DIFFERENCE IN THE UPTAKE OF LOW AND HIGH CONCENTRATIONS OF ARACHIDONIC ACID INTO MURINE PERITONEAL MACROPHAGES

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The kinetics of arachidonic acid (AA) incorporation into macrophages was investigated in experimental and theoretical models. The difference in the uptake between low physiological concentrations (10^{-9}–10^{-7} M) and high inflammatory concentrations (10^{-6}–10^{-5} M) of the AA were compared. The mathematical model fitting the AA incorporation into macrophages was proposed and analyzed. We suggested that inflammatory concentrations of AA activated the metabolism of AA and change the mechanism of its incorporation into cells. Experimentally it has been shown that (i) during the [^{3}H]AA incorporation into the cells the steady state was reached at 10 hours; the levels of incorporation for high and low concentrations were different; (ii) high concentrations of AA stimulated [^{3}H]AA release from intracellular stores of the prelabeled cells, and phospholipase A_2 was involved in these processes.

Polyunsaturated fatty acid (PUFAs) are components of phospholipids and play an important role in regulation of functional and regulatory properties of cellular membranes. Both incorporation of PUFAs into membranes and their release under the action of different substances are important for the regulation of functioning of humans and mammalians in normal and pathological conditions [1, 2]. Arachidonic acid (AA; 20:4 ω-6) is an outstanding substance among PUFAs because AA is a substrate for the biosynthesis of physiologically active eicosanoids (prostanoids, leucotrienes, lipoxins) [3, 4]. So, processes of AA transport, synthesis and metabolism are under intensive investigation. It is known that under normal physiological conditions AA is included into lipids of cellular membranes or forms complexes with proteins. The content of free AA does not exceed nanomolar concentrations [2]. During the inflammation the concentration of free AA in extracellular medium (so called exogenous AA) raises up to several micromoles [2]. The interaction of exogenous AA with cells includes their transport across membranes, incorporation into phospholipids, and metabolism into physiologically active compounds. Although there have been many studies on the transport of AA across membranes, the mechanism of this process is still under active discussion [5, 6]. The absorption on the cell plasma membrane, moving across the phospholipid bilayer and dissociation into the cytosol are steps of the process. Next AA becomes a component of bound into diverse phospholipids 5]. It is to be noted, that transmembrane movement of AA from extracellular space into cells takes time within second range. At the same time AA metabolism and eicosanoid biosynthesis are significantly more longer procedures (they take minutes, hours). The mechanism of these processes is far from understanding.

In this report we investigate the kinetics of AA incorporation within a wide range of time and compare the process of AA incorporation under low physiological and high inflammatory concentrations of exogenous AA. The mathematical model fitting behavior of the experimental system is proposed.

Experimental Procedure

Murine peritoneal macrophages were obtained from 5- to 6-week-old F1 male mice [7]. The mice were killed by cervical dislocation. The macrophages were collected from 4–5 untreated mice by peritoneal lavage with ice-cold PRMI-1640, supplemented with 2 mM L-glutamin, sodium bicarbonate 100 units/ml penicillin and 100 μg/ml streptomycin. Then fetal calf serum (FCS) was added to achieve the final concentration of 7.5%. Cells 1×10^6/ml 2×10^5/well were incubated in 96-well plates Nunk (Denmark) in atmosphere with 5% O_2 at 37°C. After 2 h nonadherent cells were removed and fresh medium was added to the macrophage monolayers.

For the investigation of incorporation kinetics RPMI-1640 medium with different concentrations of AA but the same concentration of [^{3}H]AA (10^{-10} M, 1.92×10^{-8} Ci/ml) was added to the cells. The 180 μl aliquots of culture medium were taken out in time. Radioactivity in the samples was measured by liquid scintillation counting.

To study AA release the cells were labeled with [3H]AA (10^{-10} M, 1.92×10^{-8} Ci/ml) during 22-24 hours in RPMI-1640 with 2% of FCS. Then cells were washed two times by phosphate saline buffer. Next fresh medium with different concentration of AA or A23187 was added. The 180 μl aliquots of culture medium were taken out in time. Radioactivity in the samples was measured by liquid scintillation counting.

The mathematical model was computed using the program written in *Transfom* language which is a part of *Sigma Plot for Windows v1.0* program.

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Results and Discussion

Experimental investigation of AA uptake by macrophages. To study the kinetics of AA uptake by murine peritoneal macrophages the solutions with different concentrations of AA but with the same quantity of [^{3}H]AA were added to the cells. The radioactivity in the cell culture medium was detected as a function of time (Fig. 1). The different sets of curves corresponded to low (10^{-10}–10^{-7} M) and high (10^{-6}–10^{-5} M) AA concentrations. The amount of incorporated AA was linearly depended on the concentration of added AA in the intervals 10^{-10}–10^{-7} M and as well in the interval 10^{-6}–10^{-5} M. Thus part of AA incorporated into the cells was the same for each of these intervals (Fig. 2). This results point that at least two processes with large difference in the rates are involved in AA uptake. At 20 h of AA incubation with cells 47–50% and 28–30% of radioactivity was incorporated into cells in presence of low and high AA concentrations respectively (Fig. 1). Basing on this results one can suggest that additional pathways of AA metabolism were activated by inflammatory AA concentrations.

Modeling of AA uptake by the cells. The process of AA incorporation into the cells can be subdivided to the next stages [5, 8]: (i) transport of AA to the cell surface; (ii) AA penetration through plasma membrane; (iii) AA incorporation into phospholipids.

In the used experimental system AA was added directly to the cells. Thus, we omitted the transport stage. Incorporation of AA into phospholipids is a multistep process comprising several concordant stages of acylation and deacylation [2]. In the most cases the rate of this process limits the overall rate of AA uptake by cells. Assuming that AA uptake by cells has no saturation with the respect to investigated AA concentration range and the rate constant of AA liberation from phospholipids depends on exogenous AA concentration, the uptake of AA by cells can be described by mechanism depicted as follows:

$$\mathrm{AA} + \mathrm{Mem} \overset{K_1}{\leftrightarrow} \mathrm{AA_{Mem}} \underset{k_{-2}}{\overset{k_2}{\leftrightarrow}} \mathrm{AA_{PL}}; \quad k_{-2} = f([\mathrm{AA}]), \qquad (1)$$

where AA is arachidonic acid in the extracellular space; Mem is the regions of membrane interacting with AA; $\mathrm{AA_{Mem}}$ is free or membrane bound AA; $\mathrm{AA_{PL}}$ is AA bound to phospholipid; K_1 is equilibrium constant; k_2 and k_{-2} are the observed rate constants of acylation and deacylation. In our work we determined the quantity of radioactive label in the medium. All labeled compounds are marked with symbol (*).

From the accepted condition that incorporation process is not saturated by AA we obtain $[\mathrm{AK}] \ll [\mathrm{Mem}]$. Then $[\mathrm{Mem}] = [\mathrm{Mem}]_0 = \mathrm{const}$, and [Mem] could be included into equilibrium:

$$K_1' = \frac{[\mathrm{AA}]}{[\mathrm{AA_{Mem}}]}; \quad K_1' = \frac{[\mathrm{AA}^*]}{[\mathrm{AA^*_{Mem}}]}, \qquad (2)$$

$$\frac{k_{-2}}{k_2} = \frac{[\mathrm{AA_{Mem}}]}{[\mathrm{AA_{Phl}}]}; \quad \frac{k_{-2}}{k_2} = \frac{[\mathrm{AA^*_{Mem}}]}{[\mathrm{AA^*_{Phl}}]}. \qquad (3)$$

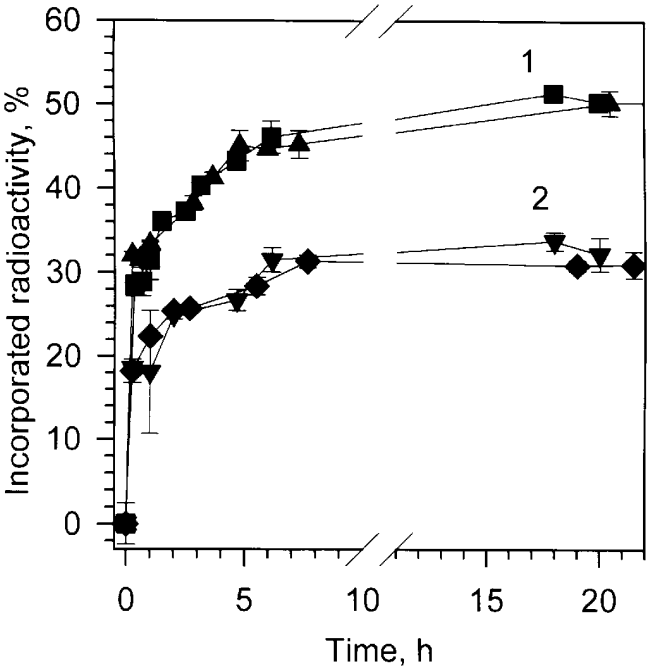

Fig. 1. The kinetic of [^{3}H]AA incorporation into murine peritoneal macrophages. Concentrations of added AA (M): from 10^{-10} to 10^{-7}—curve *1*; from 10^{-6} to 10^{-5}—curve *2*. 100% correspond to radioactivity added to the culture medium at the initial time.

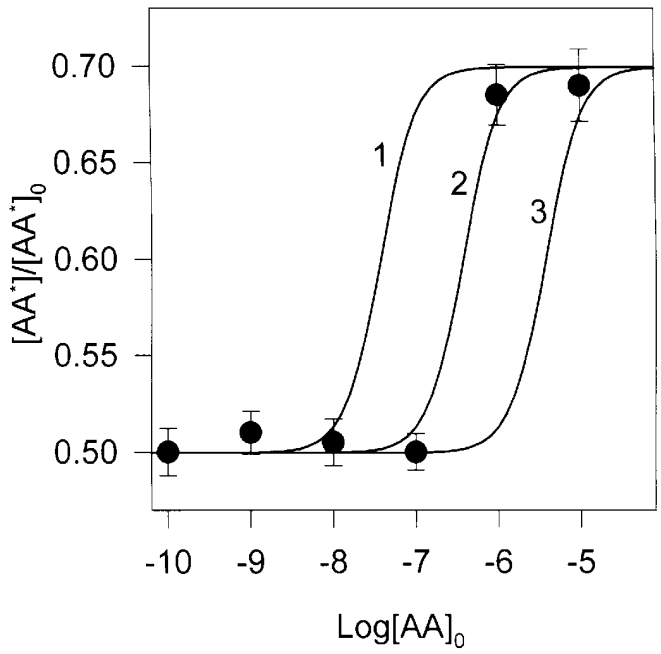

Fig. 2. Radioactivity level in culture medium after 22 h of AA incorporation. Initial medium contained different concentrations of AA but the same concentration of [^{3}H]AA (10^{-10} M, 1.92×10^{-8} Ci/ml). The experimental results (•) were compared with calculated data (lines) at α: *1*—10^{14}, *2*—10^{12}, and *3*—10^{10}. Calculations were performed for scheme 1) under conditions: $\dfrac{k_2}{k_{-2}} = \dfrac{\beta}{\alpha[\mathrm{AA}]_0^2 + 1}$, $[\mathrm{AA}]_0 \gg [\mathrm{AA}^*]_0$; $[\mathrm{Mem}] \gg [\mathrm{AA}]$; $\beta = 1.33$.

So:

$$[\mathrm{AA}^*]_0 = [\mathrm{AA}^*] + \frac{[\mathrm{AA}^*]}{K_1'} + \frac{[\mathrm{AA}^*]}{K_1'}\,\frac{k_2}{k_{-2}} \qquad (4)$$

or:

$$\frac{[AA^*]}{[AA^*]_0} = \left[1 + \frac{1}{K_1'} \cdot \left(1 + \frac{k_2}{k_{-2}}\right)\right]^{-1}. \quad (5)$$

At high AA concentrations k-2¿¿k2, so:

$$\frac{[AA^*]}{[AA^*]_0} = \frac{1}{1 + 1/K_1'} \quad (6)$$

and K_1' can be calculated using experimental data. At $[AA^*]/[AA^*]_0 = 0.7$, $K_1' = 2.3$. Taking this value (5) and taking $[AA^*]/[AA^*]_0 = 0.5$ (Fig. 1), at low AA concentrations, we have $k_2/k_{-2} = 1.33$.

Giving k_{-2} as a lineal function from $[AA]_0$ ($k_{-2} = a[AA]_0 + b$) we have:

$$\frac{k_2}{k_{-2}} = \frac{\beta}{\alpha[AA]_0 + 1}, \quad (7)$$

where $\beta = k_2/k_{-2}$ at low AA concentrations ($\alpha \cdot [AA]_0 \ll 1$) or $\beta = 1.33$, and α is a parameter responsible for the initial point of the change of the equilibrium amount of incorporated AA. One can see that there are two ranges of AA concentration with two levels of AA uptake (Fig. 2). The calculated result with two levels of incorporated AA corresponded to the experimental one with normal and inflammatory AA concentrations. However, the change of incorporation level occurred within two orders of AA concentration change in the model results, whereas experimental change was much more sharper. One can see from experimental results in Fig. 2 that the change of the levels corresponded to the change of AA concentration from 10^{-7} to 10^{-6} M.

It is well known for biological systems that many of physiologically active substances can act as triggers, i. e., small changes in their concentrations can provoke considerable changes of rates of different processes. In other words, the order of velocity dependence of many processes on the trigger concentrations can be considerably higher then one. It is well known for the activation of receptors, ionic channels. If we assume that the order of dependence of k_{-2} from AA is two, we have:

$$\frac{k_2}{k_{-2}} = \frac{\beta}{\alpha \cdot [AA]_0^2 + 1}, \quad (8)$$

In this case the theoretical change of AA incorporation level occurs within one order of concentration of added AA and the curve with $\alpha = 10^{12}$ coincides practically with experimental results (Fig. 2). Thus the model taking into consideration the activation of AA liberation from intracellular pools by exogenous AA becomes well fitting to experimental results.

Possible reasons of difference in AA metabolism under the action of normal and inflammatory concentrations of exogenous AA. AA release in cells occurs mainly as a result of phopholipid hydrolysis by different phospholipases of A2 families. Activation of these enzymes follows their phosphorilation and/or elevation of Ca^{2+} concentration in cell [10]. Recently it was shown that in different types of cells AA can change permeability of plasmatic membrane for different ions. Influence Ca^{2+} release from inner stores [11] and protein kinase activity 12], regulate gene expression on transcription level [12]. Our results allow to suggest that endogenous AA in high inflammatory concentrations may play a role of trigger of one or several mechanisms of activation of phospholipase A2 and thus modulate intracellular metabolism of AA. It is known that AA metabolism from different pools can considerably differ depending the activator [7, 13–15]. We have shown that inflammatory concentrations of exogenous AA stimulate release of endogenous AA from intracellular stores. Thus exogenous AA participates in synthesis of eicosanoids not only as substrate but also as regulator of this process. To confirm this suggestion we investigated AA influence on its release from intracellular stores. "Cold" AA in concentrations 10^{-8}–10^{-5} M was added to the [^{3}H]AA prelabeled cells. The radioactivity in the medium was detected. It was observed that physiological concentrations of AA (10^{-8}–10^{-7} M) did not affect the AA release from intracellular stores (Table 1). But the rate of AA release from macrophages was significantly increased under stimulation by inflammatory concentrations of AA (10^{-6}–10^{-5} M). Thus, the described above decrease in AA incorporation into the cells in the range of inflammatory concentrations can be assumed as activation of AA release from intracellular stores. The inhibition of phospholipase A_2 by 4-bromophenacylbromide leaded to significant decrease of AA-stimulated release of AA from cells (Table). These data point to phospholipase A_2 participation in AA release under stimulation by exogenous inflammatory concentrations of AA. Further investigation of mechanism of such phospholipase A_2 activation could be useful for understanding of eicosanoid synthesis correction during human phatophysiological processes and diseases.

Table 1

The influence of different stimuli on AA release from murine peritoneal macrophages

Stimulus	Changing of radioactivity in the medium, cpm
— (Control)	118 ± 32
AA, 10^{-8} M	102 ± 54
AA, 10^{-7} M	100 ± 30
AA, 10^{-6} M	384 ± 59
AA, $10^{-6,5}$ M	637 ± 55
AA, 10^{-5} M	785 ± 79
A23187 (5 μM)	1314 ± 58
AA, 10^{-5} M + 4-Bromophenacyl bromide, 2×10^{-5} M	244 ± 27

The cells were labeled with [^{3}H]AA for 22 h next washed 3 times with phosphate saline buffer. 1 h later the stimuli were added and after 2 h the radioactivity was measured in the cell supernatant.

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