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Effects of Extracellular Calcium and Starvation on Biochemical Indices of the Rat Hepatocytes

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ABSTRACTS : *The focus of this study was to investigate that cellular parameters and glucose uptake might be altered by extracellular calcium and starvation. Addition of 1 mM Ca^{++} to hepatocytes (equalling to the free calcium concentration of blood) significantly increased intracellular K^+ and decreased Na^+ & LDH leakage. This pertains to the hepatocytes of control rats as well as those of rats fasted for 24 and 48 hr. These effects might be come from the membrane-stabilizing effects of calcium. But calcium had no effects on cell volumes, superoxide-formation and glucose uptake. Actually hepatocytes of starved rats showed changes in several cellular parameters. Starvation increased LDH leakage, glucose uptake and the total concentration of Na^+ and K^+, whereas it markedly decreased cell volumes. Since total tonicity remained unchanged, intracellular Na^+ and K^+ could contribute to a higher share of total osmolarity in starvation. Starvation increased the cytoplasmic pH because R-NH^{3+}ions and their corresponding counterions disappeared. This increase may be related to suppress the protonization of amino groups in proteins. Starvation decreased hepatic glycogen, a major compound that affects cytosolic volume of hepatocytes. The data indicate that starvation increases the glucose transport activity. The possible molecular basis will be discussed.*

Key Words : *Hepatocytes, Calcium, Starvation, Sodium, Potassium, Cell- and dextran volumes, LDH leakage, Chemiluminescence*

I. INTRODUCTION

Although several studies have suggested that the reduced activity of the Na^+-K^+ pump during starvation is a likely source of energy saving in human starvation (Patrick & Golden, 1977), the hypothesis has not been tested in intact cells, nor has the contribution of passive permeability been considered in a controlled animal study. Starvation indicates decrease in both active and passive components of ion turnover of erythrocytes of rats (Zhao & Willis, 1988). And Na^+ and K^+ transport damage was induced by oxygen free radicals in red cell membrane (Maridonneau *et al.*, 1983).

We studied effects of calcium and starvation on the superoxide-formation of hepatocytes (Kim, 1993). In hepatocytes and isolated plasma membranes of normal rat hepatocytes, we could not observe any influence of added calcium. The cytoplasmic side of superoxide-forming system may also be partially accessible in plasma membrane. For some effectors, which decrease the CL (chemiluminescence) such as ATP, it is known that cytoplasmic calcium is increased. It is questionable, however, whether this is causative for CL decrease since some effectors inducing "respiratory burst" in neutrophils act by the increase of intracellular calcium concentrations (Dale & Penfield, 1984).

But we found that NADH-induced oxygen free radicals yield of the isolated plasma membranes of starved rats was increased several fold as compared to the isolated plasma membranes of control rats (Kim, 1993).

The purpose of the present study, therefore, was to investigate the several cellular parameters and glucose uptake in rat hepatocytes by extracellular calcium and starvation.

II. MATERIALS AND METHODS

1. Materials

Collagenase A, lucigenin, NAD(P)H, NAD(P)$^+$, SOD were purchased from Boehringer (Mannheim, Germany). D-(U-^{14}C)-glucose was from Amersham Buchler (Braunschweig, Germany). ^{14}C-carboxydextran, HTO were obtained from Du Pont de Nemours (Dreieich, Germany). D(+)-glucose was a product of Merck (Darmstadt, Germany).

2. Isolation of Liver Cells

Liver cells were isolated according to the procedure with a modification (Berry & Friend, 1969; Baur *et al*, 1975). Female Wistar rat (170-200 g) was narcotized by ether. After opening the abdomen and tubing of the vena cava inferior and vena portae, the liver was perfused with 1.2 mM Ca^{++} perfusion medium I (121 mM NaCl, 18 mM $NaHCO_3$, 5.9 mM KCl, 0.74 mM NaH_2PO_4, 0.6 mM $MgSO_4$, 5 mM D-glucose, pH 7.2 at 37°C, perfusion speed: 3 $ml \times min^{-1} \times g^{-1}$ liver). All perfusion media were aerated with carbon dioxide. After opening the thorax, a tube was inserted into the proximal part of the vena cava inferior, the liver was perfused with Ca^{++}-free perfusion medium at 37°C via the vena portae for 20 min. Perfusion of the isolated liver was carried out with a recirculating medium containing 0.47 mM Ca^{++}, 2 mM pyruvate and 25 mg collagenase (Boehringer collagenase A 0.21 U/mg) in 50 ml Ca^{++}-free medium. Then, it was continued through the vena cava for 10-15 min. The liver was disintegrated after 25-30 min of perfusion. After shredding the liver, the tissue was transfered to a round-bottomed flask and enzymic treatment (collagenase medium) was continued for another 6 min with carbon dioxide under slow rotation (in water bath at 37°C). The treated tissue was filtrated (300 µm gauze) and washed (Washing medium: 137 mM NaCl, 5.2 mM $CaCl_2$, 0.9 mM $MgSO_4$, 5 mM D-glucose, 3 mM Na_2HPO_4/KH_2PO_4, pH 7.2 at 0°C) by centrifugation 3 times (70×g, 20 sec.) The cells were stored in suspension at 0°C in the standard medium containing 137 mM NaCl, 5 mM KCl, 0.4 mM $MgSO_4$, 0.12 mM $CaCl_2$, 3 mM KH_2PO_4/Na_2HPO_4, and 20 mM morpholino-3-propanesulfonic acid (MOPS) adjusted to pH 7.3 with Tris base.

The protein concentration was 30-50 mg protein/ml and cell viability was determined by tryphan blue staining, LDH and intracellular Na^+/K^+ concentration.

Hepatic plasma membranes were isolated as previously described (Emmelot *et al*, 1974).

3. Incubation of Liver Cells

Incubation of cells was carried out at 37°C. Normal incubation medium was identical with standard medium (pH 7.3), with 1 mg cellular protein/ml. Determination of cell volume and adherent fluid volume was carried out with 9.25 KBq ^{14}C-carboxydextran and 74 KBq tritium marked water (HTO) per 1 ml incubation medium applying the silicon centrifugation method.

The silicon centrifugation was performed according to the procedure described elsewhere (Pfaff, 1965; Klingenberg, 1967).

4. Determination of Intracellular Na^+/K^+ Concentration, Cellular Volume and Adherent Fluid

D-glucose (16 mM) was added to the incubation medium (containing ^3H_2O and ^{14}C-carboxydextran) either alone (control) or together with calcium. Apart from that, the same procedures as in glucose uptake studies were used.

5. ^{14}C-D-glucose Uptake into the Isolated Cells

A cell suspension containing 1 mg cellular protein/ml was preincubated for 8 min at 37°C. Then ^{14}C-D-glucose (8 KBq/200 µl) and 16 mM glucose were added to the incubation medium. 200 µl samples were withdrawn after 5-, 15-, 25-, 35-, 45-, and 60 seconds. The effects of calcium on uptake were compared with controls.

6. Lucigenin Chemiluminescence (CL)

Chemiluminescence assay was carried out with lucigenin (Albrecht *et al*, 1990; Allen 1981, 1982). In the physiological pH range, superoxide radicals are detected selectively by lucigenin-CL. The experiment was performed at 37°C with 1 mg/ml hepatocyte or 50 µg plasma membrane/ml in an automated luminometer (LB9500, Firma Berthold, Wil-

bad, Germany) interfaced with a PC-compatible computer. The program for kinetics was developed in institute of pharmacology and toxicology in Tuebingen, Germany. The lucigenin-enhanced reactions were followed by a highly sensitive photomultiplier (375-620 nm). Each polyethylene minivial contained a final volume of 1.5 ml, including buffer.

III. RESULTS AND DISCUSSION

Several parameters of isolated hepatocytes were changed by addition of calcium. It might be come from the membrane-stabilizing effects of calcium. Calcium (1 mM) increased intracellular K^+ and decreased Na^+. This pertains to the hepatocytes of control rats as well as those of rats fasted for 24 hr and 48 hr (Fig. 1, 2). But calcium had no effect on cell volumes and glucose uptake (Fig. 4, 5). It appears that the assayed parameters are more strongly influenced by the state of nutrition than by calcium-addition.

Starvation increased the total concentrations of Na^+ and K^+, while it clearly decreased cell volumes (Fig. 4). Since total tonicity remained unchanged,

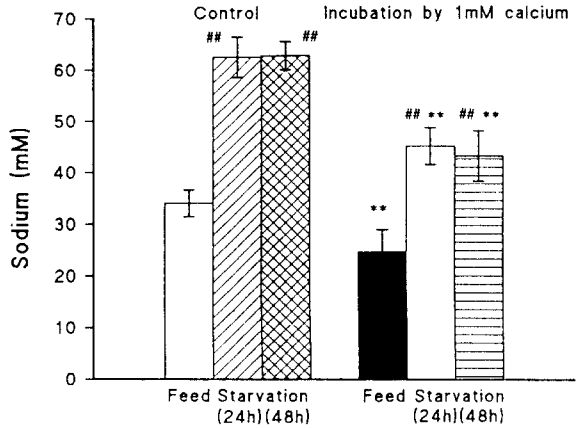

Fig. 1. Changes of intracellular sodium in hepatocytes from control and starved rats by extracellular calcium. 1 mM calcium was added to the incubation medium at 2 min before silicon centrifugation. Samples were withdrawn after 6 min preincubation with 1 mg hepatocyte protein/ml at 37°C. Data were derived from 7 rats. *, ** are significantly different ($p < 0.05$, $p < 0.01$) from control group. #, ## are significantly different ($p < 0.05$, $p < 0.01$) from feeding group.

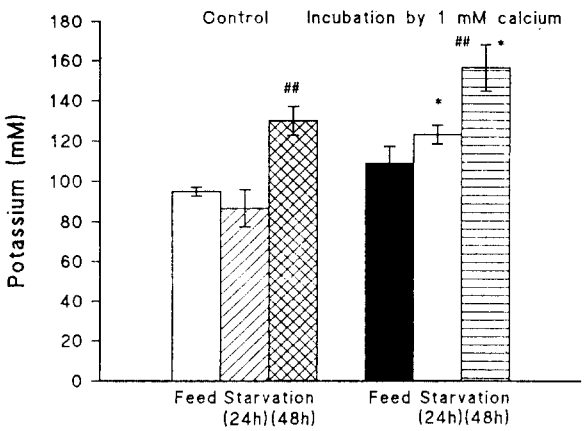

Fig. 2. Changes of intracellular potassium in hepatocytes from control and starved rats by extracellular calcium.

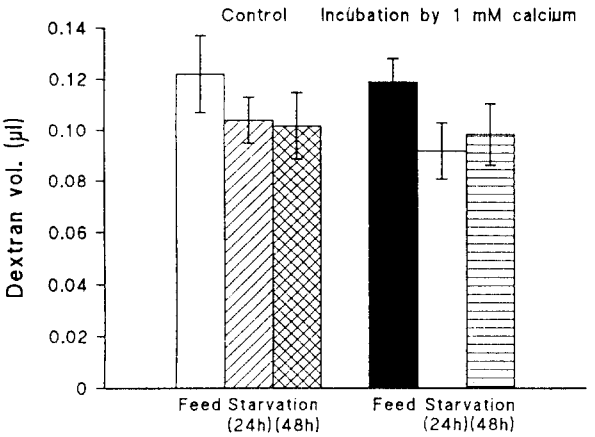

Fig. 3. Changes of dextran volumes in heaptocytes from control and starved rats by extracellular calcium. 1 mM calcium was added to the incubation medium at 2 min before silicon centrifugation. Samples were withdrawn after 6 min preincubation with 1 mg hepatocytes protein/ml at 37°C. Data were derived from 7 rats. *, ** are significantly different ($p < 0.05$, $p < 0.01$) from control group. #, ## are significantly different ($p < 0.05$, $p < 0.01$) from feeding group.

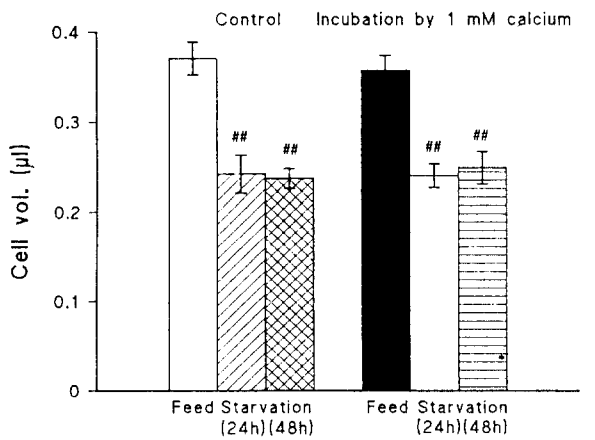

Fig. 4. Changes of cell volumes in hepatocytes from control and starved rats by extracellular calcium.

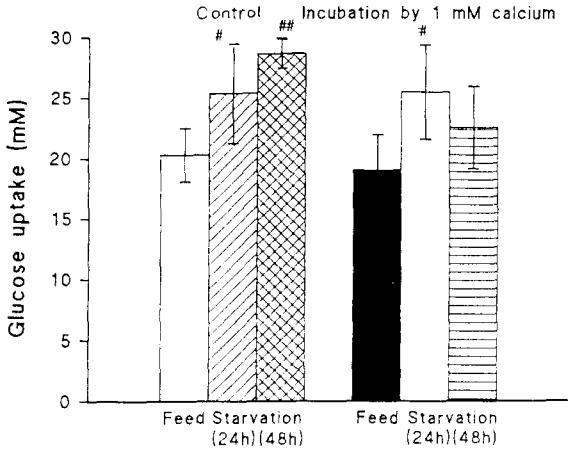

Fig. 5. Changes of glucose uptake in hepatocytes from control and starved rats by extracellular calcium. 1 mM calcium was added to the incubation medium at 2 min before silicon centrifugation. Samples were withdrawn after 6 min preincubation with 1 mg hepatocyte protein/ml at 37°C. Data were derived from 6 rats. *, ** are significantly different ($p < 0.05$, $p < 0.01$) from control group. #, ## are significantly different ($p < 0.05$, $p < 0.01$) from feeding group.

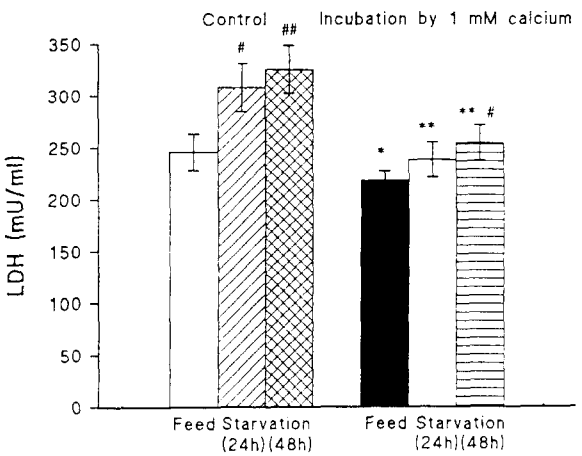

Fig. 6. Changes of LDH leakage in hepatocytes from control and starved rats by extracellular calcium.

intracellular Na^+ and K^+ might be contributed to the higher share of total osmolarity in starvation. This means the increase of intracelullar pH on starvation suppressing the protonization of amino groups in proteins. LDH leakage was significantly increased in the absence of calcium and more strongly by starvation (Fig. 6). Glucose uptake of starved rat hepatocytes for 24 hr was remarkably increased against the control. Calcium had no additional effects. Glucose uptake of starved rat hepatocytes for 48 hr was even more increased (Fig. 5).

We observed the stimulatory effect of D-glucose on the chemiluminescence was generally increased in starved hepatocytes. And there were good arguments for such a change. Because we found that NADH-induced chemiluminescence yield of the isolated plasma membranes of starved rats was increased several fold as compared to the isolated plasma membranes of control rats (Kim, 1993).

The increased reduction of pyridine nucleotides in starvation (Gumma *et al.*, 1971) seemed to have consequences on the glucose transport. It was clearly increased after starvation. The increase of chemiluminescence appeared to persist for a while. Since membranes isolated from starved rats convert the reduction equivalents by NADH much faster into superoxide formation than that of fed rats. These effects were more strongly observed in the absence of calcium. Calcium (1 mM) weakened it but did not completely suppress it (data not shown).

Starvation has decreased hepatic glycogen, a major compound that affects cytosolic volume of hepatocytes. Starvation for 24-48 hr per se decreased cellular glycogen levels without any significant damages of hepatocytes, but starvation increased the glucose transport activity.

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