Trivalent chromium inhibits TSP-1 expression, proliferation, and O-GlcNAc signaling in vascular smooth muscle cells in response to high glucose in vitro

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Submitted 22 July 2014; accepted in final form 22 October 2014

Ganguly R, Sahu S, Chavez RJ, Raman P. Trivalent chromium inhibits TSP-1 expression, proliferation, and O-GlcNAc signaling in vascular smooth muscle cells in response to high glucose in vitro. Am J Physiol Cell Physiol 308: C111–C122, 2015. First published October 29, 2014; doi:10.1152/ajpcell.00256.2014.—Trivalent chromium (Cr3+) is a mineral nutrient reported to have beneficial effects in glycomic and cardiovascular health. In vitro and in vivo studies suggest that Cr3+ supplementation reduces the atherogenic potential and lowers the risk of vascular inflammation in diabetes. However, effects of Cr3+ in vascular cells under conditions of hyperglycemia, characteristic of diabetes, remain unknown. In the present study we show that a therapeutically relevant concentration of Cr3+ (100 nM) significantly downregulates a potent proatherogenic matricellular protein, thrombospondin-1 (TSP-1), in human aortic smooth muscle cells (HASMC) stimulated with high glucose in vitro. Promoter-reporter assays reveal that this downregulation of TSP-1 expression by Cr3+ occurs at the level of transcription. The inhibitory effects of Cr3+ on TSP-1 were accompanied by significant reductions in O-glycosylation of cytoplasmic and nuclear proteins. Using Western blotting and immunofluorescence studies, we demonstrated that reduced protein O-glycosylation by Cr3+ is mediated via inhibition of glutamine: fructose 6-phosphate amidotransferase, a rate-limiting enzyme of the hexosamine pathway, and O-linked N-acetylglucosamine (O-GlcNAc) transferase, a distal enzyme in the pathway that controls intracellular protein O-glycosylation. Additionally, we found that Cr3+ attenuates reactive oxygen species formation in glucose-stimulated HASMC, suggesting an antioxidant effect. Finally, we report an antiproliferative effect of Cr3+ that is specific for high glucose and conditions triggering elevated protein O-glycosylation. Taken together, these findings provide the first cellular evidence for a novel role of Cr3+ to modulate aberrant vascular smooth muscle cell function associated with hyperglycemia-induced vascular complications.

trivalent chromium; thrombospondin-1; vascular smooth muscle cells; O-glycosylation; reactive oxygen species

DIABETIC PATIENTS ARE PREDISPOSED to an increased risk of vascular complications. Hyperglycemia, characteristic of diabetes, is an independent risk factor attributing to atherosclerotic complications associated with diabetes (30, 41). Previous studies from our laboratory (47) and others (37, 51) have proposed the matricellular protein thrombospondin-1 (TSP-1) as a putative link between hyperglycemia and diabetic atherogenesis. TSP-1 is a potent proatherogenic and antiangiogenic protein that regulates cell-cell and cell-matrix interactions (1, 5), is significantly upregulated in the injured vascular wall and early-stage atherosclerotic lesions (48, 49), and stimulates vascular smooth muscle cell (VSMC) growth (39, 42). Previous genetic association studies have linked the TSP protein family to atherosclerotic cardiovascular disease (53, 55). Animal studies have further suggested that TSP-1 deficiency has protective effects during early stages of plaque formation (40, 52). TSP-1 expression has also been shown to be significantly increased in diabetic patients and diabetic animal models (4, 51) as well as in different vascular cells (VSMC, endothelial cells, and fibroblasts) exposed to high glucose in vitro (47, 51). We previously reported that activation of the hexosamine pathway and protein O-glycosylation (O-GlcNAcylation) mediate upregulation of TSP-1 by glucose in cultured human aortic smooth muscle cells (HASMC) (46, 47). Moreover, we have shown that this metabolic pathway regulates HASMC proliferation under high glucose-stimulated conditions in vitro (47).

Trivalent chromium (Cr3+) is a mineral nutrient that regulates carbohydrate and lipid metabolism. Although numerous studies have suggested a beneficial role of Cr3+ in glycomic and cardiovascular health (2, 36, 50, 54), several inconsistencies have arisen due to lack of an effect in clinical trials using Cr3+ at doses well below those used in rodent studies (14, 56). However, such discrepancies have been somewhat reconciled on the basis of reports that Cr3+ was active only at pharmacologically relevant doses, while clinical response to Cr3+ was dependent on the glycomic environment of the patient (60). Diabetic patients have low circulating and tissue Cr3+ levels (17, 31). Previous studies have reported that Cr3+ deficiency may lead to elevated blood glucose, insulin, triglyceride, and cholesterol, accompanied with a decrease in HDL levels and an increase in incidence of heart attacks (38, 59). Growing evidence demonstrates that Cr3+ supplementation increases insulin sensitivity viaactivation of the insulin signaling pathway (9, 57). Cr3+ has also been shown to attenuate dyslipidemia and inhibit lipid peroxidation and secretion of proinflammatory cytokines, including TNF-α, IL-6/8, monocyte chemoattractant protein 1, C-reactive protein, and ICAM-1, in streptozotocin-induced diabetic rats in vivo and cultured monocytes exposed to high glucose in vitro (23, 24, 27). Epidemiological studies have further revealed that chromium picolinate (CrP), the most bioavailable form of Cr3+, in combination with biotin, significantly lowers the plasma atherogenic index in type 2 diabetic patients (18). Also, animal studies using a hypercholesterolemic rabbit model of atherosclerosis revealed that Cr3+ supplementation causes plaque regression (44). Despite a strong therapeutic potential of Cr3+ in diabetic vascular disease, the precise effects of Cr3+ on vascular cells under hyperglycemic conditions remain unknown. In the present study we have investigated the effects of Cr3+ on TSP-1 expression and smooth muscle cell proliferation in glucose-stimulated HASMC cultures. In addition, we have reported that...
Cr³⁺ supplementation attenuates O-linked N-acetylgalactosamine (O-GlcNAc) signaling and decreases reactive oxygen species (ROS) formation in HASMC exposed to high glucose in vitro.

MATERIALS AND METHODS

Chemicals. CrCl₃ was purchased from Sigma, and CrP was provided as a gift from Nutrition 21 (Purchase, NY). The cell proliferation reagent WST-1 and sodium 3,3′-[1-(phenylamino)carbonyl]-3,4-tetrazolium-bis(4- methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) cell viability kit were purchased from Cayman Chemicals and Cell Signaling Technology, respectively; 5- and 6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CMH; DCFDA) was purchased from Molecular Probes. All other reagents, solutions, and buffers were of the highest analytical grade commercially available.

Cell culture. Primary isolates of HASMC were obtained as a gift from Dr. Olga I. Stenina (Cleveland Clinic, Cleveland, OH). Primary HASMC cultures were grown in DMEM/F12 supplemented with 10% FBS (Atlanta Biologicals) and 1% penicillin-streptomycin (Cellgro). HASMC cultures were grown in DMEM/F12 supplemented with 10% FBS (Atlanta Biologicals) and 1% penicillin-streptomycin (Cellgro) at 37°C in a 5% CO₂ environment. In all experiments, cells were used at passages 8–15. At 24 h prior to stimulation, 80–90% confluent cell cultures were placed in low-glucose (5 mM) DMEM supplemented with 0.2% FBS. Cells were preincubated for 16–18 h in the presence or absence of 100 nM CrCl₃ or CrP, stimulated with 30 mM glucose, 2 mM glucosamine, or 100 μM O-(2-acetamido-2-deoxy-D-glucopyranosylidene)-N-phenylcarbamate (PUGNac), and incubated for an additional 24 h. Cr³⁺ was not removed from the medium during this last incubation period. To examine the specificity of the Cr³⁺ effects, multiple Cr³⁺ complexes were utilized.

Preparation of whole cell lysate and nuclear cell fractions and Western blotting. Whole cell lysates were prepared using 1× RIPA buffer supplemented with protease inhibitors. In some experiments, nuclear cell fractions were isolated using a nuclear extraction kit (Cayman Chemicals) according to the manufacturer’s instructions. Total protein concentration was determined in cell lysates using a Bio-Rad protein assay. Equal amounts of protein lysates were resolved in 8–10% SDS-polyacrylamide gel under reducing conditions. The resolved proteins were transferred to a PVDF membrane. For Western blotting, membranes were blocked in 5% nonfat dry milk for 1 h at 25°C and incubated overnight at 4°C with the following antibodies: human anti-TSP-1 (1:1,000 dilution; clone AB11, Thermo Fisher), anti-O-GlcNAc (1:1,000 dilution; Abcam), anti-glutamine:fructose 6-phosphate amidotransferase (GFAT, 1:1,000 dilution; Pro-Fisher), anti-OGT antibodies: human anti-TSP-1 (1:1,000 dilution; clone AB11, Thermo Fisher), anti-O-GlcNAc (1:1,000 dilution; Abcam), anti-glutamine:fructose 6-phosphate amidotransferase (GFAT, 1:1,000 dilution; Proteintech), anti-OGT primary antibody (OGT, 1:1,000 dilution; a gift from Dr. Gerald Hart, John Hopkins University School of Medicine, Baltimore, MD), and anti-O-linked N-acetylgalactosaminidase (OGA; 1:1,000 dilution; Sigma). The membranes were washed in Tris-buffered saline-Tween 20 three times for 5–15 min each and then incubated with horseradish peroxidase-conjugated secondary antibody in 5% milk for 1 h at 25°C. The membranes were washed again in Tris-buffered saline-Tween 20 three times for 5–15 min each, incubated with chemiluminescence detection reagents (Supersignal West Pico, Thermo Scientific), and developed using autoradiography. The membranes were subsequently stripped and reprobed with anti-β-actin (1:1,000 dilution, Cell Signaling Technology) to control for nuclear protein loading. Equal protein loading of samples was further confirmed by staining the membranes with Ponceau S. Densitometric quantification of all Western blots was performed using ImageJ software, and data are presented as fold increase vs. controls.

Cell transfection and luciferase assay. Primary HASMC cultures were plated in 24-well cell culture plates in DMEM/F12 supplemented with 10% FBS. About 70–80% confluent cells were transiently transfected with ~1270/+66 TSP-1 gene (THBS1) promoter linked to luciferase reporter construct or pGL3 vector control using Lipofectin reagent (Invitrogen), as reported elsewhere (8). Transfected cells were incubated with 30 mM glucose with or without 100 nM CrCl₃ or CrP for 42 h. Luciferase activity was measured in cell extracts as previously reported (8). Protein concentration in cell extracts was measured using bicinchoninic acid (BCA) protein assay reagent (Pierce), and luciferase activity was normalized to the total protein content in the cell extracts.

Immunocytochemistry. HASMC were grown on coverslips in six-well cell culture plates in DMEM/F12 supplemented with 10% FBS. At 24 h prior to stimulation, 60–70% confluent cells were placed in low-glucose (5 mM) DMEM supplemented with 0.2% FBS. After cells were preincubated for 16–18 h with 100 nM CrCl₃ or CrP, glucose or glucose + PUGNac was added, and cell incubation continued for 24 h. At the end point, medium was aspirated, and cells were fixed in a solution containing 4% paraformaldehyde and 0.2% Triton X-100 for 20 min at 25°C and then blocked in a solution containing 5% donkey serum for 60 min at 25°C. All immunofluorescence images were quantified using ImageJ software.

O-GlcNAc immunostaining. Immunofluorescence detection of O-GlcNAc epitope of intracellular protein substrates was performed as reported elsewhere (47). Briefly, cells treated and processed as described above were incubated for 1 h at 25°C with anti-O-GlcNAc (1:100 dilution; clone RL2, Abcam), a mouse monoclonal antibody to O-GlcNAc moieties on proteins. To control for nonspecific staining, parallel cultures of cells treated with glucose + PUGNac were incubated in the absence of anti-O-GlcNAc primary antibody. Cells were washed three times in PBS for 15 min each, incubated with a 1:50 dilution of Rhodamine Red-X-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch) for 1 h at 25°C, and washed three times in PBS for 15 min each. Coverslips were mounted using Vectashield mounting medium containing DAPI (Vector Laboratories) for identification of the cell nuclei. The mean red fluorescence intensity depicting O-GlcNAc (RL2) staining was measured for each image.

GFAT immunostaining. Cells treated and processed as described above were fixed in a solution containing 4% formaldehyde for 10 min at 25°C. The cells were rinsed in PBS and then permeabilized by incubation in a solution containing 0.2% Triton X-100 for 5 min at 25°C. Permeabilized cells were blocked in 5% donkey serum for 1 h at 25°C and then incubated with anti-GFAT (1:100 dilution; Proteintech) for 16–18 h at 4°C. As a control for nonspecific labeling, parallel cultures of glucose-treated cells were incubated in the absence of anti-GFAT primary antibody. The cells were briefly washed in PBS and then further incubated with Alexa Fluor 594-conjugated anti-rabbit IgG secondary antibody (1:1,500 dilution; Molecular Probes) for 1 h at 25°C. Coverslips were mounted using DAPI-containing mounting medium for nuclear identification. The red fluorescence intensity indicative of GFAT staining was measured for each image.

OGT immunostaining. Cells were treated and processed as described above. For detection of OGT immunofluorescence, cells were incubated with anti-OGT antibody (1:100 dilution; Abcam) for 16–18 h at 4°C in 5% donkey serum. Concurrently, parallel cultures of glucose-treated cells were incubated in the absence of anti-OGT primary antibody to control for nonspecific staining. The cells were washed in PBS three times for 10–15 min each and further incubated with Alexa Fluor 594-conjugated anti-rabbit IgG secondary antibody (1:1,500 dilution; Molecular Probes) for 1 h at 25°C. Coverslips were mounted using a DAPI-containing mounting medium for identification of cell nuclei. Quantitation of all immunofluorescence images was performed using ImageJ software by measurement of the mean red fluorescence intensity depicting OGT staining for each image.

Measurement of ROS. Primary cultures of HASMC were grown in six-well cell culture plates in DMEM/F12 supplemented with 10% FBS. At 24 h prior to initiation of the experiment, 60–70% confluent
cells were placed in serum-free, low-glucose (5 mM) DMEM. Under these serum-free conditions, cells were preincubated with or without 100 nM CrCl3 or CrP for 16–18 h and 30 mM glucose was added. At 24 h after glucose stimulation, medium was aspirated, and cells were washed in PBS (with Ca2+ and Mg2+) and further incubated in 1 μM CMH2DCFDA (Invitrogen) for 20 min at 37°C. Cells were washed briefly in PBS, and oxidation of the probe was determined by measurement of cell fluorescence, as detected by live cell imaging. Six to eight images were collected for each treatment group within an independent experiment, with at least two replicates for each treatment in an independent experiment. Quantitation of all fluorescence images was performed using ImageJ software.

**Proliferation of cultured smooth muscle cells.** Primary HASMC cultures were plated in 96-well culture plates (2,000 –3,000 cells/well) in DMEM/F12 supplemented with 10% FBS. Cells were allowed to attach overnight and then placed in serum-free, low-glucose (5 mM) DMEM and further incubated with 30 mM glucose in the presence or absence of 100 nM CrCl3 or CrP for 72 h. To simulate conditions with a stronger driving force promoting persistent O-GlcNAcylation of protein substrates, parallel cell cultures were treated with 30 mM glucose + 50 μM Thiamet-G (TG) with or without CrCl3 or CrP for 72 h. Cell proliferation was measured at the end point using the cell proliferation reagent WST-1, a nonradioactive colorimetric assay, according to the manufacturer’s instructions (Cayman Chemicals).

**XTT cell viability assay.** Cytoxicity of the Cr3+ complexes used in this study was evaluated using the XTT cell viability assay according to the manufacturer’s instructions (Cell Signaling Technology). Briefly, HASMC cultures were seeded in 96-well tissue culture plates at confluence. Cells were plated in 100 μl of low-glucose (5 mM) DMEM containing 0.2% FBS for 24 h. Cells were then treated with different stimulants in the presence or absence of CrCl3 or CrP for 24–72 h. At relevant end points, the activated XTT solution was added to each well, and cell incubations were continued for 2–5 h. The sample absorbance was then measured at 490 nm using a microplate reader.

**Statistical analysis.** Each experiment was repeated at least three times with two to five replicates for each treatment within an independent experiment. For immunofluorescence experiments, six to eight images were collected for each individual treatment within an independent experiment. Immunofluorescence images were quantified using ImageJ software. Data are presented as fold increase vs. controls. For proliferation experiments, at least four to five replicates were used for each treatment group within an independent experiment. For representative Western blots,_lane_images show proteins detected on a single blot; however, for the purpose of clarity of presentation, lanes were rearranged as indicated. Values are means ± SE. Significant differences between mean values were detected using the unpaired Student’s t-test (2-tailed); P ≤ 0.05 was considered statistically significant.

**RESULTS**

**Cr3+ inhibits TSP-1 expression in HASMC stimulated with high glucose and conditions promoting protein O-GlcNAcylation in vitro.** To investigate whether Cr3+ modulates TSP-1 expression under conditions of hyperglycemia in a cell type found in the large vessels, primary cultures of HASMC were stimulated with 30 mM glucose in the presence or absence of CrCl3 and CrP for 24 h. Initially, a dose-response study was performed for CrCl3 and CrP at 1 mM–1 nM. Immunoblot experiments showed that while high glucose increased TSP-1 expression (~3.7-fold), treatment with Cr3+ significantly inhibited glucose-induced TSP-1 expression at millimolar to nanomolar concentrations (data not shown); this effect was further sustained at a clinically relevant concentration of Cr3+ (100 nM). Specifically, incubation with Cr3+ resulted in >70% decrease in high glucose-induced TSP-1 expression compared with cells treated with glucose alone (Fig. 1A). To further determine the effect of Cr3+ on TSP-1 expression under conditions of elevated protein O-GlcNAcylation, HASMC cultures were stimulated with glucosamine (GlcN), a downstream metabolite of the hexosamine pathway that serves as a physiological substrate of O-GlcNAcylation, with or without Cr3+ for 24 h. Densitometric quantification of Western blots revealed that CrCl3 and CrP significantly inhibited TSP-1 expression in 2 mM GlcN-stimulated HASMC cultures (Fig. 1B; >78% vs. GlcN alone). Similar results were also obtained when cells were stimulated with a hexosaminidase inhibitor, PUGNAc, which inhibits removal of O-GlcNAc residues from cellular proteins, thereby stabilizing the O-GlcNAc protein modification. Specifically, while PUGNAc stimulus markedly increased TSP-1 expression (~4-fold), treatment with Cr3+ produced a statistically significant downregulation of TSP-1 expression in PUGNAc-stimulated cells (Fig. 1C; ~78% vs. PUGNAc alone). Incubation with Cr3+ alone in the absence of any stimulants did not affect TSP-1 expression (data not shown). The cytotoxicity of the Cr3+ complexes used in this study was further assessed using the XTT cell viability assay. At all time points, Cr3+ did not have a detrimental effect on cell viability, measured in terms of mitochondrial enzyme activity (see MATERIALS AND METHODS). Furthermore, microscopic observation of cells revealed that incubation with Cr3+ does not alter the morphology of HASMC cultures. Together, these findings clearly demonstrate that Cr3+ has a strong inhibitory effect on expression of the prothrombotic protein TSP-1 in HASMC under hyperglycemic conditions in vitro.

**Cr3+ attenuates protein O-GlcNAcylation in HASMC under hyperglycemic conditions in vitro.** To assess the effect of Cr3+ on high glucose-induced TSP-1 transcription, HASMC cultures were transiently transfected with pGL3 control vector or −1270/+66 pTHBS1 promoter linked to a luciferase reporter construct and then incubated with high glucose in the presence or absence of Cr3+. As shown in Fig. 2, while glucose increased THBS1 promoter activity (~4-fold), treatment with Cr3+ resulted in a significant decrease in high glucose-induced THBS1 promoter activity compared with HASMC treated with glucose alone (60–67%). On the other hand, incubation with Cr3+ did not have an effect on cells transfected with the control vector. These data demonstrate that downregulation of TSP-1 expression by Cr3+ in glucose-stimulated HASMC occurs at the level of transcription.
Cr\(^{3+}\) inhibits thrombospondin 1 (TSP-1) expression in human aortic smooth muscle cells (HASMC) stimulated with high glucose and conditions promoting O-glycosylation (O-GlcNAc). Primary HASMC cultures in low-glucose (5 mM) DMEM supplemented with 0.2% FBS were pretreated with or without 100 nM CrCl\(_3\) or chromium picolinate (CrP) for 16–18 h and then stimulated with 30 mM glucose (A), 2 mM glucosamine (GlcN; B), and 100 μM O-(2-acetamido-2-deoxy-\(\beta\)-glucopyranosylidene) amino-N-phenylcarbamate (PUGNAc; C) for 24 h. Whole cell lysates were used in Western blot experiments. Top: representative Western blots for TSP-1 and \(\beta\)-actin (loading control). Bottom: densitometric quantification of Western blots from 3–5 independent experiments. Values are means ± SE. *P ≤ 0.003 vs. control; **P ≤ 0.002 vs. glucose (A). +P ≤ 0.02 vs. control; ++P ≤ 0.01 vs. GlcN (B). ♦P ≤ 0.01 vs. control; ♦♦P ≤ 0.002 vs. PUGNAc (C).

expected, increased O-GlcNAcylated protein levels compared with untreated controls (Fig. 3; ~2- to 4-fold), Cr\(^{3+}\) significantly decreased the level of O-GlcNAcylated proteins in stimulated cells (Fig. 3; 53–68% vs. stimulants alone). Moreover, this reduction in the level of O-GlcNAcylation was observed on several proteins ranging from ~165 to 65 kDa. Together, these findings demonstrate that Cr\(^{3+}\) prevents increased protein O-GlcNAcylation in HASMC stimulated with high glucose and conditions triggering O-GlcNAc modification of intracellular proteins.

We next evaluated the effect of Cr\(^{3+}\) on the cellular localization of O-GlcNAcylated proteins in cells stimulated with high glucose (30 mM) + PUGNAc (100 μM), mimicking hyperglycemia and insulin resistance in vitro. Consistent with the Western blot data, high glucose + PUGNAc increased O-GlcNAcylated protein levels compared with untreated cells; on the contrary, incubation with Cr\(^{3+}\) significantly decreased the RL2 immunofluorescence compared with glucose + PUGNAc (Fig. 4A). Specific quantification of the red fluorescence intensity indicative of the RL2 immunostaining further revealed that while high glucose + PUGNAc increased O-GlcNAcylation by 2.3-fold vs. control cells, Cr\(^{3+}\)-treated HASMC showed significantly attenuated levels of O-GlcNAcylation (Fig. 4B; 45–50% vs. glucose + PUGNAc). Importantly, this reduction in the level of O-GlcNAcylation was observed on cytoplasmic and nuclear proteins, the latter shown by colocalization of nuclear RL2 and DAPI staining, depicted by the magenta color (Fig. 4A, merge images). Parallel processing of glucose + PUGNAc-treated cells without the RL2 primary antibody failed to show immunofluorescence staining, confirming specificity of the results (Fig. 4C).

Fig. 1. Cr\(^{3+}\) inhibits thrombospondin 1 (TSP-1) expression in human aortic smooth muscle cells (HASMC) stimulated with high glucose and conditions promoting O-glycosylation (O-GlcNAc). Primary HASMC cultures in low-glucose (5 mM) DMEM supplemented with 0.2% FBS were pretreated with or without 100 nM CrCl\(_3\) or chromium picolinate (CrP) for 16–18 h and then stimulated with 30 mM glucose (A), 2 mM glucosamine (GlcN; B), and 100 μM O-(2-acetamido-2-deoxy-\(\beta\)-glucopyranosylidene) amino-N-phenylcarbamate (PUGNAc; C) for 24 h. Whole cell lysates were used in Western blot experiments. Top: representative Western blots for TSP-1 and \(\beta\)-actin (loading control). Bottom: densitometric quantification of Western blots from 3–5 independent experiments. Values are means ± SE. *P ≤ 0.003 vs. control; **P ≤ 0.002 vs. glucose (A). +P ≤ 0.02 vs. control; ++P ≤ 0.01 vs. GlcN (B). ♦P ≤ 0.01 vs. control; ♦♦P ≤ 0.002 vs. PUGNAc (C).

Fig. 2. Cr\(^{3+}\) attenuates high glucose-induced TSP-1 transcription in HASMC in vitro. Primary HASMC cultures were transiently transfected with pGL3 vector control or −1270/+66 pTHBS1 promoter-luciferase reporter construct. After 6 h of recovery, medium was aspirated and cells were treated with 30 mM glucose in the presence or absence of 100 nM CrCl\(_3\) or CrP for 42 h. Luciferase activity was measured in cell extracts. Values are means ± SE; n = 5–8. *P < 0.005 vs. control (THBS1 promoter); +P < 0.005 vs. glucose (THBS1 promoter).
Downregulation of GFAT and OGT signaling mediates reduced protein O-GlcNAcylation by Cr³⁺ in HASMC. To elucidate the signaling mechanism responsible for the reduced protein O-GlcNAcylation in response to Cr³⁺, the effect of Cr³⁺ on expression of GFAT, the rate-limiting enzyme of the hexosamine pathway, was investigated in glucose-stimulated cells. Immunofluorescence experiments demonstrated that while high glucose increased GFAT expression, revealed by the cytoplasmic red staining, incubation with Cr³⁺ with or without 100 nM CrCl₃ or CrP for 16–18 h and then stimulated with 30 mM glucose (A), 2 mM GlcN (B), and 100 μM PUGNAc (C) for 24 h. Whole cell lysates were used in Western blot experiments. Top: representative Western blot for O-GlcNAc and β-actin (loading control). Bottom: densitometric quantification of Western blots from 3–6 independent experiments. Values are means ± SE. Lane images in A show proteins detected on a representative single blot; however, they were rearranged for clarity of presentation. *P ≤ 0.0001 vs. control; **P ≤ 0.00017 vs. glucose (A). +P ≤ 0.0004 vs. control; ++P ≤ 0.00008 vs. GlcN (B). ●P ≤ 0.05 vs. control; ♦♦P ≤ 0.02 vs. PUGNAc (C).

We next ascertained the effects of Cr³⁺ on two major distal enzymatic regulators of O-GlcNAcylation, OGT and OGA. Immunofluorescence studies demonstrated that while high glucose increased OGT expression compared with untreated cells, depicted by the enhanced red staining (Fig. 6A), Cr³⁺ significantly inhibited glucose-induced OGT expression compared with cells treated with glucose alone (Fig. 6A). Notably, treatment with Cr³⁺ reduced OGT expression within the cell nuclei, shown by colocalization of the nuclear OGT and DAPI staining (Fig. 6A, merge images). Quantitation of the OGT fluorescence intensity revealed a 1.7-fold increase in high glucose-induced OGT expression vs. controls, which was attenuated following incubation with Cr³⁺ under similar experimental conditions (Fig. 6B; 43–47% vs. glucose alone). In parallel experiments, the specificity of OGT immunofluorescence was confirmed in glucose-treated cells incubated in the absence of the primary OGT antibody (data not shown). These microscopic observations were also validated in Western blot experiments using nuclear extracts prepared from glucose-stimulated HASMC treated with or without Cr³⁺. Consistent with these immunostaining data, whereas high glucose increased OGT expression by 1.9-fold vs. untreated controls,
incubation with Cr$_{3}^{+}$ significantly abrogated glucose-induced OGT expression (Fig. 6, C and D; 65–76% vs. glucose alone). In contrast to these results, Western blot experiments showed that Cr$_{3}^{+}$ did not have a statistically significant effect on OGA expression in HASMC under hyperglycemic conditions in vitro (Fig. 6E). Together, these results clearly demonstrate that reduced protein O-GlcNAcylation by Cr$_{3}^{+}$/H11001 is mediated via inhibition of GFAT and OGT expression in glucose-stimulated HASMC.

Cr$_{3}^{+}$ suppresses high glucose-induced ROS formation in HASMC in vitro. To explore the possibility that Cr$_{3}^{+}$ lowers vascular inflammation in diabetes via an antioxidant effect, HASMC cultures pretreated with or without Cr$_{3}^{+}$ were incubated with 30 mM glucose under serum-free conditions (see MATERIALS AND METHODS). Consistent with earlier findings, glucose stimulation significantly increased ROS levels vs. untreated cells (Fig. 7A). As illustrated in Fig. 7B, quantification of the CMH$_{2}$DCFDA fluorescence intensity showed a 1.8-fold increase in ROS generation under these conditions. On the other hand, incubation with Cr$_{3}^{+}$ resulted in a 60–66% decrease in glucose-induced intracellular ROS formation compared with HASMC incubated with glucose alone (Fig. 7, A and B); as such, fewer ROS-producing cells could be detected among the Cr$_{3}^{+}$-treated cells. Importantly, treatment with Cr$_{3}^{+}$ alone, in the absence of glucose stimulation, did not affect the intracellular ROS levels in HASMC cultures (data not shown).

Cr$_{3}^{+}$ inhibits proliferation of HASMC stimulated with high glucose and conditions triggering O-GlcNAcylation in vitro. To determine whether Cr$_{3}^{+}$ modulates VSMC function induced by high glucose, HASMC cultures were stimulated with 30 mM glucose under serum-free conditions in the presence or absence of Cr$_{3}^{+}$. As shown in Fig. 8A, Cr$_{3}^{+}$ supplementation
significantly attenuated high glucose-induced HASMC proliferation. This inhibitory effect of Cr³⁺ on smooth muscle cell proliferation was blocked under serum stimulation. Specifically, while high glucose (30 mM) and FBS (10%) increased smooth muscle cell proliferation by 2.0- and 2.9-fold, respectively, compared with untreated cells, incubation with Cr³⁺/H₁₁₀₀₁ specifically inhibited glucose-induced HASMC proliferation (Fig. 8A; 37–55% vs. glucose alone). In contrast, this antiproliferative response to Cr³⁺ was abrogated under serum-stimulated conditions (Fig. 8A). To further assess the antiproliferative response to Cr³⁺ under culture conditions promoting persistent O-GlcNAc modification of intracellular proteins, HASMC cultures were incubated with high glucose + TG, the latter used as a potent and more specific inhibitor of OGA that increases cellular O-GlcNAcylation, as OGA cannot remove O-GlcNAc residues from protein substrates. Interestingly, while glucose + TG increased HASMC proliferation (3.59-fold vs. untreated controls), incubation with Cr³⁺ significantly attenuated glucose + TG-induced HASMC proliferation (Fig. 8B; ~68% vs. glucose + TG alone). Similar findings were also obtained in PUGNAc-treated cells following exposure to Cr³⁺ (data not shown). Together, these findings clearly demonstrate an antiproliferative effect of Cr³⁺ in HASMC that is “specific” for high glucose and conditions triggering O-GlcNAc modification of cellular proteins.

**DISCUSSION**

The present study demonstrates, for the first time, that the micronutrient Cr³⁺, at relatively low concentrations represen-
Downregulates expression of the proatherogenic protein TSP-1 and inhibits \( \text{O}\text{-GlcNAc} \) signal transduction in HASMC cultures under hyperglycemic conditions in vitro. Cr\(^{3+}\) supplementation decreased high glucose-induced ROS formation, suggesting an antioxidant effect. The current work also reveals an antiproliferative effect of Cr\(^{3+}\) in glucose-stimulated HASMC in vitro. Interestingly, these inhibitory effects of Cr\(^{3+}\) occurred only under glucose stimulation, supporting the notion that Cr\(^{3+}\) action is contingent on the diabetic milieu (60). Moreover, incubation with Cr\(^{3+}\) did not have a similar inhibitory effect on expression of GAPDH and phosphorylated STAT3 (data not shown), used as specificity controls from other signaling cascades, suggesting that the effects on \( \text{O}\text{-GlcNAc} \) signaling are not related to a generalized Cr\(^{3+}\) response. Importantly, use of multiple Cr\(^{3+}\) complexes in this study illustrates the specificity of Cr\(^{3+}\) effects on VSMC, independent of the anionic ligands in the complex. Overall, this study reveals a novel role of Cr\(^{3+}\) in modulation of VSMC function that may have an important therapeutic relevance in diabetic atherogenesis.

As a protein with several proatherogenic properties, the importance of TSP-1 in the development of atherosclerosis and restenosis is highlighted in evidence from multiple sources (7, 21, 55). Previous studies from our laboratory as well as others have proposed TSP-1 as a possible link between hyperglycemia and accelerated atherosclerosis associated with diabetes (37, 47, 51). We further reported a cell-specific regulation of TSP-1 transcription by high glucose that was dependent on...
formation and recruitment of specific transcriptional complexes to cell-specific response elements of the TSP-1 gene (THBS1) promoter (46). Wang et al. (58) demonstrated that binding of the upstream stimulatory factors USF1 and USF2 to THBS1 promoter increased TSP-1 transcription in response to glucose in mesangial cells. Recent studies from our laboratory (8) also revealed a transcriptional mechanism of activation of THBS1 by high leptin concentrations in vitro, characteristic of hyperleptinemia in diabetes and obesity. The current findings are consistent with these earlier reports and suggest regulation of TSP-1 transcription as an important target of Cr3+/H11001 in VSMC under conditions of hyperglycemia.

Protein O-GlcNAcylation, characterized by an O-linked attachment of the sugar moiety β-N-acetylglucosamine to specific serine/threonine residues on nucleocytoplasmic proteins, is a unique posttranslational modification that serves an important regulatory mechanism for intracellular glucose signaling. O-GlcNAcylation modulates the response of numerous intracellular proteins, including signaling mediators, metabolic enzymes, cytoskeletal proteins, and transcription factors (19, 33, 62). Previous studies have shown that high glucose regulates the activity of several nuclear proteins, including SP1, YY1, and cAMP response element-binding protein, via increased O-GlcNAcylation (12, 33, 35). Moreover, an augmentation or

Fig. 7. Cr3+/H11001 attenuates high glucose-induced reactive oxygen species (ROS) levels in HASMC in vitro. Primary cultures of HASMC were grown in 6-well clusters in DMEM-F12 supplemented with 10% FBS. At 24 h prior to stimulation, 60–70% confluent cells were placed in serum-free, low-glucose (5 mM) DMEM. Cells were preincubated with 100 nM CrCl3 or CrP and then incubated with 30 mM glucose for 24 h. At the end point, medium was aspirated, and cells were washed in PBS (with Ca2+ and Mg2+), incubated in 1 μM 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CMH2DCFDA) for 20 min at 37°C, and washed in PBS, and intracellular ROS levels were detected by measurement of cell fluorescence via live cell imaging. A: representative ROS fluorescence images. Original magnification x20. All microscopic slides were processed simultaneously. B: quantification of ROS fluorescence intensity from 3 independent experiments. Values are means ± SE. *P ≤ 0.01 vs. control; +P ≤ 0.01 vs. glucose.

Fig. 8. Cr3+/H11001 inhibits proliferation of HASMC stimulated with high glucose and Thiamet-G (TG) in vitro. After an overnight attachment, HASMC cultures seeded in 96-well clusters were incubated in low-glucose (5 mM) serum-free DMEM in the presence or absence of 100 nM CrCl3 or CrP with 30 mM glucose or 10% FBS (A) and 30 mM glucose + 50 μM TG (B). After 72 h of incubation, cell proliferation was assessed by measurement of colorimetric absorbance at 450 nm using WST-1 cell proliferation reagent. Values are means ± SE; n = 4. #P ≤ 0.0001 vs. control; *P < 0.0007 vs. glucose (A); *P ≤ 0.002 vs. control; +P ≤ 0.0008 vs. glucose + TG (B).
suppression of gene transcription via O-GlcNAc modification is largely dependent on recruitment of specific groups of coactivators/repressor proteins to distinct gene promoter fragments. Earlier studies, including work from our laboratory, demonstrated that O-GlcNAcylation is the underlying mechanism for glucose-induced upregulation of TSP-1 transcription in VSMC and endothelial cells (13, 46). The current findings that Cr³⁺ inhibited the transcriptional activation of THBS1 concomitant with reduced O-GlcNAcylation of nuclear proteins prompt us to speculate that attenuation of O-GlcNAc modification of specific transcription factors(s) is a plausible mechanism that mediates downregulation of TSP-1 by Cr³⁺. Indeed, a recent study reported that CR³⁺ modulates transcriptional activation of cholesterol synthesis via attenuation of O-GlcNAcylation of SP1 (43). Studies currently underway warrant identification of the specific nuclear protein(s) that are modulated by Cr³⁺ via O-GlcNAcylation and how this affects the TSP-1 transcriptional response to Cr³⁺.

An enhanced flux through the hexosamine biosynthetic pathway (HBP) triggering an increased availability of UDP-N-acetylglucosamine is a critical modulator of glucose-induced gene expression. Regulation of glucose flux through this pathway, mediated via GFAT, was previously associated with increases in transforming growth factor-β1, plasminogen activator inhibitor type 1 synthesis, and NF-κB-dependent promoter activation in glucose-stimulated mesangial cells (28, 29, 61). Concurrently, increased nutrient flux through HBP leads to an enhanced cycling of O-GlcNAc residues on intracellular proteins, regulated by two key distal enzymes in the pathway, OGT and OGA (12). Emerging data have further indicated two different OGT isoforms, the nucleocytoplasmic and the mitochondrial variant, both of which have catalytic activity and induce O-GlcNAcylation of numerous nuclear and mitochondrial proteins, respectively (19, 33). The present study has identified the signaling mechanism by which Cr³⁺ modulates cycling of O-GlcNAc residues on nucleocytoplasmic proteins in hyperglycemic VSMC, with both GFAT and OGT being targets of Cr³⁺. Notably, we propose that while GFAT inhibition is required for regulation of O-GlcNAc by Cr³⁺, it is not completely sufficient for the reduced O-GlcNAc modification of cellular proteins in response to Cr³⁺. Together, these data suggest OGT as a critical upstream target of Cr³⁺ that may play a critical role in regulation of nuclear proteins controlling TSP-1 gene transcriptional response to Cr³⁺.

Clinical studies have revealed an antioxidant effect of Cr³⁺ in diabetic patients, assessed by the total serum antioxidant status and glutathione peroxidase activity (11, 34). In vitro and in vivo studies have demonstrated that Cr³⁺ attenuates lipid peroxidation under hyperglycemic conditions (24–27). Moreover, Cr³⁺ supplementation has been reported to upregulate antioxidant and DNA repair genes in human keratinocytes (20). It is well appreciated that oxidative enzymes, including NADPH oxidase and xanthine oxidase, induce increased ROS generation (32) in the arterial wall under hyperglycemic conditions, which can potentially lead to severe vascular damage (15). Consistent with these earlier reports, the present study provides evidence for reduced ROS levels as an underlying mechanism responsible for the antioxidant effects of Cr³⁺ in glucose-stimulated VSMC. Emerging data further support the concept that O-GlcNAc signaling is a major redox-sensing pathway capable of modulating the oxidative stress response (32). Previous studies have shown that dysregulation of intracellular glucose signaling resulting in activation of the hexosamine pathway and protein O-GlcNAcylation disrupts the activity and normal functioning of key atherogenic lipoproteins, including LDL particles; the net result of this effect could be altered LDL clearance or enhanced LDL uptake by aortic intimal cells and macrophages with a subsequent increase in foam cell formation (3), a hallmark of atherosclerosis. Also, O-GlcNAcylation of LDL has been reported to increase susceptibility to oxidative modifications, in turn attributing to its atherogenic properties (3). Recent reports have further demonstrated that hyperglycemia-induced increases in HBP flux trigger O-GlcNAcylation of mitochondrial proteins, resulting in elevated mitochondrial ROS formation. For example, a glucose-induced increase in O-GlcNAcylation of endothelial nitric oxide synthase was associated with enhanced mitochondrial superoxide generation in cultured endothelial cells (22). In vitro studies using renal mesangial cells and cardiomyoblasts have also shown that augmentation of O-GlcNAc levels significantly elevated ROS and mitochondrial superoxide production (10, 45); one possible explanation is that increased O-GlcNAcylation of antioxidant proteins such as superoxide dismutase enhances their susceptibility to proteosomal degradation, in turn accounting for attenuated antioxidant capacity (45). The current findings that Cr³⁺ decreased high glucose-induced ROS generation concomitant with reduced protein O-GlcNAcylation strongly favor the hypothesis that O-GlcNAc signaling mechanisms mediate the antioxidant effect of Cr³⁺ in VSMC. Cogent to the observation that Cr³⁺ lowered O-GlcNAc modulation of cytoplasmic proteins, it would be feasible to propose that reduced O-GlcNAcylation of mitochondrial proteins likely contributes to the attenuated ROS formation by Cr³⁺ in VSMC under hyperglycemic conditions. VSMC migration and proliferation represent a key pathological trigger attributing to initiation and progression of atherosclerosis. Diabetic patients have a much higher incidence of abnormal VSMC migration and proliferation than nondiabetic individuals (16). Earlier studies have indicated that, in response to vascular injury, TSP-1 stimulates VSMC activation, which leads to increased migration and proliferation of smooth muscle cells into the medial layer of the aortic vessel wall, ultimately eliciting enhanced neointimal formation (39). Our earlier study demonstrated that anti-TSP-1 antibodies and siRNAs targeted against TSP-1 significantly blocked high glucose-induced HASMC proliferation (47). In the context of the present findings, we speculate that the inhibitory effects of Cr³⁺ on TSP-1 expression may block VSMC activation under glucose stimulation. Of particular importance, the present study reveals a novel “glucose-specific” antiproliferative response to Cr³⁺ that may have a significant bearing on diabetic vascular disease. While HBP flux and sustained increases in protein O-GlcNAcylation are important pathogenic contributors of hyperglycemia-induced insulin resistance and diabetes-related vascular complications (6), the contribution of O-GlcNAc signal transduction to VSMC migration and proliferation associated with diabetic atherogenesis remains incompletely understood. This study, while lending support to our earlier hypothesis (47), provides initial cellular evidence of an antiproliferative effect of Cr³⁺, dependent on O-GlcNAc-signaling mechanisms, in hyperglycemic VSMC in vitro.
In conclusion, the present study reveals an important inhibitory role of Cr\(^{3+}\) in expression of the proatherogenic protein TSP-1 in glucose-stimulated HASMC, which may account for its antiproliferative response. In addition, Cr\(^{3+}\) downregulated O-GlcNAc signaling mechanisms and prevented ROS generation in glucose-stimulated HASMC. Taken together, these results suggest O-GlcNAc signal transduction as a pertinent link through which Cr\(^{3+}\) may modulate abnormal VSMC function and oxidative stress response under hyperglycemic conditions. Future in vivo studies will investigate the role of O-GlcNAc signaling as a putative therapeutic target in hyperglycemia-induced macrovascular complications.

ACKNOWLEDGMENTS

We thank Dr. Olga I. Stenina (Cleveland Clinic) for providing the THBS1 promoter construct.

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GRANTS

This study was supported in part by a grant from the Diabetes Action Research and Education Foundation and Northeast Ohio Medical University start-up funds to P. Raman.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

R.G., S.S., R.J.C., and P.R. performed the experiments; R.G., S.S., and P.R. analyzed the data; R.G. and P.R. interpreted the results of the experiments; R.G. and P.R. prepared the figures; R.G. and P.R. drafted the manuscript; R.G. and P.R. edited and revised the manuscript; R.G., S.S., R.J.C., and P.R. approved the final version of the manuscript; P.R. is responsible for conception and design of the research.

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