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A FURTHER STUDY OF THE KINETICS OF CHOLESTEROL UPTAKE AT THE ENDOTHELIAL CELL SURFACE OF THE RABBIT AORTA *IN VITRO*

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SUMMARY

This study is concerned with a further development of the kinetics of the uptake *in vitro* of [4-^{14}C]cholesterol by intima-media layers of thoracic aortas from normal rabbits.

1. Separation of the intima-media [^{14}C]cholesterol into the free and esterified forms revealed that the majority of the labelled serum cholesterol, bound initially at the luminal endothelial cell membrane, was in the esterified form. Following the initial binding phase, no significant increase was observed in esterified [^{14}C]cholesterol of the intima-media, whereas the intima-media content of free [^{14}C]cholesterol increased with time at a rate equal to that for the uptake of total [^{14}C]cholesterol in the second phase. A transport of esterified [^{14}C]cholesterol across the endothelial cell surface would thus imply a hydrolysis following uptake.

2. These findings are in agreement with the demonstration that less than 3 % of the non-labelled intima-media cholesterol pool of the rabbits is found in the esterified form.

3. Based on the above findings a model is shown for the uptake of cholesterol by intima-media. The mathematical treatment of the model indicates that the previously found relationship between cholesterol transfer rate and concentration of total cholesterol in serum is in agreement with the model. An estimation of the amount of cholesterol, which maximally can be bound per unit surface area, shows this amount to be of the same order of magnitude as the amount of cholesterol found in a lipoid monolayer of the same area. The rate constant for the cholesterol transfer across the endothelial cell surface is about the same order of magnitude as that for the ingestion in rabbit macrophages.

4. The kinetics presented are compatible with morphological descriptions of a pinocytotic uptake process.

INTRODUCTION

The uptake of serum lipoprotein-bound [^{14}C]cholesterol at the endothelial cell surface of the rabbit aorta was previously shown to proceed biphasically in time, in so far as an initial, rapid, temperature-independent uptake was followed by a slower, temperature-dependent uptake[1]. The relationship between the transfer rate of total cholesterol corresponding to the second phase and the concentration of total cholesterol

in the serum used as incubation medium might point to saturation kinetics. According to this, a limited number of cholesterol-binding sites at the luminal endothelial cell membrane was suggested. Although separation of intima-media [^{14}C]cholesterol into free and esterified cholesterol was not performed, it could be concluded that a preferential uptake of one of the two forms of cholesterol could not explain the observed relationship.

In studies on the uptake of labelled cholesterol by the intima-media of the intact but atheromatous rabbit aorta[2] and by normal rat aorta *in vitro*[3], it has been tentatively assumed that the finding of more labelled cholesterol in the free fraction of the aortic tissue than could be accounted for on the basis of the distribution of labelled cholesterol between free and esterified serum cholesterol, was an indication of a preferential uptake of the non-esterified form. However, in the former study[2] it was admitted that hydrolysis of esterified cholesterol after uptake might explain the observations.

It is the aim of this study to elucidate how much free and esterified cholesterol contribute to cholesterol uptake at the endothelial cell surface, taking into account the existence of two phases in the uptake process.

MATERIALS AND METHODS

White female rabbits of uniform breed were obtained from The Danish State Serum Institute. They were used at an age of 4–6 months and their weights ranged from 2.42 to 2.52 kg.

The preparation of aortic hemisegments, the serum labelling procedure, the incubation technique and the analytical details have been described previously[4]. The label used was ^{14}C in the biologically stable 4-position of the cholesterol molecule. Following incubation of the aortic hemisegments in the [^{14}C]cholesterol-containing serum intima-media layers were prepared from the central parts of right and left hemisegments, cut out at two levels of the thoracic aorta. The incubations took place at 38° for different times (1 to 240 min).

The cholesterol of the aortic lipid extract was separated into free and esterified cholesterol according to the method of CARROLL[5] as earlier described for separation of serum cholesterol[4]. The intima-media content of free and esterified non-labelled cholesterol was determined on non-incubated aortic hemisegments.

RESULTS

Intima-media content of free and esterified non-labelled cholesterol

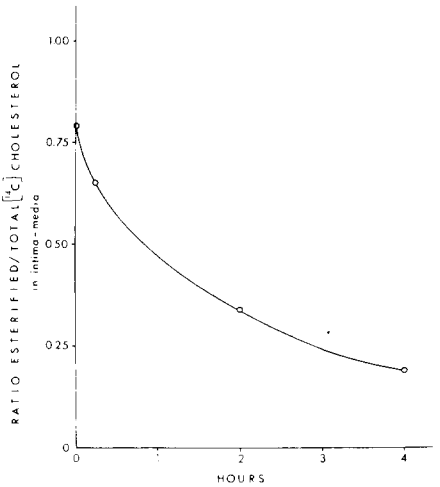
Separation into free and esterified non-labelled cholesterol was performed on eight intima-media layers. The results (means ± S.E.) are: free cholesterol, 5.21 ± 0.13 mg per g dry wt.; esterified cholesterol, 0.14 ± 0.01 mg per g dry wt. Thus it appears that esterified cholesterol makes up only 2–3 % of the intima-media cholesterol pool.

Intima-media content of free and esterified labelled cholesterol following incubation in [^{14}C]cholesterol-containing serum

Fig. 1 shows the ratio between esterified and total [^{14}C]cholesterol found in intima-media following various incubation times. In the serum used as incubation

medium the corresponding ratio was found to be 0.73 by chemical determination and 0.70 by radioactive determination.

In a previous study[1] equations were calculated for the relative uptake of free + esterified [^{14}C]cholesterol by (1) upper and (2) lower intima layers as a function of



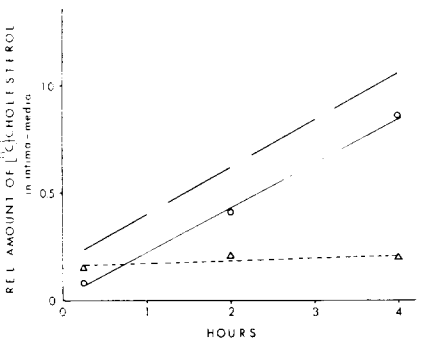


Fig. 1. Ratio esterified to total [^{14}C]cholesterol within intima-media as a function of incubation time. Each point is a mean value of two derterminations originating from 3–5 pooled intima-media layers from the upper and lower part of the thoracic aorta.

Fig. 2. The amount of free and esterified labelled cholesterol within intima-media at different incubation times. The values are expressed relative to a control by combination of the values shown in Fig. 1 and the equation for the relative uptake of total [^{14}C]cholesterol as a function of time (see text). — —, total [^{14}C]cholesterol; ○—○, free [^{14}C]cholesterol; △—△, esterified [^{14}C]cholesterol.

time. Combining these two equations we get the following equation for upper + lower intima-media layers:

$$\frac{^{14}\text{C activity}(t)}{^{14}\text{C activity}(4\ h)} = 0.22\ t + 0.18$$

This equation will be used here, because each point in Fig. 1 originates from analysis on the same number of intima-media layers from upper and lower parts of the thoracic aorta. As the rabbits used are of uniform breed and age and the above relationship is based on fifty observations, it is assumed that this relationship also applies to the intima-media layers used in this study. If we combine the ratios in Fig. 1 (the ordinate values) with the equation given above, the intima-media content of free and esterified [^{14}C]cholesterol can be calculated relative to the 4-h uptake of [^{14}C]cholesterol (total) at different incubation times. The results are shown in Fig. 2.

As the above equation for relative uptake of [^{14}C]cholesterol as a function of time is calculated for t values between 15 and 240 min ordinate values in Fig. 2 can only be shown within this time interval.

Fig. 2 indicates that the intima-media content of esterified, labelled cholesterol does not increase significantly following the initial binding phase. The amount of free, labelled cholesterol within intima-media increases with the time of incubation at a

rate equal to that for the uptake of total cholesterol corresponding to the second phase.

DISCUSSION

It appears from Fig. 1 that esterified cholesterol constitutes the greatest part of the [^{14}C]cholesterol bound in the rapid, initial phase. As the curve has a relatively steep course at low time values it cannot be judged from Fig. 1 whether the initially bound [^{14}C]cholesterol consists only of esterified cholesterol or esterified and free cholesterol in a ratio similar to that found in the serum used for incubation. Fig. 2, however, indicates that the amount of esterified [^{14}C]cholesterol within intima-media does not increase significantly after the initial binding phase, while the amount of free [^{14}C]cholesterol increases with time of incubation at a rate equal to that for the uptake of total [^{14}C]cholesterol corresponding to the second phase. In a previous study it was shown that the cholesterol uptake by intima-media in this phase represents the unidirectional transport of cholesterol across the endothelial cell surface from serum. The fact that all the serum [^{14}C]cholesterol transported to intima-media is found in the free form is in accordance with the finding that more than 97 % of the intima-media tissue cholesterol is in the non-esterified form.

The results shown in Fig. 2 thus indicate the following possibilities: (1) that only serum [^{14}C]cholesterol in the free form is transported across the endothelial cell membrane; (2) that if the [^{14}C]cholesterol which has passed across the endothelial cell membrane, is also derived from the esterified form in serum, then a rapid and complete hydrolysis takes place. Such a hydrolysis may occur within the intima-media following a transport of cholesterol in the esterified form across the endothelial cell surface, or it may be an essential step in the transport process for esterified cholesterol in the serum used for incubation. Both possibilities will enter into the following kinetic considerations.

Assume that free cholesterol (F) and esterified cholesterol (E) in serum react with endothelial cell membrane loci (M) to form reversibly two surface complexes (MF) and (ME), from which free and esterified cholesterol are transported into intima-media (F^+) and (E^+) at the same rate, and that intima-media cholesterol in the esterified form (E^+) undergoes a fast and complete hydrolysis to free cholesterol (F^+):

$$F + M_f \underset{k_2}{\overset{k_1}{\rightleftharpoons}} MF \xrightarrow{k_3} F^+ + M$$

$$E + M_f \underset{k'_2}{\overset{k'_1}{\rightleftharpoons}} ME \xrightarrow{k_3} E^+ + M$$

$$E^+ \xrightarrow{k_4} F^+$$

M_f = concentration of free endothelial cell membrane loci. If M_t = total concentration of endothelial cell membrane loci, then by conventional kinetic postulates:

$$\frac{dMF}{dt} = k_1 \cdot M_f \cdot F - k_2 \cdot MF - k_3 \cdot MF \qquad (1)$$

$$\frac{dME}{dt} = k'_1 \cdot M_f \cdot E - k'_2 \cdot ME - k_3 \cdot ME \qquad (2)$$

For steady-state conditions ($dMF/dt = 0$ and $dME/dt = 0$) M_f can be expressed in MF + ME:

$$M_f = (MF + ME)\frac{K_F \cdot K_E}{K_E \cdot F + K_F \cdot E} \quad (3)$$

in which

$$K_F = \frac{k_2 + k_3}{k_1} \quad (4)$$

and

$$K_E = \frac{k'_2 + k_3}{k'_1} \quad (5)$$

The transfer rate for total cholesterol across the endothelial cell membrane is given by the equation:

$$v = dF^+/dt = k_3 \cdot MF + k_4 \cdot E^+ \quad (6)$$

According to the preliminary assumptions, $k_4 \cdot E^+ = k_3 \cdot ME$ and therefore

$$v = k_3(MF + ME) \quad (7)$$

By definition

$$M_t = M_f + MF + ME \quad (8)$$

Substituting M_f in this equation with the right side of Eqn. 3 and multiplying on both sides of the equation with k_3, we get the following expression:

$$v_{max} = v\left(\frac{K_F \cdot K_E}{K_E \cdot F + K_F \cdot E} + 1\right) \quad (9)$$

or

$$\frac{1}{v} = \frac{1}{v_{max}}\left(\frac{K_F \cdot K_E}{K_E \cdot F + K_F \cdot E} + 1\right) \quad (10)$$

in which v_{max} has been substituted for the product $k_3 \cdot M_t$ and represents the maximal rate of transport at an infinite concentration of total cholesterol irrespective of the ratio between F and E.

Fig. 2 seems to indicate that steady state is obtained after the initial 15-min period at least for esterified cholesterol, because the intima-media content of esterified [^{14}C]cholesterol remains constant from $t = 15$ to 240 min. The finding of non-significant changes in the specific activity of serum cholesterol and the components entering into this quantity[4], and of a constant rate of increase in free [^{14}C]cholesterol (Fig. 2) during incubation, seems to justify further the assumption of steady-state conditions in the experiments during the second phase of cholesterol uptake.

It appears from Eqn. 10 that a rectilinear relationship between $1/v$ and the reciprocal concentration of total cholesterol in serum should be expected when the concentration of free cholesterol (F) and esterified cholesterol (E) are proportional to the concentration of total cholesterol.

In a previous study[1] the following relationship was found between rate (v) of cholesterol transfer across the endothelial cell surface and the concentration [Ch] of total cholesterol in serum used for incubation:

$$\frac{1}{v} = 11.9\frac{1}{[Ch]} + 17.1 \quad (11)$$

in which v has the dimension $\mu g \cdot cm^{-2} \cdot h^{-1}$ and [Ch] the dimension $\mu g \cdot \mu l^{-1}$. As the specific activity of free cholesterol always is equal to that of esterified cholesterol in the sera used as incubation media, v will express the transfer rate for cholesterol, whether it is derived from the free form, the esterified form or both in serum. No significant variation was found in the ratio esterified/total cholesterol of serum as a function of total cholesterol concentration[1]. This means that the concentration of free as well as esterified cholesterol is proportional to the concentration of total cholesterol in serum. The above relationship, $1/v$ and $1/[Ch]$, is thus in agreement with the model presented.

By inserting $1/[Ch] = 0$ in Eqn. 11 v_{max} can be calculated to be 0.058 $\mu g \cdot cm^{-2} \cdot h^{-1}$. The rate constant k_3 can be calculated from the afore-mentioned relationship between relative uptake of [^{14}C]cholesterol and time, [^{14}C]cholesterol (t)/[^{14}C]cholesterol (4 h) = $0.22\,t + 0.18$, assuming that the extrapolated uptake for $t = 0$ represents the relative amount of [^{14}C]cholesterol bound to the endothelial surface loci. Thus $k_3 = 0.22/0.18 = 1.2\ h^{-1}$. This may lead to an estimation of the amount of cholesterol which maximally can be bound per cm^2 intimal cell surface, or of the mean area per molecule cholesterol. The calculated values are 0.05 $\mu g/cm^2$ and 130 Å^2/molecule, respectively.

The cholesterol amount per cm^2 stroma surface area of rabbit erythrocytes is stated to be 0.12 μg (ref. 6). Assuming that the erythrocyte membrane is composed of bimolecular lipoid layers the cholesterol amount per cm^2 monolayer is 0.06 μg. In experiments on monolayers consisting of lecithin and cholesterol the mean area per cholesterol molecule is stated to vary from 56 Å^2 at a lecithin mole fraction of 0.25 to 288 Å^2 at a lecithin mole fraction of 0.75 (surface pressure 5 dynes$\cdot$cm^{-1})[7,8]. The agreement as to the order of magnitude between these figures and the above-estimated mean area per molecule cholesterol maximally bound to the luminal endothelial cell membrane may indicate that the endothelial cell surface loci to which cholesterol is bound correspond to the sites to which the membrane-cholesterol is attached in the luminal membrane monolayer.

Michaelis–Menten kinetics similar to the kinetics reported in this study have been shown to describe the pinocytotic mechanism involved in the uptake of radioactive colloidal gold by macrophages from the peritoneal cavity of rabbits[9]. The kinetics of the cholesterol uptake *in vitro* at the endothelial cell surface as reported here are thus compatible with morphological descriptions of a pinocytotic uptake process, and the rate constant k_3 in the above model may therefore be interpreted as a rate constant for the membrane ingestion process. k_3 (1.2 h^{-1}) is of the same order of magnitude as the rate constant of ingestion in the macrophages (average 5 h^{-1} at 37°).

Concerning the finding that all the serum [^{14}C]cholesterol transported to intima-media is in the free form, it is of interest to mention that it has been suggested that cholesterol enters the mucosal cells of the small intestine only as unesterified cholesterol[10,11], and that almost all of the hydrolytic activity against cholesteryl oleate is present in the brush-border fraction of the mucosal cells[12].

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REFERENCES

1 J. JENSEN, *Biochim.Biophys. Acta*, 135 (1967) 544.
2 H. A. I. NEWMAN AND D. B. ZILVERSMIT, *J. Biol. Chem.*, 237 (1962) 2078.
3 S. HASHIMOTO AND S. DAYTON, *J. Atherosclerosis Res.*, 6 (1966) 580.
4 J. JENSEN, *Biochim. Biophys. Acta*, 135 (1967) 532.
5 K. K. CARROLL, *J. Lipid Res.*, 2 (1961) 135.
6 W. S. SPECTOR, *Handbook of Biological Data*, Saunders, Philadelphia–London, 1956, p. 47.
7 L. DE BERNARD, *Bull. Soc. Chim. Biol.*, 40 (1958) 161.
8 E. N. WILLMER, *Biol. Rev.*, 36 (1961) 368.
9 R. E. GOSSELIN, *Federation Proc.*, 26 (1967) 987.
10 M. FRIEDMAN, S. O. BYERS AND S. ST. GEORGE, *Ann. Rev. Biochem.*, 25 (1956) 613.
11 C. R. TREADWELL, L. SWELL AND G. V. VAHOUNY, *Federation Proc.*, 21 (1962) 903.
12 J. S. K. DAVID, P. MALATHI AND J. GANGULY, *Biochem. J.*, 98 (1966) 662.