related MAPK (20). In the present study, we used JNKI-1 as a relatively specific inhibitor for JNK1, JNK2, and JNK3. JNKI-1 works by inhibiting the interaction between JNK and its substrate (21). JNKI-1 almost completely suppressed HG-induced IL-12 mRNA expression without effect on basal IL-12 mRNA expression. In contrast, the JNK inhibitor 1 control peptide, an inactive analogue of JNKI-1, did not block HG-induced IL-12 mRNA expression, further supporting the conclusion that JNKI-1 specifically inhibited and JNK mediated HG-induced IL-12 mRNA expression.

JNK has been directly linked to NF κ B regulation (22). Furthermore, evidence shows that IL-12 is an inducible, heterodimeric disulfide-linked cytokine composed of p35 and p40 subunits. Expression of the p35 subunit is constitutive and ubiquitous (23), while the biological activity of IL-12 is regulated mainly by the induction of p40, which reported to be regulated primarily at the level of transcription including NF κ B (24). For NF κ B activation, phosphorylation of I κ B kinase (IKK) appears to be critical. Therefore, we chose the phosphorylation of IKK as an indicator of NF κ B activation. Our data clearly show an increase in the amount of phosphorylated NF κ B in HG conditions vs in NG condition, demonstrating a stimulatory effect of HG on NF κ B activation. NF κ B inhibitor which contains the nuclear localization sequence residues 360-369 of NF κ B p50 and inhibits translocation of the NF κ B active complex into the nucleus (25), was used for evaluating whether NF κ B is involved in the regulation of HG-induced IL-12 mRNA expression. Our results demonstrate that blockade of the NF κ B translocation completely suppresses HG-induced IL-12 mRNA expression, strongly suggesting an involvement of NF κ B in the action of glucose.

We haven't pursued in this study whether there is a signaling linearity connecting PKC, p38 MAPK, JNK, and NF κ B. Data have shown that pretreatment of neutrophils with the structurally distinct PKC α/β inhibitors Go6976 or GF109203X decreased nuclear translocation of NF- κ B and production of the pro-inflammatory cytokine TNF- α . These inhibitors also prevented LPS-induced phosphorylation of IKK α/β , phosphorylation and degradation of I κ B- α , as well as phosphorylation of the 65 subunit of NF κ B. Activation of p38 and JNK also was diminished in neutrophils in which PKC α/β was inhibited (26). These data clearly suggest a cascade involving PKC to

MAPK to NFκB to cytokine production in neutrophils. The signal transduction linking PKC, MAPK, NFκB in HG-induced IL-12 mRNA expression will require further investigation. However, elegant studies have shown that a role for mitochondrial induced oxidative stress as a common pathway leading to glucose-induced complications (14). It will be interesting in future studies to explore the role of the mitochondria in glucose-induced IL-12 production in macrophages. Our studies also are supported by a recent report showing a key role of oxidative stress in generating IL-12 in mouse peritoneal macrophages (27).

Summary

In the present study, high glucose induced an increase in multiple pro-inflammatory cytokines in MPM, *in vitro* and in 2 diabetic mouse models. This increase induced by high glucose was not due to osmotic pressure, suggesting that this is a specific effect by high glucose. IL-12 has been taken as an example and our data show that PKC, p38 MAPK, JNK, and NFκB are involved in the regulation of HG-induced IL-12 gene expression. These results suggest that glucose-induced macrophage generation of pro-inflammatory could be involved in both short term and long term vascular complication of diabetes.

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FIGURE LEGENDS:

Figure 1. Effects of HG on mRNA expression of cytokine genes in MPM.

MPM were treated under HG conditions for the indicated time and total cellular RNA was extracted for real time RT-PCR analysis. Data were calculated by $2^{-\Delta\Delta CT}$ method and presented as fold induction of transcript for IL-12 gene normalized to GAPDH in cells treated under HG condition over that under NG condition. Results shown are mean \pm SE from three experiments run in triplicate. ** $p < 0.01$ vs. NG.

A: Dose-dependent effects of HG on mRNA expression of cytokine genes in MPM. **B:** Time-dependent effects of HG on mRNA expression of cytokine genes in MPM. **C:** High osmolality did not induce IL-12 mRNA expression. MPM were incubated under NG, NG plus 15 mmole/L 3OMG or 15 mmole/L mannitol, or NG plus 15 mmole/L D-glucose (HG) conditions for 3 hours. **D:** IL-12 protein production. MPM were seeded in 24-well plates with each well containing 1×10^6 cells. Cells were treated with NG or HG for 4 sequential intervals. The conditioned medium was analyzed for secreted IL-12 protein by ELISA using a specific IL-12 p40 antibody. HG induced IL-12 protein release to the medium at the time interval of 0-16 h. Values are the mean \pm SE of triplicate cultures. Representative of 3 experiments. * $P < 0.05$ vs NG, ** $P < 0.01$ vs NG, *** $P < 0.001$ vs NG.

Figure 2. Enhanced mRNA expression of cytokine genes in STZ-treated diabetic mice and db/db mice.

A: STZ or saline was injected IP into 4 mice each. Thioglycollate was injected IP to control or STZ groups on day 8. MPM were isolated and total cellular RNA was

extracted for real time RT-PCR analysis as in Fig 1. Results shown are mean \pm SE from three experiments run in triplicate. **** $P < 0.001$ vs. control. **B:** Peritoneal macrophages were isolated from diabetic db/db mice and non-diabetic db/+ littermates. Total RNA extracted from these macrophages was subjected to relative RT-PCR analysis using gene-specific primers (for mouse IL-1 β , TNF- α , IL-6 and IL-12) and 18S primers as internal control. PCR products were fractionated on 1.5% agarose gels and photographed using an Alphamager 2000 Documentation and Analysis system. Density of DNA bands corresponding to amplified products and 18S RNA was determined with Quantity One software (BioRad). Results are expressed as ratio of specific band to 18S RNA internal standard. Results shown are mean \pm SEM from three separate experiments, db/db vs control is $p < 0.001$.

Figure 3. HG activates PKC activity and the inhibitory effect of PKC inhibitors on HG-induced IL-12 mRNA expression. **A:** PKC activity was measured by detecting the density changes of phosphor-proteins using western blot method. 50 μ g of macrophage protein was resolved on 10% SDS-polyacrylamide gel and the proteins on the gel were transferred on Immun-Blot PVDF membrane and detected by specific phosphor antibody. **A:** Time-course of HG stimulatory effect on the amount of phosphor-PKC (pan) (γ Thr514). **B:** Time-course of HG stimulatory effect on the amount of phosphor-MARCKS. **C:** The inhibitory effects of PKC inhibitors on HG-induced IL-12 mRNA expression.

MPM were pre-treated with calphostin C (100 nmole/L) or GF-109203X (100 nmole/L) in NG medium for 30 min. The cells were then incubated in NG or HG media for an

additional 6 h. Total cellular RNA was extracted for real time RT-PCR analysis as in Fig 1. Results are mean \pm SE of three independent experiments run in triplicate. ** p < 0.01 vs. NG; ***p <0.01 vs. HG.

Figure 4. HG activates p38 kinase and effect of p38 kinase inhibitor on HG-induced IL-12 mRNA expression. **A:** Time course of HG stimulatory effects on phosphor-p38 protein detected by western blot. The western blot conditions were the same as in Fig 3A. **B:** Inhibitory effects of p38 inhibitor on HG-induced IL-12 mRNA expression. MPM were pre-treated with either SB 202190 (2 μ mole/L) or vehicle in NG or HG medium for 30 min. The cells were then incubated in NG or HG media for an additional 3 h. Total cellular RNA was extracted for real time RT-PCR analysis as in Fig 1. Results are mean \pm SE of three independent experiments run in triplicate. * p < 0.05 vs. NG; *p <0.05 vs. HG.

Figure 5. HG activates JNK activity and effect of JNK inhibitor on HG-induced IL-12 mRNA expression. **A:** Time course of HG stimulatory effects on phosphor-JNK protein detected by western blot. The western blot conditions were the same as in Fig 3A. **B:** Inhibitory effects of JNK inhibitor on HG-induced IL-12 mRNA expression. MPM were pre-treated with or without JNKI-1 (10 μ mole/L) or its control peptide (10 μ mole/L) in NG or HG medium for 30 min. The cells were then incubated in NG or HG media for an additional 3 h. Total cellular RNA was extracted for real time RT-PCR analysis as in Fig 1. Results are mean \pm SE of three experiments run in triplicate. ** p < 0.01 vs. NG; ***p <0.01 vs. HG.

Figure 6. HG activates IKK activity and effect of NFκB inhibitor on HG-induced IL-12 mRNA expression. **A:** Time course of HG stimulatory effects on phosphor-IKK protein detected by western blot. The western blot conditions were the same as in Fig 3A. **B:** Inhibitory effects of NFκB inhibitor on HG-induced IL-12 mRNA expression. MPM were pre-treated with NFκB inhibitor 1 its control peptide (75μg/ml) in NG or HG medium for 30 min. The cells were then incubated in NG or HG media for an additional 3 h. Total cellular RNA was extracted for real time RT-PCR analysis as in Fig 1. Results are mean ± SE of three experiments run in triplicate. ** p < 0.01 vs. NG; **p <0.01 vs. HG.

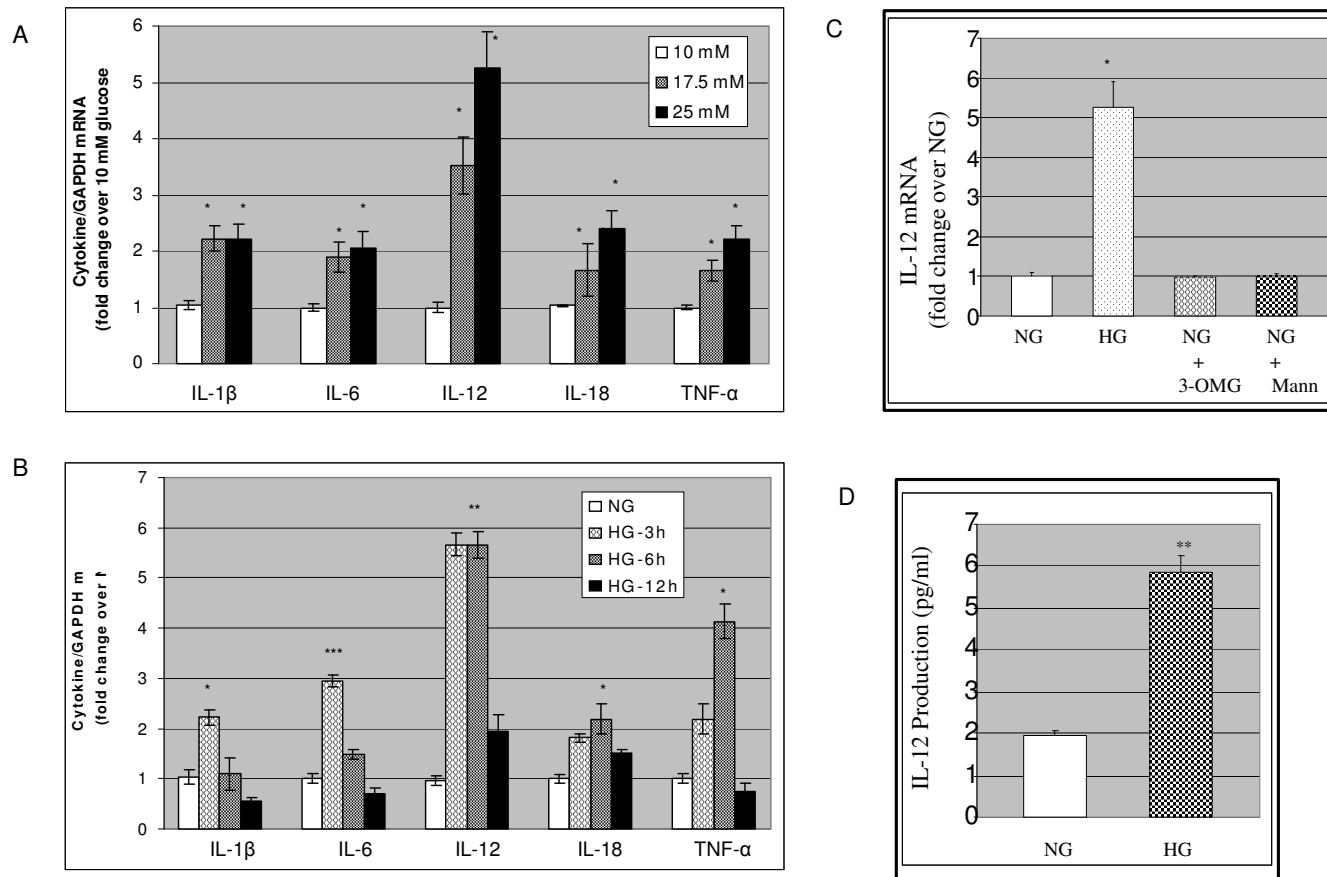


Fig.1

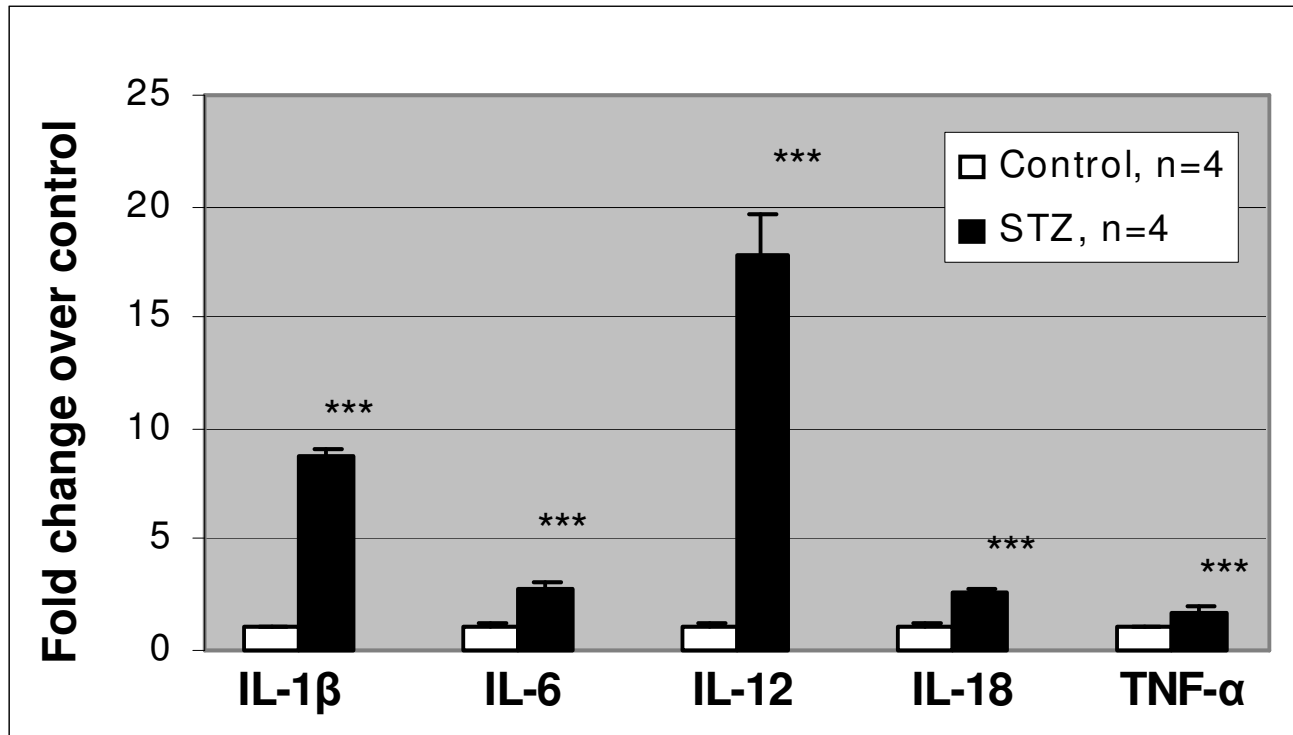


Fig. 2A

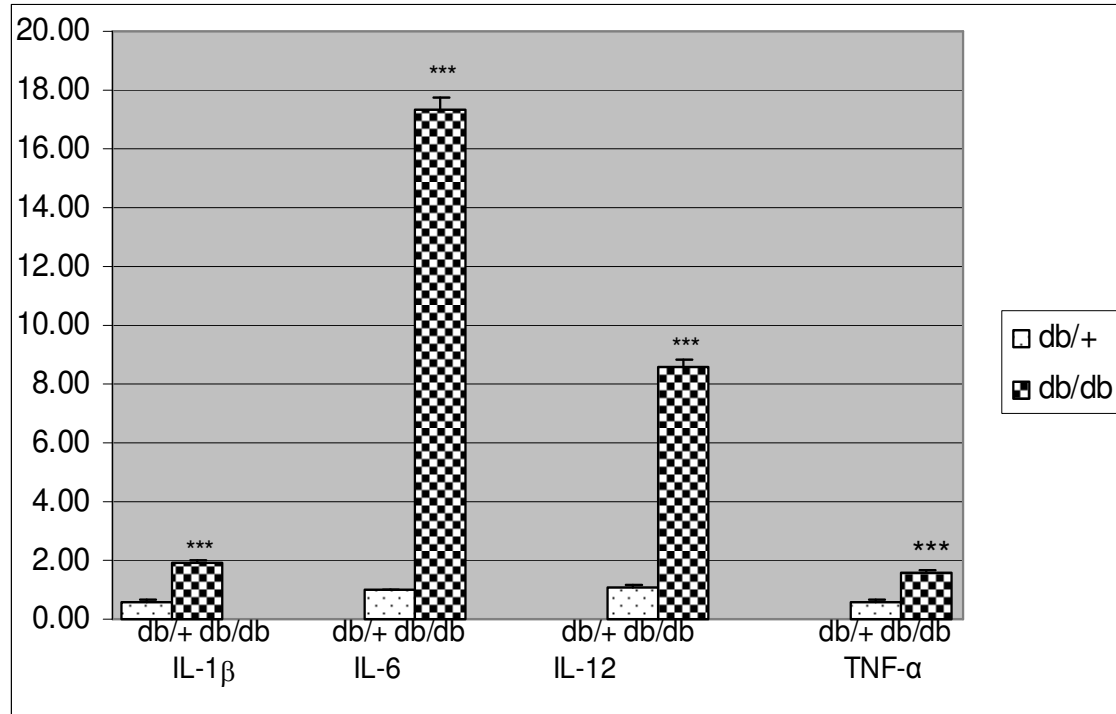


Fig 2B

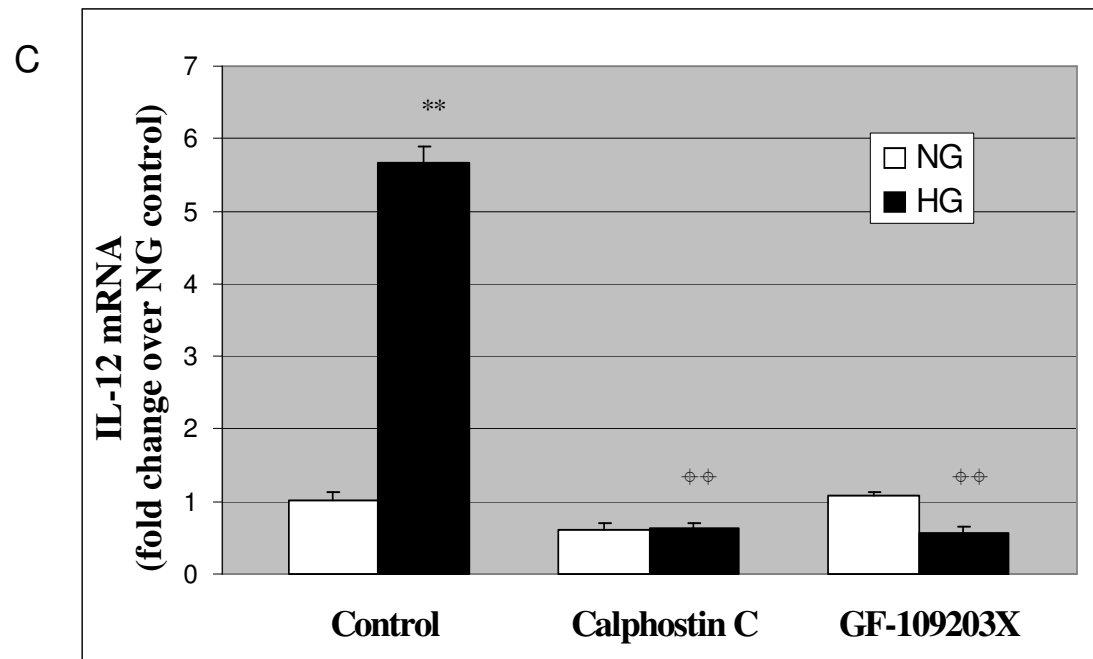
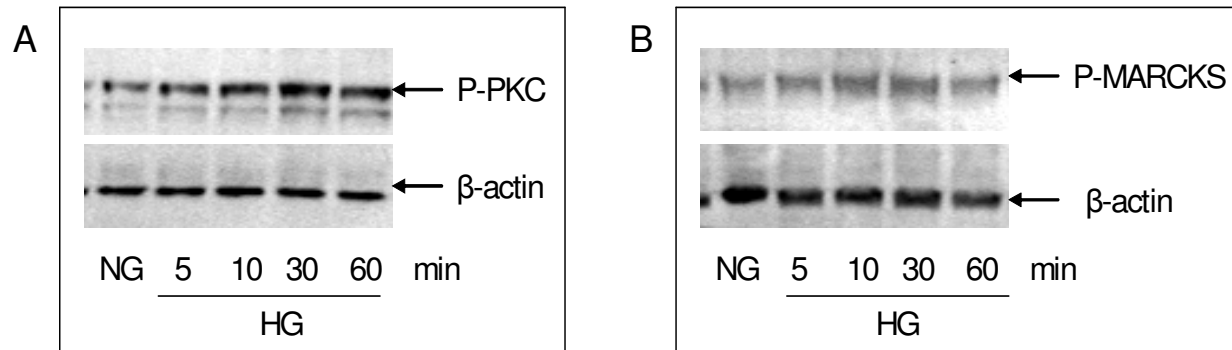


Fig 3

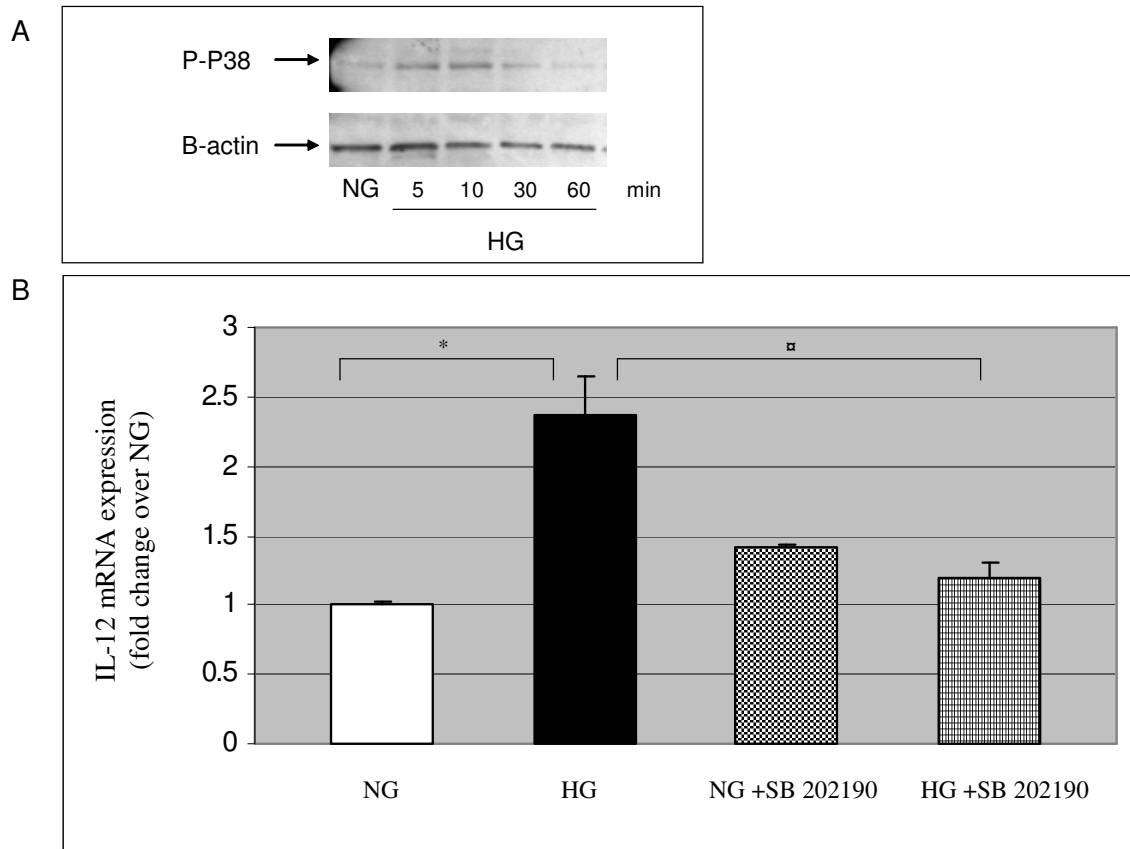


Fig.4

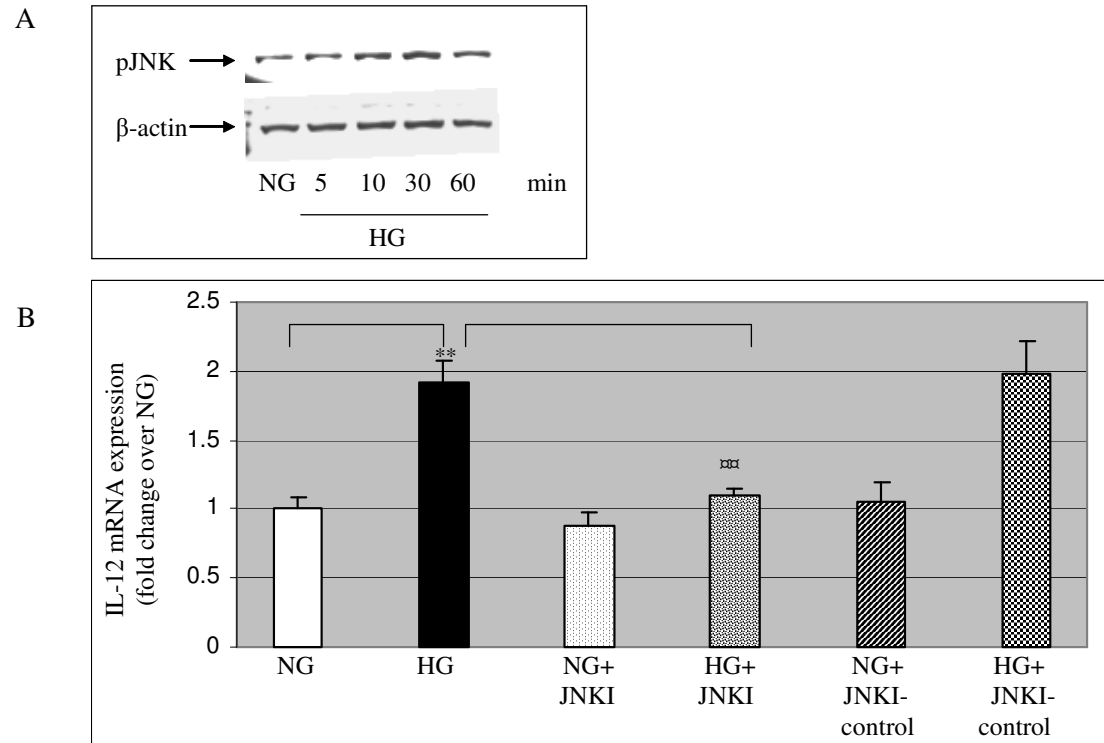


Fig.5

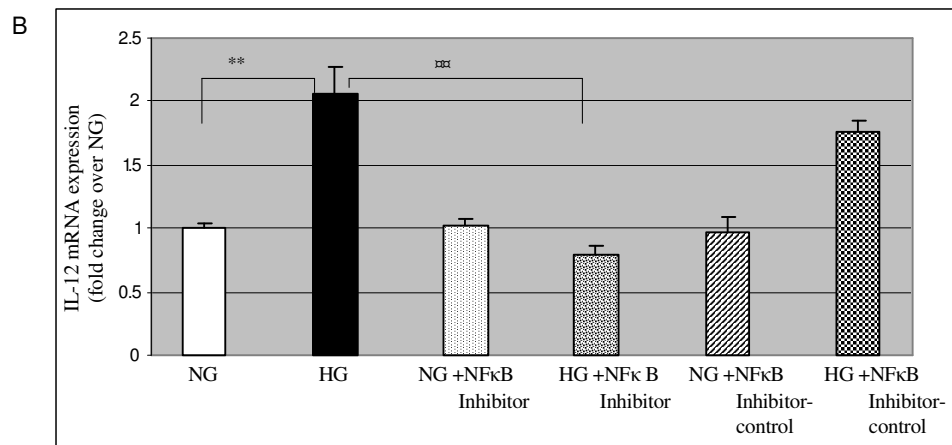
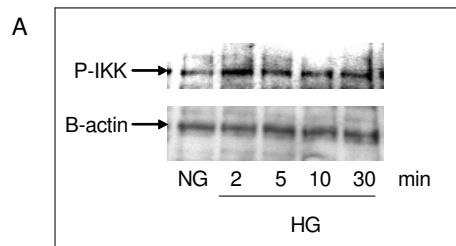


Fig.6