Developmental Alteration in Adrenergic Regulation of Hepatic Glycogen Phosphorylase

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Abstract—The effects of development upon adrenergic regulation of glycogenolysis were characterized using isolated hepatocytes from 3 different age groups of male rats (6 week-old, 8 week-old and 30 week-old). The phosphorylase a response in isolated hepatocytes to alpha-adrenergic stimulation decreased moderately with advancing age; whereas, that to beta-adrenergic stimulation declined more rapidly and almost disappeared at the age of 30 weeks. This developmental alteration in relative contribution of alpha- and beta-adrenergic regulation of phosphorylase was further confirmed by the experiments with specific antagonists. Also, the dramatic decrease of beta-adrenergic response on glycogen phosphorylase activity was found to be closely associated with a similar change of cAMP generation. In addition, the glucagon effect on cAMP production was found to be declined with advancing animal age. These results demonstrate that the glycogenolytic response of isolated rat hepatocytes to catecholamines can be mediated by different pathways according to the age of the animal; thus, juvenile male rats exhibit both the alpha- and beta-adrenergic mechanism for activation of phosphorylase and the maturation is associated with a modest decline of alpha receptor-mediated effect and a dramatic attenuation of a beta-adrenergic/cAMP response.

The regulation of liver glycogen metabolism is one of the factors which plays an important role in the control of carbohydrate metabolism (1). The rate-limiting enzyme for the regulation of glycogenolysis is glycogen phosphorylase. This enzyme exists in an interconvertible active and inactive form and are controlled by a variety of hormones and neural stimulation (2, 3). In particular, catecholamines and glucagon have profound effects on the process (4).

Hepatic glycogenolysis induced by catecholamines occur through both alpha- and beta-adrenergic receptor mediated pathways (4, 5). The beta-adrenergic activation of glycogen phosphorylase is blocked by propranolol and is presumed to result from an increase of intracellular cAMP. The alpha-adrenergic pathway, on the other hand, activates phosphorylase without detectable increases in intracellular cAMP levels or the activation of cAMP-dependent protein kinase. and is effectively blocked by phentolamine, phenoxybenzamine and prazosin, but is unaffected by propranolol. According to the current lines of evidence, the alpha-receptors governing hepatic glycogenolysis in isolated rat hepatocytes belong to the alpha₁-subclass (6, 7), and this alpha-adrenergic receptor-mediated pathway involves a rise in cytosolic Ca²⁺ which will stimulate phosphorylase kinase and lead to the activation of glycogen phosphorylase (8–10).

Abbreviations: cAMP, Adenosine 3',5'-cyclic monophosphosphate; EDTA, Ethylenediaminetetraacetic acid; MEM, Minimum essential media; IBMX, 3-Isobutyl-1-methylxanthine; MOPS, 3-(N-Morpholino)-propanesulfonic acid; Hepes, 4-(2-Hydroxyethyl)-1-piperazine-ethanesulfonic acid.

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These adrenergic regulations in adult rat liver, however, have been shown to be modified under a number of physiological and pathological conditions, including sex (11), adrenalectomy (12), hypothyroidism (13), regeneration (14), cholestasis (15, 16), preneoplasia (17) and during primary culture (18–20). Developmental maturity and the aging process have been known to be associated with reduced responsiveness of the hepatic beta-adrenergic receptor/cAMP system as reflected in assays of adenylate cyclase activity and cAMP accumulation (21–24). In addition, the alpha-adrenergic receptor-mediated pathway has been known to predominate in the activation of hepatic phosphorylase in the adult male rats (8, 25, 26). However, it is still uncertain what kind of alteration may develop during maturation in the relative functional significance of alpha- and beta-adrenergic receptor pathways in regulation of glycogenolysis.

The aim of the present work was to characterize the developmental effect on the relative contribution of alpha- and beta-adrenergic pathways in the control of hepatic glycogenolysis by using specific agonists and antagonists. The results demonstrate that both alpha- and beta-receptor mediated phosphorylase activation declines progressively with advancing age, in particular beta-receptor mediated response reduces more rapidly. Compared with the dramatic drop of beta-adrenergic receptor-mediated response, the age-related decline of alpha-adrenergic receptor-mediated response is so small that apparently hepatic glycogenolysis seems to be switched over from beta- to alpha-regulation with maturation.

Materials and Methods

Materials: Alpha-D-(U-14C) glucose 1-phosphate was purchased from New England Nuclear (Boston, MA). Reagents for radioimmunoassay of cAMP, i.e., 125I-labeled cAMP tyrosine methyl ester, anti-cAMP rabbit antiserum, and cAMP were kind gifts from Yamasa Shoyu Company (Chiba, Japan). Prazosin was a gift from Pfizer Pharmaceutical Company. Collagenase (Type II) was purchased from Worthington Biochemical Company (Freehold, NJ); tissue culture medium 199 (Earl’s salt), DMEM and MEM were from Gibco. Other chemicals and reagents were obtained from standard commercial sources.

Animals: Three groups of male Sprague-Dawley rats representing different ages were used. The first group, representing immature juveniles, weighed between 140 and 160 g and were 35 to 42 days old (6 weeks). An intermediate group, representing young adults, were 50 to 60 days old (8 weeks) and weighed 240 to 260 g. The oldest group, representing fully mature adults, weighed from 490 to 510 g and were 180 to 240 days old (30 weeks). The rats were fed standard laboratory chow ad libitum and were kept on a 12 hr light-12 hr dark schedule. All rats were kept on this schedule for at least 7 days. Isolation of hepatocytes was routinely initiated 3 to 4 hr into the light period.

Isolation of hepatocyte: Hepatocytes were isolated by the collagenase perfusion method of Berry and Friend (27) as modified by Bissel (28). Briefly, the liver was perfused at 37.0°C with 150 ml of Ca2+ free-MEM buffered with bicarbonate containing 0.06% (W/V) collagenase. Medium alone was infused over a period of 6-7 min. Collagenase was then introduced. After 10–15 min, the liver was carefully cut from its ligaments and transferred to a sterile bottle, opened with scissors and shaken in MEM containing 0.002% (w/v) deoxyribonuclease in a rotary-action shaking water bath at 37.0°C for 10 min. After filtration through a double layer of sterile cotton gauze, the suspension was centrifuged at 50 g for 2.5 min. Two cycles of washing with medium 199 or Hepes-buffered medium (134 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 2.5 mM CaCl2, 5 mM NaHCO3 and 10 mM Hepes, pH 7.4) were carried out, and cells were suspended finally in Hepes-buffered medium supplemented with 40 mM glucose. The yield of cells from one liver averaged 1.5×10⁸, with 90–95% viability as estimated by trypan blue exclusion.

Incubation of hepatocytes: Freshly isolated hepatocytes (6 to 15×10⁶ cells) were incubated in plastic tubes in a total volume of 1 ml of Hepes-buffered medium supplemented with 40 mM glucose and shaken in
an orbital water bath shaker at 150 to 200 rpm at 37°C under an atmosphere of O₂/CO₂ (95:5). Hepatocytes were preincubated for 30 min before the stimulation by agonists, while prazosin or propranolol were added as required after 20 min of preincubation. At the end of incubation, 200 µl aliquots of the cell suspension were transferred to a plastic tube containing 50 µl of drugs as indicated. The tubes were then shaken in a bath at 37°C for 2 min and then immediately immersed into the liquid nitrogen to terminate the reaction. The frozen hepatocytes were stored at -70°C until assay. For the glycogen phosphorylase assay, the frozen hepatocytes were first mixed with a half volume of 30 mM MOPS, 150 mM NaF, 15 mM EDTA and 3 mM dithiothreitol (pH 7.0). The samples were homogenized with Polytron cell disrupter (Brinkman Instruments, Westbury, NY) at a setting of 8 for 20 sec. The homogenates were centrifuged at 12,000 g max for 5 min at 4°C, and the supernatant was used for the assay of glycogen phosphorylase and cAMP as described below.

Assays of glycogenolysis and cAMP formation: Glycogen phosphorylase a activity was measured using a filter disc assay similar to that of Gilboe et al. (29) as modified (30). Briefly, caffeine (0.5 mM) was included in the assay to suppress phosphorylase b activity. Data are expressed as micromoles of (14C) glucose from alpha-D-(U-14C) glucose 1-phosphate incorporated into total assay glycogen per min per 100 mg of protein. For assay of cAMP, 100 µl of the supernatant described above were added to 50 µl of 0.4 N HCl. Samples were heated in a boiling water bath for 1 min and then neutralized with 4 N NaOH. Cyclic AMP was measured by radioimmunoassay (31). Data are expressed as pmol/mg protein.

Protein concentration was determined by the method of Lowry et al. (32) using bovine serum albumin as standard.

The experimental data given in the text are expressed as the mean±S.E.M. Statistical differences between two means (P<0.05) were determined by Student's t-test for unpaired observations.

Results

Figure 1 demonstrates that treatment of isolated hepatocytes with phenylephrine (alpha adrenergic agonist, Fig. 1A), isoproterenol (beta adrenergic agonist, Fig. 1B) and...
epinephrine (mixed alpha and beta adrenergic agonist, Fig. 1C) elicited a dose-dependent increase in stimulation of phosphorylase activity which varied markedly in magnitude according to the age of the animals. Also, as shown in Fig. 1, the extent of this age-related alteration was found to be different according to the agonist. Thus, the phosphorylase a response to phenylephrine and epinephrine decreased moderately with advancing age (Fig. 1A and 1C). On the other hand, the response to isoproterenol markedly declined with age, and it almost disappeared in isolated hepatocytes from 30 week-old rats (Fig. 1B). The sensitivity of phosphorylase a stimulation to epinephrine was not significantly different in the three groups (ED50's were 1 × 10^{-7} M in all age groups), whereas those to phentlephrine (ED50 was 1 × 10^{-7}, 3 × 10^{-6} and 3 × 10^{-6} M for 150 g, 250 g and 500 g body weight rats, respectively) and isoproterenol (ED50 was 2 × 10^{-8} M for 150 g body weight rats, whereas those for the other two groups were not detected) were greater in hepatocytes from older animals.

This age-related alteration in relative contribution of alpha- and beta-adrenergic regulation of phosphorylase was further characterized by the experiments with specific antagonists, propranolol (10^{-6} M) and prazosin (10^{-6} M) (Table 1). In preliminary studies, it was found that these concentrations of antagonists exert maximal antagonistic effect on epinephrine-induced activation of phosphorylase; therefore, further experiments with antagonists were conducted using these concentrations of antagonists. In these experiments, phenylephrine (10^{-6} M) was preferentially antagonized by prazosin (10^{-6} M) rather than propranolol (10^{-6} M) in all age groups. Isoproterenol's stimulation was effectively blocked by both propranolol and prazosin in juvenile animals, whereas in older rats, this was preferentially blocked by prazosin rather than propranolol. These data confirm that the alpha adrenergic response predominates over beta adrenergic activation of glycogen phosphorylase as rats age.

In addition, the dramatic decrease of beta-adrenergic activation of glycogen phosphorylase was found to be associated with blunted cAMP accumulation in response to isoproterenol (Fig. 2B) or epinephrine (Fig. 2C) in hepatocytes from older rats. This change is in good agreement with the previous report of Morgan et al. (24); however, we did not detect increased cAMP accumulation in response to the alpha agonist phenylephrine in older rats (Fig. 2A; 23, 24). Furthermore, the ability of glucagon, another important cAMP-generative hor-

### Table 1. Effect of alpha- and beta-adrenergic blockade on epinephrine-, phenylephrine- and isoproterenol-stimulated glycogen phosphorylase activation in isolated hepatocytes from male rats of 150 g, 250 g and 500 g body weight

<table>
<thead>
<tr>
<th>Animal weights (g)</th>
<th>Stimulants</th>
<th>% Inhibition</th>
<th>10^{-8} M Prazosin</th>
<th>10^{-8} M Propranolol</th>
</tr>
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<tr>
<td>150</td>
<td>Epinephrine</td>
<td>45.7±14.9</td>
<td>34.3±13.3</td>
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<tr>
<td></td>
<td></td>
<td>83.2±2.8</td>
<td>21.5±11.3</td>
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<tr>
<td></td>
<td>(10^{-6} M)</td>
<td>84.6±7.1</td>
<td>3.5±3.4</td>
<td></td>
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<tr>
<td>150</td>
<td>Phenylephrine</td>
<td>92.1±5.8</td>
<td>18.3±17.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>80.9±5.7</td>
<td>19.6±9.6</td>
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<td>(10^{-6} M)</td>
<td>89.7±4.9</td>
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<tr>
<td>Isoproterenol</td>
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<td>40.9±3.7</td>
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<tr>
<td></td>
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* Experimental conditions were identical with those of Fig. 1 except that the hepatocytes were preincubated with each antagonist for 10 min at the time of addition of the indicated concentration of agonist.  
* Results are means±S.E.M. from four different experiments.
mone, to activate cAMP production in the rats of various ages was examined. As shown in Fig. 3, there was a significant decrease in maximal accumulation of cAMP by glucagon with advancing age.

Fig. 2. Dose-response curves for phenylephrine (A), isoproterenol (B) and epinephrine (C) stimulation of cAMP accumulation in hepatocytes from male rats of 150 g, 250 g and 500 g body weight. Experimental conditions were identical with those of Fig. 1 except that cAMP accumulation was measured in the presence of 0.2 mM IBMX. Results are the mean±S.E.M. of 3–5 different experiments.

Fig. 3. Dose-response curves for glucagon stimulation of cAMP accumulation in hepatocytes from male rats of 150 g, 250 g and 500 g body weight. Experimental conditions were identical with those of Fig. 2. Results are the mean±S.E.M. of 3–5 different experiments.

Fig. 4. Dose-response curves for glucagon stimulation of cAMP accumulation in hepatocytes from male rats of 150 g, 250 g and 500 g body weight. Experimental conditions were identical with those of Fig. 2. Results are the mean±S.E.M. of 3–5 different experiments.

Discussion

In the present experiments, we found that the relative contribution of alpha- and beta-adrenergic control of the glycogenolytic response to catecholamines in isolated rat hepatocytes can be varied as rats grow; thus, juvenile rats exhibit the dual mechanism for activation of phosphorylase (both alpha- and beta-adrenergic responses), and the maturation is associated with an attenuation of both alpha- and beta-adrenergic responses. Interestingly, the decrease in alpha adrenergic activation with age is more modest than the marked fall in beta adrenergic one.

A great deal of interest has focused on the altered responsiveness to catecholamines that occurs with postnatal development and aging (33). Diminished responsiveness to both alpha- and beta-adrenergic stimulation has been found in a number of tissues (for general review, see 33). In the male rat liver, catecholamine-induced glycogenolysis is found to be mediated mainly by beta-adrenergic stimulation during the fetal and neonatal period (22, 34, 35), whereas in the adult stage, it is predominantly mediated by alpha-adrenergic stimulation (7, 22, 23, 25). Thus, it has been generally thought that maturation of alpha-adrenergic response
might be a postnatal event, which is coupled with functional loss of beta-adrenergic response by an unknown mechanism. Our data confirmed the findings of earlier studies (23, 24) that juvenile male rats exhibit both alpha- and beta-adrenergic receptor activation of glycogen phosphorylase, whereas in older rats, the alpha-adrenergic response becomes predominant. However, our present studies show that both alpha- and beta-adrenergic activation of phosphorylase declines progressively with advancing age, and especially beta-adrenergic response decreases so markedly that it overshadows the relatively modest decline of the alpha-adrenergic one. Thus, apparently the adrenergic regulation of glycogen phosphorylase seems to convert from an alpha-type to a beta-type as rats grow.

Although the explanation for the dramatic reduction of beta adrenergic response with age is uncertain from the present study, a possible post-receptor mechanism might be implicated. As glucagon, another hormone receptor system coupled with cAMP generation, was also found to be decreased with age in stimulating cAMP production, one could conjecture that attenuation in the stimulatory regulation or conceivably potentiation of inhibitory regulation of adenylate cyclase. In particular, the importance of considering the latter possibility that an enhanced inhibitory mechanism may be responsible for the diminished action of stimulatory hormones should be emphasized, since this possibility has been found to play a major role in the age-related blunting response to stimulatory hormones in activating lipolysis of rat adipocytes (36). Also, recent work by Itoh et al. (20) has strongly supported this view, showing that the function of the inhibitory guanine nucleotide regulatory component of adenylate cyclase can be modified in exactly the same model system of rat hepatocytes that we studied in the present work.

In conclusion, our present studies confirm and extend results indicating dynamic changes in adrenergic regulation of glycogen phosphorylase in rat liver with development. Although studies are now in progress as to the mechanism responsible for these changes, the underlying explanation for the alteration in expression of alpha- and beta-adrenergic responses with age awaits further investigation.

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