Effect of Age and Dietary Restriction on the Expression of α2u-Globulin*

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The effect of aging on the expression of α2u-globulin was studied in liver tissue from 6–30-month-old male Fischer F344 rats. The synthesis of α2u-globulin by suspensions of isolated hepatocytes decreased 90% between 6 and 22 months of age. The levels of α2u-globulin mRNA and the transcription of α2u-globulin genes by isolated liver nuclei decreased 80±5% between 5 and 24 months of age. Because α2u-globulin has been suggested to be a "senescence marker protein," the expression of α2u-globulin was measured in rats fed a diet restricted in calories. This dietary restriction procedure has been shown to increase significantly the longevity of rodents. The expression of α2u-globulin was compared in liver tissue from 18-month-old rats fed ad libitum and a restricted diet (40% restriction of total calories). The synthesis, mRNA levels, and transcription of α2u-globulin were 1.8–3-fold higher for liver tissue from restricted rats compared to liver tissue from rats fed ad libitum. Therefore, dietary restriction alters the age-related change in the expression of α2u-globulin. Our results demonstrate that the changes in α2u-globulin expression that arise during aging or dietary restriction are regulated at the level of transcription.

α2u-Globulin is a protein (Mr = 18,700) that is synthesized by hepatic parenchymal cells, secreted into the blood stream, and excreted in the urine of adult male rats (1, 2). The synthesis of α2u-globulin is under complex multihormonal control, e.g. androgens (3, 4), glucocorticoids (5), thyroid hormone (6, 7), insulin (8), and growth hormone (9, 10) are stimulatory, whereas estrogens are inhibitory (11). In addition, the synthesis of α2u-globulin changes as a function of malignant transformation, development, and senescence. For example, α2u-globulin synthesis increases during development reaching peak values at 9–12 weeks of age (4, 12, 13), and ceases when hepatocytes undergo malignant transformation (13) or senescence (4, 14).

During the past two decades, a considerable amount of research has shown that gene expression in the liver of rodents declines with increasing age. e.g. the rates of protein synthesis by isolated hepatocytes (15, 16) and the transcriptional activity of liver nuclei (17) decreases between 40 and 60% with increasing age. However, very little is known about the effect of aging on the expression of specific genes. Roy et al. (14) proposed that α2u-globulin was a "senescence marker protein" in male rats because the levels and synthesis of α2u-globulin decrease dramatically with increasing age. Preliminary studies by Roy's laboratory (14, 18) suggest that the decrease in α2u-globulin synthesis with age is due to changes in mRNA levels, which indicates that aging is acting at a pretranslation site in the regulation of α2u-globulin expression. The age-related changes in α2u-globulin mRNA levels might be a consequence of an alteration in either gene transcription, altered processing of the primary transcript, or changes in mRNA turnover. In this study, we compared the synthesis mRNA levels, and the relative transcriptional rate of α2u-globulin by the hepatocytes of male rats during senescence and after dietary restriction. Dietary restriction increases the survival of laboratory rodents and appears to retard aging and senescence (19, 20). Our experiments demonstrate that the age-related decrease in α2u-globulin expression occurs through the regulation of transcription of the α2u-globulin genes and that dietary restriction results in an increase in the expression of α2u-globulin.

MATERIALS AND METHODS

Animals—Male Fischer F344 rats were used in this study. The rats used to study the effect of aging on the expression of α2u-globulin were obtained from the animal colony maintained by the National Institute on Aging. These rats are fed a commercial lab chow ad libitum and have a mean and maximum survival of 27 and 35 months, respectively (21). The male Fischer F344 rats used to study the effect of dietary restriction on α2u-globulin expression were maintained on two diets as described by Birchenall-Sparks et al. (22). Briefly, rats were fed a semi-synthetic, casein diet, which was obtained from Teklad Test Diets (Madison, WI). At 6 weeks of age the rats were placed on two dietary regimens; the rats were either fed ad libitum or were fed 60% of the amount of diet (restricted diet) consumed by the rats fed ad libitum. Yu et al. (19) have shown that the mean and maximum survival of male Fischer F344 rats are increased approximately 40% using a dietary restriction procedure similar to that described by Birchenall-Sparks et al. (22).

Isolation of Hepatocytes and Protein Synthesis—Hepatocytes were obtained by the in situ collagenase perfusion of liver as described by Engelmann et al. (23). Hepatocyte viability, as determined by trypan blue exclusion, was consistently greater than 90%, and the number of hepatocytes was determined using a hemocytometer. Protein synthesis was determined using a system similar to that described by Ricca et al. (24). Hepatocytes (3 million/ml) were suspended in Eagle's minimal essential medium (Sigma) and incubated for 15 min at 37 °C under an atmosphere of oxygen/carbon dioxide (95:5). Either 35S-methionine (1000 Ci/mmol) or [3H]-valine (10 Ci/mmol) was added to the suspension at a concentration of 20 or 50 μCi/ml, respectively. The hepatocytes were incubated at 37 °C under an atmosphere of oxygen/carbon dioxide (95:5) for 20 min unless otherwise indicated. The reaction was terminated by placing the cells on ice and pelleting

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The cells by low-speed centrifugation. The incorporation of radioactive activity into each extracellular protein (supernatant) or intracellular protein (cell pellet) by hepatocytes is shown in Fig. 1A. There is a short lag period of 30-45 min before radioactivity appears in extracellular proteins; this corresponds to the time to synthesize and transport extracellular proteins (25). The synthesis of a2,-globulin by hepatocytes was determined by taking the extracellular supernatant from hepatocytes incubated with either [35S]methionine or [3H]valine and separating the proteins by SDS-polyacrylamide gel electrophoresis (26). In a few cases, the isoelectric variants of a2,-globulin were analyzed by two-dimensional gel electrophoresis and the radioactive activity migrating as albumin or a2,-globulin was determined.

FIG. 1. Time course for the synthesis of proteins by suspensions of hepatocytes. Hepatocytes were isolated from a 5-month-old rat. A shows the incorporation of [3H]valine into extracellular (O) and extracellular (O) proteins. B shows the incorporation of [3H]valine into albumin (square) and a2,-globulin (A) in the extracellular medium. Proteins in the extracellular medium were subjected to SDS-polyacrylamide gel electrophoresis, and the radioactive activity migrating as albumin or a2,-globulin was determined.

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The gels were either analyzed by fluorography (28) or the amount of radioactivity incorporated into a2,-globulin was determined with a liquid scintillation counter and corrected for background by measuring the activity incorporated into albumin or a2,-globulin in samples of medium taken immediately after the addition of [3H]valine. Fig. 1B shows the incorporation of [3H]valine into albumin and a2,-globulin in hepatocytes in suspension. The appearance or radioactivity in a2,-globulin is linear between 45 and 120 min. Therefore, when measuring a2,-globulin synthesis by hepatocytes, the amount of radioactivity incorporated into a2,-globulin was determined after incubating hepatocytes with the radioactive amino acid for 120 min.

The autoradiogram in Fig. 2 shows that a2,-globulin can be resolved by SDS-gel electrophoresis from other extracellular proteins synthesized by hepatocytes. It is also evident from Fig. 2 that the incorporation of [35S]methionine into a2,-globulin decreases markedly with age. The synthesis of a2,-globulin was quantified by measuring the amount of [3H]valine incorporated into a2,-globulin by suspensions of hepatocytes isolated from 5-7.5 months of age.

FIG. 2. Effect of age on the synthesis of a2,-globulin. Hepatocytes were isolated from 5-, 13-, 22-, and 30-month-old rats and incubated with [35S]methionine. The culture supernatants from four animals for each age were pooled. The proteins in the supernatants were separated by SDS-polyacrylamide gel electrophoresis, and the gel was analyzed by fluorography. The migration of protein standards of known molecular weights is shown, and the arrow indicates the migration of a2,-globulin.
Changes in Levels and Transcription of α2-Globulin mRNA—The levels of α2-globulin mRNA in liver RNA isolated from 5-29-month-old rats was measured to determine whether the age-related decline in α2-globulin synthesis arose from pretranslational changes. Fig. 3 shows that the cDNA probe to α2-globulin hybridized to a 1.2-1.3-kilobase RNA species, which is identical to the size of α2-globulin mRNA reported in rat liver by Unterman et al. (37). Although the hybridization of the cDNA probe to the total RNA decreased markedly with age, no change in the size of the α2-globulin mRNA was observed. In addition, there was no evidence for an accumulation of α2-globulin mRNA precursors or a degradation of α2-globulin mRNA with increasing age (Northern blot in Fig. 3). Identical results were obtained with poly(A)⁺ RNA isolated from the livers of 5-29-month-old rats (data not shown). Therefore, the decline in α2-globulin mRNA levels is not correlated with a loss in the poly(A) sequence at the 3′-end of the mRNA.

The autoradiograph of the dot blot in Fig. 3 shows that α2-globulin mRNA was essentially undetectable in the livers of 29-month-old rats. It also should be noted that the levels of α2-globulin mRNA in 24-month-old rats were quite variable; in some rats it was relatively high, whereas in others it was undetectable. Table I gives the relative levels of α2-globulin mRNA in total RNA isolated from rats of various ages. The level of α2-globulin mRNA in hepatic RNA decreased over 90% between 5 and 22 months of age. No change in α2-globulin synthesis was observed after 22 months of age.

Table I: Age-related changes in the expression of α2-globulin

<table>
<thead>
<tr>
<th>Age</th>
<th>Synthesis*</th>
<th>mRNA levels*</th>
<th>Transcription*</th>
</tr>
</thead>
<tbody>
<tr>
<td>months</td>
<td>dpm/10⁶ cells</td>
<td>area/8 μg RNA</td>
<td>ppm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-6</td>
<td>25.2 ± 1.8</td>
<td>2.07 ± 0.06</td>
<td>1420</td>
</tr>
<tr>
<td>12-14</td>
<td>17.8 ± 1.4</td>
<td>1.46 ± 0.08</td>
<td>1066</td>
</tr>
<tr>
<td>22-24</td>
<td>3.3 ± 0.4</td>
<td>0.29 ± 0.13</td>
<td>298</td>
</tr>
<tr>
<td>29-30</td>
<td>3.3 ± 0.4</td>
<td>0.06 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

*Values represent the mean ± S.E. of determinations from four animals for each age. Values for synthesis and mRNA levels were obtained from two different groups of animals. The decrease in the synthesis of α2-globulin and a2-globulin mRNA levels between 5 and 14 months and between 14 and 24 months was significant at the p < 0.001 level.

The incorporation of [3H]valine into α2-globulin was significantly greater for hepatocytes isolated from the restricted rats. Roy et al. (14) reported that five major isoelectric variants of α2-globulin could be detected by two-dimensional gel electrophoresis, and the synthesis of all isoelectric variants decreased with age in α2-globulin mRNA levels was common to other RNA species, the levels of 2S S rRNA and the mRNAs for albumin and α1-antitrypsin were determined (data not shown). The relative levels of 28S RNA did not change significantly with age, whereas the relative levels of both albumin and α1-antitrypsin increased significantly.

The decline in α2-globulin mRNA levels could arise from age-related changes in either the transcription of α2-globulin genes, the processing of initial α2-globulin transcript, or the turnover of α2-globulin mRNA. To elucidate the mechanism responsible for the age-related decline in α2-globulin mRNA levels, the transcription of α2-globulin mRNA was measured using isolated nuclei. Table I shows that the synthesis of RNA transcripts that hybridize to the α2-globulin cDNA probe decreased 80% between 5 and 23 months of age.

Effect of Dietary Restriction on Expression of α2-Globulin—In this series of experiments, the expression of α2-globulin in two groups of 18-month-old rats was measured: rats fed ad libitum and rats that were placed on a restricted diet (60% of ad libitum) at 6 weeks of age. It should be noted that at 18 months of age a significant decline in α2-globulin expression was observed (Table I). The incorporation of [3H]valine into α2-globulin was measured (Fig. 4, panel 2, and Table II). The incorporation of [3H]valine into α2-globulin was significantly greater for hepatocytes isolated from the restricted rats. Roy et al. (14) reported that five major isoelectric variants of α2-globulin could be detected by two-dimensional gel electrophoresis, and the synthesis of all isoelectric variants decreased with age.

FIG. 3. Assay of α2-globulin mRNA levels in the livers of rats of various ages. The hybridization of the ³²P-labeled α2-globulin cDNA probe to a Northern blot is shown on the left. Five μg of RNA, which was pooled from four rats for each age, was applied to the gel, and the migration of rRNA standards is shown. The hybridization of the ³²P-labeled probe to RNA blots (8 μg of RNA) from four individual rats (A, B, C, and D) for each age and from a pooled sample (P) is shown on the right.
Regulation of $\alpha_{2u}$-Globulin Expression

**Table II**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Protein synthesis dpm/$10^6$ cells</th>
<th>mRNA levels area/8 $\mu$g RNA ppm</th>
<th>Transcription</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad libitum</td>
<td>7.5 ± 2.4</td>
<td>0.37 ± 0.08</td>
<td>554</td>
</tr>
<tr>
<td>Restricted</td>
<td>22.0 ± 4.8$^a$</td>
<td>0.66 ± 0.12$^a$</td>
<td>1306</td>
</tr>
</tbody>
</table>

$^a$ Value is significantly greater than ad libitum value ($p < 0.05$).

**Fig. 5.** Effect of dietary restriction on the synthesis of isoelectric variants of $\alpha_{2u}$-globulin. Hepatocytes isolated from 5- and 22-month-old rats fed ad libitum and 22-month-old rats fed a restricted diet were incubated with [35S]methionine. The culture supernatants from four animals for each group were pooled, equal amounts of radioactively labeled proteins from the supernatants were subjected to two-dimensional gel electrophoresis, and the regions of the gels containing the isoelectric variants of $\alpha_{2u}$-globulin were shown for the 22-month-old rats. The fluorographs were overlaid to enhance the detection of the isoelectric variants of $\alpha_{2u}$-globulin. The migration of protein standards of known molecular weight and pl values is shown. The isoelectric variants of $\alpha_{2u}$-globulin, which are shown by the arrows, were identified according to the description given by Roy et al. (14). It should be noted that the migration of the proteins at the acidic end of the gel for the restricted rats was slightly different from the gels for rats fed ad libitum.

Increasing age. Therefore, we were interested in determining whether the increase in $\alpha_{2u}$-globulin synthesis by restricted rats was due to an increase in the synthesis of all isoelectric variants or whether the synthesis of only a few variants was affected by dietary restriction. The data in Fig. 5, which confirm the study by Roy et al. (14), show that all five isoelectric variants of $\alpha_{2u}$-globulin decreased with age. In addition, Fig. 5 shows that the increase in $\alpha_{2u}$-globulin synthesis in restricted rats was due to an increase in the synthesis of all five isoelectric variants.

The effect of dietary restriction on the expression of $\alpha_{2u}$-globulin was also studied at the transcriptional level. Fig. 4 (panel 3) shows that dietary restriction had no effect on the size of the $\alpha_{2u}$-globulin mRNA; however, the levels of $\alpha_{2u}$-globulin mRNA in the livers of restricted rats were 1.8-fold greater than those in the liver of rats fed ad libitum (Table II). Table II also shows that the synthesis of RNA transcripts that hybridized to the $\alpha_{2u}$-globulin cDNA probe was almost 2-fold greater for liver nuclei isolated from the restricted rats.

**Discussion**

We have demonstrated that the age-related decline in $\alpha_{2u}$-globulin expression arises predominantly through the regulation of $\alpha_{2u}$-globulin transcription. An excellent correlation was found between the age-related decline in the synthesis, mRNA levels, and transcription of $\alpha_{2u}$-globulin genes in liver (Table I). Because these three parameters were measured in three different groups of rats at different times, the correlation is even more striking. Recently, Kulkarni et al. (32) showed that the control of $\alpha_{2u}$-globulin expression during development and hormonal stimulation occurred predominantly through the regulation of the transcription of $\alpha_{2u}$-globulin genes. Thus, our results and the data by Kulkarni et al. (32) show that the regulation of $\alpha_{2u}$-globulin expression occurs primarily at the level of transcription.

If $\alpha_{2u}$-globulin is a senescence marker protein, as proposed by Roy et al. (14), one should be able to change the age-related decline in $\alpha_{2u}$-globulin expression by procedures that alter the aging process, i.e. senescence. Dietary restriction (underfeeding not malnutrition) is the only experimental manipulation known to increase the longevity of mammals (20, 39, 40). The classic experiments by McCay et al. (41) in 1935 showed that a drastic restriction of total calories increased the survival of rats. Later studies by Berg (42, 43) showed that the longevity of rats could be increased by dietary restriction procedures less severe than those used by McCay et al. (41). Because dietary restriction has consistently been shown to increase the life expectancy of rodents, it has been suggested that this intervention retards the aging process. However, increasing life expectancy of an organism does not necessarily indicate that an intervention has influenced the process by which the organism ages. Several lines of evidence strongly suggest that the increase in longevity resulting from dietary restriction is the result of a retardation of the aging process (39), e.g. dietary restriction (a) increases the maximum as well as the mean survival, (b) retards the incidence and severity of a wide variety of diseases, (c) retards the decline in a variety of physiological processes, and (d) retards the decline in the function of a variety of organs/tissues.

In our study, we used a restriction regimen similar to that described by Yu et al. (19). They showed that this dietary restriction regimen increased the mean and maximum survival of male Fischer F344 rats 45–50%. We compared the expression of $\alpha_{2u}$-globulin by hepatic tissue from rats fed ad libitum and rats fed the restricted diet at 18 months of age. In rats fed ad libitum, we observed a dramatic (80–90%) decrease in the expression of $\alpha_{2u}$-globulin by 22 months of age (Table I). In contrast, at 18 months of age, rats maintained on the restricted diet had higher levels of $\alpha_{2u}$-globulin in their urine, and the expression of $\alpha_{2u}$-globulin by liver tissue from the restricted rats was higher than rats fed ad libitum (Fig. 4, Table II), e.g. the synthesis, mRNA levels, and transcription of $\alpha_{2u}$-globulin were 1.8–3-fold higher for hepatic tissue from the restricted rats. Thus, our study supports the suggestion by Roy et al. (14) that $\alpha_{2u}$-globulin can serve as a senescence marker protein because dietary restriction, which increases the longevity of the rats, retards the age-related decline in $\alpha_{2u}$-globulin expression.

The data obtained in this study clearly show that the regulation of $\alpha_{2u}$-globulin expression during senescence and dietary restriction occurs predominantly at the level of transcription. However, the molecular basis for the changes in $\alpha_{2u}$-globulin transcription is unknown. Because $\alpha_{2u}$-globulin
is regulated by a variety of hormones, the changes in α_{2u}-globulin expression during senescence and dietary restriction could arise from age-related changes in the hormonal status of the animal. On the other hand, the decrease could arise from changes at the cellular level, i.e., cells could be unable to express α_{2u}-globulin.

Although it is well documented that dietary restriction increases the longevity of rodents, the mechanism underlying the effect of dietary restriction on longevity is not understood. Because the methodology used to increase longevity involves restricting calories, most investigators have assumed that dietary restriction acts through some type of metabolic mechanism. However, research conducted during the past 5 years shows that the increase in longevity is not due to retardation of development/maturity (40, 44, 45), reduced adiposity (46, 47), or reduced metabolic rate (39). Because dietary restriction has a profound effect on most tissues and diseases, the mechanism must involve a process that has the potential to impact on most, if not all, cells in an organism. The regulation of gene expression represents one site of regulation common to all cells in a living organism, and changes in gene expression can have a profound effect at both the cellular and organismic level. Our study is the first evidence to show that dietary restriction has an effect at the level of gene expression, specifically transcription. Future studies should be conducted with other cDNA probes and other tissues to determine whether changes in the expression of other genes occur with dietary restriction.

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