Cholesteryl Ester-Transfer Protein inhibitors stimulate aldosterone biosynthesis in adipocytes through Nox-dependent processes

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Running Title: CETP inhibitors stimulate aldosterone in adipocytes

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Abstract

Hyperaldosteronism and hypertension were unexpected side effects observed in trials of torcetrapib, a Cholesteryl Ester-Transfer Protein (CETP) inhibitor that increases HDL. Given that CETP inhibitors are lipid soluble and accumulate in adipose tissue, have binding sites for proteins involved in adipogenesis and that adipocytes are a source of aldosterone, we questioned whether CETP inhibitors, (torcetrapib, dalcetrapib, and anacetrapib) influence aldosterone production by adipocytes. Studies were performed using human adipocytes (SW872), which express CETP, and mouse adipocytes (3T3-L1), which lack CETP gene. Torcetrapib, dalcetrapib, and anacetrapib increased expression of CYP11B2, CYP11B1, and Steroidogenic Acute Regulatory Protein (StAR), enzymes involved in mineralocorticoid and glucocorticoid generation. These effects were associated with increased reactive oxygen species (ROS) formation. Torcetrapib, dalcetrapib, and anacetrapib upregulated STAT3 and PPARγ, important in adipogenesis, but only torcetrapib stimulated production of chemerin, a pro-inflammatory adipokine. To determine mechanisms whereby CETP inhibitors mediate effects, cells were pretreated with inhibitors of Nox1/4 (GKT137831), Nox1 (ML171), mitochondria (rotenone) and STAT3 (S3I-201). In torcetrapib-stimulated cells, Nox-inhibitors, rotenone and S31-201 downregulated CYP11B2 and StAR and reduced aldosterone. Dalcetrapib and anacetrapib effects on aldosterone were variably blocked by GKT137831, ML171, rotenone, and S31-201. In adipocytes, torcetrapib, dalcetrapib, and anacetrapib inhibit enzymatic pathways responsible for aldosterone production through Nox1/4- and mitochondrial-generated ROS and STAT3. CETP inhibitors also influence adipokine production. These processes may be CETP-independent. Our findings identify novel adipocyte-related mechanisms whereby
CETP inhibitors increase aldosterone production. Such phenomena may contribute to hyperaldosteronism observed in CETP inhibitor clinical trials.
Introduction

Cardiovascular disease (CVD) is a leading cause of death worldwide (Alwan et al., 2010). Of the many risk factors associated with CVD is dyslipidaemia, particularly low levels of High Density Lipoprotein (HDL) and high levels of Low Density Lipoprotein (LDL) (Miller and Miller, 1975; Gordon et al., 1977; Roheim, 1986.). Low HDL is a risk factor for coronary heart disease independently of LDL levels (Gordon et al., 1977). Factors implicated in low HDL include genetics (single nucleotide polymorphisms of genes regulating HDL) and environment (diet, exercise, obesity etc) (Voight et al., 2012; Luscher et al., 2014). HDL reduces cholesterol by transferring it from peripheral cells to the liver for excretion (reverse cholesterol transport). On the other hand, Cholesteryl Ester-Transfer Protein (CETP) transfers cholesterol ester from anti-atherogenic HDL to pro-atherogenic LDL or VLDL (Barter et al., 2003; Tall, 1986). As such, HDL has been considered a potential therapeutic target for the prevention and treatment of cardiovascular diseases. Various lipid-modulating drugs are currently available for clinical use, including statins, niacin and fibrates, but their HDL-elevating effects are modest. Based on genetic studies demonstrating that CETP deficiency is associated with markedly increased HDL levels (Inazu et al., 1990), drugs have been developed to inhibit CETP as a strategy to increase HDL, which may have cardiovascular protective effects, at least in some patients (Voight BF., et al, 2012). Small molecule CETP inhibitors used clinically include torcetrapib, dalcetrapib, anacetrapib, and evacetrapib, all of which increase HDL (Johns et al., 2012).

The Investigation of Lipid Level Management to Understand Its Impact in Atherosclerotic Events (ILLUMINATE) Trial, a major phase 3 secondary prevention morbidity and mortality trial in more than 15,000 subjects compared the CETP
inhibitor torcetrapib plus atorvastatin with atorvastatin alone. The trial was terminated prematurely because of higher all-cause morbidity and mortality, despite elevated HDL. Reasons for this remain unclear although elevated blood pressure and associated increase in plasma aldosterone, have been implicated (Barter et al., 2007). Aldosterone can cause cardiovascular injury independently of blood pressure elevating effects. Data from dalcetrapib trials demonstrated a modest blood pressure elevating effect (Schwartz et al., 2012), whereas anacetrapib does not seem to influence blood pressure, although clinical trials are still ongoing (Robinson and Frishman, 2014). Variable effects on plasma aldosterone have been demonstrated for these CETP inhibitors (Johns et al., 2012; Kontush et al., 2008).

Mechanisms underlying torcetrapib-induced hyperaldosteronism are unclear, but in vitro studies demonstrated that in adrenal cell lines, torcetrapib stimulated aldosterone production by increasing expression of aldosterone synthase (CYP11B2) (Forrest et al., 2008; Hu et al., 2009). We previously demonstrated that adipocytes possess the enzymatic machinery to synthesise mineralocorticoids and glucocorticoids, namely CYP11B2, CYP11B1 and StAR, and that they produce aldosterone and corticosteroids in basal and stimulated conditions (Briones et al., 2012; Briones et al., 2011). Adipocyte-derived aldosterone involves generation of reactive oxygen species (ROS) through Nox-dependent processes and plays a role in adipogenesis, adipocyte maturation and adipokine production (Briones et al., 2012).

Considering the fact that CETP inhibitors are lipophilic that may accumulate in adipose tissue (Dalvie et al., 2008; Gotto et al., 2014), we hypothesized that adipocytes may be an extra-adrenal source of CETP inhibitor-induced aldosterone production.
Material and Methods

Cell culture

Human SW872 and murine 3T3-L1 cell lines were obtained from the American Type Culture Collection (Manassas, VA). SW872 cells were cultured in DMEM, high glucose, supplemented with 10% Fetal Bovine Serum (FBS). 3T3-L1 cells were cultured in DMEM, low glucose, supplemented with 10% Calf Bovine Serum (CBS) (Life Technologies, Paisley, UK). Both cell lines were supplemented with antibiotics (0.1 mg/mL streptomycin and 100 U/mL penicillin) in 5% CO₂, at 37°C. Medium was changed every two days until confluence and two days post confluence, cells were differentiated to adipocytes. Differentiation for both cell lines was performed in DMEM supplemented with 10% FBS, 0.5 mmol/L 3-isobutyl-1-methylxanthine, 0.25 μmol/L dexamethasone (Sigma-Aldrich, St Louis, MO, USA), and 1 μmol/L insulin (Cell Applications, San Diego, CA, USA) for two days. Medium was replaced by DMEM/10% FBS, and 1 μmol/L insulin for an additional two days and then replaced by insulin-free DMEM/10% FBS. Ten days after the differentiation process, cells exhibited adipocyte morphology. One day before experiments, the medium was changed to DMEM 1% FBS or 1% CBS, for SW872 or 3T3-L1, respectively. V79 hamster cells stably transfected with human CYP11B1 or human CYP11B2, and H295R cells that express hCYP11B1 and hCYP11B2 were used as positive controls (gift from Dr Eleanor Davis, University of Glasgow).

Experimental protocols

To evaluate adipocyte effects of CETP inhibitors, cells were treated with torcetrapib, dalcetrapib or anacetrapib (1-10 μM) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 5 mins-5 hours. To determine molecular mechanisms involved in cell activation by CETP inhibitors, cells were pretreated for 30 min with N-Acetyl-L-
cysteine (ROS scavenger; Sigma-Aldrich, St Louis, MO, USA), ML171 (Nox1 Inhibitor; Calbiochem-EMD Millipore, Billerica, MA, USA), rotenone (mitochondrial inhibitor; Sigma-Aldrich, St Louis, MO, USA), GKT137831 (Nox1/4 inhibitor; Genkyotex, Geneva, SWT), and S3I-201 (Stat3 Inhibitor, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

**NAD(P)H oxidase activity and Hydrogen Peroxide Production.**

Stimulated adipocytes were washed with cold PBS and homogenized in lysis buffer (20 mmol/L of KH$_2$PO$_4$, 1 mmol/L of EGTA, 1 µg/mL of aprotinin, 1 µg/mL of leupeptin, 1 µg/mL of pepstatin, and 1 mmol/L of PMSF). Activity of NADPH oxidase was measured by chemiluminescence with lucigenin (5 µmol/L) as the electron acceptor and NADPH (100 µmol/L) as the substrate as we previously described (Briones et al., 2011). Luminescence was measured every 1.8 seconds for 5 minutes in a luminometer (AutoLumat LB 953, Berthold). A buffer blank was subtracted from each reading.

Hydrogen Peroxide was evaluated by Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Life Technologies, Paisley, UK) according to the manufacturer’s instructions. Obtained values were normalized by protein concentration in the cell lysate.

**Western Blot**

Cells were lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% SDS) supplemented with 1 mmol/L PMSF, 1 µg/mL pepstatin A, 1 µg/mL leupeptin, 1 µg/mL aprotinin (Sigma-Aldrich, St Louis, MO, USA), 10 mM sodium fluoride (AnalaR Normapur; VWR, Belgium), and 1 mM sodium orthovanadate (Alfa Aesar, Ward Hill, MA, USA). Protein concentrations were determined using the DC protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were
separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The non-specific binding was blocked with non-fat dry milk. Membranes were incubated overnight at 4°C in constant agitation with primary antibodies specific for phospho-STAT3 (#9138S), STAT3 (#9139S), phospho-CREB (#9198S), CREB (#9197S) (Cell Signaling Technology, Beverly, MA, USA), Nox2 (ab31092), Nox4 (ab133303), CYP11B2 (ab167413) (Abcam, Cambridge, UK), Nox1, Nox5 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and β-actin (Sigma-Aldrich, St Louis, MO, USA). The anti-StAR was a kind gift from Dr David Stocco (Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center) and used 1:500 dilution. As secondary antibodies, we used anti-rabbit IgG-HRP (1:2,000) and anti-mouse-HRP (1:5,000) (Jackson ImmunoResearch, West Grove, PA, USA). Protein expression were visualized using the chemiluminescent substrate SuperSignal West Pico (Thermo Scientific, Rockford, IL, ISA). The resulting autoradiograms were analyzed using the package ImageJ 1.44p (Wayne Rasband, NIH, USA) (http://imagej.nih.gov/ij).

**Real-Time RT-PCR**

Total RNA was isolated using the Trizol reagent (Life Technologies, Paisley, UK) according to the manufacturer’s instructions and diluted in nuclease-free H₂O (Ambion®, Life Technologies, Paisley, UK). cDNA was generated from total RNA using the High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Warrington, UK)). Real-time PCR was performed with the Applied Biosystems 7900HT Fast Real-Time PCR system, using Power SyBr® Green Master Mix (Applied Biosystems, Warrington, UK) and specific human primers, as follow: GAPDH, CYP11B1, CYP11B2, StAR, Mineralocorticoid Receptor (MR), Glucocorticoid Receptor (GR), PPARγ, Nox1, Nox2, Nox4, and Nox5 (Supplemental
Table 1. Relative gene expression was calculated by the $2^{\Delta \Delta CT}$ method as previously described (Livak and Schmittgen, 2001). Data are shown as the fold change in expression of the target gene relative to the internal control gene (GAPDH).

Enzymatic immunoassays

Commercially available enzyme immunoassay ELISA kits were used to measure the concentration of aldosterone (#10004377), cortisol (#500360), corticosterone (#500655) (Cayman Chemical, Ann Arbor, MI, USA), and chemerin (#DCHM00, RnD systems, Abingdon, UK) in cell supernatant according to the manufacturer’s instructions.

Statistical analysis

Results are presented as means±SEM. Statistical differences between mean values were determined by one-way ANOVA followed by Newman–Keuls test or Student t-test, as appropriate using the GraphPad Prism 5.0 software (San Diego, CA). P<0.05 values were considered as significant.

Results

Tocetrapib, dalcetrapib, and anacetrapib increase aldosterone production in human adipocytes.

Human differentiated adipocytes were treated with torcetrapib, dalcetrapib and anacetrapib. All drugs increased aldosterone concentration in the supernatant after 5 hours of stimulation (Figure 1A). Significant effects were evident at doses as low as 0.1 µM. Similar responses were observed in mouse 3T3-L1 adipocytes (Supplemental Figure 1), which lack the CETP gene. To avoid any interference that could result in a false positive effect due to cross reactivity by glucocorticoids, we
evaluated the cortisol concentration in the same samples. Cortisol levels were not significantly increased by any of the drugs (Figure 1B).

Torcetrapib, dalcetrapib and anacetrapib increased mRNA and protein expression of CYP11B2 and CYP11B1 (Figure 2A, Supplemental Figure 2), enzymes responsible for aldosterone and cortisol production respectively. The specificity of the antibody was verified using V79 cells stably transfected with either human CYP11B1 or CYP11B2, and H295 cells expressing both enzymes (Supplemental Figure 2C).

StAR transports cholesterol into the mitochondria and plays an important role in aldosterone production (Hattangady et al., 2012). Expression of StAR, at the mRNA and protein levels, was increased by CETP inhibitors (Figure 2B, Supplemental Figure 2D). These drugs also increased protein levels of the transcription factor Nurr1 (Figure 2C), associated with enhanced activity of the CYP11B2 promoter. The StAR promoter has a binding sequence for CREB/ATF-1 that acts synergically with Nurr1 to induce CYP11B2 expression and aldosterone production (Spyroglou et al., 2009). Phosphorylation of CREB was increased in adipocytes stimulated with anacetrapib (Figure 2D-F).

To evaluate whether CETP inhibitors influence adipocyte receptors through which aldosterone signals, we investigated expression of mineralocorticoid receptors (MR) and glucocorticoids receptors (GR). We found that both receptor types are increased in treated cells (Supplemental Figure 3).

CETP inhibitors influence adipocyte function

The STAT3 pathway plays an important role in adipogeneis (Zhang et al., 2011). STAT3 was rapidly phosphorylated in treated adipocytes (Figure 3A). This was associated with increased expression of PPARγ, an adipocyte differentiation marker.
Torcetrapib stimulated production of chemerin, a pro-inflammatory adipokine (Figure 3C).

**CETP inhibitor effects are mediated through ROS**

Studies in adrenal cortical cells showed that ROS contributes to aldosterone production (Rajamohan et al., 2012). As such we questioned whether CETP inhibitors influence aldosterone production by adipocytes through ROS-dependent processes. All 3 drugs increased generation of O$_2^-$ and H$_2$O$_2$ in human adipocytes (Figure 4) and mouse adipocytes (Supplemental Figure 4). In SW872 adipocytes pre-treated with the ROS scavenger N-acetyl cysteine (NAC), CETP inhibitor effects on aldosterone production and mRNA expression for CYP11B2, CYP11B1, StAR, MR, and GR were inhibited (Supplemental Figure 5).

Nox isoform expression (mRNA and protein) was variably increased by the different CETP inhibitors (Figure 5, Supplemental Figure 6). To determine if Noxs play a role in the effects observed for CETP inhibitors, human SW872 adipocytes were pretreated with ML171 (Nox1 inhibitor), GKT137831 (Nox1/4 inhibitor), rotenone (mitochondrial oxidase inhibitor), and S3I-201 (STAT3 inhibitor). PPAR$_\gamma$ expression induced by torcetrapib was inhibited by GKT and ML171 (Supplemental Figure 7A-C). Torcetrapib-stimulated chemerin production was reduced by GKT and S31-201 (Supplemental Figure 7D). In torcetrapib-treated adipocytes, GKT, ML171, rotenone, and S3I-201 decreased aldosterone production and mRNA levels of CYP11B2, StAR (Figure 6), MR, and GR (Supplemental Figure 8). In dalcetrapib-stimulated cells, aldosterone biosynthesis was inhibited by ML171 and S31-201 (Figure 6, Supplemental Figure 9), without effect of GKT or rotenone. In anacetrapib-stimulated adipocytes, aldosterone production and biosynthetic enzyme expression were reduced by GKT, ML171 and rotenone (Figure 6, Supplemental Figure 10).
Blockade of transfer of cholesterol esters from HDL to pro-atherogenic lipoproteins VLDL and LDL with CETP inhibitors has been considered a promising therapeutic strategy in the prevention and management of atherosclerosis (Barter et al., 2003; Tall, 1986). However, the ILLUMINATE trial, which evaluated cardiovascular effects in patients treated with torcetrapib plus atorvastatin versus atorvastatin alone, was terminated prematurely because of increased events in the torcetrapib group (Barter et al., 2007). Exact reasons for this remain unclear, but torcetrapib caused hypertension and hyperaldosteronism. Mechanisms whereby torcetrapib increases plasma aldosterone are elusive, although increased adrenal secretion through processes involving endogenous ouabain have been implicated (Funder, 2010).

Here we provide evidence for another source of torcetrapib-induced aldosterone production by showing that adipocytes generate aldosterone in response to CETP inhibitors. Reasons for probing adipocytes were three-fold; firstly we previously demonstrated that adipocytes have a functional renin angiotensin aldosterone system and produce aldosterone in a highly regulated manner (Briones et al., 2012; Briones et al., 2011), secondly CETP inhibitors are lipid soluble and accumulate, to variable degrees, in adipocytes (Dalvie et al., 2008; Gotto et al., 2014), and thirdly, by computational analysis, these drugs were found to bind to several endogenous proteins related to adipogenesis, such as PPARα, PPARβ, PPARγ, and LXR (Xie et al., 2009). We studied human and mouse adipocytes and found similar responses in both cell types, indicating that CETP inhibitor-induced adipocyte aldosterone production is independent of CETP, since mice lack the CETP gene. The biosynthesis of aldosterone has been very well characterised in adrenal cells and is mediated by StAR and the rate-limiting enzyme CYP11B2 (Hattangady et al., 2012).
Both enzymes are expressed in adipocytes and the three CETP inhibitors studied here significantly increased their expression. StAR, which is regulated by cAMP response-element (CRE)-binding protein (CREB) plays an important role in the acute generation of aldosterone through processes dependent on cholesterol flux into the mitochondria and conversion to pregnenolone. Aldosterone is also controlled chronically by CYP11B2 (Hattangady et al., 2012). Our results show differential regulation of these enzymes by CETP inhibitors. Torcetrapib and dalcetrapib increased expression of Nurr1, a transcription factor that regulates CYP11B2 (Spyroglou et al., 2009), and increased protein content of CYP11B2, whereas anacetrapib increased expression of CREB and StAR. Hence torcetrapib and dalcetrapib influence pathways associated with chronic aldosterone production, whereas anacetrapib influences processes associated with acute biosynthesis. Whether increased adipocyte-derived aldosterone translates into changes in plasma aldosterone levels still remains unknown. Our ongoing studies in adrenalectomized mice and in mice stimulated with CETP inhibitors will address this.

We previously showed that Ang II-induced adipocyte-derived aldosterone involves ROS. Other studies demonstrated that aldosterone production by adrenal cortical cells occurs in a ROS-dependent manner by regulating Nurr1 protein expression (Rajamohan et al., 2012). Here we extend those findings to show that CETP inhibitors modulate expression of Noxs and increase production of superoxide and hydrogen peroxide in adipocytes. Aldosterone production was reduced by N-acetyl-cysteine, a ROS scavenger, in CETP inhibitor-treated cells. To dissect the source of ROS required for aldosterone production, we used the pharmacologic inhibitors GKT137831, ML171 and rotenone, inhibitors of Nox1/Nox4, Nox1 and mitochondrial oxidases respectively. GKT137831, ML171 and rotenone inhibited
torcetrapib- and anacetrapib-induced aldosterone production, suggesting a role for Nox1, Nox4 and mitochondria in ROS-sensitive aldosterone production by these CETP inhibitors. For dalcetrapib the source of ROS is less clear, because ML171, but not GKT137831, reduced aldosterone synthesis, indicating a potential partial role for Nox1.

Associated with changes in aldosterone biosynthesis was downregulation of enzymes involved in aldosterone production, including CYP11B2 and StAR. Together these findings indicate that CETP inhibitors induce ROS generation, which stimulate aldosterone-synthesising enzymes to produce aldosterone in adipocytes. Exactly how these drugs regulate ROS-generating enzymes is unclear, but it may be possible that they have binding sites that target oxidases, as suggested by computational analysis (Chang et al., 2010).

In addition to influencing mineralocorticoid biosynthesis in adipocytes, CETP inhibitors impact on adipocyte function as evidenced by effects on differentiation processes and production of adipokines. In drug-treated adipocytes, STAT3 phosphorylation and PPARγ expression, important in adipogenesis and differentiation respectively (Chang et al., 2010) were increased and production of chemerin, a proinflammatory adipokine was stimulated. STAT3 also plays some role in aldosterone biosynthesis in CETP inhibitor-treated cells, because STAT3 inhibition blunted aldosterone production by torcetrapib and dalcetrapib.

From a functional viewpoint, our data have important clinical implications. We show that CETP inhibitors, which are lipid soluble, stimulate production of aldosterone by adipocytes through highly regulated processes. These drugs also influence adipocyte function by promoting adipocyte differentiation and production of adipokines, processes associated with the pro-inflammatory phenotype of adipose
tissue, important in cardiovascular disease. Finally, we demonstrate that adipocyte effects of torcetrapib, dalcetrapib and anacetrapib, occur through CETP-independent processes, because mouse adipocytes, which lack the CETP gene, responded in a similar manner to human adipocytes, which possess functionally active CETP.

In conclusion our study provides insights into novel molecular mechanisms whereby CETP inhibitors increase aldosterone production by adipocytes. These findings may explain, in part, the unexpected side effect of hyperaldosteronism in the large torcetrapib clinical studies. The exact contribution of adipocyte- versus adrenal-derived aldosterone is unknown, but our ongoing studies in adrenalectomized mice should provide information to address this. Our observations are important because other CETP inhibitors are in clinical development and potential injurious effects on adipocyte biology should be considered.

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Authorship Contributions

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Disclosures

No disclosures to declare.
References


Footnotes

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Figure Legends

Figure 1. Torcetrapib, Dalcetrapib, and Anacetrapib induce aldosterone production by human SW872 adipocytes. Cells were treated with 0.1, 1 and 10 µM of torcetrapib (Torc), dalcetrapib (Dalc), or anacetrapib (Anac) for 5 h. Aldosterone (A) and cortisol (B) concentration in the cell supernatant was evaluated by ELISA and normalized by total RNA or protein concentration (n=12). Data are expressed as mean ± SEM; *P<0.05 vs control vehicle group.

Figure 2. Torcetrapib, Dalcetrapib, and Anacetrapib induce the expression of CYP11B2, StAR and Nurr1, and CREB phosphorylation in human SW872 adipocytes. Cells were treated with 1 µM of torcetrapib (Torc), dalcetrapib (Dalc), or anacetrapib (Anac). The expression CYP11B2 (A), StAR (B) and Nurr1 (C) was analyzed in the cell lysate after 5 h by western blot and normalized by β-actin. Phospho-CREB (p-CREB) was evaluated by western blot after 5, 15, and 30 min and normalized by total CREB (D-F). Autoradiographs show one representative experiment. Protein expression was quantified using ImageJ software. Graph data are presented as mean ± SEM (n=6); *P<0.05 vs control vehicle group.

Figure 3. STAT3 Phosphorylation, PPARγ expression and Chemerin production by human SW872 adipocytes stimulated with Torcetrapib, Dalcetrapib, and Anacetrapib. Cells were treated with 1 µM of torcetrapib (Torc), dalcetrapib (Dalc), or anacetrapib (Anac). Phospho-STAT3 was evaluated by western blot after 5, 15, and 30 min and normalized to total STAT3 (A-C). Autoradiographs show one representative experiment. Protein expression was quantified using ImageJ software. The PPARγ mRNA expression was evaluated after 5 h and normalized to GAPDH mRNA (D). Chemerin production was evaluated...
after 5 h in the cell supernatant by ELISA and normalized to total RNA (E). Graph data are presented as mean ± SEM (n=6); *$P<0.05$ vs control vehicle group.

**Figure 4: Torcetrapib, Dalcetrapib, and Anacetrapib induce ROS generation in human SW872 adipocytes.** Cells were treated with 1 µM of torcetrapib (Torc), dalcetrapib (Dalc), or anacetrapib (Anac). ROS production was evaluated in the cell lysate by lucigenin chemiluminescence assay after 5 and 30 min (A) and Amplex Red assay after 5, 30, and 60 min (B). Values were normalized by protein concentration of the cell lysate. Data are presented as fold change of the control vehicle values and expressed as mean ± SEM (n=5); *$P<0.05$ vs control vehicle group.

**Figure 5: Nox isoforms expression are induced in human SW872 adipocytes stimulated by Torcetrapib, Dalcetrapib, and Anacetrapib.** Cells were treated with 1 µM of torcetrapib (Torc), dalcetrapib (Dalc), or anacetrapib (Anac). The expression Nox1 (A), Nox2 (B), Nox4 (C), and Nox5 (D) was analyzed in the cell lysate after 5 h by western blot. β-actin was used as a load control. The autoradiographs show one representative experiment. Protein expression was quantified using ImageJ software. Graph data are expressed as mean ± SEM (n=6); *$P<0.05$ vs control vehicle group.

**Figure 6: Effects of Torcetrapib, Dalcetrapib, and Anacetrapib on aldosterone production are affected by inhibitors of ROS production and STAT3 phosphorylation.** Cells were pre-treated with GKT137831 (Nox1/4 inhibitor) (10 µM), ML171 (Nox1 inhibitor) (1 µM), Rotenone (mitochondrial oxidase inhibitor) (10 µM) or S3I-201 (STAT3 inhibitor) (50 µM) for 30 min, then treated with 1 µM of Torcetrapib (A-C), Dalcetrapib (D-F), or Anacetrapib (G-I) for 5 h. Aldosterone
concentration in the cell supernatant was evaluated by ELISA and normalized by total RNA. The mRNA expression for CYP11B2 and StAR was evaluated and normalized to GAPDH mRNA. Graph data are expressed as mean ± SEM (n=7); *P<0.05 vs control vehicle group. + P<0.05 vs Torc, Dalc or Anac treated group
Figure 1

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Figure 3
Figure 4

A

$O_2^-$ fold increase

B

$H_2O_2$ fold increase

cont 5 30 5 30 5 30 min
torc dalc anac

5 30 60 min 5 30 60 5 30 60 min
torc dalc anac
Figure 5

![Graph A](image)

![Graph B](image)

![Graph C](image)

![Graph D](image)
Figure 6