Endothelial Surface N-Glycans Mediate Monocyte Adhesion and Are Targets for Anti-inflammatory Effects of Peroxisome Proliferator-activated Receptor γ Ligands*

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Background: Activation of peroxisome proliferator-activated receptor (PPAR) γ in endothelial cells inhibits tumor necrosis factor (TNF) α-dependent monocyte adhesion.

Results: TNFα and PPARγ target endothelial high mannose/hybrid N-glycan expression to regulate monocyte rolling adhesion.

Conclusion: High mannose/hybrid N-glycoforms on the endothelial surface mediate monocyte interactions during inflammation.

Significance: N-Glycan processing enzymes may be novel targets to control vascular inflammatory processes.

Endothelial-monocyte interactions are regulated by adhesion molecules and key in the development of vascular inflammatory disease. Peroxisome proliferator-activated receptor (PPAR) γ activation in endothelial cells is recognized to mediate anti-inflammatory effects that inhibit monocyte rolling and adhesion. Herein, evidence is provided for a novel mechanism for the anti-inflammatory effects of PPARγ ligand action that involves inhibition of proinflammatory cytokine-dependent up-regulation of endothelial N-glycans. TNFα treatment of human umbilical vein endothelial cells increased surface expression of high mannose/hybrid N-glycans. A role for these sugars in mediating THP-1 or primary human monocyte rolling and adhesion was indicated by competition studies in which addition of α-methylmannoside, but not α-methylglucoside, inhibited monocyte rolling and adhesion during flow, but not under static conditions. This result supports the notion that adhesion molecules provide scaffolds for sugar epitopes to mediate adhesion with cognate receptors. A panel of structurally distinct PPARγ agonists all decreased TNFα-dependent expression of endothelial high mannose/hybrid N-glycans. Using rosiglitazone as a model PPARγ agonist, which decreased TNFα-induced high mannose N-glycan expression, we demonstrate a role for these carbohydrate residues in THP-1 rolling and adhesion that is independent of endothelial surface adhesion molecule expression (ICAM-1 and E-selectin). Data from N-glycan processing gene arrays identified α-mannosidases (MAN1A2 and MAN1C1) as targets for down-regulation by TNFα, which was reversed by rosiglitazone, a result consistent with altered high mannose/hybrid N-glycan epitopes. Taken together we propose a novel anti-inflammatory mechanism of endothelial PPARγ activation that involves targeting protein post-translational modification of adhesion molecules, specifically N-glycosylation.

Inflammation underlies the pathogenesis of numerous vascular diseases including atherosclerosis (1–4). A central tenet of this process is increased interactions between circulating leukocytes and the endothelium, which occurs via the sequential steps of rolling, firm adhesion, and ultimately transmigration into the vessel wall (4). Typically, increased leukocyte adhesion to the endothelium comprises an early event that can be stimulated by several factors including proinflammatory cytokines (e.g. tumor necrosis factor α (TNFα)).

A widely accepted mechanism for increased leukocyte adhesion is the up-regulation of endothelial adhesion molecule expression. However, as discussed previously (5–7), many adhesion molecules are glycosylated (via O- or N-linkages) or sulfated. These post-translational modifications may be important for correct trafficking to the plasma membrane and in many cases are the actual ligands that mediate adhesive interactions with leukocytes. Importantly, incorrect glycosylation can render the adhesion molecule inactive with respect to rolling and/or adhesion of circulating leukocytes. It has been suggested that under basal conditions not all expressed adhesion molecules are functional due to incorrect glycosylation (7). Interestingly, differential expression of glycans on the luminal surface at specific and distinct regions in the vasculature has been associated with different vascular inflammatory diseases, thus the glycan profile is analogous to a molecular zip code that regulates leukocyte trafficking (8).

Although evidence for both O- and N-glycans in mediating inflammatory processes exists, less is known about N-glycans. Some studies have demonstrated the importance of N-glycans in providing endothelial ligands for cognate receptors on neutrophils and lymphocytes (9–11). However, relatively little is known about how this post-translational modification of adhesion molecules is regulated during inflammation (12) and whether this occurs in a manner that overlaps with, or is dis-
distinct from mechanisms that control up-regulation of adhesion molecule protein expression.

Peroxisome proliferator activated receptor γ (PPARγ)\(^3\) is a nuclear receptor/transcription factor, which upon activation by a ligand, binds to PPAR-response elements on target genes resulting in either activation or inhibition of transcription. Transcriptional activity of these complexes is regulated by PPAR ligands. PPARγ activation has been investigated largely from the perspective of the regulation of genes that control lipid and glucose metabolism (13–15), with recent data suggesting critical roles in inhibiting vascular inflammation (15–18). The anti-inflammatory mechanisms remain poorly defined, and depending on the experimental conditions employed, can be associated with an inhibition of cytokine-dependent expression of endothelial adhesion molecules (16). Our recent studies suggest that PPARγ ligand treatment of endothelial cells under static conditions attenuates subsequent TNFα-dependent monocyte rolling and adhesion. Moreover, this effect is independent of TNFα-dependent increased adhesion molecule expression (19, 20). However, the mechanism for this effect remains unclear. In this study we provide evidence that PPARγ activation can prevent monocyte rolling and adhesion to TNFα-activated endothelial cells by selectively targeting adhesion molecule N-glycosylation. Specifically, we show that (i) TNFα stimulates endothelial expression of N-glycans (specifically of the high mannose and/or hybrid type) at cell junctions, (ii) that these epitopes play a role in modulating monocyte rolling and adhesion to the endothelium, and (iii) reveal a novel anti-inflammatory effect of PPARγ ligands in inhibiting TNFα-dependent up-regulation of endothelial surface N-glycans.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human umbilical vein endothelial cells (HUVEC), human aortic endothelial cells, and the human monocyte cell line, THP-1, were purchased from ATCC (Manassas, VA) or Lonza Corporation (NJ). Primary human monocytes were isolated using histopaque density gradients from healthy volunteers according to Institutional IRB approved protocols as previously described (20). Cell Tracker Green (CMFDA, C2925) fluorescent dye was purchased from Molecular Probes Inc., Eugene, OR. TNFα, RPMI 1640, and sugars and glycan synthesis inhibitors were purchased from Sigma. Endothelial basal medium and supplements (CC-3124) were purchased from Lonza. Rosiglitazone, ciglitazone, troglitazone, 15-deoxy-\(\Delta\)-12,14-prostaglandin J2 (15d-PGJ\(_2\)), and GW 9662 were purchased from Cayman Chemical Co. Nitrolinoleic acid (LNO\(_2\)) was a kind gift from Dr. Bruce Freeman (University of Pittsburgh). Fluorescently tagged lectins were purchased from Vector Labs.

**Cell Culture and Viability**—HUVEC and HAEC were cultured as previously described (20) and used between passages 3 and 7. All experiments were performed within 1 day of cells reaching confluence. THP-1 cells were maintained in RPMI 1640 containing 10% FBS, 1 mg/ml of penicillin-streptomycin at 0.5–1.0 \(\times\) 10\(^5\) cells/ml to maintain them in the log cell growth phase. For adhesion assays or processed for lectin fluorescence analysis below. Due to varying specific activities of TNFα from one batch to another, concentrations that increased monocyte adhesion to endothelial cells under static conditions by 2-fold was determined (varied between 2 and 10 ng/ml) and those concentrations were used in this study. Results from either HUVEC or human aortic endothelial cells were similar with respect to the TNFα-dependent changes in N-glycan profiles and inhibitory effects of rosiglitazone toward monocyte rolling and adhesion.

**Real-time RT-PCR**—Total RNA was extracted using the RNeasy Mini Kit (Qiagen), genomic DNA was removed using DNase I (Invitrogen) and reverse transcribed using the SuperScript III First-strand Synthesis Kit (Invitrogen). Sense and antisense primers, respectively, were designed using Primer Blast for ICAM-1 (5′-ACGGATGCCAGTGGGCCAC-3′, 5′-GGGAGCTCCTGAGGGCCAGA-3′), VCAM-1 (5′-GCCACACACAGGTGGACACA-3′, 5′-ACAGCCTGTGTCGTTGAAT-3′) and β\(_2\)-microglobulin (5′-AGACTCGGGCCGAGATGTCT-3′, 5′-TGCTGATGAGTCGTTGAGTA-3′). mRNA expression was analyzed via real-time PCR using SYBR Green master mix (Bio-Rad) and a 7300 Fast Real-time PCR system (Applied Biosystems). Data are presented as difference in C\(_T\) between adhesion molecule and β\(_2\)-microglobulin. Determination of human glycosylation related genes was conducted using a SABioscience human glycosylation PCR array (PAHS-046) according to the manufacturer’s protocol. Fold-changes were calculated using the \(\Delta\Delta\)C\(_T\) method compared with β\(_2\)-microglobulin and control expression.

**α-Mannosidase Activity**—α-Mannosidase activity was determined as described with minor modifications (21). Cells were treated as described and lysed in PBS containing 1% Triton X-100 for 10 min on ice before clarification at 10,000 \(\times\) g for 10 min. For each reaction, 50 μl of lysate (corresponding to 25–40 μg of protein) was combined with 10 μl of acetate buffer (1 mM sodium acetate, pH 6.5) and 40 μl of 25 mM p-nitrophenol-α-mannopyranoside in a microtiter plate. Plates were incubated for 2 h at 37 °C and the reaction was stopped by the addition of 100 μl of stop solution (133 mM glycine, 67 mM NaCl, 83 mM sodium carbonate, pH 10.4) and absorbance was measured at 405 nm. To determine specificity of reaction some samples also included 0.5 mM swainsonine or 0.1 μg/μl of kifunensine. Activity is reported as the relative absorbance per μg of protein. Each sample was run in duplicate and each treatment condition was tested 4–6 times.

**Static Adhesion Assay**—Static adhesion assays were performed as previously described (20). Briefly, HUVEC were grown in 48-well plates and treated with PPARγ ligand for 16 h

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\(^3\) The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; HUVEC, human umbilical vein endothelial cell; HAEC, human aortic endothelial cells; ANOVA, analysis of variance; ICAM, intercellular adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1.
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(All cases PPARγ ligand stock concentrations were diluted to ensure identical volumes were added, and where appropriate vehicle controls included) and during the last 4 h coincubated with or without TNFα. Cells were then washed twice with warm sterile PBS and incubated with monocytes at a final monocyte:endothelial cell ratio of 6:1 for 30 min at 37 °C in a 5% CO₂ incubator. The bound and unbound fractions were collected separately and fluorescence was measured using a PerkinElmer fluorescent plate reader (excitation = 485 nm, emission = 535 nm) and the percent of monocytes bound was determined.

**In Vitro Flow Assay—**Leukocyte rolling and firm adhesion during flow were determined as previously described (19, 20) using the Glycotech flow chamber system (Rockville, MD). Briefly, HUVEC were cultured in 35-mm dishes and treated with vehicle or PPARγ ligand for 16 h and during the last 4 h coincubated with or without TNFα. HUVEC were washed twice with warm sterile PBS and THP-1 monocytes labeled with Cell Tracker Green, then flowed over the endothelium at 100–300 µl/min corresponding to a wall shear rate (or shear stress the endothelial cells experience) of 0.5–1.5 dynes/cm² in RPMI basal media (without serum) containing calcium and magnesium. The flow system forms a laminar flow on the endothelial cell monolayer. HUVEC and the labeled monocytes were maintained at 37 °C throughout the duration of the experiment. The cells were viewed on a Leica inverted fluorescence microscope equipped with a Hamamatsu Orca ER digital CCD camera (Compix, Cranberry Township, PA). Real-time images of each field were captured at 33 frames/s for 2 min, and the resulting time lapse images were analyzed to calculate average rolling velocities. This was performed by motion tracking analysis using the Automated Image Capture and Motion Tracking and Analysis software (Simple PCI, Compix Inc., Cranberry Township, PA). Any cell that did not move for 5 s or more was considered to be firmly bound and numbers were calculated per min of data acquired. For experiments designed to determine the number of rolling monocytes and monocyte rolling velocities, 100,000 cells/ml were used. For experiments determining the number of adhered monocytes during flow, 250,000 cells/ml were used. To distinguish cells rolling on the endothelium via adhesive interactions from those freely flowing in the perfusion buffer close to the endothelium, the critical rolling velocity for THP-1 cells will be calculated as previously described (19, 20). Any cell traveling at a velocity below the critical rolling velocity is considered in contact with the endothelium, and as such is able to engage in rolling adhesions.

**Sugar Competition Experiments—**For sugar inhibition experiments, either α-methylglucoside or α-methylmannoside (0–200 mM) were mixed with THP-1 immediately (<30 s) prior to initiation of flow. In this protocol, the total exposure time of monocytes to sugars before exposure of monocytes to endothelium was <2 min. Viability of THP-1 exposed to the highest concentration of sugar used for 10 min was >95% assessed by Trypan blue exclusion (not shown). Note, interactions of monocytes and endothelium were followed for 2 min only (see above) and no changes in endothelial morphology or viability were observed over this period after exposure to the highest concentration of sugar used. Osmolarity of media (measured by vapor pressure osmometry) increased with increasing sugar concentrations, but was not different between mannose and glucose being 298 ± 5 (PBS), 295 ± 3.2 (RPMI), 297 ± 7.8 and 295 ± 0.5 (RPMI + 2 mM glucose and mannose, respectively), 312 ± 1.5 and 310 ± 3 (RPMI + 20 mM glucose and mannose, respectively), and 500 ± 0.7 and 500 ± 3.5 (RPMI + 200 mM glucose and mannose, respectively).

**Lectin Staining—**HUVECs were grown on microscope coverslips in endothelial basal medium containing 2% FBS. After treatments, cells were washed using ice-cold PBS containing 1 mM each CaCl₂ and MgCl₂ (2 ml, 2 times) and then stained with 10 µg/ml of lectin for 15 min on ice. Cells were washed with PBS (2 times) and fixed using 4% paraformaldehyde in PBS for 10 min at room temperature. Fixed cells were washed, DNA was stained with Hoechst 33342 (Molecular Probes), and coverslips were mounted for viewing. For different lectin-fluorescent conjugates, pilot studies were performed to define doses and incubation times for optimal binding. Images were acquired using a Leica fluorescent microscope and confocal images were acquired by the University of Alabama at Birmingham, Center for Developmental and Functional Imaging on a Zeiss LSM 710 confocal microscope. For 96-well plate-based staining, HUVECs were grown to confluence and were stained as above (lectin concentration was lowered to 1 µg/well). After fixation, plates were read on a Victor² plate reader (PerkinElmer Life Sciences). The different lectins used and their specificities are shown in Table 1.

**Immunofluorescent Staining—**HUVECs were stained with lectins as above, fixed, permeabilized with 0.1% Triton X-100 for 5 min at room temperature, and then blocked with 5% goat serum in PBS. After blocking, coverslips were incubated with mouse anti-occludin (1:200, Abcam) in PBS containing 5% goat serum overnight at 4 °C. Following washing, cells were incubated with goat anti-mouse Alexa Fluor 594 (1:1000, Molecular Probes) in PBS with 5% goat serum for 1 h at RT. DNA was stained with Hoechst 33342, coverslips were mounted, and images were acquired using a Leica fluorescent microscope.

**Statistical Analysis—**In vitro flow and static adhesion experiments were conducted >3 times with 3 replicates per experiment. In some studies, due to differing potencies of different batches of TNFα, fold-change in the number of adhered monocytes during flow relative to TNFα in each experiment were calculated and then averaged. Significance was assessed by either paired t test or by one-way ANOVA with post hoc analysis using Tukey test as indicated. Significance was set at a value of p < 0.05. Statistical analyses were performed using GraphPad software.

**RESULTS**

**TNFα Stimulates Expression of High Mannose and/or Hybrid N-Glycans at Endothelial Cell Junctions—**The hypothesis that inflammatory stimuli alter the expression of glycans on the endothelial cell surface was tested by administration of TNFα to HUVEC and monitoring changes in glycoforms using a panel of fluorescently tagged lectins. The lectins used and their specificities for specific carbohydrate epitopes/linkages are described in Table 1. Fig. 1 shows representative fluorescence micrographs and Fig. 1C their quantitation and demonstrates...
that TNFα increased binding of ConA, DSL, and LCA but not jacalin, VVL, SNA lectin, or UEAI. Specificity of lectin binding to N-glycans, and to high mannose specifically, was indicated by the attenuation of TNFα-dependent effects by addition of tunicamycin (5 μM), an inhibitor of the rate-limiting step in N-glycosylation (not shown) or inclusion of α-methylmannnoside during the staining procedure (Fig. 1B). Together, these data indicate increased expression of high mannose and/or hybrid N-glycans in response to TNFα, but not complex N-glycans nor O-glycans. Fig. 1 suggests that increased N-glycans are localized at the plasma membrane and endothelial junctions. This was confirmed by co-localization of ConA, DSL, and LCA binding with the junctional protein occludin (Fig. 2A) and for ConA and LCA (not shown) by confocal microscopy (Fig. 2B).

Endothelial Expression of High Mannose/Hybrid N-Glycans Modulate Monocyte-Endothelial Interactions—To test if increased high mannose/hybrid N-glycans contribute to TNFα-dependent leukocyte rolling and adhesion to endothelial cells, a competition experiment was performed in which dynamic flow-dependent THP-1 monocyte adhesion to TNFα-activated HUVEC was evaluated in the absence and presence of increasing concentrations of α-methylmannose or α-methylglucose. The latter was included as an osmotic and negative control because it will not compete with mannose residues expressed on the endothelial cell surface. Fig. 3 shows that THP-1 adhesion during flow was not affected by α-methylglucose, but were significantly blunted when α-methylmannose (20 and 200 mM) was included in flow media. Inhibition was not complete, reaching maximal levels of ~60%, and neither α-methylmannose nor α-methylglucose affected THP-1 adhesion to TNFα-stimulated HUVEC evaluated under static conditions (Fig. 3B).

Rosiglitazone Inhibits Monocyte-Endothelial Interactions during Flow—Our previous studies demonstrated that exposure of HUVEC to the synthetic PPARγ ligand rosiglitazone (2 μM, 16 h) had no effect on TNFα-dependent adhesion of THP-1 monocytes under static conditions. However, under the same experimental conditions, rosiglitazone did inhibit monocyte adhesion when assessed in the presence of flow (20). Fig. 4 extends these data to show that rosiglitazone inhibits THP-1 adhesion in a dose-dependent manner (Fig. 4A) and occurs at all flow rates tested (Fig. 4B). Fig. 4, C and D, shows that TNFα increased expression of E-selectin and ICAM-1, two candidate adhesion molecules important in mediating rolling and adhe-

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**TABLE 1**

**Lectins used and their specificity**

| Lectin                                    | Glycan recognized        | Sugar moiety recognized         |
|-------------------------------------------|--------------------------|---------------------------------|
| Concanavalin A (ConA)                     | High mannose N-glycan    | α-Linked mannose                |
| Lens culinaris agglutinin (LCA)           | High mannose/hybrid N-glycan | α-Linked mannose          |
| Datura stramonium lectin (DSL)            | Complex N-glycan         | (1,4)-Linked N-acetylglucosamine |
| Ulex europeaus agglutinin I (UEA I)       | O-Glycan                 | α-Linked fucose                 |
| Jacalin                                   | O-Glycan                 | Galactose (β1,3)-N-acetylgalactosamine |
| Vicia villosa lectin (VVL)                | Complex N-glycan         | α or β-linked terminal N-acetylgalactosamine (GalNAcα(3)Gal) |
| S. nigra lectin (SNA)                     |                          | α2,6-Sialic acid                |

**FIGURE 1.** TNFα stimulates expression of high-mannose and/or hybrid N-glycans. Panel A, HUVEC were treated with TNFα (10 ng/ml, 4 h) and glycan expression was determined using lectin fluorescence. Shown are representative fluorescence micrographs (×40 magnification) for lectins as indicated. Green, lectin binding; blue, Hoechst (nuclei). Panel B shows ConA and DSL staining in TNFα-treated HUVEC in the absence and presence of α-methylmannose. Panel C shows quantitation of lectin binding (from panel A). Values shown mean ± S.E. (n = 3). *, p < 0.02 and **, p < 0.01 relative to corresponding control by Student’s t test.
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**FIGURE 2.** TNFα enriches N-glycans at cell junctions. Panel A, HUVECs were treated with TNFα (10 ng/ml, 4 h) and N-glycans were stained using the indicated lectins. Cells were then stained for the tight junction marker occludin. Representative images (×63 magnification) from three separate experiments are shown. Green, lectin; red, occludin; blue, nuclei. Panel B, HUVECs were treated with TNFα (10 ng/ml) and stained with FITC-ConA and imaged by confocal microscopy. A representative three-dimensional reconstruction is presented.

**FIGURE 3.** TNF-α up-regulated endothelial N-glycans mediate THP-1 rolling and adhesion only during flow. HUVEC were untreated (control) or treated with TNFα (10 ng/ml) and THP-1 adhesion during flow was assessed at 1.5 dyn/cm² (panel A), or under static conditions (panel B) and in the absence (hatched bars) or presence of either α-methylglucose or α-methylmannose at the indicated concentrations. For panel A data are presented as fold-change relative to TNFα and represent mean ± S.E. (n = 3–7). #, p < 0.05 relative to TNFα; *, p < 0.01 relative to TNFα by ANOVA with Tukey post-test. The number of firmly adhered cells for the TNFα group ranged from 4 to 10. For panel B data show mean ± S.E. (n = 4–8). *, p < 0.05 relative to TNFα by ANOVA with Tukey post-test.

**Effects of Mannose and Rosiglitazone on Primary Human Monocyte Adhesion during Flow**—Differences between THP-1 cells (human leukemia cell line) and primary human monocytes have been documented. To test if monocyte cell transformation endows sensitivity to mannose and the rosiglitazone effects described above, primary human monocytes were freshly isolated and flow-dependent adhesion to TNFα-activated HUVEC ± rosiglitazone or in the presence of α-methylmannose (as described above) was determined. Fig. 5 shows that competition assays with α-methylmannose in the flow media or pre-treatment of HUVEC with rosiglitazone inhibited monocyte adhesion during flow in a similar manner to that observed with THP-1 cells.

**PPARγ Ligands Inhibit TNFα-dependent Expression of Endothelial N-Glycans**—We hypothesized that one mechanism by which rosiglitazone may attenuate monocyte-endothelial interactions only during flow and in the absence of adhesion molecule expression changes, is to regulate adhesion molecule N-glycosylation. Fig. 6A shows that rosiglitazone inhibits ConA binding to TNFα-treated HUVEC in a dose-dependent manner that parallels inhibitory effects on THP-1 adhesion during flow. These effects of rosiglitazone were reversed by co-addition of the PPARγ antagonist GW9662 (Fig. 6B). The PPARα agonist benzofibrate had no effect on TNFα-dependent increased ConA binding (not shown). Furthermore, various structurally distinct PPARγ ligands, but not vehicle controls, all inhibited TNFα-dependent increases in ConA binding (Fig. 6C). These data reveal a novel molecular target for anti-inflammatory effects of PPARγ ligands directed toward endothelial N-glycoforms.

**Rosiglitazone Reverses TNFα-dependent Down-regulation of α-Mannosidase Activity**—To determine potential targets for (i) how TNFα modulates protein N-glycosylation and (ii) how PPARγ activation reverses this, a targeted gene array for human glycosylation genes was performed. Fig. 7A shows a heat map indicating relative expression changes of 24 genes involved in N-glycan processing in control, rosiglitazone, TNFα, and
TABLE 2
Effects of rosiglitazone on TNFα-dependent up-regulation of adhesion molecule mRNA

|                  | Control | Rosiglitazone | TNFα | TNFα + Rosiglitazone |
|------------------|---------|---------------|------|----------------------|
| ICAM-1           | 10.4±0.74 | 10.8±0.21    | 2.3±0.26 | 3.1±0.35             |
| VCAM-1           | ND      | ND            | 7.5±0.41 | 8.7±0.15             |

*ND, not detected.

DISCUSSION

The anti-inflammatory effects of PPARγ activation in endothelial cells can occur via several mechanisms prominent of which is inhibition of proinflammatory cytokine-dependent up-regulation of adhesion molecule expression (25, 26). Our previous studies (19, 20), which were confirmed herein, have shown that activation of PPARγ can also attenuate TNFα-dependent THP-1 rolling and adhesion to endothelial cells without altering adhesion molecule expression. In this study, evidence is provided for a novel PPARγ-dependent anti-inflammatory mechanism that involves regulation of endothelial N-glycosylation.

Protein N-glycosylation is a multiple step process that ultimately results in 3 types of N-glycoforms: high mannose, hybrid, or complex-type (27). Generally, N-glycosylated proteins are targeted for secretion or expression on the cell surface. Despite the knowledge that adhesion molecules are N-glycosylated (6), relatively little is known about the regulation or functional effects of N-glycosylation in immune cell-endothelial interactions. However, a role in the TNFα-dependent effects can be postulated based on findings that this proinflammatory cytokine modulates the pattern of N-glycosylation in synovioocytes (28), mediates interconversion between high mannose and hybrid N-glycans in epithelial cells (29), and affects expression of genes encoding enzymes responsible for protein N-glycosylation in endothelial cells (29, 30). Similarly, tumor-conditioned media-dependent alteration of endothelial N-glycan composition has been proposed to be important in tumor extravasation (31). Several studies have also demonstrated the functional effects of altered endothelial N-glycosylation. For example, aberrant N-glycosylation of E-cadherin is associated with carcinogenesis secondary to altered cell-cell adhesion and...
communication (32). With respect to leukocyte interactions specifically, increased expression of carboxylated \( \text{N} \)-glycans (33) or \( \text{N} \)-linked high mannose sugars (10, 11) on endothelial cells have been implicated in mediating neutrophil adhesion under static conditions. Furthermore, inhibition of \( \text{N} \)-glycan synthesis decreases IL-1-mediated lymphocyte binding to endothelial cells (34) and high endothelial venular expression of \( \text{N} \)-glycans has been shown to be critical to support L-selectin-mediated lymphocyte trafficking (9). Interestingly, in the latter case, evidence that alteration of \( \text{N} \)-glycan composition independent of the adhesion molecule expression, was important in lymphocyte homing was provided.

Our data support and develop these concepts by demonstrating that TNF-\( \alpha \)-dependent modulation of \( \text{N} \)-glycosylation is regulated by pathways distinct to those that modulate expression of adhesion molecules. Specifically, lectin staining studies showed that TNF-\( \alpha \) treatment of endothelial cells increases high mannose/hybrid \( \text{N} \)-glycan expression, which was inhibited by PPAR\( \gamma \) ligands. Increased high mannose/hybrid \( \text{N} \)-glycan expression was observed at endothelial cell junctions, which are also sites where adhesive interactions with leukocytes occur (35–38). Moreover, TNF-\( \alpha \) also increased expression of ICAM-1, VCAM-1, and E-selectin, which are important mediators of monocyte rolling and adhesion. However, the increased expression of these adhesion molecules was not affected by PPAR\( \gamma \) ligands, yet THP-1 adhesion was decreased, suggesting increased adhesion molecule expression alone is insufficient to mediate monocyte adhesion. Finally, the observation that THP-1 adhesion was attenuated by coincubation of \( \alpha \)-methyl-
This model is also consistent with the fact that adhesion molecules are the scaffolds for the actual ligands (namely glycosylated and sulfated epitopes) that mediate adhesive interactions with cognate receptors. It has also been suggested that under basal conditions not all expressed adhesion molecules are functional due to incorrect glycosylation. These concepts have led to the suggestion that the glycosylation process may be regulated by distinct pathways to those that regulate adhesion molecule expression, which is supported by data reported herein.

Little is known on how TNFα and PPARγ activation modulate endothelial glycosylation. Previous gene array studies (29) suggest that the myriad of glycosyl and mannosyltransferases that act in concert to control the pattern of protein N-glycosylation in the endoplasmic reticulum and Golgi complex are potential targets. This was confirmed by targeted N-glycan processing gene array studies that identified α-mannosidases MANA2 and MANC1, and B4GALT1 and ST6GAL1 as proteins whose expression was modulated by TNFα and reversed by rosiglitazone. MANA2 and MANC1 are α,1,2-mannosidases that catalyze the earliest steps of mannose removal required for the conversion of high mannose to hybrid and subsequently complex N-glycans. B4GALT1 (facilitates galactose addition) and ST6GAL1 (adds sialic acid in α2,6-conformation to galactose residues) catalyze latter steps in N-glycan maturation. It is important to note that the sequential nature of the N-glycosylation process ensures that the end pattern of N-glycosylation is regulated by enzymes catalyzing the earlier steps. For example, Fig. 7B shows that ST6GAL1, which catalyzes the terminal addition of α1,2-sialic acid onto galactose residues on N-glycan structures, increases in response to TNFα. However, binding of S. nigra lectin, a lectin specific for α2,6-sialic acid, did not increase after TNFα treatment (see Fig. 1). This is likely explained by the fact that expression of the upstream mannosidasises MANA2 and MANC1 decreased (a result consistent with increased ConA binding (Fig. 1)), which is predicted to prevent N-glycan maturation beyond high mannose types. Importantly, PPARγ activation reversed TNFα-dependent α-mannosidase down-regulation and gene expression changes translated into concomitant changes in α-mannosidase activity (Fig. 7C). Interestingly, four distinct α1,2-mannosidases exist, and despite the redundancy in their activities, down-regulation of two of these still significantly (~20%) decreased total cellular α1,2-mannosidase enzymatic activity.

We have not identified the specific protein(s) whose N-glycosylation status is affected by TNFα and PPARγ activation. One potential candidate is ICAM-1, which can mediate both rolling and adhesive phases of leukocyte adhesion to endothelial cells (39). Appropriate ICAM-1 N-glycosylation ensures correct protein folding and trafficking to the cell surface. Also the pattern of N-glycosylation on the third extracellular IgG-like domain of ICAM-1 is key for binding the cognate receptor, CD11b (Mac-1) (22, 23). Specifically, inhibiting the processing of high mannose to complex N-glycans increased CD11b binding to ICAM-1 (22). The aforementioned study concluded that by decreasing the branching and hence size of N-glycans (by preventing complex N-glycan formation), steric effects were minimized leading to enhanced CD11b binding. Previous data
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(10) and data presented herein using a α-methylmannose-based competition offer an alternative explanation whereby high mannose/hybrid N-glycans are directly engaged in binding of cognate receptors. Consistent with this, monocyte integrins (e.g. MAC-1) have been shown to possess lectin sites were man- nose binding can occur (40). Unlike previous studies, however, our data only revealed a role for high mannose/hybrid N-glycans when THP-1 adhesion was assessed during flow. The basis of this difference is not clear and we note that competition experiments showed maximal inhibition of ~60% suggesting that high mannose N-glycans are not the only mediators of leukocyte recruitment. This is consistent with functional redundancy among multiple binding sites within a given endothelial adhesion molecule, and between different adhesion mol- ecules themselves and we note that further studies are required to determine the proteins whose N-glycosylation status is reg- ulated during inflammation.

Emerging data are highlighting the importance of the glyco- calyx as a key site for regulating the inflammatory cascade with the concept being that proinflammatory stimuli (e.g. TNFα, hyperglycemia or oxidized low-density lipoprotein) induce shedding of glycosaminoglycans thereby decreasing the width and size of the endothelial glyocalyx (13, 41–47). This in turn allows greater accessibility of circulating macromolecules to underlying glycoproteins (e.g. adhesion molecules) that present their epitopes to which circulating leukocytes roll and adhere. The model presented here suggests that in concert with decreased glyocalyx size, TNFα also modulates the pattern of N-glycosylation at endothelial junctions, which in turn mediates higher affinity interactions with circulating monocytes.

In summary, evidence is provided that TNFα increases high mannose/hybrid N-glycans expression at endothelial junctions and that these sugars are important in controlling monocyte trafficking by mediating rolling and adhesion. This model is consistent with a recent hypothesis that forward a key role for core N-glycan structures (i.e. mannose) in providing signals to the innate immune system for recognizing cells under inflam- matory stress (48, 49). We extend this model further by show- ing that endothelial PPARγ activation may be an important regulator of this process, which also provides a novel anti-in- flammatory mechanism for activation of these nuclear receptors.

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