The Natural Protective Mechanism Against Hyperglycemia in Vascular Endothelial Cells: Roles of the Lipid Peroxidation Product 4-Hydroxydodecadienal (4-HDDE) and PPARδ

Short title: 4-HDDE and PPARδ downregulate glucose uptake in VEC

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**Objective**- Vascular endothelial cells (VEC) downregulate their rate of glucose uptake in response to hyperglycemia by decreasing the expression of GLUT-1. Hitherto we discovered critical roles for the protein calreticulin and the arachidonic acid metabolizing enzyme 12-lipoxygenase in this autoregulatory process. The hypothesis that 4-hydroxydodeca-(2E,6Z)-dienal (4-HDDE), the peroxidation product of 12-lipoxygenase, mediates this downregulatory mechanism by activating PPARδ was investigated.

**Research Design And Methods**- Effects of 4-HDDE and PPARδ on the glucose transport system and calreticulin expression in primary bovine aortic endothelial cells were evaluated by pharmacological and molecular interventions.

**Results**- Using GW501516 (PPARδ agonist) and GSK0660 (PPARδ antagonist) we discovered that high glucose-induced downregulation of the glucose transport system in VEC is mediated by PPARδ. A PPAR-sensitive luciferase reporter assay in VEC revealed that high glucose increased markedly luciferase activity, while GSK0660 abolished it. HPLC analysis showed that high glucose incubation substantially elevated the generation of 4-HDDE in VEC. Treatment of VEC, exposed to normal glucose, with 4-HDDE mimicked high glucose and downregulated the glucose transport system and increased calreticulin expression. Like high glucose, 4-HDDE significantly activated PPARδ in cells overexpressing hPPARδ, but not hPPARα, -γ1 or -γ2. Moreover, silencing of PPARδ prevented high glucose-dependent alterations in GLUT-1 and calreticulin expression. Finally, specific binding of PPARδ to a PPAR response element in the promoter region of the calreticulin gene was identified by employing a specific ChIP assay.

**Conclusions**- Collectively our data show that 4-HDDE plays a central role in the downregulation of glucose uptake in VEC by activating PPARδ.
Hyperglycemia is a major and independent risk factor in the development of cardiovascular disease and atherosclerosis in diabetes (1;2). Vascular endothelial cell (VEC) dysfunction precedes the development of atherosclerotic plaques (3-6). These adverse functions in VEC result from impaired carbohydrate metabolism, mitochondrial dysfunction, oxidative stress, excessive protein glycation, malfolding of proteins and altered expression of various genes (3;7).

For reasons not well understood, some diabetic patients never develop long-term vascular complications (8;9). We discovered an autoregulatory mechanism that protected VEC against deleterious effects of hyperglycemia by downregulating the level of their principal glucose transporter GLUT-1 mRNA and protein, and its plasma membrane abundance (10-12). We have linked this protective mechanism to an augmented expression of the enzyme 12-lipoxygenase (12-LO), which produces 12-hydroxyeicosatetraenoic acid (12-HETE) from arachidonic acid. Pharmacological inhibition of 12-LO completely blocked this downregulatory interaction, suggesting a critical role for 12-HETE in this process. We also found that GLUT-1 downregulation resulted from destabilization and degradation of GLUT-1 mRNA via a specific interaction with the protein calreticulin (13) whose expression was significantly increased in VEC and blood vessels under hyperglycemic glucose conditions (13).

The enzyme 12-LO converts arachidonic acid to 12-hydroperoxyeicosatetraenoic acid (12-HpETE), which is effectively reduced by glutathione peroxidase (GPx) to 12-HETE (14). Reactive oxygen species (ROS), which are produced under hyperglycemic conditions (7), inactivate GPx and slow this reaction. This renders 12-HpETE susceptible to radical induced-peroxidation and a chain-breaking reaction to generate the corresponding reactive hydroxyalkenal, 4-hydroxododeca-(2E, 6Z)-dienal (4-HDDE) (15;16).

High levels of 4-hydroxyalkenals are cytotoxic due covalent adduct formation with macromolecules. Yet, these lipid peroxidation products function at low concentrations as signaling molecules (17;18). For instance, 4-hydroxy-2E-nonenal (4-HNE), the peroxidation product of 15-HpETE, binds to and activates the nuclear receptor PPARδ in 3T3-L1 preadipocytes (19).

The present study aimed at investigating the hypothesis that 12-HETE and/or 4-HDDE, the oxidation and peroxidation products of 12-HpETE, respectively, interact with PPARδ and regulate calreticulin expression to operate the downregulatory machinery of the glucose transport system in VEC.

**RESEARCH DESIGN AND METHODS**

**Materials:** Glucose–free Dulbecco’s modified Eagle’s medium (DMEM) was from Gibco (Grand Island, NY). Biological Industries (Beth-Haemek, Israel) supplied antibiotics, bovine fibronectin, fetal calf serum and soybean trypsin inhibitor. Calbiochem (Darmstadt, Germany) supplied GW501516 and 4-HNE. 4-HDDE was synthesized as described (1). Sigma-Aldrich (Israel) supplied 12-HETE, WY14643, baicalein, cytochalasin B, GSK0660, L-glucose, troglitazone and the anti α-tubulin antibody. The following polyclonal antibodies were used: rabbit anti-calreticulin (Stressgen Biotechnologies, Victoria, BC, Canada), rabbit anti-GLUT-1 (courtesy of Dr. H-G. Joost, the Institute of Human Nutrition, Bergholz-Rehbrücke, Germany), anti (C-terminus) GLUT-3 and GLUT-4 (Millipore, Billerica, MA), anti PPARα, PPARγ and PPARδ (Cayman chemicals. Ann Arbor, MI);
anti PPARδ (H-74; Santa Cruz Biotechnology, Santa Cruz, CA) and horseradish peroxidase-conjugated anti rabbit IgG (Jackson ImmunoResearch, West Grove, PA). Abcam (Cambridge, UK) supplied the ChIP assay kit. American Radiolabeled Chemicals (St. Louis, MO) supplied 2-[1,2-^3^H(N)]deoxy-D-glucose (1.48 TBq/mmol). Mirus Bio-Corporation (Madison, WI) supplied the TransIT-LT1reagent and Biontex Laboratories (Munich, Germany) the Metafectene. Promega Madison, WI) supplied the dual luciferase reporter assay system, ligase, Pfu DNA polymerase, pGEM T-easy plasmid, random primers, restriction enzymes and RNA polymerase RNasin was from TaKaRa (Tokyo, Japan). Real Time PCR reagents were purchased from Applied Biosystems (Carlsbad, California). ReddyMix PCR Master was from Thermo Scientific (Epsom, Surrey, UK). The PPARδ siRNA was from Dharmacon (Lafayette, CO). The pcDNA3 and pEGFP-N1 plasmids and the MCF10 cDNA library were kindly provided by Dr. R. Hertz and Dr. R. Reich, respectively, (the Hebrew University, Jerusalem, Israel). The pSVPORT1-hRXR vector and the 3xPPRE-TK-luciferase plasmid were donated by Dr. B.M. Spiegelman (Dana Farber Cancer Institute, Boston, MA). The plasmids pCMX-hPPARγ1 and pCMX-hPPARγ2 and the respective empty plasmids were kindly provided by Dr. R. Evans (Howard Hughes Medical Institute, LaJolla, CA). The pSG5 and pSG5-hPPARα vectors were prepared in Dr. B. Staels’ laboratory. All primers were synthesized by Sigma-Aldrich (Israel). Organic solvents were from Frutarom Ltd. (Haifa, Israel) and Mallinckrodt Baker B.V. (Deventer, Holland) supplied organic solvents.

**Cell cultures:** Primary cultures of bovine aortic endothelial cells were prepared and characterized as described previously (20). EA.hy926 cells were cultured and maintained as described (21).

**METHODS**

**Hexose uptake assay:** The [H^3^]dGlc uptake assay in VEC cultures was conducted as previously described (22). The non-specific [H^3^]dGlc uptake that was determined in the presence of 10 µmol/L of cytochalasin B in the uptake mixture was less than 4% of the total uptake. Cell numbers were determined microscopically in a hemocytometer following cell detachment with a trypsin-EDTA solution for endothelial cells (Sigma-Aldrich, Israel). Soybean trypsin inhibitor (50 µg/ml) was used to stop the reaction. Trypan Blue exclusion tests showed less than 1-3% dead cells following trypsinization.

**Western blot analyses:** Cell lysates were prepared and Western Blot analyses of GLUT-1 calreticulin, PPARδ and α-tubulin were performed as previously described (22) or according to the antibody suppliers’ protocols. Cell surface biotinylation of VEC and determination of plasma membrane associated GLUT-1 were carried as described (23).

**Real Time PCR analysis:** RNA isolation, cDNA synthesis and real time PCR analyses of calreticulin and GLUT-1 mRNA in VEC were performed as described by Totary-Jain et al. (13) using the same primers. Both mRNA levels were normalized to 18S rRNA.

**Cell transient transfections:** VEC cultures , at 60% confluency, were cotransfected with DNA complexed to TransIT-LT1reagent in 2 ml of growth medium according to the manufacturer’s instructions. The DNA consisted of 165 ng of pSG5-hPPARα, or pCMX-hPPARγ1, or pCMX-hPPARγ2, or pcDNA-hPPARδ or the corresponding empty plasmids. It also contained the following plasmids: pSVPORT1-hRXR (82.5 ng), 3xPPRE-TK-Luciferase reporter (500 ng), the Renilla luciferase (100 ng) and pEGFP-N1 (82.5 ng).
The latter served to assess the yield of transfection by fluorescence microscopy (>80%; excitation, 490 nm; emission, 515 nm). After 24 h, the cultures were washed, received fresh medium, and incubated for additional 24 h. Firefly luciferase-induced luminescence was determined with the dual luciferase reporter assay in Mithras LB-940 luminometer, and normalized to Renilla luciferase activity as an internal control according to the kit’s instructions (Berthold Technologies, Bad Wilbad, Germany).

**Extraction of polar lipids and HPLC analysis:** Polar lipid extraction from culture media or plasma and HPLC analysis were performed according to Zanardi et al. (24) with some modifications. Briefly, VEC cultures in 15-cm plates were incubated for 24 h with serum-free media and the indicated additions. The media were then collected, cleared by centrifugation and loaded on pre-washed Supelclean LC-18 SPE tubes (6mL/1gr.; Supelco, Bellefonte, PA) and washed with 15 mL of cold water and petroleum ether (boiling range 40-60 °C). The polar lipid fraction was then eluted with 3 mL of cold methanol, dried under N₂, dissolved in 300 μL of cold methanol, filtered through a Teflon syringe filter (0.45 μM; National Scientific, Rockwood, TN), sealed under N₂ and kept at -70°C. HPLC analysis was performed in a Merck Hitachi machine with a Supelcosil LC-18-DB column (5μM particle size, 25-cm x 10-mm; Supelco, Bellefonte, PA) connected to an UV detector (295 nm). For 4-HDDE, the elution (1.0 mL/min) started with a gradient of acetonitrile:water (42:58), flowed by a linear gradient that progressed over 25 min to 100% acetonitrile. For 4-HNE detection the initial ration in the mixture was 30:70. Pure 4-HDDE and 4-HNE were used to resolve relevant peaks at 10.0 and 4.2 min, respectively. The recovery of the standards was 85-90%. Clarity-Lite software was used to analyze and quantify HPLC data.

**Chromatin immuneprecipitation (ChIP):** VEC at 5.5 mmol/L glucose were transfected with pcDNA-hPPARδ, pSVPORT1-hRXR and pEGFP-N1 plasmids, as described above, and incubated for 24 h. The cells were then treated with 100 nmol/L of GW501516 or with DMSO and incubated for additional 24 h. Following treatment with GW501516 or with DMSO, cell lysates were prepared, sonicated, fractionated, immunoprecipitated with anti-PPARδ (H-74) and anti-histone H3 (positive control, Abcam, ab-1791) and taken for PCR with specific primers, according to the protocol of the ChIP assay kit (Abcam, Cambridge, UK), and as described in the online Data Supplement.

**PPARδ silencing with siRNA:** Subconfluent bovine aortic EC cultures were transfected with 100 nmol/L of the small interference RNA (siRNA) 5′- AAATGATGTCACTGAAGGGC-3′ targeted to bovine PPARδ using metafectene according to the manufacturer’s instructions. Control cells were treated with metafectene only. Cells were harvested 72 h after transfection and lysates were prepared and used for Western blot analyses.

**Statistical analysis:** Results are given as Mean ± SEM. Statistical analyses were performed using the nonparametric Mann-Whitney test.

**RESULTS**

Effects of PPAR agonists on the rate of glucose uptake in VEC: The potential role of PPARα, -γ or -δ in the regulation of glucose uptake in VEC was elucidated by studying the capacity of their respective selective agonists, WY14643, troglitazone and GW501516 (25) to downregulate the rate of glucose uptake in VEC under normal glucose levels (Figure 1A). The control treatment (25 mmol/L glucose) shows the characteristic high glucose-induced ~50% reduction in the rate of hexose uptake in comparison to the 5.5 mmol/L glucose
incubation (10). GW501516, but not troglitazone or WY14643, mimicked high glucose and comparably downregulated the uptake rate in cells under normal glucose, indicating that only PPARδ participates in the downregulatory response. Maximal effect of GW501516 was obtained between 1 to 100 nmol/L and within 36-48h (Supplemental Figures S1A and S1B which can be found in an online appendix athttp://diabetes.diabetesjournals.org). The vehicle (DMSO) had no significant effect on the rate of glucose uptake.

Figure 1B shows that the PPARδ antagonist GSK0660 (26) effectively prevented the downregulation of glucose uptake induced by 25 mmol/L glucose in VEC, whereas the basal high rate of glucose uptake in cells at 5.5 mmol/l glucose was not altered. Supplemental Figure S1C confirms the specificity and the competitive nature of GSK0660-induced inhibition of PPARδ.

Baicalein, a specific 12-LO inhibitor was used to test the hypothesis that 12-LO metabolites and PPARδ cooperate in the downregulatory response Figure 1C shows that the inhibition of 12-LO with baicalein prevented high glucose-induced downregulation of glucose uptake. Yet, GW501516 downregulated the uptake in the presence of baicalein. No synergistic interactions between GSK0660 and baicalein in high glucose VEC cultures were found, suggesting that 12-LO and PPARδ act in sequence (Figure 1D). The cellular content of PPARδ was not altered by the ambient glucose, GW501516 or baicalein (Supplemental Figure S2).

Supplemental Figure S3 indicates that the slight increase in the osmotic pressure in the high glucose-containing culture medium had no effect on the glucose transport system.

**GW501516 reduces GLUT-1 expression and plasma membrane abundance in VEC.**
PPARδ is this endogenous receptor that is activated under hyperglycemic conditions.

12-HETE activates PPARδ in VEC:
High glucose increases 12-LO expression in VEC and consequently augments the synthesis and secretion of 12-HETE in VEC (10). When tested in the present luciferase reporter assay, 12-HETE (3 µmol/L, 24 h) increased luciferase activity 1.70±0.03-fold (n=3) in hPPARδ-expressing VEC, in comparison with control cells. This is most likely an underestimated value because 12-HETE is prone to a rapid oxidation in the air [t_{1/2} = 10-12 min (10)]. This degradation is most likely the reason for the failure of exogenously added 12-HETE to downregulate the rate of hexose transport in VEC cultures (10).

4-HDDE activates PPARδ and modulates the rate of hexose transport in VEC: Radical-induced peroxidation of the 12-LO and 15-LO products, 12-HpETE and 15-HpETE, respectively, generate the corresponding biologically active aldehydes 4-HDDE and 4-HNE (16;18). It has been reported that 15-HETE and 4-HNE activate PPARδ (19). We asked whether 4-HDDE could activate PPARδ in VEC. Figure 4A shows a remarkable 4.3±0.1-fold increase in hPPARδ (but not hPPARα-, hPPARγ1- or hPPARγ2-) dependent stimulation of the reporter luciferase activity in cells treated with 100 nmol/L 4-HDDE. Like high glucose and GW501516 (Figures 1 and 2), 4-HDDE (50 nmol/L) significantly reduced the rate of hexose uptake (Figure 4B) and the cell content of GLUT-1 mRNA and protein (Figure 4C and 4D) in VEC under 5.5 mmol/L glucose. The upregulatory effect induced by baicalein in high glucose cultures was also reversed by 4-HDDE (Figure 4, B-D). The inhibitor GSK0660 abolished 4-HDDE-induced downregulation of the rate of hexose transport in VEC exposed to 5.5 mmol/L glucose (Fig. 4E). The concentrations of 4-HDDE used in these experiments (50-100 nmol/L) did not compromise VEC viability (Supplemental Figure S7). The similar effects of 4-HDDE and GW501516 suggest that the former is an endogenous ligand for PPARδ.

Extraction of polar lipid and HPLC analyses were performed to measure 4-HDDE production in VEC exposed to 2 or 25 mmol/L glucose. High glucose increased the secretion of 4-HDDE 5.9±3.1-fold higher than at the low glucose incubation (Figure 5A), while baicalein blocked this effect, confirming that 4-HDDE is indeed derived from a 12-LO metabolite. The initial step in lipid peroxidation is a hydrogen atom abstraction by reactive oxygen species (16); therefore antioxidants can attenuate this process (27). Figure 5 (A&B) shows that the antioxidant N-acetylcysteine completely prevented high glucose-dependent generation of 4-HDDE and the downregulation of hexose transport. Figure 5C depicts a comparable increase in HDDE generation in the plasma of hyperglycemic Zucker diabetic rats (details on the animals are given in the online Data Supplement. Representative HPLC tracings and 4-HDDE peaks in culture medium and plasma extracts are shown in Supplemental Figure S8. Noteworthy, similar HPLC analyses of the same extracts did not reveal significant differences in 4-HNE levels between the low- and high glucose incubations (data not shown).

PPARδ regulates calreticulin expression in VEC:
Calreticulin, whose expression is significantly increased in VEC exposed to high glucose, destabilizes GLUT-1 mRNA (13). Figures 6 (A-D) confirm previous findings (13) on high glucose-induced increased expression of calreticulin mRNA and protein levels in VEC under high glucose. This effect was eliminated in the presence of baicalein, suggesting that 12-LO metabolites participate in the regulation of calreticulin expression. Both GW501516 (Figures 6A and 6B) and 4-HDDE (Figures
both compounds also reversed the effect of baicalein and restored calreticulin mRNA and protein expression to the levels measured under the high glucose incubation. Inversely, the inhibition of PPARδ with GSK0660 prevented high glucose-induced increased expression of calreticulin in VEC (Figure 6E). These data suggest that PPARδ participates in the regulation of calreticulin expression in VEC.

The potential of PPARδ to interact with PPAR-regulatory elements (PPREs) in the calreticulin gene was determined. We identified four PPRE sequences in the promoter region of the bovine calreticulin gene (3,000 bp upstream of the coding sequence) located at: 5′-1710-1732, 1964-1986, 2139-2161 and 2255-2277 bp. Chromatin immunoprecipitation (ChIP) assay was employed to determine whether PPARδ binds to these elements. DNA samples from VEC overexpressing hPPARδ and hRXR and treated with 100 nmol/L of GW501516 or the vehicle were immunoprecipitated with an anti-PPARδ antibody and taken for the respective PCR analyses. Figure 6F depicts a specific binding interaction of PPARδ with the PPRE located at 5′-2139-2161 bp in this promoter region.

PPARδ expression in VEC was silenced with a specific siRNA and the expression levels of calreticulin and GLUT-1 were determined (Figure 7). A 50-60% reduction in PPARδ expression in VEC exposed to 25 mmol/L led to a 30-40% decrease in the content of calreticulin and a 30-40% increase in GLUT-1 expression in comparison with the respective controls. Similar effects were observed in VEC exposed to 5.5 mmol/L glucose (data not shown).

Finally, we used the EA.hy926 cell line to determine whether human-derived endothelial cells regulate the glucose transport system like bovine VEC. This cell line, which was derived from primary cultures of human umbilical cord vascular endothelial cells (21), has been shown in many studies to preserve markers and functions of the primary cells. High glucose downregulated the rate of glucose transport and total GLUT-1 level and increased calreticulin expression in a PPARδ-dependent manner in these cells in [Supplemental Figure S9 (A-C)]. EA.hy926 cells under normal glucose condition and exposed to 4-HDDE mimicked the effect of high glucose and reduced the rate of hexose transport, while GSK0660 prevented this effect (Fig. S9D). These data suggest that both bovine and human vascular endothelial cells employ a similar mechanism to downregulate the glucose transport system.

DISCUSSION

Two key factors that mediate high glucose-induced downregulation of the glucose transport system in bovine aortic endothelial cells and in the human-derived EA.hy926 cells have been identified: the lipid peroxidation product 4-HDDE, and its cognate nuclear receptor PPARδ. The latter increases the expression of the protein calreticulin that was shown before to destabilize GLUT-1 mRNA (13).

The augmented production of 4-HDDE results from high glucose-induced 12-LO expression and activity and glucose-derived ROS. The mechanism responsible for the increase expression of 12-LO is not yet known. Numerous studies have proven that hyperglycemia promotes the generation of ROS (3;7). We showed before an augmented production of ROS in bovine aortic EC primary cultures under high glucose conditions (28). Two observations confirm the role of ROS in the generation of 4-HDDE from 12-LO metabolites. First, the inhibition of 12-LO activity with baicalein significantly reduced 4-HDDE secretion from VEC under
high glucose conditions. Second, the antioxidant N-acetylcysteine blocked 4-HDDE production in VEC under similar conditions. The capacity of baicalein and N-acetylcysteine to prevent high glucose-induced downregulation of glucose uptake is attributed to the impeded production of 4-HDDE. Collectively, these data indicate that high glucose-induced overexpression of 12-LO and overproduction of ROS underlie the augmented generation of 4-HDDE.

The physiological, pathophysiological or cytotoxic effects of 4-hydroxyalkenals depend on their absolute concentrations. Esterbauer et al. (18) concluded that at concentration higher than 20 µmol/L 4-HNE exhibited cytotoxic effects due to its chemical reactivity and the formation of stable adducts with macromolecules. Bacot et al. (16; 29) linked 4-hydroxyalkenals to membrane disorders due to the formation of stable adducts with ethanolamine phospholipids (PE). It is important to emphasize that the effective concentrations of 4-HDDE (1-100 nmol/L) used in the present study were not cytotoxic to VEC. The present study shows that a moderate production of ROS, sufficient to initiate the generation of non-toxic levels of 4-HDDE in VEC, is required for an effective cellular defense against harmful effects of hyperglycemia. Notwithstanding, an excessive and uncontrolled oxidative stress in VEC may lead to the production of cytotoxic levels of 4-hydroxyalkenals. Many studies have indeed suggested that an excessive 4-hydroxyalkenal production underlies various pathological conditions (30;31).

Tissue-specific production of 4-hydroxyalkenals correlates with the particular pattern of expression of the various lipoxygenases and synthesis of their corresponding HpETEs. For example, the peroxidation products 4-HHE and 4-HNE, but not 4-HDDE were found in the diabetic rat retina, which predominantly expresses 5-LO and 15-LO (29;32). Our present findings on 4-HDDE- and PPARδ-dependent downregulation of the glucose transport system in human vascular endothelial cells correlate well with previous reports on high glucose-induced expression of 12-LO in human VEC (4).

We found before that high glucose levels increased the expression of 12-LO, but not of 15-LO in VEC (10). Subsequently, high glucose promotes the generation of 4-HDDE from 12-HpETE but not of 4-HNE, which is derived from 15-HpETE. Nevertheless, it has been shown before that 4-HNE also activates PPARδ (19). Using the luciferase reporter assay we found that the potency of 4-HDDE was 500-higher (50 nmol/L) than that reported above for 4-HNE. These disparate potencies suggest that binding affinity of 4-HDDE to the ligand binding domain (LBD) in PPARδ is markedly higher than that of 4-HNE. This may reflect the increased hydrophobicity of the 4-HDDE in comparison with 4-HNE (LogP values 3.48 and 2.45, respectively, calculated with the Molinspiration Chemoinformatics software). X-ray analyses of crystal structures of the PPARδ LBD identified a network of hydrogen bonds with His413, Tyr427, His287 and Thr253 that are involved in the binding interaction of eicosapentanoic acid (33). His413 has also been implicated in a hydrogen-bonding interaction with the 4-hydroxy group of 4-HNE (19). The specific molecular binding kinetics and affinity of 4-HDDE to the PPARδ LBD remain to be analyzed.

Many studies over the last decade have assigned PPARδ key metabolic regulatory functions (34). For instance, it augments lipogenesis and glycolysis in the liver (35), increases fatty acid oxidation in adipocytes (36) and increases oxidative metabolism in skeletal muscles (37;38). Of interest are recent studies on protective effects of PPARδ ligands against the development of atherosclerosis by regulating lipid homeostasis, decreasing the expression of
inflammatory genes and attenuating macrophage migration. We speculate that 4-HDDE-activated PPARδ may mediate some of these effects.

This study links PPARδ activation to the transcription of the calreticulin gene. We have previously found that the final step in the downregulation of GLUT-1 expression in VEC is calreticulin-mediated destabilization of the transporter mRNA (13). Because calreticulin is a multifunctional protein (39) it is feasible that PPARδ-regulated transcription of calreticulin may affect some of these functions (e.g., calcium storage, cell adhesion, chaperoning of malfolded proteins). The present study has identified a PPRE in the promoter of the calreticulin gene that seems to interact with PPARδ. The precise nature of this interaction and its contribution to the assembly of an active transcription complex of calreticulin need further investigations.

Recently Gross and Staels (40) have stressed the need for the development of novel PPARα and PPARγ agonists for the treatment of type-2 diabetes. We suggest that novel PPARδ agonists may reduce the risk of dysfunctional endothelial cells in blood vessels by downregulating the rate of glucose uptake and protecting them from the deleterious effects of increased influx of glucose. Clearly, 4-HDDE is not considered a potential pharmacological agonist due its severe side effects when present at concentrations that allow irreversible covalent binding and cross-linking of macromolecules. Nonetheless, molecular and chemical analyses of the interaction of 4-HDDE with the PPARδ LBD may provide a platform for a rational design of potent and safe PPARδ agonists.

Finally, our previous and current findings on the autoregulation of the glucose transport system in VEC are summarized in the model shown in Figure 8: High glucose levels increase the expression of the arachidonic acid metabolizing enzyme 12-LO, which leads to an augmented production of 12-HpETE and its immediate metabolite 12-HETE. Concomitantly, high glucose-derived ROS initiate the peroxidation of 12-HpETE and the generation of 4-HDDE. This molecule, and possibly 12-HETE, interact specifically with and activate PPARδ, which in turn binds to a PPRE element in the promoter region of the calreticulin gene and augments its transcription. Calreticulin binds to a specific 10-nucleotide sequence located in the 3'-UTR region of GLUT-1 mRNA, destabilizes and renders it susceptible to degradation. Consequently the cell content of GLUT-1 protein and its plasma membrane abundance are significantly reduced and downregulation of glucose uptake ensues.

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FIGURE LEGENDS

Figure 1. Activation of PPARδ reduces the rate of hexose transport in VEC.
Confluent VEC cultures were exposed to 5.5 or 25 mmol/L glucose for 48 h. (A), The 5.5 mmol/L cultures were treated without or with GW501516 (100 nmol/L), troglitazone (30 µmol/L) or WY14643 (60 µmol/L). (B), Both 5.5 and 25 mmol/L glucose cultures were incubated for 48 h in the absence or presence of 1 µmol/L of GSK0660. (C), VEC cultures were incubated at 5.5 or 25 mmol/L glucose and treated with GW501516 (100 nmol/L) and/or baicalein (80 µmol/L), as indicated. The latter was present during the last 10 h of incubation. (D), VEC cultures were incubated at 5.5 or 25 mmol/L glucose for 48 h without or with GSK0660 (1 µmol/L, 48 h) and or baicalein (80 µmol/L, last 10 h). At the end of incubations, cells were washed and taken for the standard [3H]dGlc uptake assay. The rates of dGlc uptake at 5.5 mmol/L glucose [(A) 70 ± 8, (B) 54 ± 5 (C) 96 ± 1 and (D) 65 ± 8 pmol dGlc/10^6 cells/min] were taken as 100%. *P<0.05 for differences from the respective controls (n=4).

Figure 2. GW501516 decreases the level of GLUT-1 mRNA and protein as well as its plasma membrane abundance in VEC.
VEC cultures were treated with GW501516 and baicalein as described under the legend to Figure 1C. Cells were then processed and taken for (A) real-time PCR analysis of GLUT-1 mRNA, (B) Western blot analysis of GLUT-1 or (C) for cell surface biotinylation to determine the relative plasma membrane (PM) abundance of GLUT-1. *P<0.05, for differences from the respective controls (n=4).

Figure 3. High glucose selectively activates PPARδ in VEC.
(A), VEC cultures were transfected with the 3xPPRE-TK-Luciferase reporter- and the Renilla luciferase plasmids and incubated at 2 or 25 mmol/L glucose for 48 h. GSK0600 (1 µmol/L) was present as indicated during the last 24 h of incubation. P<0.05 in comparison with the *2- or **25 mmol/L glucose control incubations (n=4) (B), VEC cultures were transfected with the following expression plasmids: pSG5-hPPARα or pCMX-hPPARγ1 or pCMX-hPPARγ2 or pcDNA-hPPARδ. Control cells were transfected with the corresponding empty plasmids. Cells were also co-transfected with pSVPORT-hRXR, pEGFP-N1 plasmid, 3xPPRE-TK-Luciferase reporter plasmid and the Renilla luciferase plasmid. The cells were incubated for 48 h at 2 or 25 mmol/L glucose and then harvested, lysed and taken for the luciferase activity assay. Results are given as the relative luciferase activity in comparison with the 2 mmol/L glucose incubation. *P<0.05, (n=4).

Figure 4. 4-HDDE specifically activates PPARδ and downregulates the glucose transport system in VEC.
(A), VEC cultures that had been maintained at 5.5 mmol/L glucose were transfected with the various hPPAR expression vectors and other plasmids and incubated as described in the legend to Figure 3B. Cultures received the indicated concentration of 4-HDDE, during the last 24 h of incubation. The cells were then lysed and the relative luciferase activity was determined and standardized to untreated control. *P<0.05, for differences from the untreated cells (n=4). (B-D), Confluent VEC cultures were incubated for 48 h with 5.5 or 25 mmol/L glucose in the absence or presence of 4-HDDE (50 nmol/L). Baicalein (80 µmol/L) was added to cultures for the last 10 h of incubation. The cells were then washed, lysed and taken for (B) the standard [3H]dGlc
4-HDDE and PPARδ downregulate glucose uptake in VEC

uptake assay, (C) real-time PCR analysis of GLUT-1 mRNA or (D) Western blot analysis of total GLUT-1. The rate of dGlc uptake at 5.5 mmol/L glucose (79 ± 3 pmol dGlc/10⁶ cells/min), was taken as 100%. *P<0.05, for differences from the respective controls (n=4). (E), Confluent VEC cultures were incubated at 5.5 or 25 mmol/L glucose for 48 h in the absence or presence of 4-HDDE (50 nmol/L) and or GSK0660 (1 µmol/l), as indicated. At the end of incubation the cells were washed and taken for the standard [³H]dGlc uptake assay. The rate of dGlc uptake at 5.5 mmol/L glucose [65 ± 8 pmol/10⁶ cells/min] was taken as 100%. *P<0.05, for differences from the respective controls (n=4).

Figure 5. 4-HDDE level is increased in high glucose-VEC cultures and in the plasma of Zucker Fatty Diabetic rats.

(A), Media (18 ml) of 2 or 25 mmol/L glucose VEC, without or with 80 µmol/l of baicalein or 1 mmol/L of N-acetylcysteine, were collected after 48 h of incubation, extracted and taken for HPLC analysis of polar lipids. The average level of 4-HDDE in the 2 mmol/L medium extracts was taken as 1 unit. P<0.05, for differences from the *2 or **25 mmol/L glucose incubations (n=4). (B), VEC cultures at 25 mmol/L glucose were treated similarly and taken at the end of the 48 h incubation period to the standard [³H]dGlc uptake assay. The rate of uptake at 5.5 mmol/L glucose (70 ± 1 pmol dGlc/10⁶ cells/min) was taken as 100%. P<0.05, for differences from the *2 or **25 mmol/L incubations (n=4). (C), The levels of 4-HDDE in extracts of plasma samples from ZDF and normoglycemic rats (respective blood glucose levels were 354± 28 and 92± 6, n=4) were determined by HPLC and the value of the average levels of 4-HDDE in the plasma of the non-diabetic rats was taken as 1 unit. *P<0.05, for differences from the respective controls (n=4).

Figure 6. PPARδ regulates calreticulin expression in VEC.

Confluent VEC cultures were treated with (A and B) GW501516 (100 nmol/L) or (C and D) 4-HDDE (50 nmol/L) as described in the legends to Figures 1A and 4B, respectively. Cells were then processed for a real-time PCR analysis of calreticulin mRNA (A and C) or a Western blot analysis of calreticulin (B and D). *P<0.05, for differences from the respective controls (n=4). (E), Confluent VEC cultures were treated with GSK0660 (1 µmol/L) as indicated for 48 h. At the end of incubation the cells were processed for a Western blot analysis of calreticulin. (F) VEC overexpressing hPPARδ and hRXR, which were prepared as described in the legend to Figure 3B, were treated with GW501516 or the vehicle and processed for PPARδ-ChIP analysis as described under “Methods”.

Figure 7. Partial silencing of PPARδ reduces calreticulin- and increases GLUT-1 expression in VEC.

Subconfluent cultures of VEC maintained at 25 mmol/L glucose were transfected with siRNA targeted to the bovine PPARδ mRNA, as described under “Methods”, and incubated for 72 hr. The cultures were then washed, lysed and taken for Western blot analyses of (A) PPARδ, (B) calreticulin and (C) GLUT-1. *P<0.05, for differences from the metafectene controls (n=4).

Figure 8. A model for high-glucose-induced downregulation of the glucose transport system in VEC.
4-HDDE and PPARδ downregulate glucose uptake in VEC

Figure 1 (A-D)
Figure 2 (A-C)

A

GLUT-1 mRNA
(relative quantification)

B

Total GLUT-1

C

PM GLUT-1

Glucose, mmol/L:
5.5
25
GW501516:
- +
- +
- +

Baicalein:
- -
- +
+ +
Figure 3 (A-C)

- **Figure 3A**: Graph showing luciferase activity in VEC with different glucose concentrations and the effects of GSK0060.

- **Figure 3B**: Bar graph comparing luciferase activity across various conditions with different constructs.

Caption: 4-HDDE and PPARδ downregulate glucose uptake in VEC.
4-HDDE and PPARδ downregulate glucose uptake in VEC
4-HDDE and PPARδ downregulate glucose uptake in VEC

Figure 4 (B-D)
4-HDDE and PPARδ downregulate glucose uptake in VEC

**Figure 4E**

- Glucose, mmol/L: 5.5, 25
- dGlc uptake (percent of control)
- 4-HDDE: - - - + +
- GSK0600: - - + - +
**Figure 5 (A-C)**

4-HDDE and PPARδ downregulate glucose uptake in VEC.

A. VEC culture medium

B. 4HJDE CONTENT (fold increase)

Glucose mmol/L: 2, 25, 25, 25
Baicalein: - - + -
N-acetylcystein: - - - +

C. Plasma

4-HDDE CONTENT (fold increase)

Rats: C ZDF

**Figure 5 (A-C)**

4-HDDE and PPARδ downregulate glucose uptake in VEC.

A. VEC culture medium

B. 4HJDE CONTENT (fold increase)

Glucose mmol/L: 2, 25, 25, 25
Baicalein: - - + -
N-acetylcystein: - - - +

C. Plasma

4-HDDE CONTENT (fold increase)

Rats: C ZDF
Figure 6 (A-E)
Figure 7 (A-C)

A

PPARδ

TOTAL PPARδ (percent of control)

B

CAL Reticulin

TOTAL CALRETIULIN (percent of control)

C

GLUT-1

TOTAL GLUT-1 (percent of control)

Metafectene: - + +

PPARδ siRNA: - - +
Figure 8