Nicotinic acid and DP1 blockade: Studies in mouse models of atherosclerosis

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Running Title: DP1 antagonism in ApoE⁻/⁻ and Ldlr⁻/⁻ mice

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Abstract

The use of nicotinic acid to treat dyslipidemia is limited by induction of a "flushing" response, mediated in part by the interaction of prostaglandin D₂ (PGD₂) with its G-protein coupled receptor DP1 (Ptgdr). The impact of DP1 blockade (genetic or pharmacologic) was assessed in experimental murine models of atherosclerosis. In Ptgdr⁻/⁻ ApoE⁻/⁻ mice vs. ApoE⁻/⁻ mice, both fed a high-fat diet, aortic cholesterol content was modestly higher (1.3- to 1.5-fold, \( P<0.05 \)) in Ptgdr⁻/⁻ ApoE⁻/⁻ mice at 16 and 24 weeks of age, but not at 32 weeks. In multiple ApoE⁻/⁻ mouse studies, a DP1-specific antagonist L-655 generally had a neutral to beneficial effect on aortic lipids in the presence or absence of nicotinic acid treatment. In a separate study, a modest increase in some atherosclerotic measures was observed with L-655 treatment in Ldlr⁻/⁻ mice fed a high-fat diet for 8 weeks; however, this effect was not sustained for 16 or 24 weeks. In the same study, treatment with nicotinic acid alone generally decreased plasma and/or aortic lipids, and addition of L-655 did not negate those beneficial effects. These studies demonstrate that inhibition of DP1, with or without nicotinic acid treatment, does not lead to consistent or sustained effects on plaque burden in mouse atherosclerotic models.

Keywords: nicotinic acid, dyslipidemia, DP1 antagonist, atherosclerosis model
Introduction

Nicotinic acid produces beneficial effects on low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglycerides (TG) and decreases cardiovascular events in high-risk patients (1, 2). Despite demonstrated efficacy in cardiovascular disease, nicotinic acid is poorly tolerated and underused in clinical practice, largely due to the adverse effect of flushing—cutaneous vasodilation and attendant discomfort in the face, neck, trunk, and arms (2). Nicotinic acid-induced flushing is thought to be mediated, at least in part, by the release of prostaglandin D$_2$ (PGD$_2$) in the skin (3, 4), leading to vasodilation of blood vessels and consequent symptoms of redness, warmth, tingling, and itching. Studies in mice have shown that the relevant skin cell type is the epidermal Langerhans' cell (5-7). Consistent with a role for PGD$_2$ in flushing, the administration of laropiprant, a high-affinity antagonist of the PGD$_2$ receptor DP1, has been shown to significantly inhibit nicotinic acid-induced vasodilation in mice (8) and humans (9). While a recent study has suggested that nicotinic acid induces PGE$_2$ formation in isolated keratinocytes (10), inhibition of PGE$_2$ and its effects on flushing have not been demonstrated. Conversely, there is clear demonstration that nicotinic acid-induced vasodilation is suppressed in mice that have been genetically engineered to lack DP1 (5, 8).

DP1 antagonism represents a strategy for improving the tolerability of nicotinic acid, and laropiprant is currently used in the clinic in combination with nicotinic acid to treat dyslipidemia (8). A necessary condition of this strategy is that pharmacological blockade of DP1 should not in itself cause significant adverse effects. However, there are no published reports of preclinical models that have measured potential effects of DP1 receptor antagonists on atherosclerosis endpoints, including plaque burden, in the presence or absence of nicotinic acid. The effects of nicotinic acid alone on plasma lipids have been explored in several animal models, including the
mouse, rat, guinea pig, rabbit, dog, mini pig, and Rhesus monkey. However, for those species in which HDL-C metabolism has been examined, nicotinic acid generally lacks the effects seen in humans, with the possible exception of the mouse model, in which the human cholesteryl ester transfer protein (CETP) transgene is expressed (11). Recent studies in both rabbits and mini pigs suggest that nicotinic acid has benefits in atherosclerosis over and above its effects on serum lipids (12).

This report describes genetic and pharmacological approaches to antagonize DP1 function and measure the impact on mouse models of atherosclerosis. In addition, we utilize well-established ApoE<sup>-/-</sup> and Ldlr<sup>-/-</sup> mouse models of atherosclerosis to study the effects of nicotinic acid treatment, with and without the presence of a specific DP1 antagonist. The DP1 antagonist L-655 (Figure S1) was chosen due to its selectivity for murine DP1 over murine thromboxane A<sub>2</sub> (TxA<sub>2</sub>) receptor (TP). TP has an often-used pro-atherogenic role in mouse models (13-16); therefore, laropiprant was not chosen because it displays significant affinity for the murine TP. We assessed a battery of atherosclerotic endpoints in these mouse studies, including aortic cholesterol levels, en face staining of the aorta, and cross-sectional histological analyses that examine plaque composition.

**Materials and Methods**

**In vitro characterization of L-655**

The affinity of L-655 for DP1, DP2, and TxA<sub>2</sub> receptors was determined using membrane-based radioligand binding assays employing recombinant murine receptors (17). The antagonist potency of L-655 was determined in a functional assay employing cells expressing recombinant mouse DP1 (whole-cell cyclic adenosine monophosphate (cAMP)-based assay) (18).
Antagonist activity at platelet TP was monitored in human platelet assays that measure the inhibition of U46619 (TxA$_2$ mimetic)-induced platelet aggregation in platelet rich plasma (19).

**Animals**

_ApoE$^{-/-}$_ mice (B6.129P2-<i>ApoE</i>$^{tm1Unc}$ /J) used in these studies were purchased from The Jackson Laboratory (Bar Harbor, Maine) or bred in house using the descendants of a breeding pair originally purchased from The Jackson Laboratory. _Ptgdr$^{-/-}$ApoE$^{-/-}$_ double-deficient mice were bred in house from the above mentioned stock of _ApoE$^{-/-}$_ mice and _Ptgdr$^{tm1Dgen}$_ mice (deficient in the prostaglandin 2 DP1 receptor) obtained from Deltagen, San Carlos, CA (8). _Ldlr$^{-/-}$_ mice used in these studies were bred from strain B6.129-<i>Ldlr</i>$^{tm1Her}$ /J, stock no. 002207, from the Jackson Laboratory. Both the _ApoE_ and the _Ldlr_ knockout mice were backcrossed into C57BL/6 at least ten generations before being used in the study.

Both strains of mice, particularly on a Western diet, were prone to develop skin lesions, which were exacerbated by fighting among cage mates; however, skin lesion development may also be facility-dependent. We excluded animals with large or unhealed lesions (~5% of ApoE knockouts and ~15% of Ldlr knockouts) because the presence of these skin lesions is associated with decreased plaque volume (K Cheng, unpublished data), presumably because of the immune responses generated. Mice with small skin lesions that had healed may have been included; however, an attempt was made to closely monitor the mice across the duration of the experiments to minimize this potential variable.

All animals were kept in a controlled environment, with a 12-hour light/dark cycle, with constant temperature and humidity, and had access to food and water _ad libitum_. The studies were conducted in conformity with the Public Health Service (PHS) Policy on Humane Care and
Use of Laboratory Animals. All procedures described here were approved by the Merck Research Laboratories (Rahway) Institutional Animal Care and Use Committee.

**Determination of L-655 dose**

Vasoconstriction/vasodilatation studies were designed to determine the dose of L-655 that would effectively block the DP1 receptor, without having measureable off target effects at the TP receptor. Male and female *ApoE<sup>-/-</sup>* and *Ldlr<sup>-/-</sup>* mice were tested separately (*n*=5-6 per gender per group). *Ldlr<sup>-/-</sup>* mice were weaned at 4 weeks of age, and at 8 weeks of age were placed on the high-fat Western diet (HFWD) TD88137 (21% fat and 0.15% cholesterol; Harlan Teklad, Madison, Wisconsin) for at least 4 weeks prior to the beginning of the studies. *ApoE<sup>-/-</sup>* mice were weaned at 4 weeks of age and placed on HFWD immediately after weaning and for at least 12 weeks prior to the beginning of the studies. These feeding conditions mimicked those selected for the long-term atherosclerosis studies (see below), with the limitation that mice had to be at least 12 weeks old to perform vasoconstriction/vasodilatation studies.

U46619, a full TP agonist (Cayman Chemical, Ann Arbor, Michigan), was supplied by the manufacturer as a 10 mg/ml stock in methyl acetate. The compound was dried under nitrogen and resuspended in 100% dimethylsulfoxide (DMSO) to 8 mg/ml, and then diluted with water to 0.2 mg/ml to provide a final dose of 2 mg/kg. Solid SQ-29,548, a TP antagonist (Cayman Chemical) was resuspended in 0.1M sodium carbonate to a concentration of 2 mg/ml and then diluted in physiologic balanced saline (PBS) to 0.3 mg/ml to provide a final dose of 3 mg/kg. PGD<sub>2</sub> (Sigma-Aldrich, St Louis, Missouri) was prepared as a stock solution at 40 mg/ml in 100% ethanol, and diluted to 0.2 mg/ml in saline to provide a final dose of 2 mg/kg.
One week prior to performing the vasodilatation/vasoconstriction studies, mice were switched to milled HFWD, which contained an admixture of L-655 at 0 (chow-alone control), 0.003%, 0.01% or 0.03% (w/w). Mice had access to the diets ad libitum.

The first series of studies was performed to test for the lack of TP antagonism in mice fed L-655 for 1 week. To demonstrate that these strains of mice were indeed responsive to TP antagonism, mice that had not been exposed to L-655 were used to monitor the effects of the TP antagonist SQ-29,548 on U46619-induced vasoconstriction. Studies were carried out in the morning, immediately following the end of the feeding cycle (starting at 8:00 AM and not extending past 11:00 AM), when plasma levels of L-655 were expected to be the highest.

To study the effects of L-655 on vasoconstriction, mice were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneal injection) 10 minutes prior to the start of the vasoconstriction studies. Cutaneous blood flow in the mouse ear was measured by laser Doppler perfusion imaging, employing a PeriScan PIMII laser Doppler Perfusion Imager system (Perimed, Inc., North Royalton, Ohio). The settings of the system were: repeated mode, image size 12 × 12, start method auto interval, 40 images, images every 30 seconds, medium resolution, intensity 7-9V, range 0-3V. All measurements were taken from the ventral side of the right ear. Mice were placed in left lateral recumbency, and the right ear was taped to a flat surface, exposing the ventral side for measurements. After a 3-minute baseline reading, U46619 (2 mg/kg) or vehicle (5% DMSO in water) was administered by subcutaneous injection, and cutaneous blood perfusion in the ear was measured for another 17 minutes. Perfusion values for the baseline in each animal were averaged and subsequent values after injection of vehicle or U46619 were expressed as the percentage change over this initial value. Negative percentage changes were indicative of reduced blood perfusion (i.e., vasoconstriction). All procedures were
performed under dim lights. In the control group of animals not exposed to L-655, SQ-29,548 (3 mg/kg) was administered by intraperitoneal injection after a 3-minute baseline reading. Five minutes later, animals were challenged with a subcutaneous dose of U46619 at 2 mg/kg. Vasoconstriction was then monitored for another 17 minutes.

The second series of studies was designed to determine the minimal dose of L-655 that would inhibit PGD2-induced vasodilatation in male and female ApoE<sup>−/−</sup> or Ldlr<sup>−/−</sup> mice. Mice were fed the HFWD as described above. One week prior to performing the vasodilatation studies, mice were switched to milled HFWD, which contained an admixture of L-655 at 0 (chow-alone control), 0.003%, 0.1%, or 0.03% (w/w). Mice had access to the diets ad libitum. After feeding on medicated diets for 1 week, the vasodilatatory response to 2 mg/kg PGD2 was tested in the mice using the same Laser Doppler perfusion technique described above, with the following modifications: on the day of study, mice were placed in clean cages in the morning (between 7:30-8:00 AM), and switched to HFWD lacking L-655 to prevent any further exposure to the compound-containing diet. Vasodilatation was studied in the afternoon (after 3:00 PM), after the medicated diet had been removed for at least 7 hours. Studying vasodilatation in the afternoon, many hours after the last possible dose of compound could be ingested, provided the most stringent test of in vivo DP1 antagonism since this time point likely reflected the minimal plasma concentration of L-655 that would occur when mice were not actively feeding. After a 3-minute baseline reading of cutaneous blood perfusion, PGD<sub>2</sub> (2 mg/kg) or vehicle (0.5% ethanol in saline) were administered by intraperitoneal injection and measurement of cutaneous perfusion continued for another 17 minutes. Perfusion values for the baseline in each animal were averaged and subsequent values after injection of vehicle or PGD<sub>2</sub> were expressed as percent change over
this initial value. Positive percentage changes were indicative of increased blood perfusion (i.e. vasodilatation).

Treatment groups

Genetic studies

To study the effect of DP1 deficiency on the development of atherosclerosis, parallel colonies of male ApoE−/− and Ptgdr−/−ApoE−/− double-deficient mice were weaned at 4 weeks of age and then fed pelleted HFWD. Groups of animals were terminated at 16, 24, and 32 weeks of age, and aortic cholesterol content and plasma cholesterol and TG were analyzed. Groups of 8 to 19 animals were analyzed per genotype and time point.

A second study comparing male ApoE−/− and Ptgdr−/−ApoE−/− double-deficient mice out to 40 weeks of age was also conducted (n=15–19, per genotype and time point). Only plasma cholesterol and TG were available for these animals.

Pharmacological studies

For the series of studies assessing whether pharmacological antagonism of DP1 had any effect on the anti-atherogenic properties of nicotinic acid in ApoE−/− mice, male and female ApoE−/− mice were weaned at 4 weeks of age, and immediately fed pelleted HFWD. At 15 weeks of age, mice were switched to milled HFWD for 1 week. At 16 weeks of age, animals were assigned to different treatment groups, depending on study design. All studies consisted of a baseline group (terminated at 16 weeks of age); a vehicle group, which received HFWD for 8 weeks; a nicotinic acid group, which received HFWD supplemented with 3% nicotinic acid for 8 weeks; and one or more groups receiving either L-655 alone (0.003%, 0.01%, or 0.3% in
HFWD) or a combination of L-655 plus 3% nicotinic acid. All groups received the admixed diets for 8 weeks. Most studies used male mice; one included both males and females. Ten to 36 animals were analyzed per gender and treatment group.

To study whether pharmacological antagonism of DP1 had any effect on the development of atherosclerosis or on the anti-atherogenic properties of nicotinic acid in Ldlr−/− mice, a large 3-part study was carried out (Figure 1A). In all parts, male and female Ldlr−/− mice were kept on regular mouse chow until 8 weeks of age; n=41–56 animals per gender per group were analyzed. The same dietary admixture of drugs was used as described for the ApoE−/− mice, except that all treatments with L-655 were at 0.01%. The three parts were performed concurrently so that the vehicle groups of the first part could be used for all three parts.

In Part 1, the time course of L-655 effects was examined. A baseline group, terminated at 8 weeks of age, was used to establish baseline values; vehicle and L-655 groups were treated for 8, 16, and 24 weeks, starting at 8 weeks of age. Lesion development as a function of time (histology) in the vehicle treated group is shown in Figure 1B.

Parts 2 and 3 were focused on assessment of the effects of nicotinic acid treatment ± L-655 on animals on a HFWD for 16 (early therapeutic) and 24 weeks (late therapeutic), respectively. In Part 2, vehicle, L-655, nicotinic acid, and an L-655/nicotinic acid combination were administered to respective groups for 8 weeks, starting at 16 weeks of age and ending at 24 weeks. In Part 3, the same four treatment groups were also treated for 8 weeks, but this time beginning at age 24 weeks and ending at 32 weeks. Of the animals in each group, 7 to 12 were analyzed for aortic en face and aortic root histology; the remaining animals were analyzed for aortic lipids. Plasma from all animals was saved for measurement of cholesterol and TG.
**Aortic and Plasma Lipid Analysis**

After the *in vivo* treatment phase, mice were euthanized with carbon dioxide, and blood was immediately collected from the vena cava into syringes rinsed with 0.5 M EDTA. Plasma was prepared via centrifugation at 850 × g for 15 minutes at 4°C and stored at -80°C until analysis. Levels of total cholesterol (TC) and TG were determined on an automated Roche P Module Clinical Chemistry Analyzer (Roche Diagnostics, Indianapolis, Indiana).

Aortic lipid content was determined as described in a published report (20). Briefly, after mice were euthanized and blood collected, the vasculature was perfused through the left ventricle with cold PBS supplemented with 5 mM EDTA. A section of aorta from the aortic root to the right renal artery was used for biochemical determination of cholesterol after being cleaned of adventitious fat. Total and free cholesterol (FC) were determined by an enzymatic fluorometric assay, and cholesterol ester (CE) was calculated as the difference between TC and FC.

**Aorta En Face Analysis**

En face analysis was performed according to previously published procedures (21). Briefly, after euthanasia and blood collection, mice were perfused with PBS through the left ventricle for 3 to 5 minutes, and aortas (from the point where the aorta exits the top of the heart to the iliac bifurcation) were collected, cleaned of adventitious fat, opened longitudinally, and placed in 10% formalin for up to 10 days until the time of Sudan IV staining. To prepare for the Sudan IV staining, the aortas were rinsed in PBS for 5 minutes and in 1 ml of 70% ethanol for 3 minutes, followed by gentle mixing in 70% ethanol two more times. The aortas were then immersed in 1 ml of 0.5% Sudan IV in a solution of ethanol:acetone:water (35:50:15) for 8 minutes, followed by rocking. The aortas were destained in 1 ml of 80% ethanol for 3 minutes.
twice and washed with PBS twice. The Sudan IV-stained aorta image was captured with a digital camera and analyzed with Image-Pro Plus software (Media Cybernetics, Inc., Bethesda, Maryland) to quantify Sudan IV stained surface area. Imaging and analysis were performed by a blinded operator.

**Aortic Root Histomorphometric Analysis**

After removal of the aortas as described above, the intact heart was removed and transected through a plane perpendicular to the aorta. The section containing the aortic root was placed in a standard cryomold, covered with OCT cryo-embedding media and immediately frozen. The whole specimen was cut in 8-µm sections, and every 4th section was processed for hematoxylin and eosin staining. The area (in µm²) of atherosclerotic lesions was then measured on digital images of the slides using computer based image analysis. Concurrent with image acquisition, the position of the distal end of the aortic valves was determined by a human operator on the basis of direct observation. This point was arbitrarily defined as zero microns. Lesion volume in the 300 µm of aorta distal to the zero point was calculated as the summation of the lesion area times the section thickness for all the sections in 300 µm.

**Statistical Methods**

Comparisons between treatment groups were made using two-sample \( t \)-tests, using the Welch-Satterthwaite method to avoid assuming equal variances. A two-sided \( P \)-value of less than 0.05 was required to call a difference statistically significant. No multiplicity adjustments were made to \( P \)-values.
Results

Genetic Knockdown of DP1 in Apoe<sup>−/−</sup> mice

The time course of aortic and plasma lipids in male ApoE<sup>−/−</sup> and Ptgdr<sup>−/−</sup>ApoE<sup>−/−</sup> double-deficient mice fed a HFWD from 4 weeks of age until 32 weeks of age was examined. Aortic TC (Figure 2) and CE (data not shown) were significantly higher in the Ptgdr<sup>−/−</sup>ApoE<sup>−/−</sup> mice at 16 and 24 weeks, and aortic FC (data not shown) was significantly higher at 24 weeks. However there were no significant differences in any of those atherosclerosis measures at 32 weeks. Plasma TC was significantly higher in the double-deficient Ptgdr<sup>−/−</sup>ApoE<sup>−/−</sup> mice at 24 and 32 weeks of age, and plasma TG were significantly higher at all three ages (Figure S2).

A second similar study (Figure S3) found significantly higher plasma cholesterol at 16 and 32 weeks of age, although not at 24 or 40 weeks. In contrast to the increase in TGs observed in Ptgdr<sup>−/−</sup>ApoE<sup>−/−</sup> mice in the first study described above (Figure S2), plasma TGs were not significantly different in these double-deficient mice at 16, 24, and 32 weeks of age in the second study (Figure S3).

Characterization of L-655, a selective DP1 antagonist

The affinity of L-655 for murine DP2, murine and human DP1, and TP indicated that L-655 binds more tightly to DP1 than to TP and murine DP2 (Table 1). L-655 also inhibited PGD<sub>2</sub>-induced cAMP formation in human platelet-rich plasma (PRP) with an IC<sub>50</sub> value of 2.51 ± 0.76 nM (n=13). In comparison, in human PRP, L-655 inhibited U46619 (1.36 µM)-induced platelet aggregation in a dose-dependent manner, with a mean IC<sub>50</sub> ± SD of 19.4 ± 8.6 µM, indicating a high degree of selectivity for DP1 over TP (19). In addition, U46619 demonstrated >400× selectivity of binding over EP1, EP2, EP3, EP4, FP, and IP receptors (19).
Similarly, L-655 exhibited a high degree of selectivity in vivo between functional DP1 and TP antagonism in ApoE<sup>−/−</sup> mice (Figure 3). At doses of L-655 ≥100 ppm, trough afternoon plasma levels completely blocked PGD<sub>2</sub>-induced vasodilation, whereas morning peak levels had no off-target effects in blocking induction of cutaneous vasoconstriction by the TP agonist U44619 (Figures 3 and 4). In comparison, a 2 mg/kg dose of the TP antagonist SQ-29548 completely blocked the vasoconstriction in this model. The finding that vasodilation was blocked ~7 hours after drug-containing chow had been removed suggests that DP1 antagonism occurs throughout the day using this route of administration. Conversely, no evidence for TP antagonism was observed at any dose of L-655 tested (Figures 3 and 4), including a dose of 1000 ppm that resulted in high plasma levels of this compound (5.06 ± 1.14 µM, data not shown).

Given that L-655 had high selectivity between functional DP1 and TP antagonism in ApoE<sup>−/−</sup> mice, doses of 30, 100, and 300 ppm admixed in high-fat chow were chosen for subsequent atherosclerosis studies.

PGD<sub>2</sub> at 2 mg/kg induced a robust vasodilatory response in both male Ldlr<sup>−/−</sup> mice (Figure 4) and female Ldlr<sup>−/−</sup> mice (data not shown). In both genders, pre-treatment with L-655 for 1 week resulted in a dose-dependent inhibition of PGD<sub>2</sub>-induced vasodilation. L-655 at 30 ppm only partially inhibited vasodilation, with one animal out of four in both genders responding to PGD<sub>2</sub>. Doses of 100 and 300 ppm completely abrogated the vasodilatory response to PGD<sub>2</sub> in both male and female Ldlr<sup>−/−</sup> mice.

Doses of L-655 that partially (30 ppm) or totally (100 and 300 ppm) blocked PGD<sub>2</sub>-induced vasodilation failed to block U46619-induced vasoconstriction in male Ldlr<sup>−/−</sup> mice (Figure 4) and female Ldlr<sup>−/−</sup> mice (data not shown). As a control, the use of SQ-29,548 indicated that the vasoconstriction induced by U46619 could be prevented by TP antagonism.
These results indicate that, in \( Ldlr^{-/-} \) mice, L-655 at doses \( \geq 100 \) ppm can completely block PGD\(_2\)-induced vasodilation (Figure 4), without measureable off target effects on TP. Thus, the dose selected for our atherosclerosis studies in \( Ldlr^{-/-} \) mice (male and female) was 100 ppm.

**Effects of L-655 ± nicotinic acid in ApoE\(^{-/-} \) mice**

In three independent studies, 4-wk-old \( ApoE^{-/-} \) mice were fed a HFWD for 16 weeks followed by 8 weeks of treatment with L-655 alone or in combination with nicotinic acid. Two additional studies included a dose of 1000 ppm L-655, which was considered too high because of potential non-specific effects and are therefore not reported here. However, results at 30, 100, and 300 ppm of L-655 indicated that plasma levels at these doses were sufficient to inhibit vasodilation but not high enough to affect vasoconstriction (Figure 3; Table S1); therefore, results from these studies are presented here and summarized in Table 2. Note that the presence of nicotinic acid did not alter the plasma levels of L-655 (Table S1), which were comparable to levels obtained in the dose finding study (Figure 3).

Two studies used only male mice, while the third included both males and females (Table 2; Table S2). Nicotinic acid alone reduced aortic CE and total aortic cholesterol (Figure 5) compared with vehicle; the reduction was statistically significant in two of three studies (Table 2). L-655 alone produced statistically significant reductions in aortic CE (Table 2), TC and FC (Table 2; Table S2) in female mice at a dose of 100 ppm. An analogous beneficial effect by L-655 was not induced in male mice suggesting a gender specific effect. In two studies, CE was similar or significantly less in the nicotinic acid/L-655 combination group compared with the nicotinic acid-only group (Table 2). In summary, L-655 produces a neutral to beneficial
effect on aortic CE and does not attenuate the anti-athero effects of nicotinic acid (Table 2). Similar patterns were observed for aortic TC and FC (Table S2).

In the one study that included histologic assessments of aortic lesions (Table S2), there were no significant differences between treatment groups for male mice, but female mice receiving L-655 plus nicotinic acid had significantly smaller lesions than the vehicle-only group (-20%, \(P = 0.045\)) or the niacin only group (-22%, \(P = 0.021\)).

In the same study (Study 3), L-655, with or without nicotinic acid, caused statistically significant increases of 17% to 25% in plasma TG levels in female mice but not in males. Conversely, in male mice there was a significant 16% increase in plasma cholesterol when L-655 was combined with nicotinic acid, compared with nicotinic acid alone, but it was not significant in females. In females there was also a significant decrease in plasma cholesterol with L-655 alone (Table S2).

**Effects of L-655 ± nicotinic acid in Ldlr\(^{-/-}\) mice**

Based on the results from the dose finding study shown in Figure 4, a dose of 100 ppm of L-655 was chosen for the large study carried out in Ldlr\(^{-/-}\) mice. The plasma levels of L-655 were sufficient to see inhibition of vasodilation without affecting vasoconstriction (Figure 4; Table S3). In Ldlr\(^{-/-}\) mice, there were sporadic but no consistent or sustained increases in atherosclerotic measures across the 24-week period tested in both males and females (Figures 6 & 7; Table S4). In female mice, L-655 treatment resulted in 1.2- to 1.3-fold increases compared to vehicle in aortic lipids, and plasma cholesterol was significantly higher, by 7%. In males, aortic lesions were significantly higher with L-655 than with vehicle, by 1.5- to 5.1-fold. However, after 16 and 24 weeks of treatment, there were no significant differences between
vehicle and L-655 in any of the measures for either males or females, except for a decrease in plasma TG for females after 16 weeks (Figure 7; Table S4).

In Part 2 of the Ldlr−/− mouse study, examining 8 weeks of drug treatment that occurred between 16 and 24 weeks of age, similar decreases of plasma TC and TGs were observed with nicotinic acid alone and with the nicotinic acid/L-655 combination in both males and females when compared with vehicle (Figure 8A; Table S5). Aortic CE, FC, and TC significantly decreased both with nicotinic acid treatment alone and with the nicotinic acid/L-655 combination in males, but only with the nicotinic acid/L-655 combination in females (Figure 8B; Table S5). Statistically significant reductions in % aortic lesion area, as measured by en face, were observed with treatment by nicotinic acid alone or in combination with L-655 in female but not male mice (Figure 8C, Figure 9, Table S5). Moreover, the nicotinic acid/L-655 combination, although not nicotinic acid alone, significantly decreased lesion area of the aortic root, as measured by histology, in females only (Table S5).

In part 3 of the Ldlr−/− mouse study, examining 8 weeks of drug treatment that occurred between 24 and 32 weeks of age (Table S6), there were statistically significant reductions in plasma TC in both males and females with nicotinic acid alone and with the nicotinic acid/L-655 combination. Overall, very similar trends were observed with plasma TGs with statistically significant lower TGs measured with nicotinic acid alone in both males and females and with the nicotinic acid/L-655 combination in males. Consistent with the reductions in plasma TGs and TGs, percent aortic lesion area was significantly reduced by L-655 and the nicotinic acid/L-655 combination in males. Furthermore, aortic TC and FC were significantly reduced by nicotinic acid. There were some sporadic and gender-specific increases in atherosclerotic measures;
specifically, L-655 alone significantly increased aortic CE in male mice only, and plasma TC in females only (Table S6)

**Discussion**

In the present study, genetic ablation of the DP1 receptor in *ApoE*−/− mice fed a high-fat diet resulted in a modest increase in aortic cholesterol at early time-points (16 and 24 weeks of age), but not at 32 weeks. These early but modest increases in aortic lipids are consistent with results obtained by another group in double-deficient *Ptgdr*−/− *Ldlr*−/− mice (22). In comparison, pharmacological inhibition with the DP1 antagonist L-655 did not increase aortic lipids in multiple *ApoE*−/− mouse studies. In *Ldlr*−/− mice, L-655 produced a modest increase in aortic lipids and aortic root histology at one early time point that was not sustained at later time points. For plasma lipids, both TG and cholesterol levels in *Ptgdr*−/− *ApoE*−/− mice were slightly elevated; however, pharmacological inhibition of DP1 in *ApoE*−/− mice had no consistent effect on plasma TG or cholesterol levels, suggesting a potential developmentally linked effect of DP1 inhibition on plasma lipids. When combined with nicotinic acid, L-655 did not negate the effect of nicotinic acid on aortic lipids in either *ApoE*−/− or *Ldlr*−/− mice. The combination did have significantly higher plasma cholesterol in male *ApoE*−/− mice and higher plasma TG in female *ApoE*−/− mice.

Mouse models have been used to study the effects of lipid-lowering and anti-atherosclerotic agents, and provide practical tools for examining the biology of several targets and the pharmacological effects of drugs. As with all animal studies, there are inherent limitations with respect to extrapolating the findings to human disease and important distinctions between the *ApoE*−/− and *Ldlr*−/− mice that are noteworthy. For example, high-fat feeding of *ApoE*−/−...
mice results in accumulation of lipids mostly in VLDL particles, unlike in humans, whereas high-fat feeding of Ldlr\(^{+/−}\) mice results in accumulation of cholesterol in LDL particles, similar to humans (23). The ApoE\(^{+/−}\) mouse has elevated circulating cholesterol and develops plaque even when fed a chow diet, whereas the Ldlr\(^{+/−}\) mouse model develops substantive dyslipidemia and plaque only after exposure to a Western style, high-fat, cholesterol-containing diet. In the context of studying plaque, Ldlr\(^{+/−}\) and ApoE\(^{+/−}\) models tend to accumulate plaque in the aorta, unlike humans who localize plaque predominantly to the coronary arteries (24). Early stages of plaque formation are similar between these mouse models and humans; however, the size, composition and histology of the plaques are quite dissimilar. Importantly, unlike humans with advanced coronary plaque and elevated plasma lipids, ApoE\(^{−/−}\) and Ldlr\(^{−/−}\) mice do not generate end-stage ischemic lesions or coronary heart disease (23, 24). Moreover, for a large number of drugs tested for cholesterol lowering and other indications, ApoE\(^{−/−}\), Ldlr\(^{−/−}\) and other mouse models do not provide consistent responses for several endpoints, relative to one another and to humans (23, 25, 26). Given these differences, caution must be exercised in the interpretation of data from murine models of disease. Nevertheless, these studies provide important mechanistic insight into atherosclerosis.

Whereas the effects of nicotinic acid on circulating lipids and lipoprotein particles have been well studied both clinically and in multiple preclinical species (1, 11, 12, 27), few studies in preclinical species address the potential effect of nicotinic acid on plaque. In an 8-week study of male rabbits on a cholesterol-enriched (2% w/w) diet, the addition of nicotinic acid (0.4% w/w) reduced serum, liver, and aortic cholesterol (by 60%, 77%, and 64%, respectively) compared to a cholesterol diet alone (28). Nicotinic acid has also been studied in high-fat fed ApoE\(^{−/−}\) mice (29, 30). In that study, nicotinic acid admixed in chow (0.5% w/w) had no effect on plasma TG or
HDL-C, nor did it affect atherosclerosis as measured by histologic and morphometric analysis of aortic lesions. Given that relatively high doses of nicotinic acid were required to alter plasma lipids in other studies (e.g., 1% in the study employing CETP transgenic mice above (31) and 3% in this work), it is very likely that a nicotinic acid dose of 0.5% w/w is not sufficient to impact plasma or aortic lipids.

In our studies in ApoE\(^{-/-}\) and Ldlr\(^{-/-}\) mice, treatment with nicotinic acid (3% w/w), in the presence or absence of L-655, generally significantly lowered both plasma and aortic lipid levels by approximately 20% to 30%. Also notable was that both strains of mice, particularly on a Western diet, were prone to developing skin lesions, which were exacerbated by fighting of cage mates. We eliminated animals with large or unhealed lesions because we have found that the presence of these skin lesions is associated with decreased plaque volume (K Cheng, unpublished data), presumably because of the immune responses generated. Mice with small skin lesions that had healed over may have been included; however, an attempt was made to closely monitor the mice across the duration of the experiments to minimize this possible variable. However, in both models this dose of nicotinic acid tended to have smaller and less statistically significant effects on aortic lesions as compared with aortic lipids. A caveat to this comparison is the use of measurement techniques; specifically, a greater number of samples were used when studying aortic lipids by the biochemical method as compared with aortic lesions, which were measured by \textit{en face} staining or histology. Historically, \textit{en face} analysis has been most commonly used in the field to visually assess aortic lesions. Although \textit{en face} provides detailed spatial information about the lesion location, it cannot delineate the three dimensional structure of the lesion as provided by histological assessment. Variability in these studies may arise from multiple sources, including the relatively short half-life of nicotinic acid which is administered in
the feed. While niacin effects were comparable across males and females, there may be a slight gender effect with L-655 treatment alone. However, while statistical significance was met in one gender (females) and not the other (males), in general, the directionality of changes was similar for both genders. There were some anomalous gender-specific effects of L-655 on TGs and plasma cholesterol levels in female and male ApoE\(^{-/-}\) mice; however, similar effects were not seen in Ldlr\(^{-/-}\) mice. Given that the mice were prone to developing skin lesions, as discussed in the methods section, despite our efforts to eliminate animals with lesions, there could have been immune responses from mice with transient lesions which could have subsequently led to sporadic effects on decreased plaque volume.

There has been no reported direct link between DP1 biology and cardiovascular risk; nevertheless, in vitro data have indirectly suggested that DP1 antagonism in platelets could promote platelet aggregation and be pro-atherogenic (22). Specifically, in vitro studies have shown that PGD\(_2\) inhibits platelet aggregation, and platelet activation results in the release of PGD\(_2\). It has been speculated that antagonism of DP1 could block the ability of endogenous PGD\(_2\) to inhibit further platelet aggregation and thereby may indirectly enhance the reactivity of platelets. However, several clinical studies have indicated that treatment with the DP1 antagonist laropiprant has no platelet aggregatory effect; in fact, a transient and mild increase in bleeding time has been reported (32-34). One caveat in interpreting this observation is that laropiprant has been shown to antagonize both DP1 and TP in humans (32-34), unlike the DP1-specific antagonist L-655 used in this mouse work. Therefore, it is likely that the mild anti-platelet effects in humans are a result of TP antagonism and theoretically represent a net effect of antagonizing both DP1 and TP on platelets. As such, the DP1- and TP-specific effects on platelets cannot be teased out from cited clinical studies of laropiprant.
In this work, *in vivo* dose selection of the DP1 antagonist L-655 (that was 20-fold more selective for DP1 over TP binding *in vitro*) was made such that trough afternoon plasma levels were sufficient to completely block PGD₂-induced vasodilation (a measure of DP1-specific activity), while morning peak levels had no off target effects in blocking TP-induced vasoconstriction. Assuming that vasodilation and vasoconstriction were reasonable measures of all DP1-specific and TP-specific activity, the DP1 antagonist had no consistent or sustained effects on multiple measures of atherosclerosis. In agreement with findings of others (22), we have observed that mouse platelets do not respond to PGD₂ (as measured by cAMP production) but do respond to forskolin and iloprost treatment, suggesting very low or undetectable levels of murine DP1 expression in mouse platelets. Therefore, the overall lack of effects of L-655 on atherosclerotic measures in mice reported in this work does not necessarily apply to DP1 antagonism of human platelets and the potential effect on atherosclerosis in the clinic. However, clinical treatment with large amounts of PGD₂ that engage the DP1 receptor did not reduce bleeding times, supporting the hypothesis that engagement of the DP1 receptor does not promote pro-aggregatory platelet effects in humans (35-37).

In summary, the present work presents a comprehensive analysis of the effects of DP1 blockade (genetic and pharmacologic) on multiple endpoints of atherosclerosis in mice. Despite the limitations of using mouse models, the data indicate a lack of consistent or sustained effects of DP1-specific antagonism on atherosclerosis both in the presence and absence of treatment with nicotinic acid, a commonly used lipid-altering agent with a mechanism of action that is still unclear (38). The safety and efficacy of DP1 blockade, along with the lipid-altering effects of extended-release niacin treatment, is the focus of an ongoing ~25,000-patient coronary outcomes study (39). This is a topic of interest, especially in view of the recent results of the
‘Atherothrombosis Intervention in Metabolic Syndrome with Low HDL/High Triglycerides: Impact on Global Health’ (AIM-HIGH) study, which showed that extended-release niacin offered no benefits beyond statin therapy alone in reducing cardiovascular events.(40)

Acknowledgments

Editorial assistance was provided by Christine McCrary Sisk and Kathleen Newcomb of Merck Sharp & Dohme Corp., a subsidiary of Merck & Co., Inc., Whitehouse Station, NJ
References


(39) HPS-2 THRIVE. Available at: http://www.ctsu.ox.ac.uk/~thrive . 2012. 8-14-2012.


Table 1. Characterization of L-655, a selective DP1 antagonist(41)

<table>
<thead>
<tr>
<th></th>
<th>L-655</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Human (IC50)</td>
</tr>
<tr>
<td>DP1 binding</td>
<td>DP1: 0.43 nM</td>
</tr>
<tr>
<td>TP binding</td>
<td>TP: 339 nM</td>
</tr>
<tr>
<td></td>
<td>Mouse (IC50)</td>
</tr>
<tr>
<td>DP1 binding</td>
<td>DP: 1.57 ± 0.48 nM&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>TP binding</td>
<td>TP: 31.8 ± 12.1 nM&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Inhibition of PGD2-induced cAMP accumulation in platelet-rich human plasma</td>
<td>Human: 2.51 ± 0.76 nM&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Mouse: 3.16 ± 2.61 nM&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Inhibition of TP agonist-induced aggregation in platelet-rich human plasma</td>
<td>19400 ± 8600 nM&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fold selectivity over mouse receptors</td>
<td>DP2: 10,000</td>
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<tr>
<td></td>
<td>EP1: 1867</td>
</tr>
<tr>
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<td>EP2: 1411</td>
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<td></td>
<td>IP: &gt;30,000</td>
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<td></td>
<td>FP: &gt;6000</td>
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<sup>a</sup>Data shown as mean ± standard deviation

IC50=amount required for 50% inhibition; TP=thromboxane A2 receptor
Table 2. Summary of the effects of L-655 ± nicotinic acid in *Apoe*−/− mice on aortic cholesterol ester content from three studies

<table>
<thead>
<tr>
<th></th>
<th>Study 1 Males</th>
<th>Study 2 Males</th>
<th>Study 3 Males</th>
<th>Study 3 Females</th>
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<tbody>
<tr>
<td>Aortic cholesterol ester (nmol/aorta)</td>
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<td></td>
<td></td>
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<tr>
<td>Baseline mean</td>
<td>152.8</td>
<td>146.4</td>
<td>86.6</td>
<td>85.6</td>
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<tr>
<td>Vehicle mean</td>
<td>465.4</td>
<td>469.9</td>
<td>254.9</td>
<td>264.2</td>
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<tr>
<td>% change from vehicle</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>-13.1</td>
<td>-23.6*</td>
<td>-18.6*</td>
<td>-28.0*</td>
</tr>
<tr>
<td>L-655 (300 ppm)</td>
<td>-1.5</td>
<td>-1.1</td>
<td>-7.0</td>
<td>-43.0*</td>
</tr>
<tr>
<td>Nicotinic acid + L-655 (300 ppm)</td>
<td>0.9</td>
<td>-28.3*</td>
<td>-23.0*</td>
<td>-33.0*</td>
</tr>
<tr>
<td>% change from nicotinic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotinic acid + L-655 (300 ppm)</td>
<td>16.2</td>
<td>-6.3</td>
<td>-5.0</td>
<td>-7.0</td>
</tr>
</tbody>
</table>

*P<0.05
Legends for figures

**Figure 1.** L-655 ± nicotinic acid in an *Ldlr*<sup>−/−</sup> mouse model of atherosclerosis (A). Study was designed to include male and female *Ldlr*<sup>−/−</sup> mice (*n* = 41-56 animals per gender) kept on regular mouse chow until 8 weeks of age. A baseline group, terminated at 8 weeks of age, was used to establish baseline values; vehicle and L-655 groups were treated for 8, 16, and 24 weeks, starting at 8 weeks of age. In Part 1, the time course of L-655 effects was examined. Parts 2 and 3 were nicotinic acid treatment ± L-655 on animals on a high fat diet for 16 (early therapeutic) and 24 weeks (late therapeutic), respectively. In Part 2, vehicle, L-655, nicotinic acid, and an L-655/nicotinic acid combination were administered to respective groups for 8 weeks, starting at 16 weeks of age and ending at 24 weeks. In Part 3, the same four treatment groups were treated for 8 weeks, beginning at age 24 weeks and ending at 32 weeks. The three parts were performed concurrently so the vehicle groups of the first part could be used for all three. Lesion development as a function of time (histology) in the vehicle-treated group is shown (B).

**Figure 2.** Effects of a DP1 knockout in an *ApoE*<sup>−/−</sup> athero mouse model. At early time points, *Apoe*<sup>−/−</sup> × DP1<sup>−/−</sup> mice have a modest (1.2 fold) increase in aortic lipids compared to *ApoE*<sup>−/−</sup>; this effect is not maintained at 32 weeks and 40 weeks (data not shown).

**Figure 3.** L-655 is a functional antagonist of the DP1, but not TP, receptor in male *ApoE*<sup>−/−</sup> mice. Vasoconstriction studied immediately following feeding; vasodilation studied ≥7 hrs post-feeding. Vasoconstriction induced by TP agonist (U46619) is blocked by TP antagonist SQ29548 but not by morning peak levels of L-655 (A). At doses of L-655 ≥100 ppm, trough afternoon plasma levels completely block PGD<sub>2</sub>-induced vasodilation (B).

**Figure 4.** L-655 is a functional antagonist of the DP1, but not TP, receptor in male *Ldlr*<sup>−/−</sup> mice. Vasoconstriction studied immediately following feeding; vasodilation studied ≥7 hr post feeding. Vasoconstriction induced by TP agonist (U46619) is blocked by TP antagonist SQ_29548 but not by morning peak levels of L-655 (A). At doses of L-655 ≥100 ppm, trough afternoon plasma levels completely block PGD<sub>2</sub>-induced vasodilation (B).
**Figure 5.** Effects of the DP1 antagonist, L-655 ± niacin in *ApoE*<sup>−/−</sup> mice on plasma total cholesterol (A) and aortic total cholesterol (B). Nicotinic acid treatment (from 16 weeks to 24 weeks of age) lowers plasma and aortic cholesterols; however, L-655 had no effect on either measure ± nicotinic acid.

**Figure 6.** DP1 antagonist (L-655) does not enhance atherosclerosis overall in *Ldlr*<sup>−/−</sup> mice. In both males (top panels) and females (bottom panels) L-655 has little overall impact on aortic total lipids (A), aortic lesions by *en face* (B) and aortic lesions by histology (C). At 8 weeks of treatment, transient increases were seen in males in measures of aortic lesions (B) and histology (C) and in females by total aortic cholesterol (A).

**Figure 7.** Representative figures of aortic *en face* from male mice treated for 8 (a, c) or 24 weeks (b, d) with vehicle (a, b) or L-655 (c, d).

**Figure 8.** Eight weeks of DP1 antagonist (L-655) treatment started at 16 weeks of age does not affect the anti-atherosclerotic effects of nicotinic acid in *Ldlr*<sup>−/−</sup> male (top panels) and female (bottom panels) mice. Nicotinic acid significantly lowers plasma (A) and aortic total cholesterol (B) and has no effect on aortic lesions as measured *en face* (C). L-655 alone has no effect and does not alter the effects of nicotinic acid.

**Figure 9.** Representative examples of aortic *en face* from male mice treated for 8 (A, C) or 24 weeks (B, D) with vehicle (A), L-655 (B), nicotinic acid (C), or L-655 + nicotinic acid (D).
Figure 1.

A. 

![Graph showing different treatments over age (wk): Baseline, Vehicle, DP1 antagonist time course Part 1, Therapeutic Early (16 wk) Part 2, Late (24 wk) Part 3.]

B. 

![Images showing mammalian heart sections at different time points: Baseline (8 wk of age), 16 wk HFWD, 8 wk HFWD, 24 wk HFWD.]

Nicotinic acid (3% in feed); DP1 antagonist, L-655 (0.01%, 100 ppm) in feed.
**Figure 2.**

Western (HF) Diet

4 wk 16 wk 24 wk 32 wk

**ApoE<sup>−/−</sup>**

**Ptgd<sup>−/−</sup>ApoE<sup>−/−</sup>**

Aortic TC<sup>i</sup> (nmoles/aorta)

Weeks of treatment

<sup>*</sup>P<0.05 vs. ApoE<sup>−/−</sup>.

<sup>+</sup>Aortic TC = aortic total cholesterol; similar results with aortic cholesterol esters and aortic free cholesterol.
Figure 3.

A. **TP-induced vasoconstriction**

Male ApoE KO mice

<table>
<thead>
<tr>
<th>Condition</th>
<th>AM</th>
<th>PM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (6)</td>
<td>0.143 ± 0.046</td>
<td>0.059 ± 0.008</td>
</tr>
<tr>
<td>U46619 2 mg/kg (6)</td>
<td>0.374 ± 0.105</td>
<td>0.265 ± 0.144</td>
</tr>
<tr>
<td>SQ29548 3 mg/kg+U46619 2 mg/kg (6)</td>
<td>0.872 ± 0.326</td>
<td>0.393 ± 0.072</td>
</tr>
<tr>
<td>L-655 30 ppm+U46619 2 mg/kg (6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-655 100 ppm+U46619 2 mg/kg (6)</td>
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<td></td>
</tr>
<tr>
<td>L-655 300 ppm+U46619 2 mg/kg (7)</td>
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</tr>
</tbody>
</table>

B. **PGD2-induced vasodilation**

Male ApoE KO mice

<table>
<thead>
<tr>
<th>Condition</th>
<th>AM</th>
<th>PM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (6)</td>
<td>0.143 ± 0.046</td>
<td>0.059 ± 0.008</td>
</tr>
<tr>
<td>PGD2 2 mg/kg (6)</td>
<td>0.374 ± 0.105</td>
<td>0.265 ± 0.144</td>
</tr>
<tr>
<td>L-655 30 ppm+PGD2 2 mg/kg (6)</td>
<td>0.872 ± 0.326</td>
<td>0.393 ± 0.072</td>
</tr>
<tr>
<td>L-655 100 ppm+PGD2 2 mg/kg (5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-655 300 ppm+PGD2 2 mg/kg (9)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.

A. TP-induced vasoconstriction

Male LDLr KO

B. PGD2-induced vasodilation

Male LDLr KO

<table>
<thead>
<tr>
<th>Male (µM)</th>
<th>AM</th>
<th>PM</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 ppm</td>
<td>0.623 ± 0.143</td>
<td>0.277 ± 0.107</td>
</tr>
<tr>
<td>100 ppm</td>
<td>2.978 ± 2.619</td>
<td>0.774 ± 0.428</td>
</tr>
<tr>
<td>300 ppm</td>
<td>8.495 ± 4.573</td>
<td>3.197 ± 2.635</td>
</tr>
</tbody>
</table>
Figure 5.

A. Total cholesterol (mg/dL)

B. Aortic total cholesterol (nmol/aorta)

NA = nicotinic acid; *P<0.05 vs vehicle.
Figure 6.

A. Aortic Total Cholesterol*

B. Aortic Lesion Area (En face staining)

C. Histological Assessment of Lesions

*P<0.05 vs vehicle.
Figure 7.

A) Vehicle 8 wk

B) L-655 8 wk

C) Vehicle 24 wk

D) L-655 24 wk
Figure 8.

A. Plasma Total Cholesterol
   Males

B. Aortic Total Cholesterol
   Males

C. Aortic Lesions (En face)
   Males

D. Plasma Total Cholesterol
   Females

E. Aortic Total Cholesterol
   Females

F. Aortic Lesions (En face)
   Females

Similar results with aortic cholesterol esters and free cholesterol; *P< 0.05 vs vehicle. NA = nicotinic acid.
Figure 9.

A) Vehicle

B) L-655

C) NA

D) NA + L-655