# Accumulation of ATP by Plasma Membranes of Human and Rat Hepatocytes Induced by Some Growth Factors and Phosphatidylcholine

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Using the method of ATP luminometry it is shown that crude membrane preparations from human and rat hepatocytes accumulate ATP 20-100 nmol/mg protein during a 1-min incubation under conditions of oxidative phosphorylation. Application of appropriate inhibitors shows that a possible contamination of the membrane preparations with mitochondria does not contribute to this ATP accumulation. Phosphatidylcholine, tumor necrosis factor, and cell proliferation factor markedly stimulate the accumulation of ATP by plasma membrane-enriched particles isolated from rat and human liver. The hepatocyte plasma membrane is shown to be able to synthesize ATP from inorganic phosphate and ADP using the aerobic mechanism. ATP in the plasma membrane is assumed to participate in the transmembrane signal transduction from growth factors to the cell effector systems.

**Key Words:** *hepatocytes; ATP; growth factors; oxidative phosphorylation, hemangioma*

Preservation of the regeneration capacity of hepatocytes is an indispensable condition for extensive resection of the liver [1]. This capacity is known to be controlled by hepatocyte growth factors such as glycyl-histidyl-lysine (GHL), cell proliferation factor, endothelial growth factor, and epidermal growth factor. Some of these factors may participate in the autocrine mechanism of hepatocyte malignant growth and stimulate the growth of vessels of solid tumors of the liver (hemangiomas). Previously [2,11] we have reported that the earliest cell response in various tissues to the receptor-mediated effect of insulin and peptide growth factors of the mitogen and cytokine families is a rapid (45 sec-1 min) aerobic formation of adenosine-5'-triphosphate (ATP) on the plasma membranes of target cells coupled with electron transfer from cytoplasmic NADH to impermeable external redox-acceptors. At present, a large body of experimental data suggests that the plasma membranes of all eucaryotic cells contain a cyanide-resistant redox-system which transports electrons to such external acceptors as ascorbate free radical, $Fe^{2+}$, $Cu^{2+}$, diferritransferrin, and oxygen [13, 16]. It has been speculated that rapid ATP production may occur during the transduction of the signal for hepatocyte growth and proliferation from external cell receptors to effector kinases residing on the cytoplasmic surface of the cell membrane. The present investigation confirms this assumption.

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## MATERIALS AND METHODS

The study was carried out on plasma membrane-enriched particles (PMEP) isolated from various target cells of rat liver as described earlier [14] with some

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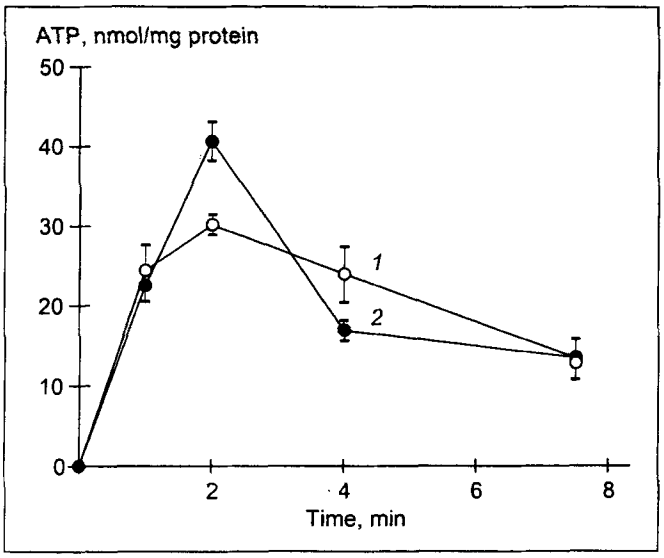


**Fig. 1.** Time course of ATP accumulation by plasma membrane-enriched particles isolated from rat liver during incubation in the absence (*1*) and presence (2) of glycyl-histidyl-lysine (0.2 μg/ml). Each value is the mean of 4 experiments. Vertical lines show confidence intervals at $p$=0.05.

modifications. The liver was removed from random-bred male rats with an average weight of 200 g and minced on ice, and a 10% homogenate in 1 mM $NaHCO_3$ was prepared using a Downs homogenizer with a loosely fitted pestle (50 poundings). The homogenate was filtered through one layer of gauze and centrifuged at 2500 g and 4°C for 10 min. The pellet was resuspended in cold 0.25 M sucrose and recentrifuged at 2000 g for 10 min under the same conditions. From the resultant two-layer pellet the upper light gray loose layer was harvested and resuspended again in 0.25 M sucrose. The above procedure was repeated twice, the top layer being collected each time. The final pellet was suspended in 0.25 M sucrose and used in further experiments. The preparations contained 1.7-2 mg protein/ml and, judging from 5'-nucleotidase activity [9], was 18-fold enriched with plasma membranes in comparison with the initial homogenate. PMEP from human liver was isolated analogously. The preparations contained 1-1.2 mg protein/ml. PMEP from human liver hemangioma cells were isolated as described previously [3] from hemangioma tissue excised during surgery. Experiments on ATP biosynthesis were carried out as follows. The reaction was performed in 20-ml Erlenmeyer flasks. Incubation medium in a volume of 0.7 ml was placed in each flask previously cooled on ice. The incubation medium contained 0.04 M Tris-HCl buffer (pH 7.5), 2.5 mM $MgSO_4$, 0.2 mM ADP potassium salt, 5 mM $KH_2PO_4$, 0.2 mM NaF, 0.1 mM NADH, 10 mM β-oxybutyrate, 277 μg/ml bovine serum albumin, 0.1 mM cytochrome C (oxidized), 76 mM sucrose, and 2 μM 5'-fluorosulfonylbenzoyladenosine, a protein kinase inhibitor. The medium also contained 1.5 μM antimycin A, 1 mM KCN, and 7.61 mM rotenone as inhibitors of the mitochondrial electron-transport chain. These inhibitors were omitted in the study of the effect of mitochondria on ATP accumulation. The test growth factors in the required concentration were added to the experimental samples in 20 μl of 1 mM Tris-glycine buffer and the same volume of buffer (pH 7.5) was added to the control samples. The flasks were placed in a water bath with a shaker, the PMEP suspension (0.5 ml in 0.25 M sucrose) was added to each sample, and the samples were incubated for 1 min at 30°C with constant shaking. After the 1-min incubation a 100 μl aliquot from each sample was transferred to 500 μl dimethyl sulfoxide (DMSO) for termination of the reaction and extraction of the accumulated ATP. The concentration of ATP was determined by the luciferin-luciferase method using a Mikrolyum luciferin-luciferase reagent produced at the Department of Enzymology, Moscow State University on a KLIMBI computer-assisted luminometer (Russia). To this end a 20 μl sample in DMSO was transferred to a measuring cuvette containing 10 μl Mikrolyum reagent in 0.9 ml of 10 mM $MgSO_4$ and, after measurement of the background luminescence, a standard solution of ATP (0.1 μM) was added to the cuvette. In the study of the dynamics of ATP accumulation DMSO was not used, and after certain intervals an aliquot of the incubation mixture (20 μl) was immediately transferred from the incubation flask to the cu-

**TABLE 1.** Effect of Mitochondrial Inhibitors on Accumulation of ATP by Crude Fraction of Plasma Membranes Isolated from Rat Hepatocytes in the Presence and Absence of Glycyl-Histidyl-Lysine (GHL, $n$=4, $M\pm m$)

| Experimental conditions | Accumulation of ATP, nmol/mg protein during the first minute of incubation | ATP (E-C) |
|---|---|---|
| *Without inhibitors of mitochondrial oxidation* | | |
| Control (without GHL) | 28.47±1.26 | |
| Experiment (with GHL) | 31.43±1.73* | 6.47 |
| *In the presence of inhibitors of mitochondrial oxidation* | | |
| Control (without GHL) | 27.20±0.49 | |
| Experiment (with GHL) | 38.06±7.12* | 10.76 |

**Note.** *$p$<0.02 in comparison with the control; $n$ is the number of experiments. Here and in Tables 2 and 3: E-C denotes the difference between experimental and control values.

**TABLE 2.** Effect of Phosphatidylcholine on Accumulation of ATP by Plasma Membranes Isolated from Rat Liver in the Presence and Absence of Glycyl-Histidyl-Lysine (GHL, *M±m*)

| Experimental conditions | Accumulation of ATP, nmol/mg protein during the first minute of incubation | ATP (E-C) |
|---|---|---|
| Control | 27.19±1.12 (6) | |
| GHL, 0.2 μg/ml | 38.78±5.77 (6)* | 11.59 |
| Phosphatidylcholine, 100 μg/ml | 42.20±0.78 (4)* | 15.01 |
| GHL+phosphatidylcholine | 49.57±2.11(4)* | 22.38 |

**Note.** *$p$<0.001 in comparison with the control. Number of experiments is presented in parentheses.

vette. The content of ATP in all experiments was calculated using Clim test software (KLIMBI).

The data were processed statistically according to the Student $t$ test using CSS-STATISTICA software.

## RESULTS

Using the method of ATP-luminometry, we found that the incubation medium initially contained 2.00±0.08 nmol/mg protein ATP. The ATP was present as an admixture in the ADP preparation added to the medium. PMEP isolated from rat and human liver initially did not contain measurable concentrations of ATP. Measuring the content of ATP (without DMSO) in the course of incubation, we found that ATP accumulates in the medium during the first minute of incubation (30.2±0.46 and 40.64±2.35 nmol/mg protein in the absence and presence of GHL, respectively) and gradually diminishes in quantity during the subsequent incubation (Fig. 1). This removal of ATP from the medium may be due to the presence of some ATP consumers on the plasma membrane, which are not inhibited by 5'-fluorosulfonylbenzoyladenosine. The addition of DMSO after a 1-min incubation did not affect the amount of ATP (Tables 1-3), suggesting that ATP synthesized on PMEP is not adsorbed on the membranes and is completely released to the medium. The absence of inhibitors of the mitochondrial electron-transport chain (antimycin, rotenone, and cyanide) did not markedly affect the ATP accumulation (Table 1). On the contrary, the accumulation of ATP in the presence of GHL was somewhat increased. These data confirm that mitochondria were not involved in ATP accumulation in our incubation mixture. Phosphatidylcholine considerably enhanced the ATP-accumulation ability of PMEM from rat liver and slightly potentiated the effect of GHL when both substances were added to the incubation medium simultaneously (Table 2). Growth factors markedly affected the ATP-generating activity of PMEP of various origin (Table 3). Cell proliferation factor exhibited the maximal effect and stimulated ATP production in PMEP from both rat liver and, albeit to a lesser extent, normal human liver tissue. GHL stimulated ATP formation in PMEP from rat liver but inhibited it in PMEP from human liver. Human tumor necrosis factor-α enhanced ATP production in both these preparations. PMEP isolated from human liver hemangioma accumulated ATP only in the presence of endothelial growth factor and were unable to produce ATP in the absence of this factor. These data suggest

**TABLE 3.** Effect of Some Growth Factors on Accumulation of ATP by Plasma Membrane-Enriched Particles Isolated from Human and Rat Liver (*M±m*)

| Growth factor, final concentration, μg/ml | Accumulation of ATP, nmol/mg protein during the first minute of incubation | | Effect of growth factor | |
|---|---|---|---|---|
| | control | experiment | ATP (E-C) | E/C, % |
| *Rat liver* | | | | |
| GHL, 0.2 | 27.19±1.12 (6) | 38.78±5.77 (6)*** | 11.59 | 143 |
| Cell proliferation factor, 0.3[1] | 2.98±0.30 (4) | 18.58±4.04 (4)*** | 15.60 | 623 |
| Tumor necrosis factor-α, 2 | 27.19±1.12 (6) | 30.72±2.77 (4)** | 3.53 | 113 |
| Human liver | | | | |
| GHL, 0.2 | 112.25±12.53 (5) | 84.03±5.92 (5)*** | -28.22 | 75 |
| Epidermal growth factor, 2 | 112.25±12.53 (5) | 130.19±7.13 (5)* | 17.94 | 116 |
| Tumor necrosis factor-α, 2 | 112.25±12.53 (5) | 145.58±2.24 (5)*** | 30.33 | 130 |
| Cell proliferation factor, 0.3[1] | 19.15±3.56 (4) | 26.29±2.48 (4)** | 7.14 | 138 |
| *Hemangioma from human liver* | | | | |
| Endothelial growth factor, 50[1] | 0.00±0.00 (4) | 11.22±2.57 (4) | 11.22 | - |

**Note.** [1]ATP was isolated by ion-exchange chromatography and measured spectrofluorimetrically after Kornberg as described previously [4]. Number of experiments shown in parentheses, *$p$=0.01, **$p$=0.02, ***$p$=0.001. E/C is the experiment to control ratio.

a substantial ATP-accumulation capacity of liver plasma membranes under conditions of oxidative phosphorylation and modulation of this process by growth factors. Recent investigations have demonstrated that the liver growth factor receptor is transmembrane tyrosine kinase, a heterodimer consisting of an extracellular a-subunit and intramembrane β-subunit linked via a disulfide bridge. After ATP-dependent autophosphorylation, this tyrosine kinase may bind with various signal molecules such as phospholipase-C-γ, GTPase-activating protein, *src* kinases, and phosphatidylinositol-3-kinase [6]. ATP generated on the plasma membrane may be used for initiating the autophosphorylation of the receptor tyrosine kinases and, consequently, may play an important role in the transduction of a regulatory signal from the growth factor into the hepatocyte. This assumption is confirmed by reports that ATP-binding proteins, predominantly kinases, may be specifically labeled and detected using 5'-fluorosulfonylbenzoyladenosine, a structural analog of ATP [8,14]. In our previous experiments we found that the accumulation of ATP induced by various growth factors in PMEP isolated from some target cells dramatically increased in the presence of 5'-fluorosulfonylbenzoyladenosine. The revealed stimulation of ATP accumulation by phosphatidylcholine (Table 2) may be due to the fact that hydrolysis of phosphatidylcholine on the membrane stimulates the binding of growth factors with specific receptors [5,10] and thereby potentiates their effect on ATP synthesis. Moreover, phosphatidylcholine and products of its hydrolysis may participate in the signal transduction through activation of protein kinase C [7].

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