Pharmaceutical Research, Vol. 5, No. 7, 1988

Research Article

The Effects of Phthalimide and Saccharin Derivatives on Low-Density Lipoprotein (LDL) and High-Density Lipoprotein (HDL) Receptor Activity and Related Enzyme Activities

Iris H. Hall,[1,2] Oi T. Wong,[1] and Steven D. Wyrick[1]

Received September 1, 1987; accepted January 30, 1988

The hypolipidemic agents, phthalimide, saccharin, *o*-(*N*-phthalimido) acetophenone, *N*-(*p*-chlorobenzoyl) sulfamate, and *o*-chlorobenzylsulfonamide affected low-density lipoprotein (LDL) and high-density lipoprotein (HDL) receptor activity and lipoprotein degradation. In isolated rat hepatocytes, rat aorta foam cells, and human fibroblasts, LDL receptor activity, which is dependent on apo-B and -E, was inhibited by the drugs in a dose-dependent manner. LDL degradation was accelerated in the hepatocytes, while it was inhibited in aorta cells and fibroblasts. The drugs enhanced HDL receptor activity, dependent on apo-E and -A1, and HDL degradation in the hepatocytes, whereas in fibroblasts and aorta cells HDL receptor binding and degradation were suppressed. In parallel, activities of acyl CoA acyl transferase, *sn*-glycerol-3-phosphate acyl transferase, and heparin-induced lipoprotein lipase decreased and activities of HMG–CoA reductase and cholesterol oleate-ester hydrolase increased. In fibroblasts the presence of drugs enhanced HDL binding of intracellular cholesterol. *In vivo* studies demonstrated that phthalimide and saccharin treatment enhanced the clearance of HDL and decreased the clearance of LDL from the serum of rats. The results suggest that the mode of action of the agents is to modulate the lipoprotein receptor and, thereby, the clearance of lipids from peripheral tissue as part of the hypolipidemic activity.

KEY WORDS: low-density lipoprotein (LDL) receptor activity; high-density lipoprotein (HDL) receptor activity; lipid regulatory enzymes; hypolipidemic agents; cyclic imides.

INTRODUCTION

The disease state of atherosclerosis is contributed to by hyperlipidemia in humans, in which case the low-density lipoprotein (LDL)-cholesterol content is high and the high-density lipoprotein (HDL)-cholesterol content is low. LDL conducts cholesterol to the peripheral tissues including the plaques, and removal of cholesterol from the tissues is suppressed, leading to less cholesterol being removed by the liver (1). Commercially available agents are not effective in elevating HDL-cholesterol, e.g., no effect was observed with cholestyramine, probucal, and neomycin sulfate, whereas a 4 to 16% increase was observed with clofibrate. Niacin treatment elevated levels of HDL cholesterol (2,3). Modulation of the LDL and HDL in the blood is important because the lipid mobilization and clearance from the body are regulated by LDL receptors which are specific for apo-B and apo-E and by HDL receptors which are specific for apo-E and apo-A1 (4–7). Selected cyclic imides have potent hypolipidemic activity, lowering both serum cholesterol and triglyceride levels greater than 40% in rodents at 20 mg/kg/day, orally or i.p. (8–12). These agents lowered LDL-cholesterol and very low-density lipoprotein (VLDL)-cholesterol while raising the HDL-cholesterol content over long periods of dosing (13–16). These derivatives did not follow a dose–response curve, but rather a hyperbolic effect was observed, with 20 mg/kg/day affording the maximum pharmacological effect and 40 and 60 mg/kg/day causing less lipid lowering effect. Thus the present study was undertaken to investigate the effects of cyclic imides on LDL and HDL receptor activities and enzymes reported to be regulated by the LDL receptor in (i) rat isolated hepatocytes, since the liver clears cholesterol and excretes it and its metabolites into the bile; (ii) human fibroblasts, since this tissue represents a peripheral tissue; and (iii) isolated rat aorta foam cells, since these cells present proliferating aorta plaque cells.

MATERIALS AND METHODS

Source of Compounds and Reagents

Phthalimide (1) was purchased from Eastman Organic Chemicals. Saccharin (2) was obtained from Ruger Chemical Co., and the standard, clofibrate (6), from Ayerst Pharmaceutical Co. *o*-(*N*-Phthalimido) acetophenone (3) (8), the sodium salts of *N*-(*p*-chlorobenzoyl)sulfamate (4) (17,18), and *o*-chlorobenzylsulfonamide (5) (17,18) were synthesized as outlined and the physical and chemical characteristics were identical to those reported. Isotopes were purchased from

[1] Division of Medicinal Chemistry and Natural Products, School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514.

[2] Author to whom correspondence should be addressed.

0724-8741/88/0700-0413$06.00/0

New England Nuclear. Substrates and cofactors for the enzyme assays were obtained from Sigma Chemical Co. ^{125}I was counted using a Baird atomic counter and ^{14}C and ^{3}H were counted using a Packard beta counter with correcting for quenching of the sample.

Separation of Lipoproteins

Blood (10 ml) was collected from the abdominal vein of Sprague Dawley rats (400 g) using ether anesthesia. Serum lipoproteins were separated by ultracentrifugation. Human serum from healthy male subjects was obtained from the North Carolina Memorial Hospital Blood Bank. Human low-density lipoprotein (LDL; density, 1.019–1.063 g/ml), high-density lipoprotein (HDL; density, 1.063–1.210 g/ml), and lipoprotein-deficient serum (LPDS; density, 1.215 g/ml) were separated by differential ultracentrifugation (20). Rat LDL and HDL lipoproteins were separated by the method of Mookerjea *et al.* (21) modified for rat lipoproteins.

Labeling of Lipoproteins

Radiolabeling of LDL and HDL fractions was conducted by a modification of the iodine monochloride method (22). Purified LDL and HDL lipoproteins were iodinated with 250 μCi of carrier-free ^{125}I (New England Nuclear, 350 mCi/ml) for 10 min at 4°C in 0.5 *M* glycine–NaOH buffer, pH 10, with 4 Eq of ICI reagent/mol of lipoprotein. Labeled lipoproteins were dialyzed against 0.15 *M* NaCl–0.1% EDTA, pH 7.4, for 48 hr, changing the dialysis medium six times. Labeled lipoprotein was sterilized using a 0.45-μm filter.

Tissue Culture Cells

Normal human fetal foreskin (BG 9) fibroblasts (North Carolina Cancer Research Center) were maintained as a monolayer culture in Eagles minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin in a humidified 5% carbon dioxide incubator at 37°C.

Isolated rat hepatocytes were obtained from Sprague Dawley male rats (~350 g) by perfusing the livers with calcium-free Hepes buffer, pH 7.4, for 5 min and then collagenase buffer (50 ml/min) for 10 min, which afforded a single-cell suspension. Parenchymal cells were purified by centrifugation and plated in organ tissue dishes at 1.5×10^6 cells in MEM supplemented with antibiotics and 10% fetal calf serum for 24 hr in the incubator (23).

Aorta foam cells were obtained from Sprague Dawley male rats (~400 g) anesthetized with ether. The thoracic cavity was opened, and the aorta (2 cm) was excised and placed in cold MEM, 10% newborn calf serum, nonessential amino acids, and antibiotics (22). The aorta was cut into small circular segments which were cut longitudinally to expose the intimal surface. This surface was carefully stripped from the adventitia, placed in 6 ml of fresh medium, and incubated. After 3 days, new MEM medium was added (10 ml). Medium was changed twice a week. Only foam cells will proliferate, and these cells were confluent within 2 weeks. The cells were treated with 0.25% trypsin, and 1×10^3 cells were transferred to petri dishes (35 mm) and allowed to grow until they were confluent, usually 3 days (24).

Tissue Culture Receptor Activity for LDL and HDL

Receptor activity binding and internalization as well as lipoprotein degradation were determined by the method of Goldstein and Brown (4). To 1.5×10^6 hepatocytes, 2×10^5 fibroblasts or 1×10^4 aorta foam cells, 2 ml of MEM, 10% LPDS, and 10 μCi ^{125}I-LDL or ^{125}I-HDL (100 μg) were added. Sterile drugs (25 to 250 μ*M*) in phosphate-buffered saline (PBS) were added and incubated for 18 hr. Lipoprotein degradation was assessed by removing the medium and treating it with 10% trichloroacetic acid (TCA), KI, and hydrogen peroxide and extracting the lipids with chloroform. Whole cells were washed repeatedly, taken up into 0.1 *M* NaCl, and counted. Protein content was assayed by the Lowry procedure (25), and receptor activity is expressed as counts per minute (cpm) per milligram of protein.

Enzyme Activity

Initial studies were performed to determine if the drugs altered the viability of the cells over the 18 hr. Hepatocytes, fibroblasts, and aorta foam cells were incubated with drugs from 25 to 250 μ*M* for 18 hr, and then the cells were treated with 0.25% trypsin containing Evan blue, counted using a hemocytometer, and compared to control untreated cells (number of cells per milliliter).

Enzyme assays were performed by incubating cells for 18 hr with drugs, MEM, and 10% LPDS, pH 7.4, and the cells were scraped off the dishes and pooled in PBS. The cells were fractionated according to the literature procedure used for each enzyme assay. Cholesterol synthesis (HMG–CoA reductase activity) was determined using the method of Haven *et al.* (26) and isolated by the procedure of Wada *et al.* (27). Acyl CoA cholesterol acyl transferase activity was determined by the procedure of Balasubramaniam *et al.* (28) using 20 μCi 1-^{14}C-oleic acid (57.3 mCi/mmol) and an albumin complex of human or rat LDL (100 μg protein/ml). Cholesterol ester hydrolase activity was determined in an analogous manner using 10 μCi of cholesterol-oleate-1-^{14}C (56.6 mCi/mmol). Hepatocyte cholesterol-7-α-hydroxylase activity was determined by the method of Shefer *et al.* (29) using 1,2-^{3}H-cholesterol (54.8 Ci/mmol).

sn-Glycerol-3-phosphate acyl transferase activity was determined using *sn*-[1,3-^{14}C-glycerol-3-phosphate (144 mCi/mmol) according to Lamb *et al.* (30). Lipoprotein lipase activity was determined by the method of Chait *et al.* (31) using glycerol-tri-(^{14}C)-palmitic acid(64 mCi/mmol) emulsified with lecithin. The cells were treated with 5 IU/ml heparin, 4% albumin, and 10% glycerol for 30 min prior to the enzyme assay to release the lipase from the membrane surface. The protein content of each of the subcellular fractions was determined by the Lowry technique (25). Enzyme activities are expressed as disintegrations per minute (dpm) per milligram of protein. Protein synthesis was determined by incubating cells with 2 μCi of L-^{3}H-4,5-leucine (59.8 Ci/mmol) for 18 hr (32).

HDL Update of ^{3}H-Cholesterol from Fibroblasts

To confluent human fibroblasts (BG-9) ^{3}H-cholesterol (10 μCi) was added in fresh medium and incubated for 24 hr. The medium was decanted and the cells were washed four times in PBS, pH 7.4. Fresh MEM plus 10 μl of human HDL

Table I. The Effects of Phthalimide, Saccharin, Sulfamate, and Sulfonamide Derivatives on LDL Receptor Activity and LDL Degradation in Rat Isolated Hepatocytes

Compound (N = 6)	Percentage of control (X ± SD)							
	LDL receptor binding				LDL degradation			
	25 μM	50 μM	100 μM	250 μM	25 μM	50 μM	100 μM	250 μM
1	76 ± 3*	72 ± 4*	53 ± 2*	48 ± 3*	129 ± 4*	195 ± 7*	182 ± 4*	157 ± 6*
2	85 ± 6	81 ± 5*	72 ± 3*	71 ± 3*	98 ± 3	128 ± 5*	138 ± 3*	95 ± 6
3	66 ± 4	64 ± 5*	64 ± 3*	55 ± 1*	115 ± 6	180 ± 3*	158 ± 4*	146 ± 5*
4	71 ± 3	68 ± 2*	65 ± 3*	51 ± 2*	129 ± 5*	127 ± 5*	105 ± 3	73 ± 6*
5	108 ± 4	92 ± 5	92 ± 6	55 ± 4*	78 ± 7*	116 ± 6	147 ± 5*	128 ± 5*
6	79 ± 6	66 ± 7*	66 ± 2*	65 ± 6*	126 ± 6*	100 ± 6	86 ± 4	64 ± 3*
Control	100 ± 6	100 ± 6	100 ± 6	100 ± 6	100 ± 7	100 ± 7	100 ± 7	100 ± 7
Control value	954 cpm/mg protein				1094 cpm/mg protein			

* $P \leq 0.001$ as determined by Student's t test.

and antibiotics were added and incubated for 24 hr. The medium was collected and the HDL was isolated by the ultracentrifugation differential technique. HDL protein was denatured with trichloroacetic acid, collected on Whatman No. 1 filters, washed, and counted. Preliminary studies demonstrated that BG-9 fibroblast intracellular ^{3}H-cholesterol binds to extracellular HDL in a concentration-dependent manner from 1 to 100 μl of human HDL (33).

In Vivo Clearance of HDL and LDL Lipoproteins

In vivo ^{125}I-HDL and ^{125}I-LDL uptake from the blood of Sprague Dawley male rats was determined after treating for 14 days with phthalimide or saccharin at 20 mg/kg/day, orally. Twenty-four hours prior to surgery, the animals were placed on a 0.2% Lugal's solution in their drinking water to prevent ^{125}I uptake by the thyroid gland. After anesthesia (ether) the femoral vein was surgically exposed and 0.1 ml of ^{125}I-HDL or ^{125}I-LDL was injected i.v. Blood was collected at 0, 1, 2.5, 4.5, 7, and 10 hr and centrifuged to obtain the serum. Aliquots were counted, and the clearance by the tissues is expressed as the log percentage of the administered radiolabeled lipoprotein at time zero [34].

RESULTS

The phthalimide, saccharin, sulfamate, and sulfonamide derivatives as well as the standard clofibrate reduced hepatocyte LDL receptor activity in a concentration-dependent manner from 25 to 250 μM (Table I). These agents elevated hepatocyte LDL degradation, with 50 or 100 μM affording the highest elevation. The agents lowered human fibroblast LDL receptor binding, e.g., at 250 μM, greater than 50% inhibition was achieved (Table II). Fibroblast LDL degradation over 18 hr was reduced with test agents; however, clofibrate had no effect at higher concentrations. All agents at 100 μM reduced rat aorta foam cell LDL receptor activity and degradation with the exception of the sulfamate derivative (Table III).

Hepatocyte HDL receptor binding activity and degradation were elevated in a concentration-dependent manner, with 250 μM causing the highest elevation (Table IV). Fibroblast HDL receptor binding and degradation were reduced in a concentration-dependent manner, with 250 μM affording the lowest values (Table V). Aorta cell HDL receptor activity demonstrated a mixed effect, with phthalimide, saccharin, *o*-(*N*-phthalimido)acetophenone, and clofibrate affording a 13–19% reduction, whereas the sulfamate and the sulfonamide derivatives elevated activity (Table III). The latter two agents caused significant elevations in HDL degradation in aorta cells. The test agents had no effect on cell survival.

A comparison of the effects of the agent on lipoprotein receptor activities and rate-limiting enzymes showed that

Table II. Effects of Phthalimide, Saccharin, Sulfamate, and Sulfonamide Derivatives on Human Fibroblast LDL Receptor Activity and LDL Degradation

Compound (N = 5)	Percentage of control (X ± SD)							
	LDL receptor binding				LDL degradation			
	25 μM	50 μM	100 μM	250 μM	25 μM	50 μM	100 μM	250 μM
1	85 ± 6	50 ± 4*	44 ± 3*	44 ± 2*	87 ± 6	75 ± 6*	60 ± 5*	62 ± 4*
2	107 ± 7	72 ± 6*	64 ± 4*	43 ± 4*	89 ± 5	72 ± 4*	65 ± 4*	62 ± 5*
3	88 ± 4	64 ± 7*	46 ± 2*	46 ± 2*	106 ± 4	85 ± 5	85 ± 5	48 ± 3*
4	85 ± 5	83 ± 5*	67 ± 3*	42 ± 5*	88 ± 4	84 ± 6	61 ± 3*	37 ± 4*
5	102 ± 3	89 ± 6	88 ± 5	39 ± 3*	108 ± 6	96 ± 5	97 ± 6	42 ± 3*
6	85 ± 6	63 ± 5	45 ± 6*	45 ± 5*	98 ± 5	68 ± 4*	107 ± 5	103 ± 6*
Control	100 ± 3	100 ± 3	100 ± 3	100 ± 3	100 ± 5	100 ± 5	100 ± 5	100 ± 5
Control value	577 cpm/mg protein				2744 cpm/mg protein			

* $P \leq 0.001$.

Table III. Effects of Phthalimide, Saccharin, Sulfamate, and Sulfonamide Derivatives on Rat Aorta Foam Cells at a 100 μM Concentration

Compound (N = 5)	Percentage of control (X ± SD)			
	LDL receptor binding	LDL degradation	HDL receptor binding	HDL degradation
1	16 ± 2*	27 ± 5*	87 + 5	70 + 5*
2	21 ± 3*	30 ± 4*	83 + 4*	101 + 6
3	21 ± 4*	29 ± 5*	81 + 4*	84 + 6
4	98 ± 3	104 ± 6	137 + 6*	228 + 8*
5	76 ± 3*	85 ± 5	132 + 7*	213 + 6*
6	14 ± 2*	32 ± 3*	83 + 7	118 + 5
Control	100 ± 4	100 ± 6	100 + 4	100 + 4
Control value	350 cpm/mg protein	712 cpm/mg protein	277 cpm/mg protein	1722 cpm/mg protein

	HMG–CoA reductase	Acyl CoA acyl cholesterol transferase	Cholesterol oleate-ester hydrolase
1	82 ± 6	55 ± 5*	1268 ± 7*
2	120 ± 5	63 ± 4*	647 ± 6*
3	98 ± 7	57 ± 5*	1241 ± 5*
4	130 ± 4*	67 ± 3*	1233 ± 5*
5	135 ± 6*	73 ± 6*	801 ± 6*
6	136 ± 5	5 ± 1*	1586 ± 7*
Control	100 ± 7	100 ± 4	100 ± 7
Control value	607 dpm/mg protein	122 dpm/mg protein	63 dpm/mg protein

	sn-Glycerol-3-phosphate acyl transferase	Heparin-induced lipoprotein lipase	Leucine incorporation into protein
1	14 ± 4*	49 ± 5*	252 ± 6*
2	13 ± 2*	39 ± 4*	591 ± 6*
3	33 ± 4*	55 ± 6*	256 ± 4*
4	63 ± 5*	115 ± 5	122 ± 6
5	92 ± 4	100 ± 4	185 ± 5*
6	18 ± 2*	34 ± 3*	223 ± 7*
Control	100 ± 5	100 ± 7	100 ± 6
Control value	242 cpm/mg protein	41 dpm/mg protein	760 dpm/mg protein

* $P \leq 0.001$.

hepatocyte HMG–CoA reductase activity was unchanged for most of the agents below 100 μM (Table VI). Clofibrate and the sulfonamide derivative caused increased activity at 50 μM and higher. In fibroblasts, concentrations of 250 μM were required to elevate reductase activity (Table VII). Saccharin and phthalimide at 100 μM did not cause an elevation in aorta reductase activity (Table III); however, the other derivatives elevated HMG–CoA reductase activity. Acyl CoA cholesterol transferase, *sn*-glycerol-3-phosphate acyl transferase, and heparin-induced lipoprotein lipase activities were inhibited in heptocytes and fibroblasts in a concentration-dependent manner by the agents. Aorta acyl CoA cholesterol transferase activity was inhibited significantly by the agents at 100 μM. *sn*-Glycerol-3-phosphate acyl transferase

Table IV. The Effects of Phthalimide, Saccharin, Sulfamate, and Sulfonamide Derivatives on HDL Receptor Activity and HDL Degradation by Isolated Rat Hepatocytes

Compound (N = 6)	Percentage of control (X ± SD)							
	HDL receptor binding				HDL degradation			
	25 μM	50 μM	100 μM	250 μM	25 μM	50 μM	100 μM	250 μM
1	95 ± 4	133 ± 6*	147 ± 5*	167 ± 4*	82 ± 6	108 ± 6	146 ± 5*	148 ± 6*
2	115 ± 3	128 ± 2*	133 ± 3*	154 ± 4*	103 ± 5	108 ± 4	122 ± 7*	154 ± 3*
3	119 ± 2	127 ± 4*	133 ± 3*	143 ± 5*	132 ± 4*	142 ± 3*	149 ± 2*	176 ± 5*
4	104 ± 6	137 ± 6*	142 ± 4*	183 ± 6*	105 ± 6	110 ± 7	146 ± 5*	146 ± 4*
5	117 ± 7	120 ± 4*	175 ± 7*	201 ± 7*	100 ± 3	135 ± 2*	176 ± 5*	206 ± 7*
6	115 ± 5	131 ± 5*	147 ± 6*	171 ± 7*	114 ± 6	165 ± 5*	178 ± 7*	198 ± 4*
Control	100 ± 5	100 ± 5	100 ± 5	100 ± 5	100 ± 6	100 ± 6	100 ± 6	100 ± 6
Control value	322 cpm/mg protein				1327 cpm/mg protein			

* $P \leq 0.001$.

Table V. Effects of Phthalimide, Saccharin, Sulfamate, and Sulfonamide Derivatives on Human Fibroblast HDL Receptor Activity and HDL Degradation

Compound (N = 5)	Percentage of control ($X \pm SD$)							
	HDL receptor binding				HDL degradation			
	25 μM	50 μM	100 μM	250 μM	25 μM	50 μM	100 μM	250 μM
1	76 ± 5*	65 ± 6*	60 ± 7*	43 ± 4*	87 ± 7	62 ± 4*	52 ± 6*	48 ± 3*
2	89 ± 4	66 ± 3*	65 ± 4*	53 ± 3*	74 ± 5*	44 ± 3*	43 ± #*	37 ± 2*
3	76 ± 3*	70 ± 3*	63 ± 2*	63 ± 4*	95 ± 7	88 ± 5	78 ± 5*	46 ± 4*
4	85 ± 4	83 ± 5	67 ± 3*	42 ± 2*	88 ± 6	84 ± 6	61 ± 4*	37 ± 5*
5	102 ± 6	89 ± 4	88 ± 4	39 ± 2*	106 ± 6	117 ± 8	85 ± 4	48 ± 3*
6	77 ± 6*	45 ± 3*	41 ± 4*	30 ± 3*	81 ± 6*	62 ± 4*	46 ± 4*	32 ± 4*
Control	100 ± 5	100 ± 5	100 ± 5	100 ± 5	100 ± 4	100 ± 4	100 ± 4	100 ± 4
Control value	493 cpm/mg protein				654 cpm/mg protein			

* $P \leqq 0.001$.

activity was inhibited by the agents except the sulfonamide derivative. Heparin-induced lipoprotein lipase activity was inhibited by phthalimide, *o*-(*N*-phthalimido)acetophenone, saccharin, and clofibrate. Hepatocyte cholesterol-7-α-hydroxylase activity was inhibited in a concentration-dependent manner by the agents. Fibroblast cholesterol-ester hydrolase activity was elevated two- to threefold by the agents at 250 μM. Aorta cholesterol-ester hydrolase activity was

Table VI. Effects of Phthalimide, Saccharin, Sulfamate, and Sulfonamide Derivatives on Isolated Hepatocyte Enzyme Activities Involved in Lipid Metabolism

Compound (N = 6)	Percentage of control ($X \pm SD$)							
	HMG–CoA reductase				Acyl CoA cholesterol acyl transferase			
	25 μM	50 μM	100 μM	250 μM	25 μM	50 μM	100 μM	250 μM
1	107 ± 6	113 ± 6	148 ± 4*	184 ± 7*	67 ± 6*	55 ± 5*	41 ± 5*	23 ± 6*
2	102 ± 6	112 ± 5	144 ± 5*	215 ± 6*	114 ± 5	57 ± 5*	49 ± 4*	34 ± 4*
3	103 ± 3	147 ± 5*	150 ± 4*	183 ± 5*	113 ± 4	71 ± 6*	69 ± 6*	50 ± 3*
4	99 ± 6	96 ± 5	120 ± 6	193 ± 7*	101 ± 3	96 ± 3	77 ± 4*	54 ± 4*
5	123 ± 6*	139 ± 4*	139 ± 6*	147 ± 6*	119 ± 6	67 ± 4*	59 ± 5*	57 ± 4*
6	88 ± 5	123 ± 4*	125 ± 5*	187 ± 3*	107 ± 6	116 ± 5	78 ± 5*	45 ± 4*
Control	100 ± 5	100 ± 5	100 ± 5	100 ± 5	100 ± 4	100 ± 4	100 ± 4	100 ± 4
Control value	6556 dpm/mg protein				685 dpm/mg protein			
	Cholesterol-7-α-hydroxylase				*sn*-Glycerol-3-phosphate acyl transferase			
	25 μM	50 μM	100 μM	250 μM	25 μM	50 μM	100 μM	250 μM
1	104 ± 6	77 ± 7*	73 ± 5*	16 ± 3*	92 ± 6	68 ± 5*	67 ± 6*	43 ± 4*
2	109 ± 7	79 ± 6*	62 ± 5*	30 ± 4*	87 ± 7	80 ± 6*	63 ± 4	39 ± 5*
3	82 ± 6	80 ± 7*	65 ± 5*	42 ± 4*	86 ± 6	81 ± 6	51 ± 8*	21 ± 4*
4	91 ± 7	80 ± 6*	43 ± 4*	18 ± 3*	115 ± 7	85 ± 6	55 ± 3*	33 ± 6*
5	122 ± 3*	91 ± 4	73 ± 5*	20 ± 2*	152 ± 6*	124 ± 5*	71 ± 4*	48 ± 5*
6	56 ± 4*	33 ± 5*	33 ± 2*	29 ± 3*	121 ± 7*	107 ± 3	88 ± 7	62 ± 3*
Control	100 ± 5	100 ± 5	100 ± 5	100 ± 5	100 ± 6	100 ± 6	100 ± 6	100 ± 6
Control value	788 dpm/mg protein				1804 dpm/mg protein			
	Heparin-induced lipoprotein lipase				Leucine incorporation into protein			
	25 μM	50 μM	100 μM	250 μM	25 μM	50 μM	100 μM	250 μM
1	83 ± 6	62 ± 5*	38 ± 4*	33 ± 3*	98 ± 6	103 ± 4	105 ± 5	109 ± 6
2	32 ± 5*	22 ± 2*	21 ± 3*	11 ± 4*	116 ± 6	99 ± 5	97 ± 4	96 ± 3
3	70 ± 6*	60 ± 4*	45 ± 5*	31 ± 4*	102 ± 7	104 ± 5	103 ± 5	100 ± 5
4	59 ± 5*	49 ± 4*	39 ± 3*	22 ± 4*	97 ± 6	105 ± 4	112 ± 7	98 ± 6
5	74 ± 6*	60 ± 6*	43 ± 5*	40 ± 2*	110 ± 5	126 ± 4*	108 ± 4	112 ± 5
6	89 ± 4	52 ± 5*	52 ± 4*	38 ± 3*	109 ± 6	89 ± 7	83 ± 5	108 ± 7
Control	100 ± 4	100 ± 4	100 ± 4	100 ± 4	100 ± 6	100 ± 6	100 ± 6	100 ± 6
Control value	251 dpm/mg protein				980 dpm/mg protein			

* $P \leqq 0.001$.

Table VII. The Effects of Phthalimide, Saccharin, Sulfamate, and Sulfonamide Derivatives on Human Fibroblast Enzyme Activities of Lipid Metabolism

Compound (N = 6)	Percentage of control (X ± SD)							
	HMG-CoA reductase activity				Acyl CoA cholesterol transferase			
	25 μ*M*	50 μ*M*	100 μ*M*	250 μ*M*	25 μ*M*	50 μ*M*	100 μ*M*	250 μ*M*
1	100 ± 5	102 ± 4	109 ± 4	129 ± 5*	65 ± 6*	50 ± 5*	48 ± 1*	44 ± 2*
2	93 ± 4	95 ± 5	105 ± 2	130 ± 6*	59 ± 5*	42 ± 3*	43 ± 2*	18 ± 3*
3	97 ± 3	98 ± 4	101 ± 3	117 ± 5	64 ± 5*	58 ± 3*	53 ± 2*	47 ± 3*
4	105 ± 6	121 ± 4	122 ± 4*	211 ± 7*	79 ± 6*	59 ± 5*	54 ± 4*	26 ± 2*
5	106 ± 5	121 ± 7	122 ± 4*	180 ± 6*	133 ± 6*	45 ± 4*	42 ± 3*	20 ± 2*
6	102 ± 6	139 ± 6*	140 ± 6*	143 ± 5*	82 ± 4*	68 ± 5*	61 ± 3*	41 ± 3*
Control	100 ± 7	100 ± 7	100 ± 7	100 ± 7	100 ± 5	100 ± 5	100 ± 5	100 ± 5
Control value	2589 dpm/mg protein				2326 dpm/mg protein			
	Cholesterol oleate-ester hydrolase				*sn*-Glycerol-3-phosphate acyl transferase			
	25 μ*M*	50 μ*M*	100 μ*M*	250 μ*M*	25 μ*M*	50 μ*M*	100 μ*M*	250 μ*M*
1	137 ± 5*	192 ± 4*	331 ± 5*	332 ± 7*	94 ± 5	82 ± 5	77 ± 6*	59 ± 6*
2	101 ± 4	114 ± 3	158 ± 4*	221 ± 6*	97 ± 6	56 ± 4*	32 ± 4*	32 ± 5*
3	109 ± 7	134 ± 5*	213 ± 5*	358 ± 5*	92 ± %	71 ± 5*	46 ± 4*	36 ± 4*
4	88 ± 7	89 ± 4	178 ± 5*	309 ± 6*	144 ± 4	69 ± 4*	66 ± 5*	33 ± 3*
5	136 ± 6*	159 ± 6*	178 ± 5*	290 ± 7*	119 ± 5	89 ± 5	80 ± 4*	38 ± 5*
6	149 ± 5*	200 ± 5*	303 ± 6*	307 ± 6*	108 ± 3	90 ± 4	84 ± 5	57 ± 6*
Control	100 ± 5	100 ± 5	100 ± 5	100 ± 5	100 ± 6	100 ± 6	100 ± 6	100 ± 6
Control value	68 dpm/mg protein				1473 dpm/mg protein			
	Heparin-induced lipoprotein lipase				Leucine incorporation into protein			
	25 μ*M*	50 μ*M*	100 μ*M*	250 μ*M*	25 μ*M*	50 μ*M*	100 μ*M*	250 μ*M*
1	117 ± 7	74 ± 5*	71 ± 6*	43 ± 5*	135 ± 6*	127 ± 7*	127 ± 7*	120 ± 6
2	80 ± 6*	77 ± 6*	57 ± 5*	56 ± 3*	142 ± 7*	144 ± 4*	127 ± 6*	105 ± 5
3	95 ± 7	78 ± 5*	65 ± 6*	29 ± 4*	146 ± 6*	124 ± 4*	113 ± 5	80 ± 3*
4	103 ± 7	91 ± 6	61 ± 7*	30 ± 4*	118 ± 4	112 ± 6	108 ± 7	64 ± 5*
5	111 ± 6	99 ± 5	86 ± 5	56 ± 5*	98 ± 7	91 ± 6	85 ± 6	59 ± 5*
6	63 ± 6*	38 ± 4*	37 ± 5*	23 ± 4*	109 ± 7	92 ± 6	91 ± 7	69 ± 5*
Control	100 ± 4	100 ± 4	100 ± 4	100 ± 4	100 ± 7	100 ± 7	100 ± 7	100 ± 7
Control value	269 dpm/mg protein				1380 dpm/mg protein			
	HDL uptake of cholesterol from inside the cells (100 μ*M*)							
1	145 ± 4*							
2	155 ± 6*							
3	118 ± 4							
4	75 ± 2							
5	116 ± 3							
6	124 ± 5							
Control	100 ± 5							
Control value	529 dpm/100 μl							

* $P \leqq 0.001$.

elevated 6- to 15-fold at 100 μ*M*. Hepatocyte protein synthesis was not altered by the agents. Fibroblast protein synthesis was increased 27 to 44%, whereas aorta protein synthesis was increased two- to fivefold by the agents with the exception of the sulfamate derivative.

The agents at 100 μ*M* increased HDL lipoprotein uptake of intracellular cholesterol from fibroblasts 15 to 55% (Table VII). *In vivo* administration of phthalimide or saccharin at 20 mg/kg/day for 14 days caused slower clearance at 4.5 hr ($P \leqslant 0.05$) of ^{125}I-LDL from the serum of rats, while ^{125}I-HDL was cleared more rapidly in the treated rats over a 10-hr period ($P \leqslant 0.001$) (Fig. 1).

In Tables I–VII, the average mean value of the control is expressed as 100% ± SD, and test values are given as a percentage of the control. The control values reflect the actual enzyme units. N equals the number of samples determined. Probability, P, values were determined using Student's t test between the control and each data point.

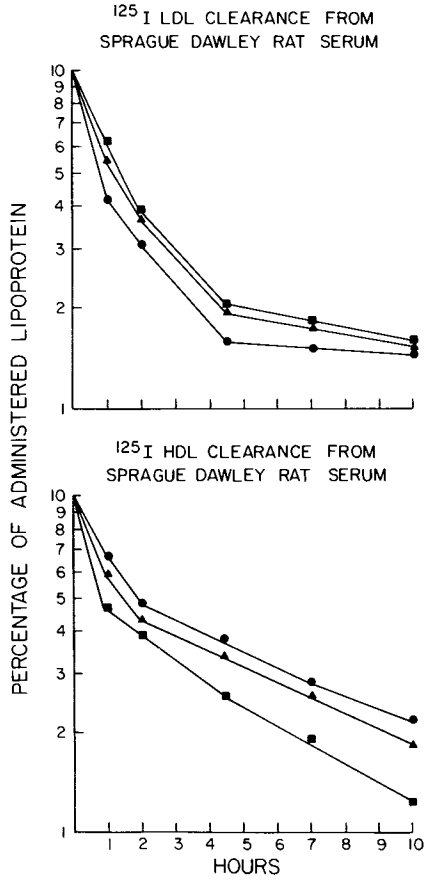

Fig. 1. The clearance of ^{125}I-LDL or -HDL over 10 hrs after i.v. administration from serum of Sprague Dawley rats after 14 days of treatment with phthalimide (■——■) or saccharin (▲——▲) were compared to the control values (●——●). The relative standard deviations from the means did not exceed 4% for all values. $N = 6$.

DISCUSSION

A clinical hypolipidemic agent needs to eliminate the disease state in the arteries rather than modulating hepatic lipid synthesis. In hyperlipidemic patients, serum LDL which conducts cholesterol to the plaques is elevated and HDL-cholesterol is reduced. The effect of hyperlipidemic agents on lipoprotein receptors which regulate lipid movement into cells as well as hepatic lipid clearance from the blood is important. Apo-B-containing lipoproteins are the causative factor in atherogenesis (36). Phthalimide, saccharin, *o*-(*N*-phthalimido)acetophenone, *N*-(*p*-chlorobenzoyl)sulfamate, and *o*-chlorobenzylsulfonamide reduce serum cholesterol and triglycerides by 40% (8,11,17); nevertheless, they do not suppress hepatic HMG-CoA reductase activity (8,11,18,19) as many hypercholesterolemic agents. Suppressing hepatic, fibroblast, and aorta foam cell LDL receptor activity mediated by apo-B reduces the uptake of LDL-cholesterol by peripheral tissue. LDL is also transported across cell membranes by a non-receptor-mediated process. Increased Apo-B concentrations and activity of acyl CoA cholesterol acyl transferase are positively linked with growth of the arterial plaque (42). These studies indicate that the drugs reduced degradation of LDL entering fibroblasts and aorta foam cells over 18 hr, thus releasing less free cholesterol.

A regulatory process in human fibroblasts showed that elevated LDL receptor activity reduces HMG-CoA reductase activity and elevates acyl CoA cholesterol acyl transferase activity (1,2,4). Increased hepatic LDL receptor activity elevates cholesterol-7α-hydroxylase activity (37–40). These studies indicate that the LDL receptor binding activity can be modulated by hyperlipidemic agents negatively so that suppression of the LDL receptor activity stimulates HMG-CoA reductase activity and reduces acyl CoA cholesterol acyl transferase and cholesterol-7-α-hydroxylase activities, resulting in less storage of tissue cholesterol esters and accelerating biliary excretion of cholesterol metabolites. Previous *in vivo* rat studies after 2-week administration of imides at 20 mg/kg/day showed an increase in bile excretion of cholesterol and its metabolites and a decreased total tissue cholesterol content, i.e., liver, small intestine (8,11,18,19). Rat *in vivo* studies demonstrated that serum ^{125}I-LDL clearance was reduced by saccharin and phthalimide, suggesting that LDL receptor binding and uptake were reduced by the agents at the therapeutic dose. Furthermore, these agents reduce the activity of the rate-limiting enzyme of triglyceride synthesis, i.e., *sn*-glycerol-3-phosphate acyl transferase, and heparin-induced lipoprotein lipase. Tissue triglycerides have been demonstrated to be reduced by these agents after *in vivo* administration (8,11,18,19). The relationship between the LDL receptor activity and the regulation of these enzyme activities is presently unknown. These agents pass into the cell, thus direct inhibition of enzymatic activity cannot be ruled out as a possible mode of action of the agents.

The HDL lipoprotein is responsible for the uptake of cholesterol from peripheral tissues and conduction of cholesterol to the liver for excretion via the bile, which is mediated by a heptocyte receptor dependent on apo-E and apo-A1 (35,41). These agents accelerated cholesterol ester hydrolysis and the uptake of cholesterol by HDL from fibroblasts, hepatocyte HDL receptor activity, and HDL lipoprotein degradation to release free cholesterol, which should accelerate excretion from the body. Fibroblast and aorta HDL receptor activity and HDL lipoprotein degradation were reduced by most of the agents. Concentrating HDL inside peripheral cells would be contrary to its role in conducting cholesterol to the liver for excretion. *In vivo* these agents increase fecal and biliary cholesterol and its metabolites (8,11,18,19), which is consistent with the present findings that HDL lipoprotein receptors are modulated by the agents. Increased clearance of serum ^{125}I-HDL after *in vivo* treatment supports the idea that the agents act on the membrane lipoprotein receptors. These agents decrease LDL-cholesterol and elevate HDL-cholesterol *in vivo* in rats (9,13–15), which led to increased clearance of cholesterol from the arterial plaques (9,15).

ACKNOWLEDGMENT

Research support for this project was from National Institute of Heart, Lung and Blood Grant HL 25680.

REFERENCES

1. W. B. Kannel and P. A. Sytkowski. *Pharm. Ther.* **32**:207–235 (1987).
2. T. A. Miettinen, J. K. Huttunen, C. Ehnholm, T. Kumlin, S. Mattim, and V. Naukkarinen. *Atherosclerosis* **36**:249–259 (1980).
3. B. H. Blankenhorn. In *Preventive Treatment of Atherosclerosis*. Addison–Wesley, Reading, Mass., 1984, pp. 131–158.
4. J. L. Goldstein and M. S. Brown. *J. Biol. Chem.* **249**:5153–5162 (1974).
5. N. E. Miller, D. S. Weinstein, and D. Steinberg. *J. Lipid Res.* **18**:438–450 (1977).
6. D. W. Bilheimer. *Agents Actions (Suppl.)* **16**:191–201 (1984).
7. M. S. Brown, S. E. Dana, and J. L. Goldstein. *Proc. Natl. Acad. Sci.* **70**:2162–2166 (1973).
8. J. M. Chapman, Jr., P. J. Voorstad, G. H. Cocolas, and I. H. Hall. *J. Med. Chem.* **28**:237–243 (1983).
9. S. D. Wyrick, P. J. Voorstad, G. H. Cocolas, and I. H. Hall. *J. Med. Chem* **27**:768–772 (1984).
10. P. J. Voorstad, G. H. Cocolas, and I. H. Hall. *Pharm. Res.* **1**:250–255 (1984).
11. I. H. Hall, J. M. Chapman, Jr., P. J. Voorstad, and G. H. Cocolas. *J. Pharm. Sci.* **74**:956–961 (1984).
12. J. M. Chapman, Jr., S. D. Wyrick, P. J. Voorstad, J. H. Maguire, G. H. Cocolas, and I. H. Hall. *J. Pharm. Sci.* **73**:1428–1484 (1984).
13. I. H. Hall, D. J. Reynolds, O. T. Wong, C. B. Oswald, and A. R. K. Murthy. *Pharm. Res.* (in press) (1987).
14. A. R. Murthy, S. D. Wyrick, and I. H. Hall. *J. Med. Chem.* **28**:1591–1596 (1985).
15. I. H. Hall, O. T. Wong, and D. J. Reynolds. *Biomed. Biochim. Acta* (in press) (1988).
16. I.H. Hall, A. R. K. Murthy, P. A. Day, and J. Calvin. Submitted for publication).
17. S. D. Wyrick, I. H. Hall, and A. Dubey. *J. Pharm. Sci.* **73**:374–377 (1984).
18. I. H. Hall, S. D. Wyrick, P. J. Voorstad, and A. Dubey. *Pharm. Res.* **1**:28–32 (1984).
19. I. H. Hall, S. D. Wyrick, P. J. Voorstad, and A. Dubey. *J. Pharm. Sci.* **73**:378–381 (1984).
20. R. L. Havel, H. A. Eder, and J. M. Bragdom. *J. Clin. Invest.* **34**:1345–1353 (1955).
21. E. S. Mookerjea, C. E. Parks, and A. Kuksis. *Lipids* **10**:374–382 (1975).
22. A. S. McFarlane. *Nature (Lond.)* **182**:53 (1958).
23. P. O. Segler. *Methods Cell Biol.* **13**:29–83 (1976).
24. E. L. Bierman, O. Stein, and Y. Stein. *Circ. Res.* **35**:136–149 (1974).
25. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. L. Randall. *J. Biol. Chem.* **193**:265–275 (1951).
26. G. T. Haven, J. R. Krzemien, and T. T. Nquyen. *Res. Comm. Chem. Path. Pharmacol.* **6**:253–261 (1973).
27. F. Wada, K. Kirata, and Y. Sakamoto. *J. Biol. Chem.* **193**:6273–6280 (1968).
28. S. Balasubramaniam, K. A. Mitropoulos, and S. Venkatesan. *Eur. J. Biochem.* **90**:377–383 (1978).
29. S. Shefer, S. Hauser, and E. M. Mosbach. *J. Lipid Res.* **9**:328–333 (1968).
30. R. G. Lamb, C. K. Wood, B. M. Landa, P. S. Guzelian, and H. J. Fallon. *Biochim. Biophys. Acta* **489**:318–329 (1977).
31. A. Chait, P. H. Iverius, and J. D. Bunzell. *J. Clin. Invest.* **69**:490–493 (1982).
32. I. H. Hall, W. L. Williams, Jr., S. Chaney, C. Gilbert, D. J. Holbrook, O. Muraka, H. Kiyokawa, and K. H. Lee. *J. Pharm. Res.* **74**:250–254 (1985).
33. D. C. K. Roberts, C. Cortese, A. Laville, L. Masuna, and B. Lewis. *Biochem. J.* **226**:319–323 (1985).
34. A. VanTol. *Agents Action (Suppl)* **16**:69–83 (1984).
35. G. M. Dallinga-Thie and A. VanTol. *Agents Action (Suppl.)* **16**:87–92 (1984).
36. S. Eisenberg. *J. Lipid Res.* **25**:1017–1058 (1984).
37. J. L. Goldstein and M. S. Brown. *Metabolism* **26**:1257–1275 (1977).
38. C. J. Packard and J. Shepherd. *Atherscler. Rev.* **11**:29–63 (1983).
39. M. S. Brown and G. L. Goldstein. *J. Clin. Invest.* **72**:743–747 (1983).
40. J. M. Dietscky and D. K. Spady. *Agents Action (Suppl.)* **16**:177–188 (1984).
41. E. Trezzi, F. M. Maggi, G. Maione, and A. L. Catapaus. *Agents Action (Suppl.)* **16**:47–50 (1984).
42. R. J. Havel and J. P. Kane. *Annu. Rev. Med.* **33**:417–433 (1982).