Nicotinic acid and DP1 blockade: studies in mouse models of atherosclerosis[S]

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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 D_2 (PGD_2) 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 versus *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.—Strack, A. M., E. Carballo-Jane, S-p. Wang, J. Xue, X. Ping, L. A. McNamara, A. Thankappan, O. Price, M. Wolff, T. J. Wu, D. Kawka, M. Mariano, C. Burton, C. H. Chang, J. Chen, J. Menke, S. Luell, E. I. Zycband, X. Tong, R. Raubertas, C. P. Sparrow, B. Hubbard, J. Woods, G. O'Neill, M. G. Waters, and A. Sitlani. **Nicotinic acid and DP1 blockade: studies in mouse models of atherosclerosis. *J. Lipid Res.* 2013.** 54: **177–188.**

Supplementary key words dyslipidemia • DP1 antagonist• atherosclerosis model

Nicotinic acid produces beneficial effects on LDL cholesterol (LDL-C), HDL cholesterol (HDL-C), and triglycerides (TGs) 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, i.e., 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). Although 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

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Abbreviations: CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; FC, free cholesterol; HDL-C, HDL cholesterol; HFWD, high-fat Western diet; LDL-C, LDL cholesterol; PGD_2, prostaglandin D_2; PRP, platelet-rich plasma; TC, total cholesterol; TG, triglyceride; TP_1, thromboxane A_2 receptor.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of three figures and six tables.

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 to measure the impact on mouse models of atherosclerosis. In addition, we utilize well-established *ApoE*$^{-/-}$ and *Ldlr*$^{-/-}$ 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 (see supplementary Fig. I) was chosen because of its selectivity for murine DP1 over murine thromboxane A_2 (TxA_2) 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_2 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 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-*ApoE*^{tm1Unc}/J) used in these studies were purchased from The Jackson Laboratory (Bar Harbor, ME) 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-*Ldlr*^{tm1Her}/J, stock no. 002207, from the Jackson Laboratory. Both the *ApoE* and the *Ldlr* knockout mice were backcrossed into C57BL/6 at least 10 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 observations), 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 h 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 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*$^{-/-}$ and *Ldlr*$^{-/-}$ mice were tested separately (n = 5–6 per gender per group). *Ldlr*$^{-/-}$ 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*$^{-/-}$ mice were weaned at 4 weeks of age and placed on a 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, MI), was supplied by the manufacturer as a 10 mg/ml stock in methyl acetate. The compound was dried under nitrogen and resuspended in 100% 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 0.1 M sodium carbonate to a concentration of 2 mg/ml and then diluted in PBS to 0.3 mg/ml to provide a final dose of 3 mg/kg. PGD_2 (Sigma-Aldrich; St. Louis, MO) 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 a 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 min 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, OH). The settings of the system were: repeated mode, image size 12 × 12, start method auto interval, 40 images, images every 30 s, 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 min 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 min. 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 min 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 min.

The second series of studies was designed to determine the minimal dose of L-655 that would inhibit PGD_2-induced vasodilatation in male and female *ApoE*$^{-/-}$ *or Ldlr*$^{-/-}$ mice. Mice were fed the HFWD as described above. One week prior to performing the vasodilatation studies, mice were switched to a 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 PGD_2 was tested in the mice using the same laser doppler perfusion technique described above, with the following modifications: on the day of the study, mice were placed in clean cages in the morning (between 7:30 and 8:00 AM), and switched to a 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 h. 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, because this time point probably reflected the minimal plasma concentration of L-655 that would occur when mice were not actively feeding. After a 3 min baseline reading of cutaneous blood perfusion, PGD_2 (2 mg/kg) or vehicle (0.5% ethanol in saline) was administered by intraperitoneal injection, and measurement of cutaneous perfusion continued for another 17 min. Perfusion values for the baseline in each animal were averaged, and subsequent values after injection of vehicle or PGD_2 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 a 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 a pelleted HFWD. At 15 weeks of age, mice were switched to a 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 a HFWD for 8 weeks; a nicotinic acid group, which received a 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 a 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, three-part study was carried out (**Fig. 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 Fig. 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 min 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, IN).

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. TC and free cholesterol (FC) were determined by an enzymatic fluorometric assay, and cholesteryl 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 min, and aortas (from the point where the aorta exits the top of the heart to

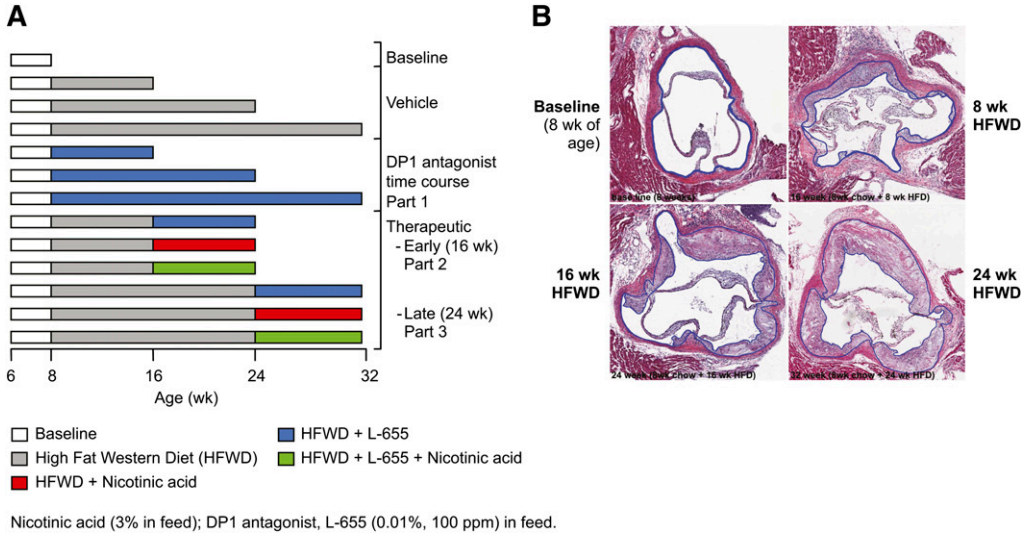


Fig. 1. L-655 ± nicotinic acid in an $Ldlr^{-/-}$ mouse model of atherosclerosis (**A**). Study was designed to include male and female $Ldlr^{-/-}$ mice (n = 41–56 animals per gender) kept on regular mouse chow until 8 weeks of age. A baseline group, terminated at 8 weeks of age, was used to establish 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 in 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).

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 min and in 1 ml of 70% ethanol for 3 min, 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 min, followed by rocking. The aortas were destained in 1 ml of 80% ethanol for 3 min 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.; Bethesa, MD) to quantify the 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 into 8 μm sections, and every 4th section was processed for hematoxylin and eosin staining. The area (in μm^2) 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$^{-/-}$ mice

The time course of aortic and plasma lipids in male $ApoE^{-/-}$ and $Ptgdr^{-/-}ApoE^{-/-}$ double-deficient mice fed a HFWD from 4 weeks of age until 32 weeks of age was examined. Aortic TC (**Fig. 2**) and CE (data not shown) were significantly higher in the $Ptgdr^{-/-}ApoE^{-/-}$ 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 $Ptgdr^{-/-}ApoE^{-/-}$ mice at 24 and 32 weeks of age, and plasma TGs were significantly higher at all three ages (see supplementary Fig. II).

A second, similar study (see supplementary Fig. III) 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 $Ptgdr^{-/-}ApoE^{-/-}$ mice in the first study described above (see supplementary Fig. II), plasma TGs were not significantly different in these double-deficient mice at 16, 24, and 32 weeks of age in the second study (see supplementary Fig. III).

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

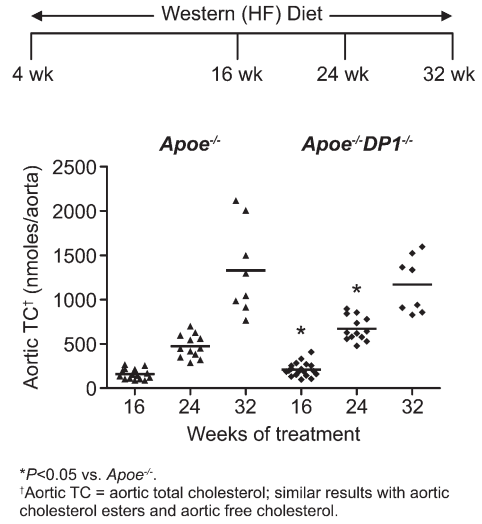


Fig. 2. Effects of a DP1 knockout in an $ApoE^{-/-}$ athero mouse model. At early time points, $Apoe^{-/-}$ ×$DP1^{-/-}$ mice have a modest (1.2-fold) increase in aortic lipids compared with $ApoE^{-/-}$; this effect is not maintained at 32 weeks and 40 weeks (data not shown).

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

	L-655
Human (IC_{50})	DP1: 0.43 nM
DP1 binding	TP: 339 nM
TP binding	
Mouse (IC_{50})	DP: 1.57 ± 0.48 nM[a]
DP1 binding	TP: 31.8 ± 12.1 nM[a]
TP binding	
Inhibition of PGD2-induced cAMP accumulation in platelet-rich human plasma	Human: 2.51 ± 0.76 nM[a]
	Mouse: 3.16 ± 2.61 nM[a]
Inhibition of TP agonist-induced aggregation in platelet-rich human plasma	19,400 ± 8,600 nM[a]
Fold selectivity over mouse receptors	DP2: 10,000
	EP1: 1,867
	EP2: 1,411
	EP3: 230
	EP4: >9,500
	IP: >30,000
	FP: >6,000

IC_{50} = amount required for 50% inhibition; TP = thromboxane A2 receptor.
[a]Data shown as mean ± standard deviation.

than to TP and murine DP2 (**Table 1**). L-655 also inhibited PGD_2-induced cAMP formation in human platelet-rich plasma (PRP) with an IC_{50} 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_{50} ± 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 $ApoE^{-/-}$ mice (**Fig. 3**). At doses of L-655 ⩾100 ppm, trough afternoon plasma levels completely blocked PGD_2-induced vasodilation, whereas morning peak levels had no off-target effects in blocking induction of cutaneous vasoconstriction by the TP agonist U44619 (**Figs. 3, 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 h 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 (Figs. 3, 4), including a dose of 1,000 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 $ApoE^{-/-}$ mice, doses of 30, 100, and 300 ppm admixed in high-fat chow were chosen for subsequent atherosclerosis studies.

PGD_2 at 2 mg/kg induced a robust vasodilatory response in both male $Ldlr^{-/-}$ mice (Fig. 4) and female $Ldlr^{-/-}$ mice (data not shown). In both genders, pretreatment with L-655 for 1 week resulted in a dose-dependent inhibition of PGD_2-induced vasodilation. L-655 at 30 ppm only partially inhibited vasodilation, with one animal out of four in both genders responding to PGD_2. Doses of 100 and 300 ppm completely abrogated the vasodilatory response to PGD_2 in both male and female $Ldlr^{-/-}$ mice.

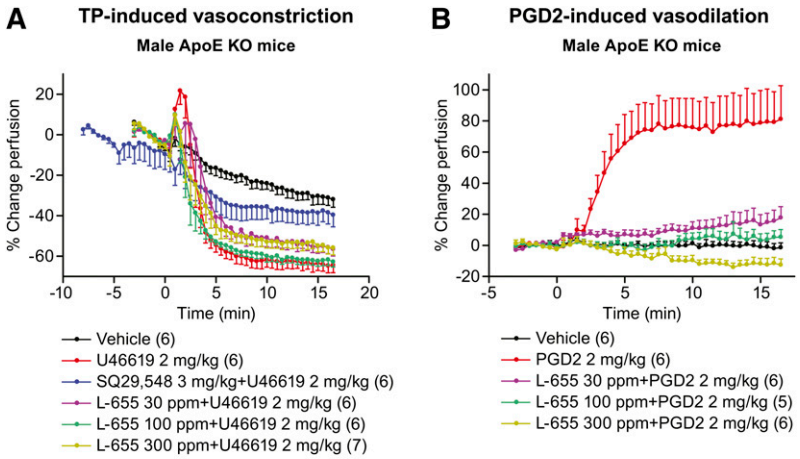
Doses of L-655 that partially (30 ppm) or totally (100 and 300 ppm) blocked PGD_2-induced vasodilation failed to block U46619-induced vasoconstriction in male $Ldlr^{-/-}$ mice (Fig. 4) and female $Ldlr^{-/-}$ mice (data not shown). As a control, the use of SQ-29,548 indicated that the vasoconstriction induced by U46619 could be prevented by TP antagonism.

These results indicate that in $Ldlr^{-/-}$ mice, L-655 at doses ⩾100 ppm can completely block PGD_2-induced vasodilation (Fig. 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 1,000 ppm L-655, which was considered too high because of potential nonspecific 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 (Fig. 3; supplementary Table I); 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 (see supplementary Table I), which were comparable to levels obtained in the dose-finding study (Fig. 3).

Two studies used only male mice, whereas the third included both males and females (Table 2; see supplementary Table II). Nicotinic acid alone reduced aortic CE and total aortic cholesterol (**Fig. 5**), compared with vehicle; the reduction was statistically significant in two of three studies



	Male (μM)	
	AM	PM
30 ppm	0.143 ± 0.046	0.059 ± 0.008
100 ppm	0.374 ± 0.105	0.265 ± 0.144
300 ppm	0.872 ± 0.326	0.393 ± 0.072

Fig. 3. L-655 is a functional antagonist of the DP1, but not TP, receptor in male *ApoE*$^{-/-}$ mice. Vasoconstriction studied immediately following feeding; vasodilation studied ≥7 h postfeeding. 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_2-induced vasodilation (B). Error bars indicate SEM.

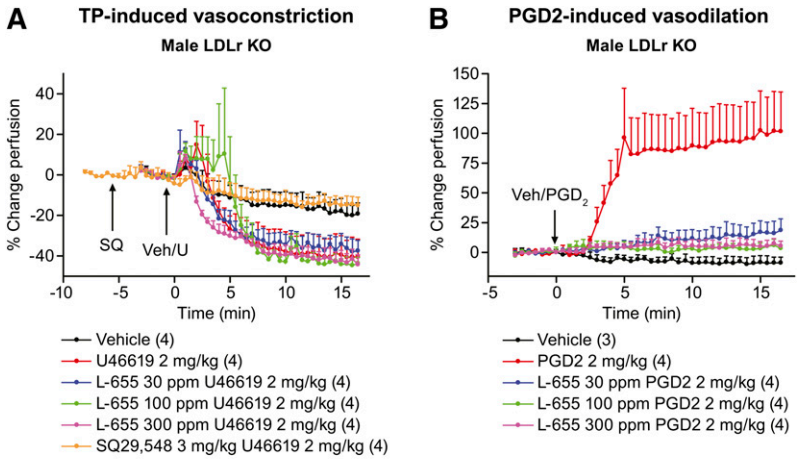
(Table 2). L-655 alone produced statistically significant reductions in aortic CE (Table 2), TC, and FC (Table 2 and supplementary Table II) in female mice at a dose of 100 ppm. An analogous beneficial effect of L-655 was not induced in male mice, suggesting a gender-specific effect. In two studies, 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 (see supplementary Table II).

In the one study that included histologic assessments of aortic lesions (supplementary Table II), 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 (see supplementary Table II).

Effects of L-655 ± nicotinic acid in Ldlr$^{-/-}$ mice

Based on the results from the dose-finding study shown in Fig. 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



	Male (μM)	
	AM	PM
30 ppm	0.623 ± 0.143	0.277 ± 0.107
100 ppm	2.978 ± 2.619	0.774 ± 0.428
300 ppm	8.495 ± 4.573	3.197 ± 2.635

Fig. 4. L-655 is a functional antagonist of the DP1, but not TP, receptor in male *Ldlr*$^{-/-}$ mice. Vasoconstriction studied immediately following feeding; vasodilation studied ≥7 h 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_2-induced vasodilation (B). Error bars indicate SEM.

TABLE 2. Summary of the effects of L-655 ± nicotinic acid in *Apoe*$^{-/-}$ mice on aortic cholesterol ester content from three studies

Study 1		Study 2			Study 3	
	Males		Males		Males	Females
		Aortic cholesterol ester (nmol/aorta)				
Baseline mean	152.8	Baseline mean	146.4	Baseline mean	86.6	85.6
Vehicle mean	465.4	Vehicle mean	469.9	Vehicle mean	254.9	264.2
		% change from vehicle				
Nicotinic acid	−13.1	Nicotinic acid	−23.6[a]	Nicotinic acid	−18.6[a]	−28.0[a]
L-655 (300 ppm)	−1.5	L-655 (30 ppm)	−1.1	L-655 (100 ppm)	−7.0	−43.0[a]
		L-655 (300 ppm)	−6.7			
Nicotinic acid + L-655 (300 ppm)	0.9	Nicotinic acid + L-655 (30 ppm)	−28.3[a]	Nicotinic acid + L-655 (100 ppm)	−23.0[a]	−33.0[a]
		Nicotinic acid + L-655 (300 ppm)	−24.7[a]			
		% Change from nicotinic acid				
Nicotinic acid + L-655 (300 ppm)	16.2	Nicotinic acid + L-655 (30 ppm)	−6.3	Nicotinic acid + L-655 (100 ppm)	−5.0	−7.0
		Nicotinic acid + L-655 (300 ppm)	−1.5			

[a] $P < 0.05$.

without affecting vasoconstriction (Fig. 4 and supplementary Table III). 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 (**Figs. 6 and 7**; supplementary Table IV). In female mice, L-655 treatment resulted in 1.2- to 1.3-fold increases compared with 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 TGs for females after 16 weeks (Fig. 7 and supplementary Table IV).

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 (**Fig. 8A** and supplementary Table V). 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 (Fig. 8B and supplementary Table V). Statistically significant reductions in percent aortic lesion area, as measured en face, were observed with treatment by nicotinic acid alone or in combination with L-655 in female but not male mice (Fig. 8C,

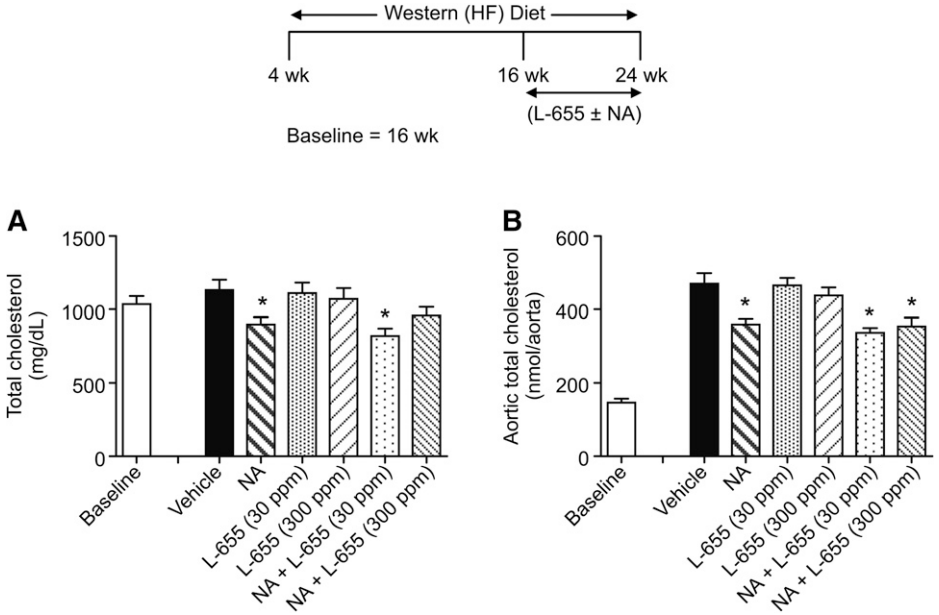


Fig. 5. Effects of the DP1 antagonist, L-655 ± niacin in *ApoE*$^{-/-}$ mice on plasma total cholesterol (A) and aortic total cholesterol (B). Nicotinic acid treatment (from 16 weeks to 24 weeks of age) lowers plasma and aortic cholesterols; however, L-655 had no effect on either measure ± nicotinic acid. Error bars indicate SEM. * $P < 0.05$ versus vehicle. NA, nicotinic acid.

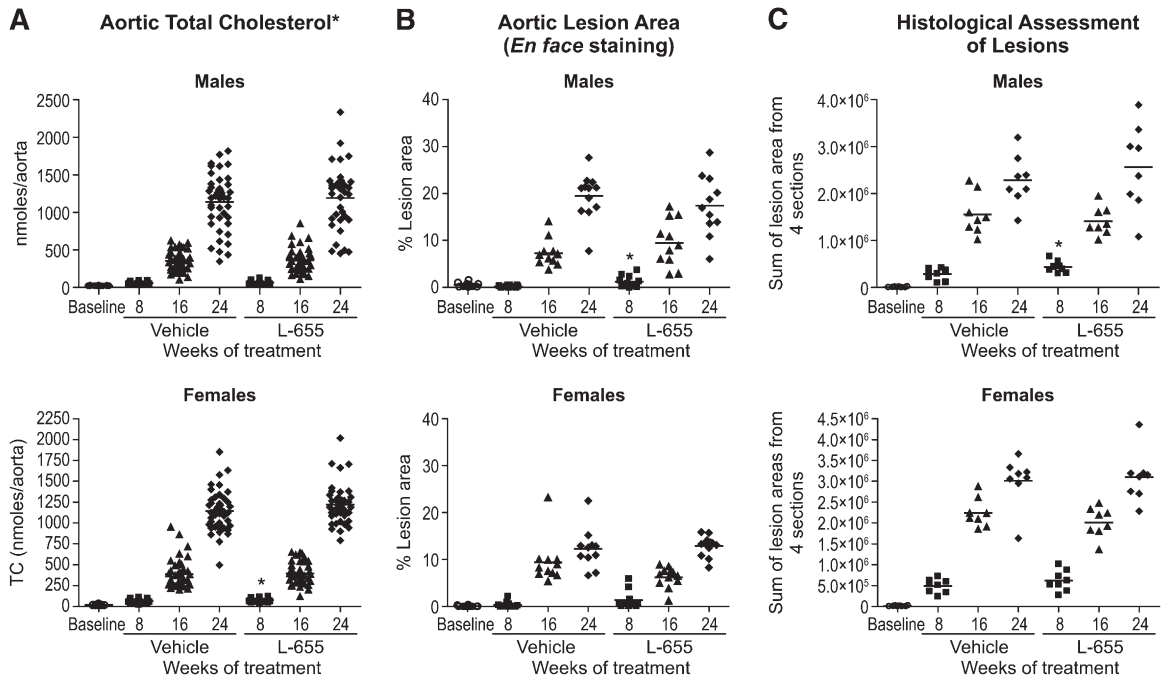


Fig. 6. DP1 antagonist (L-655) does not enhance atherosclerosis overall in *Ldlr*$^{-/-}$ mice. In both males (top panels) and females (bottom panels) L-655 has little overall impact on aortic total lipids (A), aortic lesions by en face (B), and aortic lesions by histology (C). At 8 weeks of treatment, transient increases were seen in males in measures of aortic lesions (B) and histology (C) and in females by total aortic cholesterol (A). * $P < 0.05$ versus vehicle.

Fig. 9, and supplementary Table V). 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 (see supplementary Table V).

In Part 3 of the *Ldlr*$^{-/-}$ mouse study, examining 8 weeks of drug treatment that occurred between 24 and 32 weeks of age (see supplementary Table VI), 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 (see supplementary Table VI)

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

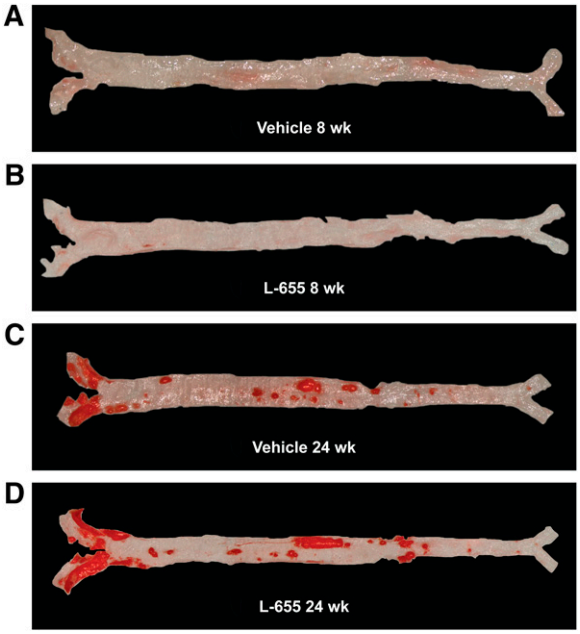
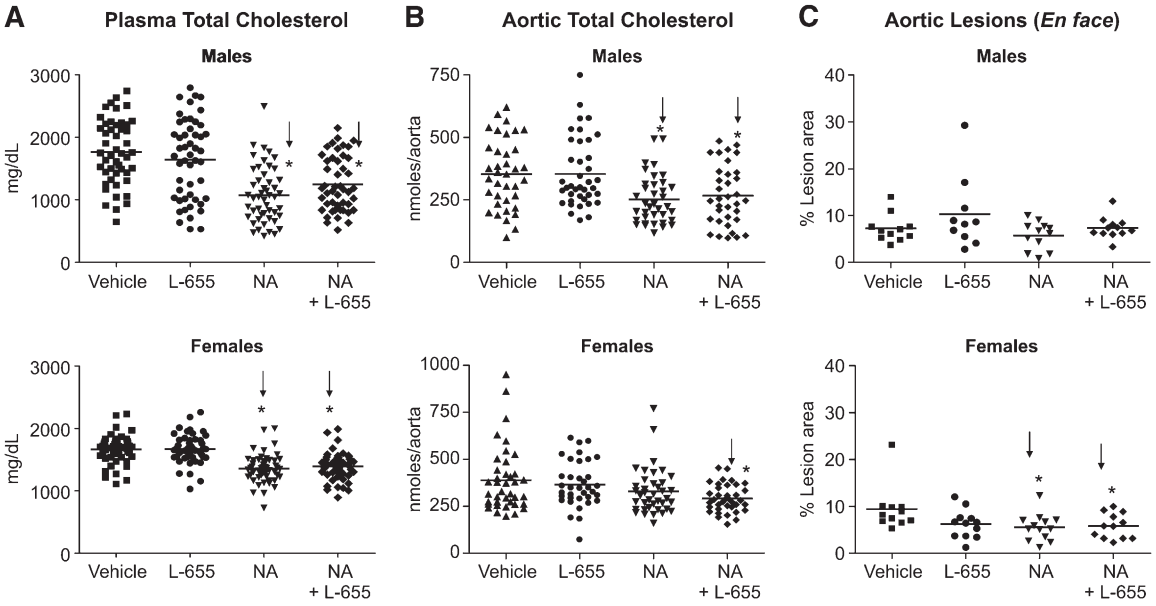


Fig. 7. Representative figures of aortic en face from male mice treated for 8 (A, C) or 24 weeks (B, D) with vehicle (A, B) or L-655 (C, D).



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

Fig. 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 $Ldlr^{-/-}$ 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. $P < 0.05$ vs. vehicle. NA, nicotinic acid.

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, $ApoE^{-/-}$,

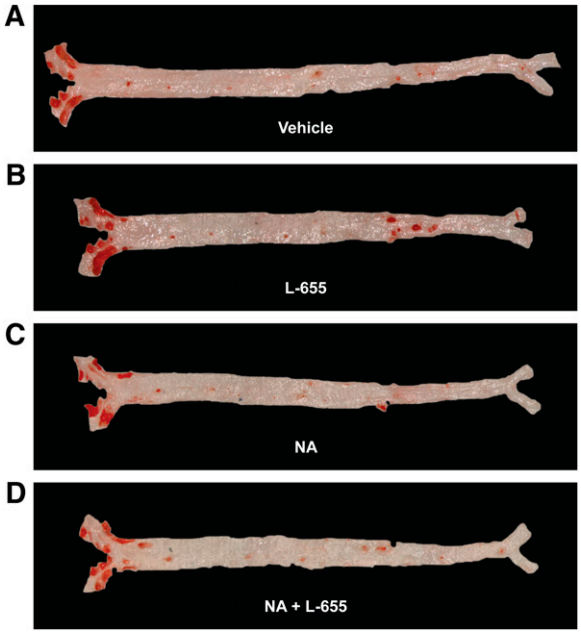


Fig. 9. Representative examples of aortic en face from male mice treated for 8 (A, C) or 24 weeks (B, D) with vehicle (A), L-655 (B), nicotinic acid (C), or L-655 + nicotinic acid (D).

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 with 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 observations), 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 en face staining or histology. Historically, en face analysis has been most commonly used in the field to visually assess aortic lesions. Although en face analysis 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 the nicotinic acid that is administered in the feed. Although niacin effects were comparable across males and females, there may be a slight gender effect with L-655 treatment alone. However, although 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 that 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 could 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 PGD_2-induced vasodilation (a measure of DP1-specific activity), whereas 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 PGD_2 (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 PGD_2 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 in the 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).

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