Inhibitory Effect of Extracts of Muscles of Mackerel (*Scomber japonicus* HOUTTUYN) on Hepatic Glycogenolysis in Rats

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The effects of extracts of muscles of mackerel (*Scomber japonicus*; M-ext) on hepatic glycogenolysis were investigated by a rat liver perfusion method. M-ext inhibited glucagon- and cyclic adenosine monophosphate (AMP)-induced glycogenolysis but was ineffective on phenylephrine-induced glycogenolysis.

The contents of hepatic glycogen and cyclic AMP, and phosphorylase and glycogen synthase activities in liver were measured after perfusion with glucagon. M-ext inhibited the increase of cyclic AMP and activation of phosphorylase.

It is considered that M-ext inhibits hepatic glycogenolysis caused by glucagon through a cyclic AMP-dependent mechanism.

Keywords hepatic glycogenolysis; *Scomber japonicus*; liver perfusion; cyclic AMP-dependent mechanism; phosphorylase

Introduction

High protein meals and the extracts from the muscles of various animals have been shown to possess hypoglycemic effects. In the preceding paper,[1] we have reported that the extracts of muscles of pig (*Sus scrofa*), chicken (*Gallus domesticus*) and mackerel (*Scomber japonicus*) decreased blood glucose levels in normal mice and alloxan-induced diabetic mice, and that among these extracts, mackerel extract proved to be weak in insulin-like activity and in insulin secretion-promoting effect, but strong in inhibitory effect on hepatic glycogenolysis induced by epinephrine (α-, β-adrenergic agonist) as determined by a rat liver perfusion method.

This paper reports an examination of the effects of mackerel extract on hepatic glycogenolysis by a rat liver perfusion method, using glucagon, cyclic adenosine monophosphate (AMP) and phenylephrine (α-adrenergic agonist) as stimulators of hepatic glycogenolysis.

Materials and Methods

Materials The extract of muscle of mackerel (abbreviated as M-ext) was obtained according to the method described in the previous paper.[1] The protein content in M-ext assayed by Lowry's method was 504 mg/g. In all experiments, the concentration of M-ext was represented by values converted to a protein basis.

The sources of chemicals and reagents were as follows: glucagon (Sigma Chemical Co.), adenosine-3′,5′-cyclic monophosphate (Kohjin Co., Ltd.), L-phenylephrine hydrochloride (Tokyo Kasei Co., Ltd.), porcine insulin (Sigma Chemical Co.), α-D-[U^{14}C]glucose 1-phosphate (NEN Research Prod.), uridine diphosphate 1-[^{3}H]glucose (NEN Research Prod.), glycogen from rabbit liver (Nacalai Tesque Inc.), Dowex 1×2 (acetate form, Dow Chemical Company), EGTA (ethylene glycol bis[β-aminoethylether]-*N*,*N*,*N*′,*N*′-tetraacetic acid, Nacalai Tesque Inc.), Glucose C-Test Wako (Wako Pure Chemical Ind. Ltd.) and cAMP assay system (Amersham).

Animals Male rats (8 weeks old, 230—250 g) of kwl:-Wistar strain were used. The animals were housed in a room maintained at 23±2 °C and 60±10% relative humidity for 7 d before the start of the experiment, with free access to food and water.

Glucose Output in Perfused Rat Liver Liver perfusion was performed according to the method of Kimura *et al.*[2] The Krebs-Ringer-bicarbonate (KRB) buffer was gassed with 95% O_2–5% CO_2 and maintained at pH 7.4 and 37 °C. The flow rate was kept constant at 25 ml/min. KRB buffer and sample solution were passed through membrane filters of 0.45 μm.

Glucagon (10^{-11}—5×10^{-10} M), cyclic AMP (10^{-5} M) or L-phenylephrine (10^{-6} M) was injected into the perfusion solution over a 20-min interval by means of an injection pump, starting 30 min after the beginning of perfusion. The sample solution was introduced through the side arm pump into the KRB buffer 10 min before injection of glucagon, cyclic AMP or phenylephrine.

Determination of Glucose, Glycogen, Cyclic AMP, Glycogen Phosphorylase and Glycogen Synthase Glucose content in the effluent from the inferior *vena cava* was measured by the mutarotase-glucose oxidase method (Glucose C-test Wako). For the assay of glycogen, cyclic AMP, glycogen phosphorylase and synthase, a portion of the liver was frozen quickly with carbon dioxide. The frozen tissue was stored at −80 °C until analysis. Glycogen was measured by the anthrone method[3] and cyclic AMP by a radioimmunoassay method (cAMP assay system).

For the enzyme assay, the frozen liver was homogenized by a Polytron tissue disruptor with nine volumes of ice-cold 100 mM Tris–HCl buffer (pH 7.4) containing 100 mM NaF, 1% glycogen, and 10 mM dithiothreitol. To measure the active form of phosphorylase (phosphorylase a), assay was started by adding 50 μl of the homogenate to 100 μl of the assay medium containing 15 mM α-D-[U^{14}C]glucose 1-phosphate (4×10^4 cpm/tube), 225 mM NaF and 0.75 mM caffeine. In the case of the estimation of total activity, 0.75 M Na_2SO_4 and 7.5 mM AMP were used instead of caffeine. All phosphorylase assays were performed at 37 °C for 30 min.[4,5] For termination of the reaction and isolation of labeled glycogen, the incubation mixture (50 μl) was poured onto resin (Dowex 1×2, 100—200 mesh) in a short column and the column was washed twice with 0.5 ml of water.[4] Radioactivity of the combined washings containing labeled glycogen was measured with a scintillation counter. The scintillator consisted of 60 mg of 1,4-bis-2-(5-phenyloxazol-2-yl)benzene (POPOP) and 5 g (PPO) per 1 l of a mixture of three volumes of toluene and one volume of Triton X-100.

The enzyme activity was represented in units of μmol/min/g wet tissue of [U^{14}C]glucose 1-phosphate incorporated into glycogen after incubation for 30 min.

To measure the active form of glycogen synthase, assay was started by adding 100 μl of the homogenate to 100 μl of the assay medium containing 6 mM uridine diphosphate (UDP)-1-[^{3}H]glucose (3×10^4 cpm/tube), 50 mM Tris–HCl buffer (pH 7.8), 5 mM EDTA, 1% glycogen, and 15 mM Na_2SO_4. For the total synthase, glucose 6-phosphate was used instead of Na_2SO_4. Assay was performed at 37 °C for 60 min for the active form activity and for 10 min for total activity. Termination of the reaction and isolation of labeled glycogen were done in the same manner as for phosphorylase assay.

The enzyme activity was represented in units of nmol/min/g wet tissue of UDP-1-[^{3}H]glucose incorporated into glycogen after incubation for 60 min or 10 min.[4,5]

Statistical Analysis Reference should be given for Cochlan Cox test.

Results

Effects of M-ext on Glucagon-Induced Glycogenolysis After a 30-min perfusion, the rate of glucose output from the perfused rat liver was stabilized at levels of 0.610±0.069 μmol/min/g wet tissue (mean±S.E., $n=11$) and 0.574±0.062 μmol/min/g wet tissue ($n=3$) in the presence and absence of perfusate calcium, respectively.

The effect of M-ext on glucose output induced by a half maximal concentration of glucagon (5×10^{-11} M) was exam-

ined. As shown in Fig. 1, M-ext at concentrations above 50 μg/ml significantly inhibited the glycogenolysis induced by glucagon.

As shown in the dose–response curve of glucagon-induced glycogenolysis (Fig. 2), M-ext (100 μg/ml) significantly inhibited glycogenolysis induced by 5×10^{-11} M glucagon.

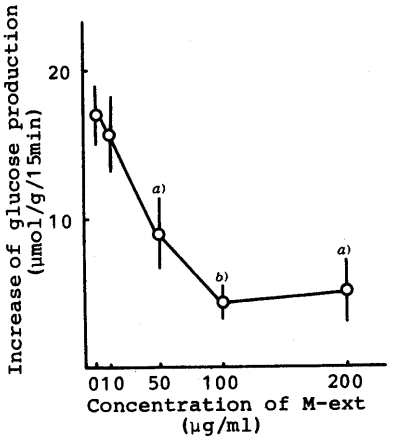

Fig. 1. Dose Dependency of the Inhibitory Effect of M-ext on Glucagon-Induced Glycogenolysis

Livers from fed rats were perfused with KRB buffer. Twenty minutes after the start of perfusion, M-ext was added to the perfusate, followed 10 min later by the addition of glucagon. The net increase in the glucose output during 15 min of glucagon (5×10^{-11} M) perfusion with various concentrations of M-ext is plotted. Each point indicates the mean ± S.E. from three to six independent experiments. *a*) $p < 0.05$ and *b*) $p < 0.01$ *vs.* glucagon only.

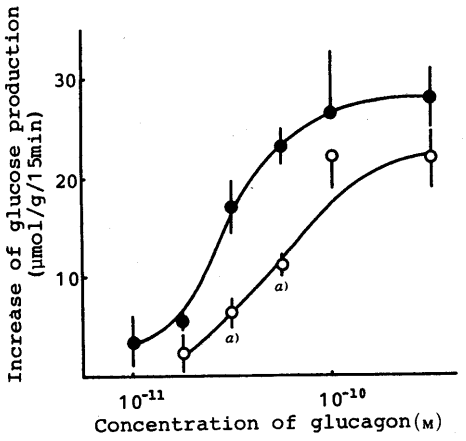

Fig. 2. Effects of M-ext on the Dose–Response Curve of Glucagon

Experimental conditions were the same as those for Fig. 1 except that the concentrations of glucagon was varied from 10^{-11} M to 5×10^{-10} M. Each point indicates the mean ± S.E. from three to nine experiments. —●—, glucagon only; —○—, glucagon + M-ext 100 μg/ml; *a*) $p < 0.01$ *vs.* glucagon only.

Porcine insulin (1 mU/ml) and M-ext (100 μg/ml) inhibited significantly the glucose output by glucagon (5×10^{-11} M) (Fig. 3). Even in Ca^{2+}-free perfusate (after addition of EGTA), M-ext (100 μg/ml) significantly inhibited glucagon-induced glycogenolysis (Fig. 4).

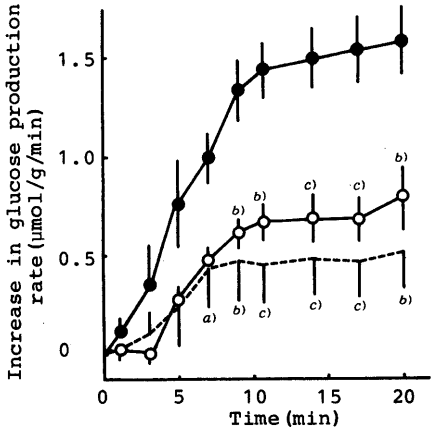

Fig. 3. Effects of M-ext and Porcine Insulin on Glucagon-Induced Glycogenolysis

Experimental conditions were the same as those for Fig. 1. Glucagon (5×10^{-11} M) was added 30 min after the beginning of perfusion (0 min in the figure). Increase in the rate of glucose output above basal is shown as a function of time. In each group, when glucagon was not added, the rate of glucose production did not change significantly during the observation period. Each point indicates the mean ± S.E. from six or nine experiments. —●—, glucagon; —○—, glucagon + M-ext 100 μg/ml; ----, glucagon + porcine insulin 1 mU/ml; *a*) $p < 0.05$, *b*) $p < 0.01$, *c*) $p < 0.001$ *vs.* glucagon only.

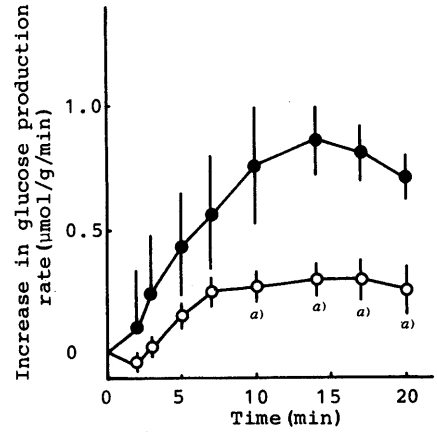

Fig. 4. Effect of M-ext on Glucagon-Induced Glycogenolysis in Perfused Rat Liver with Ca^{2+}-Free Perfusate

Experimental conditions were the same as those for Fig. 3 except that KRB buffer was modified to Ca^{2+}-free perfusate by addition of EGTA. Results are expressed as in Fig. 3. Each point indicates the mean ± S.E. from three experiments. —●—, glucagon (5×10^{-11} M); —○—, glucagon + M-ext 100 μg/ml; *a*) $p < 0.05$ *vs.* glucagon only.

TABLE I. Effects of M-ext and Porcine Insulin on Glycogen, Cyclic AMP and Phosphorylase Levels in Rat Liver at the End of Perfusion

Treatment	No. of experiments	Glycogen (mg/g liver)	Cyclic AMP (nmol/g liver)	Phosphorylase (μmol/min/g liver)		
				Phosphorylase a (A)	Total (T)	A/T (%)
KRB buffer	4	64.50 ± 3.64	0.67 ± 0.03	0.44 ± 0.08	1.76 ± 0.09	26.03 ± 5.58
Glucagon	9	42.22 ± 8.95[a)]	0.84 ± 0.04[a)]	0.93 ± 0.05[b)]	1.80 ± 0.05	52.22 ± 3.04[b)]
Glucagon + M-ext	6	106.70 ± 31.11	0.69 ± 0.02[d)]	0.70 ± 0.04[d)]	1.87 ± 0.07	37.22 ± 2.05[d)]
Glucagon + porcine insulin	5	114.02 ± 21.34[d)]	0.68 ± 0.06[c)]	0.78 ± 0.04[c)]	1.82 ± 0.03	43.15 ± 2.84[c)]

The experimental conditions and the analytical procedures were as described in Materials and Methods. Glucagon (5×10^{-11} M) was added 30 min after the beginning of the perfusion. M-ext (100 μg/ml) or porcine insulin (1 mU/ml) was added 10 min before glucagon infusion. Values shown are mean ± S.E. *a*) $p < 0.05$ and *b*) $p < 0.01$ *vs.* KRB buffer only, *c*) $p < 0.05$ and *d*) $p < 0.01$ *vs.* glucagon only.

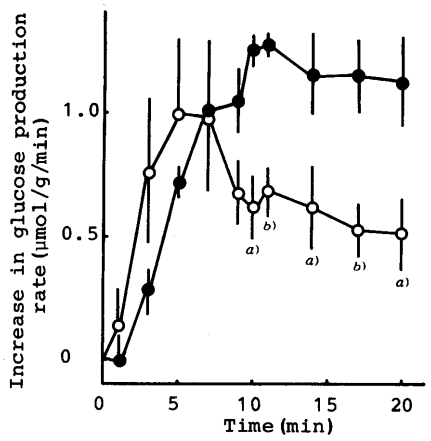

Fig. 5. Effect of M-ext on Cyclic AMP-Induced Glycogenolysis

Experimental conditions were the same as those for Fig. 3. Cyclic AMP (10^{-5} M) was added 30 min after the beginning of perfusion (0 min in the figure). Results are expressed as in Fig. 3. Each point indicates the mean ± S.E. from four experiments. —●—, cyclic AMP; —○—, cyclic AMP + M-ext 100 μg/ml; *a*) $p<0.05$, *b*) $p<0.01$ *vs.* cyclic AMP only.

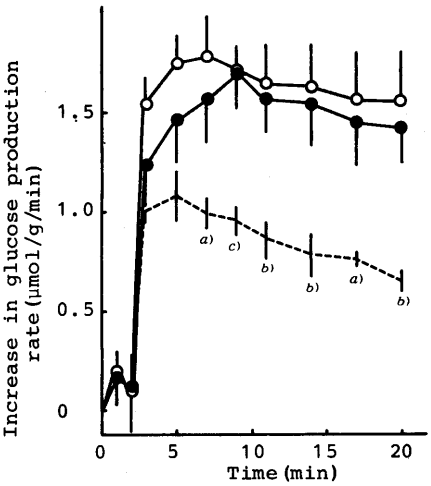

Fig. 6. Effect of M-ext on Phenylephrine-Induced Glycogenolysis

Experimental conditions were the same as those for Fig. 3. Phenylephrine (10^{-6} M) was added 30 min after the beginning of perfusion (0 min in the figure). Results are expressed as in Fig. 3. Each point indicates the mean ± S.E. from four or six experiments. —●—, phenylephrine; —○—, phenylephrine + M-ext 100 μg/ml; ----, phenylephrine + porcine insulin 1 mU/ml; *a*) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$ *vs.* phenylephrine only.

The contents of glycogen and cyclic AMP, and phosphorylase activity in the liver at the end of perfusion are shown in Table I. Glucagon resulted in the decrease of glycogen, increase of cyclic AMP and increase of phosphorylase activity, compared with those in the case of buffer alone. Porcine insulin (1 mU/ml) inhibited the decrease of glycogen, increase of cyclic AMP and activation of phosphorylase caused by glucagon. M-ext (100 μg/ml) inhibited the increase of cyclic AMP and activation of phosphorylase, and exhibited a tendency to inhibit the decrease of glycogen caused by glucagon.

Glucagon showed no significant effect on the activity of glycogen synthase. Porcine insulin (1 mU/ml) and M-ext (100 μg/ml) were also ineffective.

Effect of M-ext on Cyclic AMP-Induced Glycogenolysis The concentration of cyclic AMP having a similar action to glucagon (5×10^{-11} M) on glucose output was 10^{-5} M. Glucose output by cyclic AMP (10^{-5} M) is shown in Fig. 5. M-ext significantly inhibited the glucose output by cyclic AMP.

Effect of M-ext on Phenylephrine-Induced Glycogenolysis The effective concentration corresponding to glucagon (5×10^{-11} M) was 10^{-6} M in the case of phenylephrine. Porcine insulin inhibited the glucose output but M-ext did not inhibit phenylephrine-induced glycogenolysis.

Discussion

The synthesis and breakdown of glycogen in the liver are under strict hormonal regulation. Glycogenolysis is regulated at the site of phosphorylase. It is already known that glucagon activates phosphorylase through a cyclic AMP-dependent mechanism[6-9] and that Ca^{2+}-dependent hormones (phenylephrine, vasopressin and angiotensin II) also stimulate phosphorylase activity *via* a cyclic AMP-independent pathway.[7-10]

In this work, the effects of M-ext were investigated on both glycogenolysis induced by glucagon and phenylephrine (α-adrenergic stimulation) using a rat liver perfusion method. M-ext inhibited glucagon-induced glycogenolysis but was ineffective on phenylephrine-induced glycogenolysis. It has been reported that insulin exhibited inhibitory effects on hepatic glycogenolysis induced by both hormones. Accordingly, it is clear that M-ext has a mode of action which is different from that of insulin on glycogenolysis in rat liver. M-ext inhibited an increase in the concentration of cyclic AMP and activation of phosphorylase in the liver caused by glucagon, and also glucose output induced by cyclic AMP. From these findings, it can be considered that M-ext not only inhibits glucagon-stimulated cyclic AMP production but also enhances inactivation of the produced cyclic AMP by phosphodiesterase, or depresses the action of cyclic AMP to stimulate cyclic AMP-dependent protein kinase.

Furthermore, since M-ext showed inhibitory effects on glucagon-induced glycogenolysis in both Ca^{2+}-free and Ca^{2+}-containing perfusion, but did not inhibit glycogenolysis induced by phenylephrine (α-adrenergic stimulation), the inhibitory effect of M-ext on glycogenolysis in the liver seems not to involve a Ca^{2+}-dependent mechanism. Insulin could not be detected by radioimmunoassay or enzyme immunoassay in M-ext. The quantity of sodium phosphate which is contained in the buffer solution used for extraction of mackerel muscles did not influence hepatic glycogenolysis by glucagon (5×10^{-11} M).

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