BIOPHYSICS AND BIOCHEMISTRY

Regulation of ^{14}C-Cholesterol Uptake from Mixed Micelles by Organ Culture of the Mucosa Epithelium from Human Small Intestine: Effects of Selective ACAT Inhibitor

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The initial rate of ^{14}C-cholesterol uptake from mixed micelles is studied using organ culture of mucosal epithelium from human small intestine obtained during surgery. Intense, reproducible, and ACAT-regulated level of specific ^{14}C-cholesterol uptake is monitored during the first 3 h of culturing. The specific ^{14}C-cholesterol uptake is assessed by the difference between the total uptake of ^{14}C-cholesterol and nonspecific uptake of ^{14}C-sitosterol from the micelles. It is shown that less than 20% of absorbed ^{14}C-cholesterol is converted into cholesterol esters. C44788, a high-affinity and selective ACAT inhibitor, inhibits ^{14}C-cholesterol uptake in a dose-dependent manner and practically has no effect on ^{14}C-oleate uptake.

Key Words: *small intestine; enterocytes; absorption; lipids; cholesterol; ACAT*

Since the small intestine is the major organ responsible for absorption of dietary lipids and synthesis of intestinal lipoproteins, *in vitro* modeling of these processes is essential for further experimental studies of cell mechanisms underlying atherogenic dyslipoproteinemias and accelerated atherosclerosis. Over many years absorption of dietary cholesterol (CH) was studied in acute or chronic animal experiments [2, 6,8] or in humans by measuring the distribution of radiolabeled CH in the blood, feces, and bile [3,5]. However, physiological models provide little information for precise studies of the intralumenal and intracellular phases of the absorption of dietary lipids. Previously, we developed a technique of the culturing of mucosa explants from the small intestine of children (emergency autopsies) [9]. In the study of intestinal absorption the organ culture of mucosal epithelium offers important advantages over primary enterocyte culture and transformed colon-derived epithelial CaCo2 cells [4,7]. Mucosa explants maintain the specific spatial organization of intestinal crypts and villi with respect to the basal membrane and submucosa. The culturing is performed in a serum-free medium in order to exclude the effects of hormones and "signals" from the serum. Micelles with incorporated ^{14}C-CH are applied onto the lumi-

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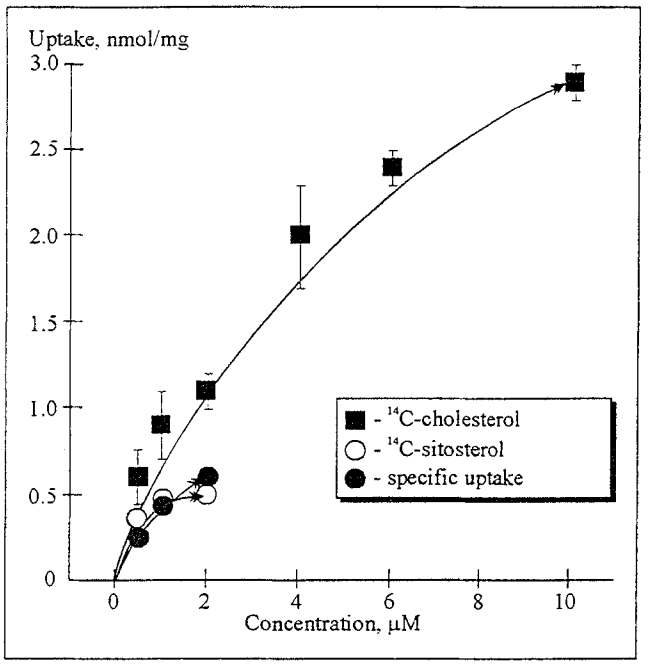

Fig. 1. Uptake of micellar ^{14}C-cholesterol by organ culture of mucosa explants from small intestine of adult humans.

nal surface, and the distribution of CH in the cell and medium is assessed. Simultaneously, the initial rate of ^{14}C-CH uptake with respect to the uptake of ^{14}C-sitosterol (^{14}C-SS) and esterification of labeled CH are measured. In the present study we used mucosa explants from human small intestine with a higher level of specific uptake of micellar ^{14}C-CH and a lower level of nonspecific ^{14}C-SS uptake [1,9].

Mucosa explants from small intestine were used in the study of the first stage of absorption — cholesterol uptake from ^{14}C-CH-labeled taurocholate micelles. Therefore, here by CH uptake is meant cholesterol uptake by enterocytes from mixed micelles [1,9].

MATERIALS AND METHODS

All duodenal segments were obtained during abdominal repair surgery at the A. V. Vishnevskii Surgery Center. Preoperative diagnosis in the majority of patients was duodenal adenocarcinoma. Autopsy specimens were obtained from 12 men aged 35-59 and 2 women (35 and 45 years old). A 5-cm-length intestinal segment was excised 15-20 cm below the ligament of Treitz and immediately placed into cold oxygenated Hanks' solution containing 20 mM HEPES, fungizone, kanamycin, and polymyxin (100 μg/ml each) [1,9]. The specimens were repeatedly washed with Hanks' solution, the mucosa was separated from the media-adventitia. Explants of standard size (2×2 mm) were transferred the mucosa surface up to 24-well plates. Each well contained 0.5 ml preoxigenated Williams' medium E supplemented with 20 mM HEPES, 4 μg/ml insulin, kanamycin, fungizone, and polymyxin (100 μg/ml each, Sigma). The specimens were preincubated for 1 h at 37°C in a CO_2-incubator, and the medium was replaced with the test solution. All manipulations were performed under sterile conditions. Taurocholate micelles containing ^{14}C-CH or ^{14}C-SS were prepared as described previously [1,9].

The mucosa explants were incubated with micelles containing 2 μM unlabeled CH and 40 μCi/ml ^{14}C-CH. After the incubation, the micelles were washed with a 100-fold volume of Williams' medium, and the explants were weighted. Lipids were routinely extracted and separated by thin-layer chromatography on Kieselgel G plates (Merck, Darmstadt). Chromatograms were stained with 10% phosphomolybdic acid. CH and CH ester spots were cut off, transferred into vials with a dioxane scintillation liquid, and counted

TABLE 1. Correlation Between Specific ^{14}C-CH Uptake from Micelles and Synthesis of ^{14}C-CE

Total uptake of ^{14}C-CH, nmol/mg	^{14}C-SS uptake, nmol/mg	Tissue ^{14}C-CE, nmol/mg	%	Medium ^{14}C-CE, nmol/mg	%
5.2±1.3	0.5±0.02	0.2±0.1	4.6	6.2±0.1	4.4
4.9±1.1	0.5±0.02	0.6±0.2	12.7	1.1±0.2	24.4
6.6±0.8	0.7±0.01	0.6±0.15	9.0	0.1±0.02	2.0
6.6±0.4	0.4±0.01	1.1±0.3	15.5	0.3±0.03	4.2
5.8±0.8	0.7±0.15	0.4±0.05	6.9	0.2±0.03	3.4
5.8±0.5	0.1±0.04	1.9±0.6	32.6	0.8±0.06	14.0
10.6±0.3	0.8±0.02	1.8±0.02	16.5	1.2±0.03	11.6
7.4±0.5	0.8±0.02	—	—	—	—
Mean values					
6.05±1.7		0.93±0.6		0.56±0.4	

Note. r=0.60, p=0.15 for tissue CE; r=0.42, p=0.35 for medium CE. Total percent of CE is 1.49±1.01=25%.

TABLE 2. Dose-Dependent Effects of C44788 on Specific Uptake of Micellar ^{14}C-CH in Organ Culture of Mucose Explants from Human Small Intestine

Parameter	Concentration of C44788, nM						
	0	25	50	75	100	200	500
Experiment 1							
^{14}C-CH uptake	4.9±1.1		2.2±0.8	0.8±0.1	0.9±0.4	1.3±0.04	1.7±0.7
% of control	100±22		45±16	16±2	18±8	26±0.8	35±14
^{14}C-SS uptake	0.5±0.02			0.2±0.04	0.2±0.04		0.17±0.1
Experiment 2							
^{14}C-CH uptake	10.6±0.3	6.0±0.2	6.0±0.8	5.1±1.0	2.0±0.02	1.3±0.15	1.2±0.13
% of control	100±3.0	56.6±2.0	57±1.9	47±9.4	18.9±2.0	12.2±1.4	11.3±1.2
^{14}C-SS uptake	0.8±0.2		0.4±0.2	0.7±0.01	0.8±0.04	0.5±0.2	0.6±0.2
Experiment 3							
^{14}C-CH uptake	7.4±0.5	5.5±0.2	3.6±0.1	2.5±0.4	5.1±2.2	1.6±1.2	1.3±0.04
% of control	100	73±3	49±1	33±5	69±30	21±3	17±0.5
^{14}C-SS uptake	0.8±0.2	0.8±0.2			0.4±0.1		0.6±0.02
Experiment 4							
^{14}C-CH uptake	6.9±0.4	4.8±0.1	4.1±0.02	3.9±0.4	4.4±0.3	2.0±0.1	1.8±0.1
% of control	100	70±1	59±0.2	56±5	63±4	29±1	26±1
^{14}C-SS uptake	0.5±0.05		0.4±0.1		0.5±0.08		
M±SE	7.4±2.4	5.4±0.6	4.0±1.6	3.5±1.8	3.1±2	1.5±0.3	1.5±0.3
%	100	72±8	53±21	47±24	42±27	21±4.4	20±3.8

in a Rack-Beta β-counter (LKB). Specific CH uptake by the mucosa segments was determined from the difference between the total ^{14}C-CH uptake and ^{14}C-SS uptake and expressed in nM CH per 1 mg wet tissue [1,9].

Each measurement was repeated 3-4 times. The significance of the differences was evaluated by the Student test.

RESULTS

In preliminary experiments we studied ^{14}C-CH and ^{14}C-SS uptake by cultured mucosa explants as a function of time. Both the total and specific uptake of ^{14}C-CH rose during the first 3 h and attained a plateau between 3 and 6 h, whereas ^{14}C-SS uptake remained practically unchanged throughout the incubation. During the first 3 h of incubation, the nonspecific uptake of ^{14}C-SS constituted about 5% (data not shown). Taking these data into account, we have chosen the first 2 h as the best suiting for measuring the initial rate of ^{14}C-CH micelle uptake.

Figure 1 shows the dose-effect curves for the total and specific uptake of ^{14}C-CH micelles. This dependence was almost linear in a concentration range of 0.5-2.0 μM and non-linear from 2.0 to 6.0 μM without reaching a plateau, whereas the uptake of ^{14}C-SS micelles was much less dependent on the concentration of the plant sterol. In view of this, we have chosen a CH concentration of 2 μM for measurements of the initial rates of the CH micelle uptake.

In the next experimental series, we analyzed the correlation between the total and specific uptake of ^{14}C-CH micelles and the level of CH esters (CE) synthesized from exogenous micellar ^{14}C-CH. Table 1 summarizes the data on the total and specific uptake of ^{14}C-CH and the level of ^{14}C-CE in the mucosa and in the incubation medium for 8 mucosa explants from different donors. There was only a weak correlation between ^{14}C-CH uptake from the taurocholate micelles and the intensity of CH synthesis in cells and culture medium. Judging from the mean values of ^{14}C-CH and ^{14}C-CE, only 20-25% of micellar CH is converted into CE. When the intestinal mucosa explants from children obtained during acute autopsy were incubated under standard conditions with ^{14}C-CH-labeled micelles, no synthesis of ^{14}C-CE was detected (Table 1).

These data suggest that in mucosa explants obtained from adults less than 25% of micellar ^{14}C-CH are converted into CE and only half of them is located in the enterocytes.

For elucidation of the role of ACAT in the uptake and esterification of micellar dietary CH, mucosa explants were preincubated in the presence of ascending concentrations of C44788 (Wellcome), a

TABLE 3. Dose-Dependent Effect of C44788 on Synthesis of CE from ^{14}C-Oleate—Bovine Serum Albumin

Parameter	Concentration of C44788, nM					
	0	50	75	100	200	500
Tissue CE, nmol/mg	12±0.6	10.4±0.3	4.12±1.04	6.03±1.1	7.0±1.7	8.74±3.2
	11.9±0.43	1.8±0.17	1.9±0.1	1.7±0.04	1.7±0.2	—
	3±1.7	3.4±0.4	2.6±0.3	2.8±0.3	3±0.1	2±0.1
Medium CE, nmol/mg	24.2±7.5	14.3±2	7.5±0.2	11.9±2.9	22.5±8	8.18±1.3
	26.5±6.1	21.7±4.4	19.08±2.7	16.98±2.7	18.9±1.2	13.8±1.4
	31±1.7	34±0.4	26±0.3	28±0.3	30±0.1	20±1
M±SE	27.2±3.5	23.3±10	17.5±9.4	18.9±8.2	23.8±6	13.9±6

selective ACAT inhibitor (kindly provided by Dr. R. Arrowsmith). The explants were incubated for 2 h in Williams' medium in the presence of C44788, transferred to a fresh portion of Williams' medium, and incubated for 2 h with micelles containing 2 μM unlabeled CH and 0.02 μCi/ml ^{14}C-CH. Nonspecific uptake was measured after a 2-h incubation of some explants with micelles containing 2 μM unlabeled SS and 0.02 μCi/ml ^{14}C-SS. As seen from Table 2, in all four experiments the inhibitor (25-500 nM) reduced specific ^{14}C-CH uptake in a dose-dependent manner. IC_{50} was within the range of 50-100 nM. The maximum inhibition (~80%) was observed at a C44788 concentration of 200-500 nM. Unlike CH, the uptake of micellar ^{14}C-SS after preincubation with C44788 remained practically unchanged. The synthesis of CE from micellar ^{14}C-CH was inhibited by C44788 in much the same manner (IC_{50}=50-100 nM), which attests to the involvement of ACAT into specific uptake of micellar ^{14}C-CH.

In the next experimental series, we studied the effects of varying concentrations of C44788 on the transport of fatty acids, the second substrate of ACAT. The explants were incubated for 2 h with ascending concentrations of C44788, then washed, transferred into a fresh portion of Williams' medium, and incubated for 2 h in the presence of 50 μl ^{14}C-oleate-bovine serum albumin (final activity 5 μCi/ml) without exogenous micellar CH. After the incubation, the explant was washed, lipids were extracted from the tissue and medium by the method of Folch, and separated by thin-layer chromatography. The radioactivity of CE spots was counted in 3-4 parallel samples by the single-point assay. As follows from Table 3, ^{14}C-oleate incorporation into CE in tissue was inhibited in one of three experiments, while in the medium C44788 inhibited CH esterification in all experiments in a dose-dependent manner, the maximum effect (40-60%) being observed at an inhibitor concentration of 100-500 nM. Since the absolute rate of CH esterification with ^{14}C-oleate lay in a picomolar range, we assume that the one order of magnitude lower rate of CE synthesis can be attributed to the deficiency or unavailability of endogenous intracellular CH for ACAT.

This assumption was verified in the next experimental series. The explants were first incubated with ascending concentrations of C44788 for 2 h,

TABLE 4. Dose-Dependent Effect of C44788 on Synthesis of CE from ^{14}C-Oleate and ^{3}H-CH

Parameter	Concentration of C44788, nM					
	0	50	75	100	200	500
Tissue CE						
^{14}C-oleate-CE, pmol/mg	3±1.7	3.4±0.4	2.6±0.3	2.8±0.3	3.0±0.1	2.0±0.1
% of control	100±15	110±13	87±8	91±43	100±43	75±30
^{3}H-CH-CE, nmol/mg	1.9±0.6	0.3±0.04	0.2±0.06	0.17±0.01	0.1±0.04	0.07±0.02
% of control	100±31	16±2.1	11±3.2	9.0±0.5	5±0.34	0.5±0.2
Medium CE						
^{14}C-oleate-CE, pmol/mg	27.2±3.5	23.3±10	17.5±9.4	18.9±9.4	23.8±6	13.9±6
% of control	100±13	86±36	64±35	69±35	88±22	51±22
^{3}H-CH-CE, nmol/mg	0.7±0.08	0.48±0.09	0.3±0.02	0.4±0.06	—	0.3±0.03
% of control	100±11	68.5±13	43±2.9	57±8.6	—	43±4.2

washed, and the medium was replaced with a fresh portion containing bovine serum albumin-^{14}C-oleate (5 μCi/ml) and taurocholate micelles (2 μM CH/5 μCi/ml ^{3}H-CH). Incubation was carried out at 37°C for 2 h, after which the explants were washed, the lipids were extracted from the tissue and medium, separated by thin-layer chromatography, and the radioactivity of ^{14}C-oleate and ^{3}H-CH in CE spots was counted. As seen from Table 4, C44788 inhibited in a dose-dependent manner the synthesis of CE from micellar ^{3}H-CH, while the synthesis of CE from ^{14}C-oleate was inhibited only in the medium but not in the mucosa explants. These findings suggest that C44788 selectively inhibits the CE synthesis from micellar exogenous CH but has no effect on the transport of exogenous ^{14}C-oleate.

In the last experimental series, we found that the selective uptake of micellar ^{14}C-CH with respect to nonspecific uptake of ^{14}C-SS occurred only in viable cells. To this end, some mucosa explants were incubated for 10-180 min in the presence of 2 μg/ml sodium azide. After the incubation, the explants were washed, and the standard aliquot of mixed micelles containing ^{14}C-CH and ^{14}C-SS was added. The uptake of both ^{14}C-CH and ^{14}C-SS decreased as soon as after 10 min of incubation with azide. During the next hour the uptake of ^{14}C-CH continued to drop, while the uptake of ^{14}C-SS gradually increased. The formation of CE from micellar ^{14}C-CH also decreased with prolongation of incubation with azide. This corresponded to the appearance of numerous trypan-positive cells in crypts and mucosa. Trypan-positive cells first appeared after a 30-min incubation, while in the control explants the viability of enterocytes (except for the marginal zones) was preserved over a 6-12-h incubation.

Thus, the proposed technique for culturing the mucosa explants from human small intestine ensure the native structure of crypts and villi during the first 6 h, when the initial rate of labeled CH uptake from dietary micelles was measured.

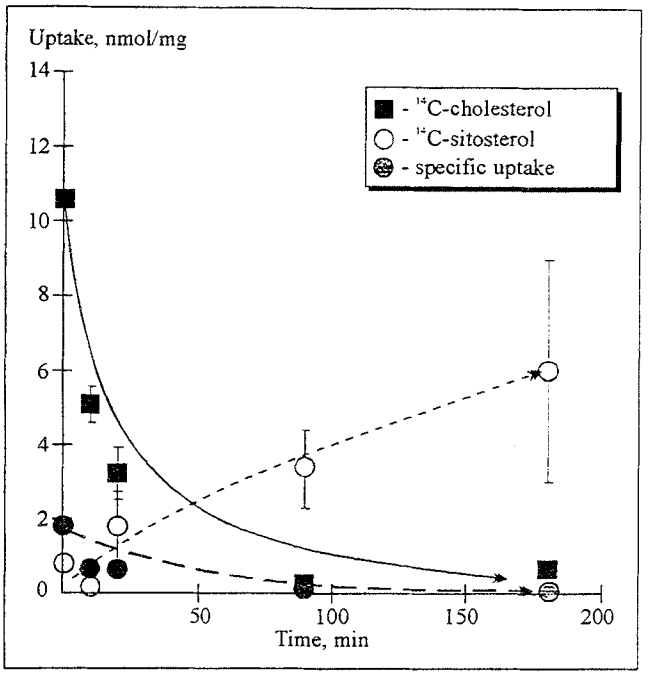

Fig. 2. Effect of sodium azide on the uptake of ^{14}C-cholesterol, ^{14}C-sitosterol, and synthesis of cholesterol esters in tissue culture of intestinal explants.

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