O-GlcNAc transferase inhibits LPS-mediated expression of inducible nitric oxide synthase through an increased interaction with mSin3A in RAW264.7 cells

So-Young Hwang, Ji-Sun Hwang, Song-Yi Kim, and Inn-Oc Han

Department of Physiology and Biophysics, College of Medicine, Inha University, Incheon, Korea

Submitted 6 February 2013; accepted in final form 10 June 2013

---

MACROPHAGES APPEAR TO BE THE major initial sensors of danger from septic or foreign signals recognized by Toll-like receptors. Such cells secrete inflammatory mediators including TNF-α, IL-1β, and nitric oxide, which in turn act to control inflammation (21, 26). Although some inflammatory stimuli have beneficial effects and are linked to tissue repair processes, excessive and severe inflammation may contribute to pathogenesis of acute septic shock and/or chronic diseases such as obesity, diabetes, arthritis, heart disease, cancer, or neurodegenerative diseases (7, 15, 26).

In our previously published study (10), we investigated the protective effect of glucosamine (GlcN) in ischemia- or LPS-induced inflammatory responses in the brain. Thus hexosamine biosynthesis pathway may play an important role in regulating the extent of inflammatory responses activated after exposure of cells to potent inflammatory stimuli. In the cell, GlcN elevates the level of UDP-N-acetylglucosamine (UDP-GlcNAC), which is one of its direct substrates in the transfer of O-GlcNAc to proteins via the action of O-linked N-acetylglucosamine (O-GlcNAc) transferase (OGT). GlcN also resulted in the hypo-GlcNAcylation of mSin3A, which was directly or indirectly associated with increased iNOS transcription by modulating histone acetylation either directly or indirectly.

OGT; O-GlcNAc; c-Rel; mSin3A

---

Address for reprint requests and other correspondence: I.-O. Han, Dept. of Physiology and Biophysics, College of Medicine, Inha Univ., 7-241, Shinheung-Dong, Jung-Ku, Incheon, 400-712, Korea. (e-mail: iohan@inha.ac.kr).

http://www.ajpcell.org 0363-6143/13 Copyright © 2013 the American Physiological Society

subsequent iNOS induction via increases in the OGT association and O-GlcNAcylation of p65 at least in BV2 mouse microglial cells and RAW264.7 macrophage cells (10). In the present study, we demonstrate that LPS-treated mouse macrophage cells show increased OGT association. This consequently induces increased recruitment of OGT and mSin3A to NF-κB binding sites, while mSin3A, in turn, targets HDAC1 and HDAC2 to iNOS promoter. In contrast, GlcN treatment decreased the recruitment of OGT, suggesting that OGT functions as a transcriptional repressor during inflammatory stimulation but not inflammatory suppression. This activity functions in concert with the activities of other transcriptional complexes to inhibit overactivation and/or fine-tune the inflammatory response.

MATERIALS AND METHODS

Reagents. Except where otherwise noted, all reagents were purchased from Sigma Chemical (St. Louis, MO) and were of the highest quality available. Cell cultures. RAW 264.7 mouse macrophage cells were maintained at 37°C at 5% CO2 in DMEM supplemented with 10% FBS (HyClone, Logan, UT), streptomycin (100 μg/ml), and penicillin (100 U/ml) all purchased from Sigma, (Ref. 13).

Immunoprecipitation and immunoblotting. Whole cell protein lysates were prepared in lysis buffer (10 mM Tris, 140 mM NaCl, 1% Triton, 0.5% SDS, and protease inhibitors, pH 8.0). For immunoprecipitation, 500 μg of cell lysates were incubated with anti-OGT (Sigma) or anti-mSin3A (Santa Cruz Biotechnology) antibody for 1 h. The antibody-protein complex was precipitated with protein G-Sepharose beads and analyzed by Western blotting. For Western blotting, protein samples (20 μg for each) were separated by SDS-PAGE and transferred to Hybond-ECL nitrocellulose membrane (Amersham Biosciences, Piscatway, NJ). The membrane was incubated with antibodies (Santa Cruz Biotechnology, otherwise noted) against OGT (Sigma), mSin3A, HDAC1, HDAC2, HDAC3, iNOS, β-actin, or anti-O-GlcNAc (Covance, Berkeley, CA) antibodies (8). Next, horse-radish peroxidase-conjugated secondary antibodies (Amersham Biosciences, Piscatway, NJ; 1:10,000 dilution in TBS Tween) were applied and developed by the ECL detection system (Amersham Biosciences). Densitometric scanning and quantification of the intensities in Western blot bands were carried out using ImageJ (National Institutes of Health; Windows version).

Galactosyltransferase labeling. Galactosyltransferase labeling assays were performed as described previously (12). In brief, protein lysates were immunoprecipitated with anti-mSin3A antibody and mixed with labeling buffer (5 mM MnCl2, 10 mM galactose, and 50 mM HEPES pH 7.4). Galactosyltransferase labeling reactions were initiated by adding 2 μCi UDP-[3H]galactose (American Radiolabeled Chemicals) in 5’-AMP solution (2.5 mM 5’-AMP) and 50 μM galactosyltransferase (Sigma) and incubating at 37°C for 1 h. Reaction mixture proteins were separated by SDS-PAGE, intensified with EN3HANCE fluor (Perkin Elmer), dried, and exposed to X-ray film.

Biotin pull-down assay. Biotin pull-down assays were performed as described previously, with minor modifications (5). The procedure measures the quantitative binding of transactivators or molecules of interest to a specific probe: a 20-nucleotide sequence containing the NF-κB binding site (5’-GCTAGGGGATTTCTCCT-3’) at position −957/−977 of the iNOS promoter. Two complementary DNAs were synthesized and biotinylated by Bioneer (Daejeon, Korea) and annealed to generate a double-stranded probe. Binding assays were performed by incubating 500 μg nuclear protein extracts with 2 μg biotinylated DNA probe and 25 μl streptavidin-conjugated agarose

Fig. 1. Regulation of promoter activation of NF-κB and inducible nitric oxide synthase (iNOS) and transcriptional activities of p65 and c-Rel by O-linked N-acetylgalactosamine (O-GlcNAc) transferase (OGT) in response to LPS and/or GlcN stimulation. RAW 264.7 cells were transfected with NF-κB reporter (A), iNOS promoter reporter (B), pFR-luc reporter containing 5 GAL4 binding sites in its promoter plus p65 (C) or c-Rel (D) chimeric protein fused to GAL4 DNA binding domain (DBD-c-Rel). In some conditions, OGT plasmid was cotransfected. Cells were then treated with GlcN (5 mM) and/or LPS (0.1 μg/ml) and Luciferase activity, in relative light units (RLU), was measured at 24 h. *P < 0.05, compared with LPS treated without OGT cotransfection. **P < 0.05, compared with LPS + GlcN treated without OGT cotransfection.
beads for 1 h. DNA-protein complexes were analyzed by Western blotting using the indicated antibodies.

**Transient transfection and luciferase assay.** The NF-κB reporter contained three copies of the κB-binding sequence fused to the firefly luciferase gene (Clontech, Mountain View, CA). An iNOS promoter containing a 973-bp region upstream from the transcription start site has been described previously (13). Expression plasmids pCMX-mSin3A and pcDNA3-HA-OGT were kindly provided by Dr. J. E. Kudlow (29). The expression vector pCI-p300 and pCMV-cAMP response element-binding protein-κB-binding protein (CBP) were obtained from Dr. E. Kieff. The pFR-luc vector was purchased from Stratagene (La Jolla, CA). Gal4-p65 (1–551) and Gal4-c-Rel (309–588) were generous gift from Dr. M. Fresno (18). Cells were transfected by electroporation (Microporator; Digital Biotechnology). Luciferase activity was assayed 24 h after transfection and normalized to transfection efficiency using a cotransfected β-galactosidase plasmid. Bioluminescence was measured using a Turner Designs luminometer (TD-20/20).

**Chromatin immunoprecipitation assay.** Chromatin immunoprecipitation (ChiP) assays were performed as described previously (10). Briefly, formaldehyde (1%) was added to the culture medium for cross-linking reaction and cell lysates were prepared. Samples were sonicated on ice (5 min/sample total sonication time) in a Bioruptor (COSMO Bio). Twenty percent of total supernatant was saved as total input control, and the rest was precleared with 20 μg normal IgG, and 50 μl protein G-Sepharose for 2 h. The precleared lysates were immunoprecipitated by specific antibodies against c-Rel, mSin3A, HDAC1, HDAC2, HDAC3, acetylated-H2A, acetylated-H2B, acetylated-H3, or acetylated-H4 (Santa Cruz Biotechnology). Rabbit Ig or Mouse Ig (Dako) was used for immunoprecipitation as a negative control. After being washed, the immunoprecipitates were eluted with elution buffer. The eluted immunocomplex was treated with RNase A overnight at 65°C, and the contained proteins were removed by treatment with EDTA, 1 M Tris-Cl (pH 6.5), and proteinase K at 42°C for 1 h. The remaining DNA was extracted using a DNA purification kit (Qiagen). The gene promoter sequences in the immunoprecipitates were amplified by PCR or quantitative real-time PCR using the primers for iNOS promoter forward (–904): GTGTACCTCAGACAAGGGC and reverse (–1058): CACACATGGCATGGAATTTT. The final PCR products were electrophoresed in 1% agarose gel in Tris-borate-EDTA buffer.

**Quantitative real-time PCR.** Precipitated DNAs were used as templates for PCR amplification, and products were quantitatively detected by measuring incorporation of fluorescent SYBR green into double-stranded DNA (iCycler iQ; Bio-Rad). Relative DNA levels were calculated from the PCR profiles of each sample using the threshold cycle (Ct), corresponding to the cycle at which a statistically significant increase in fluorescence occurred. Ct is considered the amount of template present in the starting reaction. To correct for differences in the amount of total cDNA in the starting reaction, Ct values for an endogenous control (input DNA) were subtracted from those of the corresponding sample. Each experiment was repeated at least three times, and GAPDH was used as housekeeping gene for endogenous control. The ChIp-qPCR Negative Control Primers (Qiagen) were used to measure the amount of no-specific genomic DNA that coprecipitates during a ChiP procedure.

![Fig. 2. Regulation of protein-association of OGT and mammalian Sin3A (mSin3A) in response to LPS and/or GlcN stimulation. RAW 264.7 cells were pretreated with GlcN (5 mM) and stimulated with LPS (0.1 μg/ml) for 24 h. A: Total cell lysates were prepared and lysates were immunoprecipitated (IP) with anti-OGT or anti-mSin3A antibody. Immunoprecipitated proteins were subjected to Western blotting with antibodies against OGT or mSin3A. Relative band intensities of precipitated mSin3A and OGT protein was normalized to input. B: nuclear extracts were prepared and binding proteins to the iNOS promoter-derived, biotinylated NF-κB probe was measured by streptavidin-agarose pull-down assay followed by Western blotting for OGT and mSin3A, as described in MATERIALS AND METHODS. Shown blots are representative of 3 independent experiments. Relative intensity DNA-bound OGT and mSin3A protein was normalized to input. Shown data are expressed as means ± SE (error bars) of 3 independent experiments. *P < 0.05, untreated control vs. LPS treated; †P < 0.05, LPS vs. LPS + GlcN treated.](http://ajpcell.physiology.org/ by 10.22033.6 on June 21, 2017)
Statistical analysis. The data are expressed as the means ± SE and analyzed for statistical significance using ANOVA, followed by Scheffé’s test for multiple comparison. P < 0.05 was considered significant.

RESULTS

OGT inhibits LPS-induced NF-κB/iNOS promoter activation and the transcriptional activities of p65 and c-Rel in the presence or absence of GlcN. As a previous report showed that the interaction between OGT and p65 was increased by LPS and decreased by GlcN (10), we examined the involvement of OGT in the transcriptional activities of NF-κB and iNOS. Overexpression of OGT inhibited NF-κB reporter activity and iNOS promoter activity in response to LPS and also suppressed the GlcN-mediated inhibition of NF-κB/iNOS transcription (Fig. 1, A and B). These results suggest that OGT plays an inhibitory role in the transcription of NF-κB/iNOS. To assess the influence of OGT on the transcriptional activity of p65 and c-Rel, we examined the activity of a Gal4-dependent reporter following cotransfection of GAL4-p65 or GAL4-c-Rel and OGT. Both basal and LPS-driven transcription levels from this reporter were significantly reduced by OGT (Fig. 1, C and D).

LPS increases the interaction of OGT with mSin3A but GlcN reduces their interactions. It has been shown that OGT recruits corepressor protein mSin3A to inhibit transcription (23, 29). Thus we examined the interaction between OGT and mSin3A using coimmunoprecipitation assays. An antibody against OGT

A

B

Fig. 3. Effect of mSin3A overexpression in iNOS transcription with or without OGT. RAW 264.7 cells were transfected with iNOS promoter containing luciferase plasmid. At some conditions, mSin3A and/or OGT were cotransfected. Cells were then treated with GlcN (5 mM) and/or LPS (0.1 μg/ml) for 24 h and luciferase activity, in RLU, was measured. *P < 0.05.

Fig. 4. iNOS promoter bindings of mSin3A, histone deacetylases (HDACs), and acetylated-histones in response to LPS and/or GlcN. RAW264.7 cells were pretreated with GlcN for 2 h and stimulated with LPS (0.1 μg/ml) for 24 h. Formaldehyde cross-linked chromatin from 24-h stimulated cells was immunoprecipitated with antibodies against to the indicated proteins. Immunoprecipitated DNA was purified for quantitative real-time PCR (qPCR) or standard RT-PCR analyses using specific primer for mouse iNOS promoter. Input represents PCR product obtained from 2% of the preimmunoprecipitated DNA. *P < 0.05, compared with control.
coimmunoprecipitated mSin3A in LPS-stimulated cell lysates; this interaction was, however, inhibited by GlcN (Fig. 2A, *top*). LPS and GlcN exerted similar effects on OGT and mSin3A interactions, as determined by reciprocal experiments that used an anti-mSin3A antibody to coimmunoprecipitate OGT (Fig. 2A, *bottom*). Next, we assayed binding of OGT and mSin3A to a biotinylated oligonucleotide corresponding to the distal NF-κB site (−957 to −977). Stimulation with LPS for 24 h induced an increase in OGT and mSin3A binding, and this was inhibited by GlcN pretreatment (Fig. 2B).

Transcription of iNOS is inhibited by mSin3A. To examine the influence of mSin3A on iNOS transcription, we overexpressed mSin3A in RAW264.7 cells. We found that overexpression of mSin3A inhibited LPS-induced iNOS promoter expression, but cotransfection of OGT-encoding vectors did not further inhibit iNOS promoter activation under basal, LPS-treated, and/or GlcN-treated conditions (Fig. 3).

LPS enhances mSin3A, HDAC1, and HDAC2 binding to the iNOS promoter. Despite several trials, however, we failed to demonstrate any direct and solid interaction of OGT or mSin3A with HDACs. Then, we examined whether a physical interaction between OGT and mSin3A facilitated recruitment of this complex and HDACs to the iNOS promoter. The bindings of mSin3A, HDACs, and acetylated histones to the iNOS promoter were then examined using a ChIP assay. RAW264.7 cells were stimulated with LPS and/or GlcN for 24 h. Cell lysates were subjected to immunoprecipitation using antibodies against OGT, mSinA, HDACs, and acetylated histones to the iNOS promoter. The bindings of mSin3A, HDAC1, and HDAC2 was not significantly affected by LPS and/or GlcN. HDAC3 was not precipitated by WGA beads, indicating that this protein is likely not modified by O-GlcNAcylation. The O-GlcNAcylation changes of mSin3A in response to LPS and/or GlcN were further verified by radiolabeling of the mSin3A immunoprecipitate with galactosyltransferase or by immunodetection using O-GlcNAc-specific antibody. The levels of radioactivity and immunoreactivity to O-GlcNAc antibody in mSin3A decreased following addition of LPS. Again, this effect was reversed by GlcN (Fig. 5). Whether WGA-bound HDAC1 and 2 are O-GlcNAc-modified remains to be elucidated.

HDAC activity and histone modification mediate the ability of OGT to repress LPS-induced iNOS expression. Next, we investigated whether OGT-mediated repression of iNOS induction is associated with HDAC activity. In these experiments,
we used the specific HDAC inhibitor TSA to examine the effects of HDAC inhibition on OGT-mediated repression of iNOS in response to LPS. Basal and LPS-induced iNOS transcription rates were enhanced by TSA (50 ng/ml; Fig. 6A). Further, transfection of OGT reduced the activity of the iNOS promoter, as previously observed. However, this repressive effect was eliminated by treatment with TSA (Fig. 6A).

These results suggest that histone acetylation might be involved in the regulation of iNOS in response to LPS. Furthermore, iNOS protein levels were also dose dependently increased following 24 h of TSA treatment in RAW264.7 cells in both the presence and the absence of GlcN (Fig. 6B).

To further determine the importance of histone acetylation in regulation of LPS/GlcN-induced iNOS transcription, we overexpressed the histone acetyltransferase enzymes that regulate iNOS expression. Transient transfection of p300 and CBP enhanced LPS-induced iNOS protein expression. Further, the increased levels of such proteins rescued GlcN-mediated repression of iNOS expression (Fig. 6C).

**DISCUSSION**

When the inflammatory response is sustained unchecked, the host defense function reverses and inflammation becomes damaging. A number of factors can control inflammation; these include chronic exposure to inflammatory stimuli from the environment or formation of endogenous factors perceived as foreign by the immune system. In addition, failures can occur in mechanisms that normally inhibit inflammation. Typically, such mechanisms involve attenuation of responses via induction of proteins that inhibit inflammatory signal transduction pathways. Other mechanisms include induction or activation of transcriptional repressors and production of mediators with anti-inflammatory activities, such as IL-10 or TGF-β. In our recent study, we showed that GlcN (14, 16) inhibits LPS-mediated production of inflammatory molecules in microglia and macrophage potentially through the reduced association with OGT. Unlike our expectation of OGT being a positive regulator for the LPS response, OGT overexpression led to the reduction of LPS responses. Under LPS stimulation (i.e., an inflammatory state), activated OGT appears to inhibit inflammatory responses through the inhibition of NF-κB. However, in GlcN-treated cells (in which inflammation is suppressed), the functions of OGT are abolished. Our results further indicate that GlcN inhibits LPS-driven inflammatory responses in a manner independent of HDACs.

To regulate the extent of iNOS induction, OGT seems to simultaneously interact with the relevant transcriptional activator (p65) and repressor (mSin3A). As noted in our previous report, LPS upregulates the O-GlcNAcylation of p65 and its interactions with OGT, whereas GlcN downregulates these activities. This suggests that there may be a positive correlation between transcriptional activity and increased OGT binding of p65. In the present study, however, we found that OGT overexpression reduced the transcriptional activities of NF-κB and the expression of iNOS. This was unexpected, because the OGT inducer LPS is a strong inducer of these activities. Here, we present two scenarios that may explain the complicated role played by OGT in LPS-induced transcriptional regulation of iNOS. One possibility involves that OGT association with p65 mediates transcriptional repression by 10.220.33.6 on June 21, 2017 http://ajpcell.physiology.org/ Downloaded from
Our results show that O-GlcNAcylation of mSin3A is actively regulated by LPS and/or GlcN. In this process, OGT may not be the sole regulator of its O-GlcNAcylation. For example, LPS-dependent increases in OGT interactions produced opposite results, compared with O-GlcNAcylation of p65 and mSin3A; this resulted in an increase in p65 O-GlcNAcylation and a decrease in mSin3A O-GlcNAcylation. LPS decreased the extent of O-GlcNAcylation of total nucleocytoplasmic proteins. In addition, OGT levels were unchanged following stimulation with LPS (data not shown). There might be a number of distinct mechanisms that are responsible for O-GlcNAcylation of individual proteins, including substrate availability, regulation of OGT or O-GlcNAcase enzymatic activities, or differential substrate targeting. Currently, the mechanisms by which OGT recognizes diverse substrates and the mechanisms regulating OGT activity toward different substrates remain puzzling. Regulation of OGT activity has been proposed to occur via posttranslational modification such as phosphorylation, nitrosylation, and O-GlcNA modification (14, 20). Various protein-protein interactions have also been proposed to influence OGT function, including modulator proteins that alter its activity on specific substrates (3). One simple mechanism that probably contributes to regulating O-GlcNA levels on different proteins is likely that the ratio or relative activities of OGT and O-GlcNAcase activity toward a given target protein can influence the extent of O-GlcNA modification. Thus extensive research is required in the future to understand the mechanisms regulating OGT activity toward specific target proteins.

To gain a better understanding of the mechanisms involved in repression of iNOS by OGT, we investigated the role played by HDACs in this process. OGT interacted directly with the endogenous iNOS promoter, as demonstrated by a ChIP assay. Further, LPS caused recruitment of mSin3A, HDAC1, and HDAC2 to the iNOS promoter, whereas GlcN suppressed this effect. These results support the idea that OGT mediates repression of iNOS via recruitment of a corepressor complex containing mSin3A, HDAC1, and HDAC2. Histone H4 appears to be a primary substrate of the mSin3A-HDAC complex, as indicated by reduction of acetylated histone H4 at an endogenous iNOS promoter. Acetylation of histones can be affected by treatment of cells with HDAC inhibitors such as TSA or by overexpressing the histone acetyltransferases CBP/p300 (19). We found that TSA treatment and CBP/p300 enhanced iNOS expression following LPS treatment in either the presence or absence of GlcN. In addition to modifying histones, p300/CBP also directly acetylates several transcription factors, including p65 and p50 (6). We failed to detect increased acetylation of p65 following LPS treatment (data not shown). Further, neither overexpression of CBP/p300 nor TSA treatment affected the transcriptional activity of NF-κB (data not shown). These data rule out any possible involvement of p65 acetylation in LPS- and/or GlcN-mediated iNOS expression. Although a number of studies have examined the molecular mechanisms of iNOS induction in inflammatory cells such as macrophages, only a few have considered the effect of histone modification. The HDAC inhibitors butyrate and TSA have been shown to display anti-inflammatory properties in terms of iNOS induction in macrophages (4, 17). However, these data contrast with our results; we found that TSA and CBP/p300 enhanced iNOS synthesis in microglia (data not shown) and macrophages. To date, any role for histone acetylation in iNOS gene regulation in macrophage has not been explored. Thus additional studies are needed to determine whether histone acetylation at the iNOS promoter is dynamically regulated by various inflammatory factors. Likewise, it remains unknown whether the process operates in concert with promoter occupancy by mSin3A, p300/CBP, and HDACs.

What can we expect in the future? One major goal is to define the mechanisms of inflammatory gene control, particularly in terms of resolving the pathophysiology of inflammation. Our discovery of a potential mechanism by which NF-κB/iNOS is regulated by OGT will certainly add to the fascination surrounding regulation of NF-κB during the inflammatory response. Further, our results may provide a new insight into the regulation of NF-κB-dependent physiological processes. Thus the work may lead to a method whereby the inflammatory response could be controlled to ensure appropriate physiological functioning.

GRANTS

This work is supported by a National Research Foundation of Korea grant funded by the Korean government (MEST; 2010-0014338) and Inha University.

AUTHOR CONTRIBUTIONS


REFERENCES


AJP-Cell Physiol • doi:10.1152/ajpcell.00042.2013 • www.ajpcell.org


