Increased Production of 12/15 Lipoxygenase Eicosanoids Accelerates Monocyte/Endothelial Interactions in Diabetic db/db Mice*

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Atherosclerosis is a major complication of diabetes. Up to 16 weeks of age, the db/db mouse is insulin-resistant and hyperglycemic and is a good model of Type 2 diabetes. After ~16 weeks of age, the mice develop pancreatic beta cell failure that can progress to a Type 1 diabetes phenotype. We have previously shown that glucose increases production of endothelial 12/15 lipoxygenase (12/15LO) products in vitro. In young 10-week-old Type 2 diabetic db/db mice, we found significant elevations in levels of urinary 12/15LO products, 12S-hydroxyeicosatetraenoic acid (12S-HETE) and 13S-hydroxyoctadecenoic acid (13S-HODE) in vivo compared with C57BLKS/J mice. Using isolated primary endothelial cells (ECs) from db/db mice and WEHI78/24 mouse monocyte cells in static adhesion assays, we found increased WEHI monocyte adhesion to db/db ECs (14 ± 2 monocytes/field for db/db ECs versus 4 ± 1 monocytes/field for C57BLKS/J ECs, p < 0.002). Thus, ECs from db/db mice appear to be “pre-activated” to bind monocytes. Analysis of db/db ECs revealed a 2-fold elevation in 12/15LO protein compared with C57BLKS/J EC. To determine that 12/15LO products were responsible for the increased monocye adhesion observed with db/db ECs, we inhibited expression of murine 12/15LO using either an adenovirus expressing a ribozyme to 12/15LO (AdRZ) or with the 12/15LO inhibitor cinnamyl-3,4-dihydroxy-α-cyanocinnamate. Treatment of db/db ECs for 48 h with AdRZ or 4 h with 10 μM cinnamyl-3,4-dihydroxy-α-cyanocinnamate significantly reduced monocyte adhesion to db/db endothelium (p < 0.009). Thus, inhibition of the murine 12/15LO in db/db mice significantly reduced monocyte/endothelial interactions. We also found that adhesion of monocytes to diabetic db/db ECs was mediated by interactions of α4β1 integrin on monocytes with endothelial vascular cell adhesion molecule 1 and connecting segment 1 fibronectin and interactions of β2 integrins with endothelial intercellular adhesion molecule 1. In summary, regulation of the 12/15LO pathway is important for mediating early vascular changes in diabetes. Modulation of the 12/15LO pathway in the vessel wall may provide therapeutic benefit for early vascular inflammatory events in diabetes.

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came from the studies of Funk and colleagues (19, 21, 23), who showed that disruption of the 12/15LO gene in mice significantly reduced atherosclerosis development in vivo. Several groups have shown that the human 15LO enzyme oxidizes low density lipoproteins (LDL) in vitro (20, 24, 25). Cathcart and colleagues (26) found that 12/15LO activity in monocytes produced superoxide that mediated oxidation of LDL. 12/15LO protein has been localized to aortic atherosclerotic lesions in rabbits and in humans (27, 28) and is responsible for production of oxidized lipid adducts localized within atherosclerotic plaques (29, 30).

In the current study, we examined early inflammatory events that mediate vascular complications in vivo using diabetic db/db mice. We found that db/db mice produce significant amounts of 12/15LO eicosanoid products in vivo. We found that aortic endothelial cells from these mice are “pre-activated” to bind monocytes. Blocking of the 12/15LO pathway in endothelial cells of the db/db mice prevented monocyte/endothelial interactions. The results of this study indicate that products of the 12/15LO pathway mediate monocyte/endothelial interactions in diabetes.

**EXPERIMENTAL PROCEDURES**

*Reagents—* Antibodies to endothelial adhesion molecules for flow cytometry (FITC anti-mouse CD106 (5533322), FITC anti-mouse CD94 (553252), and FITC-labeled isotype controls) were purchased from BD Biosciences. Rat anti-mouse antibodies to VLA-4 (PS2) and VCAM-1 (MK2) were provided by Dr. Klaus Ley (University of Virginia). Rabbit anti-mouse ICAM-1 antibody (YN1.1) was purchased from Chemicon. Anti-mouse β3 integrin antibody (GAME-46) was purchased from BD Biosciences. Polyclonal antibody to 12/15LO was provided by Dr. Jerry L. Nadler (University of Virginia). CS-1 peptide (EILDVPST) was purchased from American Peptide Co. WEHI 7/24 monocytes were a gift of Dr. J. A. Berliner (UCL). Cinnamon hydroxy-cyano-cinnamate (CDC) was purchased from Biomol. Mitochondrial ROS inhibitors carbonyl cyanide m-chlorophenylhydrazone and thenoylflur- orocactone were purchased from Sigma.

*Mice—* Young 10-week-old male db/db (BKS.Cg-m+/+Leprdb) mice were obtained from Jackson Laboratories (stock number 000642). The db/db mice were on a pure C57BLKS/J background strain; thus 10-week-old male C57BLKS/J mice from Jackson Laboratories (stock number 000662) were used as controls. Mice were fed rodent chow and housed in micro-isolator cages in a pathogen-free facility. All experiments followed Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) guidelines and approval for use of rodents was obtained from University of Virginia.

**Eicosanoid Measurement in Urine—** For extraction of lipids from urine, 0.5 ml of urine from each mouse was added to 40 nmol of internal standard nonphysiological 8-HETE in siliconized screw-capped glass tubes. The pH was adjusted to pH 3 using 1 M HCl. 2.25 ml of ethyl acetate was added and tubes were centrifuged at 9000 rpm. The ethyl acetate phase was transferred to a 5-ml glass vial and dried under nitrogen. The dried lipid phase was dissolved in 0.2 ml of acetonitrile and diluted with 0.8 ml of water. Free fatty acids were separated from phospholipids and neutral lipids using two C18 Bond-Elute columns, (1 ml, 50 mg; Varian). Samples were loaded onto the first column, and polar phospholipids were eluted with 30% acetonitrile. Free fatty acids and neutral lipids were eluted with 0.4 ml of 90% acetonitrile. These fractions were diluted with 0.9 ml of water and reapplied to a fresh column, and the free fatty acids alone were eluted using 0.6 ml of ethyl acetate.

The fluorescence derivatives of the free fatty acids were formed using 0.5% 2,3-naphthalimide/ethanolamine/sulfonamide dissolved in 0.5 ml of acetonitrile. The reaction mixtures were dried with nitrogen, resuspended in 0.4 ml of acetonitrile, diluted with 0.6 ml of water, and applied to a third Bond-Elut column. The fatty acid derivatives were eluted with 0.6 ml of ethyl acetate, evaporated under nitrogen, and resuspended in 100 µl of methanol for HPLC analysis. HPLC separation and analysis was performed using a C18 Waters symmetry column and a Waters 510 pump for 100 min with B = 90% methanol/tetrahydrofuran plus 0.1% acetic acid and A = 0.1% acetic acid following related protocols of Roman et al. (31). Peaks were detected fluorometrically at an excitation wavelength of 259 nm and emission wavelength of 394 nm. The area ratio of sample HETE area/internal standard (8-HETE) area was plotted against nanograms of HETE injected, and unknown sample HETE values were calculated from their area ratios. All HETEs (5-, 12-, and 15-HETE) and HODEs (9- and 13-HODE) were baseline separated using this elution protocol. HETE and HODE measurements in urine were normalized to milligrams of creatinine.

**Isolation of Mouse Aortic Endothelial Cells—** Aortic endothelial cells from C57BLKS/J and db/db mice were harvested from aortic root under sterile conditions. The aorta was excised, all peri-adventitial fat was removed, and the aortic pieces were placed onto Matrigel in DMEM plus 15% heat-inactivated FBS following the methods outlined by Shi and colleagues (32, 33). After 3 days, the aortic explants were removed, and the endothelial cells were allowed to grow in DMEM plus 15% fetal bovine serum (FBS) supplemented with 180 µg/ml endothelial cell growth supplement. At confluency, the cells were passaged using Dispase and then cultured for 2 days in DMEM plus 15% heat-inactivated FBS containing n-valine to eliminate possible fibroblast contamination. After 2 days, the ECs were returned to growth medium without n-valine and allowed to grow to confluency.

Mouse endothelial cell cultures were tested for purity at passage 2 using either Von Willebrand factor staining or di-acetylated LDL uptake and were used in experiments from passages 3 to 6.

**Mouse Monocyte Adhesion Assay—** Our laboratory has recently developed a monocyte adhesion assay that utilizes primary MAECs and WEHI7/24 cells. WEHI7/24 cells are a mouse monocytic cell line that has been fully characterized by McEvoy and colleagues (34, 35). WEHI were cultured in DMEM plus 10% heat-inactivated FBS. WEHI cells are labeled with calcein-AM using standard methods described by the manufacturer (Molecular Probes). For the adhesion assay, MAECs were cultured to confluency in a 48-well plate and incubated with 35,000 calcein-labeled WEHI cells/well for 30 min at 37 °C. Nonadher- ent cells were rinsed, and adherent cells were fixed with 1% glutaraldehyde. The number of adherent monocytes within a 10 × 10 eyepiece grid at ×40 magnification was counted using epifluorescence microscopy. As a positive control for monocyte adhesion, MAECs were incubated with 10 units/ml recombinant murine TNFα (R&D Systems #410- MT) for 4 h. For studies using blocking antibodies or peptides, WEHI cells were incubated for 15 min at 37 °C with 10 µg/ml EILDVPST, antibody to α, integrin (clone PS2, 20 µg/ml), antibody to β3 integrin (clone GAME-46, 20 µg/ml), or isotype control antibody prior to adding to MAECs for adhesion assay. In some studies, VCAM-1 antibody (clone MK2.7, 20 µg/ml) block endothelial VCAM-1, ICAM-1 antibody (clone YN1.1, 20 µg/ml) to block endothelial ICAM-1, or iso- type control antibody was added to ECs for 4 h at 37 °C prior to performing a monocyte adhesion assay.

**Cytofluorimetry—** C57BLKS/J and db/db MAECs at passage 4 were collected in PBS by gentle scraping using a cell scraper. 150,000 cells per sample were analyzed for each antibody. Cells were incubated for 30 min at 4 °C with 1:100 dilution of antibody (FITC anti-mouse VCAM-1 and FITC anti-mouse ICAM-1 or isotype control antibody). After incubation, cells were rinsed 3× in PBS and fixed in paraformaldehyde. Samples were analyzed using a FACSCalibur cell sorter. Analyses were performed using a single FITC-labeled antibody per tube. Unstained and isotype control antibodies were included in analyses as controls.

**Human Endothelial Cell Culture—** Human aortic endothelial cells (HAECs) were obtained from aortic rings of explanted donor hearts (11). HAECs were cultured for 7 days in Medium 199 containing 20% heat-inactivated FBS, 20 µM H2O2, 100 µg/ml gentamicin, 90 µg/ml heparin in the presence of 5.5 mM glucose (NG) or 25 mM glucose (HG) for 7 days. The 7-day, 25 mM HG incubation condition was chosen because monocyte adhesion to endothelial cells was maximal at this concentration of glucose and time of incubation (11). For studies using chemical uncouplers of mitochondrial function, HAECs were cultured for 7 days above and treated with 2.5 mM 2,4-dinitrophenol, 50 µM methanol/tetrahydrofuran plus 0.1% acetic acid or 0.1% acetic acid following related protocols of Roman et al. (31). Peaks were detected fluorometrically at an excitation wavelength of 259 nm and emission wavelength of 394 nm. The area ratio of sample HETE area/
this kit is specific for 12S-HETE, with less than 0.1% cross-reactivity with other eicosanoids.

**Statistical Analyses**—Data for all experiments were analyzed by ANOVA and Fisher’s protected least significant difference test using the StatView 6.0 software program. Data are represented as the mean ± S.E. of eight mice per group unless otherwise noted in the figure legends. Monocyte adhesion data are represented as the mean ± S.E. of six experiments unless otherwise noted in the figure legends.

**RESULTS**

Monocyte Adhesion to Endothelial Cells Is Increased in Diabetic Mice—db/db mice are hyperglycemic and insulin-resistant as early as 6 weeks of age (2). At 6–12 weeks of age, these mice are used as a model of Type 2 diabetes (21). We recently developed a technique for isolating primary endothelial cells from mouse aorta (36). Using this approach, we examined monocyte adhesion to endothelial cells from control (C57BLKS/J) and diabetic (db/db) mice. As shown in Fig. 1, we found that basal, unstimulated ECs isolated from db/db mice bound more monocytes than did control ECs in a static adhesion assay (p < 0.002). TNFα (10 units/ml) was added to C57BLKS/J ECs as a positive control to show maximal monocyte adhesion. **,** significantly higher than CTR (p < 0.0001). Data represent the mean ± S.E. of eight experiments.

Fig. 1. Monocyte adhesion to endothelial cells in increased in diabetic db/db mice. Endothelial cells were isolated from aorta of C57BLKS/J (CTR) and diabetic (db/db) mice. Cells were used from passages 3 through 6. Adhesion assays using WEHI cells, a mouse monocyte cell line, were performed as described under “Experimental Procedures.” TNFα (10 units/ml) was added to CTR cells as a positive control (CTR+TNF) to show maximal monocyte adhesion. *, significantly higher than CTR (p < 0.002); **, significantly higher than CTR (p < 0.0001). Data represent the mean ± S.E. of eight experiments.

Diabetic db/db Mice Have Increased Production of 12/15LO Eicosanoid Products—We have previously shown that HAECS cultured chronically in elevated glucose show increased production of 12S-HETE and 15S-HETE, the primary products of the 12/15LO enzyme (11). We have also shown that exogenous addition of these eicosanoids to HAECS stimulated monocyte adhesion (11). We examined whether diabetic db/db mice would have increased production of 12/15LO eicosanoid products in vivo. Urine was collected for 24 h from control C57BLKS/J and diabetic db/db mice. Using novel fluorescent HPLC techniques for quantitation of eicosanoids (see “Experimental Procedures”), we found a dramatic 5-fold increase in the amounts of 12S-HETE and 15S-HETE produced in the db/db mouse (Fig. 3). 9S-HODE and 13S-HODE production was increased 2-fold in the db/db mice in vivo (Fig. 3). These eicosanoid products are generated by the 12/15LO enzyme in mice; the platelet 12LO enzyme does not produce 9S-HODE and 13S-HODE (15). There was no change in levels of 5S-HETE (a product of the 5LO pathway; Fig. 3) or in cyclooxygenase enzyme products (data not shown) in the db/db mice.

Because we saw a large increase in the production of 12/15LO eicosanoids in db/db mice in vivo and based upon our previous work, which indicated that 12S-HETE can stimulate monocyte/endothelial adhesion, we examined whether there was an increase in 12/15LO protein in db/db mice. As shown in Fig. 4, there was approximately a 2-fold increase in the level of 12/15LO protein in aorta of db/db mice. We also found increased expression of 12/15LO in kidney of db/db mice (data not shown). Thus, ECs from db/db mice have increased expression of 12/15LO protein that leads to increased 12/15LO eicosanoid production in vivo.

**Monocyte Adhesion to ECs of Diabetic db/db Mice Is Caused by 12/15LO Expression**—To determine that 12/15LO products were directly responsible for the increased monocyte adhesion...
observed with db/db ECs, we inhibited expression of 12/15LO using an adenovirus expressing a ribozyme to 12/15LO (designated AdRZ) as well as using the 12/15LO inhibitor CDC. CDC blocks platelet 12LO and 12/15LO expression in ECs (39). We inserted the ribozyme into an adenovirus expressing either the 12/15LO ribozyme (AdRZ) or the AdLacZ to glucose (22). As shown in Fig. 5, addition of the 12/15LO ribozyme (+AdRZ) or control LacZ vector (+AdLacZ) followed by addition of labeled normal WEHI monocytes for an adhesion assay as described under “Experimental Procedures.” *, significantly higher than CTR (p < 0.001); #, significantly lower than db/db or CTR alone (p < 0.009). Data represent the mean ± S.E. of three experiments.

Fig. 5. Inhibition of 12/15LO enzyme activity in ECs of diabetic db/db mice prevents monocyte adhesion. MAECs from C57BLKS/J (CTR) or db/db (db/db) mice were incubated with either 10 units/ml mTNFα (+TNF) or CDC (+CDC) for 4 h or incubated for 48 h with recombinant adenovirus expressing either the 12/15LO ribozyme (+AdRZ) or control LacZ vector (+AdLacZ) followed by addition of labeled normal WEHI monocytes for an adhesion assay as described under “Experimental Procedures.” *, significantly higher than CTR (p < 0.001); #, significantly lower than db/db or CTR alone (p < 0.009). Data represent the mean ± S.E. of three experiments.

DISCUSSION

Vascular inflammation and damage are major events that contribute to vascular complications of diabetes (41–47). Using a mouse model of diabetes, we present novel findings that show that eicosanoid products of the 12/15LO pathway are significantly up-regulated in diabetic db/db mice in vivo and that these products mediate early events in inflammation that contribute to atherosclerosis and vascular damage.

A quite exciting finding was that diabetic db/db mice have a severalfold increase in production of 12/15LO products in vivo. The reasons for this increased production are unknown yet could relate to presumably to either 1) increased activity of the murine 12/15LO in db/db mouse (22). As shown in Fig. 5, addition of the 12/15LO inhibitor CDC or the AdRZ to db/db ECs completely blocked monocyte adhesion (p < 0.009). These data indicate that monocyte adhesion in diabetic db/db mice is mediated through the 12/15LO enzyme pathway.

We next examined expression of VCAM-1 and ICAM-1 on db/db mouse ECs using flow cytometry. Surface expression of VCAM-1 and ICAM-1 was not increased on db/db ECs compared with control ECs (50.5% expression on CTR ECs versus 49.2% expression on db/db ECs for ICAM-1, and 78.1% expression on CTR ECs versus 74.4% expression on db/db ECs for VCAM-1). We examined whether the inhibitor CDC would reduce expression of VCAM-1 or ICAM-1 and found no reduction of adhesion molecule expression on db/db ECs by CDC (Fig. 6).

Production of 12/15LO Products Is Mediated by ROS in
due to modulation of cytosolic PLA2 activity. We will examine this possibility in db/db mice. Interestingly, we found that monocyte adhesion to endothelial cells was significantly increased in diabetic db/db mice. Under normal conditions, endothelial cells do not bind monocytes unless stimulated to do so. We found a significant increase in monocyte adhesion to unstimulated db/db ECs, suggesting that the db/db mouse ECs are pre-activated to bind monocytes. In diabetes, monocyte/endothelial adhesion is accelerated due to hyperglycemia and increased oxidative stress (55–59). Our original observations in normal human aortic ECs in vitro were that glucose stimulated monocyte/endothelial adhesion and that glucose also increased production of 12/15LO products (11). Another study by our group (22) indicates that blocking of the 12/15LO pathway in HAECs in vitro blocked monocyte adhesion. In our current study, inhibition of the murine 12/15LO in diabetic db/db ECs completely prevented diabetes-mediated monocyte/endothelial interactions (Fig. 5). Taken together, these studies provide novel, strong evidence that glucose regulates monocyte/endothelial interactions in diabetes through modulation of 12/15LO.

We clearly found involvement of both α,β1 integrin interactions with VCAM-1 and CS-1 FN and β2 integrin interactions with ICAM-1 in mediating monocyte/endothelial interactions in db/db mice. Interestingly, expression of endothelial VCAM-1 and ICAM-1 was not increased in db/db mice. Currently, there are no available reagents to measure expression of CS-1 FN on

**Fig. 6.** 12/15LO inhibitor CDC does not reduce endothelial expression of adhesion molecules. Representative flow cytometry histograms showing ICAM-1 and VCAM-1 expression on the surface of db/db db/db endothelial cells are shown. Incubation of db/db ECs for 4 h with 10 μM CDC did not reduce expression of VCAM-1 or ICAM-1 on db/db ECs (db/db+CDC). Flow cytometry was performed as described under “Experimental Procedures.” Data were confirmed using four mice per group.

**Fig. 7.** Mitochondrial ROS production is linked to 12S-HETE production in HAECs. HAECs were cultured for 7 days in 5.5 mM glucose (NG) or 25 mM glucose (HG) in the presence of pharmacological inhibitors of the mitochondrial electron transport chain (+TTFA and +CCCP) as described under “Experimental Procedures.” 12S-HETE was measured in cell media using an EIA. Values were normalized to total cell protein. ***, significantly higher than NG (p < 0.0001); *, significantly lower than HG by ANOVA (p < 0.001).
mouse endothelium, so we could not determine whether CS-1 FN was increased on ECs of db/db mice. We did find that both CS-1 FN and VCAM-1 contributed to monocyte/endothelial adhesion (Fig. 2). Blocking of both epitopes for CS-1 FN and VCAM-1 on WEHI monocytes completely prevented monocyte adhesion. We also found that ICAM-1 and β2 integrin played a role in db/db-mediated monocyte adhesion. The exact quantification of αβ1 integrin-mediated interactions with CS-1 FN and VCAM-1 versus β2 integrin-mediated interactions with ICAM-1 in contributing to db/db-mediated monocyte adhesion was not measured in the current study. However, although not done in a quantitative manner, we did find that blocking of αβ1 integrin completely prevented monocyte/endothelial adhesion in db/db ECs, whereas blocking of β2 integrins did not completely prevent adhesion. Additional studies are necessary to address this issue. Nevertheless, we were clearly able to illustrate that all three of these key receptor-counter-receptors on endothelial cells (CS-1 FN, VCAM-1, and ICAM-1) are important in regulating monocyte adhesion in db/db mice.

The signaling pathways by which 12/15LO products stimulate endothelial activation are unclear but probably involve reactive oxygen species production. Brownlee and colleagues (40) have shown that glucose stimulates ROS production in endothelial cells. Stimulation of 12/15LO activity generates superoxide as well (26, 29). As shown in Fig. 7, inhibitors of ROS production in ECs blocked 12/15LO-mediated production. These data suggest that 12/15LO activity is modulated through ROS production. Thus, our hypothesis is that glucose activates ROS production in ECs, which subsequently activates 12/15LO. Studies have shown regulation of the 12/15LO gene by the transcription factors Sp1 and AP-1 as well as by specific mito-

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Additions and Corrections

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