Effect of Metabolic Stress on the Expression of mTOR Kinase in Mouse Liver Cells

E. A. Martinova, A. V. Vasiliev, and M. M. Gapparov

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The time course of changes in mTOR kinase protein and Akt and HSP70 molecular chaperone in hepatic cells was studied in a model system of metabolic stress (48-h food deprivation with subsequent restoration of normal volume of nutrition) on C57Bl/6 mice. The fluorescence intensity of mTOR protein stained with antibodies decreased in all liver cells during fasting. The expression of mTOR protein in different populations of liver cells was heterogeneous. The percentage of cells with initially high expression of mTOR protein decreased during 48-h food deprivation, while the percentage of cells with low mTOR expression increased. The levels of mTOR, Akt, and HSP70 returned to normal only on day 4 after normalization of nutrition.

Key Words: *mTOR kinase; hepatocytes; lymphocytes; stress; nutrition*

mTOR kinase (mammalian target of rapamycin) coordinates signal pathways regulating anabolism and catabolism in eukaryotic cells. mTOR interacts with protein synthesis and cell cycle initiation factors in response to nutrients or changed energy status of the cells [5]. Cell survival during metabolic stress (MS) depends on activation of signals regulating the efficiency of mRNA translation in response to stress and triggering phosphoinositol-3-kinase (PI3K), protein kinase B (PKB/Akt), mTOR kinase, and their targets [8]. The PI3K/Akt signal pathway transmits signals providing normal level of liver cell regeneration [2]. mTOR forms two multicomponent complexes, mTORC1 and mTORC2, coordinating functionally different cell responses to nutrients and growth factors. The complexes differ by sensitivity/resistance to rapamycin and by coupling with the heat shock protein (HSP70) signal pathways. Ribosomal kinase p70 S6K1 and eIF-4EBP-1 complex of eukaryotic transcription initiation factor and its binding protein are the effectors of mTOR signal pathway [9].

Basal activities of mTOR and S6K1 kinases in rodent liver depend on the type of nutrition. Rats receiving fat-rich rations develop obesity; this is associated with significant elevation of mTOR and S6K1 kinase activities in the liver [4]. Mice exposed to similar conditions develop fatty degeneration of the liver and insulin resistance, positively associated with an increase in protein synthesis and mTOR and S6K1 kinase phosphorylation. In rats maintained on a protein-rich ration, initiation of protein synthesis translation is activated, which is determined by activation of the mTOR signal pathway and its second messengers p70 S6K1 and eIF-4EBP-1 [3]. Rations with low protein content lead to reduction of mTOR kinase phosphorylation in hepatocytes and the formation of a stable eIF-4EBP-1 complex, which suppresses protein synthesis. Normalization of nutrition after 48-h food deprivation in rats is associated with phosphorylation of p70 SbK1 and e4E-BP1 proteins, dissociation of protein translation initiation factor, stimulation of protein synthesis, and liver weight recovery [1].

We studied changes in the expression of mTOR kinase protein in mouse liver cells during MS.

MATERIALS AND METHODS

The study was carried out on female C57Bl/6 mice aged 10 weeks. Experiments were carried out in accor-

Institute of Nutrition, the Russian Academy of Medical Sciences, Moscow, Russia. ***Address for correspondence:*** eamartinova@lycos.com. E. A. Martinova

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dance with the international regulations on handling of laboratory animals. Complete food deprivation for 48 h with free access to water, followed by normalization of nutrition, served as the model stress system. Protein expression was measured at the beginning of experiments, on day 1 of food deprivation, after 48 h of fasting, and on days 1 and 4 of subsequent normal nutrition.

Primary cultures of mouse hepatic cells were incubated in RPMI-1640 with L-glutamine (Flow) containing 10 mM HEPES (Sigma) and 10% FCS (Flow). The cells were fixed (20 min) in 2% paraformaldehyde in Hanks solution with 0.1% saponin for cell permeabilization, washed, and stained with antibodies to mTOR (Sigma), Akt/PKB (GenWay), and HSP70 (Calbiochem). The percentage of antigen-positive cells and fluorescence intensity were evaluated on a FACSCalibur flow cytometer (Becton Dickinson) using the CellQuest program. FITC fluorescence was measured at λ=530±15 nm. The cell cycle was evaluated after propidium iodide staining at λ=610±5 nm. A total of 10,000 events were analyzed in each sample. The relative density of the antigen in experimental *vs.* control samples was estimated by the formula:

$$(Fm'\text{-}Fn')exp/(Fm''\text{-}fn'')cont= \rho Agexp/\rho Agcont\times 100\% \text{ [7]},$$

where Fm' is fluorescence intensity in the sample, Fn' is nonspecific fluorescence intensity, exp and cont denote experiment and control, respectiely; ρAg is the relative density of the antigen.

Experiments were repeated 3 times, 8 animals per point. The data were analyzed using ANOVA. The groups were compared using Student's test. Nonparametric analysis of several groups was carried out using Newman–Keuls test (null hypothesis on equality of all means is verified by the analysis of dispersions; if it is rejected, all means are ranged in increasing order and compared by pairs; $p \leq 0.05$ reflects a statistically significant difference between the groups.

RESULTS

Complex mTORC2 phosphorylated PKB/Akt kinase by Ser^{473}, which led to its complete activation (Fig. 1). Changed activity of Akt in the primary rat hepatocyte culture in response to insulin stimulation inversely correlated with the time course of mTOR kinase activity [6]. The level of protein and activity of PKB/Akt in liver cells continued to increase during the initial period of nutrition normalization after stress (Fig. 1). The time course of PKB/Akt level positively correlated with changes in the level of HSP70, Rictor protein molecular chaperone in the mTORC2 complex, conjugated with protein synthesis regulation during energy stress. Metabolic stress increased the percentage of liver cell death, stimulating the compensatory activation of proliferation (Fig. 1), which was confirmed by staining for cell cycle markers.

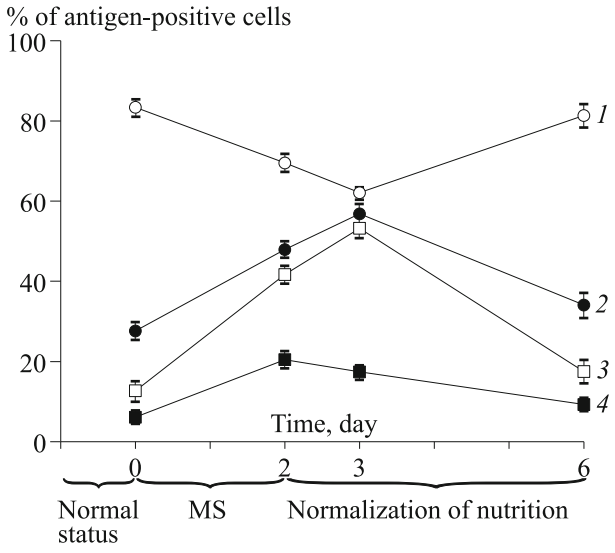

Fig. 1. Time course of mTOR kinase protein (*1*), PHB/Akt kinase protein (*2*), and HSP70 heat shock protein (*3*) in mouse hepatic cells during 48-h MS (food deprivation) and subsequent normalization of nutrition. *4*) cell cycle.

Changes in the expression of mTOR protein in mouse hepatic cells in MS depended on the cell population and were maximally expressed in hepatocytes (Fig. 2, *a*), which was confirmed by flow cytometry data on cell distribution in FSC and SSC (forward and side laser scatter). Gate R1 corresponded to hepatocytes (according to staining for C/EBPα marker). Gate R2 contained lymphocytes, stellate cells, macrophages, and other hepatic cells, but not hepatocytes (confirmed by staining with antibodies to CD3, CD45, F4/80 receptors). The level of mTOR protein in hepatocytes in MS decreased less markedly than in lymphocytes, which could be due to different content of mitochondria (Fig. 2, *c*).

The level of mTOR protein in hepatic cells normalized only on day 4 after normalization of nutrition (Fig. 2, *b*). We detected two pools of cells with high and low expression of mTOR protein in mouse liver (Fig. 3). The fluorescence intensity of each of these cell pools varied by 2-5% under conditions of changed metabolic status. The percentage of cells with low level of mTOR protein increased in MS and returned to normal on day 4 after normalization of nutrition (Fig. 3, *c*). The percentage of liver cells with high level of mTOR protein decreased during food deprivation and on day 1 after normalization of nutrition (Fig. 3, *d*). The presence of cell pools with high and low expression of mTOR reflected heterogeneity of cellular com-

position of the liver and the characteristics of mTOR kinase expression regulation in different cells.

Hence, changes in protein expression in liver cells under conditions of MS are systemic. Food deprivation for 48 h leads to reduction of mTOR protein level paralleled by an increase in the content of Akt kinase protein and HSP70 chaperone and compensatory activation of mitosis under conditions of their more intense death.

The expression of mTOR kinase during food deprivation and subsequent normalization of nutrition are determined by the cell population. The percentage of cells with initially high level of mTOR protein expression decreases during fasting, while the percentage of cells with initially low level of mTOR protein increases. Restoration of nutrition after a sharp violation of metabolism leads to normalization of mTOR protein on day 4.

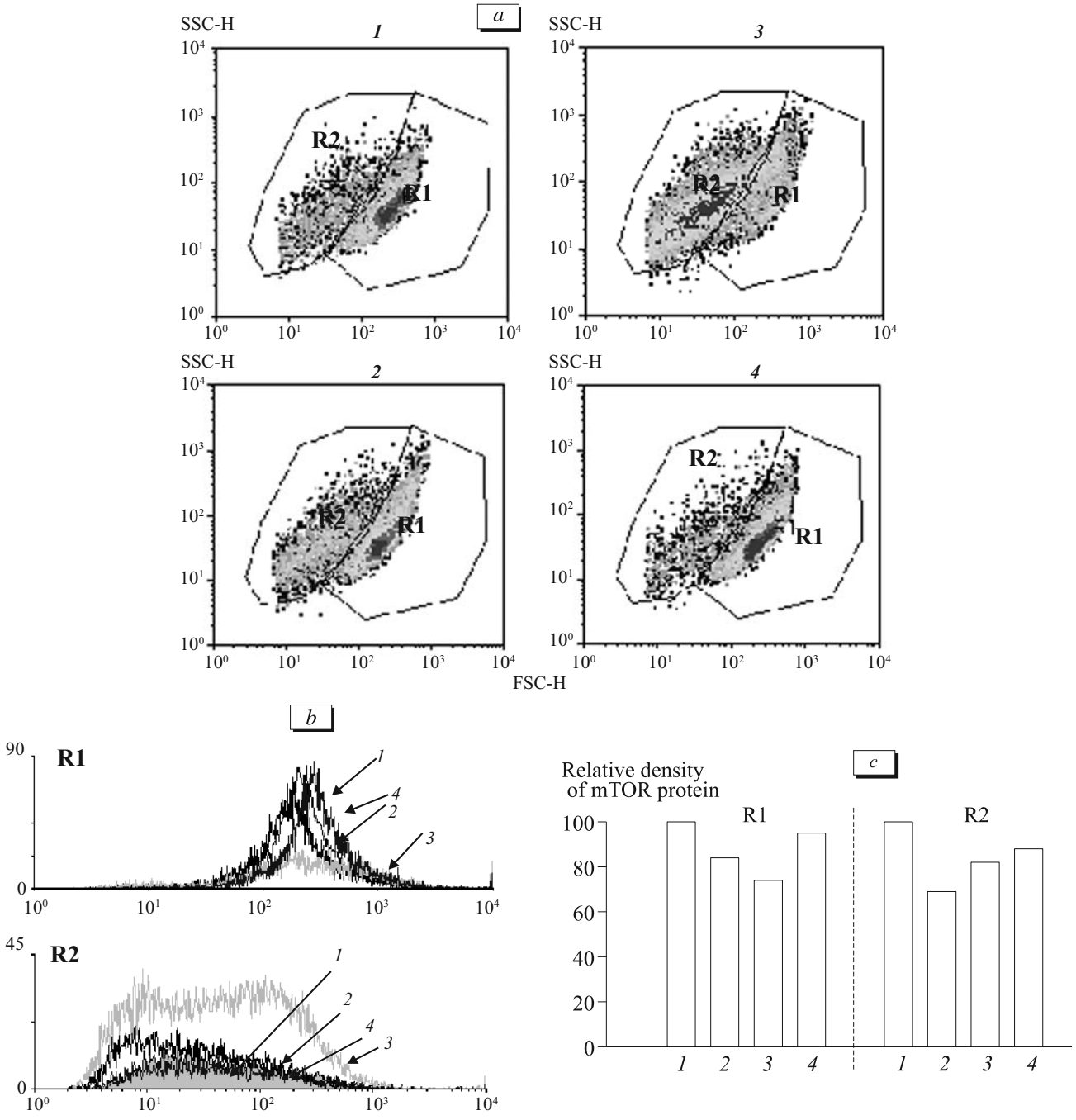

Fig. 2. Flow cytometry data, illustrating the time course of mTOR kinase expression in different populations of liver cells during MS and normalization of nutrition. *a*) changed population of hepatocytes (gate R1) and other hepatic cells (gate R2) in 48-h food deprivation and normalization of nutrition; *b*) time course of mTOR kinase; *c*) relative density of mTOR antigen in liver hepatocytes and lymphocytes. Here and in Fig. 3: *1*) normal status; *2*) MS; *3*) normalization of nutrition, day 1; *4*) normalization of nutrition, day 4.

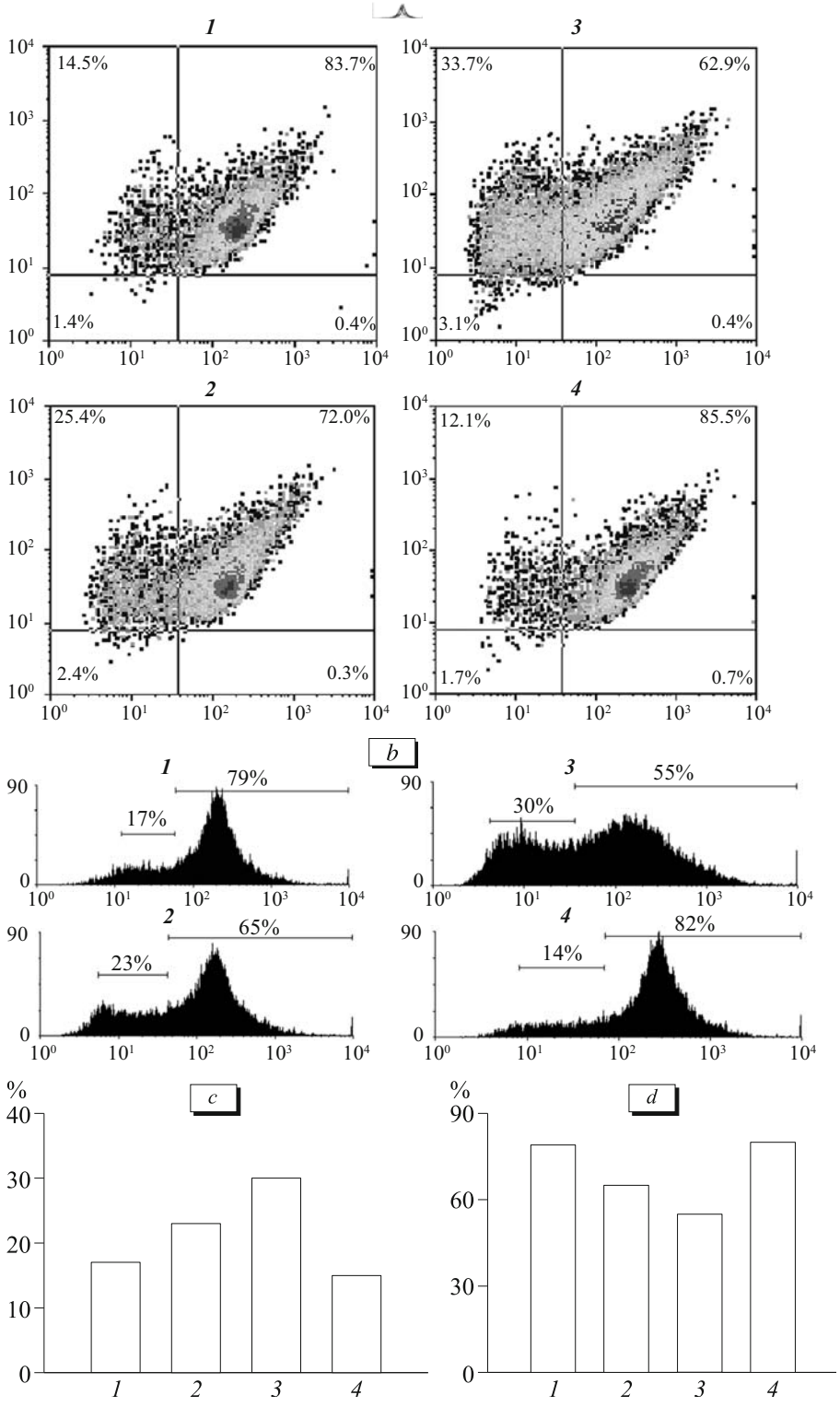

Fig. 3. Flow cytometry data illustrating changed percentage of liver cells with initially high or low level of mTOR kinase expression in MS and after normalization of nutrition. *a*) distribution of cells with high expression of mTOR kinase (upper right section of each sample); *b*) time course of cells with different density of mTOR; *c*, *d*) percentage of cells with initially low density of mTOR (*c*) and initially high level of mTOR (*d*) during food deprivation and normalization of nutrition.

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