Cellular Internalization, Transport, and Esterification of Iodine-125-NP59 by MA-10 Leydig Tumor Cells

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The present studies were directed toward understanding the cellular processing of the cholesterol analogue, NP59. NP59 readily entered MA-10 Leydig tumor cells. The cholesterol analogue entered the cells by binding to the plasma membrane and becoming internalized along with plasma membrane cholesterol. Internalized NP59 was readily esterified to NP59 ester. Transport of NP59 within the cell was indistinguishable from transport of cholesterol. Cholesterol and NP59 transport were under the control of cAMP, however, only cholesterol entered the mitochondria and was converted into progesterone. Thus, internalized NP59 could not be removed from the cell by conversion into steroid hormones. Esterified NP59 was metabolically inert and could not be converted back to free NP59 and free fatty acid. Since NP59 was not a substrate for the cholesteryl ester hydrolase, it became trapped in the cell as NP59 ester.

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Iodinated analogues of cholesterol are used for imaging normal adrenal or tumors of the adrenal or ovary (*1*). The analogue in most widespread clinical use, NP59, has been studied very little in animals or isolated cells. It is not known how it penetrates target cells, why it stays there, or how closely it mimics cholesterol.

Another cholesterol analogue, 19-iodocholesterol, has been studied in animals. Injected 19-iodocholesterol is associated with serum lipoprotein fractions within minutes and is recovered primarily in the free and to a lesser extent in the esterified form (*2*). Since this compound can be readily esterified by cells (*3*), these findings suggest that most tracer passively associates with circulating lipoproteins. It is thought likely that the 19-iodocholesterol enters the cell along with the lipoprotein. Once within the cell the tracer is esterified but becomes trapped as an ester since it is a poor substrate for the cholesteryl ester, hydrolase (*4*).

In the present study, we investigated the uptake and metabolism of iodine-125- (^{125}I) NP59 by the MA-10 Leydig tumor cells. The MA-10 cells are a stable, clonal cell line derived from a Leydig cell tumor that responds to gonadotropin or cAMP with increased progesterone synthesis (*5*). The metabolism of cellular cholesterol (*6–10*) and lipoproteins (*11*) has been extensively investigated using the MA-10 cells. Using these cells, we find that NP59 can enter the cell independently of lipoproteins by binding to the plasma membrane and becoming internalized. Internalized NP59 is transported and esterified like cholesterol but becomes trapped as an ester since it is a poor substrate for the cholesteryl ester hydrolase.

MATERIALS AND METHODS

Materials

Iodine-125-NP59 was synthesized and iodinated as described previously (*3,12*). [1,2-^{3}H]-cholesterol (65.5 Ci/mmol) and 26-^{14}C-cholesterol (54 Ci/mole) were from DuPont New England Nuclear (Boston, MA); cholesterol was from Steroloids (Wilton, NH); dibutyryl-cAMP was from Sigma (St. Louis, MO); and Eastman Kodak thin-layer sheets were purchased from Fisher (Plano, TX). All other materials were reagent grade.

Methods

Cell Culture. Cell culture conditions for MA-10 cells have been described previously (*5*). For experiments in which cells were to be cholesteryl-ester loaded with low-density lipoprotein (LDL), the medium was changed to medium containing lipoprotein-deficient horse serum and 50 μg/ml of LDL cholesterol 14 hr before beginning the experiment. The LDL was prepared as described previously (*6,11*).

For experiments cells were incubated in assay medium (Waymouth 752/1 with 20 m*M* HEPES, 1.2 g/l $NaHCO_3$ and 1% BSA, pH 7.4). Dibutyryl-cAMP was added from a 40-fold concentrated solution in sodium PBS, pH 7.4 containing 1 mg/ml BSA. ^{3}H-cholesterol (65.5 Ci/mmol) or ^{125}I-NP59 (3.9-

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9 Ci/mmol) were added in 10 μl of ethanol to dishes of cells containing 2 ml of assay medium.

Lipid Methods. Cells were scraped from dishes and the cellular residue extracted with chloroform:methanol; 2:1. The organic phase of this extract was concentrated and spotted on thin-layer chromatography (TLC) sheets. Extracts of cells treated with ^{3}H-cholesterol also induced carbon-14-cholesterol as an internal recovery standard. After lipids were separated by TLC developed in heptane:ethyl ether, acetic acid, 85:15:2, the area of the TLC sheet corresponding to either cholesterol or cholesteryl esters were cut out and either counted by liquid scintillation counting for ^{3}H-cholesterol or by gamma counting for ^{125}I-NP59. ^{3}H-cholesterol counts were corrected for recovery of the internal recovery standard, ^{125}I-NP59 counts were uncorrected. Iodine-125-NP59 and ^{125}I-NP59 ester migrated at approximately the same Rf as cholesterol and cholesteryl ester, respectively.

RESULTS

Recently, it has become clear that the cholesterol label will bind to the plasma membrane of cells and subsequently become internalized (*13–15*). In macrophages (*13*) or fibroblasts (*14*), the cholesterol label becomes esterified while in steroid synthesizing cells (such as MA-10 cells) the label will either be esterified in quiescent cells (*15*) or converted into progesterone in stimulated cells (*8,9*). Figure 1 data show that both ^{125}I-NP59 and ^{3}H-cholesterol bound to the cell surface and were internalized into the cell. Although qualitatively the labeled compounds behaved similarly, there were some significant quantitative differences. There was less NP59 radioactivity but more mass bound to the plasma membrane compared to ^{3}H-cholesterol. Significant amounts of ^{125}I-NP59 radioactivity but not ^{3}H-cholesterol radioactivity were loosely bound and washed off during the washes between the binding period and the uptake period of the experiment. The data shown in the lower panels of this figure indicate that bound radioligand of either tracer is subsequently internalized and appears as NP59 or cholesteryl esters. As the data in Figure 1 show, a greater proportion of NP59 bound to the cell becomes incorporated into cholesteryl esters. Thus, 13% of surface bound ^{125}I-NP59 is internalized and esterified within 6 hr while 7.3% of surface bound ^{3}H-cholesterol is internalized and esterified over this time period. The data in Figure 2 quantitate the recovery of bound ^{125}I-NP59 as intracellular esters. By 6 hr, more than 24% of the surface bound ^{125}I-NP59 is recovered as NP59 ester. In contrast, only 6.5% of surface-bound ^{3}H-cholesterol is recovered as cholesteryl esters over the same time period (Fig. 1 and Ref. *10*).

Table 1 data show experiments in which cells were surface-labeled with either ^{125}I-NP59 or ^{3}H-cholesterol

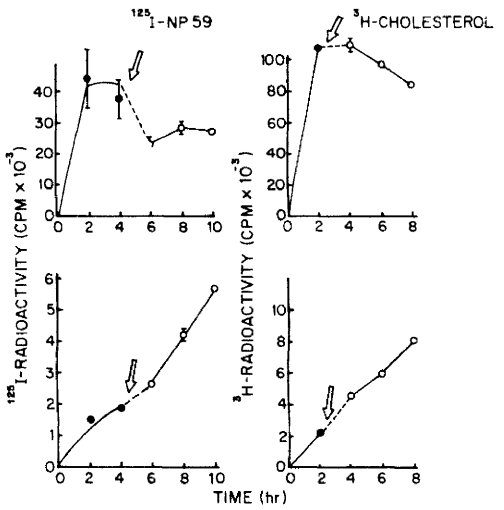

FIGURE 1
Dishes of cells (30 × 15 mm) were washed twice with 1 ml of Wa/BSA. One milliliter of Wa/BSA containing either 2 μCi ^{125}I-NP59 (9 Ci/mmol, left panels) or 0.5 μCi of ^{3}H-cholesterol (65.5 Ci/mmol, right panels) was added. The cells were incubated with tracer for 4 hr (^{125}I-NP59) or 2 hr (^{3}H-cholesterol) at which time the dishes were washed three times with Wa/BSA and then incubated further in 1 ml Wa/BSA without tracer. At the times indicated, dishes of cells were removed, washed three times with Wa/BSA, and the cell pellet extracted with chloroform:methanol (2:1). Free and esterified sterols were separated by chromatography on TLC plates developed in heptane:diethyl ether:acetic acid (85:15:1). The radioactivity corresponding to free (upper panels) and esterified sterols (lower panels) was cut out and counted. (●) indicates sterol radioactivity from dishes of cells incubated with medium containing radioactive NP59 or cholesterol, (⇒) indicates where the medium radioactivity was removed and the cells washed, and (○) indicates sterol radioactivity in the cells placed back in medium without any tracer. The data shown are from a representative experiment, duplicate points per experiment.

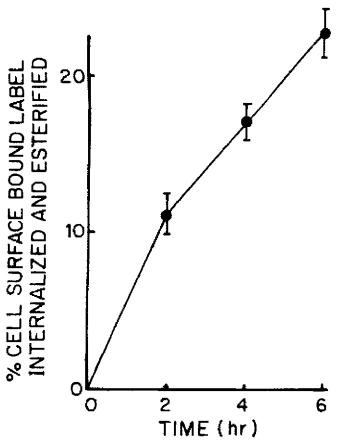

FIGURE 2
Dishes of cells (30 × 15 mm) were washed twice with 1 ml of Wa/BSA and placed in 1 ml of Wa/BSA containing 2 μCi NP59 (9 Ci/mmol) for 4 hr. At this time, the dishes were washed three times with Wa/BSA and placed in 1 ml of Wa/BSA. After the indicated periods of time, cells were extracted, the extract chromatographed, and the free and esterified sterol radioactivity quantitated. The esterified cholesterol radioactivity is presented as the percentage of bound free cholesterol radioactivity. Data shown are the mean ± range of two independent experiments, duplicate points per experiment.

TABLE 1
Cyclic AMP Directs Transport of ^{125}I-NP59

	Radioactivity in cholesteryl esters cpm/dish (% T = 4)		
	T = 4	T = 10	T = 10 + Bu_2cAMP
^{125}I-NP59			
Exp. 1	2803 ± 325	5511 ± 268 (197)	3704 ± 519 (132)
Exp. 2	3899 ± 255	9066 ± 288 (233)	3558 ± 46 (91)
^{3}H-cholesterol			
Exp. 1	5635 ± 404	16903 ± 3459 (300)	4320 ± 416 (77)
Exp. 2	11384 ± 734	31297 ± 5575 (275)	8605 ± 839 (76)

Dishes of MA-10 cells (30 × 15 mm) were washed twice in 1 ml of Wa/BSA and then placed in Wa/BSA containing 2 μCi ^{125}I-NP59 (3.9 Ci/mmol) or 0.5 μCi ^{3}H-cholesterol (65.5 Ci/mmol). After 4 hr at 37°C, all dishes were washed three times with 1 ml of Wa/BSA and then were either extracted immediately (T = 4) or placed in 2 ml of fresh Wa/BSA alone (T = 10) or containing 1 m*M* dibutyryl-cAMP (T = 10 + Bu_2cAMP). After six more hours at 37°, the latter two groups were removed and the cell pellets extracted in chloroform:methanol (2:1). All cell extracts were chromatographed and quantitated as described Figure 1. The data shown are the mean ± s.d. of triplicate points in each experiment.

after which unbound label was washed off and then the cells were allowed to accumulate label in the ester fraction in either the absence or presence of dibutyryl-cAMP stimulation. In this way, we could measure the extent to which cAMP inhibited either radioligands incorporation into cholesteryl ester (*7,8*). In cells surface-labeled with ^{125}I-NP59 but not stimulated by cAMP, the ester radioactivity increased on average 2.2-fold over the next 6 hr. Dibutyryl-cAMP treatment prevented this increase almost completely. Cells surface labeled with ^{3}H-cholesterol behaved much the same. In unstimulated cells radioactivity in the ester fraction increased by an average of 2.9-fold. Dibutyryl-cAMP stimulated cells, however, ended this incubation with less radioactivity than they started with, on average 77%. These later results were expected since it is known that the MA-10 cells contain a cAMP-stimulated cholesteryl ester hydrolase enzyme (*7*). In these experiments, we also tried to detect ^{125}I-steroid hormones that might be synthesized from ^{125}I-NP59. The MA-10 cells readily internalize ^{3}H-cholesterol and utilize this label to synthesize progesterone (*8,9*). Experiments directed towards finding an ^{125}I-steroid analogue in the medium of ^{125}I-NP59 treated, dibutyryl-cAMP stimulated cells were unsuccessful.

It is technically very difficult to measure the rate of ester hydrolysis in cells containing the small amounts of cholesteryl esters normally stored in MA-10 cells (*7*). This measurement can be made, however, if the cellular cholesteryl ester content is augmented by preincubating the cells with LDL. Table 2 data show experiments designed in a manner similar to the experiments of Table 1 but employing cells containing 3-fold more cholesteryl esters than in the previous experiments. Cholesteryl ester loaded ^{125}I-NP59 labeled cells internalized and esterified more ^{125}I-NP59 after the first 4-hr incubation than did cells with the basal level of cholesteryl esters. Allowing these cells to incubate an additional 6 hr in the absence of dibutyryl-cAMP caused on average 2.2-fold more radioactivity accu-

TABLE 2
Iodine-125-NP59 Is a Poor Substrate for the Cholesterol Ester Hydrolase Enzyme for MA-10 Cells

	Radioactivity in cholesteryl esters cpm/dish (% T = 4)		
	T = 4	T = 10	T = 10 + 58035 + Bu_2cAMP
^{125}I-NP59			
Exp. 1	7808 ± 735	19302 ± 761 (247)	8741 ± 744 (112)
Exp. 2	8137 ± 1291	16150 ± 1834 (199)	7280 ± 1799 (90)
^{3}H-cholesterol			
Exp. 1	9159 ± 1117	20392 ± 3450 (223)	4306 ± 316 (47)
Exp. 2	13875 ± 252	32663 ± 2044 (235)	6420 ± 721 (46)

Fourteen hours prior to T = 0 all dishes of cells were placed in growth medium supplemented with 50 μg/ml LDL cholesterol. At T = 0, dishes were washed two times with 1 ml Wa/BSA and placed in 1 ml WA/BSA containing 2.6 μCi of ^{125}I-NP59 (3.9 Ci/mmol) or 0.5 μCi ^{3}H-cholesterol (65.5 Ci/mmol). After 4 hr at 37°C, all dishes were washed three times with 1 ml Wa/BSA and either extracted immediately (T = 4) or placed in 1 ml of fresh Wa/BSA alone (T = 10) or Wa/BSA containing 2 μg/ml 58035 and 1 m*M* dibutyryl-cAMP (T = 10 + 58035 + Bu_2cAMP). After 6 hr more at 37°C, the latter two groups were removed and the cell pellets extracted, chromatographed, and quantitated as described in Figure 1. The data shown are the mean ± s.d. of triplicate points in each experiment.

mulation in the ester fraction. Cells incubated for 6 hr with dibutyryl-cAMP did not accumulate any more radioactivity in the ester fraction but also did not mobilize radioactivity from the esters; on average the radioactivity at the end of this incubation is the same as at the beginning, 101%. The cellular handling of the ^{3}H-cholesterol surface label is similar to the handling of ^{125}I-NP59 during the basal labeling period of 4 hr and during the subsequent 6 hr for the unstimulated cells. Cholesterol ester-loaded cells internalized more ^{3}H-cholesterol during the 4-hr labeling incubation than cells not cholesteryl ester-loaded. During the subsequent 6 hr in the absence of dibutyryl-cAMP, the radioactivity in the ester fraction increased an average of 2.3-fold. If, however, dibutyryl-cAMP was present in the second incubation, the radioactivity in the ester fraction decreased to 47% of the levels present at the beginning of the incubation. Thus, esters present in this fraction at the beginning of the incubation were hydrolyzed and used for steroid hormone synthesis. The data contrast sharply with the ^{125}I-NP59 data where no mobilization of radioactivity from the ester fraction occurred. Thus, ^{3}H-cholesteryl ester is hydrolyzed while ^{125}I-NP59 ester is not.

DISCUSSION

Although compounds such as NP59 have achieved usefulness as clinical tools to image adrenal or tumors of steroidogenic tissues (*1*), little is known about the processing of this radioisotope by animals or cells. The present study was directed towards understanding the cellular processing of this compound. We found that ^{125}I-NP59 was readily taken up into MA-10 Leydig tumor cells. This compound entered the cells independently of lipoproteins. Uptake was dependent on binding to the cell surface and subsequent internalization of tracer. Once inside the cell, ^{125}I-NP59 was readily esterified but could not be converted into steroid hormones nor could it be mobilized once stored as an ester.

The steroidogenic tissue of man as well as several other species uses circulating lipoproteins as the major source of cholesterol for steroidogenesis (reviewed in *16*). The best evidence would indicate that 19-iodocholesterol probably enters the adrenals of rats along with lipoprotein cholesterol (*2*). It seems likely that NP59 may be taken up by more than this single mechanism. Studying the uptake of NP59 and NP59 incorporated into lipoproteins revealed that the compound itself was taken up much more completely than when it was incorporated into lipoproteins (*17*). The present data may explain this discrepancy. Direct cellular uptake by the adrenal may account for the differences detected in this study.

In these experiments, ^{125}I-NP59 appeared to bind the cell surface less well than ^{3}H-cholesterol. Thus, a significant portion of ^{125}I-NP59 radioactivity would dissociate from the cell while all ^{3}H-cholesterol remained tightly bound. This apparent lower affinity binding might be explained by the different specific radioactivities of two radioligands. The specific radioactivity of ^{125}I-NP59 is about 10-fold less than ^{3}H-cholesterol. An even lower specific radioactivity ligand, ^{14}C-cholesterol (10^{3}-fold less than ^{3}H-cholesterol) binds minimally to the cell surface of MA-10 cells.

Once inside the cell, NP59 behaves much like 19-iodocholesterol. Like 19-iodocholesterol (*3*), NP59 is readily esterified. A much greater proportion of cell surface bound ^{125}I-NP59 becomes trapped in the ester fraction than does ^{3}H-cholesterol. This occurs not because NP59 is a better substrate for esterification but because esterified NP59 is trapped as an ester. The steroidogenic cell can neither excrete NP59 as steroid hormones nor mobilize NP59 ester from stores. In this respect, NP59 again resembles 19-iodocholesterol (*4*) in that it also appears to be a poor substrate for the cellular cholesteryl ester hydrolase. It seems that any successful adrenal or steroidogenic cell scanning agent must possess this property of becoming trapped by the cell.

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SELF-STUDY TEST
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ANSWERS

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radiographs will often show the mass, computed tomography may be warranted in this situation when the standard radiographs are normal. Frequently, ventilation will be normal, although a large mass may impinge sufficiently on the bronchus to cause a ventilatory abnormality, as well.

Pulmonary embolism is not likely in this case and should be placed lower on the list of possibilities than the other entities. Because emboli are usually multiple and frequently shower into both lungs, it would be unusual to observe a massive embolus entirely occluding flow to one lung without detecting any perfusion deficit in the other lung. In one recent revew of 525 perfusion scintigrams, there were 16 cases with massive unilateral perfusion defects. Pulmonary embolism was the cause in only one of these patients.

Other causes of massive unilateral perfusion defects include pulmonary artery agenesis or stenosis, Swyer-James syndrome, bronchial adenoma or foreign body, pulmonary artery sarcoma, massive pleural effusion and pneumothorax, and shunt procedures for congenital heart disease.

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ITEM 4: The "Stripe Sign"

ANSWERS: **A,** T; **B,** T; **C,** T

The perfusion defect seen in Figure 1 exhibits a "stripe sign," i.e., a rim of activity (well maintained perfusion) between the perfusion defect and the adjacent pleural surface. Acute pulmonary embolism is uncommon in the artery supplying a defect with an associated "stripe sign," as might be expected, because embolism should interrupt perfusion to an entire segment or subsegment and cause a defect extending to the pleural surface. In a study of 18 lung segments with the "stripe sign" reported by Sostman et al., only one was found by angiography to be due to a pulmonary embolus. Many (but not all) of these defects were matched with ventilation abnormalities.

In a study of serial lung imaging in patients undergoing angiography for suspected pulmonary embolism, Alderson et al. found that a peripheral rim of activity developed adjacent to perfusion defects during resolution of documented emboli in 2 of 30 patients. The initial studies in both of these patients had shown pleural-based segmental defects in the same regions. Thus, resolving pulmonary embolism can cause the "stripe sign." In the same study, however, there were eleven perfusion defects with "stripe signs" that did not change in appearance between the first and second perfusion studies; none of these were associated with pulmonary embolism.

Murata et al. studied eleven lung segments showing "stripe signs" on conventional perfusion scintigrams. In their evaluation employing chest radiography, positron-tomographic ventilation imaging with ^{13}N and CT imaging, they found ventilation abnormalities in all eleven segments, with a central distribution in ten. Most of their patients had CT evidence of emphysematous changes. Thus, the finding in this patient is most likely due to centrally located emphysema.

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