Off-Target Vascular Effects of Cholesteryl Ester Transfer Protein Inhibitors Involve Redox-Sensitive and Signal Transducer and Activator of Transcription 3–Dependent Pathways


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ABSTRACT

Elevated blood pressure was an unexpected outcome in some cholesteryl ester transfer protein (CETP) inhibitor trials, possibly due to vascular effects of these drugs. We investigated whether CETP inhibitors (torcetrapib, dalcetrapib, and anacetrapib) influence vascular function and explored the putative underlying molecular mechanisms. Resistance arteries and vascular smooth muscle cells (VSMC) from rats, which lack the CETP gene, were studied. CETP inhibitors increased phenylephrine-stimulated vascular contraction (logEC50 6.6 ± 0.1, 6.4 ± 0.06, and 6.2 ± 0.09 for torcetrapib, dalcetrapib, and anacetrapib, respectively, versus control 5.9 ± 0.05). Only torcetrapib reduced endothelium-dependent vasorelaxation. The CETP inhibitor effects were ameliorated by N-acetylcysteine (NAC), a reactive oxygen species (ROS) scavenger, and by S3I-201 [2-hydroxy-4-[2-(4-methylphenyl)sulfonyloxyacetamido]benzoic acid; STAT3, signal transducer and activator of transcription 3; VSMC, vascular smooth muscle cells; N-acetylcysteine; O2•−, superoxide anion; PE, phenylephrine; ROS, reactive oxygen species; S3I-201, 2-hydroxy-4-[2-(4-methylphenyl)sulfonyloxyacetamido]benzoic acid; STAT3, signal transducer and activator of transcription 3; VSMC, vascular smooth muscle cells; WKY, Wistar Kyoto.

Introduction

The cholesteryl ester-transfer protein (CETP) plays a key role in the metabolism of lipoproteins through the bidirectional transfer of neutral lipids between triglyceride-rich lipoproteins and high-density lipoproteins (HDL) (Tall, 1986; Barter et al., 2003). Human genetic studies demonstrated that CETP deficiency is associated with increased HDL levels (Inazu et al., 1990), and this led to the hypothesis that pharmacologic inhibition of CETP may be an effective therapeutic strategy to elevate HDL cholesterol and reduce cardiovascular risk in patients with dyslipidemia (Okamoto et al., 2000).

The Investigation of Lipid Level Management to Understand Its Impact in Atherosclerotic Events (ILLUMINATE) trial was the first designed to investigate whether CETP inhibition would increase HDL concentrations and reduce cardiovascular events (Barter et al., 2007). The study was prematurely terminated, not only because torcetrapib failed to achieve the clinical benefits, which was further confirmed in three additional large trials (ILLUSTRATE, RADIANCE 1, and RADIANCE 2) (Bots et al., 2007; Kastelein et al., 2007; Nissen et al., 2007), but also because there were unexpected adverse effects of elevated blood pressure, increased cardiovascular events, and an increased death rate, despite an increase in HDL cholesterol and decrease in low-density lipoproteins. Most clinical studies using CETP inhibitors have

ABBREVIATIONS: ACh, acetylcholine; CETP, cholesteryl ester transfer protein; DMEM, Dulbecco’s modified Eagle medium; ERK, extracellular signal-regulated kinase; GKT137831, [2-(2-chlorophenyl)-4-[3-(dimethylamino)phenyl]-5-methyl-1H-pyrazolo[4,3-c]pyridine-3,6-dione] (Nox1/4 inhibitor); HDL, high-density lipoproteins; MAPK, mitogen-activated proteins kinase; MLC, myosin light chain; MYPT1, myosin phosphatase target subunit 1; NAC, N-acetylcysteine; O2•−, superoxide anion; PE, phenylephrine; ROS, reactive oxygen species; S3I-201, 2-hydroxy-4-[2-(4-methylphenyl)sulfonyloxyacetamido]benzoic acid; STAT3, signal transducer and activator of transcription 3; VSMC, vascular smooth muscle cells; WKY, Wistar Kyoto.
demonstrated elevated blood pressure as a major adverse event (Barter et al., 2007; Schwartz et al., 2012). The mechanisms behind this remain unclear, but the increased levels of plasma aldosterone that are observed in some patients treated with CETP inhibitors may play a role (Barter et al., 2007).

Other small molecule CETP inhibitors have been clinically evaluated—dalcetrapib, anacetrapib, and evacetrapib—all of which increase HDL. The Dal-OUTCOMES trial of dalcetrapib was terminated in phase 3 as well; no therapeutic benefit was found, and a small but significant increase in systolic blood pressure and C-reactive protein levels was observed (Schwartz et al., 2012). The trial for evacetrapib was recently discontinued, but the trial for anacetrapib is ongoing (Gotto et al., 2014; Teramoto et al., 2014; Brinton et al., 2015).

Although the mechanisms underlying the adverse blood pressure actions induced by torcetrapib and other CETP inhibitors remain elusive, in vivo and in vitro data have demonstrated that there may be off-target effects in different cells. Torcetrapib stimulates aldosterone production in adrenal cells (Forrest et al., 2008; Hu et al., 2009) and adipocytes (Rios et al., 2015), which could contribute to hyperaldosteronism and blood pressure elevation. However, treatment with aldosterone/mineralocorticoid blockers demonstrated only partial effects on torcetrapib-induced hypertension (Forrest et al., 2008). Other putative mechanisms include impairment in endothelial function due to decreased nitric oxide synthase activation and reduced nitric oxide production (Simic et al., 2012). Increased vascular reactive oxygen species (ROS) production, which stimulates aldosterone production, may also be important (Simic et al., 2012; Rios et al., 2015).

These studies suggest that CETP inhibitors may influence endothelial function, but it is unclear whether they directly impact on vascular contraction and whether the effects are CETP dependent. Accordingly, we investigated the effects of torcetrapib, dalcetrapib, and anacetrapib on vascular reactivity in isolated rat arteries and explored the potential molecular mechanisms underlying the vascular effects of CETP.

![Fig. 1](image-url) Torcetrapib, dalcetrapib, and anacetrapib increase sensitivity to phenylephrine in small mesenteric arteries from WKY rats. Resistance mesenteric arteries were isolated from WKY rats, mounted in a wire myograph, and treated with (A) torcetrapib, (B) dalcetrapib, or (C) anacetrapib (1 μM for 1 hour). Concentration-response curves to phenylephrine (PE) or acetylcholine (ACh) were derived from endothelium-intact arteries. Results indicate the mean ± S.E.M. of five to eight vessels of different animals. *P < 0.05 versus control vehicle group.
inhibitors in cultured rat vascular smooth muscle cells (VSMC). In particular, we focused on the redox-sensitive mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription 3 (STAT3) signaling pathways, which are important in VSMC regulation. We studied rats as our experimental model because rodents lack the CETP gene, which makes the effects of the drugs independent of the enzyme.

**Materials and Methods**

**Animals.** Male Wistar Kyoto (WKY) rats, 16 to 20 weeks old, were studied (n = 8–10). Mesenteric small arteries were isolated and used to study vascular function by wire myography. These arteries were also used for primary culture of VSMC. All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health, and were approved the United Kingdom Home Office Guidance on the operation of the Animals (Scientific Procedures) Act 1986 and institutional ethics approval.

**Cell Culture.** VSMC derived from rat mesenteric arteries were isolated and characterized, as we described before (Touyz et al., 1994). Briefly, mesenteric arteries were cleaned of adipose and connective tissue, and VSMC were dissociated by the enzymatic digestion of the drugs independent of the enzyme.

**Preparation and Study of Small Arteries.** Third-order superior mesenteric arteries (approximately 2 mm in length) from WKY rats were dissected and placed in cold physiologic salt solution containing (mM): NaCl 120, NaHCO3 25, KCl 4.7, KH2PO4 1.18, MgSO4 1.18, CaCl2 2.5, EDTA 0.026, and glucose 5.5. The vessels were mounted on two stainless-steel wires in standard organ chambers for isometric tension recording and left to equilibrate for about 30 minutes in Krebs–Henseleit solution. The arterial integrity was assessed by stimulation with 120 mM of KCl.

After washing and a new stabilization period, endothelial function was assessed by stimulation with acetylcholine (ACh, 1 μM) on vessels previously contracted with phenylephrine (PE, 1 μM). Cumulative concentration–response curves to PE or ACh (0.001–10 μM) were obtained in vessels preincubated with torcetrapib, dalcetrapib, or anacetrapib (1 μM for 1 hour) or vehicle. The CETP inhibitor concentrations used in this study were based on our previous findings (Rios et al., 2015) and on dose–response experiments where we evaluated the effect of the drugs on superoxide anion (O2−) production in VSMC from WKY (Supplemental Figure 1). The concentration used in our study is similar to or lower than the Cmax in humans treated with torcetrapib, dalcetrapib, or anacetrapib (Dalvie et al., 2008; Krishna et al., 2009; Derks et al., 2010), which may have (patho)physiologic significance.

**Experimental Protocols.** To evaluate the effect of CETP inhibitors, we treated VSMC with torcetrapib, dalcetrapib, or anacetrapib (1 μM) (Santa Cruz Biotechnology, Dallas, TX) for 5 minutes to 24 hours. To determine the molecular mechanisms involved in cell activation by CETP inhibitors, we pretreated the cells for 30 minutes with the ROS scavenger N-acetyl-L-cysteine (NAC; Sigma-Aldrich, St. Louis, MO), the Nox1 inhibitor GKT137831 (2-(2-chlorophenyl)-4-[3-(dimethylamino)phenyl]-5-methyl-1H-pyrazolo[4,3-c]pyridine-3,6-dione) (Genkyotex, Geneva, Switzerland), or the STAT3 inhibitor S3I-201 (2-hydroxy-4-[[2-(4-methylphenyl)sulfonyl]acetyl]aminobenzonic acid) (Santa Cruz Biotechnology).

**NADPH Oxidase Activity and Hydrogen Peroxide Production.** Stimulated VSMC were washed with cold phosphate-buffered saline and homogenized in lysis buffer (20 mM of KH2PO4, 1 mM of EGTA, 1 μg/ml of aprotinin, 1 μM of leupeptin, 1 μg/ml of pepstatin, and 1 mM of phenylmethylsulfonyl fluoride) and transferred to microtubes. The lucigenin-derived chemiluminescence assay was used to determine the NADPH oxidase activity in total protein cell homogenates. The reaction was started by the addition of NADPH (0.1 mM) to the sample-containing suspension (50 μl), lucigenin (5 μM), and assay phosphate buffer (50 mM KH2PO4, 1 mM EGTA, 150 mM sucrose, 1 mM of phenylmethylsulfonyl fluoride) and transferred to microtubes.

**Fig. 2.** Phenylephrine-induced contraction increased by CETP inhibitors is dependent on ROS production and STAT3 activation. Resistance mesenteric arteries were isolated from WKY rats and mounted in a wire myograph. Vessels were pretreated with (A) NAC, a ROS scavenger (10 μM), or (B) S3I-201 (SI), a STAT3 inhibitor (50 μM), for 30 minutes, followed by treatment with torcetrapib, dalcetrapib, or anacetrapib (1 μM for 1 hour). Concentration–response curves to phenylephrine (PE) derived from endothelium-intact arteries. Results indicate the mean ± S.E.M. of five to eight vessels of different animals. *P < 0.05 versus control vehicle group; #P < 0.05 versus NAC or S3I-201 pretreated group.
pH 7.4), 250 μl final volume. Luminescence was measured every 1.8 seconds for 3 minutes in a luminometer (Lumistar Galaxy; BMG Labtechnologies, Ortenberg, Germany). Buffer blank was subtracted from each reading. Hydrogen peroxide was evaluated by using the Amplex Red Hydrogen Peroxide/ Peroxidase Assay Kit (Molecular Probes/Life Technologies, Paisley, United Kingdom) according to the manufacturer’s instructions. Obtained values were normalized by protein concentration in the cell lysate and are expressed as the fold change related to the vehicle control group.

**Western Blot Analysis.** Cells were lysed in radiomunoprecipitation assay (RIPA) buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% SDS) supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 μg/ml aprotinin (Sigma-Aldrich), 10 mM sodium fluoride (AnalaR Normapur; VWR International, Leuven, Belgium), and 1 mM sodium orthovanadate (Alfa Aesar, Ward Hill, MA). Protein concentrations were determined using the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad Laboratories). The nonspecific binding was blocked with nonfat dry milk. The membranes were incubated overnight at 4°C in constant agitation with primary antibodies specific for phospho-STAT3 (Tyr705), total-STAT3, phospho-MLC (Ser19), total-MLC, phospho-extracellular signal-regulated kinase (p-ERK1/2) (Thr202/Tyr204), total ERK1/2, phospho-P38 (Thr180/Tyr182), total-p38MAPK (Cell Signaling Technology, Beverly, MA), phospho-myosin phosphatase target subunit 1 (p-MYPT1) (Thr696), total-MYPT1 (Santa Cruz Biotechnology), and β-actin (Sigma-Aldrich). As secondary antibodies, we used anti-rabbit IgG–horseradish peroxidase (1:2000) and anti-mouse–horseradish peroxidase (1:5000) (Jackson ImmunoResearch, West Grove, PA).

Protein expression was visualized using the chemiluminescent substrate SuperSignal West Pico (Thermo Scientific, Rockford, IL). The resulting autoradiograms were analyzed using ImageJ 1.44p software (Wayne Rasband, National Institutes of Health, http://imagej.nih.gov/ij).

**Statistical Analysis.** The results are presented as mean ± S.E.M. Statistical differences between mean values were determined by one-way analysis of variance followed by the Newman-Keuls test or Student’s t test, as appropriate, using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA). *P < 0.05 was considered statistically significant.

**Results**

**CETP-Inhibitors Increase Phenylephrine-Induced Vascular Contraction.** To investigate effects of CETP inhibitors on vascular function, we treated endothelium-intact mesenteric arteries from WKY rats with torcetrapib, dalcetrapib, and anacetrapib. The membranes were incubated overnight at 4°C in constant agitation with primary antibodies specific for phospho-STAT3 (Thr180/Tyr182), total-p38MAPK (Cell Signaling Technology, Beverly, MA), phospho-myosin phosphatase target subunit 1 (p-MYPT1) (Thr696), total-MYPT1 (Santa Cruz Biotechnology), and β-actin (Sigma-Aldrich). As secondary antibodies, we used anti-rabbit IgG–horseradish peroxidase (1:2000) and anti-mouse–horseradish peroxidase (1:5000) (Jackson ImmunoResearch, West Grove, PA).

Protein expression was visualized using the chemiluminescent substrate SuperSignal West Pico (Thermo Scientific, Rockford, IL). The resulting autoradiograms were analyzed using ImageJ 1.44p software (Wayne Rasband, National Institutes of Health, http://imagej.nih.gov/ij).

**Statistical Analysis.** The results are presented as mean ± S.E.M. Statistical differences between mean values were determined by one-way analysis of variance followed by the Newman-Keuls test or Student’s t test, as appropriate, using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA). *P < 0.05 was considered statistically significant.

**Results**

**CETP-Inhibitors Increase Phenylephrine-Induced Vascular Contraction.** To investigate effects of CETP inhibitors on vascular function, we treated endothelium-intact mesenteric arteries from WKY rats with torcetrapib, dalcetrapib, and anacetrapib (1 μM). Phosphorylated (A) MLC, (B) MYPT1, (C) STAT3, and (D) c-Src were evaluated by Western blot analysis after 5, 15, and 30 minutes and were normalized to total forms of MLC, MYPT1, STAT3, and c-Src. Results are presented as fold change of the vehicle control group. Graphs show the mean ± S.E.M. (n = 6), and the autoradiographs show one representative experiment. *P < 0.05 versus control vehicle group.
and anacetrapib. Endothelium-intact mesenteric arteries from WKY rats treated with torcetrapib, dalcetrapib, and anacetrapib were more sensitive to contractions induced by phenylephrine compared with vehicle-treated vessels, as evidenced by the leftward shift of the concentration–response curve (Fig. 1). The logEC50 for tocetrapib, dalcetrapib, and anacetrapib was 6.6 ± 0.1, 6.4 ± 0.06, and 6.2 ± 0.09, respectively, compared with the vehicle control (5.9 ± 0.05) (P < 0.05). ACh-induced vasorelaxation was not significantly modified by dalcetrapib or anacetrapib but was significantly reduced by torcetrapib, as indicated by the slight rightward shift of the dose–response curve (Fig. 1A).

**Increased Phenylephrine-Induced Contraction by CETP Inhibitors Is Dependent on ROS Production and STAT3 Activation.** To investigate putative molecular mechanisms underlying CETP inhibitor–induced vascular effects, we probed redox-sensitive and STAT3-dependent pathways by pre-exposing vessels to NAC, a ROS scavenger, and S3I-201, a STAT3 inhibitor. As shown in Fig. 2, the PE-induced contraction in vessels stimulated by CETP inhibitors was normalized by NAC (Fig. 2A) and S3I-201 (Fig. 2B) pretreatment.

**CETP Inhibitors Induce Phosphorylation of MLC, MYPT1, and STAT3 in Rat VSMC.** Vascular smooth muscle contraction is dependent on phosphorylation of Ser19 and Thr18 of MLC kinase, and sustained by phosphorylation of MYPT1 (Thr696), which inhibits MLC phosphatase (Webb, 2003). Both MLC and MYPT1 were significantly phosphorylated in VSMCs stimulated with torcetrapib, dalcetrapib, and anacetrapib (Fig. 3, A and B) versus control conditions (P < 0.05). STAT3 phosphorylation is associated with vascular reactivity (Johnson et al., 2013). We found that torcetrapib, dalcetrapib, and anacetrapib induced significant phosphorylation of STAT3 (P < 0.05) (Fig. 3C). No differences were observed for c-Src phosphorylation (Fig. 3D) or for ERK1/2 and p38MAPK activation (Fig. 4, A and B), which are upstream signaling proteins involved in VSMC activation. CETP inhibitors had no effect on proliferating cell nuclear antigen (PCNA) expression, an index of cell growth (Supplemental Figure 2).

**CETP Inhibitors Induce ROS Production in Rat VSMC.** Because ROS play an important role in the regulation of VSMC function, we questioned whether the effects of CETP inhibitors observed in our study are mediated through ROS-dependent processes. All three drugs increased generation of O2− and hydrogen peroxide (H2O2) by 2- to 4-fold in VSMC (Fig. 5, Supplemental Figure 1). The kinetics for ROS generation differed between the CETP inhibitors. Torcetrapib and dalcetrapib induced a rapid response within 5 to 10 minutes, with dalcetrapib having a biphasic effect on O2− production (Fig. 5, A and B). Maximal O2− and H2O2 effects of anacetrapib were evident at 60 and 10 minutes, respectively (Fig. 5C).

To determine whether ROS play a role in the effects observed with CETP inhibitors, VSMC were pretreated with NAC (a ROS scavenger) or GKT137831 (an inhibitor of Nox1 and Nox4, major sources of ROS in VSMCs). Both NAC and GKT137831 inhibited the phosphorylation of MLC induced by torcetrapib, dalcetrapib, and anacetrapib (Fig. 6A). No significant effects were observed for the expression of pMYPT1 or pSTAT3 (Supplemental Figure 3 and Fig. 6B). To assess the role of STAT3, VSMCs were pretreated with the STAT3 inhibitor S3I-201 before stimulation with CETP inhibitors. As shown in Fig. 6C, the MLC phosphorylation induced by torcetrapib and dalcetrapib was inhibited by S3I-201 without an effect on p-MYPT1 (Supplemental Figure 4).

**Discussion**

The major findings from our present study demonstrate that torcetrapib, dalcetrapib, and anacetrapib have vasoconstrictor actions and that phenylephrine-induced contractile responses in small mesenteric arteries from rats are enhanced by these agents. Molecular mechanisms underlying vascular actions of CETP inhibitors involve ROS- and STAT3-dependent processes. These vascular effects are independent of CETP inhibition because rats lack the CETP gene and thus do not express the enzyme.

Findings from clinical trials have shown that elevated blood pressure is an adverse off-target effect of CETP inhibitors. The prohypertensive and CETP-independent actions of these agents are further supported by two preclinical studies where
torcetrapib induced a significant increase in blood pressure in mice and rats, which lack CETP (Forrest et al., 2008; Simic et al., 2012). The mechanisms underlying these actions are poorly understood, although some studies suggest that hyperaldosteronism, down-regulation of endothelial nitric oxide synthase, oxidative stress, and increased endothelin-1 production could be possible contributory factors (Forrest et al., 2008).

Here we show that CETP inhibitors directly influence VSMC function and vascular contractility. Torcetrapib, dalcetrapib, and anacetrapib increased the sensitivity of vessels to phenylephrine, indicating increased contractility. These findings differ from those of Forrest et al., (2008), who failed to demonstrate a direct action of torcetrapib on vascular tone in isolated arterial vascular segments from mice infused with the drug. In that study, torcetrapib increased blood pressure but did not enhance contractile responses to vasoactive agents, did not influence endothelium-dependent vasorelaxation, and did not increase pressure in an isolated perfused hindlimb preparation. Reasons for the differences in results between our study and those of Forrest et al., (2008), where it was concluded that blood pressure effects are due to increased aldosterone production, may relate to the variations in drug doses used and to differences in the experimental protocols. We studied direct in vivo effects of CETP inhibitors on small vessel function at a concentration of 1 μM whereas Forrest and colleagues used torcetrapib and anacetrapib at 10 mg/kg administered intravenously over 30 minutes.

Vascular contraction is a highly regulated process involving many signaling pathways, such as calcium-dependent regulation of MLC, activation of redox-sensitive proteins due to increased oxidative stress, and activation of Rho kinase (Reho et al., 2014). Maintaining VSMCs in a mature differentiated contractile phenotype is important for vascular contraction and involves JAK-STAT3 (Kirchmer et al., 2014). We found that exaggerated contractile responses to CETP inhibitors were attenuated in vessels treated with a ROS scavenger and an inhibitor of STAT3, indicating the importance of redox-sensitive processes and STAT3 signaling in CETP inhibitor vascular effects. Similar to others (Simic et al., 2012), we found that endothelium-dependent vasorelaxation was impaired by torcetrapib, as evidenced by the shift of the acetylcholine-stimulated vasorelaxation curve.

To investigate in greater detail some of the molecular mechanisms associated with vascular effects of CETP inhibitors, we studied VSMCs stimulated with torcetrapib, dalcetrapib, and anacetrapib in the absence or presence of pharmacologic...
modulators. VSMC contraction is dependent on the increase of intracellular concentration of calcium ([Ca^{2+}]), which binds to calmodulin. This complex phosphorylates MLC kinase, enabling the actin–myosin interaction and subsequent contraction. An additional mechanism underlying VSMC contraction is activation of Rho kinase, which phosphorylates and inactivates MLC phosphatase (MYPT1), thereby increasing the stability of the MLC kinase, resulting in contraction (Webb, 2003; Hilgers and Webb, 2005). Here we show that CETP inhibitors induced phosphorylation of both MLC and MYPT1. Despite all three compounds having a different chemical structure, they have similar molecular actions, possibly because they are all small molecules that target multiple common signaling pathways. Although we did not examine the effects on [Ca^{2+}], others have shown that torcetrapib increases the expression of L-type calcium channels and [Ca^{2+}] (Hu et al., 2009; Clerc et al., 2010), which may be another mechanism whereby CETP inhibitors influence VSMC function and vascular contractility.

We previously showed that torcetrapib, dalcetrapib, and anacetrapib stimulate ROS production in adipocytes (Rios et al., 2015). Here we advance those findings by demonstrating that these drugs also increase ROS generation in VSMCs. Although all three agents significantly increased ROS production, the time course was variable, with torcetrapib and dalcetrapib having acute effects within 10 minutes of cell stimulation whereas anacetrapib actions were slightly delayed. These diverse responses may relate, in part, to differential activity of Nox, the primary source of vascular ROS. ROS effects of CETP inhibitors have functional significance because procontractile signaling pathways are sensitive to changes in intracellular redox status (Montezano and Touyz, 2014). This is further supported in our studies where we show that ROS scavenging by NAC ameliorates hypercontractility induced by CETP inhibitors and that NAC and GKT137831 reduce CETP inhibitor effects on MLC phosphorylation. Together these findings indicate that in VSMC torcetrapib, dalcetrapib, and anacetrapib stimulate Nox-derived ROS production, which may induce phosphorylation of MLC leading to vascular contraction. Exactly how CETP inhibitors regulate ROS-generating enzymes remains 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 ROS production, CETP inhibitors affected the activation of STAT3, a transcription factor involved in VSMC activation. We found that the MLC phosphorylation induced by CETP inhibitors was reduced by STAT3 inhibition. STAT3 inhibition was also associated with attenuated contractile responses to torcetrapib, dalcetrapib, and anacetrapib. Similar findings were shown by Johnson et al. (2013) in angiotensin II-induced hypertension. Whether the effects mediated by STAT3 involve gene transcription are unclear, but the fact that responses were rapid suggests that the effects are likely nongenomic. Activation of STAT3 by CETP inhibitors appears to be independent of redox-sensitive processes because NAC and GKT137831 did not influence STAT3 activation. Others have also shown that STAT3 may be independent of ROS (Johnson et al., 2013). The effects of CETP inhibitors on VSMC signaling pathways are highly regulated and are not generalized phenomena because activation of ERK1/2, p38MAPK, and c-Src were unaffected by these.

Fig. 6. Effects of torcetrapib, dalcetrapib, and anacetrapib on phosphorylated MLC are affected by inhibitors of ROS and STAT3. Cells were pretreated with NAC, a ROS scavenger (10 μM), GKT137831, a Nox1/4 inhibitor (10 μM), or S3I-201, a STAT3 inhibitor (50 μM), for 30 minutes, followed by stimulation with torcetrapib, dalcetrapib, or anacetrapib (1 μM, 5 minutes). Phosphorylated MLC and STAT3 were evaluated by Western blot analysis and normalized to the total forms of MLC or STAT3. Results are presented as fold change of the vehicle control group. Graphs show mean ± S.E.M., and the autoradiographs show one representative experiment. (n = 6). *P < 0.05 versus control vehicle group. #P < 0.05 versus NAC, GKT137831, or S3I-201 pretreated group.
agents. Moreover, VSMC growth was not influenced by CETP inhibitors, as evidenced by no effect on proliferating cell nuclear antigen expression.

In conclusion, our findings indicate that torcetrapib, dalcetrapib, and anacetrapib have CETP-independent effects that directly influence vascular function. In particular, we show that these drugs promote vasoconstriction through redox-sensitive and STAT3-dependent pathways. Although most vascular effects were similar for the three different agents, only torcetrapib impaired endothelial function. These differential effects may relate to chemical structural differences of the drugs. Overall, our findings suggest that off-target vascular effects may explain, at least in part, some of the adverse cardiovascular events, such as hypertension, observed in CETP inhibitor clinical trials.

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Authorship Contributions

\textbf{Participated in research design}: Rios, Montezano, Touyz.

\textbf{Conducted experiments}: Rios, Montezano, Lopes, Camargo, Neves.

\textbf{Contributed new reagents or analytic tools}: Touyz.

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\textbf{Wrote or contributed to the writing of the manuscript}: Rios, Montezano, Touyz.

References


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