ONLINE DATA SUPPLEMENT

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

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SUPPELEMNTAL RESEARCH DESIGN AND METHODS

Animals

Zucker diabetic fatty rats (ZDF, homozygous for the leptin receptor gene mutation, *fa/fa*) and normoglycemic Sabra rats were purchased from Charles River Laboratories (Wilmington, MA) and Harlan Laboratories (Jerusalem, Israel), respectively. The rats (2-4 months old) weighted 356.8 ± 51.3 (n=4) and 176.6 ± 26.1 gr (n=4) respectively. Their respective mean blood glucose levels at sacrifice were 354 ± 28 and 92 ± 6 mg/dL. Both groups were fed a normal rat chow *ad libitum*. Blood samples were collected from the aorta of anesthetized animals (ketamine hydrochloride), immediately mixed with 0.2% w/v EDTA and centrifuged for 10 min at 6,000 x g at room temperature. The collected plasma was separated, sealed under N_2 and frozen at -70ºC. Blood glucose levels were determined with a Freestyle Freedom glucometer (Abbott, Alameda, CA). The joint ethics committee (IACUC) of the Hebrew University and Hadassah Medical Center approved the study protocol for animal welfare. The Hebrew University is an AAALAC International accredited institute.

Construction of pcDNA-hPPARδplasmid

The coding sequence of hPPARδ was amplified from a MCF10 cDNA library, using *Pfu* DNA polymerase and the following forward 5'-CCCGACAGTGTTTT-3' and reverse 5'-TAGTACATGTCCTTG-3' primers. PCR products were submitted to Taq polymerase reaction for the addition of adenine overhangs at each blunt end and then sub-cloned into pGEM T-Easy vector. pGEM T-easy-hPPARδ plasmid was digested with Not-1 and the insert was ligated to Not1 linearized pcDNA3 plasmid. Sequence analysis was performed at the Hebrew University Center for Genomic Technologies.

Chromatin immuneprecipitation (ChIP)

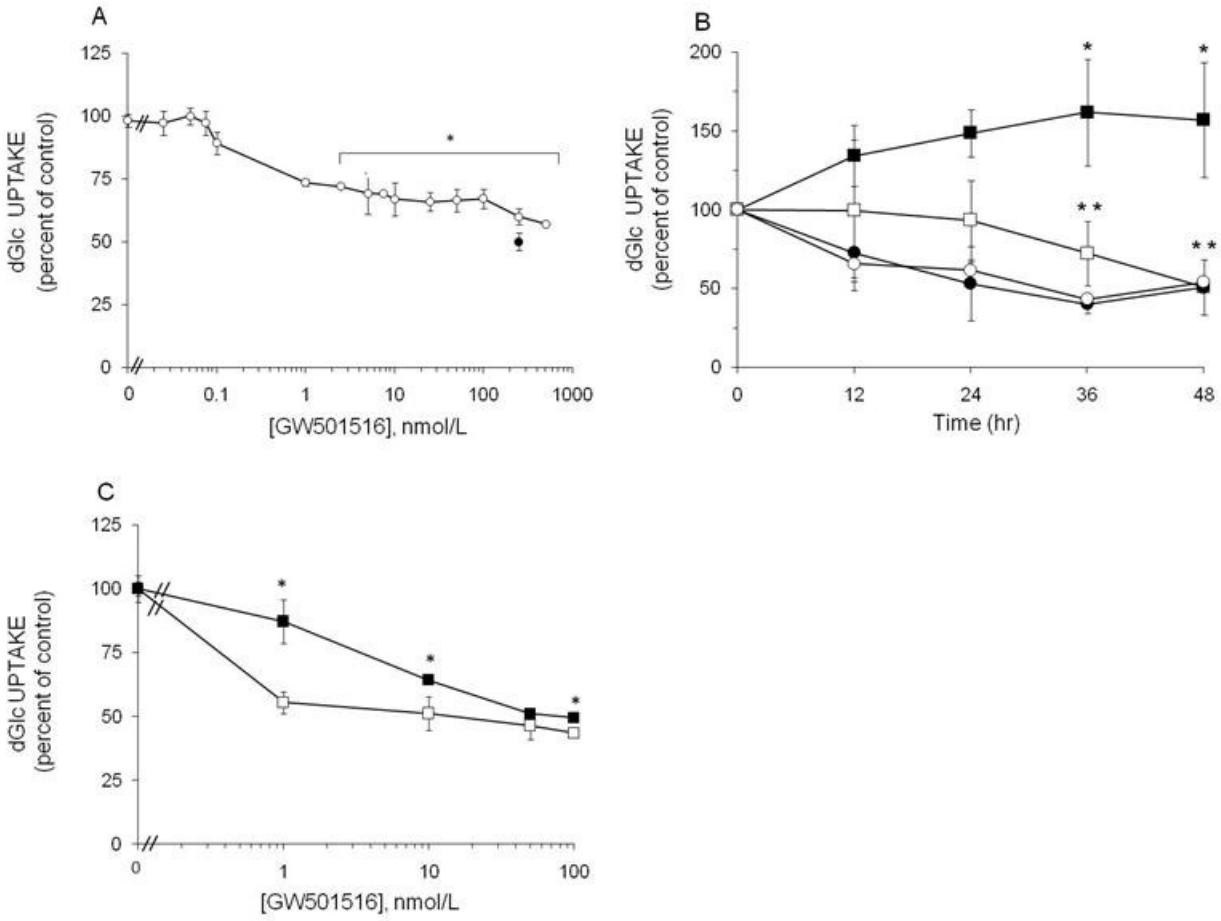
Following cell transfection with pcDNA-hPPARδ, pSVPORT1-hRXR and pEGFP-N1 plasmids and incubation with GW501516 or with DMSO, 3 $x10^6$ cells per treatment were processed for the ChIP assay according to the manufacturer's instructions. Cell lysates were sonicated for 15 min (amplitude 70%, 30 sec pulse on, 30 sec pulse off) in Sonics VC 750 instrument (Sonics & Materials, Newtown, CT, USA) to shear chromatin DNA to ~200-1000 bp-fragments. A fraction of 5% of the soluble chromatin (pre-IP) was taken for the input template-DNA control. Anti-PPARδ (H-74) and anti-histone H3 (positive control, Abcam, ab-1791) antibodies (5 µg/IP) were used for immuneprecipitation. The negative controls were the eluates of non-immunoprecipitated DNA samples (Beads). The primers for the four PPRE regions in the promoter of the bovine

calreticulin gene (F, forward; R, reverse; numbers indicate base-pair positions relative to the beginning of the 3,000 bp 5'-untranslated region) were:

PPRE LOCATION	PRIMER SEQUENCE
1710-1732	F, 5'- TCCAGCTCTTCCGTTAGGGAT -3' R, 5'- GCCTTACTCTGGTCTGGCCA -3'
1964-1986	F, 5'- CATGGGTAGGACTCCTTCCGA -3' R, 5'- CCCTAACGGAAGAGCTGGATT -3'
2139-2161	F, 5'- TGGCAGATAATGGTCCGTTCA -3' R, 5'- TCGGAAGGAGTCCTACCCATG -3'
2255-2277	F, 5'- GCCTGTGGGCCTGTAGGTT -3' R, 5'- GCCACCTCACTCCTTACCCAA -3'

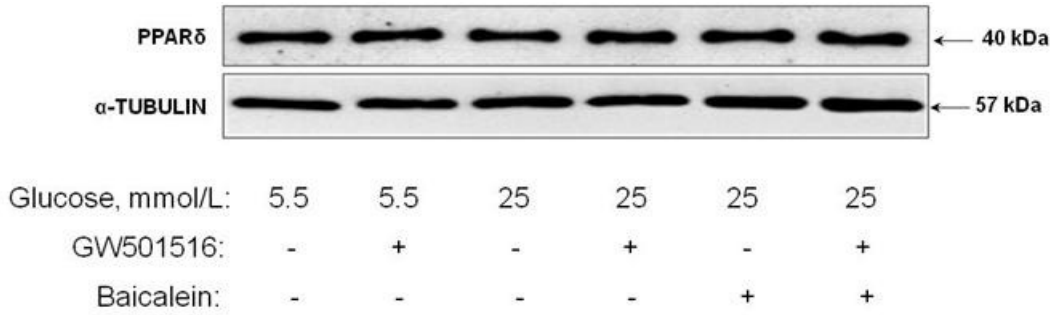
The presence and location of PPREs in the promoter region of the bovine calreticulin gene were obtained using the MatInspector software (http://www.genomatix.de/products/MatInspector/index.html). The precipitated DNA and input were amplified by PCR (5 min at 94°C followed by 30 cycles of 15 sec at 94°C and 30 sec at 60°C and 20 sec at 72°C and one final incubation at 72°C for 10 min). The PCR products were separated on a 1.5% agarose gel stained with ethidium bromide.

SUPPLEMENTAL FIGURES



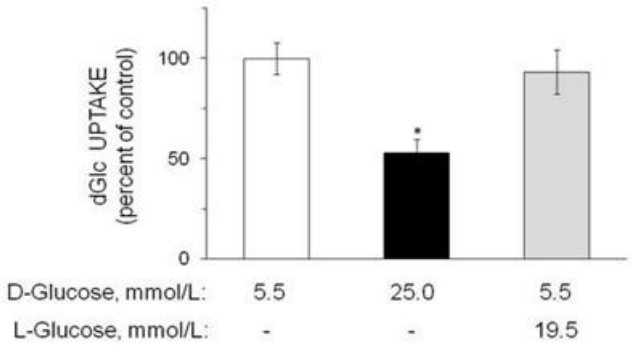
Supplemental Figure S1. Dose-response and time-course analyses of GW501516-dependent stimulation of hexose transport in VEC.

(*A*), Confluent VEC cultures were exposed to 5.5 mmol/L glucose for 48 h without or with the indicated concentrations of GW501516. The single black circle represents the value of dGlc uptake in VEC following incubation with 25 mmol/L glucose for 48 h. (*B*), Similar VEC cultures were incubated for 48 h with 5.5 mmol/L (squares) or with 25 mmol/L glucose (circles) in the absence (black symbols) or presence (open symbols) of 100 nmol/L of GW501516. (*C*), Similar cultures were exposed to increasing concentrations of GW501516 in the absence (open squares) or presence of 1 µmol/L of GSK0660 (black squares). At the end of incubations, the cells were washed and taken for the standard [^{3}H]dGlc uptake assay. Data are presented as the relative change in dGlc uptake in comparison with control cells that received DMSO only. The rate of dGlc transport at 5.5 mmol/L glucose (58 ± 5 pmol dGlc/10^6 cells/min for *A* and *B* and 77 ± 2 pmol dGlc/10^6 cells/min for *C*) was taken as the 100% value. P<0.05, *A*, * in comparison with the control in the absence of GW501516; *B*, in comparison with the *25- or **5.5 mmol/L glucose incubations or *C*, with the corresponding incubation with GW501516 only (n=4).



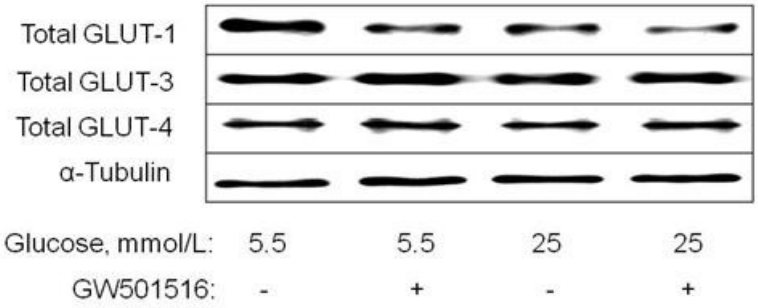
Supplemental Figure S2. PPARδ expression in VEC is not affected by ambient glucose, GW501516 or baicalein.

Confluent VEC were incubated with 5.5 or 25 mmol/L glucose for 48 h in the absence or presence of GW501516 (100 nmol/L). Baicalein (80 µmol/L) was present during the last 10 h of incubation. The cells were then lysed and taken for Western blot analyses of PPARδ and α-tubulin. The latter served as a protein loading control.

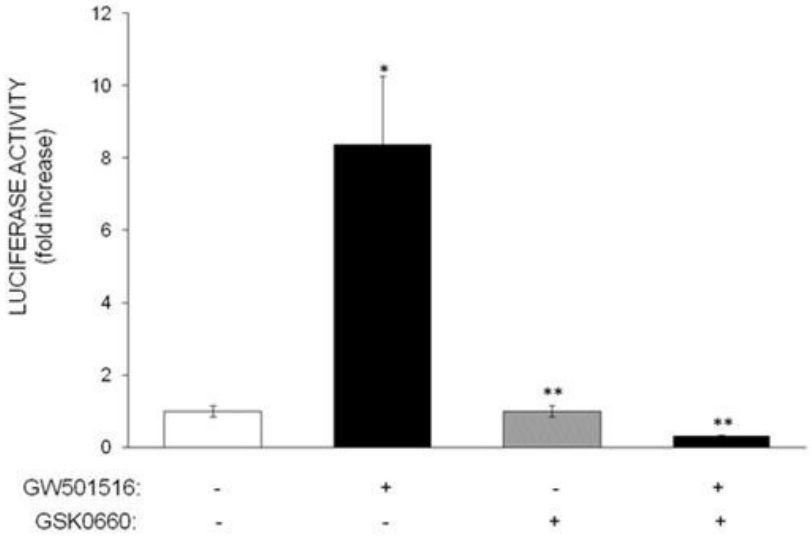


Supplemental Figure S3. Osmolarity control.

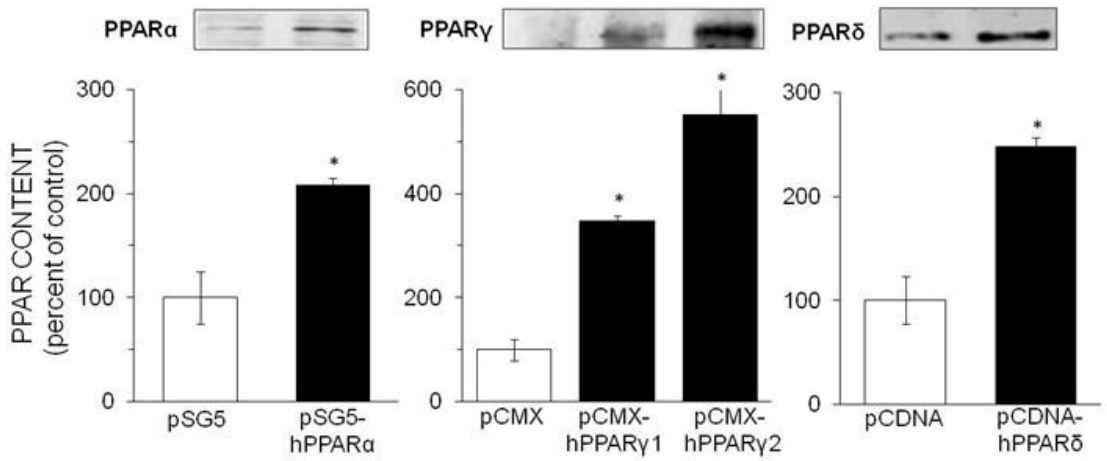
Confluent VEC cultures were exposed to 5.5 mmol/L D-glucose, 25 mmol/L D-glucose or to 5.5 mmol/L D-glucose plus 19.5 mmol/L L-glucose for 48 h. The cells were then washed and taken for the standard [^{3}H]dGlc uptake assay. The rate of uptake in cells incubated with 5.5 mmol/L glucose (55 ± 1 pmol dGlc/10^6 cells/min) was taken as 100%. *P<0.05, for difference from this control (n=4).



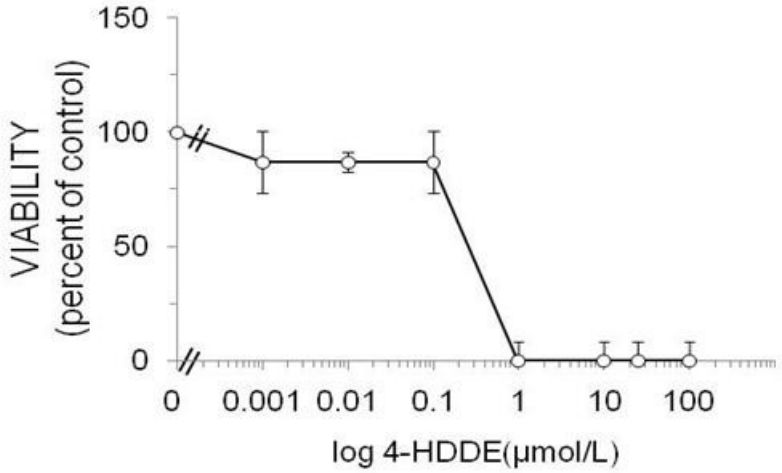
Supplemental Figure S4. High glucose and GW501516 selectively reduce GLUT-1, but not GLUT-3 and GLUT-4, content in VEC.
VEC were incubated with 5.5 or 25 mmol/L glucose for 48 h, without or with 100 nmol/L of GW501516. The cells were then washed, lysed and taken for Western blot analyses of the various GLUTs and α-tubulin. The latter served as a protein loading control.



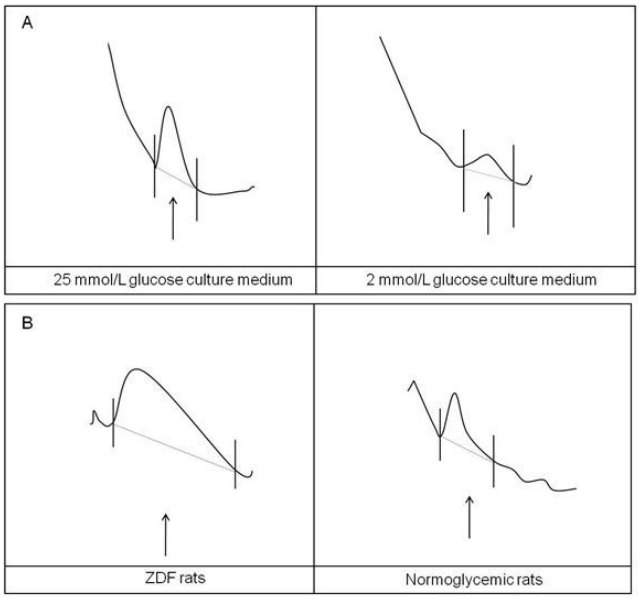
Supplemental Figure S5. PPARδ antagonistic activity of GSK0660 in a 3xPPRE-TK-Luciferase reporter assay.
VEC cultures at 2 mmol/L glucose were transfected with the 3xPPRE-TK-Luciferase reporter plasmid and the Renilla luciferase plasmid and then incubated for 24 h with 1 nmol/L of GW501516 and/or 1 µmol/L of GSK0600, as indicated. The cells were then washed, harvested, lysed and taken for the luciferase activity assay. Results are given as the relative increase of luciferase activity in comparison with the 2 mmol/L glucose (vehicle) incubation. P <0.05 for differences from the 2 mmol/L glucose incubation *without or ** with GW501516 (n=4).



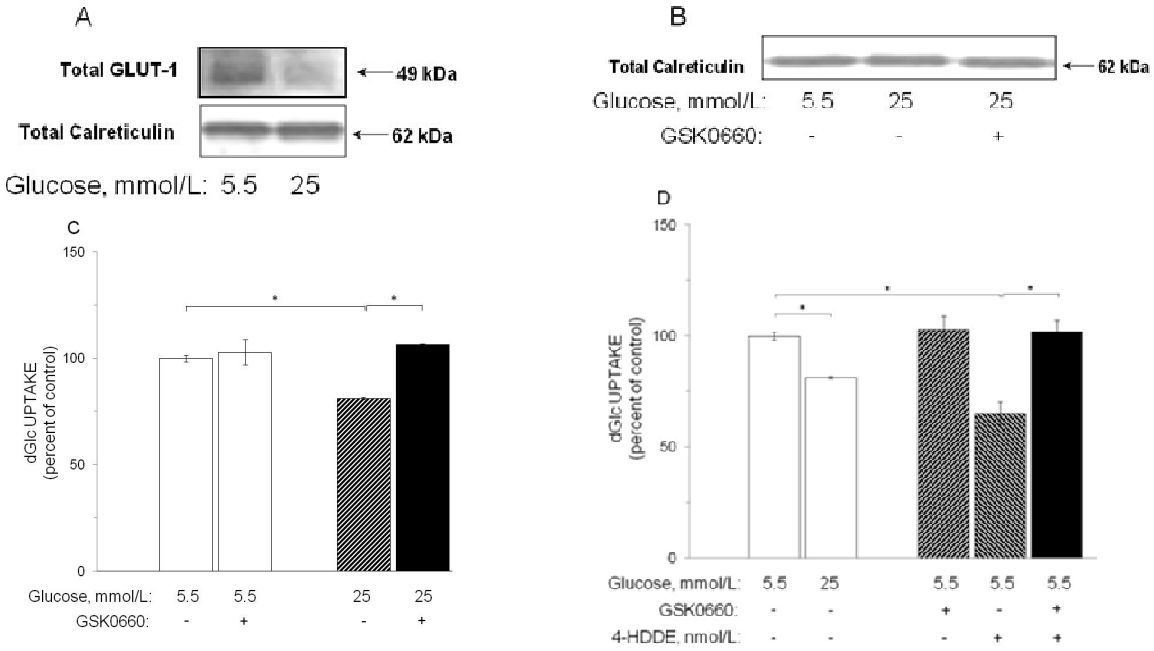
Supplemental Figure S6. Overexpression of hPPARα, -γ1, -γ2 and -δ in VEC.
VEC cultures were transfected with the following expression plasmids: pSG5-hPPARα or pCMX-hPPARγ1 or pCMX-hPPARγ2 or pcDNA-hPPARδ. Control cells were transfected with the corresponding empty plasmids. Cells were also co-transfected with pSVPORT-hRXR, pEGFP-N1 plasmid, 3xPPRE-TK-Luciferase reporter plasmid and the Renilla luciferase plasmid. After 48 h the cells were harvested, lysed and taken for a Western blot analyses with specific antibodies against the various hPPAR isotypes.



Supplemental Figure S7. Effects of 4-HDDE on the viability of VEC.
Confluent VEC cultures that had been pre-conditioned at 5.5 mmol/L glucose received the same fresh medium without or with the indicated concentrations of 4-HDDE and were incubated for 48 h. The cultures were then washed 3 times with PBS at room temperature, lysed and taken for protein determination. The protein content in the absence of 4-HDDE (0.21 ± 0.03 mg/well) was taken as 100% (n=4).



Supplemental Figure S8. HPLC profiles of 4-HDDE analysis.
Representative HPLC tracings of 4-HDDE in extracts of cell culture media (*A*) and rat plasma samples (*B*). Arrows indicate peaks of 4-HDDE, as resolved with the pure compound.



Supplemental Figure S9. The autoregulation of glucose transport system in EA.hy926 cells.
Confluent EA.hy926 cultures were exposed to 5.5 or 25 mmol/L glucose for 48 h. (*A, B*), in the absence or presence of 1 µmol/L of GSK0660, then washed and taken for Western blot analysis of GLUT-1 and Calreticulin. *(C, D)* Similar cells were incubated with 1 µmol/L of GSK0660 and/or 4-HDDE, then washed and taken for the standard [^{3}H]dGlc uptake assay. The rate of dGlc

uptake at 5.5 mmol/L glucose (73 ± 8 pmol dGlc/10^6 cells/min) was taken as 100%. *P<0.05 for differences from the respective controls (n=4).

REFERENCES

1. Bacot S, Bernoud-Hubac N, Baddas N, Chantegrel B, Deshayes C, Doutheau A, Lagarde M, Guichardant M: Covalent binding of hydroxy-alkenals 4-HDDE, 4-HHE, and 4-HNE to ethanolamine phospholipid subclasses. *J Lipid Res* 44:917-926, 2003
2. Zanardi E, Jagersma CG, Ghidini S, Chizzolini R: Solid phase extraction and liquid chromatography-tandem mass spectrometry for the evaluation of 4-hydroxy-2-nonenal in pork products. *J Agric Food Chem* 50:5268-5272, 2002