**Summary.** The effects of glucagon, adrenalin or rapamycin on glycogen autophagy in the liver and heart of newborn rats were studied using biochemical determinations and electron microscopy. Glucagon or adrenalin increased autophagic activity in the hepatocytes and myocardiocytes, glycogen-hydrolyzing acid glucosidase activity in the liver and heart and degradation of glycogen inside the autophagic vacuoles. Glucagon or adrenalin also increased the maltose-hydrolyzing acid glucosidase activity in the liver, but not in the heart. Similar effects were produced in the newborn heart by rapamycin.

These observations support previous studies suggesting that the cellular machinery which controls glycogen autophagy in the liver and heart of newborn animals, is regulated by the cyclic AMP and the mTOR pathways.

**Key words:** Glycogen autophagy, Glucagon, Adrenalin, Rapamycin

**Introduction**

Newborn animals require ample energy substrates to confront their metabolic requirements. After delivery, newborn rats are sustained during this period of nutrient deprivation by the precipitous degradation of liver glycogen to produce glucose. This degradation takes place both in the hyaloplasmic and autophagosomal compartments of hepatocytes and the phosphorolytic degradation of glycogen is assisted by the hydrolytic degradation of polysaccharide. Postnatal burst of autophagic activity and consumption of liver glycogen stores are triggered by the hypoglycemia that follows birth (Rosenfeld, 1964; Shelley and Neligan, 1966; Kotoulas and Phillips, 1971; Kotoulas et al., 1971, 2004; Pfeiffer, 1971). Increased hyaloplasmic and autophagosomal glycogen catabolism has also been demonstrated in the newborn rat myocardiocytes. In this period, glucose represents a chief myocardial fuel substrate (Viragh et al., 1982; Kondomerkos et al., 2004).

Postnatal glycogen autophagy in the liver and heart is accompanied by increased glycogen-, maltose- and isomaltose-hydrolyzing acid alpha 1,4-glucosidase activities to effect the total hydrolysis of glycogen to glucose. Cyclic AMP or cyclic AMP-elevating agents induce glycogen autophagy and glycogen-hydrolyzing acid glucosidase activity (Hers, 1964; Rosenfeld, 1964; Kotoulas, 1984, 1986; Kotoulas et al., 1991; Kalamidas et al., 1994; Kalamidas and Kotoulas, 1999; Kondomerkos et al., 2004). Rapamycin, an inhibitor of mTOR kinase, has similar effects (Kalamidas et al., 2004). In the newborn rat liver, the effects of glycogen autophagy-inducing agents could be associated with an acid mannone 6-phosphatase deficient state of lysosomes (Kalamidas and Kotoulas, 2000b; Kalamidas et al., 2002; Kotoulas et al., 2003).

In this work, we have extended our previous studies with glucagon, adrenalin or rapamycin (Kotoulas and Phillips, 1971; Kotoulas, 1984; Kalamidas et al., 2004; Kondomerkos et al., 2004) to include further biochemical and morphological observations in the liver and heart of newborn rats. Our results support previous studies suggesting that glycogen autophagy is regulated positively by the cyclic AMP and negatively by the mTOR pathways.

**Materials and methods**

**Chemicals**

DL-ethionine (E-5139), glucose 6-phosphate (G-7250), glycogen type III (G-8876), maltose hydrate grade I (M-5885), mannone 6-phosphate (M-6876), rapamycin (R-0395) and Triton X-100 (X-100) were obtained from Sigma (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany). Glucose and protein determinations were carried out using the kits 510-DA
and P-5656 respectively, from Sigma. Adrenalin hydrochloride (Lot. 98007) was purchased from Demo Pharmaceuticals SA (Krioneri, Greece). Glucagon hydrochloride (GlucaGen, recombinant glucagon, 1 mg/ml solution, Lot. LW60114) was from Novo Nordisk A/S (Bagsvaerd, Denmark). Actinomycin D (10710) was from Serva (Heidelberg, Germany). Reagents for electron microscopy were obtained as before (Kalamidas et al., 1994).

Animals and experimental design

Newborn rats were obtained from pregnant Wistar females. Animals were housed and handled according to the EEC-European Council Directive 86/609 (1986) and the Institute of laboratory animals resources, USA (1996). The newborns were separated from their mothers within few minutes from delivery and transferred to a 37°C incubator. The animals were humanely killed at the age of 6 hours unless otherwise specified, by rapid decapitation according to a standard protocol (Kotoulas and Phillips, 1971); before sacrificing, blood samples were obtained from the cervical veins for determining blood glucose. After decapitation, the liver and heart were immediately excised and either were frozen until homogenization or fixed in osmium tetroxide.

Glucagon-treated animals were injected with 0.1 ml solution of GlucaGen, at birth and 3 hours after birth, a dose of 16.7 mg/kg each time. Adrenalin-treated animals were injected with 0.03 ml of adrenalin, at birth and 3 hours after birth, a dose of 0.5 mg/kg each time. Rapamycin-treated animals were injected with 0.3 ml of rapamycin in 10% DMSO at birth, 2 and 4 hours after birth, a dose of 4.75 mg/kg each time. Actinomycin D-treated animals were injected with 0.1 ml of solution at birth and three hours after birth, a dose of 0.8 mg/kg each time. Ethionine-treated animals were injected with 0.03 ml of adrenalin, at birth and 3 hours after birth, a dose of 16.7 mg/kg each time. Adrenalin-treated animals were injected with 0.1 ml of solution at birth and 3 hours after birth, a dose of 100 mg/kg. Control animals from the same litters were injected with carrier only (Kotoulas and Phillips, 1971; Kalamidas and Kotoulas, 2000a; Kalamidas et al., 2004; Kondomerkos et al., 2004).

Biochemical determinations

Homogenization of liver and heart tissues was carried out with distilled water in a 10% and 5% dilution respectively, using a glass homogenizer with a rotating Teflon-coated pestle, in an ice-bath.

Glycogen- and maltose-hydrolyzing acid alpha 1,4-glucosidase activities were determined as previously described (Kalamidas and Kotoulas, 1999; Kondomerkos et al., 2004) using substrate solutions of glycogen (15 mg/ml) and maltose (7.5 mg/ml) respectively. For the assay, 0.2 ml of homogenate were pre-incubated with Triton X-100 for 5 min at 37°C. Then, 0.4 ml of substrate solution in 0.1 M acetate buffer pH 5.0, were added. The reaction mixture was incubated at 37°C in a water bath and 0.1 ml aliquots were removed for glucose determination. Protein was determined according to Lowry et al. (1951). Acid mannose 6-phosphatase and acid glucose 6-phosphatase activities were determined in the homogenates according to previously published methods (Kotoulas et al., 2003; Kalamidas et al., 2004). The statistical evaluation of the results was performed manually by Student’s t-test, according to Hill (1966).

Electron microscopy

Electron microscopy and morphometric analysis were performed as before (Kotoulas and Phillips, 1971; Kalamidas and Kotoulas, 1999; Kondomerkos et al., 2004). Excised liver or heart tissue was fixed for 1 hour at 4°C in 1% osmium tetroxide in 0.1 M phosphate buffer pH 7.2. Tissue was embedded in Epon-Araldite and sectioned with an LKB Ultratome. Ultrathin sections of gray interference color were stained with uranyl acetate and lead citrate. For the morphometric work, a lattice composed of horizontal and vertical lines was superimposed onto the prints and the fractional volume of the cytoplasmic constituents was estimated.

Results

Biochemical results

The administration of glucagon or adrenalin significantly elevated the blood glucose levels of newborn rats at the age of 6 hours (p<0.05). Such an elevation was not observed when actinomycin D or ethionine was added (p>0.05, Table 1). Liver glycogen-hydrolyzing and maltose-hydrolyzing acid glucosidase activities increased in both glucagon- and adrenalin-treated rats (Table 2). Heart glycogen-hydrolyzing acid glucosidase activity also increased in these animals, whereas maltose-hydrolyzing activity did not (Table 3). The administration of rapamycin had similar effects on these enzyme activities (Table 4).

Table 1. Blood glucose after the administration of glucagon or adrenalin and the protein synthesis inhibitors actinomycin D or ethionine to newborn rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 hrs</th>
<th>3 hrs</th>
<th>6 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>59.3±17.3</td>
<td>56.7±17.0</td>
<td></td>
</tr>
<tr>
<td>Glucagon</td>
<td>132.6±24.0 *</td>
<td>90.7±20.3 *</td>
<td></td>
</tr>
<tr>
<td>Glucagon + Actinomycin D</td>
<td>74.0±18.6</td>
<td>43.9±17.1</td>
<td></td>
</tr>
<tr>
<td>Glucagon + Ethionine</td>
<td>66.7±16.8</td>
<td>63.6±16.0</td>
<td></td>
</tr>
</tbody>
</table>

Results are means ± standard deviations and expressed as mg/100 ml. Each value includes 5 observations. *: p<0.05
μmoles phosphate/hr/mg protein; n=4). The difference between 0 and 3 hours was statistically significant (p<0.05). The administration of rapamycin lowered the heart acid mannose 6-phosphatase activity at the age of 6 hours (controls: 30.1±10.9 μmoles phosphate/hr/mg protein, rapamycin-treated: 18.0±7.9 μmoles phosphate/hr/mg protein, n=7, p<0.05). Rapamycin also lowered the heart acid glucose 6-phosphatase activity (controls: 41.2 ± 9.0 μmoles phosphate/hr/mg protein, rapamycin-treated: 20.0 ± 7.8 μmoles phosphate/hr/mg protein, n = 6, p<0.01).

Table 2. Liver glycogen-hydrolyzing and maltose-hydrolyzing acid glucosidase activities after glucagon or adrenalin treatment of newborn rats.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Glucagon-treated</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogen-hydrolyzing activity</td>
<td>0.210±0.063</td>
<td>0.360±0.110</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>(5)</td>
<td></td>
<td>(5)</td>
<td></td>
</tr>
<tr>
<td>Maltose-hydrolyzing activity</td>
<td>0.286±0.018</td>
<td>0.321±0.010</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>(5)</td>
<td></td>
<td>(5)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Heart glycogen-hydrolyzing and maltose-hydrolyzing acid glucosidase activities after glucagon or adrenalin treatment of newborn rats.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Glucagon-treated</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogen-hydrolyzing activity</td>
<td>0.283±0.076</td>
<td>0.437±0.108</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>(9)</td>
<td></td>
<td>(9)</td>
<td></td>
</tr>
<tr>
<td>Maltose-hydrolyzing activity</td>
<td>0.368±0.026</td>
<td>0.320±0.024</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>(5)</td>
<td></td>
<td>(5)</td>
<td></td>
</tr>
</tbody>
</table>

Electron microscopic results

Controls and glucagon- or adrenalin-treated hepatocytes at the age of 6 hours were as described before (Kotoulas and Phillips, 1971; Kotoulas, 1984; Kondomerkos et al., 2004). Control hepatocytes presented hyaloplasmic glycogen stores of various sizes and a number of autophagic vacuoles. These organelles were solitary or in groups and usually situated at the margins of hyaloplasmic glycogen stores. They often contained glycogen and amorphous material. (Fig.1a). Engulfed mitochondria or peroxisomes (either intact or partly digested) were rarely seen. The ultrastructural appearance of hepatic macrophages (Kupffer cells) was as described before (Blouin et al., 1977; Pino et al., 1981; Van Bossuyt and Wisse, 1988; McCuskey and McCuskey, 1990).

Glucagon- or adrenalin-treated hepatocytes showed hyaloplasm moderately depleted of glycogen. Increased number and size of autophagic vacuoles were noted. Their fractional volume was also increased. They contained only small quantities of glycogen (lysosomal

Table 4. Heart glycogen-hydrolyzing and maltose-hydrolyzing acid glucosidase activities after rapamycin treatment of newborn rats.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Rapamycin-treated</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogen-hydrolyzing activity</td>
<td>0.257±0.090</td>
<td>0.439±0.176</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>(8)</td>
<td></td>
<td>(8)</td>
<td></td>
</tr>
<tr>
<td>Maltose-hydrolyzing activity</td>
<td>0.254±0.068</td>
<td>0.151±0.038</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>(4)</td>
<td></td>
<td>(4)</td>
<td></td>
</tr>
</tbody>
</table>

Results are means ± standard deviations and expressed as μmoles of glucose produced per hour per mg protein. Numbers in parentheses signify the number of observations (n) included in the results.

Table 5. Comparison of the fractional volumes of hyaloplasmic glycogen, autophagic vacuoles and lysosomal glycogen from the hepatocytes of control and glucagon- or adrenalin-treated newborn rats.

<table>
<thead>
<tr>
<th></th>
<th>HYALOPLASMIC GLYCOGEN</th>
<th>AUTOPHAGIC VACUOLES</th>
<th>LYSOSONMAL GLYCOGEN</th>
<th>% VOLUME OF AUTOPHAGIC VACUOLES OCCUPIED BY GLYCOGEN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.6±1.9</td>
<td>1.38±0.11</td>
<td>0.25±0.05</td>
<td>18</td>
</tr>
<tr>
<td>Glucagon</td>
<td>7.7±1.6</td>
<td>1.71±0.20</td>
<td>0.13±0.06</td>
<td>8</td>
</tr>
<tr>
<td>Adrenalin</td>
<td>7.8±1.6</td>
<td>1.60±0.14</td>
<td>0.12±0.04</td>
<td>7</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

Results are means ± standard errors and computed from a total of 40 micrographs and an area of 6000 μm² for the hepatocytes of controls or treated newborn animals.
Fig. 1. Rat hepatocytes at the age of 6 hours. a. Control: An autophagic vacuole (arrow) contains a large quantity of undigested glycogen. b. Glucagon-treated: Several autophagic vacuoles (arrows) contain negligible quantities of undigested glycogen. c. Adrenalin-treated: Several autophagic vacuoles (arrows) contain only small quantities of undigested glycogen. Bars: 0.5 µm.
glycogen) and amorphous material. (Table 5; Fig. 1b,c). Engulfed mitochondria or peroxisomes were rarely seen. Hepatic macrophages (Kupffer cells) in the glucagon- and adrenalin-treated animals showed increased irregularity of the nuclear contour, as observed before (Kondomerkos et al., 2004). This may be related to the lobulation of the nucleus observed in adrenalin-treated isolated macrophages (Kondomerkos et al., 2003a,b). Hepatic macrophages in the adrenalin-treated animals also showed decreased number of the rounded, uniformly dense organelles, presumably representing

Fig. 2. Rat myocardiocytes at the age of 6 hours. a. Control: An autophagic vacuole (arrow) is filled with undigested glycogen. b. Glucagon-treated: Several autophagic vacuoles (arrows) contain moderate quantities of undigested glycogen. c. High magnification of glucagon-treated: An autophagic vacuole (arrow) is delimited by a double membrane and contains a small quantity of undigested glycogen in the form of β-particles. Bars: 0.5 µm.
primary lysosomes (Van Bossuyt and Wisse, 1988).

Control myocardiocytes at the age of 6 hours presented hyaloplasmic glycogen stores of various sizes. A number of autophagic vacuoles were noted, frequently containing glycogen and amorphous material (Fig. 2a). These organelles were mostly solitary and rarely in groups and usually situated at the margins of hyaloplasmic glycogen stores.

Glucagon-treated myocardiocytes showed a moderate depletion of the hyaloplasmic glycogen stores and increased number and size of autophagic vacuoles. The autophagic vacuole fractional volume was also increased. The cell glycogen contained in the autophagic vacuoles (lysosomal glycogen) occupied a smaller proportion of the autophagic vacuole volume than in the controls. (Table 6; Fig. 2b,c).

### Discussion

Glucagon is known to promote autophagy. Early reports showed that in the adult rat liver, glucagon promoted cellular autophagy and degradation of organelles, the mitochondria predominating. This process was considered to represent mainly a morphological expression of gluconeogenesis (Ashford and Porter, 1962; Rosa, 1971). However, under our experimental conditions with newborns, the autophagy-promoting effects of glucagon on the liver and heart appear to be rather selective and related to the breakdown of glycogen. In the immediate postnatal period, the gluconeogenic mechanisms are not fully developed (Dawes and Shelley, 1968). Engulfed mitochondria or other organelles (either intact or partly degraded) are rarely seen. Autophagic vacuoles are deployed at the borders of hyaloplasmic glycogen stores and usually contain glycogen.

Glucagon or adrenaline administration are capable of elevating tissue cyclic AMP and enhance glycogen autophagy (Kotoulas et al., 2004). Preferential autophagy of glycogen particles over other cell constituents in newborns treated with cyclic AMP-elevating agents has been previously observed. Autophagy significantly contributes to the overall degradation of intracellular macromolecules (Kotoulas and Phillips, 1971; Pfeifer et al., 1978; Kalamidas et al., 2002; Kondomerkos et al., 2004). The changes in lysosomal glycogen-hydrolyzing enzyme activity observed in our experiments were consistent with increased capability of these hormones to induce the degradation of sequestered polysaccharide to produce glucose. The volume of autophagic vacuoles increased, yet the volume of glycogen inside these organelles did not proportionally increase. On the contrary, it decreased, apparently due to the concomitant autophagosomal degradation of glycogen. The protein synthesis inhibitors actinomycin D and ethionine, known to inhibit autophagy and/or glycogen degradation inside the autophagic vacuoles, also inhibited the hyperglycemic effect of glucagon and adrenaline in the newborns. Ethionine could inhibit autophagy also through a change in the cellular ATP levels (Kotoulas, 1988; Ohsita, 2000).

The differential changes in the glycogen-hydrolyzing and maltose-hydrolyzing glucosidase activities observed in the postnatal heart support previous studies suggesting that these two activities may be due to different enzymes. Their changes may not depend on the same mechanism (Lundquist, 1986; Skoglund et al., 1987; Kotoulas et al., 1991; Kalamidas and Kotoulas, 1999; Kondomerkos et al., 2004).

The stimulatory effect of rapamycin, an mTOR inhibitor, on glycogen-hydrolyzing acid glucosidase activity in the liver (Kalamidas et al., 2004) and heart, indicates a role of the mTOR pathway on glycogen autophagy. This pathway functions as a sensor of nutrient abundance and may negatively regulate this process. The cyclic AMP and the mTOR pathways may converge on a common target to regulate glycogen autophagy (Kalamidas et al., 2004; Kotoulas et al., 2004).

The inhibitory effects of rapamycin on the acid mannosase 6-phosphatase and acid glucose 6-phosphatase activities in the heart are in accordance with previous observations in the liver (Kalamidas et al., 2004). These activities may be due to one common enzyme and have been postulated to co-participate in a regulatory mechanism of glycogen autophagy-derived glucose levels inside the autophagic vacuoles (Kalamidas and Kotoulas, 2000b; Foster and Nordlie, 2002; Kalamidas et al., 2002; Kotoulas et al., 2003, 2004).

### Table 6. Comparison of the fractional volumes of hyaloplasmic glycogen, autophagic vacuoles and lysosomal glycogen from the myocardiocytes of control and glucagon-treated newborn rats.

<table>
<thead>
<tr>
<th></th>
<th>HYALOPLASMIC GLYCOCEN</th>
<th>AUTOPHAGIC VACUOLES</th>
<th>LYSOSMAL GLYCOCEN</th>
<th>% VOLUME OF AUTOPHAGIC VACUOLE OCCUPIED BY GLYCOCEN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.4±2.0</td>
<td>0.20±0.05</td>
<td>0.13±0.02</td>
<td>65</td>
</tr>
<tr>
<td>Glucagon</td>
<td>5.6±1.6</td>
<td>0.49±0.07</td>
<td>0.15±0.02</td>
<td>30</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&gt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

Results are means ± standard errors and computed from a total of 40 micrographs and an area of 6500 µm² for the myocardiocytes of controls or treated newborn animals.
The importance of hepatic glycogen mobilization in neonatal physiology is well established; the significance of neonatal cardiac glycogen mobilization requires further investigation. The fetal heart strongly relies on maternal glucose and placental lactate for myocardial fueling. With birth, the newborn is cut off from its maternal supply and faces a temporary fuel shortage. It must sustain cardiac contractility while also confronting various energy-consuming situations. The hydrolytic degradation of glycogen in the autophagic vacuoles may represent an accessory mechanism for supplying fuel, assisting the phosphorylolytic degradation of glycogen in the hyaloplasm. Myocardial glycogen degradation could be related to confronting anoxia at birth, to avoid circulation failure (Dawes et al., 1959, 1963; Makinde et al., 1998). This polysaccharide contributes significantly to aerobic glucose use under acute increases in energy demand. Cyclic AMP or adrenalin have been found to enhance glycogen breakdown and preferential oxidation of glycogen-derived glucose over exogenous glucose (Henning et al., 1996; Stanley et al., 1997; Depré et al., 1998; Goodwin et al., 1998). Our findings with the glucagon- and adrenalin-induced glycogen autophagy and glycogen-hydrolyzing acid glucosidase activity, are in accordance with these studies. Glycogen autophagy in newborn myocardiocytes may represent a mechanism for locally supplying nonphosphorylated glucose, as has been proposed for newborn hepatocytes (Kondomerkos et al., 2004; Kotoulas et al., 2004). The electron microscopic and biochemical effects of adrenalin and other cyclic AMP-modifying agents on the myocardiocytes are currently under investigation.

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References

Dawes G.S., Mott J.C. and Shelley H.J. (1959). The importance of cardiac glycogen for the maintenance of life in fetal lambs and newborn animals during anoxia. J. Physiol. 146, 516-538.

Glycogen autophagy and glucagon, adrenalin or rapamycin


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