Amelioration of Glucose Intolerance by the Synthetic Androstene HE3286: Link to Inflammatory Pathways

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Antidiabetic effects of novel androstene HE3286

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Non-standard abbreviations used:

DHEA – Dehydroepiandrosterone
MCP-1 – Monocyte chemoattractant protein-1
LPS – Lipopolysaccharide
NF-κB – Nuclear factor kappa-B
IkB – Inhibitor of kappa-B protein
IKK – IkB kinase
JNK – c-Jun NH2-terminal kinase
AR – Androgen receptor
GR – Glucocorticoid receptor
ER – Estrogen receptor
PR – Progesterone receptor
PPAR – Peroxisome proliferator activated receptor
WAT – White adipose tissue
TZD – Thiazolidinedione
OGTT – Oral glucose tolerance test
DIO – Diet-induced obese
MAPK – Mitogen-activated protein kinase
TLR4 – Toll-like receptor-4
ECL – Enhanced chemiluminescence

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Abstract

Insulin resistance, the major metabolic abnormality underlying type 2 diabetes, is associated with chronic inflammation and heavy macrophage infiltration in white adipose tissue (WAT). The therapeutic properties of the synthetic adrenal steroid $\Delta^5$-androstene-17$\alpha$-ethynyl-3$\beta$,7$\beta$,17$\beta$-triol (HE3286) were characterized in metabolic disease models. Treatment of diabetic $db/db$ mice with HE3286 suppressed progression to hyperglycemia and markedly improved glucose clearance. Similar effects were also observed in insulin resistant, diet-induced obese C57BL/6J mice and in genetically obese $ob/ob$ mice. This effect appeared to be a consequence of reduced insulin resistance because HE3286 lowered blood insulin levels in $db/db$ and $ob/ob$ mice. Treatment with HE3286 was accompanied by suppressed expression of the prototype macrophage-attracting chemokine MCP-1 in WAT, along with its cognate receptor CCR2. Exposure of mouse macrophages to HE3286 in vitro caused partial suppression of endotoxin (LPS)-induced NF-$\kappa$B-sensitive reporter gene expression, NF-$\kappa$B nuclear translocation and NF-$\kappa$B/p65 serine phosphorylation. Proinflammatory kinases including I$\kappa$B kinase (IKK), c-Jun NH$_2$-terminal kinase (JNK) and p38, were also inhibited by HE3286. In ligand competition experiments, HE3286 did not bind to classical sex steroid or corticosteroid receptors, including AR, PR, ER$\alpha$ or ER$\beta$ and GR. Likewise, in cells expressing nuclear receptor-sensitive reporter genes, HE3286 did not substantially stimulate transactivation of AR, ER, GR or PPAR$\alpha$, PPAR$\delta$ and PPAR$\gamma$. These findings indicate that HE3286 improves glucose homeostasis in diabetic and insulin resistant mice, and suggest that the observed therapeutic effects result from attenuation of proinflammatory pathways, independent of classic sex steroid, corticosteroid or PPAR nuclear receptors.
Introduction

Insulin resistance is associated with a state of low-grade, chronic inflammation with increased expression of proinflammatory mediators, cytokines and chemokines in white adipose tissue (WAT) (Hotamisligil et al., 1995; Kern et al., 1995). Consistent with this observation, high blood levels of proinflammatory cytokines (e.g. TNFα, IL-6) and C-reactive protein have been reported in diabetic and insulin resistant subjects (Shoelson et al., 2007). The proinflammatory responses initiated by these proteins occur primarily through the c-Jun NH2-terminal kinase (JNK)/activator protein 1 (AP1) and the IκB kinase (IKK)/NF-κB signaling pathways (Schenk et al., 2008). Accordingly, the signaling activities of both JNK and IKK are elevated in skeletal muscle from insulin resistant humans and mice, thereby leading to chronic activation of the AP1 and NF-κB pathways with the consequent expression of proinflammatory genes (Yuan et al., 2001; Hirosumi et al., 2002; Cai et al., 2005). In mice, genetic ablation (KO models) or inhibition of JNK1 or IKKβ results in improved insulin sensitivity in vivo and in vitro (Yuan et al., 2001; Hirosumi et al., 2002; Arkan et al., 2005; Cai et al., 2005; Solinas et al., 2007). In humans, treatment with salicylates (Salsalate), which inhibit the IKKβ/NF-κB axis, results in improved glucose and lipid homeostasis (Goldfine et al., 2008). Thus, chronic activation of these proinflammatory pathways is associated with and contributes to the pathogenesis of obesity-related insulin resistance and their pharmacological modulation appears to produce therapeutic benefit to enhance insulin sensitivity.

Recent studies have shown that WAT from obese animals or humans is heavily infiltrated with macrophages that express most of the detectable proinflammatory
molecules, suggesting that they play an important role in insulin resistance and obesity-related inflammation (Weisberg et al., 2003; Xu et al., 2003). Mice in which IKKβ or JNK1 genes have been disrupted specifically in the myeloid lineage (which includes macrophages) exhibit increased insulin sensitivity and they are protected from high fat diet-induced glucose intolerance (Arkan et al., 2005; Solinas et al., 2007). Since the major proinflammatory JNK1/ or IKKβ/NF-κB axis is disabled within macrophages in these animal models, the resulting insulin sensitive phenotype suggests an important contribution of the macrophage NF-κB pathway to inflammation-induced insulin resistance (Arkan et al., 2005). Accordingly, insulin sensitizers of the thiazolidinedione (TZD) class that act through PPARγ are known to attenuate production of inflammatory mediators that promote insulin resistance (Ricote et al., 1998). Moreover, mice deficient in macrophage-specific PPARγ exhibit significant glucose intolerance with systemic insulin resistance and respond only partially to TZD treatment (Hevener et al., 2007; Pascual et al., 2007).

A major potential contributor to macrophage recruitment into WAT during obesity is the soluble mediator monocyte chemoattractant protein-1 (MCP-1), which can be produced by adipocytes, macrophages and other cells (Charo and Taubman, 2004). Importantly, MCP-1 is an NF-κB target gene that is expressed at high levels in WAT from obese mice and humans (Sartipy and Loskutoff, 2003; Charo and Taubman, 2004; Bruun et al., 2005). Studies with adipocytes in vitro indicate that MCP-1 inhibits insulin-stimulated glucose uptake, inducing an “insulin-resistant” state in cultured cells (Sartipy and Loskutoff, 2003). Moreover, genetic deficiency of the MCP-1 receptor CCR2
reduces food intake, delays development of obesity and is accompanied by decreased number of macrophages in WAT, while enhancing systemic glucose homeostasis and insulin sensitivity (Weisberg et al., 2006). Thus, it appears that adipocyte-derived MCP-1 plays a major role in promoting macrophage recruitment and inflammation in the insulin resistant state.

We have examined a chemically modified version of a naturally occurring 7β-hydroxylated metabolite of Δ⁵-androstenediol, which in turn is derived from dehydroepiandrosterone (DHEA). We refer to the resulting compound as HE3286 (Δ⁵-androstene-17α-ethynyl-3β,7β,17β-triol), which is metabolically stable (Auci et al., 2009). Although the precise pharmacological role of DHEA is still controversial, its administration is associated with multiple therapeutic effects when administered to rodents at high doses (Coleman et al., 1984). This diversity of biological actions may reflect the well-known extensive metabolic biotransformation of DHEA in rodents, from which discrete steroidal metabolites possessing intrinsic and distinct biological activities are generated. In this light, DHEA may act as a prohormone or metabolic precursor of biologically active hormonal effectors (Leiter et al., 1987; Marwah et al., 2002). Although DHEA has been shown to improve insulin resistance and glucose homeostasis in diverse animal models of metabolic disease, it has not been possible to demonstrate similar activities in human studies (Nair et al., 2006). It is thought that the lack of efficacy in humans may reflect large species-specific differences in metabolic transformation of DHEA and limited oral bioavailability (Auci et al., 2009). The metabolically stabilized, orally bioavailable compound HE3286 has been previously
studied in diverse mouse models of inflammation and septic shock and shown to possess anti-inflammatory properties without immune-suppressive activity (Auci et al., 2009).

In this report, we show that HE3286 exhibits antidiabetic effects in mice, causing glucose lowering, improving glucose intolerance and delaying progression to hyperglycemia. This therapeutic activity was associated with reduced expression of MCP-1 and its receptor CCR2 in adipose tissue. Since HE3286 also attenuated NF-κB activity and phosphorylation of proinflammatory kinases in LPS-stimulated macrophages, the therapeutic effects shown here may result from broad anti-inflammatory properties that enhance insulin sensitivity.
Methods

Animals. Male BKS.Cg-m +/+ Lepr<sup>db</sup>/J (db/db) mice (5 or 7 weeks old), or male B6.V-Lepr<sup>ob</sup>/J (ob/ob) mice (6-7 weeks old) were purchased (Jackson Laboratory, Bar Harbor, ME) and housed in an environmentally-controlled room under a 12 hr light/dark cycle, with free access to a standard mouse diet and water. After a seven-day acclimation period, blood samples were collected by tail nick for glucose measurements and a baseline OGTT was performed, after which mice were randomized to treatment groups according to equivalence of body weight, non-fasting and fasting blood glucose levels. Age-matched db/+ or ob/+ heterozygous animals were used for lean controls. For studies with DIO-mice, male C57BL/6J mice (5 weeks old) were first fed a high fat diet (20% kcal protein, 60% kcal fat, 20% kcal carbohydrate; ResearchDiets<sup>TM</sup>, New Brunswick, NJ) for 8 weeks, until reaching a target body weight of ≥30 g. Blood glucose levels and a baseline OGTT were performed, and animals were randomized as described above. All test articles for animal dosing consisted of soluble formulations of HE3286 prepared shortly before each study in a cyclodextrin-based vehicle consisting of 30% (w/v) sulfobutyl-β-cyclodextrin (Captisol<sup>®</sup>; CyDex, Lenexa, KS) dissolved in water at 10 mg/mL and adjusted with NaOH or HCl to a final pH of 6.5 ± 1. Stability of the test articles was verified by HPLC analysis at least for equivalent periods of time to the duration of the efficacy studies. All animal procedures were performed following protocols designed to adhere to the Guide for Care and Use of Laboratory Animals of the U.S. National Institutes of Health and approved by the Institutional Animal Care and Use Committee (IACUC) of Hollis-Eden Pharmaceuticals, Inc.
Oral glucose tolerance tests. Glucose tolerance was assessed by standard OGTT after an overnight fast. Mice received a bolus of glucose (2 g/kg on days 0 and 14 or 1 g/kg on days 28 or longer) by oral gavage and blood samples collected by tail nick 15, 30, 60 and 120 min thereafter. A blood sample for baseline glucose (time 0) was also collected, before initiating the OGTT. Blood glucose levels were measured using a glucometer (OneTouch® Ultra Meter; LifeScan, Milpitas, CA), except for samples that were >600 mg/dL, which were collected separately using heparin-coated microcapillary tubes and processed by a standard enzymatic method (Sigma, St. Louis, MO).

Serum MCP-1 and insulin levels. Insulin levels were measured in serum by ELISA using 96-well microtiter plates coated with mouse-specific anti-insulin monoclonal antibodies [Insulin (Mouse) Ultrasensitive EIA; Alpco Diagnostics, Salem, NH]. Serum MCP-1 levels were determined by ELISA with an affinity-purified anti-MCP-1 polyclonal antibody (Quantikine® Mouse CCL2/JE/MCP-1 Immunoassay; R&D Systems, Minneapolis, MN). Assays were conducted following the manufacturer’s protocol.

RAW264.7 cells and transient transfections. Murine RAW264.7 macrophages (ATCC #TIB-71) were maintained in Dulbecco’s modified Eagle medium (DMEM; Mediatech, Manassas, VA), with 10% fetal bovine serum (FBS; InVitrogen, Carlsbad, CA) in a humidified incubator at 37°C. For transient transfections, cells were seeded and transfected after 24 hr using the Lipofectamine™ reagent (InVitrogen) with pNF-κB luciferase plasmid (Stratagene, Santa Clara, CA) and pRLTK Renilla luciferase control.
plasmid (Promega, Madison, WI). After 24 hr, cells were exposed to the compounds indicated for 1 hr and then stimulated with 100 ng/mL LPS for 6 hr. Cell lysates were prepared and both firefly and *Renilla* luciferase activity determined sequentially using the Dual-Luciferase™ reporter assay system (Promega). Luminescence was measured with a GENios Pro plate reader (Tecan, San Jose, CA). Results were corrected for well-to-well relative transfection efficiency with respect to *Renilla* luciferase activity.

**Real time, quantitative RT/PCR.** Epidydymal adipose tissue was dissected and immediately placed in RNAlater® solution (Ambion, Austin, TX) until processed. Total RNA was extracted by using the RiboPure™ RNA purification kit (Ambion), following the manufacturer’s instructions. Quality and integrity of RNA was confirmed by OD260/OD280 ratios >1.9 and by denaturing agarose gel electrophoresis. First strand cDNA synthesis was accomplished with 100 ng of RNA using the iScript cDNA Synthesis kit (BioRad, Hercules, CA) and PCR amplification of the resulting RT products was performed in the presence of iQ™SYBR® Green I Supermix dye (BioRad) and target-specific primer pairs. The mouse acidic ribosomal protein P0 (RPLP0) was used as a reference, housekeeping gene in the same PCR reactions to normalize expression of target genes to a suitable endogenous standard (Dheda et al., 2004). PCR primers used were as follows:

- **MCP-1:** 5’-ACTCACCTGCTGCTACTCATTCAC-3’ (forward)
  5’-CTTCTTTGGGACACCTGCTGCT-3’ (reverse);

- **TNFα:** 5’-CTTGTCTACTCCAGGTTCTCTT-3’ (forward)
  5’-GATAGCAAATCGGCTGACGG-3’ (reverse);
CCR2:  5'-GAGCCTGATCCTGCCTCTACTTG-3' (forward)
      5'-CTCTTCTTCTCATTCCTACAGCGA-3' (reverse).
RPLP0:  5'-CTGAGATTCCGGGATATGCTGTTG-3' (forward)
       5'-GTCCTAGACCAGTGTTCTGAGC-3' (reverse);

Thermocycling conditions included an initial 3-min denaturing step at 95°C followed by 40 successive cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 30 sec and extension at 72°C for 20 sec. Each PCR amplification was routinely followed by a 15-min melting curve program (95°C for 1 min, 55°C for 1 min and ramping from 55°C to 94°C in 0.5°C increments in 80 cycles of 10 sec) and a final cooling step to 4°C. Real time detection of PCR amplification products was determined by fluorescence with an iCycler iQ™ Multicolor Detection System (BioRad). Single T_m peak melting curves showed no evidence of primer-dimer formation. Relative quantification of target gene expression was calculated based on real-time PCR efficiency of amplification and the relative difference in threshold crossing points between a sample and a control (Livak and Schmittgen, 2001). Results were expressed as a ratio in comparison to the reference gene (Pfaffl, 2001).

Immunostaining procedures. RAW264.7 macrophages were cultured on glass cover-slips and treated with 100 nM HE3286 or vehicle (0.1% DMSO) for 1 hr, followed by 100 ng/mL LPS for 15 minutes. Cells were fixed with 4% paraformaldehyde in PBS, washed, and permeabilized in 0.1% Triton X-100 (Sigma). Cells were washed again and blocked with 1% normal goat serum (Santa Cruz, Santa Cruz, CA) for 1 hr, followed by addition of an anti-NF-κB/p65 monoclonal antibody (IgG1; Santa Cruz) and stained with
FITC-conjugated goat-anti-mouse IgG1 (Santa Cruz). The resulting immunofluorescence was visualized and captured at 40X with a fluorescence microscope.

**Immunoblot analysis.** Murine intraperitoneal (IP) macrophages were elicited by thioglycolate and isolated as described (Welch et al., 2003). Briefly, cells were seeded in 6-well plates (7 x 10⁶/10-cm plate) and maintained in DMEM with 15 mM glucose and 10% FBS for three days. Media was changed every 24 hr and cells were then serum-starved in DMEM (15 mM glucose) with 0.5% FBS overnight. For experiments with RAW264.7 mouse macrophages, cells were cultured as described above and serum-starved (0.5% FBS) overnight. In both cases, cells were pretreated with DMSO control (0.01% final) or 100 nM HE3286 for 2 hr (IP macrophages) or overnight (RAW264.7 macrophages), followed by 100 ng/mL LPS stimulation for various times. Cell lysates were collected in RIPA buffer [20 mM Tris-HCl pH 7.4, 0.15 M NaCl, 1 mM EDTA, 1% Triton X-100 and a cocktail of protease (Roche) and phosphatase (Sigma) inhibitors]. Proteins in cell lysates were resolved by 10% SDS-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% non-fat dry milk in TBST (10 mM Tris-HCl pH 8, 0.15 M NaCl, 0.05% Tween-20) and developed for specific proteins using appropriate primary antibodies: Phospho-IKKα-Ser180/IKKβ-Ser181 (Cell Signaling, Danvers, MA); phospho-SAPK/JNK-Thr183/Tyr185 (Cell Signaling); phospho-p38 MAPK-Thr180/Tyr182 (Cell Signaling); and phospho-NF-κB-p65/Ser536 (Cell Signaling). For loading controls, blots were stripped and developed with anti-α-tubulin rabbit polyclonal antibody (Cell Signaling). Immune complexes were detected by enhanced
chemiluminescence (ECL). Relative band intensities were quantified by densitometry scanning and normalized to untreated controls.

**Nuclear Receptor Binding assays.** Assessment of binding activity for various nuclear receptors was performed by homogeneous competition assays using the PolarScreen™ fluorescence polarization system (InVitrogen). Briefly, serial dilutions of HE3286 (or a reference competitor) were incubated on 384-well plates for 2 hr at room temperature in the presence of an appropriate fluorescent ligand (Fluormone) and a nuclear receptor of recombinant origin (AR, #P3018; GR, #P2816; ERα, #P2614; ERβ, #P2615; PR, #P2895) in a total volume of 30 μL, essentially following the manufacturer’s protocol. Fluorescence polarization (mP) in each well was determined with a GENios Pro reader (Tecan, San Jose, CA) and based on the extent of mP suppression detected, IC₅₀ competition values were derived using the GraphPad Prism software (GraphPad, San Diego, CA). Most nuclear receptors employed in these assays are full-length recombinant proteins of human origin, with the exception of AR (His/GST-tagged rat AR ligand binding domain) and PR (GST-tagged human PR ligand binding domain). Reference ligands used for each receptor are indicated in Table 1.

**Transactivation assays.** Transactivation activity of sex steroid or corticosteroid receptors was mostly assessed in established stable transfectant human cancer cell lines expressing nuclear receptor-sensitive luciferase reporter genes. For AR and GR, the cell line MDA-kb2 (ATCC #CRL-2713) harboring an MMTV/luciferase cassette was used (Wilson et al., 2002). For ERα and ERβ, the cell line T47D-kBluc (ATCC #2865) stably
transfected with a synthetic plasmid containing three copies of an estrogen response element (ERE) fused upstream of a luciferase gene was used (Wilson et al., 2004). Briefly, cells were plated at 20,000 cells/well/100 μL in 96-well clear bottom white assay microtiter plates (Costar) and kept in phenol red-free RPMI supplemented with 4 mM l-glutamine and 10% charcoal-stripped FBS (CHAR-DEX; InVitrogen). Cells were exposed to the various compound dilutions as needed and following an overnight incubation at 37°C, media was aspirated, cells were lysed and luciferase activity was then determined. In some cases, transactivation of ERβ and GR was also performed by transient transfection of HEK293 fibroblasts using expression plasmids encoding full-length human GR or ERβ and appropriate luciferase reporter vectors (see below). For transactivation activity of the human peroxisome proliferator-activated receptors PPARγ, PPARδ and PPARα, the fluorimetric Gene Blazer® β-lactamase assay system (InVitrogen) was used following the manufacturer’s instructions. Assays were conducted on 384-well plates using a fluorogenic substrate (CCF4-AM, InVitrogen). Reference compounds (Tocris, Ellisville, MO) used for each receptor were: Rosiglitazone and GW1929 for PPARγ (EC_{50} = 1.3 nM); L165,041 for PPARδ (EC_{50} = 1.1 nM); and GW7647 for PPARα (EC_{50} = 0.3 nM).

cDNA expression constructs. Full-length cDNA fragments encoding GR and ERβ were cloned by PCR using double-stranded PCR-ready cDNA templates (QUICK-Clone™, Clontech, Mountain View, CA) from human adipose tissue (GR) or prostate & ovary (ERβ). Appropriate PCR primers were designed to obtain blunt-end, full-length target amplicons using AccuPrime™ Taq polymerase (Invitrogen), thereby making them
compatible with topoisomerase-mediated directional Gateway® cloning (pENTR™-TOPO®, Invitrogen). Primers used were

GR:  5’- CACCTGATATTCACTGATGGACTC-3’ (forward);
    5’- GGCAGTCACCTTTTGATGAAAC-3’ (reverse);

ERβ:  5’- CACCTCTCAAGACATGGATATAAA-3’ (forward);
      5’- TCACTGAGACTGTGGGTTCT-3’ (reverse).

Recombinant inserts were subsequently transferred to a pDEST40 expression vector by LR recombination (Gateway®-LR Clonase, Invitrogen). The identity of the inserts was verified by full bidirectional sequencing and their functional competency assessed by transient co-transfection of HEK293 fibroblasts, using appropriate GRE- or ERE-reporter plasmids (pGRE-SEAP or pERE-TA-SEAP, Clontech; or ERE-/GRE-luciferase constructs generated in pGL3 vectors; Promega, Madison, WI). Cells were exposed to increasing concentrations of reference ligands (dexamethasone for GR and 17β-estradiol for ERβ) and reporter enzyme activity determined in whole cell extracts. These titration experiments revealed concentration-dependent increases in reporter activity for each reference ligand and cognate receptor (EC50 = 0.06 nM for 17β-estradiol in ERβ-transfected cells; EC50 = 8 nM for dexamethasone in GR-transfected cells; see Table 1).

**Statistical methods.** All data are expressed as mean ± SEM. Statistical significance was assessed by one-way ANOVA with a Bonferroni post-test and in some cases via the non-parametric Mann-Whitney test. Calculations were performed using the GraphPad Prism software. In general, P values < 0.05 were considered statistically significant.
Results

Pharmacokinetic considerations and dose rationale. Although a full description of the pharmacokinetic profile of HE3286 is beyond the scope of this paper, there are a few key observations that bear on the rationale for the dosing schedule used in the studies described here. In mice, HE3286 has an absolute oral bioavailability of 16% and drug exposure is linearly dose proportional in the 20-80 mg/kg range when administered orally (Ahlem, C., Kennedy, M. Page, T. & Frincke, J. Manuscript in preparation). A mean AUC of approximately 2240 ± 960 ng·hr/mL is typically achieved following oral administration of a 40 mg/kg HE3286 dose. Since the terminal half-life of HE3286 in mice is relatively short (2.04 ± 1.2 hr) and its toxicological evaluation has shown low potential for acute systemic toxicity, the daily dose was primarily limited by volume considerations. In the absence of an established minimum effective dose or optimized dosing schedule, a dose of 80 mg/kg twice daily (BID) was chosen to provide maximum exposure using our standard research formulation. This dose level is near the maximum practical dose volume (about 5 mL/kg for obese mice) and the BID schedule was adopted to compensate for the short half-life of HE3286 to avoid relatively long periods of negligible or no drug exposure (drug holiday), which could negate efficacy if HE3286 were administered only once daily. No evidence of hepatic or renal dose limiting toxicity has been found in the course of the studies reported here. Furthermore, the doses used are below the minimum non-toxic dose of 200 mg/kg established in 90-days toxicology studies in mice.
Delayed progression to hyperglycemia and enhanced glucose tolerance in db/db mice by HE3286. Glucose lowering activity of HE3286 was initially evaluated in two studies using C57BLKs/J-m Lepr<sup>db</sup> (db/db) mice in which the drug was orally administered (gavage) in doses ranging from 20 to 80 mg/kg BID. In the first study, treatment of 8-wk old diabetic db/db mice with HE3286 (40 or 80 mg/kg BID for 28 days) significantly reduced progression of hyperglycemia (P<0.01 at days 10 and 21). In contrast, vehicle-treated animals showed a steady increase in blood glucose levels, reaching 350-400 mg/dL (Figure 1A). In the second study with younger (6-wk old), “pre-diabetic” db/db mice, HE3286 (20 or 40 mg/kg BID) maintained blood glucose levels below 200 mg/dL at all times, comparable to lean db/+ control animals (Figure 1C; P<0.001 vs. vehicle). As expected, animals treated with rosiglitazone (25 mg/kg BID) showed normal glucose levels throughout the study (Figure 1C). In contrast, vehicle-treated mice became hyperglycemic with glucose levels exceeding 450 mg/dL after 32 days. These glucose lowering effects occurred in the absence of significant changes in body weight as its rate of accretion was virtually the same among all groups in both studies (Figures 1B and 1D). In addition, oral glucose tolerance tests indicated that HE3286, like rosiglitazone, markedly enhanced glucose clearance in db/db mice after 14 days (P<0.05) or 28 days (P<0.01) of treatment (Figures 2A and 2B). To determine if these effects may have resulted from amelioration of insulin resistance, serum insulin levels were also measured (Figure 2C). Treatment with HE3286 for 4 weeks, like rosiglitazone, caused a significant reduction in insulin levels compared to vehicle (P<0.01-0.001).
Suppression of glucose intolerance in insulin-resistant DIO and ob/ob mice by HE3286. The previous results suggested that HE3286 might mitigate insulin resistance before the β-cell deficit becomes a major contributor to impaired glucose homeostasis. Therefore, the effect of HE3286 on two insulin resistant, non-diabetic models, namely diet-induced obese (DIO) C57BL/6J mice, as well as genetically obese B6.V-Lep\textsuperscript{ob}/J (ob/ob) mice was examined. As shown in Figure 3A and 3B, treatment of DIO-mice with HE3286 significantly enhanced glucose clearance ($P<0.05-0.001$). Moreover, a reduction of basal blood glucose levels was already apparent in the HE3286-treated mice prior to gavaging the glucose load (see Figure 3A, time 0). OGTT studies indicated that treatment of ob/ob mice with HE3286 significantly improved ($P<0.05-0.001$) glucose intolerance, in a similar fashion as rosiglitazone (Figure 3C and 3D), and also markedly reduced serum insulin levels (see below).

Association of anti-inflammatory activity of HE3286 with reduced insulin resistance. Given the link between insulin resistance and MCP-1-induced macrophage infiltration and inflammation in adipose tissue (Sartipy and Loskutoff, 2003; Weisberg et al., 2006), it was of interest to assess parallel changes that might occur in the expression of MCP-1 and its receptor CCR2. Expression of mRNA levels of MCP-1 and CCR2 in adipose tissue of diabetic db/db mice receiving vehicle only was markedly increased relative to non-diabetic lean db/+ littermates (Figure 4A). However, MCP-1 and CCR2 expression was significantly reduced in db/db mice treated with HE3286 or rosiglitazone ($P<0.05$) relative to vehicle-treated animals (Figure 4A). This HE3286-induced inhibition of MCP-1 expression in adipose tissue was accompanied by reduced serum levels of MCP-1.
(P<0.05) in the same animals, but not rosiglitazone (Figure 4B). The anti-inflammatory activity of HE3286 was also evident in obese insulin-resistant ob/ob mice treated with HE3286, in which serum MCP-1 protein levels decreased (P<0.01; Figure 5A). In addition, the marked increase in serum insulin levels observed in obese ob/ob mice relative to lean ob/+ mice was significantly abrogated in animals treated with HE3286 (P<0.05) but not with vehicle (Figure 5B), suggesting that insulin resistance was ameliorated. These effects were accompanied by markedly reduced expression of MCP-1 mRNA expression (P<0.05) in white adipose tissue (Figure 5C). Thus, amelioration of insulin resistance and improved glucose utilization by HE3286 treatment is associated with decreased MCP-1 production in white adipose tissue.

Modulation of macrophage activation by HE3286. Since genes encoding MCP-1 and other proinflammatory effectors are under the control of the key transcription factor NF-κB, it was of interest to explore a possible effect of HE3286 on this central regulator of the inflammatory response. LPS stimulation of cultured RAW 264.7 mouse macrophages pre-treated with HE3286 displayed markedly reduced p65 nuclear staining, indicating decreased nuclear translocation of NF-κB (Figure 6A). The effect of HE3286 on NF-κB activation was also demonstrated in time-course experiments using freshly isolated mouse peritoneal macrophages, which revealed that the extent of NF-κB/p65 serine phosphorylation was decreased by HE3286 (Figures 6B). A similar effect was also observed in RAW264.7 macrophages (see below and Figure 7). To confirm that these effects translate into a functional change in NF-κB-driven gene expression, experiments were conducted with RAW264.7 cells transiently transfected with NF-κB-sensitive
promoter/reporter constructs. Prior treatment of RAW264.7 cells with HE3286 decreased subsequent activation of NF-κB-driven reporter gene expression in response to LPS stimulation by 50-60% (Figure 6C). As expected, NF-κB-dependent luciferase expression was also inhibited by dexamethasone or the MAP kinase inhibitor PD98059. Taken together, these observations indicate that HE3286 limits activation of NF-κB in macrophages in response to LPS, and suggest that this anti-inflammatory action may contribute to the observed amelioration of glucose intolerance in vivo.

**Suppression of proinflammatory signaling kinase cascades.** To gain further insight into the mechanism for the observed anti-inflammatory activity of HE3286, the impact of the compound on major proinflammatory kinase cascades engaged by endotoxin stimulation was studied in RAW264.7 macrophages. As expected, stimulation with LPS led to increased phosphorylation of IKK and NF-κB/p65 (see Figure 6B), as well as two major pro-inflammatory MAP kinase signaling cascades, namely c-Jun NH₂-terminal kinase (JNK) and p38 (Figure 7). However, prior exposure to HE3286 resulted in marked suppression in the extent of LPS-induced phosphorylation of these proteins (P<0.05 for all kinases and NF-κB/p65 at least after 60 min of LPS stimulation; Figure 7). Although it was not possible to distinguish between IKKα and IKKβ in these experiments given the specificity of the antibodies used (phospho-IKKα/β-Ser 180/181), it is of interest to note that IKKβ has been implicated in downregulation of insulin receptor substrate-1 (IRS-1) signaling through increased phosphorylation on serine residues (Arkan et al., 2005). These findings suggest that HE3286 causes a broad anti-inflammatory effect characterized by impaired LPS-induced upstream activation of IKK
and attendant suppression of NF-κB activation, as well as reduced activation of other TLR4-sensitive pro-inflammatory signaling kinase cascades.

**Nuclear receptor activity profile of HE3286.** The possibility that the observed HE3286 effects might occur through nuclear receptors known to influence inflammation and/or glucose homeostasis (i.e. GR, ERs and PPARs) was addressed by binding and transactivation experiments. As shown in Table 1, HE3286 does not bind to the major sex steroid or corticosteroid receptors AR, ERα, ERβ, PR and GR, as indicated by IC$_{50}$ values $>10,000$ nM in competition binding assays. In addition, transactivation assays revealed no activity of HE3286 in MDA-kb2 cells coexpressing AR and GR (EC$_{50}$ $>10,000$ nM), and essentially no transactivation in transiently transfected HEK293 fibroblasts expressing ERβ (EC$_{50}$ $>3,600$ nM). In contrast, evidence for weak activity (EC$_{50}$ = 268 nM) was found in T47D-kBluc cells coexpressing ERα and ERβ (Table 1), suggesting preferential but weak potential transactivation of ERα. However, under the conditions of these assays, the natural ER ligand 17β-estradiol transactivates ERα/ERβ (T47D-kBluc cells) with an EC$_{50}$ = 0.002 nM. Finally, as shown in Figure 8, HE3286 did not cause transactivation of PPARα, PPARγ or PPARδ. Assuming that the transactivation experiments described here are equivalent in other cell types, the combined results suggest that the observed therapeutic effects of HE3286 to improve glucose metabolism *in vivo* do not result from transactivation of sex steroid, corticosteroid or PPAR receptors.
Discussion

In this report, we have studied the novel, synthetic compound HE3286, a 17α-ethynyl-substituted analogue of the naturally occurring DHEA metabolite Δ5-androstene-3β,7β,17β-triol (Marwah et al., 2002) and show that HE3286 improves glucose metabolism and exhibits antidiabetic effects. Thus, when orally administered to diabetic db/db mice twice-a-day for 4 weeks, HE3286 delays progression to hyperglycemia in the absence of any significant changes in body weight. Treatment with HE3286 also led to markedly enhanced glucose tolerance in diabetic db/db mice, insulin resistant diet-induced (DIO) mice and in genetically obese ob/ob mice. These effects were accompanied by decreased hyperinsulinemia and reduced expression levels of MCP-1 mRNA in WAT, which is consistent with diminished serum levels of MCP-1 as well. Likewise, reduced mRNA expression of the MCP-1 receptor CCR2 in WAT from db/db mice was observed. These results indicate that, in addition to ameliorating glucose intolerance and insulin resistance, HE3286 possesses anti-inflammatory properties that act to abrogate excessive MCP-1 production in WAT1.

Since the MCP-1 pathway is known to play a role in recruiting macrophages into adipose tissue and in the development of obesity-related insulin resistance (Xu et al., 2003; Bruun et al., 2005; Weisberg et al., 2006), it is possible that the HE3286-induced improvement in glucose metabolism is, at least in part, a result of anti-inflammatory effects via decreased MCP-1 production. The link between MCP-1 and overall glucose metabolism and insulin action has been uncovered by a number of studies. Thus, transgenic mice overexpressing the MCP-1 gene under the control of the aP2
promoter/enhancer develop insulin resistance and hepatic steatosis, whereas an insulin sensitive phenotype is observed in homozygous MCP-1 KO mice or in animals in which 7ND, a dominant-negative mutant of MCP-1, is acutely expressed after injection of an expression plasmid (Kanda et al., 2006). Furthermore, treatment of DIO-C57BL/6J mice with INCB3344, a selective CCR2 antagonist (Brodmerkel et al., 2005), results in enhanced glucose disposal and insulin sensitivity (Weisberg et al., 2006). Therefore, it seems reasonable to suggest that a drug that attenuates expression and production of MCP-1 can have therapeutic benefit, possibly as a result of diminished macrophage infiltration into WAT with attendant improved glucose homeostasis and increased insulin sensitivity.

The ability of HE3286 to inhibit MCP-1 expression appears to result from broader anti-inflammatory actions as indicated by our results on NF-κB function in macrophages. We found that LPS-induced nuclear translocation of NF-κB was inhibited by HE3286 in cultured RAW264.7 mouse macrophages. HE3286 also caused inhibition of NF-κB-dependent reporter gene expression, as well as a decrease in LPS-induced p65 phosphorylation. These three observations strengthen the conclusion that HE3286 attenuates NF-κB function in macrophages, leading to reduced MCP-1 gene expression. On the other hand, attenuation of NF-κB function per se could translate into therapeutic effects on glucose metabolism. Other reports have shown that inhibition of NF-κB by adenoviral-mediated expression of the IκBα superrepressor SR-IκBα prevents insulin resistance in cultured hepatocytes in vitro (Iimuro et al., 1998) and improves hepatic insulin action and glucose homeostasis in db/db mice in vivo (Tamura et al., 2007).
These studies provide strong support to the notion that inhibition of NF-κB activity in diabetes/insulin resistance can improve disordered glucose homeostasis.

Although HE3286 inhibits activation and function of NF-κB in LPS-stimulated macrophages, the basis for this inhibition appears to reside upstream of NF-κB activation. Thus, prior exposure of macrophages to HE3286 resulted in suppression of LPS-induced activation of the IKK/NF-κB axis, as well as two major pro-inflammatory MAP kinase pathways (JNK and p38). In macrophages, these kinase signaling cascades are typically activated by pattern-recognition Toll-like receptors, of which TLR4 is the major target of LPS (Medzhitov, 2001). Since it has been shown that TLR4 can transduce the proinflammatory signals of fatty acids (Shi et al., 2006), which are often elevated in insulin resistant states, it is tempting to speculate that HE3286 might interfere with TLR4 function, leading to suppression of proinflammatory cascades.

Despite the structure of HE3286, which defines it as a C-19 Δ5-androstene species different from glucocorticoids, we tested its ability to bind or transactivate GR and found a lack of activity (see Table 2). Similarly, HE3286 does not exhibit any detectable binding activity towards AR, ERα, ERβ or PR. The low transactivation activity (EC50 = 268 nM) observed in cells coexpressing ERα and ERβ, but not ERβ alone, suggested a weak potential transactivation of ERα, albeit this activity is virtually negligible when compared with 17β-estradiol (EC50 = 0.002 nM). Furthermore, preliminary results indicate that HE3286 inhibits macrophage NF-κB function in the presence of ERα-
selective antagonists and its beneficial effects on glucose homeostasis in vivo are preserved in ERα-deficient (ERKO) mice (data on file).

In contrast to TZD-based insulin sensitizers that target PPARγ, HE3286 did not induce PPARγ-mediated transactivation activity compared to rosiglitazone or GW1929 (see Fig. 7). This is an important pathway to explore since it is likely that at least part of the insulin sensitizing effects of TZDs involve PPARγ-mediated anti-inflammatory responses due to transrepression of inflammatory mediators (Ricote et al., 1998; Welch et al., 2003). Moreover, deletion of the PPARγ gene from macrophages results in impaired glucose tolerance, insulin resistance and increased expression of inflammatory effectors, indicating that macrophage PPARγ is required for the full insulin sensitizing effects of TZDs (Hevener et al., 2007). The fact that HE3286 fails to transactivate PPARγ suggests that its anti-inflammatory and antidiabetic effects occur through a PPARγ-independent pathway.

Although GR, ER and PPAR receptors are important in metabolic regulation and inflammation, there are certain nuclear receptor subtypes which also play an important role in these functions. For example, the liver X receptors LXRα and β serve as oxysterol sensors that can suppress hepatic gluconeogenesis and attenuate NF-κB function through transrepression mechanisms (Bensinger and Tontonoz, 2008). Likewise, the estrogen receptor-related receptors ERRβ and γ function through ligand-independent binding of inducible coactivators involved in the liver gluconeogenesis program and mitochondrial function, such as PGC-1α and β (Mootha et al., 2004).
Similarly, the retinoic acid receptor-related orphan receptor RORα is a key modulator of lipid homeostasis that can increase fatty acid oxidation in skeletal muscle (Lau et al., 2004), and can also suppress inflammation and NF-κB function (Delerive et al., 2001). Thus, it is clear that additional work will be necessary to assess the possibility that the beneficial effects of HE3286 might also reflect modulation of non-classic nuclear receptors.

A major drawback of efficacy studies in rodent models of metabolic disease is the possibility that the pharmacological activity of a new drug may fail to translate in humans. In the particular case of HE3286, we believe that this concern is diminished in light of emerging clinical data in obese, insulin resistant human subjects, in whom daily treatment with HE3286 for 4 weeks is associated with improved whole body insulin sensitivity as determined by glucose clamp studies (work in progress). In these studies, it will be important to continue to explore the underlying hypothesis of the link between inflammation and insulin resistance by determining whether the beneficial effects of HE3286 on glucose homeostasis are associated with anti-inflammatory activity. Furthermore, completion of phase II clinical trials with HE3286 in type 2 diabetic subjects currently in progress should provide additional support for the potential translation of the therapeutic effects described here to long-term glycemic control.

In summary, the current studies demonstrate the antidiabetic and anti-inflammatory properties of HE3286, a synthetic analogue of a naturally occurring Δ^5^-androstene metabolite of DHEA. In rodent models of diabetes and insulin resistance
HE3286 ameliorated glucose intolerance and suppressed hyperinsulinemia, as well as serum levels and adipose tissue expression of MCP-1. In addition, \textit{in vivo} expression of MCP-1 and its receptor CCR2 in adipose tissue was abrogated by HE3286 in \textit{db/db} mice. These effects do not appear to result from binding or transactivation of nuclear receptors known to influence inflammation and/or glucose homeostasis, including PPAR\textgamma, GR, ER\textalpha or ER\textbeta. In LPS-activated macrophages, HE3286 attenuated nuclear translocation of NF-\kappaB, NF-\kappaB-p65 phosphorylation and NF-\kappaB-dependent reporter gene expression. Likewise, phosphorylation of IKK, JNK and p38 was also inhibited by HE3286, possibly reflecting upstream disruption of TLR4 signaling. We propose that the antidiabetic profile of HE3286 stems from broad anti-inflammatory activity, which ultimately mitigates the negative impact that obesity-related inflammatory mediators have to impair insulin action and cause insulin resistance.
Acknowledgments

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References


Footnotes

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1Preliminary clamp studies in db/db mice suggest that HE3286 enhances insulin-stimulated glucose uptake in skeletal muscle and improves hepatic insulin action by suppressing excessive liver glucose output.
Legends for Figures

FIGURE 1. HE3286 suppresses development of hyperglycemia in diabetic db/db mice. Non-fasting blood glucose levels and daily body weight in 8-wk old male db/db mice (A and B, respectively; N = 10 per group) or 6-wk old male younger db/db mice (C and D, respectively; N = 9 per group) treated with vehicle (empty circles), 20 mg/kg (filled circles), 40 mg/kg (empty squares), 80 mg/kg HE3286 (filled squares) or 25 mg/kg rosiglitazone (triangles) by oral gavage BID for 28 days. Arrows in panel D indicate the day of OGTT studies. Data shown as mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001 with respect to vehicle).

FIGURE 2. HE3286 reduces glucose intolerance in diabetic db/db mice. Glucose tolerance was evaluated by OGTT in 6-wk old db/db mice (N = 9 per group) before dosing or after 14 and 28 days of treatment with vehicle or HE3286. (A) OGTT profile in mice treated with vehicle (empty circles), 20 mg/kg (filled circles), 40 mg/kg HE3286 (empty squares) or 25 mg/kg rosiglitazone (triangles) for 4 weeks. (B) Blood glucose AUC values during OGTTs performed at indicated days of treatment. Data are expressed as area (mean ± SEM) under each of the OGTT curves for animals treated with vehicle (V), 20 mg/kg HE3286 (20) 40 mg/kg HE3286 (40) or 25 mg/kg rosiglitazone (Rosi). (C) Serum insulin levels after 4 weeks of treatment with HE3286. For comparison, insulin levels in db/+ lean animals are shown. Data shown as mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001 with respect to vehicle).
FIGURE 3. **HE3286 reduces glucose intolerance in obese, insulin resistant mice.** Glucose utilization was assessed by OGTT in male C57BL/6J DIO-mice (A and B) or genetically obese *ob/ob* mice (C and D) treated with vehicle (empty circles, *N* = 7), 80 mg/kg HE3286 (filled squares, *N* = 7) or 10 mg/kg rosiglitazone (triangles, *N* = 9) BID for 4 weeks. Blood glucose AUC values during OGTTs performed after treatment for DIO (B) or *ob/ob* mice (D). Data are shown as mean ± SEM (*P* < 0.05, **P* < 0.01, ***P* < 0.001 with respect to vehicle).

FIGURE 4. **HE3286 treatment decreases expression of MCP-1 and CCR2 mRNA in white adipose tissue from *db/db* mice.** (A) Expression levels of MCP-1 and CCR2 mRNA were determined by RT/PCR in total RNA prepared after a 6-hr fast from epididymal adipose tissue from diabetic *db/db* mice treated with vehicle, 40 mg/kg HE3286 or 25 mg/kg rosiglitazone BID for 4 weeks (*N* = 6 for each group). Lean *db/+* mice are shown for comparison. Relative mRNA expression is normalized with respect to vehicle. (C) Serum levels of MCP-1 in *db/db* mice after 4 weeks of indicated treatments. Data shown as mean ± SEM (*N* = 6-9) (**P* < 0.01; *P* < 0.05 relative to vehicle).

FIGURE 5. **HE3286 reduces excessive production of MCP-1 and hyperinsulinemia in *ob/ob* mice.** Insulin resistant *ob/ob* mice were treated with 80 mg/kg HE3286 BID for 4 weeks and serum levels of MCP-1 (A) and insulin (B) were measured. Expression of mRNA MCP-1 in epididymal white adipose tissue (C) was determined by RT/PCR. Data are expressed as mean ± SEM (*N* = 5) (*P* < 0.05, **P* < 0.01 relative to vehicle).
FIGURE 6. HE3286 suppresses NF-κB activation in mouse macrophages. (A) Cultured RAW264.7 mouse macrophages were grown on glass coverslips and left untreated (1), treated with 100 ng/mL LPS for 15 min (2) or treated with 100 nM HE3286 for 1 hr followed by LPS for 15 min (3). Cells were then incubated with an anti-p65 monoclonal antibody (IgG1), stained with a FITC-conjugated goat-anti-mouse IgG1 and visualized by immunofluorescence (40X). (B) Freshly isolated, thioglycolate-elicited murine intraperitoneal macrophages were seeded and allowed to recover for 3 days in culture. Cells were then pretreated with 0.01% DMSO or 100 nM HE3286 for 2 hr followed by LPS stimulation for the indicated times. Untreated cells served as the control (C) and shown on the first lane. Cell lysates were prepared and proteins immunoblotted with a phospho-NF-κB p65 (Ser536) antibody. (C) Cultured RAW264.7 macrophages transiently transfected with an NF-κB/luciferase reporter vector were left untreated (basal) or exposed to 1, 10 and 100 nM HE3286, 10 nM PD98059 or 10 nM dexamethasone for 1 hr, followed by stimulation with 100 ng/mL LPS for 6 hr. The resulting activation of NF-κB was assessed by luciferase activity in cell lysates. Data shown as mean ± SEM and normalized with respect to LPS-treated cells.

FIGURE 7. HE3286 attenuates proinflammatory kinase pathways in RAW264.7 macrophages. Murine RAW264.7 macrophages were pretreated with DMSO or 100 nM HE3286 overnight and then challenged with 100 ng/mL LPS for the times indicated. (A) Total lysates were prepared and the phosphorylation status of specific proinflammatory kinases was visualized by immunoblotting. Equivalent protein loading was determined
by immunoblotting with anti-α-tubulin antibodies. (B) Relative band intensities in two independent experiments were quantified by densitometry and normalized with respect to the extent of phosphorylation at time 0 in DMSO-treated cells. Data are expressed as mean ± SD (*P < 0.05).

**FIGURE 8. HE3286 does not cause transactivation of PPAR receptors.**

Transactivation activity for each of the PPAR receptors was determined by adding the indicated concentrations of HE3286 or reference ligands (see insets) onto HEK293 cells stably transfected with isoform-specific PPAR-LBD/Gal4-DBD chimeric proteins and also harboring a PPAR-sensitive β-lactamase reporter gene (GeneBlazer®; see Methods). Cells were loaded with the CCF4-AM β-lactamase substrate and following a 24-hr incubation period with these ligands, the resulting activity was determined by fluorescence. Data are shown as relative transactivation normalized with respect to the maximal response observed in each case after subtracting background activity (symbols represent mean ± SD). (A) PPARγ; (B) PPARδ; (C) PPARα.
Tables

**TABLE 1.** Nuclear receptor competition binding and transactivation profile of HE3286

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Binding (IC$_{50}$, nM)$^b$</th>
<th>Transactivation (EC$_{50}$, nM)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Reference Ligand</strong></td>
<td><strong>Receptor(s)</strong></td>
</tr>
<tr>
<td>AR</td>
<td>&gt;10,000 15 ± 4.2 [DHT]</td>
<td>AR/GR$^d$ 14 ± 9.3 [Dex]</td>
</tr>
<tr>
<td>ER$\alpha$</td>
<td>&gt;10,000 8 ± 5.4 [E$_2$]</td>
<td>ER$\alpha$/ER$\beta$$^e$ 268 ± 25 0.002 [E$_2$]</td>
</tr>
<tr>
<td>ER$\beta$</td>
<td>&gt;10,000 7 ± 1.6 [E$_2$]</td>
<td>ER$\beta$$^f$ 3,648 ± 540 0.06 ± 0.02 [E$_2$]</td>
</tr>
<tr>
<td>PR</td>
<td>&gt;10,000 15 ± 2.1 [P]</td>
<td>GR$^g$ &gt;10,000 8 ± 6.8 [Dex]</td>
</tr>
<tr>
<td>GR</td>
<td>&gt;10,000 8 ± 0.8 [Dex]</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Abbreviations used: AR – Androgen receptor; ER$\alpha$ – Estrogen receptor $\alpha$; ER$\beta$ – Estrogen receptor $\beta$; PR – Progesterone receptor; GR – Glucocorticoid receptor; DHT – Dihydrotestosterone; E$_2$ – 17$\beta$-estradiol; P – Progesterone; Dex – Dexamethasone.

$^b$Data are expressed as IC$_{50}$ values (mean ± SEM in nM units; N = 3-4). Reference ligands are indicated in brackets. See Methods for details of experimental procedures.

$^c$Data are expressed as EC$_{50}$ values (mean ± SEM in nM units; N = 4-9). Reference ligands are indicated in brackets. See Methods for details of experimental procedures.

$^d$MDA-kb2 – Cells stably transfected with a sex steroid receptor-sensitive promoter/reporter construct (MMTV promoter) fused upstream of a luciferase gene. These cells endogenously express both AR and GR (Wilson et al., 2002).

$^e$T47D-kBluc – Cells stably transfected with an estrogen-sensitive synthetic promoter/reporter construct (ERE) fused upstream of a luciferase gene. These cells express endogenously both ER$\alpha$ and ER$\beta$ (Wilson et al., 2004).

$^f$ER$\beta$-HEK293 – HEK293 fibroblasts transiently co-transfected with an estrogen-sensitive promoter/reporter construct and a cDNA expression vector encoding the full-
length human ERβ. These cells exhibit virtually undetectable levels of endogenous sex steroid receptors (see Methods).

GR-HEK293 – HEK293 fibroblasts transiently co-transfected with a glucocorticoid-sensitive promoter/reporter construct and a cDNA expression vector encoding the full-length human GR (see Methods).
FIGURE 1

A) Blood glucose (mg/dL) vs. Treatment day

B) Body weight (g) vs. Treatment day

C) Blood glucose (mg/dL) vs. Treatment day

D) Body weight (g) vs. Treatment day
FIGURE 3

A

DIO mice

Time (min)

Blood glucose (mg/dL)

B

DIO mice

Time (min)

AUC (mg/dL min)

C

ob/ob mice

Time (min)

Blood glucose (mg/dL)

D

ob/ob mice

Time (min)

AUC (mg/dL min)
FIGURE 5

A

Serum MCP-1 (pg/mL)

Lean Veh HE Rosi

B

Serum insulin (ng/mL)

Lean Veh HE Rosi

C

Relative expression MCP-1

Lean Veh HE3286
FIGURE 6

A

No LPS + LPS + LPS + HE3286

1 2 3

B

DMSO HE3286

C

Relative NF-kB activity (%)
FIGURE 7

A

<table>
<thead>
<tr>
<th>LPS (min)</th>
<th>DMSO</th>
<th>HE3286</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
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</table>

- P-p38
- P-JNK1/2
- P-p65 (NF-κB)
- P-IKKα/β
- α-tubulin

B

- P-p65 (NF-κB)
- P-IKK
- P-JNK
- P-p38

Relative Phosphorylation vs. Time (min)
Figure 8

A

Relative transactivation (%)

[Compound] (M)

Rosiglitazone

HE3286

GW1929

PPARγ

B

Relative transactivation (%)

[Compound] (M)

L165041

HE3286

PPARδ

C

Relative transactivation (%)

[Compound] (M)

GW7647

HE3286

PPARα