The Single Dose Poloxamer 407 Model of Hyperlipidemia; Systemic Effects on Lipids Assessed Using Pharmacokinetic Methods, and its Effects on Adipokines

Hetal R. Chaudhary and Dion R. Brocks.

Faculty of Pharmacy and Pharmaceutical Sciences, 3-142H Katz Group Centre for Pharmacy & Health Research, University of Alberta, Edmonton, Canada.

Abstract - Purpose: The induction of hyperlipidemia using poloxamer 407 (P407) is gaining use for studying the effect of the condition on drug pharmacokinetics. Although a single intraperitoneal dose of P407 causes a rapid onset of hyperlipidemia, the initial lipid concentrations are much higher than seen in humans. The hyperlipidemia is also reversible in nature. Here, pharmacokinetic methods were used to assess the P407 dose response on serum lipids, adipokines and cytokines. **Methods:** Single 0.5 and 1 g/kg doses of P407 were injected into rats followed by blood collection at various times for up to 12 d. Serum was assayed for lipids, selected adipokines and cytokines. **Results:** As expected, large increases in lipid levels were seen by 36 h after dosing. Using area under the concentration vs. time curve as a measure of systemic lipid exposure, P407 increased serum baseline corrected serum lipids in a nearly dose proportional fashion. The maximum increase in lipids was observed at ~36 h, with most lipids remaining elevated for up to ~180 h, although for the 1 g/kg dose triglyceride concentrations had still not quite returned to baseline by 12 days postdose. In addition to changes in lipids, P407 significantly increased serum leptin and decreased the serum adiponectin concentrations but did not affect cytokine levels. Conclusion: Depending on study aims, for the use of the model it may be beneficial to perform single-dose assessments at time points later than 36 h when the lipoprotein concentrations will be more similar to those seen in patient with hyperlipidemia.

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INTRODUCTION

Hyperlipidemia (HL), a condition of elevated serum lipoproteins, total cholesterol (TC) and triglycerides (TG), is a major risk factor for atherosclerosis, myocardial infarction and stroke. Because it normally is devoid of perceptible symptoms to the afflicted individual, monitoring of serum lipids provides an invaluable tool for the monitoring of risk and prevention of the associated serious cardiovascular events. Various animal models have been used to study the pathophysiological effects of HL. Genetic variants of HL, such as the obese Zucker rat are available. Alternatively HL can be induced by dietary means (e.g. chronic feeding of a high fat diet) (11,13,40,43) or by treatment with compounds such as Triton(14,26) or poloxamer 407 (P407) (16). P407, a non-ionic synthetic copolymer surfactant, provides an attractive means of inducing HL because of its rapid onset and seeming lack of overt toxicity; within 24 h of its intraperitoneal (i.p.) injection a profound HL state is achieved. It has been used to induce experimental HL in several rodent species including rat(16), mouse(32) and rabbit (3). With chronic administration it has been shown to induce atherosclerosis in the mouse, a species which is quite resistance to its onset (15).

P407 increases serum lipoproteins via its actions at various levels in lipid metabolism, largely by inhibiting lipoprotein lipase, which facilitates the hydrolysis of triglycerides (TG) (16). Johnston et al investigated the effect of P407 on lipoprotein lipase activity and found that after 3 h of P407 i.p. injection in rats the enzyme activity decreased by 95% compared to a normal saline treated controls (16). P407 also causes indirect stimulation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase which is involved in cholesterol biosynthesis (3,42).

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Correspondence Author: Dion R. Brocks, Ph.D.; Katz Group Centre for Pharmacy and Health Research; Faculty of Pharmacy & Pharmaceutical Sciences; University of Alberta; Edmonton, AB, Canada. Email: dbrocks@ualberta.ca

Several groups have used the P407 model of HL for the study of HL on pharmacokinetics and pharmacodynamics of a variety of drugs (4,7-8,20,22-23,25,33,35). In most of these assessments, the pharmacokinetics of the drugs were studied after dosing at 24 or 36 h from the time of P407 i.p. injection. At this time, very large increases present themselves in serum. This provides for a convenient screening tool for the influence of lipoproteins on drug behavior. However, the downside is that the changes occurs over a short period of time, and as such, longer term changes associated with HL might not be present at the time of assessment. Another issue is that lipoprotein concentrations at this time are many fold-higher than are seen in humans, and as such it is possible that the pharmacokinetic changes are exaggerated compared to what would be seen in humans with HL.

The HL induced by a single dose of P407 reversible in nature; within 2-3 days after injection it is visually evident that HL subsides, as the plasma/serum gradually changes from a milky white to a normal clear appearance. The plasma concentrations of lipids have been followed after dosing of rats with a single dose of 0.4 g/kg. Concentrations of total cholesterol (TC), triglyceride (TG) and high density lipoprotein cholesterol (HDL-C) were observed to fall after 12-24 h of dose administration. However, low density lipoprotein cholesterol (LDL-C) was still rising and the other serum lipids were still substantially higher than baseline levels at 48 h from the time of P407 dosing (24). In examining the literature, there is little direct information as to how long the increased lipoprotein levels persist until they return to baseline after each of the two most commonly used single dose levels of P407. More importantly, the only reports of dose responsiveness are based on the comparative maximum lipid concentrations in plasma/serum after use of different doses. A complete measure of systemic responsiveness, such as the area under the concentration vs. time curve of the respective serum lipids, has not to date been employed; use of a single concentration at a single point in time is suboptimal in assessing the full systemic effect, and under some situations could be misleading.

The purpose of this study was, using pharmacokinetic methods, to clarify the level of exposure and duration of elevated serum lipids after dosing of rats with the two most commonly used doses of P407. Because adiponectin, leptin and TNF-α are associated with cardiometabolic syndrome, we also investigated the effect of the P407 model on serum adipokine and cytokine concentrations. It was hoped that the information elicited could be useful in guiding use of the model for studies examining the effect of hyperlipidemia on drug pharmacokinetics.

METHODS

Materials and reagents
Poloxamer 407 was obtained from Sigma Aldrich (St. Louis, MO). A kit for quantitative determination of HDL cholesterol (HDL-C) was purchased from Wako chemicals (Richmond, VA). Enzymatic assay kits for measurement of TG and TC in serum were obtained from Genzyme Diagnostics (Charlottetown Canada). A calibrator for TG, DC-CAL calibrator, was also purchased from Genzyme diagnostics. ELISA kits for adiponectin and leptin were obtained from Alpco Diagnostics (Salem, NH) and the TNF-α and IL-6 kits were purchased from Invitrogen Corporation (Camarillo, CA).

Animals
The animal study was approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. The male Sprague–Dawley rats (weight: 250-350 g) were purchased from Charles River Laboratories, Montreal Canada. A total of 10 rats were used in the study.

Dosing and collection of serum
A colloidal solution of P407 was prepared by dissolving 7 g in 52 mL of cold normal saline (0.13 g/mL), which was stored at 4º C. Rats were anesthetized with isoflurane and oxygen and the P407 dose was carefully injected intraperitoneally at a dose of 0.5 g/kg or 1 g/kg. Blood samples (up to 0.2 mL per sample, up to 5 samples per rat) were collected serially from the tail veins at various times ranging from 0 to 282 h after each dose. There were between 3 and 5 samples obtained at each time point, which were used for determination of indices of serum lipid exposure. After leaving the blood to clot for ~30 min at room temperature, serum was separated by centrifugation and stored at -20º C until assayed for analytes.

Lipid, adipokine and cytokine measurement
The serum concentrations of TG, TC and HDL-C were measured according to the manufacturer's instructions. The lipid concentrations in each

sample were calculated using standards provided in the kits, taking into account any dilution factors required. Adiponectin, leptin and TNF-α were measured using ELISA kits as per the manufacturer's direction. For analysis of lipids, where necessary dilution of the serum from P407 treated rats was accomplished using deionized water.

Data analysis

The Friedewald equation was used to attempt to measure the concentrations of LDL-C ([LDL-C] = TC – [HDL-C] – TG/5) in the rats (17). The non-HDL-cholesterol (non-HDL-C) concentrations, indicating the cholesterol concentrations in lower density lipoproteins (chylomicrons, low and very low density lipoproteins) was calculated as the difference between TC and HDL-C. The highest measured concentrations of the lipids (C_{max}) and the time at which they nominally occurred were recorded, as was the area under the serum concentration vs. time curve, which was calculated using the linear trapezoidal rule. The terminal phase half-lives of TG and total cholesterol were determined by regression analysis of the baseline-corrected log-transformed concentrations of lipids during the terminal phase. The AUC was determined using the total concentrations of each lipid component measured, as well as for that of baseline corrected concentrations (measured as [total concentration] – [P407 pre-dose concentration]). The increase in mean AUC of each measured lipid component attributed to dosing of rats with P407 was estimated as $increase = \frac{AUC_{total}}{AUC_{total} - AUC_{predose}}$. A return to baseline lipid concentrations was defined as the time after dosing that the mean concentrations had fallen from maximum values to within 150% of the initial baseline (predose) mean concentrations.

Significance of differences between serum lipid, adipokine and cytokine concentrations at baseline (predose) to those after each dose of P407 was determined using Student's t-test for unpaired samples. Because of difficulties in obtaining blood from the tail vein of all rats and time points, it was not possible to determine the full AUC of lipids in each rat. Hence to permit statistical comparison of the AUC between groups, the Bailer's method was used. α was set at 0.05 and critical value of Z (Zcrit) for a two sided test was adjusted at 2.24.The Z value calculated as mentioned previously (2).

RESULTS

Lipid and lipoprotein concentrations

As expected, both doses of P407 caused relatively rapid and noticeable changes in the serum lipid concentrations (Figure 1). For both dose levels, the C_{max} of triglyceride and total cholesterol were substantially higher after P407 compared to predose values; the apparent time of Cmax (t_{max}) of both lipids was seen at 36 h after dosing. For the C_{max}, the maximum fold increases in serum TC for the 0.5 and 1 g/kg doses were 20 and 44, respectively, increasing in a nearly dose-proportional fashion. The TG also increased substantially by 41- and 48-fold after doses of 0.5 and 1 g/kg P407, respectively (Table 1). The C_{max} of HDL-C was also higher but the increase was noticeably less (6.7 and 17-fold for the 0.5 and 1 g/kg doses, respectively) than that of the other lipid components.

Compared to baseline concentrations (Table 1), the increase in TC AUC attributable to P407 was found to be 4.5 and 16-fold for the 0.5 and 1 g/kg doses, respectively. Thus, this increase in TC was somewhat higher than the relative increase in dose. In contrast, the corresponding increase in TG was nearly proportional to the difference in dose (6.5 and 15 for the 0.5 and 1 g/kg doses, respectively). As for TC, the corresponding increase in HDL-C AUC was somewhat higher than the relative increase in dose (3.1 vs 8.8-fold for the 0.5 and 1 g/kg P407 dose levels, respectively). Unlike AUC, the relative increase in Cmax of TG was very similar and relatively less than proportional when comparing the 0.5 and 1 g/kg doses. In comparison for TC and non-HDL-C the increase in Cmax was at least proportional to the increase in dose.

In all rats the HL was reversible in nature, although there were some differences in the duration of time over which elevated lipid concentrations presented between the lower and higher dose of P407. For example the HDL-C had returned to baseline conditions within 5.5 and 6.5 days for the 0.5 and 1 g/kg doses, respectively. In contrast, the TC took 7.5 days to return to baseline conditions with both dose levels. In comparison, the increase in TG was more sustained. With a dose of 0.5 g/kg, TG concentrations took about 10 days to return to baseline. With the dose of 1 g/kg, mean TG concentrations were still >1.5-fold higher than baseline between the last measured pair of TG concentrations (see inset, Figure 1).

Table:1. P407 altered concentrations of total cholesterol, triglyceride and HDL cholesterol as (mean ± SD) and other calculated parameters.

Parameters	TC	TG	HDL-C	Non HDL-C	TC	TG	HDL-C	Non HDL-C
	0.5 g/kg				1 g/kg			
C_0, mg/dL	60.7±10.8	179±40.5	29.5±8.24	31.2±7.43	60.7±10.8	179±40.5	29.5±8.24	31.2±7.43
C_{max}, mg/dL	1272±182*^	7005 ±1176*^	197±22.0*	1076±143	2391±273*	9973±1004*	508±344*	2256±195
BC C_{max}, mg/dL	1211±173*^	6826±1142*^	168±20.7*	1045±143	2330±273*	9794±1006*	479±341*	2224±195
$AUC_{0\text{-tlast}}$, mg·h/mL[§]	827±27.0^	3410±165^	156±14.6^	581±22.2[T]	1974±81.2	7165±373	298±40.9	1385±66.3[T]
BC $AUC_{0\text{-tlast}}$, mg·h/mL[§]	676±25.8^	2954±161^	118±13.9^	540±22.0[T]	1858±84.6	6698±576	256±41.8	1344±66.3[T]
$t_{\frac{1}{2}}$, h	12.6	13.2	ND	ND	18.3	15.0	ND	ND

C_0, Baseline (pre-dose) concentration at time 0 h; C_{max}, maximum serum concentration; BC, baseline corrected; $AUC_{0\text{-tlast}}$, area under the concentration vs time curve from time of dosing to last measured concentration.
^ Significant difference between 0.5 g/kg and 1 g/kg dose (P<0.05), * Significant difference in concentration from C_0 concentration (P<0.05). § The last measured concentration (tlast) was that at which concentrations returned to predose conditions (250-280 h for TC and TG, and 132 to 180 h for HDL-C).
T= truncated to 132 h to allow for calculation of the parameter in samples drawn at the same time points

Because most of the calculations yielded negative values it was not possible to estimate the LDL-C concentrations using the Friedewald equation.
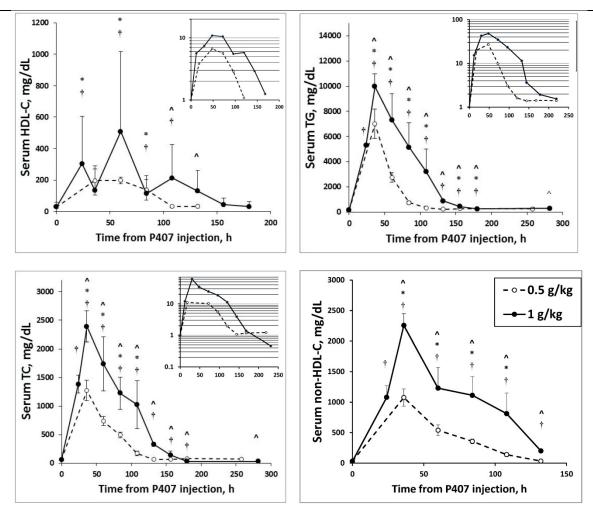
Adipokines and cytokines

The serum concentrations of TNFα and IL6 were near the lower limits of quantitation of the assay in all NL and HL rats, and there was no apparent increase in the concentrations of either cytokine in response to P407 administration. Changes were discernible, however, in the adipokine concentrations in the rats after dosing with P407 (Figure 2). For adiponectin, a similar decrease was observed in the serum concentrations after both dose levels. Mean pre-dose adiponectin concentrations were 8.0±0.72 and 10.7±2.4 ng/mL for rats in the 0.5 and 1 g/kg P407 dose groups, respectively. When expressed as percent change from pre-dose values, the mean concentrations tended to decrease in a similar pattern in both groups with a nadir occurring at 50-100 h post-dose, followed by a rise in concentrations back to predose concentrations. Baseline concentrations tended to be reached again at 150-200 h after P407 administration. The decrease in the AUC was similar for both dose levels, with the average concentration (calculated as AUC0-t$_{last}$/t$_{last}$) being 24% less for both doses levels compared to predose concentrations.

Baseline concentrations of leptin were 280±62 and 502±168 pg/mL in the P407 0.5 and 1 g/kg dose groups, respectively (Figure 2). With respect to the percent change in concentrations from baseline, in contrast to adiponectin, leptin concentrations rose after P407 in both dose groups.
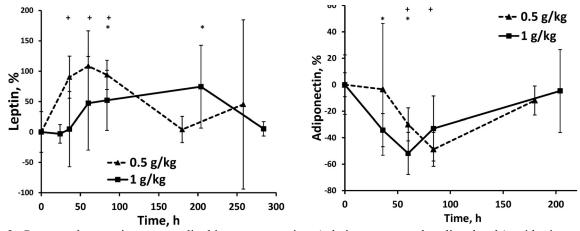
As seen for adiponectin, leptin concentrations eventually returned to baseline conditions in both groups, although the increase in concentrations from baseline seemed to take longer with the higher dose of P407. Over the time course following P407 back to baseline, the mean leptin concentrations were increased 50 and 44% for the 0.5 and 1 g/kg dose levels.

DISCUSSION

Most investigations involving P407 as a rodent model of HL have used doses of 0.5 or 1 g/kg. Accordingly with these doses and our sampling schedule, we found that serum TG and TC concentrations reached a peak at approximately 36 h after dosing (Figure 1). There has been some attention paid to the reversible nature of HL after P407. For example, Blonder et al (3) reported lipid concentrations in New Zealand albino rabbits given P407 doses ranging from 5.5 to 137.5 mg/kg, much lower than used by most investigators. Afterwards they followed the serum TG and TC concentrations for up to 14 d. A significant increase in serum lipids was seen only at one time point (2 days postdose), and only with the highest dose level. In rats given P407 at i.p. doses of approximately 1 g/kg, after sampling at 24, 48 and 96 h, peak serum TC and TG occurred at 48 h postdose (42).

Figure 1. Serum lipid concentration vs. time curves after i.p. administration of P407 (each data point is represented by the mean of 3-5 rats). The inset shows the ratio of the average concentration over each interval to the predose concentration (where the ratio = 1), vs. the midpoint of each sampling interval, plotted on semi logarithmic scale. ^Significant difference between 0.5 (open symbols) and 1g/kg (closed symbols) doses (P<0.05); *significant difference from predose concentration for 0.5 g/kg dose (P<0.05); †significant difference from predose (0 h) concentration for 1 g/kg dose; ^ significant difference between 0.5 and 1 g/kg dose levels.

Figure 2. Percent changes in serum adipokine concentration (relative to mean baseline levels) with time after i.p. administration of P407 (each data point is represented by the mean of 4-5 rats). +significantly different from predose (0 h) concentration for 0.5 g/kg dose; *significantly different from predose concentration for 1 g/kg dose (P<0.05).

Similarly, in the present study it was found that rats achieved maximum concentrations of TG and TC after 36 h of P407 dose. At 96 h Wout et al. found that the serum concentrations of both lipid components were still much higher than predose concentrations (42). After administration of 0.4 g/kg to 3 male Sprague-Dawley rats, Lee et al. recently reported that mean TG, TC and HDL-C plasma concentrations peaked at 12, 24 and 36 h after i.p. dosing (21). At 48 h postdose, similar to the current findings in serum, plasma concentrations had started to subside in magnitude.

In the current assessment the reversible nature of the HL that ensued from the i.p. administration of P407 was fully characterized for TG, HDL-C and CHOL; to our knowledge, such an in depth examination of this essential aspect of the model has not been previously performed. Similar to us, Palmer et al. had previously described that after administration to an unspecified species of rat, plasma TC was still slightly above normal at 120 h after a 0.5 g/kg dose of P407, although exposure after 1 g/kg, and calculation of AUC, was not performed.

Based on our results at the frequently used dose of 1 g/kg it takes at least 12 days for each of the serum lipid components to return to their pre-dose concentrations. Using a comprehensive measure of systemic exposure of lipids after P407 (AUC) it was observed that increases in systemic concentrations of TC and TG occurred, in a manner that was equal to or larger than that relative to the dose level. It would appear that the rise in lipid concentrations lags slightly behind the rise in plasma concentrations of P407 in rats given 1 g/kg (which peaks at about 12 h post-dose), but decline accordingly as the concentrations of the agent decline in plasma (27). The terminal half-life of P407 has been estimated at 20.9 h based on urine concentrations (34), which was close to the estimated half-life of decline of TC and TG after 1 g/kg doses (Table 1). The use of the Friedewald equation was unsuccessful in our rats probably due to a combination of the known higher-than-human concentrations of HDL-C in control rats, coupled with, in P407 treated rats, the very high concentrations of TG (39). Although it could not be estimated here, it is known through direct measure of cholesterol in the LDL fraction of plasma that LDL-C concentrations are very high at 36 h after P407 (37).

The P407 model has been used quite frequently to assess the effect of HL on the pharmacokinetics and pharmacodynamics of drugs; this has been the main focus of our research group in using the model. In the conduct of these evaluations, the test drug has usually been administered at 36 h after the administration of the dose, to coincide with the maximum concentrations of lipids. One of the limitations of this approach is that compared to basal concentrations in rodents, the relative increases in serum TC and TG concentrations that occur at this time are considerably higher than would be normally seen in HL vs. NL patients (31). The data obtained from such studies are still quite useful in that they demonstrate the possibility of HL causing changes in pharmacokinetics in humans. However, because the lipid concentrations are so high at 24 to 36 h, the exact response of HL in a human population on the pharmacokinetics of that drug might be exaggerated. This is particularly true of an examination of the unbound fraction of drug, and consequent effects on total (bound+unbound) drug clearance and volume of distribution. On the other hand, by initiating the pharmacokinetic study at 36 h after P407, the duration of time over which lipoprotein concentrations have been elevated is relatively brief. In a HL patient population the high lipoprotein concentrations are chronically sustained, which might have a more significant impact on other factors such as gene expression of proteins involved in drug disposition. Hence, in some aspects single doses of P407 might also underestimate the impact of the condition on certain aspects of drug pharmacokinetic behaviour. This is especially relevant for drug transport or metabolism, because it is known that in HL a downregulation of both types of proteins may occur (5,19,36).

The current data may be helpful in design of future drug pharmacokinetic assessments that use the P407 model. For example, by performing the pharmacokinetic study at a later time point from the time of dosing of the P407, two advantages might be realized. First, serum concentrations of the serum lipoproteins and lipids might be present at levels closer to those seen in people. Secondly, by performing the pharmacokinetic assessment at a later time point, the duration of HL would be more sustained prior to conducting the study, thereby more closely mimicking the situation experienced by patients with HL. In It can be seen that even if the pharmacokinetic studies are initiated at least 108 h after dosing of 1 g/kg P407, HL is still apparent for many hours thereafter. It is difficult to find in the literature known ranges (minimal and

maximal) of serum lipids in a human population, but it would appear that the ratio of maximal to minimal TG and TC concentrations can vary by at least 14- and up to 3-fold, respectively (31). Depending on the dose of P407, these increases correspond roughly with at least 5 days after administration of P407 (see insets Figure 1).

Hyperlipidemia is commonly present in obese patients, in which the excess of white adipose tissue is associated with an accentuated release of bioactive peptides including adipokines and pro-inflammatory cytokines (12). Obesity-related disorders including the cardiometabolic syndrome (with components of diabetes, atherosclerosis, hypertension, and coronary artery disease) are associated with decreased plasma concentrations of adiponectin (38). Leptin is a crucial factor involved in regulation of food intake, body weight, energy expenditure, maintenance of insulin sensitivity, anti-inflammatory response and regulation of blood pressure (6,12,41). In obesity hyperleptinemia is common, thought to ensue from resistance to leptin receptors for the hormone (6). Higher serum concentrations of the proinflammatory cytokines IL-6 and TNF-α commonly occur in obesity, which contributes to insulin resistance and which further contributes to the development of cardiometabolic syndrome (1,9,30). An understanding of the impact of these components is important in assessing the outcome of pharmacokinetic experiments involving the P407 HL model.

A potential modifying influence on drug disposition is inflammation, which is often associated with a decrease in the expression of drug metabolizing enzymes and transport proteins (18,39). We could not determine any significant increase in TNF-α concentrations from baseline over the 12 day assessment period, nor were measurable concentrations of IL-6 detected in any of the rats. Indeed, even with a 21 day repeated dose administration protocol of P407, Joo et al. did not observe any increase in IL-1 or TNF-α plasma concentrations (17). This suggests that the P407 model is not overtly inflammatory, and that inflammation is an unlikely contributor to the changes observed in pharmacokinetics of drugs studied using the model.

In humans there appear to be relationships between the concentrations of adipokines and dyslipidemia (10,29). Gannag-Yared et al investigated the association between adiponectin and leptin with the markers of cardiometabolic syndrome including lipid concentrations, insulin sensitivity and steroid concentrations in healthy non-diabetic Lebanese men. They found a negative correlation between serum adiponectin, waist size and TG. However, HDL cholesterol was positively correlated with adiponectin (10). In their subjects the correlation between leptin and lipid profile was poor. Matsubara et al investigated the effect of dyslipidemia on adiponectin concentrations in non-diabetic women (29). It was found that high triglyceride and low HDL cholesterol were associated with low plasma adiponectin concentrations. Some of these associations were likewise present in our rats given P407. For example, the elevated serum concentrations of atherogenic lipids (TG and CHOL) were associated with a decrease in adiponectin concentrations. Although HDL-C concentrations were observed to be increased, the magnitude of increase in that component was dwarfed by the high concentrations of the lower density categories of lipoprotein after P407 (the non HDL-C fraction). It was of interest that the concentrations of leptin were increased in accordance to the increase in TG and TC concentrations, and that like adiponectin, its concentrations similarly returned to baseline concentrations with the progression of time. In rats administered a high fat diet for 12 weeks, leptin concentrations were reported to be decreased rather than increased (28). In that study, there were reported increases in TNF-α as well, unlike what we observed with single doses of P407.

In conclusion, single doses of P407 caused significant increases in the AUC of TG, TC, HDL-C and non-HDL-C which were nearly proportional to the dose administered. Changes were also observed in two adipokines, adiponectin and leptin. The single dose administration of P407 leads to reversible yet sustained increases in plasma lipids that persist for up to 12 days afterwards. There was no indication based on assay of TNF-α or IL-6 that the model is associated with inflammation. Transient changes were seen in in serum adiponectin and leptin. The information elicited may be helpful in designing experiments that more closely mimic patients with HL.

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