Dietary Restriction of Single Essential Amino Acids Reduces Plasma Insulin-Like Growth Factor-I (IGF-I) but Does Not Affect Plasma IGF-Binding Protein-1 in Rats

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ABSTRACT The effects of dietary restriction of a single essential amino acid (EAA) on insulin-like growth factor-I (IGF-I) and IGF-binding protein (IGFBP)-1 were investigated in rats. Rats were fed experimental diets containing amino acid (AA) mixtures in which the concentrations of all EAA were at levels recommended by the National Research Council (control), in which a single EAA was restricted to 20% of that of the control diets (Leu$^-$, Lys$^-$, Met$^-$ or Thr$^-$), or in which the diet was devoid of amino acids (AA$^-$). To eliminate the effect of differences in energy intake, rats were fed the mean amount of food as consumed by the AA$^-$ group on the previous day. Growth was significantly retarded in rats fed diets restricted in just one EAA compared with that of rats fed the control diet, and further growth retardation was observed in rats fed the AA$^-$ diet. On the other hand, the plasma IGF-I concentrations in the groups with a single EAA restriction or in the AA$^-$ group were 66% ($P < 0.05$) and 50% ($P < 0.05$) of that of the control group, respectively. The effect of any single EAA restriction was not significantly different from that of total AA deprivation. The plasma IGFBP-1 concentration in the control group did not differ from that of rats fed diets with the single EAA restrictions except for methionine restriction, but it was ~6-fold greater in the AA$^-$ group. Differences in plasma IGFBP-1 concentration under these conditions could be explained by differences in hepatic IGFBP-1 mRNA contents. Based on these results, we conclude that restriction of single EAA does not affect IGFBP-1 synthesis in vivo, although the deprivation of a single EAA has been reported to increase IGFBP-1 production in hepatocyte cultures. Our results also indicated that a single EAA restriction decreased IGF-I production but did not affect IGFBP-1 production. The present study suggests that not only plasma IGF-I, but also IGFBP-1, affects the magnitude of growth retardation in vivo. J. Nutr. 130: 2910–2914, 2000.

KEY WORDS: • *essential amino acid* • *insulin-like growth factor–binding protein-1* • *mRNA* • *rats*

Insulin-like growth factor-I (IGF-I)[4] is a hormone that plays an important role in the regulation of whole body protein synthesis (Clemmons 1992). This hormone is complexed with several specific IGF binding proteins (IGFBP) in plasma. At least six distinct IGFBP (IGFBP-1 through -6) have been characterized, and these IGFBP have been suggested to modify the biologic activity of IGF-I (Jones and Clemmons 1995, Rajaram et al. 1997, Zapf 1995).

Previous studies showed that plasma concentration and liver mRNA content of IGFBP-1 are regulated by nutritional factors such as fasting, restriction of energy intake and protein intake (Busby et al. 1988, Ooi et al. 1990, Murphy et al. 1990, Straus et al. 1993, Lemozy et al. 1994, Takenaka et al. 1996). Hepatic IGFBP-1 mRNA content increases during protein deprivation, and this is attributed to the higher transcription rate of its gene (Miura et al. 1993, Takenaka et al. 1996, Tseng et al. 1992). Although rats fed a protein-free diet had greater mRNA content of IGFBP-1 [~11-fold compared with those fed a control (Con) diet], rats fed a diet containing 12% gluten, which was deficient in Lys and Thr, had same IGFBP-1 mRNA level as the Con group (Takenaka et al. 1993).

By contrast, the effect of EAA starvation on IGFBP-1 synthesis in vitro has been reported using hepatoma cell lines and primary cultured rat hepatocytes (Arany et al. 1993, Jousse et al. 1998, Pao et al. 1993, Straus et al. 1993, Thissen et al. 1994), and it was revealed that the deprivation of a single essential amino acid (EAA) greatly increased IGFBP-1 gene expression (Jousse et al. 1998, Straus et al. 1993). There is a discrepancy between the conclusions from in vivo and in vitro systems.

In the present study, we performed animal experiments to investigate the effect of the restriction of a single EAA on plasma concentrations of IGF-I and IGFBP-1 and on liver content of IGFBP-1 mRNA, as well as the effect on animal growth, and showed that the response of IGFBP-1 synthesis in vivo may be different from that in cultured liver cells.

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[4] Abbreviations used: AA, amino acid; Con, control; EAA, essential amino acid; IGFBP-1, insulin-like growth factor–binding protein-1; IGF-I, insulin-like growth factor-I; PEG, polyethylene glycol; RIA, radioimmunoassay.

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MATERIALS AND METHODS

Drugs. Recombinant human IGF-I and anti-human IGF-I polyclonal antibody were kindly donated by Fujisawa Pharmaceutical (Osaka, Japan). Anti-rabbit γ-globulin polyclonal antibody and rabbit serum were purchased from Daiichi Radioisotope Labs (Chiba, Japan). Labeled sodium iodide (Na-^{125}I) and 5′-[α-^{32}P]dCTP were obtained from Amersham Life Science (Buckinghamshire, U.K.). Other chemicals were of reagent grade and available commercially. Rat IGFBP-1 was purified from the cell culture medium of H4IIE rat hepatoma cells. IGFBP-1 antiserum was obtained by immunization of adult female rabbit with the purified rat IGFBP-1 protein.

Diets. The experimental diets contained different nitrogen sources. The Con diet contained the complete amino acid (AA) mixture recommended by the National Research Council (1978). The diets deficient in one EAA (Leu$^-$, Lys$^-$, Met$^-$ or Thr$^-$) contained an AA mixture in which the single EAA was reduced to 20% of the level of the Con diet; the AA-free diet (AA$^-$) contained no AA. The compositions of the diets are shown in **Table 1**. To eliminate the effect of reduced food intake in rats fed the AA-deficient diets, the food intake of rats fed diets restricted in a single EAA and of rats fed the Con diet (Con/paired) was restricted to that of rats fed the AA$^-$ diet. One group was fed the Con diet ad libitum (Con/ad libitum).

Rats. Male rats of the Wistar strain with a mean body weight of 120 g (6 wk old) were purchased from Charles River Japan (Kanagawa, Japan). The rats were housed under constant conditions of temperature (22 ± 1°C) and light (on at 0800 h, off at 2000 h), with free access to water. After the rats were fed a Con diet from 1000 to 1800 h for 4 d, they were divided into seven experimental groups (n = 5) and were fed the experimental diets from 1000 to 1800 h for 7 d. On d 8 at 1130 h, the rats were killed under pentobarbital anesthesia (50 mg of pentobarbital/kg of body weight). The livers were removed, quickly frozen in liquid nitrogen and kept at −70°C until the time of analysis. Blood was collected in tubes containing 10 mg EDTA on ice. Samples were centrifuged, and the plasma was kept at −20°C until assayed. Body weight changes, food intake and plasma IGF-I concentrations of the rats are shown in **Table 2**. The procedures for animal research were approved by the Committee on Laboratory Animal Care, Graduate School of Agricultural and Life Sciences, The University of Tokyo.

Radioimmunoassay (RIA) of plasma IGF-I. Acid gel chromatography was used as described previously (Crawford et al. 1992, Uchijima et al. 1995) to eliminate the effect of IGFBP. RIA for IGF-I was performed as described previously (Higashi et al. 1998).

RIA of plasma IGFBP-1. An excess amount of IGF-I was added to the RIA reaction to eliminate the endogenous IGF-I in plasma, because binding of ^{125}I–IGFBP-1 to the antibody was affected by IGF-I (Lewitt et al. 1992). Two hundred μL of plasma or the standard solution (3.3×10^{-13} to 6.6×10^{-11} mol IGFBP-1/L) was added to 100 mL of 6.5×10^{-3} g IGF-I/L, 10,000 dpm of ^{125}I–IGFBP-1 and 100 mL of 1000× diluted anti–IGFBP-1 antiserum and reacted in RIA buffer (2.5 g bovine serum albumin, 0.1 g Triton X-100, 0.2 g sodium azide and 0.1 mol phosphate buffer, pH 6.5, per L) for 18 h at 4°C. Then, 10 mL of 10× diluted anti-rabbit immunoglobulin antiserum and 100 mL of 40× diluted normal rabbit serum were added and incubation was conducted for 30 min at 4°C. After the 30-min incubation with 1 mL of polyethylene glycol (PEG) solution [60 g PEG 6000 and 1.5 g NaCl per L], the samples were centrifuged at 1000 × g for 20 min, and the radioactivity of the precipitant was measured with a γ-counter.

Rat IGFBP-1 was purified from the cell culture medium of H4IIE rat hepatoma cells by ammonium sulfate precipitation, phenyl-Toyopearl and DEAE-Toyopearl chromatography (Tosoh, Tokyo, Japan) and C4 reverse phase HPLC. Purified rat IGFBP-1 was used as the standard and for the preparation of ^{125}I-IGFBP-1. IGFBP-1 was labeled using Na-^{125}I and chloramine-T (as described by Iwaki, Takahashi and Noguchi, unpublished). Anti–IGFBP-1 antiserum was prepared by immunization of female New Zealand White rabbits with the purified IGFBP-1. Purified rat IGFBP-1 (100 mg) was dissolved in 1 mL of phosphate-buffered saline, homogenized with an equal volume of Freund's complete adjuvant and injected subcutaneously into female rabbit. At 2 and 5 wk after the first injection, a second and a third injection were carried out using Freund's incomplete adjuvant. Blood was collected 2 wk after the third injection, and this serum was used as an antiserum for rat IGFBP-1 (as described in Iwaki, Takahashi and Noguchi, unpublished). Cross-reactivity of the antiserum against other IGFBP and the parallel displacement curve of IGFBP-1 standard and serum was evaluated (Iwaki et al. unpublished). Our replacement curve confirmed the results reported by Lewitt et al. (1992).

Preparation of total RNA from rat liver followed by Northern blot analysis of IGFBP-1. Total RNA was prepared from liver according to the modified method of acid guanidinium thiocyanate-

TABLE 1

Composition of the diets

	Con[1]	Leu$^-$	Lys$^-$	Met$^-$	Thr$^-$	AA$^-$ [2]
	g/100 g					
Cornstarch	71.41	71.41	71.41	71.41	71.41	85.3
Cellulose	5	5	5	5	5	5
Soybean oil	5	5	5	5	5	5
Mineral mixture[3]	3.5	3.5	3.5	3.5	3.5	3.5
Vitamin mixture[3]	1	1	1	1	1	1
Choline chloride	0.2	0.2	0.2	0.2	0.2	0.2
AA mixture						
Arg	0.6	0.6	0.6	0.6	0.6	0
Asn	0.4	0.4	0.4	0.4	0.4	0
Glu	5	5.6	5.56	5.48	5.4	0
His	0.3	0.3	0.3	0.3	0.3	0
Ile	0.5	0.5	0.5	0.5	0.5	0
Leu	0.75	0.15	0.75	0.75	0.75	0
Lys	0.7	0.7	0.14	0.7	0.7	0
Met	0.6	0.6	0.6	0.12	0.6	0
Phe	0.8	0.8	0.8	0.8	0.8	0
Pro	0.4	0.4	0.4	0.4	0.4	0
Thr	0.5	0.5	0.5	0.5	0.1	0
Trp	0.15	0.15	0.15	0.15	0.15	0
Val	0.6	0.6	0.6	0.6	0.6	0
Gly	0.8	0.8	0.8	0.8	0.8	0
Ala	1	1	1	1	1	0
Ser	0.79	0.79	0.79	0.79	0.79	0

[1] Con, control diet containing the complete amino acid (AA) mixture.
[2] AA$^-$, AA-free diet containing no AA.
[3] The mineral and vitamin mixtures (obtained from Oriental Yeast Co., Tokyo, Japan) were prepared according to the American Institute of Nutrition (1997).

TABLE 2

Body weight, food intake and plasma insulin-like growth factor-I (IGF-I) concentration of rats fed a control diet (Con) or a single essential amino acid–deficient diets (Leu$^-$, Lys$^-$, Met$^-$, and Thr$^-$) or amino acid–free diet (AA$^-$)

	Body weight change	Food intake	Plasma IGF-I
	g/7 d		*μg/L*
Con/ad libitum	37.2 ± 2.3[a]	154.6 ± 4.2[a]	426.8 ± 39.7[a]
Con/paired	7.0 ± 2.5[b]	91.0 ± 3.5[b]	389.0 ± 27.1[a]
Leu$^-$	−16.6 ± 2.0[d]	82.2 ± 3.7[b]	256.3 ± 46.5[b]
Lys$^-$	−6.6 ± 1.0[c]	90.2 ± 3.2[b]	278.8 ± 31.5[b]
Met$^-$	−14.2 ± 2.1[d]	84.0 ± 5.2[b]	247.6 ± 45.9[b]
Thr$^-$	−13.4 ± 1.3[d]	89.2 ± 3.3[b]	236.8 ± 12.4[b]
AA$^-$	−26.8 ± 1.3[e]	91.0 ± 3.5[b]	195.8 ± 14.0[b]

[1] Values are means ± SEM, n = 5.
[2] Values with different letters in each column differ, $P < 0.05$.

phenol-chloroform extraction (Chomczynski and Sacchi 1987, Puissant and Houdebine 1990), as described previously (Miura et al. 1992).

Northern blot analysis was performed as described previously (Miura et al. 1992). The cDNAs of rat IGFBP-1 were used as probes, as described previously (Takenaka et al. 1991). The intensity of each band obtained from a Northern blot was quantified with the Fujix BAS 2000 or 3000 system (Fuji Film, Tokyo, Japan).

Statistics. Data were statistically analyzed by Duncan's multiple range test (Duncan 1955) after one-way ANOVA. Liver IGFBP-1 mRNA content and plasma IGFBP-1 concentrations were compared using the Mann-Whitney test for nonparametric data. Differences were considered significant at $P < 0.05$.

RESULTS

Food intake and body weight change. Food intake was reduced to 58.9% ($P < 0.01$) and body weight gain was reduced to 18.8% ($P < 0.01$) in the Con/paired group compared with the Con/ad libitum group (Table 2). The body weights of all groups fed EAA-restricted diets (Leu$^-$, Lys$^-$, Met$^-$ or Thr$^-$) or the diet devoid of AA (AA$^-$) were lower than that of the groups fed the Con diet, and the loss of body weight was significantly greater in the AA$^-$ group than in groups fed diets with a single EAA restriction (Table 2).

Plasma IGF-I concentration. The reduced food intake did not affect plasma IGF-I concentration. Plasma IGF-I concentration was significantly lower than that of the Con group in the groups fed the EAA-restricted diets and the AA$^-$ diet ($P < 0.05$). These groups did not differ from each another (Table 2).

Plasma IGFBP-1 concentration and liver IGFBP-1 mRNA content. The hepatic content of IGFBP-1 mRNA did not differ significantly in the rats fed diets with a single EAA restriction compared with the two Con groups, whereas the level of the AA$^-$ group was ~25-fold greater than that of the Con groups (**Fig. 1***A*). The plasma IGFBP-1 concentration was not significantly affected by single EAA restrictions, except for that of Met ($P < 0.01$); however, it was ~6-fold greater in the AA-deprived rats (AA$^-$) than in the Con rats (Fig. 1*B*).

DISCUSSION

In a series of previous studies, we demonstrated that rats fed protein-free diets had a lower plasma IGF-I concentration (Takahashi et al. 1990) and a higher liver IGFBP-1 mRNA content and plasma IGFBP-1 concentration (Takenaka et al. 1993, Umezawa et al. 1991). We have also shown in vivo that feeding rats a 12% gluten diet, which was marginally deficient in lysine and threonine, did not affect the hepatic IGFBP-1 mRNA content or the transcription rate of the IGFBP-1 gene compared with that of the rats fed a 12% casein diet (Miura et al. 1993, Takenaka et al. 1993). The plasma concentration of IGFBP-1 determined by specific RIA did not differ in the rats fed the gluten diet and was ~5- to 10-fold greater in rats fed protein-free diets (Iwaki, Takahashi and Noguchi, unpublished data). These studies indicate that deficiencies of lysine and threonine did not affect IGFBP-1 synthesis in vivo.

On the other hand, in previous reports using the rat hepatoma cell line H4IIE, IGFBP-1 mRNA was higher after deprivation of a single EAA from the experimental medium, which was explained by the greater transcription of the IGFBP-1 gene (Straus et al. 1993). A recent study by Jousse et al. (1998) demonstrated that when HEP G2 cells are deprived of each EAA from the medium, the IGFBP-1 mRNA content was higher than in the cells with control medium. These studies suggested that the effect of EAA deficiency on hepatic IGFBP-1 synthesis may be different in cultured liver cells and rat liver in vivo.

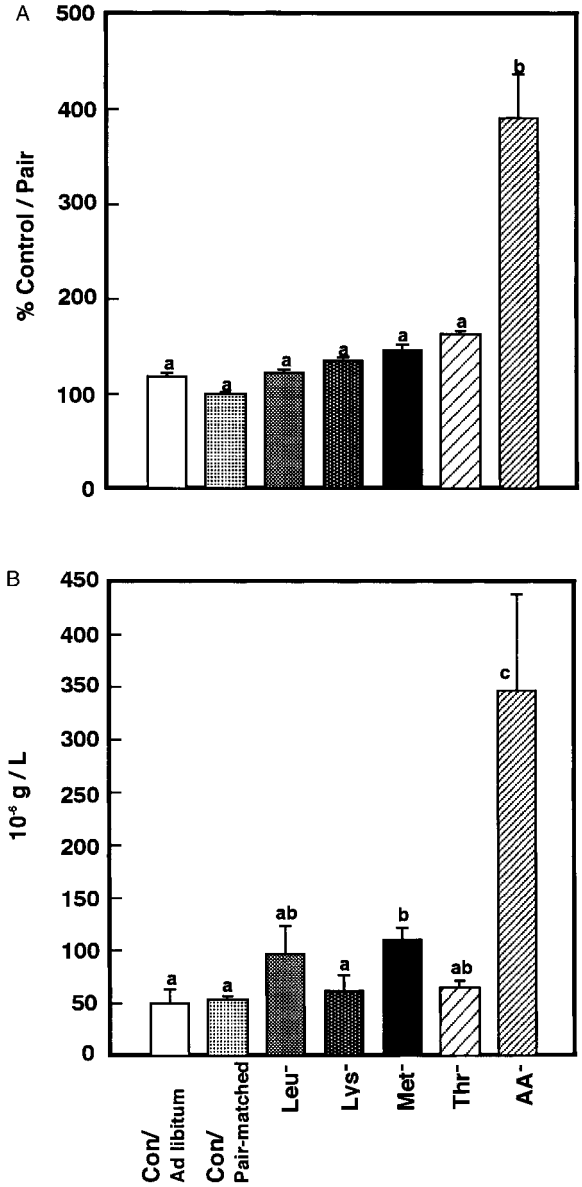

FIGURE 1 Effect of restriction in a single amino acid (Leu$^-$, Lys$^-$, Met$^-$ and Thr$^-$) or amino acid depletion (AA$^-$) on hepatic insulin-like growth factor-binding protein-1 (IGFBP-1) mRNA content (*A*) and plasma IGFBP-1 concentration (*B*) in rats fed experimental diets. (*A*) A densitometric analysis of the autoradiogram of Northern blot analysis. Total liver RNA samples (20 μg/lane) were used. The results are shown as the relative intensity of the bands, taking the mean of the Con/paired group as 100. Values are means + SEM, $n = 5$; those with different letters differ, $P < 0.05$.

To investigate the effect of single AA deficiency on hepatic IGFBP-1 synthesis in vivo, in the present study we prepared four EAA-restricted diets (Leu$^-$, Lys$^-$, Met$^-$ or Thr$^-$) that contained AA mixtures with each EAA restricted to 20% of the level of the complete AA mixture recommended by the National Research Council. In a series of studies, we used casein, gluten and soy proteins, which are marginally deficient in methionine, lysine and threonine, and methionine, respectively, as nitrogen sources (Miura et al. 1992). Therefore, we selected these AA to be restricted in the present study. Leucine was selected because its regulatory effect on IGFBP-1 mRNA content has been well

studied in hepatocyte cultures (Jousse et al. 1998, Straus et al. 1993). We restricted the EAA to only 20% to avoid energy malnutrition, which usually occurs in rats fed a diet devoid of each EAA. As a result, we found that feeding rats each of these diets restricted in one of the four AA did not affect the hepatic IGFBP-1 mRNA level or plasma IGFBP-1 concentration except Met $^{-}$. Because deprivation of each of these four AA had an effect to elevate IGFBP-1 mRNA content in cell culture system (Jousse et al. 1998), we concluded that the effect of a single EAA deficiency on IGFBP-1 synthesis differs between in vivo and cell culture systems. We could not rule out the possibility that the restriction condition in the present study may not be sufficient to induce IGFBP-1 synthesis. However, the effects of dietary AA deficiency was sufficient to affect IGF-I system in the present study because plasma IGF-I concentration of rats fed a diet with a single EAA restriction was comparable to that of total AA deprivation.

The intracellular pool of each AA in organs is thought to be affected by AA concentrations in plasma and the supply of AA degraded from endogenous proteins. Intracellular levels of each AA are one of the most likely candidates to modulate IGFBP-1 gene transcription (Takenaka et al. 2000). Accordingly, differences in intracellular pools of each EAA between liver in vivo and cultured hepatocytes may in part explain the difference between the effect of AA restriction on IGFBP-1 mRNA content in vivo and in hepatocyte culture systems.

Rats fed protein-free diets had a higher IGFBP-1 mRNA content, as well as transcription rate of the IGFBP-1 gene (Miura et al. 1993). In addition, we found that the 5′ upstream region of IGFBP-1 plays some role in increasing IGFBP-1 mRNA in response to AA deprivation in human hepatocyte cultures (Takenaka et al. 2000). These results suggest that transcription of the IGFBP-1 gene was greater in dietary AA-deprived condition and imply that transcription regulation in response to single EAA deficiency is different between liver in vivo and hepatocytes cultures.

In contrast to IGFBP-1, rats fed diets with a single EAA restriction had a dramatically lower plasma IGF-I concentration to the level of that of rats totally deprived of AA (Table 2). We have shown that the AA deficiency lowered hepatic IGF-I mRNA (Miura et al. 1992). Because the AA deficiency did not significantly affect the transcription rate of the IGF-I gene in liver in vivo, we concluded that AA deficiency lowers the stability of IGF-I mRNA (Miura et al. 1993). Because plasma IGF-I concentrations of single EAA-restricted rats were comparable to that the rats fed protein-free diets (Table 2), the restriction of a single EAA could be monitored by an unknown system and is sufficient to provide signals to destabilize IGF-I mRNA. In the case of IGF-I regulation, similar results were obtained in liver in vivo and in cell culture systems. All of these results indicate that the synthesis of IGF-I is suppressed posttranscriptionally, but transcription of IGFBP-1 gene is greater under dietary AA-deprived conditions. The different molecular mechanisms of the transmission of signals of AA deficiency to the machinery for IGF-I or IGFBP-1 synthesis may explain the different effects of the single EAA restriction on plasma concentrations of IGF-I and IGFBP-1 in the present study.

We reported that the levels of plasma IGF-I concentrations correlated well with growth rate when rats were fed diets with different protein contents (Takahashi et al. 1990). However, we show in the present study that in rats fed diets without AA, body weight loss was more severe than in a single EAA-restricted groups, despite the fact that there was no significant difference in plasma IGF-I concentration. The different responses of IGFBP-1 synthesis to restriction of a single EAA and total AA deprivation may explain this discrepancy between the body weight change and the plasma IGF-I concentration. IGFBP-1 has been shown to transport IGF-I from the vascular space or to suppress activity of serum IGF-I, resulting in a lower plasma concentration of total IGF-I or free IGF-I. We presume that with a single EAA restriction, the elevated plasma IGFBP-1 concentration suppresses the IGF-I activity. Plasma concentrations of IGFBP-3 and -4 did not differ between single AA-restricted groups and the total AA-deprived group (data not shown). On the other hand, results of immunoblotting showed that IGFBP-2 in plasma was higher in rats fed a protein-free diet, but this increase was much smaller than that of IGFBP-1 (Takenaka et al. 1996). Taken together, our results suggest that both low plasma IGF-I concentration and high plasma IGFBP-1 concentration may cause the most severe weight loss in rats fed AA-free diet.

In summary, we conclude that the effect of a single EAA deficiency on IGFBP-1 synthesis was different in rat liver in vivo from that in cell culture systems. Our results also indicated that a single EAA restriction suppressed IGF-I production but did not affect IGFBP-1 production. The present results suggest that not only plasma IGF-I but also IGFBP-1 plays an important roles in determining the magnitude of growth retardation in vivo.

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