ORIGINAL

Proteomic analysis of a diabetic congenic rat identified age-dependent alteration of an acidic protein

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Abstract : Proteomic analysis was performed in an attempt to identify a gene responsible for the expression of type 2 diabetes using a congenic rat, F.O-*Nidd2/of*, which possesses a single hyperglycemic QTL locus derived from the diabetic OLETF (Otsuka Long-Evans Tokushima Fatty) rat. Since the genetic difference between the congenic and its host strain, the F344 rat, is limited to the introgressed segment of 38 cM or ca. 2% of the rat whole genome, any discordant protein spots on two dimensional polyacrylamid gel electrophoresis (2D PAGE) will be considered strong candidate genes of this locus. Here we analyzed ca. one thousand protein spots in three different tissue types, liver, muscle and pancreas at 10, 20 and 30 weeks of age, we found that an acidic protein of 55 kD in muscle tissue shifts towards acidic end in an age dependent fashion in the congenic strain. However, the shift was not observed in the control rat, which is intriguing because the timing of the shift corresponds to the age at which hyperglycemia begins in the congenic. Future biochemical analysis should aid in elucidating the molecular mechanisms of glucose metabolism. J. Med. Invest. 54 : 289-294, August, 2007

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INTRODUCTION

Most of the quantitative traits, including those that are closely associated with common diseases, such as plasma glucose and blood pressure, are polygenic. Understanding quantitative traits requires elucidation of a complex interplay among multiple genetic and environmental factors, which remains extremely difficult even in the post genome era (1). This is partly because the effect of individual disease causative polymorphisms is often so minute and weak that it is difficult to link to traits of interest (2).

We have been studying the OLETF rat, a well-established model of obesity-associated diabetes (3, 4). Previously, our quantitative trait loci (QTL) analysis identified 14 hyperglycemic loci and subsequent construction of congenic strains, each of which possesses an OLETF-rat-derived single hyperglycemic QTL in the normoglycemic F344 rat, provided a panel of congenic strains making possible characterizing each QTL individually (5-7).

One of the QTL, *Nidd2/of*, also noted as *Niddm20* in the Rat Genome Database (RGD, http : //rgd. mcw.edu), is ca. 38 cM genome segment on the chromosome 14. This QTL is interesting and given high priority because of several reasons. First, in addition to hyperglycemic QTL the locus contains an obesity QTL as well, which was identified by our

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separate QTL analysis (8). Second, the epistasis was demonstrated between *Nidd2/of* and *Nidd1/of*, another hyperglycemic locus on the chromosome 7. Third, this congenic strain is responsive to both genetical and nutritional obesity-causing stimuli, namely leptin receptor mutation and high calorie diet. That is, the hyperglycemia becomes highly aggravated by the state of obesity.

Here, we examined protein expression profiles of the congenic strain, F.O-*Nidd2/of*. Similar proteomic study was done using the OLETF rat (9). However, the study showed that in the liver only there are numerous protein spots that showed difference between the OLETF and the LETO rat that is genetically similar yet normoglycemic control rat. Therefore, we emphasize the advantage of the use of a congenic strain, because it is expected that the molecular events closely linked to this locus only are revealed and they are likely to be much less complex than that of the OLETF rat.

MATERIALS & METHODS

Rat strains and animal procedures

The F.O-*Nidd2/of* congenic strain was described and bred in the Institute for Animal Experimentation of the University of Tokushima under SPF conditions (7, 10). F344/Slc rats were purchased from Japan SLC. All rats were kept under specific pathogen-free conditions. The temperature (21 ± 2°C), humidity (55 ± 10%), and air conditioning were all controlled. Rats had free access to tap water and standard laboratory chow and were maintained at a 12-h light and dark cycle (7 am / 7 pm). Animal procedures used in this study were approved by the University of Tokushima Animal Experimentation Committee.

Tissue sampling

Male rats of 10, 20, 30-week old were fasted overnight and anesthetized. Ca. 500 mg piece of liver, muscle and pancreas, was dissected and immediately frozen in the liquid nitrogen. The frozen tissues were kept at -80°C until use.

Protein extraction and 2D PAGE

The tissues were homogenized on ice in 10 X volume of protein extraction buffer (HEPS 20 mM, KCl 100 mM, Glycerol 10%, EDTA 10 mM, DTT 1 mM, Triton X 0.1%, pH7.3 including protease inhibitors (Aprotinin 0.25 TIU[trypsin inhibitor units], Pepstrain A 2μg/ml, Leupetin 2 μg/ml, SIGMA). The homogenate was centrifuged at 6000 g 4°C for 10 min and the resultant supernatant was analyzed by 2D PAGE using IPGphor IEF System and SE400 Sturdier Vertical Unit according to the manufacture's instruction (Amersham Biosciences). Briefly, 10 μl of protein extract was mixed with 220 μl rehydration solution (Amersham) and 40 μl 2D sample buffer (2DSB, Urea 8 M, CHAPS 4%, Tris 40 mM) and subsequently rehydrated at 20°C for 12 hours. The protein was separated on three kinds of pH gradient, pH3-10, pH4-7 and pH6-10 (Immobiline Dry Strip, Amersham). For pH3-10 and pH4-7, the gel was run at 500V for 1 hour and 800V for 8 hours. For pH6-11, 500V for 1 hour and 800V for 9 hours and 30 minutes. After IEF electrophoresis, the strip was shaken in gel buffer (Tris 50 mM, Urea 6 M, Glycerol 33%, SDS 2%, BPB 0.05%, DTT 65 mM, pH 8.8) for 15 min. at room temperature and denatured proteins were separated on 10% acrylamide gel at 50V for 14 hours. The protein spots were visualized by standard silver staining kit (WAKO). The experiment was performed at least twice using independently prepared tissue sample to confirm reproducibility.

RESULTS

Chronological comparison of protein expression profiles in congenic and control rats

To explore the differences of protein expression in liver, thigh muscle and pancreas between the F. O-*Nidd2/of* and the control rat, the proteins were resolved by 2D PAGE. A wide range pH interval of 3-10 for IGP strip gave an overview of total protein distribution (Figure 1a, b, c). Distinct protein spots were observed from small proteins of 10 kD to as large ones as over 200 kD, though the separation tended to be smeared towards acidic end in all tissue types examined. We then used IPG strips of narrower pH range of 7-4 and 11-6 to achieve a better separation in the acidic and basic regions (Figure 1d, e). In total, we were able to analyze ca. 750, 520 and 670 spots in liver, muscle and pancreas, respectively. The profiles were compared at 10, 20, 30 week of age. The overall numbers and patterns of spot distribution were unchanged among different aging stages for any tissue types examined. Among ca. 5800 spots, we found that none of these spots reproducibly changed their amount or their position, except one protein described below.

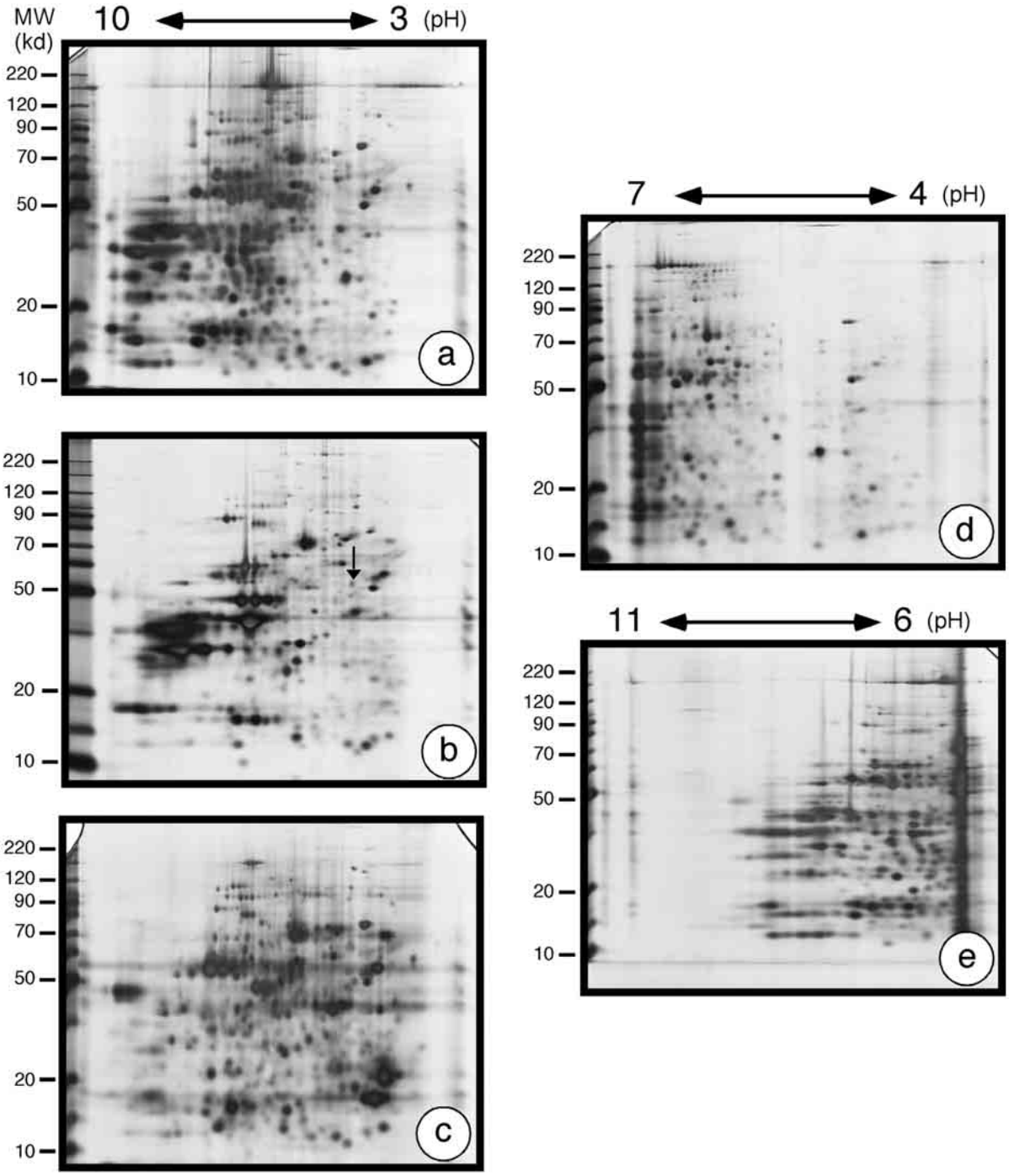

Figure 1 Representative 2D-PAGE gels showing the proteome of F344 and F.O-*Nidd2/of* rats. Shown here are animals at 20 weeks of age. Protein spots were visualized by silver staining. Protein extract was prepared from tissues of liver (a, d, e), thigh muscle (b), pancreas (c) and analyzed as described in the Methods. Three types of pH gradient strips, pH3-10(a-c), pH7-4(d) and pH6-11(e), were used. An acidic protein focused in the Fig.2 is indicated by an arrow (b). MW, molecular weight.

A 55 kD protein in muscle changes its position in the congenic rat.

In thigh muscle, we found there was a protein of 55 kD in size (Figure 1b, arrow) that changed its position along with horizontal axis as animals aged. In the congenic rat, at 30 weeks of age the protein moved toward acidic end on the basis of two nearby reference proteins, Ra and Rb (Figure 2b, d, f). In contrast, the same protein remained in the same position in the control muscle (Figure 2a, c, e). The observation is interesting because of the two reasons. First, the timing of the shift corresponds to the age that hyperglycemia starts in this congenic rat (unpublished observation). Second, it is reported that the OLETF shows insulin resistance in skeletal muscle (11).

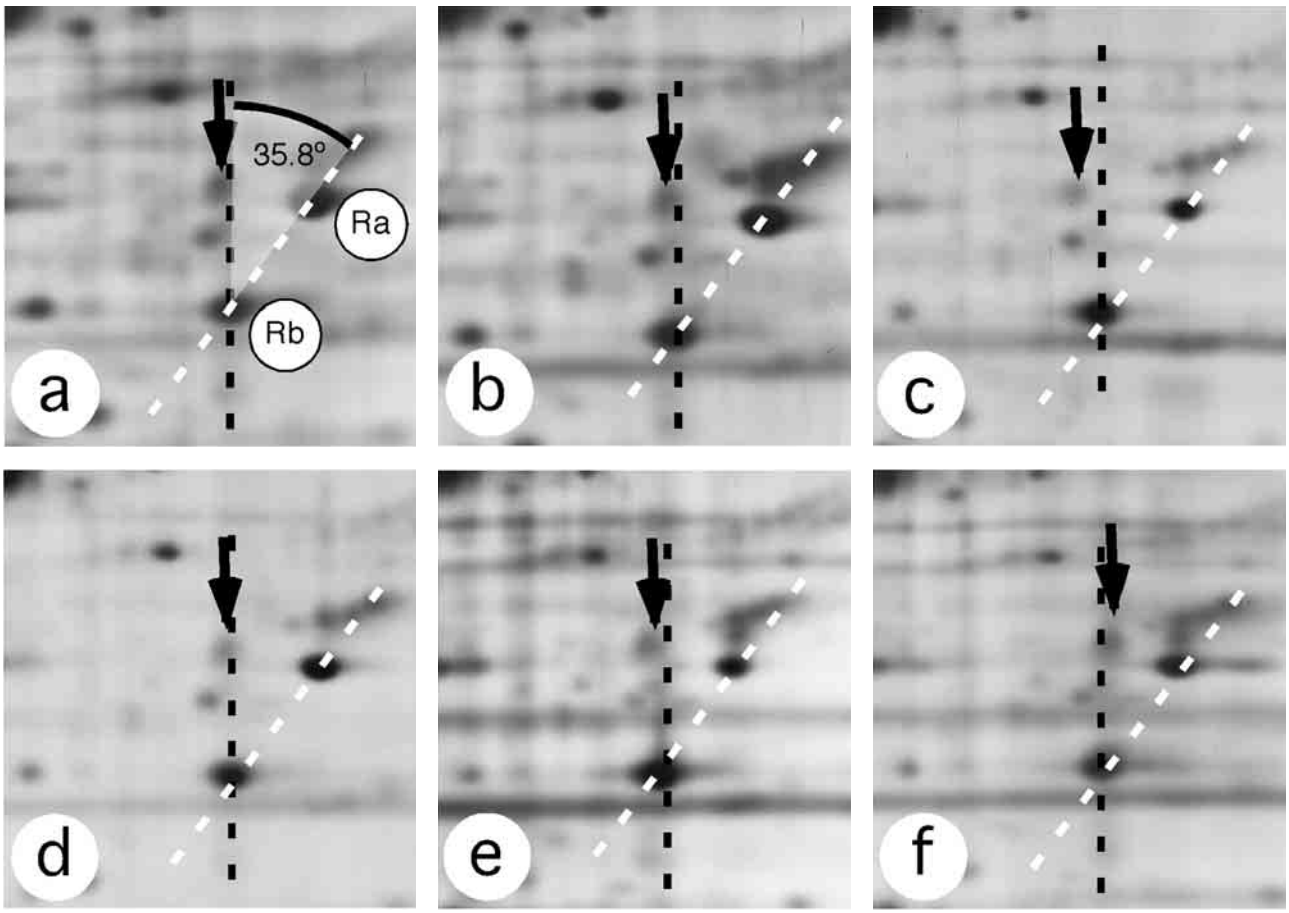

Figure 2 An age dependent alteration of an acidic protein of ca.55kD molecular mass (arrow) in thigh muscle tissue. The protein spot alteration is compared between the F344 control (a, c, e) and the F.O-*Nidd2/of* rat (b, d, f) at 10 (a, b), 20 (c, d) and 30 (e, f) weeks of age. Black dash bar represents the migration track of a reference protein (Rb). The reproducibility of the experiment is evaluated by the angle made between the black bar and the white dash bar that connects the Rb protein and another reference protein, Ra. In each gel, the angle is about 35.8°C. In the control rat, the p55 protein shifts towards acidic end at 20 weeks or older in the congenic rat (d, f). In contrast, in the control rat the protein remains in the same position (c, e).

DISCUSSION

In the present study, we analyzed the hyperglycemic congenic rat with comprehensive proteomic approach to search for molecular changes that can be crucial for understanding the mechanisms of plasma glucose regulation. Proteomics is considered as a complementary method for genomic techniques, such as microarray. One of the most significant features over transcriptome study is the ability to analyze the post-translational modifications of proteins (12). It has been said that the conventional acrylamide gel-based proteomics is problematic particularly due to the difficulty in reproducibility. We found many potentially important changes which lack reproducibility. The phenomenon of the 55 kD protein was confirmed by totally independent experiments at least twice.

In our knowledge, this is the first study to perform proteomics on an inbred congenic strain possessing a single QTL. There are reports, however, in which parental strains, the OLETF and SHR rat, were used to screen for expression differences at protein levels (9, 13, 14). Yet, these studies identified dozens of major changes. This is consistent with the hypothesis that the phenotype is polygenic and complex, and indicates that it is difficult to identify which spots play more important roles than others. In this respect, it was not surprising that the overall expression profiles were essentially identical between the congenic and the control rat, although the more sophisticated method, such as differential in-gel electrophoresis (DIGE), could have revealed more minute differences (15). Therefore, we suggest that the single difference we discovered has a high potential.

The 55 kD protein moved toward acidic end with apparently identical molecular mass. Thus, it is possible that the shift is the result of protein modifications affecting protein's pI. Another possibility is that the acidic form is due to alternative splicing. Third possibility is that the spot seen in young ani-

mals is downregulated and a new gene of an independent locus is activated. The spot seen in the younger age was completely disappeared or at least reduced below the detection limit in older rats, and vice versa. Thus we think that the phenomenon is unlikely due to the reversible modifications, such as phosphorylation, which are commonly regulated with rapid kinetics.

We searched database for candidate genes localized in the region defined by genetic markers *D14Rat23* and *D14Rat12*. Among 246 annotated genes, there is one gene predicted to encode a peptide that matches in molecular weight and pI. *Gc* is a vitamin D-binding protein precursor and described as a transporter of vitamin D and vitamin D metabolite. The peptide includes several possible sites for protein modification and is shown to be expressed in cell lines established from rat myocytes (Ensemble Peptide ID : ENSRNOP00000004174 ; http : //www.ensembl.org/Rattus_norvegicus/index.html). Thus, though the relationship between vitamin D and the diabetes remains to be established, this protein is one of the strong candidates. But it is crucial to keep in mind that the altered behavior of the 55 kD protein is not necessarily due to the direct result of polymorphisms within the QTL, but rather it may be one of the consequences downstream of the cascade.

As previously mentioned, the *Nidd2/of* locus includes obesity QTL and this congenic indeed shows increased fat mass [manuscript in preparation]. Thus it is crucial to establish the correlation between the shift of this protein and the glucose metabolism. This can be done by suppressing mild hyperglycemia by restricted diet or enforced exercise and examining if the shift of the protein is no longer observed. We expect that the future biochemical study should reveal the possible biological importance of the 55 kD protein for the glucose metabolism.

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