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MECHANISM OF POLOXAMER 407-INDUCED HYPERTRIGLYCERIDEMIA IN THE RAT

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Abstract—One 300 mg i.p. injection of the nonionic surfactant poloxamer 407 (Pluronic® F-127) produces a significant increase above control of both circulating cholesterol and triglyceride (TG) concentrations. The present study was conducted to determine the effect of poloxamer 407 (P-407) on the capacity to hydrolyze circulating TG by lipoprotein lipase (LPL) in an attempt to determine the mechanism of action of P-407. The concentration of TG in the rat following a single 300 mg i.p. injection of P-407 was marked, increasing from 84 ± 10 to 3175 ± 322 mg/dL at 24 hr. The maximal rate of TG accumulation (5.74 mg/dL/min) in the plasma of P-407-injected rats occurred between 2 and 4 hr post-injection. *In vitro* incubation of LPL with P-407 significantly inhibited enzyme activity with an inhibitory concentration at which 50% of the enzymatic activity was lost of approximately 24 μM. Concentrations of P-407 exceeding 350 μM *in vitro* completely inhibited LPL activity. The effects of P-407 on the enzymatic activity of LPL in post-heparin plasma obtained following a single 300 mg dose of P-407 to rats demonstrated greater than 95% suppression of LPL activity 3 hr post-injection compared with controls. Inhibition of LPL activity was greater than 90% as long as 24 hr following a single i.p. injection of P-407. However, while the heparin-releasable fraction of capillary-bound LPL was inhibited in the plasma, LPL activity significantly increased in cardiac and skeletal muscle in poloxamer-injected animals compared with sham-injected controls. Although there was no significant change in LPL activity in adipose tissue, testes, and lung resulting from P-407 treatment, LPL activity increased by 37% in myocardium, 69% in soleus, and 66% in gastrocnemius muscle in P-407-injected rats when compared with controls. Our studies would suggest that the predominant mechanism by which P-407 induced an increase in circulating TG was by a reduction in the rate at which TG was hydrolyzed due to inhibition of heparin-releasable LPL by the surfactant.

Coronary heart disease (CHD§) and related disorders are a leading cause of death in the United States. One primary risk factor associated with CHD is hyperlipidemia. Recently, much attention has been dedicated to controlling hyperlipidemia. Changes in both diet and lifestyle (regular exercise) combined with improved, more frequent testing and potent drugs to treat hyperlipidemia are some of the strategies being used to reduce the number of premature deaths due to CHD [1]. Hyperlipidemia has been evaluated in many experimental animal models including pigeons, rodents, rabbits, dogs, swine, and nonhuman primates [1]. Each of these animal models has advantages and disadvantages when compared for cost and ability to replicate the human condition.

We recently developed a hyperlipidemic rat model in which a single i.p. injection of the agent poloxamer 407 (P-407) resulted in elevations of total plasma cholesterol and triglycerides TG [2]. Poloxamer 407 is a block copolymer composed of a hydrophobe that is flanked on each side with hydrophillic polyoxyethylene units. Our previous findings demonstrated that elevation in plasma TG was more sensitive than elevation in total plasma cholesterol following P-407 administration. Due to an almost 10-fold larger plasma titer of TG compared with plasma cholesterol following a single injection of P-407, the present study was conducted to determine the mechanism by which P-407 causes hypertriglyceridemia in the rat.

Lipoprotein lipase (LPL), the enzyme responsible for the hydrolysis of circulating TG, is found in at least two distinct pools in tissue [3]. One fraction, which is readily released by perfusion with heparin, is localized on the surface of endothelial cells where it is directly involved in the hydrolysis of plasma lipoprotein TG. The second fraction, which remains in tissue after perfusion with heparin, is thought to represent a precursor or intracellular storage pool for the endothelium-bound enzyme. The measurement of capillary-bound enzyme activity is an estimate of the capacity to clear TG from the circulation. The heparin-releasable fraction of LPL is the best indication of the amount of capillary-bound enzyme [4].

An increase in plasma TG titer of the fasted rat occurs primarily as a result of increased TG synthesis and secretion by the liver and/or a reduction in the

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§ Abbreviations: CHD, coronary heart disease; HDL, high-density lipoprotein; LPL, lipoprotein lipase; P-407, poloxamer 407; TG, triglycerides; and VLDL, very-low-density lipoprotein.

removal or hydrolysis of TG from the peripheral circulation. Since it is reported in the literature [5, 6] that other nonionic-surfactant compounds similar to P-407 produce hypertriglyceridemia by inhibiting LPL, our initial focus was on LPL. It has been shown by a number of investigators that an i.v. injection of Triton WR-1339, a compound with a chemical structure similar to that of P-407, produces hypertriglyceridemia [7, 8]. In fact, when hepatic TG secretion rates are being quantitated, WR-1339 is the agent used to block TG removal from the circulation [9]. Therefore, we attempted to determine the role of LPL in P-407-induced hypertriglyceridemia; specifically, is the resultant increase in circulating TG the result of a reduced capacity for TG hydrolysis?

MATERIALS AND METHODS

Animal care and handling. Male Wistar rats, weighing between 200 and 250 g, were obtained from Charles Rivers Laboratories (Wilmington, MA). The animals were housed individually under controlled conditions for at least 3 weeks prior to experimentation to reduce the effects of changing circadian and feeding schedules. During this time, the animal room was maintained at a temperature between 21° and 23°. Rats were exposed to a standard 12-hr light–dark cycle (lights on 7:00 a.m. to 7:00 p.m.). Until the evening prior to experimentation, animals were provided unlimited access to a diet of Purina chow and water. At the time of the experiment, the animals weighed between 300 and 400 g.

Triglyceride accumulation. To determine the effect of P-407 on the rate of TG accumulation in the plasma, food was removed from the animal cages at 4:00 p.m. on the day prior to experimentation. The overnight fast would minimize the contribution of postprandial lipid to the plasma TG concentrations. The following morning (8:00 a.m.), rats received a 1-mL injection of 300 mg of P-407 (i.p.). Blood samples (0.4 mL) were collected from each animal into heparinized tubes by the tail clip method immediately prior to poloxamer injection and 1, 2, 4, 6, 8, 12, and 24 hr following drug administration. Plasma was prepared from the blood and stored at −80° until the time of TG analysis.

In vitro *effects of P-407 on LPL.* To determine the effects of P-407 on LPL *in vitro*, two male rats were anesthetized with ether. They each received an injection (i.v.) of 1000 I.U. of heparin in a volume of 0.3 mL. Two minutes following the heparin injection, a midline incision was made in the abdomen of each rat. The descending aorta was exposed, and a blood sample was taken from each animal using a syringe fit with a 20-gauge needle. Plasma was pooled and frozen until experimentation. During experimentation 5 μL of plasma was incubated in the cold with 95 μL of saline containing various concentrations of P-407. Following 1 hr of incubation, LPL activity was measured. Control experiments indicated that the 1-hr incubation of enzyme in normal saline alone (no poloxamer) had no effect on LPL activity.

P-407 effects on heparin-releasable LPL. To determine the effect of P-407 on heparin-releasable LPL activity, fasted rats were injected (i.p.) with 1 mL of 300 mg of P-407 at 8:00 a.m. A sham-injected control group received a similar set of injections of saline. At 3, 6, 12, and 24 hr following injection, a 0.4-mL blood sample was taken via the tail clip to assure that plasma was hyperlipidemic. Hyperlipidemic rats were then anesthetized with ether, and a 0.3-mL injection (i.v.) containing 1000 I.U. of heparin was administered to both saline and P-407-injected animals. Two minutes following heparin injection, the abdominal aorta was exposed and a blood sample was taken. Plasma was prepared and stored at −80° for subsequent analysis of LPL activity. Pilot experiments indicated that plasma could be stored for as long as 4 months without loss of enzyme activity.

Tissue LPL activity with P-407. LPL is synthesized in tissue parenchymal cells and transported to the capillary endothelium. A wide variety of physiological [10] and pharmacological [11] treatments have been shown to modify tissue LPL activity. This could reflect alterations in enzyme synthesis and/or secretion. To determine the effect of P-407 on tissue LPL, fasted rats were injected with 300 mg of P-407. Twenty-four hours later rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Tissues were taken in the following order: soleus, primarily composed of slow-twitch oxidative red fibers; gastrocnemius, made up primarily of fast-twitch glycolytic and fast-twitch oxidative glycolytic fibers; adipose tissue; testes; lung; and myocardium. Immediately following removal, tissues were weighed and put in cold 50 mM HEPES (*N*-[2-hydroxyethyl]-piperazine-*N*′-[2-ethanesulfonic acid]) buffer containing 20% glycerol (pH 7.5). The final tissue concentration was 25 mg/mL buffer for heart and soleus muscle and 50 mg/mL for all other tissues. Tissues were then homogenized for 30 sec using the Polytron (Brinkmann Instruments, Westbury, NY) set at full speed. This treatment elicits maximal tissue LPL activity [2]. Aliquots of whole tissue homogenate were then assayed for LPL activity.

Analytical techniques. The triglyceride concentration of plasma samples was determined using the coupled enzyme diagnostic kit. Lipoprotein lipase activity was measured in plasma and tissue samples using the technique described by Nilsson-Ehle and Schotz [13]. This method utilizes a lecithin stabilized [^{3}H]triolein-glycerol emulsion as substrate. Tissue and plasma enzyme activities were measured by adding enzyme to the substrate. In the *in vitro* experiments, substrate was added to the preincubated enzyme. Calf serum was included in assays of tissue LPL activity to provide a source for apoprotein C-II, a necessary cofactor for LPL activity. Free fatty acids released into the assay medium were extracted by the method of Belfrage and Vaughan [14] after 60 min of incubation at 37°. All assays were linear with respect to time and enzyme content.

Materials. Poloxamer-407 (Pluronic® F-127) was obtained from the BASF Corp. (Parsipanny, NJ). The 30% (w/w) poloxamer solution for i.p. injection was prepared by placing solutions on ice overnight to facilitate dissolution of the polymer according to the "cold method" of incorporation [15]. [^{3}H]Triolein

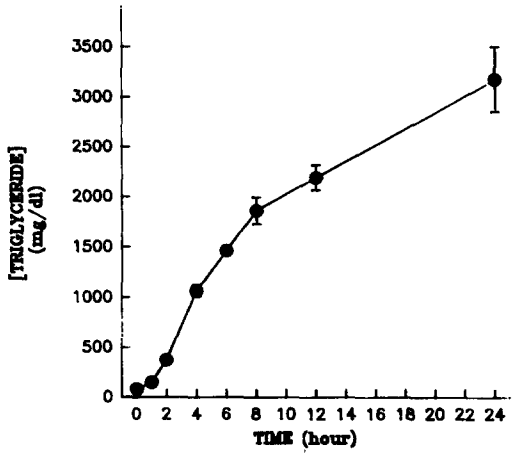

Fig. 1. Triglyceride concentration in the plasma of rats at various times following the administration of a single 300 mg dose of P-407 by i.p. injection. Values are means ± SEM (N = 6). Where no error bars are visible, error is within the symbol.

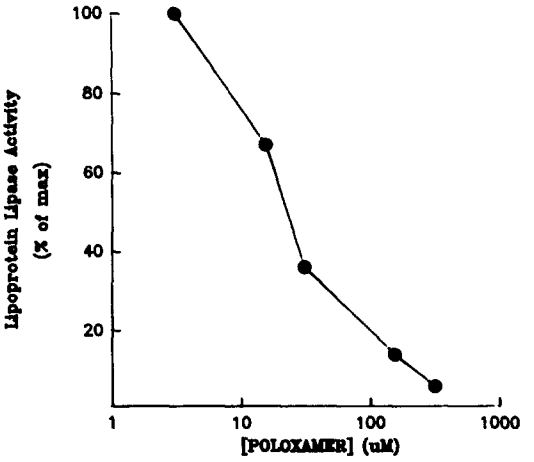

Fig. 2. Effect of incubating increasing concentrations of P-407 on the activity of lipoprotein lipase contained in post-heparin plasma from rats. Values are means of duplicate samples assayed twice. Activity equivalent to 100% was 482.66 nmol/mL/min.

(16.1 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). Heparin was from Elkins-Sinn, Inc. (Cherry Hill, NJ). Calf serum was purchased from Gibco Laboratories (Grand Island, NY). The diagnostic TG assay kit was obtained from the Sigma Chemical Co. (St. Louis, MO). All other materials were standard laboratory items.

Statistical methods. All values are given as means ± SEM. Student's *t*-test was used to compare two means [16].

RESULTS

It is quite apparent from Fig. 1 that P-407 had a profound effect on the plasma TG concentration of fasted rats. From a control value of 84 ± 10 mg/dL, TG increased throughout the 24-hr period to 3175 ± 322 mg/dL, an increase of more than 37-fold. The maximal rate of TG accumulation occurred between hr 2 and 4 following P-407 treatment. During that time, TG accumulated in the plasma at an average rate of 5.74 mg/dL/min.

Since the primary mode of TG removal from the circulation is through its hydrolysis by LPL, we determined the effect of various concentrations of P-407 on LPL activity *in vitro*. The results of this experiment are illustrated in Fig. 2. As the dose of P-407 was increased, the activity of LPL was reduced. Fifty percent of the enzyme activity was inhibited at a poloxamer concentration of 24 μM. Lipoprotein lipase activity was abolished completely at P-407 concentrations at or above approximately 350 μM.

The effect of one 300 mg injection (i.p.) of P-407 on heparin-releasable LPL activity, at various times following drug administration, is presented in Fig. 3. Three hours following P-407, heparin-releasable LPL activity was reduced more than 95% compared with plasma from control saline-treated rats. This

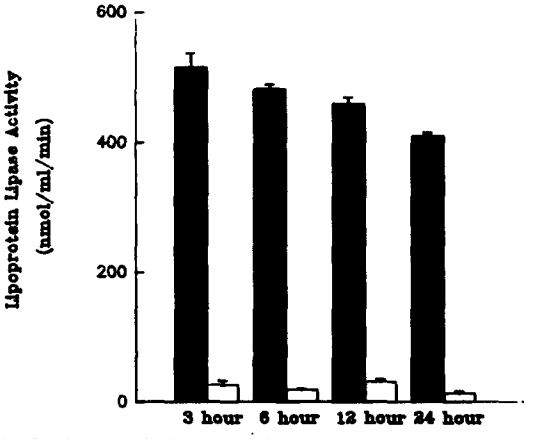

Fig. 3. Lipoprotein lipase activity contained in post-heparin plasma of rats at various times following a single 300 mg injection of P-407 (□) or normal saline (■). All values are means ± SEM (N = at least 5 animals per group). All P-407 activities were significantly different ($P < 0.01$) from the comparable saline control activity.

inhibition remained greater than 90% throughout the entire 24-hr period.

The effect of one injection of P-407 on total tissue LPL activity is shown in Fig. 4. There was a significant increase in enzyme activity in homogenates of skeletal and cardiac muscle, whereas there were no activity changes in adipose tissue, testes, and lung resulting from P-407 treatment. Activity increased 37% in myocardium, 69% in soleus, and 66% in gastrocnemius muscle in P-407-injected rats.

DISCUSSION

Wout and coworkers [2] reported that intra-

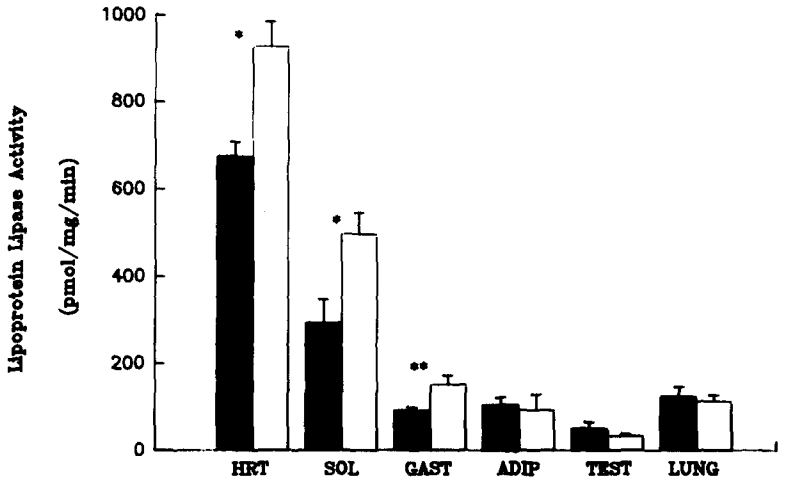

Fig. 4. Tissue lipoprotein lipase activities in rats determined 24 hr following an i.p. injection of P-407 (□) or normal saline (■). Abbreviations: HRT, heart; SOL, soleus; GAST, gastrocnemius; ADIP, adipose; and TEST, testes. All values are means ± SEM (N = 6). Asterisks indicate a significant difference between P-407 and saline activities in comparable tissue: (*$P < 0.01$, and **$P < 0.05$).

muscular and intraperitoneal administration of P-407 increases plasma TG and cholesterol concentrations in the rat in a dose-dependent manner. Our data supported their findings regarding hypertriglyceridemia.

When capillary bound LPL, released into the plasma by heparin injection, was incubated with P-407, enzyme activity was inversely proportional to the amount of P-407 in the incubation medium. Since the activity of a model enzyme, urease, was not affected by poloxamer [17], it would suggest that P-407 exhibits a level of specificity in modifying LPL activity. In addition, the design of this experiment suggests that the poloxamer affects the enzyme directly and does not reduce enzymatic activity by interfering with the interaction between LPL and the substrate at the level of the TG. This is apparent because the exogenous substrate was added in significant excess of the P-407 in our *in vitro* studies.

To determine if heparin would influence the *in vitro* inhibition of LPL by P-407, 1.5 I.U. of heparin was included during the 60-min incubation. This concentration of heparin had no effect upon the inhibition of LPL activity (data not shown). Since heparin would not reverse this inhibition, we went on to determine the *in vivo* effects of P-407 upon heparin-releasable LPL.

In the present experiment, when heparin-releasable LPL activity was calculated as an index of the capacity to clear plasma TG from the circulation 3–24 hr following P-407 administration, LPL activity was inhibited more than 90%. In an experiment where both compounds (Triton WR-1339, i.v.; and P-407, i.p.) were administered simultaneously, TG accumulation rates were approximately equivalent to those seen in animals that received only P-407 (data not shown). This would indicate that the poloxamer completely inhibits TG hydrolysis. In addition, since LPL was inhibited throughout the entire 24 hr, changes seen in the rate of TG accumulation throughout the 24 hr following drug administration probably reflect alterations in hepatic TG secretion rate.

The present study would indicate that P-407-induced hypertriglyceridemia was due to reversible or irreversible inactivation of LPL. While it has been suggested that hypertriglyceridemia resulting from Triton WR-1339 administration to rats was due to the formation of a surface layer of the detergent around lipoproteins which protected the very-low-density lipoproteins (VLDLs) from hydrolysis by LPL [18], our data would suggest a more direct effect of P-407 on the heparin-releasable fraction of LPL. This direct effect on LPL *in vitro* and *in vivo* was also reported by Hayashi *et al.* [5] using Triton WR-1339. Hayashi *et al.* demonstrated a greater than 90% inhibition of LPL activity *in vitro* at a Triton concentration of 10 mg/mL. When Triton WR-1339 was administered to rats (150 mg/100 g body weight), the activity of LPL in rat liver lysosomes determined 96 hr following Triton administration decreased 66% compared with controls [5]. Lastly, perhaps an additional reason for the increased concentration of plasma TG following injection of P-407 into rats is that cofactor protein C-II (an activator of LPL) was inactivated due to its displacement from high-density lipoprotein (HDL) following P-407 injection [8]. The presence of intact HDL cofactor complex may be an additional reason for the enormous increase in plasma TG following injection of P-407 in rats [8].

A significant reduction in the enzymatic activity of LPL *in vitro* and capillary-bound LPL in post-heparin plasma by P-407 may suggest that the surfactant denatured LPL with a subsequent loss in the enzymatic activity. However, while the denaturant effects of surfactants have long been recognized for anionic, cationic, and nonionic

surfactants, the latter class of surfactants has been shown to be relatively inert with regard to enzyme activity. Many enzymes undergo enhanced activity in the presence of nonionic surfactants, and some enzymes that have lost activity due to exposure to extremes in temperature, pH, ionic strength, etc. undergo renaturation to the native state or conformation upon addition of nonionic surfactants [19]. This effect has been exploited in the fields of biochemistry and pharmaceutics to hinder aggregation [20] and prevent precipitation and/or denaturation of proteins [19, 21]. In fact, the poloxamer family of surfactants has found use in this regard [20, 22]. Determination of whether P-407 irreversibly inactivates LPL will require future experiments.

Several different treatments have produced increases in muscle LPL activity similar to that seen in this study. The increased activity of LPL determined in cardiac and skeletal muscle of fasted rats 24 hr subsequent to an injection of P-407 could result from a reduction in the activity of the heparin-releasable LPL fraction which, in turn, increased the expression of intracellular LPL. On the other hand P-407 may potentially inhibit the transport of intracellular LPL to its location on the capillary endothelium with resultant accumulation of LPL inside myocytes. Determination of why a similar increase in intracellular LPL activity in adipose, testicular, and lung tissue was not observed in the present study will require additional experimentation. Borensztajn *et al.* [23] demonstrated that the non-releasable fraction of LPL in heart tissue of rats is not affected by Triton WR-1339 administration. In addition, it was shown [23] that LPL activity in soleus muscle of starved rats decreases 25% compared with controls following Triton administration. Intracellular modification of LPL by P-407 in the present study would appear unlikely since P-407 is not thought to cross or disrupt cellular membranes [24].

In conclusion, we have demonstrated that the effect of P-407 on LPL *in vitro* was one of continued loss in enzymatic activity as the concentration of P-407 was increased. Moreover, we have shown that P-407 injected i.p. into rats affects measured LPL activity differently, depending on the enzyme fraction analyzed. That is, the activity of the extracellular fraction of LPL bound to capillary endothelium was inhibited significantly following administration of P-407 to rats, whereas the activity of the intracellular pool of LPL found in heart and skeletal muscle was increased following a single dose of P-407. Thus, our studies would suggest that the predominant mechanism responsible for elevated concentrations of circulating TG following administration of P-407 to rats was inhibition of heparin-releasable LPL. The findings of this study are significant because a nontoxic agent has been identified that inhibits capillary-bound LPL and provides a method to block TG hydrolysis for extended time periods.

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