Gene Expression of the Three Members of Hepatocyte Nuclear Factor-3 Is Differentially Regulated by Nutritional and Hormonal Factors

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Abstract

Hepatocyte nuclear factor-3 (HNF-3) belongs to a large family of forkhead transcription factors and is made up of three members (HNF-3 α , -3 β and -3 γ). It has been shown that HNF-3 regulates a number of metabolically important genes. However, the mechanisms underlying this regulation of HNF-3 activity by hormones and nutrition have not yet been well elucidated. In attempting to explore the regulation of gene expression of HNF-3 members by physiological status, we analyzed the effects of insulin, dexamethasone and protein malnutrition on the hepatic mRNA level of each member. Male Wistar rats were fed on a 12% casein diet, 12% gluten diet (deficient in lysine and threonine) or a protein-free diet for 1 week. The protein-free diet and gluten diet caused a 3.7-fold increase in HNF-3 γ mRNA in the liver and did not affect the

mRNA level of either HNF-3 α or HNF-3 β . Daily administration of dexamethasone caused the mRNA levels of HNF-3 α and HNF-3 β to increase (2.3- and 1.4-fold, respectively), but had no effect on the HNF-3 γ mRNA level. In diabetic rats that had been injected with streptozotocin, an elevation of the hepatic mRNA levels of HNF-3 β and HNF-3 γ was observed (1.6- and 1.9-fold, respectively). Insulin replacement in the diabetic rats decreased both mRNA levels in a dose-dependent manner. HNF-3 α mRNA was not affected by insulin status. These results show that the genes of the three members of the HNF-3 family respond differently to hormonal and nutritional factors suggesting that the activities of HNF-3 members are regulated, at least in part, by the levels of their gene expression.

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Introduction

Hepatocyte nuclear factor-3 (HNF-3) belongs to a large family of forkhead transcription factors that are distinguished by the presence of a winged helix motif / forkhead domain (Lai *et al.* 1993). The HNF-3 proteins were originally observed as factors mediating the liver-specific expression of the transthyretin gene (Costa *et al.* 1989). They were later shown to be involved in the regulation of numerous liver-specific and other genes, including genes for tyrosine aminotransferase (TAT) and insulin-like growth factor binding protein-1 (IGFBP-1) (Roux *et al.* 1995, Allander *et al.* 1997). Many of these genes are regulated by insulin, glucocorticoid, and nutritional status. For instance, expression of the TAT gene is positively regulated by glucocorticoid and negatively regulated by insulin and malnutrition (Ganss *et al.* 1994). Transcription of the IGFBP-1 gene is strongly repressed by insulin and enhanced by glucocorticoid and by malnutrition (Goswami *et al.* 1994). We have shown that the IGFBP-1 gene is highly responsive to protein malnutrition (Takenaka *et al.* 1996).

Three distinct members of HNF-3 (HNF-3 α , -3 β and -3 γ) have been identified so far (Kaestner *et al.* 1994). Numerous reports have shown that each member plays distinct roles in differentiation, inflammation, cell growth and gene regulation

(Lai *et al.* 1993). It has been shown that the activity of other members of the family of forkhead transcription factors, including FKHR and AFX, are regulated by means of their phosphorylation status. For example, FKHR is phosphorylated at its serine residue following stimulation by insulin (Nakae *et al.* 1999). However, the mechanism by which HNF-3 is regulated has been less extensively studied.

In the present study, we have analyzed the effect of insulin, dexamethasone and protein malnutrition on the hepatic mRNA levels of HNF-3 α , -3 β and -3 γ . We have found that the gene expressions of members of HNF-3 respond differently to these factors.

Methods

Animals and experimental design

Male rats of the Wistar strain with a mean body weight of 120 g were purchased from Charles River Japan (Kanagawa, Japan). The rats were kept in a room maintained at 22 $\pm$ 1 °C with a 12-h light-dark cycle (12h light, 12h dark, lights on at 0800). They were given a 12% casein diet between 1000 and 1800 h for 3 days before they were given experimental diets (12% casein diet (12C), 12% gluten diet or protein-free diet (PF)). The experimental diets were given for 7 days on the same schedule, and water was available *ad libitum*. The

composition of the experimental diets has been described previously (Takahashi *et al.* 1990).

At 1130 on day 8, the rats were anesthetized with pentobarbital, and blood was taken from them at the carotid artery. The liver was excised, snap-frozen in liquid nitrogen, and stored at -80°C until the RNA was prepared.

For the analysis of the effect of dexamethasone treatment, rats were injected with 1 mg/kgBW of dexamethasone (Dex; Sigma) dissolved in 40% ethanol/60% phosphate-buffered saline at 1800 daily for 7 days. Rats of the control group were injected with vehicle. They were given 12% casein or the protein-free diet. Liver sampling on the 8th day was done as described above. Mean body weights on the 8th day were $239 \pm 5\text{g}$ (12C), $160 \pm 2\text{g}$ (12C+Dex), $171 \pm 2\text{g}$ (PF) and $146 \pm 1\text{g}$ (PF+Dex) (mean $\pm$ S.E.M.). For an insulin-deficiency study, rats were injected with 75 mg/kgBW of streptozotocin (Sigma) dissolved in 50 mM citrate buffer (pH 4.5) or vehicle only. After two days, plasma glucose levels were measured by glucose oxidase method (Glucose B-test Wako, Wako Pure Chemical, Japan) to verify diabetic symptoms. During the ensuing seven days, ten rats were given 0.8 U/day human insulin (Sigma) and another ten were given 6.4 U/day insulin intravenously at 1000. All rats in the groups described here and in the vehicle-treated groups were fed on either the control diet or the protein-free diet. On day 8, rats were given respective diets for 1.5 h after insulin treatment and then sacrificed.

All experiments were performed under the guidelines of the Animal Usage Committee of the Faculty of Agriculture, The University of Tokyo.

Solution hybridization/RNase Protection Assay

Total RNA was prepared from the liver by the acid-guanidinium-phenol-chloroform method (Chomczynski *et al.* 1987) using the TRIzolTM reagent (GibcoBRL). The concentration of RNA was determined by the absorbance at 260 nm. The integrity and amount of RNA were confirmed by visualization of ribosomal RNAs after electrophoresis on denaturing agarose gels.

Template DNAs for the synthesis of antisense RNA probes were obtained by a reverse transcription-polymerase chain reaction. The position of the amplified cDNA (nucleotide number) and the length of protected bands in the RNase protection assays were as follows: HNF-3 α ; 711-1104 (Lai *et al.* 1990), 394 bases, HNF-3 β ; 973-1280 (Lai *et al.* 1991), 308 bases, HNF-3 γ ; 519-982 (Lai *et al.* 1991) 464 bases. The amplified cDNAs were subcloned into a pCRIITM vector (Invitrogen) and the sequences were confirmed using an automated DNA sequencer (Prism 310, ABI).

A solution hybridization/RNase protection assay was performed as described previously (Kato *et al.* 1994). Forty (HNF-3s) or ten (β -actin) micrograms of the total RNA from the liver of each rat were used in the assays. The bands were visualized and quantified using a Fujix Bas 2000 system (Fuji Film Co. Kanagawa, Japan). Background value of each lane was subtracted independently. All of the data were corrected

for the intensities of the bands obtained using a rat β -actin probe. Lysate RNase protection assays were also performed using the same liver samples to obtain relative mRNA levels per wet tissue weight (Kato *et al.* 1999).

Statistics

The results were analyzed statistically using Duncan's multiple range test (Duncan 1955).

Results

Figure 1 shows the effects of a protein-free (PF) diet and a 12% gluten (12G) diet on the hepatic mRNA levels of HNF-3 α , -3 β and -3 γ . Receiving the PF diet or 12G diet for 1 week did not affect the mRNA levels of HNF-3 α and -3 β . There was a 3.7-fold increase in HNF-3 γ mRNA in the PF diet-fed group as compared with the control (12% casein) diet-fed group. In addition, feeding the 12G diet, which is deficient in lysine and

Figure 1 The Effect of a Protein-free Diet on the Hepatic mRNA Levels of the Three Members of HNF-3.

Rats were given a 12% casein (12C) diet, 12% gluten diet (12G) or a protein-free (PF) diet between the hours of 1000 to 1800 for 7 days. On the 8th day, the rats were fed their respective diets for 1.5 h, then sacrificed, and their livers were excised for the preparation of total RNA. The levels of mRNA of HNF-3 α , -3 β , -3 γ and β -actin were determined by RNase protection assay. Typical results are shown on the upper panel in which each lane represents a result from an individual rat. The quantitative representations normalized by β -actin mRNA levels are shown on the lower panel (means and S.E.M.). Means not sharing the same letter are significantly different ($P < 0.05$).

Figure 2 The Effect of Dexamethasone Treatment on Hepatic mRNA Levels of the Three Members of HNF-3. Rats were given a 12C or PF diet for 7 days as described in the legend of Figure 1. During this 7-day period, 1 mg/kgBW of dexamethasone was injected daily. The hepatic levels of mRNA of HNF-3α, -3β, -3γ and β-actin were determined by RNase protection assay. Data normalized by β-actin mRNA levels (means and S.E.M.) are shown. Means not sharing the same letter are significantly different ($P < 0.05$).

threonine, caused a similar magnitude of increase in HNF-3γ mRNA.

We next examined the effect of glucocorticoid and insulin on the hepatic mRNA levels of the three subtypes of HNF-3. Dexamethasone (1 mg/kgBW) was injected subcutaneously daily for 1 week while the control or PF diet was fed. Dexamethasone caused a 2.3-fold increase in hepatic HNF-3α mRNA levels and a 1.4-fold increase in HNF-3β mRNA levels (Figure 2). An increase in the mRNA of HNF-3γ was again observed in PF diet-fed rats. Dexamethasone alone had no effect on HNF-3γ mRNA. However, when dexamethasone was administered in PF diet-fed rats, it canceled the effect of PF diet in that the mRNA level did not differ from that of the control group.

Diabetes was induced by intraperitoneal injection of streptozotocin, and the symptoms were verified by measuring the fasting blood glucose. Two days after the injection of STZ, insulin replacement was started by daily intravenous injection (0.8 or 6.4 U/day, just before the starting of feeding). Blood glucose levels at the sacrifice (1.5 h after injection) were as

following; 191 ± 14 (normal rats), 1188 ± 97 (STZ-treated), 518 ± 108 (STZ+0.8U insulin), 273 ± 74 (STZ+6.4U insulin) (mg/100ml, mean $\pm$ S.E.M.). The blood glucose levels were not significantly different between 12C- and PF-fed groups (data not shown). Insulin deficiency did not affect HNF-3α mRNA levels, but caused drastic increases in HNF-3β mRNA in both the control and PF-fed groups (Figure 3). The increase in HNF-3β mRNA was reversed by the replacement of insulin. A similar increase in HNF-3γ mRNA was observed in diabetic rats.

The results above were confirmed by a lysate RNase protection assay where RNA probes were directly added to the lysates of liver samples. (data not shown,).

Discussion

The importance of HNF-3 in the regulation of gene expression has manifested itself in many instances. For example, targeted disruption of the HNF-3γ gene results in a decreased expression of several liver-specific genes (Kaestner *et al.* 1998). HNF-3α null mice develop a phenotype of impaired glucose homeostasis and neonatal mortality (Kaestner *et al.*

Figure 3 The Effect of Diabetes and Insulin Treatment on the Hepatic mRNA Levels of the Three Members of HNF-3. Rats were injected with 75 mg/kgBW of streptozotocin (STZ), kept on 12C diet for two days, then given a 12C (upper panel) or PF (lower panel) diet for 7 days as described in the legend of Figure 1. During this 7-day period, STZ-rats were given 0, 0.8 or 6.4U of insulin at the beginning of feeding. Hepatic levels of the mRNA of HNF-3α, -3β, -3γ and β-actin were determined by RNase protection assay. Data normalized by β-actin mRNA levels (means and S.E.M.) are shown. Means not sharing the same letter are significantly different ($P < 0.05$).

1999, Shih *et al.* 1999). HNF-3 has been shown to bind to and regulate the promoters of many genes, including transthyretin, fructose 2,6-bisphosphatase, hexokinase I, tyrosine aminotransferase and IGFBP-1 (Roux *et al.* 1995, Allander *et al.* 1997, Wang *et al.* 1999, Lin *et al.* 1997).

HNF-3 belongs to a large family of forkhead transcription factors. The mechanisms underlying the activation and inactivation of some of the forkhead transcription factors have been studied mainly with respect to the action of insulin. It has been shown that one of the other forkhead transcription factors, FKHR, is regulated by phosphorylation by Akt/protein kinase B after insulin stimulation (Nakae *et al.* 1999, Guo *et al.* 1999). In the case of AFX, another member of the family of forkhead transcription factors, nuclear translocation is also under the control of phosphorylation by protein kinase B (Takaishi *et al.* 1999). The regulation of the HNF-3 family has not been well investigated. Here we present data suggesting that the activities of HNF-3 members are regulated by alterations in the expression of their respective genes. Gene expression of HNF-3 α was elevated by dexamethasone. The expression of HNF-3 β gene was increased by dexamethasone and by diabetes, and that of HNF-3 γ was increased by protein malnutrition and diabetes. The regulation of the gene expression of HNF-3 family has not been studied extensively. A change in HNF-3 α gene expression that functioned to regulate transthyretin gene transcription has been reported in the case of an acute-phase response (Qian *et al.* 1995). The change in the mRNA levels of HNF-3 γ in response to alterations in protein nutrition shown here is reminiscent of the way that HNF-1, another liver-specific transcription factor, is regulated by amino acid limitation (Marten *et al.* 1996).

The possibility that the effect of protein malnutrition on HNF-3 gene expression is manifested by impaired insulin secretion might be excluded because the effect of STZ and protein malnutrition was no parallel. Namely, the observations that protein malnutrition caused an increase in HNF-3 γ mRNA but not HNF-3 β mRNA and that diabetes increased both mRNAs suggest that signaling mediating protein malnutrition involves a pathway that is independent of insulin signaling. Furthermore, our unpublished observation that the plasma immunoreactive insulin concentration of the rats fed the protein-free diet is not significantly different from that of those fed the casein diet favors this assumption.

The precise role of each member of HNF-3 is far from being clearly determined. A possible explanation for the presence of the three members may be the regulatory role of different genes by an individual member of HNF-3. In some instances, each member of HNF-3 affects the gene expression of a particular gene in opposite ways (Sawaya *et al.* 1994). The results of the present study suggest that these three members have different roles to play in the adaptation and/or response of animals to various physiological alterations. The distinct and overlapping patterns of activation of each HNF-3 member suggest that members of HNF-3 may integrate and tune the incoming information that indicates the nutritional and

hormonal status. Analyses of the concentrations of HNF-3 members using antibodies highly specific to the respective members will promote further understanding of their roles. In addition, a search for other mechanisms by which HNF-3 activity is regulated, including changes of phosphorylation, localization and DNA binding activity, will surely be necessary, although such mechanisms have not up to now been identified in the case of this transcription factor.

The results of the present study suggest that the effects of insulin, glucocorticoid and nutritional alteration on the gene expression of a number of genes are at least in part mediated by changes in the gene expression of distinct members of HNF-3. Further studies that elucidate the mechanisms by which these factors regulate HNF-3 gene expression are necessary in order to identify the 'master gene(s)' of the gene regulation cascade that is triggered by these hormones and nutritional changes.

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