Lipoprotein Incorporation Enhances Radioiodinated Cholesteryl Ester Uptake into Steroid Hormone-Secreting Tissues

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This study was undertaken to determine whether incorporation of radioiodinated cholesterol derivatives into plasma lipoproteins prior to administration to animals could lead to improvements in adrenal localization of radioactivity. Rat high density lipoproteins (HDL) were labeled with [^{125}I]cholesteryl iopanoate, a nonhydrolyzable ester of cholesterol. No enhancement in adrenal uptake of radioactivity was noted at 30 min following administration of the HDL preparation to control rats when compared with [^{125}I]NP-59. However, when animals were made hypolipidemic by treatment with either 4-APP or ethinyl estradiol, the adrenal radioactivity after i.v. administration of the HDL preparation was found to be over 15 times greater than that achieved with [^{125}I]NP-59. Scans of hypolipidemic rats taken at 30 min correlated well with the tissue distribution results.

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For the past two decades radioiodinated derivatives of cholesterol (*1–3*) have proven to be useful imaging agents for the diagnosis of a variety of adrenal disorders in man (*4–6*). Studies in our laboratory aimed at elucidating the mechanism(s) of radioiodinated cholesterol accumulation in the adrenal revealed that these agents rapidly become associated with native lipoproteins upon administration, and that radiotracer uptake into adrenocortical cells appears to involve a lipoprotein receptor-mediated process (*7*).

The ability of lipoproteins to interact with specific cellular receptors and then be taken up into cells by receptor-mediated endocytosis (*8*) has suggested the possible use of lipoproteins for the site-specific delivery of radiopharmaceuticals (*9*). For this purpose, investigators have labeled the apoprotein component (Fig. 1) of lipoproteins with such gamma-emitting tracers as radioiodine (*10–14*), technetium-99m (*13–16*), and iodine-131 or iodine-123 tyramine cellobiose (*13,14,17, 18*). Since the surface apoproteins serve as the recognition sites for lipoprotein-receptor interaction (*8*), and slight chemical modification of these vesicles is known to markedly alter specific receptor-mediated processes (*19,20*), our own studies have focused more on utilizing the core lipids as carriers for the gamma-emitting nuclide. Moreover, this approach has been reinforced by the more recent findings that have shown the uptake of core elements by steroid hormone-secreting tissues to be substantially greater than that of the apoprotein (*17, 18*).

Since cholesteryl esters are a major component of the lipophilic core of lipoproteins (Fig. 1), most of our studies have utilized radioiodinated analogs of cholesteryl esters (*21–26*). One such ester, radioiodinated [iodine-125 (^{125}I)] cholesteryl iopanoate ([^{125}I]CI) has been found to be resistant to in vivo hydrolysis (*24,25*), which makes it an ideal agent for monitoring the accumulation of lipoprotein lipids within cells. For example, incorporation of [^{125}I]CI into chylomicron remnants resulted in 87% of the radioactivity reaching the liver within 0.5 hr as opposed to only 31% when the agent was solubilized with Tween 20 and administered in physiological saline (*26*); a result consistent with the known ability of the liver to rapidly sequester chylomicron remnants.

Based on our earlier studies with 19-iodocholesteryl oleate (*21,22*), and the knowledge that the rat adrenal

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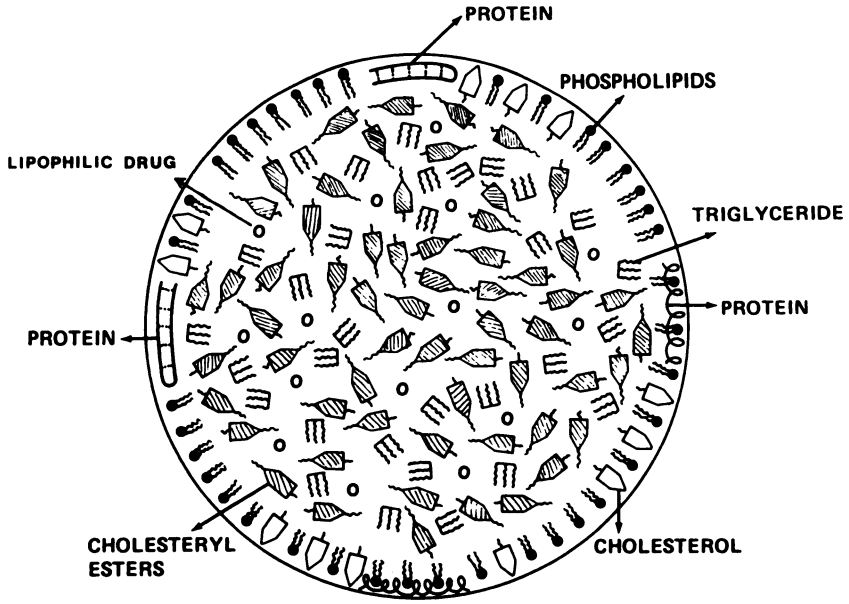

FIGURE 1
General structure of a plasma lipoprotein.

acquires most of its cholesterol through a lipoprotein receptor-mediated pathway (*27,28*), it was proposed that the adrenal uptake of [^{125}I]CI could be significantly enhanced by its incorporation into the appropriate lipoprotein fraction. Accordingly, this study describes the incorporation of [^{125}I]CI into rat high density lipoproteins (HDL) and its effect upon accumulation by the adrenal cortex. HDL were selected for these studies because they represent the major carrier for plasma cholesterol in the rat. The study also included both normal and hypolipidemic animals since hypolipidemia is known to upregulate tissue receptors for lipoproteins in laboratory animals and man (*29,30*).

MATERIALS AND METHODS

Synthesis of [^{125}I]CI and [^{125}I]NP-59

[^{125}I]CI and 6-β-iodomethyl-19-norcholest-5(10)-en-3β-01 ([^{125}I]NP-59) were synthesized as previously described (*24,31*) and stored dry under nitrogen at 0°C until used. Radiochemical purity was determined by thin layer chromatography (TLC) using silica gel plate with a fluorescent indicator (Eastman Kodak Co., Rochester, NY) developed in either benzene:ethyl acetate (9:1 v/v), petroleum ether:diethyl ether (7:2 v/v), or hexane:ethyl acetate (5:2 v/v). The plates were visualized under uv light and scanned for radioactivity using a Berthold Radiochromatogram Scanner model LB 2723.

Formulation of [^{125}I]CI and [^{125}I]NP-59 in Physiologic Saline

[^{125}I]CI was formulated for injection immediately prior to use by dissolving the compound in benzene and adding Tween 20 (polyoxyethylene sorbitan monolaurate, 0.1 ml/mg compound). The benzene was removed by evaporation under a stream of nitrogen and physiological saline solution was added. Any remaining benzene was removed until a clear solution containing 2–3% Tween 20 resulted. [^{125}I]NP-59 was formulated by adding 95% ethanol, Tween 80 (polyoxyethylene sorbitan monooleate) and physiological saline to a dried sample of the radiopharmaceutical so that the final solution contained 1.6% Tween 80 and 10% ethanol. Prior to injection, aliquots of the formulated compounds were assayed for radioactivity in a Searle 1185 Automatic Gamma System (efficiency = 82–85%). Aliquots were also analyzed by TLC as described above.

Preparation of Radiolabeled HDL

In a typical preparation, donor rats received i.v. injections of [^{125}I]CI (1.0 ml, 110 μCi, 0.122 μmol) through the tail vein. At 0.5 hr postinjection, the rats were exsanguinated by cardiac puncture while under ether anesthesia. The blood was collected in heparinized vacutainer tubes and centrifuged at low speed for 10 min to obtain plasma. Plasma from five rats was pooled (18 ml) and mixed with NaCl and KBr solutions to a final density of 1.070 g/ml as determined with a Mettler-Paar DMA 35 Densitometer following the method of Havel et al. (*32*). Ultracentrifugation and removal of VLDL, chylomicrons and LDL were performed as previously described (*21*). The resulting HDL-enriched plasma fraction [^{125}I]CI-HDL was assayed for radioactivity as described above. In order to assure that the radioactivity was associated only with HDL and to assess the radiopurity of the compound, the following analyses were performed. Polyacrylamide gel electrophoresis (PAGE), gel filtration using a column packed with Sepharose 4B (Pharmacia) and TLC of a lipid extract performed as described previously (*21*). Prior to use, the HDL fraction was dialyzed against 4 l of 0.2 *M* phosphate buffer with 0.9% NaCl, pH 7.4, for 18 hr at 4°C. In addition, a portion of the HDL fraction isolated as above was centrifuged at 100,000 *g* for 40 hr after adjusting the density of 1.210 g/ml and the top 1 ml was recovered as isolated HDL ([^{125}I]CI-HDL*).

Acetylation of [^{125}I]CI-HDL*

Isolated HDL ([^{125}I]CI-HDL*) was acetylated following the procedure of Basu et al. (*33*). Acetylation of the lysyl amino groups of the lipoprotein reduces the net positive charge of the lipoprotein particle. This modification was accomplished by adding a saturated solution of sodium acetate (1 ml/16 mg protein) to 1.5 ml of [^{125}I]CI-HDL* containing 4 mg HDL

protein with continuous stirring in an ice-water bath. Acetic anhydride (1.4 μl/mg protein) was then added in 0.2-μl increments over a 1-hr period with continuous stirring. The mixture was stirred for an additional 30 m, then dialyzed overnight at 4° against 4 l of buffer containing 5 m *M* Tris and 1 m *M* EDTA at pH 7.4. The resulting preparation was analyzed by PAGE as described below. The acetylated preparation was designated Ac[^{125}I]CI-HDL*.

Animals

Female Sprague-Dawley rats (Harlan Sprague Dawley or Charles River) weighing 200-260 g were used in all experiments. The rats were housed in temperature and light-controlled quarters and had free access to food (Purina Rat Chow) and water.

In order to deplete circulating cholesterol levels, rats were treated with either 17-ethinyl estradiol (E-E2) or 4-aminopyrazolo[3,4-d]pyrimidine (4-APP). E-E2 was dissolved in propylene glycol (1 mg/ml) and administered s.c. at a dose of 5 mg/kg/day for 5 days prior to use. 4-APP (Sigma, Chemical Corp., St. Louis, MO), in 10 m *M* sodium phosphate buffer, pH 3.3, was given i.p. for 3 days at 40 mg/kg/day. Physiological saline (3–5 ml) was also given s.c. in conjunction with the 4-APP injection in order to minimize hypovolemia.

Tissue Distribution

Rats received the following doses: [^{125}I]NP-59; 3.2–5.1 μCi, 0.017–0.027 μmol, specific activity = 96 μCi/μmol; [^{125}I]CI; 3.4–16.0 μCi, 0.020–0.096 μmol, average specific activity = 295 μCi/μmol; [^{125}I]CI-HDL 0.7–4.5 μCi; [^{125}I]CI-HDL*; 1.3 μCi; Ac[^{125}I]CI-HDL*; 0.9–1.2 μCi. Rats were injected intravenously while under a light ether anesthesia. At 0.5 hr following injection, animals were exsanguinated by cardiac puncture while under ether anesthesia. The blood was collected in a heparinized vacutainer tube. Samples of blood were counted and the remainder centrifuged at low speed for 10 min to obtain plasma. Aliquots of plasma were taken for counting. The following organs were removed, dissected free of connective tissue and fat, rinsed of blood and blotted dry: adrenal, heart, kidney, liver, lung, ovary, spleen, and thyroid. Large organs were minced with scissors. Samples were transferred to tared cellulose acetate capsules, weighed, and placed in polystyrene gamma tubes and assayed for radioactivity.

PAGE of Plasma Samples

In order to determine the association of radioactivity with lipoproteins, plasma samples from all the animals were analyzed by PAGE using the method of Narayan et al. (*34*). Briefly, this involved prestaining plasma samples with Sudan Black, a lipoprotein stain, and applying the mixture to the top of a disk polyacrylamide gel consisting of a 3.75% (w/v) acrylamide main gel with a 2% (w/v) large pore stacking gel. A current of 2.5 mA/gel was applied until the leading band migrated 1.7 cm into the main gel. Gels were then cut into five sections: the stacking gel (CM/VLDL), the top 0.5 cm of the main gel (LDL), the main gel above the leading band (HDL), the albumin band, and the remainder of the gel below albumin. Results are expressed as the percentage of total radioactivity present in each fraction.

Scintigraphic Studies

4-APP-treated animals were anesthetized with 13 mg/kg xylazine and 87 mg/kg ketamine i.m., then injected with 25–28 μCi of [^{125}I]NP-59, [^{125}I]CI, or [^{125}I]CI-HDL through the tail vein. Scintiscans were obtained of the whole body (100,000 cts/image) at 30 min postinjection with the animals lying prone on the face of a gamma camera (Siemans LEM) equipped with a high sensitivity collimator interfaced to a digital minicomputer (Siemans Microdelta).

RESULTS

Incorporation of [^{125}I]CI into HDL

Administration of [^{125}I]CI to donor rats resulted in the appearance of ~9% dose/g in the plasma at the time of sacrifice (30 min). Following ultracentrifugation, 28% of the initially administered radioactivity was recovered in the HDL fraction ([^{125}I]CI-HDL). PAGE analysis of this fraction revealed that 84% of the radioactivity migrated with the HDL band. Lipid extraction of this HDL fraction and analysis of the extract by TLC showed >94% of the radioactivity to comigrate with a CI standard. Moreover, gel filtration analysis showed a single peak of radioactivity corresponding to the leading shoulder of the main protein peak. This main protein peak has been previously shown to correspond to that for rat serum albumin (*21*).

Acetylation of Isolated [^{125}I]CI-HDL*

Acetylation of the isolated HDL fraction labeled with [^{125}I]CI resulted in alterations in the electrophoretic mobility of the HDL, with the modified preparation migrating more rapidly toward the cathode as noted previously (*22*). In addition, when the gel was sliced and assayed for radioactivity, the majority of activity was found to be associated with the more rapidly migrating bands.

Tissue Distribution of Radioiodinated Preparations

Table 1 compares the tissue distribution of [^{125}I]NP-59, [^{125}I]CI, and [^{125}I]CI-HDL in controls and estrogen-treated female rats at 30 min after administration. [^{125}I]NP-59 shows a threefold greater uptake in the adrenal than either [^{125}I]CI or [^{125}I]CI-HDL, but its uptake is only modestly affected by the estrogen-induced hypolipidemia. Uptake of [^{125}I]CI or [^{125}I]CI-HDL, on the other hand, shows an 18-fold enhancement in the estrogen-treated animals. Moreover, preincorporation of [^{125}I]CI into HDL has little effect on this uptake in the adrenal. Such is not the case for the liver or ovary, however, where preincorporation of CI into HDL caused a fourfold enhancement in uptake.

Figure 2 shows a similar comparison of five radioiodinated products (NP-59, CI, CI-HDL, CI-HDL*, and CI-AcHDL*) in the female rat made hypocholesterolemic by treatment with 4-APP. Administration of all of the HDL preparations led to higher concentrations of radioactivity appearing in the adrenals and ovaries of the hypolipidemic animals. Isolation of the radioiodinated HDL ([^{125}I]CI-HDL*) from the HDL-enriched

TABLE 1
Effect of Estrogen Pretreatment on the Biodistribution of Radioiodinated Cholesterol Derivatives in the Female Rat at 0.5 hr Following Administration

Tissue	[^{125}I]NP-59		[^{125}I]CI		[^{125}I]CI-HDL	
	Control	Treated	Control	Treated	Control	Treated
Adrenal	17.89 ± 0.90	27.11 ± 3.66	4.90 ± 0.72	88.15 ± 19.69	5.30 ± 0.80	95.90 ± 16.91
Blood	2.35 ± 0.11	5.52 ± 0.13	5.19 ± 0.44	4.44 ± 0.56	5.98 ± 0.51	2.16 ± 0.12
Heart	0.30 ± 0.02	0.49 ± 0.05	0.58 ± 0.05	0.42 ± 0.04	0.61 ± 0.07	0.24 ± 0.02
Kidney	0.45 ± 0.03	0.60 ± 0.03	0.61 ± 0.08	0.53 ± 0.05	0.65 ± 0.12	0.26 ± 0.02
Liver	4.57 ± 0.18	4.30 ± 0.23	4.04 ± 0.54	2.20 ± 0.49	1.24 ± 0.18	5.65 ± 0.38
Lung	1.52 ± 0.11	2.00 ± 0.02	1.34 ± 0.13	1.15 ± 0.30	1.41 ± 0.13	0.68 ± 0.07
Ovary	4.10 ± 0.76	7.47 ± 0.19	5.16 ± 0.72	10.77 ± 2.16	4.66 ± 0.16	22.11 ± 3.77
Spleen	1.46 ± 0.08	2.48 ± 0.11	0.78 ± 0.07	0.91 ± 0.16	0.63 ± 0.04	0.36 ± 0.05
Thyroid	1.37 ± 0.18	1.33 ± 0.30	0.87 ± 0.10	1.17 ± 0.09	0.47 ± 0.06	0.52 ± 0.12

fraction provided a product that significantly improved adrenal uptake but had little effect on ovarian uptake. As anticipated, chemical modification of this HDL preparation by acetylation ([^{125}I]CI-AcHDL*) caused a significant drop in the amount of radioactivity appearing in steroid-secretory tissues but did not effect liver uptake.

Scintigraphy

Gamma counter images taken at 30 min correlated well with the tissue distribution results. The scans obtained with 4-APP-treated rats following i.v. administration of each radioiodinated preparation are shown in Figure 3. Administration of [^{125}I]NP-59 resulted in visualization of the adrenals but not the ovaries (Fig. 3A). High liver and blood-pool radioactivity interfered with visualization of the steroid-secreting tissues following [^{125}I]CI (Fig. 3B). However, when the latter was incorporated into rat HDL, scintiscans clearly depicted both the adrenals and ovaries (Fig. 3C).

DISCUSSION

Plasma lipoproteins represent the major source of cholesterol for steroidogenesis in the rat adrenal (*27, 28*). Moreover, the ability of radioiodinated cholesterol derivatives to rapidly associate with circulating lipoproteins following i.v. administration is considered to be a major factor in the uptake of these radiopharmaceuticals by adrenocortical tissue (*7*). Hypercholesterolemia, which is known to down regulate adrenal lipoprotein receptors, has been shown to decrease the uptake of NP-59 into human adrenal cortex, an effect which can be reversed by causing a decrement in serum cholesterol (*35*).

Although appropriately labeled cholesterol derivatives are clinically useful as adrenal imaging agents, the time required to obtain adequate scans (4–5 days) is longer than desired, and there is a need to develop a novel radiopharmaceutical that will allow a reduction in the time from injection to adrenal visualization.

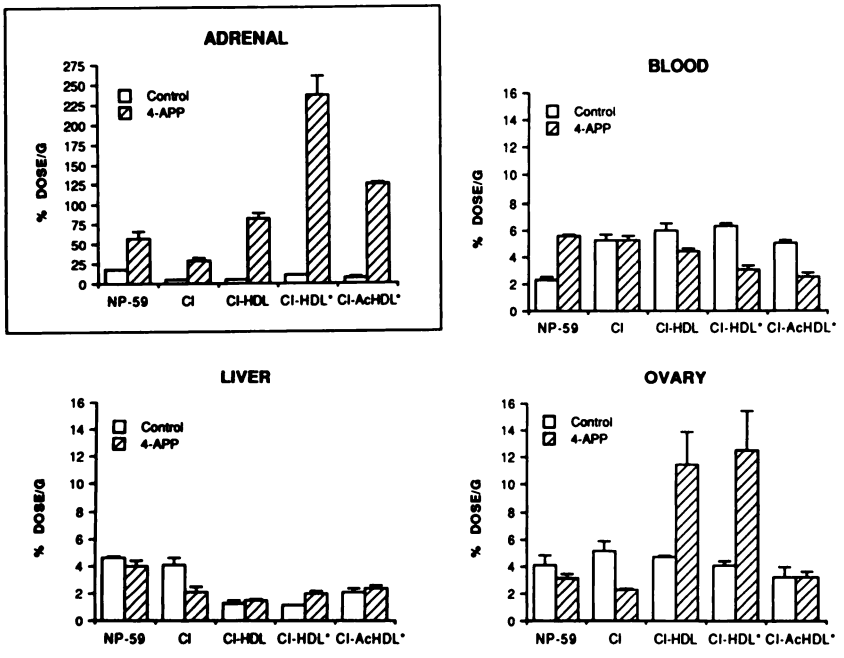

FIGURE 2
Tissue distribution in control and 4-APP pretreated rats at 0.5 hr following injection of [^{125}I]NP-59, [^{125}I]-CI, [^{125}I]-CI-HDL, [^{125}I]-CI-HDL* and [^{125}I] -CI-AcHDL*. Results expressed as mean % administered dose/g tissue ± s.e.m., n = 3–5 for each group. The adrenal panel is outlined to call attention to the different range of % dose/g.

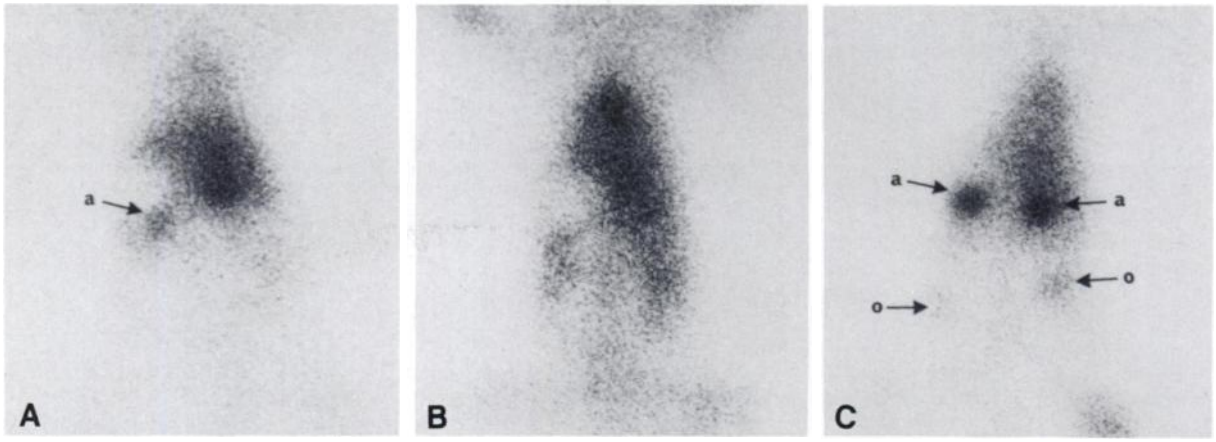

FIGURE 3
Scintiscans of 4-APP-treated rats at 0.5 hr following administration of (A) [^{125}I]-NP-59; (B) [^{125}I]-CI; (C) [^{125}I]-CI-HDL. (a = adrenal, o = ovary)

Accordingly, our laboratory has explored the potential application of various radioiodinated cholesteryl esters for this purpose since cholesteryl esters, rather than free cholesterol, are known to be a major component of plasma lipoproteins. Radioiodinated cholesteryl iopanoate has proven to be an excellent tracer for evaluating this approach due to its demonstrated resistance to in vivo hydrolysis, thus providing a means to monitor cholesteryl ester uptake by tissues (*24,25*).

Previous studies with radioiodinated cholesteryl oleate had shown that such esters rapidly associate with HDL upon i.v. administration to rats (*21*). In this study [^{125}I]CI behaved similarly and actually led to a greater recovery of radiolabeled HDL than that previously obtained with radioiodinated cholesteryl oleate (28% vs. 5%).

The greater adrenal uptake of [^{125}I]NP-59 (17.89% dose/g) over that for [^{125}I]CI-HDL (5.30% dose/g) in control rats suggests that the unesterified cholesterol analog may be taken up by the adrenals by a mechanism(s) other than one involving HDL. This observation is supported by the PAGE results (Table 2) that show only 50% of the plasma radioactivity associated with [^{125}I]NP-59 to be in the HDL fraction at 30 min. In contrast, administration of [^{125}I]CI gives rise to 75% of the radioactivity appearing in the HDL fraction at the same time point, yet a much smaller amount of radioactivity (4.90% dose/g) is present in the adrenals.

The current lack of knowledge concerning the metabolic fate of lipoproteins in animals and man makes it difficult to explain many of the observed results. On the other hand, it is known that reducing the circulating plasma cholesterol levels results in an upregulation of lipoprotein receptors in animals and man in such tissues as the liver and adrenals (*30*). Presumably such an effect accounts for the dramatic 15-fold and 18-fold enhancement in adrenal radioactivity following administration of [^{125}I]CI-HDL to 4-APP and E-E_2 treated animals, respectively. This dramatic response is much less apparent for [^{125}I]NP-59 and is only seen in the E-E_2-treated animals following administration of [^{125}I]CI. This latter result may be accounted for by the difference in the mechanisms of action of the two hypolipidemic drugs. Whereas 4-APP produces hypolipidemia by an inhibition of lipoprotein biosynthesis (*36*), estrogens exert their hypolipidemic effect by increasing the concentration of hepatic lipoprotein receptors which greatly

TABLE 2
PAGE of Plasma from Rats at 0.5 hr

	Control (n = 3)	[^{125}I]NP-59 4-APP (n = 3)	E-E2 (n = 3)
Stacking Gel (CM/VLDL)	29.7 ± 6.3*	93.5 ± 1.4	94.1 ± 0.5
LDL	16.9 ± 1.1	2.4 ± 0.6	1.8 ± 0.1
HDL	50.6 ± 6.3	1.9 ± 0.4	2.3 ± 0.2
Albumin	1.4 ± 0.1	1.3 ± 0.3	0.7 ± 0.1
Below Albumin	1.3 ± 0.5	0.8 ± 0.2	1.1 ± 0.3
		[^{125}I]CI	
	(n = 5)	(n = 5)	(n = 5)
Stacking Gel (CM/VLDL)	1.1 ± 0.2	77.1 ± 4.5	5.1 ± 2.6
LDL	23.5 ± 2.2	15.8 ± 3.4	27.2 ± 3.4
HDL	75.2 ± 2.2	4.3 ± 0.8	66.7 ± 5.9
Albumin	—	0.7 ± 0.3	—
Below Albumin	0.2 ± 0.0	2.2 ± 1.0	0.9 ± 0.2
		[^{125}I]CI-HDL	
	(n = 5)	(n = 5)	(n = 5)
Stacking Gel (CM/VLDL)	4.6 ± 0.8	6.1 ± 1.7	7.7 ± 4.3
LDL	25.7 ± 0.7	28.2 ± 2.9	22.8 ± 12.1
HDL	68.4 ± 1.2	65.0 ± 2.7	61.4 ± 13.0
Albumin	—	0.5 ± 0.1	—
Below Albumin	0.9 ± 0.4	0.2 ± 0.0	7.3 ± 4.8

* Results expressed as the % of total radioactivity in the gel, mean ± s.e.m.

accelerates lipoprotein clearance and metabolism (*37*). Thus in the E-E_2-treated animals, lipoprotein biosynthesis is not as severely compromised as in the 4-APP treated rats, therefore allowing more of the administered [^{125}I]CI to appear in the lipoprotein fractions following administration (Table 2). The radiolabeled lipoprotein formed in this way can be sequestered by the adrenal in a manner similar to that found for [^{125}I] CI-HDL.

In contrast to the adrenal, preincorporation of [^{125}I] CI into HDL does significantly enhance ovarian uptake in the estrogen-treated rat, albeit at much lower levels than the adrenal. We have no explanation for this difference between steroid-secreting tissues. It is possible that the upregulation of lipoprotein receptor is so great in the adrenal that it is not possible to discriminate between [^{125}I]CI and [^{125}I]CI-HDL, whereas the opposite is true for the ovary.

Acetylation of the surface apoproteins of human LDL has been shown to give rise to products that are no longer recognized by the LDL receptor (*20*). We have similarly shown that acetylation of radiolabeled rat HDL significantly depresses the uptake of these lipoproteins by the rat adrenal (*21*). In this study acetylation of [^{125}I]CI-HDL* was performed in order to distinguish between an HDL receptor-mediated uptake of cholesteryl ester by adrenal cells and uptake by a non-HDL receptor process.

Figure 2 compares the distribution of radioactivity following administration of CI-HDL* with that for Ac-CI-HDL*. As anticipated, acetylation resulted in a significant decrease in the amount of radioactivity appearing in the steroid-secreting tissues (most notably the adrenals) of the animals treated with 4-APP. The fact that one does not see a more complete interference with uptake upon acetylation can be explained in part on the basis that not all of the HDL are modified by this method (shown by electrophoresis). Also noteworthy, is the increased amount of radioactivity appearing in the liver of normal rats following administration of the acetylated product as opposed to [^{125}I]CI-HDL*. Increased catabolism of acetylated lipoproteins by the reticuloendothelial system (scavenger cell pathway) is well documented (*20,38*).

While these studies support a receptor-mediated uptake of cholesteryl esters similar to that described for the LDL-receptor, they provide no evidence as to whether endocytosis of the lipoprotein is an obligatory step in the accumulation process. Indeed, work in several laboratories has suggested that cholesteryl ester can transfer to cells from HDL and may not require parallel degradation of the apolipoprotein. For example, Glass and coworkers (*17,18*) labeled both apolipoproteins and core lipids and found that the uptake rate for the adrenals was sevenfold higher for the ^{3}H core lipids than the ^{125}I-labeled apolipoprotein.

Clearly, much remains unknown about the mechanisms of cholesterol and cholesteryl ester uptake by steroid-secreting cells. It is hoped that further studies with radioiodinated probes such as [^{125}I]Ci will shed additional light on the subject and lead to improvements in diagnostic imaging of steroid-secreting tissues.

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