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Stimulating Effect of Deer Antler Extract on Protein Synthesis in Senescence-Accelerated Mice *in Vivo*[1)]

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The effects of subchronic administration of Rokujo (*Cervus nippon* TEMMINCK var. *mantchuricus Swinhoe*) extract on protein, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) syntheses *in vivo* were investigated in male senescence-accelerated mice (SAM). At 20 min after intraperitoneal injection of amino acid and nucleosides labeled with radioisotopes, the incorporation of [^{14}C]leucine into the serum protein was increased in the Rokujo-treated mice, in a parallel manner to the elevation of the serum protein content. Increased incorporation of [^{14}C]uridine into RNA as well as that of [^{14}C]leucine into protein was found in the liver and kidney of the Rokujo-treated mice. Further experiments revealed that although a brief exposure to Rokujo extract *in vitro* did not directly affect the RNA polymerase activity, the activity of RNA polymerase II in solubilized liver nuclei of Rokujo-treated mice was markedly accelerated. These results suggest that enhancement of RNA polymerase II activity induced by long-term Rokujo treatment is responsible for the increase in protein synthesis *in vivo* in SAM.

Keywords——Rokujo; *Cervus nippon* var. *mantchuricus Swinhoe*; senescence-accelerated mouse; protein synthesis; RNA synthesis; DNA replication; RNA polymerase II

In our preceding paper,[3)] we demonstrated that 8-d administration of hot-water extract from *Cervus nippon* TEMMINCK var. *mantchuricus Swinhoe* (Rokujo) to male senescence-accelerated mice (SAM) led to diverse actions on age-related biochemical factors specifically in the senile-prone (SAM-P) strain. Among the effects, an increase in protein content in the liver was detectable only in the SAM-P series, and was thought to be responsible for the increased superoxide dismutase activity.[3)] Some of the anti-fatigue or restorative effects believed to result from the traditional clinical application of Rokujo may thus be related in part to activation of protein synthesis in the liver.

To determine whether or not the increase in liver protein content following Rokujo treatment reflected *de novo* translation of protein from messenger ribonucleic acid (RNA), we investigated the effects of Rokujo treatment on the metabolism of protein, RNA and deoxyribonucleic acid (DNA) *in vivo*. We also examined the effect of *in vivo* or *in vitro* Rokujo treatment on the RNA polymerase activities which regulate RNA transcription from nuclear genes.

Materials and Methods

Animals——Male SAM-P/8 mice,[4)] weighing 30—34 g, at 11 or 12 months of age were used. For one treatment, 6 mice were grouped together, maintained at 23±1 °C under an alternating 12 h light/dark cycle, and fed freely on laboratory chow and fresh water.

Chemicals——L-[U-^{14}C]Leucine ([14C]Leu; 10 mCi/mmol), [2-^{14}C]uridine ([^{14}C]uridine; 52 mCi/mmol), [methyl-^{3}H]thymidine ([^{3}H]-thymidine; 5 mCi/mmol) and [^{14}C] cytidine triphosphate ([^{14}C]CTP; 50 mCi/mmol) were purchased from Amersham. α-Amanitin was obtained from Boehringer Mannheim.

Extract from *Cervus nippon* TEMMINCK var. *mantchuricus Swinhoe* (Rokujo)——Unossified pilose antler of *C. nippon* TEMMINCK var. *mantchuricus Swinhoe* (Rokujo) was from the Jilin Province of China. Sliced Rokujo (4240 g) was extracted with 2 l of boiling water three times. The extract solution (6 l) was then concentrated to 2 l *in vacuo* and added to 6 l of 95% ethanol. The insoluble material which appeared in the ethanol mixture was filtered and dried (1137 g), but not used in this study. The filtrate was evaporated to dryness under reduced pressure. The yellow-brown residue obtained (671 g) was stored at 4 °C.

Administration of Extract——Thirty SAM-P were divided into 5 groups and orally administered the ethanol-soluble extract of Rokujo dissolved in distilled water at doses of 0 (2 groups), 200 (1 group) and 300 (2 groups) mg/kg/d, for 20 successive days. The mice were fasted for 18 h prior to the final administration of extract. At 90 min after the final administration, 3 out of the 5 groups, (0, 200, and 300 mg/kg/d) were injected intraperitoneally with a mixture of [^{14}C]Leu (3 μCi/mouse), [^{14}C]uridine (3 μCi/mouse) and [^{3}H]thymidine (1 μCi/mouse), and then at 30 min after the injection, they were sacrificed by decapitation. Radiolabeled liver, brain, heart, kidney, testis and blood were rapidly obtained and immediately subjected to various measurements as described below. The remaining 2 groups (0 and 300 mg/kg/d) were decapitated at 2 h after the final administration, and their livers were excised and used for the preparation of lysed liver nuclei as described below.

Incorporation of [^{14}C]Leu into Serum Proteins——Blood samples from the mice injected with isotopes were allowed to clot at room temperature for 4 h, and were then centrifuged (3000 rpm for 5 min; the conditions in the following precedures were the same unless otherwise stated). $HClO_4$ was added to the sera obtained to give a final concentration of 5%, and precipitates were obtained by centrifugation. The precipitated proteins were washed once with 5% $HClO_4$, rinsed in an acetone–ethyl ether mixture (1 : 1), and air-dried. The dried powders were dissolved in 0.1 N NaOH and an aliquot was used for protein determination. Another aliquot was mixed with toluene scintillation fluid and its radioactivity was counted.

Incorporation of Radioactivity into Tissues——Radiolabeled tissues were minced and homogenized with 10 volumes of distilled water by a Polytron homogenizer. Trichloroacetic acid (TCA) was added to the homogenates to give a concentration of 10% and the mixture was placed on ice. Precipitates were obtained by centrifugation, washed twice with 5% TCA, resuspended in the original volume of 5% TCA and boiled on a hot bath for 20 min. After cooling to room temperture, the suspensions were centrifuged and aliquots of the supernatants (0.25—1 ml) which contained nucleic acids were sampled for determination of the RNA/DNA contents and for measurement of the [^{14}C]uridine/[^{3}H]thymidine radioactivities. The precipitates were washed with 5% TCA, rinsed with ethyl ether, and dissolved in hot 0.1 N NaOH. The protein content of an aliquot of the solubilized solution was measured, and the radioactivity of [^{14}C]Leu in a sample corresponding to 5 mg protein was then counted.

Separation of Liver Nuclear RNA and Measurement of the Radioactivity——A portion of liver tissue (0.5 g) from labeled mice was homogenized with ice-cold 0.25 M sucrose containing 3.3 mM $CaCl_2$. Crude nuclei were obtained by low-speed centrifugation (800 ***g*** for 10 min) and further purified by ultracentrifugation at 105000 ***g*** for 60 min in 2.2 M sucrose.[5] The nuclear fractions were suspended in 20 mM Tris–HCl (pH 7.4) and washed three times with ice-cold 5% $HClO_4$. The acidified precipitates were dried after successive rinses with ethanol, ethanol–ethyl ether (1 : 1) and ethyl ether, and then incubated with 0.5 N KOH at 37 °C for 16 h. The radioactivity and the content of RNA in the solution were measured.

Determination of Protein, RNA and DNA Contents——Lowry *et al.*'s method,[6] Brown's method[7] and Burton's method[8] were employed for determination of the protein, RNA and DNA, respectively, in the samples.

Preparation of Lysed Nuclei from Liver——The preparation of lysed liver nuclei for determination of the RNA polymerase activity was carried out as described previously.[9] In brief, crude nuclear fractions obtained from frozen unlabeled liver were solubilized with 2% sodium deoxycholate by gentle stirring.[10] The clear supernatants after removal of insoluble materials were stored at −80 °C until use.

Assay of RNA Polymerase Activity——The RNA polymerase activity was measured as the incorporation of [^{14}C]CMP-moiety from [^{14}C] CTP into the acid-insoluble fraction by Hiai *et al.*'s method.[9] The reaction mixture in a total volume of 0.25 ml, containing 100 mM Tris–HCl (pH 8.0), 8 mM β-mercaptoethanol, 12 mM $MgCl_2$, 20 mM KCl, 0.25 M ammonium sulfate, 0.8 mM adenosine triphosphate 0.8 mM, guanosine triphosphate, 0.8 mM uridine triphosphate, [^{14}C]CTP and the enzyme preparation, was incubated at 37 °C, as indicated in the legends to the tables. The reaction was terminated by adding 5 ml of chilled 5% $HClO_4$ containing 50 mg of Hyflo Super-cell as the carrier. The acid-insoluble precipitate obtained by centrifugation was dispensed in 1 ml of ice-cold water. 0.15 ml of 1 N KOH was added to the suspension, followed by 10 ml of 5% $HClO_4$. The precipitate obtained by centrifugation was washed twice with 5 ml of 5% $HClO_4$, and then collected on a glass filter. The filter was successively washed with ethanol, ethanol–ether (1 : 1) and ether, and dried. The dried precipitate was solubilized by incubation with hyamine methanol solution at 45 °C for 3 h, mixed with scintillation fluid and counted using a liquid scintillation spectrophotometer.

Data Analysis——The values in Tables I and II are the means ± SEM obtained from 6 individual mice. The significance of differences *vs.* the control group were determined by applying Student's *t* test, and are indicated as

follows: *a*) $p<0.05$, *b*) $p<0.01$, or *c*) $p<0.001$.

Results

Serum Proteins

The data in Table I show that the protein content in the serum of SAM-P was significantly increased by the treatment with Rokujo extract at doses of 200 and 300 mg/kg/d for 20 successive days, being 41% and 49% above the control values. These results are consistent with our earlier finding[2] that 8-d Rokujo treatment increased the protein content in the cytosolic and mitochondrial fractions of SAM-P liver. Table I also reveals that the incorporation of [^{14}C]Leu into the serum protein was increased by the Rokujo treatment in a parallel manner (by 27% at 200 mg/kg and by 62% at 300 mg/kg) with the elevation of the serum protein content.

Protein, RNA and DNA Syntheses in Organs

Table II shows the amounts of radioactivity incorporated during 20 min into the protein, RNA and DNA in several tissues of SAM-P. In the liver and kidney, the incorporation rates of [^{14}C]Leu were physiologically higher than in the other tissues and dose-dependently increased by the Rokujo treatments. The increases in [^{14}C]Leu incorporation were parallel with

TABLE I. Effects of Rokujo Treatment on [^{14}C]Leu-Incorporation into Serum Proteins *in Vivo*

Group (mg/kg/d)	Protein content (mg/ml serum)	Incorporated [^{14}C]Leu (dpm/mg protein/min)
0	74±5.3	2529±338
200	104±6.2[b)]	3204±299
300	110±5.2[c)]	4106±482[a)]

a—*c*) See data analysis in Experimental.

TABLE II. Effects of Rokujo Treatment on the Incorporation of [^{14}C]Leu, [^{14}C]Uridine and [^{3}H]Thymidine into Protein and Nucleic Acid Fractions *in Vivo*

Organ	Group (mg/kg/d)	[^{14}C]Leu (dpm/mg protein)	[^{14}C]Uridine (dpm/mg RNA)	[^{3}H]Thymidine (dpm/mg DNA)
Liver	0	5896±711	5049± 533	102052±15066
	200	8208±727[a)]	6759±1243	107668±16244
	300	11852±368[b)]	8232± 660[b)]	69372±15172
Testis	0	2480±252	6503± 751	26413± 3650
	200	2578±188	5473±1002	26795± 2928
	300	3155±384	6779± 733	27856± 5537
Brain	0	1672±323	1470± 168	5869± 1025
	200	1974±296	1685± 160	7606± 1018
	300	1593±145	1157± 137	5338± 435
Heart	0	1803±362	2042± 191	2710± 369
	200	1889±345	2516± 222	3212± 531
	300	1658±141	2340± 169	2621± 207
Kidney	0	6445±534	2904± 273	50378±10458
	200	6806±724	4526± 601	52170± 6021
	300	7510±305[b)]	4274± 216[c)]	64161± 9102

a—*c*) See data analysis in Experimental.

the increases in [^{14}C]uridine incorporation into RNA in these tissues, but not with the [^{3}H]thymidine incorporation. There were no significant changes in all the values for the testis, brain and heart.

Nuclear RNA Synthesis in the Liver

The accelerated protein and RNA synthesis in the SAM-P liver was further characterized by measuring the incorporation of [^{14}C]uridine into the nuclear-binding RNA fraction. The nuclear RNA synthesis was also stimulated in the Rokujo-treated mice by 24% at 200 mg/kg and by 35% at 300 mg/kg compared to the control values, indicating that the increase in protein synthesis found in the liver may be due to a net increase in the RNA transcription from gene DNA.

RNA Polymerase Activity in the Liver of Rokujo-Treated Mice

The RNA polymerase activity in the solubilized liver nuclear fraction of treated mice was examined using isotope-labeled CTP as a marker of the incorporated ribonucleotide. The assay was carried out in the presence of a high concentration (0.25 M) of ammonium sulfate, in

TABLE III. Increased RNA Polymerase Activity in the Nuclear Fraction of Liver *in Vitro* after Rokujo Treatment

Treatment	[^{14}C]CMP incorporation (dpm)		
	Control	Rokujo, 300 mg/kg/d, 20 d	(% increase)
Experiment 1[a]			
Incubation 10 min	2350[b]	3740	(57)
20 min	2510	4480	(78)
30 min	3050	5610	(84)
Experiment 2[c]			
Protein 100 μg	744	1097	(47)
200 μg	998	1655	(66)
300 μg	1284	3290	(56)
Experiment 3[d]			
Control	1403	2763	(99)
+1 μM α-amanitin	1052	1259	(20)
+22 μM α-amanitin	553	725	(31)

a) Incubated with 200 μg of protein and 0.5 μCi of [^{14}C]CTP for the indicated periods. *b*) Values are the mean of 2 tubes. *c*) Incubated with the indicated amounts of protein and 0.2 μCi of [^{14}C]CTP for 20 min. *d*) Incubated with 100 μg of protein and 0.5 μCi of [^{14}C]CTP for 20 min.

TABLE IV. Lack of Effect of Rokujo Extract *in Vitro* on the RNA Polymerase Activity in Liver Nuclei

Rokujo extract (μg/ml)	[^{14}C]CMP incorporation[a] (dpm)
0	1140[b]
1	1270
10	860
100	1320
1000	1250

a) Incubated with 200 μg of protein prepared from control mice, 0.5 μCi of [^{14}C]CTP, and the indicated concentration of Rokujo extract for 20 min. *b*) Values are the mean of 2 tubes.

which RNA polymerase II activity would be preferentially detected.[11] As shown in Table III, the incorporation of [^{14}C] CMP-moiety into the newly-synthesized RNA was dependent on the incubation period and the amount of enzyme preparation. In the preparation from Rokujo-treated mice, the RNA polymerase activities were higher by 50—80% than those of the control preparation. The increase in [^{14}C] CMP-incorporation was suppressed in the presence of micromolar concentrations of α-amanitin, an RNA polymerase II inhibitor.[12].

Effect of Rokujo on RNA Polymerase Activity *in Vitro*

The data in Table IV demonstrate that the RNA polymerase activity in the solubilized liver nuclear fraction prepared from control mice was unaffected during a 20 min incubation in the presence of Rokujo extract throughout the concentration range from 1 to 1000 μg/ml.

Discussion

A stimulating effect of chronic administration of Rokujo extract on protein synthesis *in vivo* was observed in the present study. The effect was specific to the liver and kidney in the male senescence-accelerated mice employed here as a model of the senile animal. In contrast, no obvious changes were noted in the brain, testis and heart. This could be due to the relatively low incorporation rate of labeled amino acid and nucleoside in these tissues. The observed increases in serum protein content and in the incorporation of labeled amino acid may reflect an overflow of secretory proteins synthesized in the liver.

Part of the molecular basis of the stimulating effect of Rokujo on protein synthesis was also tentatively elucidated. The increase in total RNA synthesis may be related to a net synthesis of messenger RNA molecules in the liver nucleus, since the α-amanitin-sensitive RNA polymerase II activity in the lysed liver nuclear fraction was elevated after subchronic Rokujo treatment. However, it is of interest that the Rokujo extract itself did not affect the RNA polymerase activity in the solubilized nuclear membranes *in vitro*. Two interpretations from this can be suggested. One is that the effect of the Rokujo extract or its included active substances simply needs more time to influence the catalytic site(s). The other possibility (which seems more likely, although there is no clear evidence as yet) is that the Rokujo effect may require certain whole cell functions, such as the induction of enzyme molecules, activation of an enzyme with cytosoluble co-factors, *etc.* Our previous paper[3] showed that the plasma concentration of testosterone was increased by a similar subchronic treatment with Rokujo extract. An increase in the level of anabolic steroid hormones may also stimulate the synthesis of messenger RNAs in liver and kidney nuclei.[13] Whatever the real mechanism may be, active substance which controls the RNA polymerase II activity must be identified at the next step of our research.

Thus, it has been demonstrated that repeated oral administration of Rokujo extract enriches the liver and kidney proteins in SAM-P at 11—12 months of age. However, the effect of Rokujo need to be further characterized by using female SAM or normally aged animals, since some of the pharmacological effects of Rokujo extract were observed specifically in SAM-P and not in SAM-R.[3] Similar stimulating effects on protein and RNA synthesis have been reported in *Panax ginseng*,[9] another well-known traditional restorative medicine. Previous studies have clearly shown that ginsenosides are active substances which can promote RNA synthesis[10] and neurite outgrowth.[14] Since Rokujo originates from a specific animals organ (deer antler), in contrast to other natural medicines of vegetable origin, it is hoped that distinct active compounds may be identified from it.

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References and Notes

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