methods

Determining hepatic triglyceride production in mice: comparison of poloxamer 407 with Triton WR-1339

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Abstract Triglyceride (TG), a water-insoluble energy-rich lipid, is secreted by the liver as part of very low density lipoproteins (VLDLs) to supply energy to extrahepatic tissues. Overproduction of VLDL is associated with increased risk of cardiovascular heart disease; this has renewed an interest in factors that affect hepatic TG production. The TG production rate is determined by measuring temporal increases in plasma TG under conditions in which TG hydrolysis by lipoprotein lipase (LPL) is inhibited. The nonionic detergent, Triton WR-1339 (Triton), has commonly been used to inhibit LPL for this purpose. Triton, in addition to inhibition of TG hydrolysis, has properties that have the potential to adversely influence lipoprotein metabolism. Another nonionic detergent, poloxamer 407 (P-407), also inhibits LPL. In these studies, we demonstrate that P-407 is comparable to Triton in the determination of TG production but without the unwanted side effects of Triton.—Millar, J. S., D. A. Cromley, M. G. McCoy, D. J. Rader, and J. T. Billheimer. **Determining hepatic triglyceride production in mice: comparison of poloxamer 407 with Triton WR-1339. *J. Lipid Res.* 2005.** 46: **2023–2028.**

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Triglyceride (TG) is an energy-rich compound, primarily stored in liver and adipose, and is mobilized in response to various metabolic signals. In plasma, TG, which is water insoluble, circulates as the neutral lipid core of lipoproteins, mainly chylomicrons, which carry dietary fat and are secreted by the small intestine, and very low density lipoproteins (VLDLs), which carry TG from the liver. Overproduction of VLDL has been associated with a number of disease states that result in an increased risk of cardiovascular heart disease; this has renewed an interest in factors that affect hepatic TG (lipoprotein) production (1).

In the early 1950s, it was noted that intravenous injection of certain nonionic detergents resulted in milky serum that lasted up to 48 h (2). This was later shown to be due to the inhibition of TG hydrolysis by lipoprotein lipase (LPL) (3). Since then, lipolysis inhibition has been used to determine hepatic TG production rates, with Triton WR-1339 (also known as Tyloxapol) being widely used. Using this technique, the TG production rate is calculated from the increase in TG over time following detergent injection. Although this method is the basis for most studies on TG production in animals, there is considerable variation in its implementation. Variables include whether mice are fasted, fed chow or a fat-free diet, and what the plasma sampling period is (0 to 300 min) over which TG production rates are determined (**Table 1**).

In addition to inhibition of LPL, Triton has a number of other physiologic effects related to lipoprotein metabolism. Triton has been shown to cause dissociation of apolipoprotein A-I (apoA-I) and apoC-II from HDL (13). Triton is rapidly taken up by the liver, where it accumulates in the lysosomes, causes autophagic vacuole formation (14, 15), and is excreted in bile, possibly explaining a reduction in biliary phospholipid and cholesterol output (16). These hepatic and plasma effects of Triton may affect hepatic TG production, especially over extended periods of time. Our interest was to follow hepatic TG production over prolonged periods, and we sought a potential alternative to Triton with fewer side effects.

Poloxamer 407 (P-407) is a nonionic detergent that was originally used in controlled drug delivery applications (17). In the early 1990s, Johnston demonstrated that P-407 administration to mice also resulted in hyperlipidemia, later found to be due to lipase inhibition. Since then, this compound has been used to induce hyperlipidemia in various mouse models (reviewed in 18). In these studies, we compare P-407 with Triton in the determination of TG production rate and other effects on lipoprotein metabolism in wild-type mice.

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Abbreviations: apoA-I, apolipoprotein A-I; FPLC, fast-protein liquid chromatography; HLB, hydrophilic/lipophilic balance; LPL, lipoprotein lipase; P-407, poloxamer 407; TG, triglyceride.

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TABLE 1. Survey of triglyceride (TG) production rates in wild-type mice given Triton WR-1339

Strain	Diet	Sex	Age	Fast	Triton Dose	Plasma Sampling Period	TG Secretion Rate	Reference
			weeks	*h*	*mg/kg*	*min*	*μmol/kg/h*	
SJL/C57Bl	Rodent chow	NI	10–16	5	500	1–30	103	(4)
C57Bl/6 129/SvJ	Fat-free high carb	M	14	None	500	0–120	115	(5)
FVB/N	Fat-free	M,F	11–13	None	700 or 900	0–300	163	(6)
C57Bl/6	Rat chow	M	20	5	500	30–90	29	(7)
C57Bl/6	Std	F	21–22	None	300	0–120	131	(8)
KK/San	Chow	M	13–21	Time NI	400	0–180	141	(9)
C57Bl/6	Std or western	M	6–8	4	500	0–240	95	(10)
C57Bl/6	Chow	NI	NI	Overnight	500	0–240	113	(11)
Sv/129	Std or high fat	M,F	32	4	500	0–90	F∼120 M∼85	(12)

Data are expressed as μmol/h/kg using a plasma volume of 35 ml/kg body weight. NI, not indicated.

MATERIALS AND METHODS

Animals

C57BL/6, *Ldlr*$^{-/-}$, and *Apoe*$^{-/-}$ mice were obtained from Jackson Laboratories. All mice were housed under standard conditions with a 12 h light/dark cycle and free access to chow diet (Rodent Diet 5010, Lab Diet, PMI Nutrition International) and water. Mice were handled according to the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee.

Hepatic TG production rate

To measure hepatic TG production rate, mice were injected with Triton (Sigma) or P-407 (gift from BASF Customer Care) in saline approximately 6 h into the light cycle, and plasma TG was measured over a 24 h period. Triton was administered at 500 mg/kg iv, the dose most often employed (Table 1) and P-407 at 1,000 mg/kg ip, previously shown by Johnston to be optimal (19). In some cases, mice were fasted or switched to a fat-free (corn flake) diet 4 h prior to detergent injection. Immediately prior to injection, and at 1, 2, 6, and 24 h following injection, blood samples were drawn in heparin capillary tubes, plasma was prepared, and TG concentrations were determined. The TG production rate was calculated from the difference in plasma TG levels over a given interval following detergent injection and was expressed as μmol/kg/h.

Lipid analysis

Cholesterol and TG were measured using commercially available kits (Infinity Triglyceride and Cholesterol Kit, Thermo Electron Corp.). Hepatic lipids were measured following solubilization of the homogenized tissue with deoxycholate (20).

For fast-protein liquid chromatography (FPLC) analysis, pooled plasma samples from mice of the same experimental group were subjected to FPLC gel filtration by using two sequential Superose 6 columns (Pharmacia LKB Biotechnology). Samples were chromatographed at a flow rate of 0.5 ml/min, and fractions of 500 μl each were collected and assayed for TG.

Triton levels were determined in isopropanol extracts of plasma as described by Schurr, Schultz, and Parkinson (21).

Nuclear magnetic resonance (NMR) analysis of particle size was performed on pooled plasma samples by LipoScience, Inc. (Richmond, VA) according to the procedure of Otvos (22).

LPL assay

LPL was assayed as previously described by our lab using conditioned medium containing LPL as the enzyme source, except that the assay time was 15 min (23). Triton and P-407 dissolved in PBS were added at various concentrations to determine the inhibitory concentration.

VLDL apoB clearance

VLDL (d < 1.006 g/ml) was isolated from pooled human plasma and iodinated with ^{125}I. Mice were injected with either PBS, Triton, or P-407, followed by injection with ∼5 × 10^6 cpm of VLDL tracer. Blood samples were collected between 1 min and approximately 24 h following tracer injection. ApoB-specific counts were obtained following isopropanol precipitation, followed by counting of radioactivity in the apoB precipitate. Tracer data were expressed relative to the 1 min time point, and fractional catabolic rates were calculated using a two-pool model (24) using WinSAAM.

HDL apoA-I analysis

HDL was obtained by ultracentrifugation. Apolipoproteins were separated by SDS-PAGE using a 3–20% linear gradient. Protein bands were visualized by staining with Coomassie Blue R250.

Statistical analysis

Values are presented as mean ± SD or SEM. Comparisons between P-407- and Triton-treated mice were made using Student's *t*-test (two-tailed).

TABLE 2. Physiochemical properties of Triton WR-1339 and P-407

Nonionic Detergents	Molecular Weight	HLB Number (Reference)	CMC (Reference)	LPL IC_{50}	Plasma Concentration 24 h Following Injection (Reference)
			μM	*μM*	*μM*
Triton WR-1339	4,500	12.9 (25)	17 (25)	12.5	500 (21)
P-407	12,600	22 (17)	3 (17)	4	600 (26)

CMC, critical micelle concentration; HLB, hydrophilic/lipophilic balance.

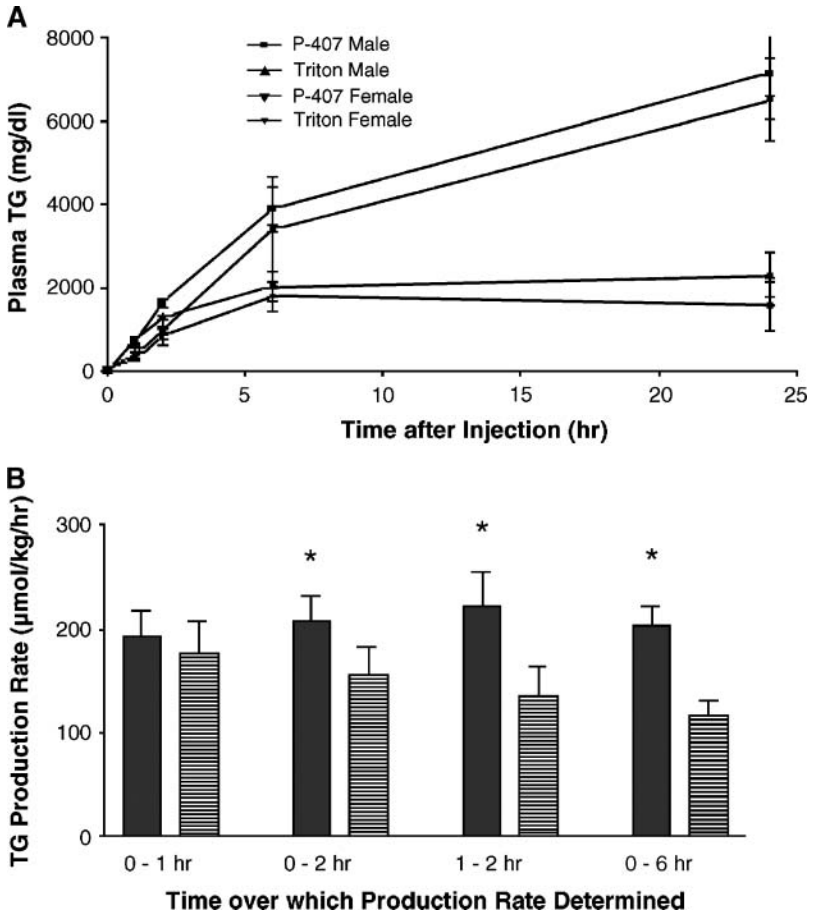

Fig. 1. Plasma triglyceride (TG) secretion rate in C57BL/6 mice administered Triton WR-1339 or poloxamer 407 (P-407). A: Representative time course of TG elevation in male and female mice upon administration of nonionic detergent. N = 4; data are average ± SD. B: Rate of TG production measured over varying time periods in Triton-treated (hatched bar) and P-407-treated (dark bar) mice. N = 6 separate experiments using either male or female mice that had been fasted or fed. Data are mean ± SEM. * Significant difference between Triton WR-1339 and P-407; $P < 0.05$.

RESULTS

The physiochemical properties of the two nonionic detergents, Triton WR-1339, a polyoxyethylene ether of alkyl phenol, and P-407, a block copolymer comprising polyoxyethylene and polyoxypropylene, are shown in **Table 2**. The two compounds are inhibitors of LPL with IC_{50}s of 12.5 and 4 μM, respectively, and the plasma concentrations of each suggest that LPL is completely inhibited for at least 24 h after administration. P-407 is relatively more hydrophilic than Triton, having a higher hydrophilic/lipophilic balance (HLB) number, 22 versus 12.9.

The accumulation of plasma TG was monitored in both male and female C57BL/6 mice administered P-407 or Triton (**Fig. 1A**). The rise in plasma TG was similar for both Triton- and P-407-treated mice over the first hour, after which the relative TG increase in Triton-treated mice declined with respect to P-407 and plateaued after 6 h (Fig. 1A). A similar effect was observed in both sexes. In contrast, the rate of TG increase in the P-407-treated animals was linear for 6 h [193 ± 25 μmol/h/kg, 0–1 h, and 203 ± 20 μmol/h/kg, 0–6 h (Fig. 1B)]. Experiments were performed in 4 h-fasted and fed mice of both sexes. Fasting for 4 h decreased the zero time plasma TG level from 65 ± 14 mg/dl to 40 ± 6 mg/dl but did not affect the rate of TG increase over time. To rule out the possibility that the difference was due to the different route of detergent administration, an experiment in which Triton and P-407 were both administered at 1,000 mg/kg ip produced similar results (data not shown).

It is possible that the decline in the rate of TG accumulation in Triton-treated mice relative to P-407-treated mice is due to the concentration of Triton in plasma falling to a level that is insufficient to inhibit LPL in plasma. We tested this in two ways. First the concentration of Triton in plasma at 24 h was found to be 650 μM, well above what is required for LPL inhibition and in agreement with that obtained in rats (Table 2). In addition, we monitored the plasma clearance of ^{125}I-labeled VLDL (**Fig. 2**). P-407 and Triton are equally effective at reducing the clearance of iodinated VLDL, both detergents capable of reducing clearance by approximately 90% when compared with saline-treated controls. This inhibitory effect of reducing VLDL clearance persisted for at least 24 h after injection of each

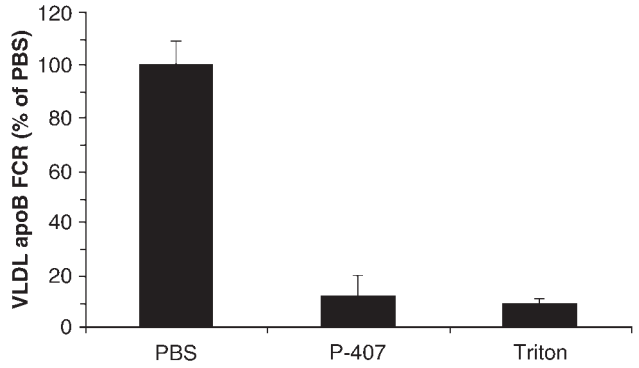

Fig. 2. The relative clearance of ^{125}I-labeled very low density lipoprotein (VLDL) apolipoprotein B (apoB) from plasma of PBS-, P-407-, and Triton-injected mice. P-407 and Triton inhibited VLDL apoB clearance to a similar extent (~90% lower fractional catabolic rate as compared with PBS-injected mice). Mice were injected with tracer 5 min following injection with detergent or PBS. Error bars represent standard deviation.

detergent. This is in agreement with the fact that both detergents at 24 h are well above the concentration required to completely inhibit LPL (Table 2). This indicates that the decline in the rate of plasma TG accumulation observed with Triton is due to a reduction in the rate of VLDL production.

To better understand the decreased TG accumulation with Triton relative to P-407, the plasma and liver lipids were analyzed after 24 h. The FPLC profile of plasma TG is shown in **Fig. 3**. In agreement with the TG accumulation data (Fig. 1A) there is about twice the amount of TG present at 24 h in the P-407-treated versus the Triton-treated mice. In addition, the P-407 VLDL particles appear larger as the chromatographic profile is shifted slightly to the left with respect to the Triton profile. This was confirmed by NMR analysis, in which the mean size of the VLDL particle in mice treated with P-407 was 79.9 nm versus 63.1 nm for Triton-treated animals. Inasmuch as LPL is thought to be completely inhibited by both Triton and P-407, this might

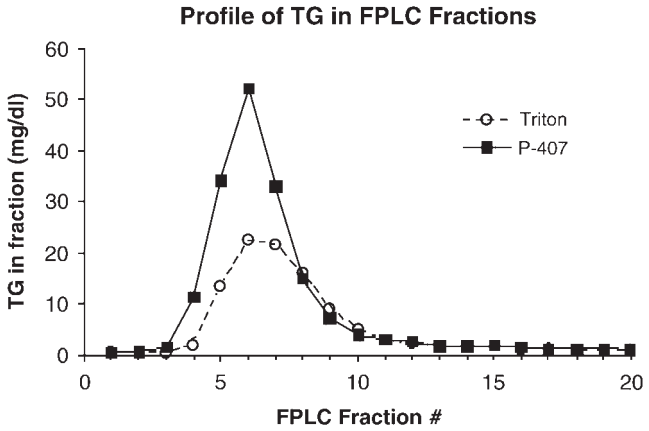

Fig. 3. Size determination of TG particles by fast-protein liquid chromatography (FPLC). Twenty-four hour pooled plasma samples from one experiment were separated by FPLC. Individual FPLC fractions were analyzed for TG as described in Materials and Methods.

suggest the secretion of smaller, less-TG-rich VLDL in the Triton-treated mice. Triton is known to accumulate in the liver (14, 15) and might affect VLDL-TG secretion.

The analysis of hepatic lipids 24 h after injection (**Fig. 4**) reveals that the TG content in Triton-treated mice is increased 4-fold relative to P-407 treatment (30.2 ± 7.0 vs. 7.7 ± 1.9 mg/g liver). This increase is specific for TG, because the hepatic cholesterol content is similar between the two treatments (Fig. 4B). Thus, Triton treatment may result in sequestration of some of the hepatic TG such that it is not available for secretion. The hepatic lipid content of untreated animals was similar to that of the P-407-treated mice (Fig. 4).

Triton has also been shown to cause dissociation of apolipoproteins from HDL (13), and we were interested in whether a similar effect is observed with P-407. P-407, unlike Triton, does not result in the destabilization of the HDL particle, again indicating fewer disruptive side effects on lipoprotein metabolism with the use of P-407 (**Fig. 5**).

Finally, because the above studies were done in wild-type mice, we tested the effect of P-407 on plasma TG accumulation in two mouse models in which VLDL production has been suggested to be altered, *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ mice. It has previously been shown that apoE is required for normal VLDL secretion (27), although the specific role in the VLDL secretory pathway has yet to be identified. Using Triton, the TG production rate is decreased by 46% in apoE knockout mice compared with wild-type controls (11). Using P-407, we found a similar 37% decrease in production rate (160 ± 21 vs. 101 ± 16 μmol/h/kg, **Fig. 6**). Twisk et al. demonstrated that in in vitro studies, TG production was increased in primary hepatocytes from LDL knockout mice compared with controls, possibly due to presecretory sequestration or rapid reuptake of newly synthesized apoB particles by the LDL receptor (28). Using Triton to measure production rates in vivo, we previously reported a normal TG production in *Ldlr* $^{-/-}$ animals (8). Similarly, using P-407 we find that the TG production rate of *Ldlr* $^{-/-}$ mice is unaffected, compared with wild-type mice (Fig. 6).

DISCUSSION

In these studies, we compared the use of two nonionic detergents, Triton and P-407, to obtain hepatic TG production rates in mice. Data combined from several experiments in both sexes in fasted or nonfasted mice were used to analyze the TG production rate (Fig. 1B). The TG production rate determined with P-407 is constant over a 6 h time period. The production rate with Triton is similar to that of P-407 when measured over the first hour but steadily decreases when measured over longer time periods. The TG production rate calculated from 0 to 2 h using P-407, 200 μmol/h/kg, is on the high end of what has been reported (Table 1). This suggests that investigators who utilize 3 to 4 h time points after Triton injection for determination of production rates may underestimate the actual rate. Some investigators have delayed collecting initial

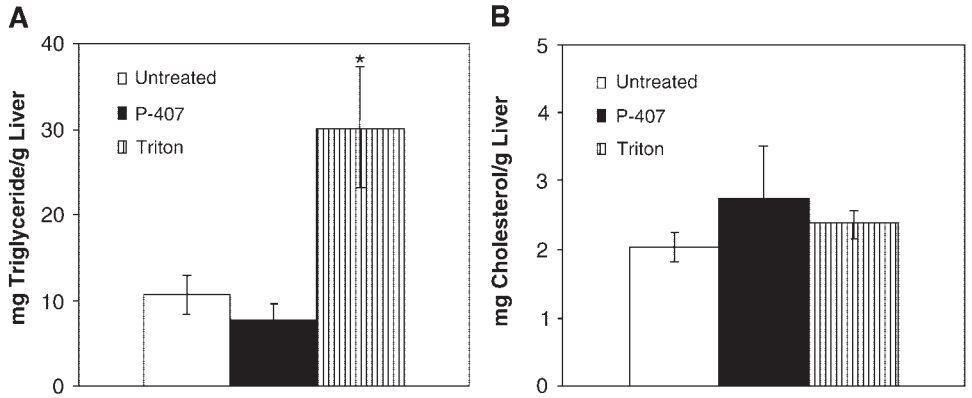

Fig. 4. Hepatic lipid content of mice treated with Triton WR-1339 or P-407 and untreated controls. Livers were excised 24 h after treatment with a nonionic detergent, and hepatic TG (A) and cholesterol (B) were determined as described in Materials and Methods. N = 4; data are average ± SD, * Significant difference between Triton WR-1339 and P-407: $P < 0.001$.

time point because of an apparent lag in LPL inhibition their studies (7). We did not determine TG levels at <1 h. However, because the production rate over the first hour is not significantly different from that over the second hour (Fig. 1B), a lag in LPL inhibition does not appear to be a significant problem. We also found that intraperitoneal injections were more practical than intravenous injections, with only about 5% failure rate. The use of P-407 allows more flexibility in the bleed times, with similar rates obtained for each timed interval used in the calculation (Fig. 1B).

The decrease in TG production rates when assessed over longer time intervals after Triton injection appears to be due not to a lack of inhibition of hydrolysis of plasma TG, but more likely to a decrease in the hepatic production rate. This is consistent with the known Triton accumulation in the lysosomes (14, 15), which may affect trafficking of TG from the lysosomes for secretion in VLDL and cause the accumulation of TG in the livers of Triton-treated mice (Fig. 4A). This increase in hepatic TG is not observed with P-407 (Fig. 4A). In addition, P-407, unlike Triton, did not cause dissolution of the HDL particle (Fig. 5). The latter two advantages of P-407 may be due to its higher HLB number. The higher HLB number may account for the different preferential route of clearance of the two compounds, renal for P-407 (29) and hepatic for Triton (26).

In summary, the use of P-407 is a preferable alternative to Triton for the determination of hepatic TG production rates and may offer advantages, particularly if experiments are carried out over an extended time period.

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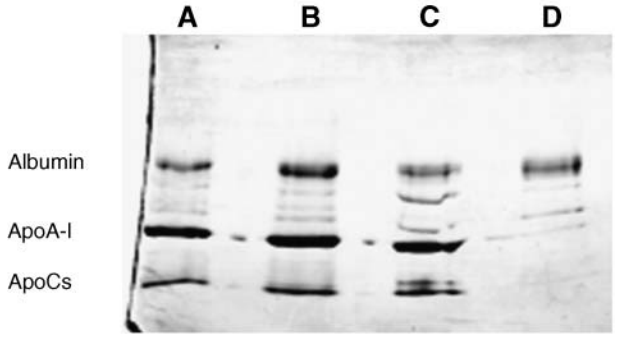

Fig. 5. SDS-PAGE of HDL fractions (d = 1.063–1.21 g/ml) isolated from mice injected with P-407 or Triton. HDL from P-407-injected mice was similar both before (A) and following (B) P-407 injection. In contrast, mice injected with Triton WR-1339 (D) had a loss of apoA-I and apoC apolipoproteins from HDL compared to uninjected mice (C).

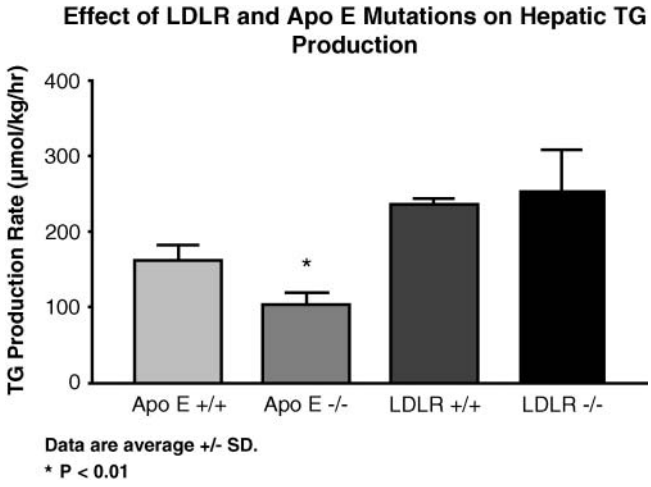

Fig. 6. TG production rate in *Apoe* $^{-/-}$ and *Ldlr* $^{-/-}$ mice treated with P-407. Plasma TG was determined as described in Materials and Methods. Rate was determined between 0 and 2 h following treatment. N = 4; data are average ± SD. * Significant difference versus *Apoe* $^{+/+}$; $P < 0.01$.

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