Proteomic analysis of serum biomarkers for prediabetes using the Long-Evans Agouti rat, a spontaneous animal model of type 2 diabetes mellitus

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Keywords
Long-Evans Agouti rat, Quantitative serum proteomics, Serine protease inhibitor A3

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J Diabetes Investig 2017; 8: 661–671
doi: 10.1111/jdi.12638

ABSTRACT
Aims/Introduction: To identify candidate serum molecules associated with the progression of type 2 diabetes mellitus, differential serum proteomic analysis was carried out on a spontaneous animal model of type 2 diabetes mellitus without obesity, the Long-Evans Agouti (LEA) rat.

Materials and Methods: We carried out quantitative proteomic analysis using serum samples from 8- and 16-week-old LEA and control Brown Norway (BN) rats (n = 4/group). Differentially expressed proteins were validated by multiple reaction monitoring analysis using the sera collected from 8-, 16-, and 24-week-old LEA (n = 4/each group) and BN rats (n = 5/each group). Among the validated proteins, we also examined the possible relevance of the human homolog of serine protease inhibitor A3 (SERPINA3) to type 2 diabetes mellitus.

Results: The use of 2-D fluorescence difference gel electrophoresis analysis and the following liquid chromatography-multiple reaction monitoring analysis showed that the serum levels of five proteins were differentially changed between LEA rats and BN rats at all three time-points examined. Among the five proteins, SERPINA3N was increased significantly in the sera of LEA rats compared with age-matched BN rats. The serum level of SERPINA3 was also found to be significantly higher in type 2 diabetes mellitus patients than in healthy control participants. Furthermore, glycated hemoglobin, fasting insulin and estimated glomerular filtration rate were independently associated with the SERPINA3 levels.

Conclusions: These findings suggest a possible role for SERPINA3 in the development of the early stages of type 2 diabetes mellitus, although further replication studies and functional investigations regarding their role are required.

INTRODUCTION
Diabetes mellitus is a growing public health problem worldwide, in which approximately 90–95% of patients are diagnosed with type 2 diabetes mellitus1. The pathogenesis of type 2 diabetes mellitus is thought to be complicated, involving multiple genetic, metabolic and environmental factors2-4. The effects of type 2 diabetes mellitus are typically limited to the detrimental loss of insulin-producing pancreatic β-cells; however, the subsequent reduction in insulin secretion can lead to multiple malfunctions, such as macrovascular and microvascular complications5. It is important to identify individuals at high risk of future
progression to diabetes, and who might benefit from interventions aimed at reducing the burden of disease associated with hyperglycemia. To identify early diagnostic markers for type 2 diabetes mellitus that are maintained throughout a diabetic phenotype, we recently carried out serum proteomics using the KK-A’ mouse model of type 2 diabetes mellitus, and identified several differentially expressed proteins in the prediabetic state. Among them, serine protease inhibitor (SERPIN) A3 level was elevated significantly in type 2 diabetes mellitus, and increased the transendothelial permeability of retinal microvascular endothelial cells, which might be involved in the pathogenesis of type 2 diabetes mellitus and/or diabetic retinopathy.

Recently, Okamura et al. established the Long-Evans Agouti (LEA) rat, a spontaneous animal model of type 2 diabetes mellitus without obesity, which was established from a Long-Evans closed colony together with the Long-Evans Cinnamon rat. Before the onset of diabetes, progressive fibrosis of islets occurs in and around the pancreatic islets. These changes are accompanied by a decrease in the number of pancreatic β-cells, resulting in defects in insulin secretion. Thus, the LEA rat could be a useful animal model for searching for biomarkers of type 2 diabetes mellitus with impaired insulin secretion.

In the present study, we carried out quantitative proteomic analysis using serum samples from LEA and control Brown Norway (BN) rats by 2-D fluorescence difference gel electrophoresis (2D-DIGE) combining liquid chromatography (LC)-multiple reaction monitoring (MRM) analysis to uncover important candidate proteins that might be linked with the pathogenesis of type 2 diabetes mellitus. We carried out replication and longitudinal studies on LEA rats, and also evaluated whether the identified proteins are also associated with type 2 diabetes mellitus in human patients. Importantly, SERPINA3 was shown to be increased significantly in type 2 diabetes mellitus patients, which could be used for the early detection of type 2 diabetes mellitus.

METHODS

Animals

Long-Evans Agouti rats, also known as LEA/SENDAl or SENDAI rats, were maintained at the Department of Laboratory Animal Medicine, Research Institute, National Center for Global Health and Medicine (NCGM)7. As the LEA rat is a spontaneous mutant derived from closed-colony Long-Evans rats, there is no suitable control strain. In the present study, we used the inbred BN strain as a reference. BN rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Animal care, use, and experimental protocols were approved by the Animal Care and Use Committee of the NCGM Research Institute, and carried out in accordance with EU Directive 2010/63/EU and the ARRIVE guidelines.

Rat serum collection

At 8, 16 and 24 weeks-of-age, bodyweight and fasting plasma glucose levels were measured as described previously. The serum levels of total cholesterol, high-density lipoprotein cholesterol and triglycerides were measured by the use of a blood biochemical analyzer (Spotchem D-Concept; ARKRAY, Inc., Kyoto, Japan). For 2D-DIGE analysis, 8- and 16-week-old male LEA and BN rats were used (n = 4/group). Fasting serum samples were prepared as described previously, and were stored at −20°C until analyzed. In a different batch, fasting serum samples were collected at 8, 16 and 24 weeks-of-age for a MRM assay (n = 4 for LEA and n = 5 for BN rats/each group).

Human serum collection

Fasting serum was acquired from 68 type 2 diabetes mellitus patients recruited from outpatients or inpatients of the Center Hospital of the NCGM, JR Tokyo General Hospital and Toyama University Hospital. Diabetes was diagnosed according to the World Health Organization criteria as described previously. We also acquired fasting serum from 98 non-diabetic participants as controls who were enrolled from an annual health check-up carried out at the Department of Complete Medical Checkup, Center Hospital of the NCGM. Each patient was assessed for clinical features, such as age, sex, body mass index and blood sample data, based on the data contained in the medical records. The indexes of homeostasis model assessment of insulin resistance and β-cell insulin secretion were calculated based on the plasma glucose and insulin concentrations as previously described. This study was approved by the ethics committee of the NCGM and of each participating institution. Informed consent was obtained from each participant, and patient anonymity was preserved.

Removal of high-abundance proteins from rat serum samples

Rat serum samples were depleted of seven abundant proteins essentially as described previously. The depleted serum samples were concentrated, and protein concentration was determined using the Bradford protein assay.

2D-DIGE analysis

A total of 50 μg of serum samples were labeled with Cy3 or Cy5 dye, according to the manufacturer’s protocol (GE Healthcare UK Ltd., Buckinghamshire, UK). An equal amount of sample from eight LEA rats and eight BN rats was pooled, labeled with Cy2 dye, and used as an internal standard. Three labeled protein samples (Cy3, Cy5 and Cy2) were combined per gel, and were subsequently applied to 24-cm immobilized pH gradient strips, pH 4–7 (GE Healthcare). Isoelectric focusing was carried out using an IPGPhor system (GE Healthcare) with a total of 95 kVh. The proteins were then separated on 10–15% gradient acrylamide gel at 1 W per gel. The gels were scanned using a Typhoon 9400 imager (GE Healthcare), and analyzed with DeCyder software (version 6.5; GE Healthcare). Protein spots with an average ratio ≥1.5 or ≤1.5 and a P-value <0.05 were considered to be differentially expressed protein spots, and were selected for identification.
Protein identification
A total of 300 µg of unlabeled internal standard sample were separated by 2-D electrophoresis for protein identification. The gel was stained by SYPRO Ruby (Bio-Rad, Hercules, CA, USA). Differentially expressed protein spots were picked from the gels and digested in trypsin (Promega, Madison, WI, USA). Tryptic digests were analyzed by LC coupled with tandem mass spectrometry. The LC coupled with tandem mass spectrometry system comprised an LCQ Deca XP Plus ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled with a Paradigm MS4 nanoLC system (Michrom BioResource, Auburn, CA, USA). Peptides were detected under the following analytical conditions: spray voltage, 2.5 kV; capillary temperature, 200°C; and mass range, 450–2,000 m/z. The raw data files from LC coupled with tandem mass spectrometry analysis were converted by Mascot distiller software 2.4.1.0 (Matrix Science, London, UK), and transferred with Mascot software version 2.4.1 (Matrix Science). Then, the UniProtKB/Swiss-Prot database was searched with Rattus norvegicus as taxonomy (release-2013_12). The search parameters were as follows: fixed modification, carbamidomethyl (C); variable modification, oxidation (M); peptide charge, 2+ and 3+; peptide and MS/MS tolerance, ±2.0 and ±0.8 Da, respectively; enzyme, trypsin; one missed cleavage; and instrument, ESI-Trap. Only significant hits, as defined by Mascot probability analysis (P < 0.05), were accepted.

MRM analysis
MRM analysis was carried out as described previously6. Briefly, 500 ng of rat serum sample were digested with trypsin and lysyl endopeptidase (Wako Pure Chemicals, Osaka, Japan) for MRM analysis. The, 10 fmol of stable isotope labeled peptide (YL*YEIAR: *Leucine is labeled with 13C and 15N) were also added to each sample as an internal standard. MRM runs were carried out using a 5500 QTRAP (AB SCIEX, Foster City, CA, USA) coupled with a Paradigm MS4 nanoLC system in the MRM mode. Data were processed using the MultiQuant program (version 2.0; AB SCIEX). Triplette analyses were carried out for each of the rat serum samples.

Measurement of human SERPINA3 levels
The serum levels of human SERPINA3 were measured using Human α 1-Antichymotrypsin ELISA Kit (ImmunoLogic Consultants Laboratory, Newberg, OR, USA) according to the protocol provided by the manufacturer.

Statistical analysis
Data are expressed as mean ± standard deviation for normally distributed variables, and median (interquartile range) for non-normally distributed variables. Differences between the two groups for normally distributed variables were tested using Student’s two-sided t-test, and non-parametric data were analyzed using the Mann–Whitney U-test. Analysis of covariance was used between groups; and multiple testing corrections were carried out using the Bonferroni method. Sex differences between the two groups were tested using the $\chi^2$-test. Correlations were calculated using Pearson’s correlation coefficient. Multiple stepwise linear regression analysis was carried out using the dependent variable, SERPINA3, and those variables showing a correlation with SERPINA3 (P-value ≤0.2) as the independent variables. All skewed variables were logarithmically transformed before analyses. Correlation and multiple stepwise linear regression analyses were carried out for type 2 diabetes mellitus patients and healthy controls. All calculations were carried out with Microsoft Excel 2011 or IBM SPSS software version 20. A P-value <0.05 was considered significant.

RESULTS
2D-DIGE analysis of serum proteomic changes in LEA and control BN rats
The characteristics of LEA and BN control rats are shown in Table S1. The average bodyweight of LEA rats increased gradually, and was significantly increased compared with that of BN rats at the age of 16 weeks. High-density lipoprotein cholesterol levels were significantly increased both in 8- and 16-week-old LEA rats, and total cholesterol was significantly increased only in 16-week-old LEA rats. Fasting plasma glucose levels did not differ between 8- and 16-week-old LEA and BN rats, although 8-week-old LEA rats showed impaired glucose tolerance compared with age-matched BN rats, as reported previously7. For the discovery of candidate protein markers of prediabetes, 2D-DIGE-based comparative proteomic analysis was carried out on samples from 8- and 16-week-old LEA vs BN rats after abundant protein depletion to enhance the visualization of lower abundance proteins. Samples containing serum from 8- and 16-week-old LEA and BN rats were labeled with Cy3 and Cy5 dyes, and run against a Cy2-labeled internal standard (Table S2). Image analysis detected a total of 2,348 protein spots, and indicated 209 and 317 protein spot features showing significant changes in abundance levels (LEA/BN ratio $\leq$1.5-fold or $\geq$1.5-fold, Student’s t-test $P < 0.05$) at 8 and 16 weeks-of-age, respectively (Figure S1). Among them, 68 protein spots were increased and 141 were decreased in 8-week-old LEA rats (Figure S1a), and 115 were increased and 202 were decreased in 16-week-old LEA rats, compared with those in age-matched BN rats (Figure S1b). Of these, 115 protein spots could be identified by LC-MS/MS, resulting in 14 and 13 unique differentially expressed proteins at 8 and 16 weeks-of-age, respectively (Tables 1 and 2). Some of the distinct spots in the 2-D gel belonged to the same protein as isofoms with different percentage coverage of the analyzed peptides, matched peptide numbers, P-values of MS and MS/MS searches, average fold-differences, t-test values, and matched peptide sequences for each identified protein. This could be due to the degradation or cleavage of the proteins or various post-translational modifications (such as α-1-inhibitor 3 [A1I3], complement component 3, and murinoglobin 2 [MUG2]). Five proteins
Validation of the identified proteins by MRM analysis

To confirm the differential expression of the identified proteins in the 2D-DIGE analysis, we carried out relative quantification of the identified proteins by MRM analysis using an independent sample set (sera obtained from 8-, 16-, and 24-week-old LEA and BN rats). We used a total of 103 transitions for targeting the 27 peptides representing the 16 differentially expressed proteins in MRM analysis (Table S3). The extracted ion chromatogram peaks of the predetermined different transitions for each target protein were detected at the same retention time (Figure S2). Each resulting MRM peak was also examined by full scan MS/MS, and the MS/MS spectrum for each MRM peak confirmed the sequence validation of the hypothesized peptide, as shown in Figure S3. The measured abundances (the ratio of the area under the most intense peak to the input internal standard) of the 27 peptides in replicate serum samples were evaluated statistically to verify the changes in the 16 proteins identified in the 2D-DIGE analysis. The amounts of 11 peptides representing the six proteins (A1I3, ApoE, C-reactive protein [CRP], MUG1, SERPINA3K and SERPINA3N) in 8-week-old LEA rats showed significant differences from those in BN rats, which validated the 2D-DIGE results (Figure 1; Table S4). Longitudinal changes of these proteins were also observed, and eight peptides representing five proteins (A1I3, ApoE, CRP, MUG1 and SERPINA3N) among them were chronologically changed in expression between LEA and BN rats at all ages examined (Figure 1; Table S4). Among these five proteins, four showed a significant increase in expression: ApoE, CRP, MUG1 and SERPINA3N; and A1I3 showed a significant decrease in expression.

In the MRM analysis for the peptide, NVVFSPLSISAALAVVSLGAK representing SERPINA3N, measured abundances in the sera derived from 8-week-old LEA and BN rats were much lower than those in 16- and 24-week-old LEA and BN rats, although there is a significant increase in expression in 8-week-old LEA rats compared with BN rats (Figure 1; Table S4). There is a possibility that the peptides derived from 8-week-old LEA and BN rats would contain post-translationally modified residues, such as glycosylation, lipidation and proteolysis, which was hardly detected by the predetermined MRM transitions. We also examined the serum SERPINA3 levels by western blotting analysis, and confirmed that SERPINA3 levels were increased in 8- and 16-week-old LEA compared with BN rats (data not shown). These results show that the five proteins are already changed at the prediabetic state in LEA rats.

Serum SERPINA3 levels in type 2 diabetes mellitus patients

To further examine the possible relationships of the validated proteins found in 2D-DIGE/MRM to type 2 diabetes mellitus, enzyme-linked immunosorbent assay was carried out to compare the serum levels of proteins in type 2 diabetes mellitus patients vs healthy control participants. Among the five differentially expressed proteins, A1I3 and MUG1 were excluded from the analysis because they are rat-specific proteins. ApoE was also excluded because high-density lipoprotein cholesterol levels were increased consistently in LEA rats (Table S1), possibly as a result of LEA rat-specific phenotypes. Therefore, enzyme-linked immunosorbent assays were carried out to measure the serum levels of CRP and SERPINA3 (the human homolog of SERPINA3N) in sex, age, and body mass index-matched type 2 diabetes mellitus patients (n = 68) and healthy control participants (n = 98). The detailed clinical characteristics of all participants are shown in Table 3. The median (interquartile) serum concentration of CRP in the control participants and type 2 diabetes mellitus patients was 0.04 mg/dL (0.02–0.07 mg/dL) and 0.08 mg/dL (0.03–0.16 mg/dL), respectively; and was significantly higher in the type 2 diabetes mellitus patients than that in the control participants (P = 3.2 × 10^-4, data not shown). The serum concentrations of SERPINA3 in the control and type 2 diabetes mellitus groups were 157.9 ± 24.1 μg/mL and 169.0 ± 38.1 μg/mL, respectively; and the difference between the groups was statistically significant (P = 0.04; Table 3).

In Table 4, Pearson’s correlation analysis suggested that SERPINA3 level was significantly correlated with creatinine (r = 0.16, P = 0.04) and estimated glomerular filtration rate (eGFR; r = -0.17, P = 0.03), and tended to be correlated with glycated hemoglobin (HbA1c; r = 0.13, P = 0.1), alanine aminotransferase (r = -0.11, P = 0.15), γ-glutamyl transpeptidase (r = -0.14, P = 0.07) and fasting insulin (r = -0.1, P = 0.2). We carried out a multiple stepwise linear regression analysis using SERPINA3 as a dependent variable and the clinical parameters (eGFR, γ-glutamyl transpeptidase, HbA1c, alanine aminotransferase and fasting insulin) as independent variables. The results showed that SERPINA3 levels were independently correlated with HbA1c (standardized β = 0.23), fasting insulin (standardized β = -0.17) and eGFR (standardized β = -0.25; Table 4).

DISCUSSION

In the present study, we carried out serum proteomic analysis of LEA rats, a spontaneous animal model of type 2 diabetes mellitus, in a prediabetic state compared with control BN rats in an attempt to uncover early diagnostic markers of diabetes that are maintained throughout a diabetic phenotype (Figure S4). We used 2D-DIGE-based serum profiling, and identified 14 and 13 unique differentially expressed proteins at 8 and 16 weeks-of-age, respectively (Tables 1 and 2). Their differential expression was confirmed by MRM analysis for relative quantitation of the candidate proteins. Using these techniques, we identified five proteins (A1I3, ApoE, MUG1, CRP and (α-2-HS-glycoprotein, apolipoprotein E [ApoE], microtubule-actin cross-linking factor 1, SERPINA3K and SERPINA3N) were found to be increased, and five proteins (A1I3, complement component 4, murinoglobulin-1 [MUG1], plasma protease C1 inhibitor, T-kininogen) were decreased both in 8- and 16-week-old LEA rats compared with BN control rats.
Table 1 | List of differentially expressed serum proteins between 8-week-old Long-evans Agouti diabetic rats and age-matched Brown Norway control rats as identified by liquid chromatography-coupled with tandem mass spectrometry after 2-D fluorescence difference gel electrophoresis analysis

| Spot number | Uniprot accession number | Protein name | Mascot score | Significant peptides | Coverage (%) | LEA/BN | P-value |
|-------------|--------------------------|--------------|--------------|---------------------|-------------|--------|---------|
| 32          | P14046                   | α₁-inhibitor 3 | 626          | 26                  | 24.6        | −6.95  | 3.2E-06 |
| 45          | P14046                   | α₁-inhibitor 3 | 398          | 12                  | 17.1        | −6.2   | 0.013   |
| 46          | P14046                   | α₁-inhibitor 3 | 347          | 17                  | 20.2        | −7.39  | 2.6E-04 |
| 47          | P14046                   | α₁-inhibitor 3 | 744          | 29                  | 27.4        | −5.4   | 0.002   |
| 57          | P14046                   | α₁-inhibitor 3 | 521          | 23                  | 30.7        | −4.59  | 2.2E-04 |
| 58          | P14046                   | α₁-inhibitor 3 | 113          | 4                   | 6           | −3.12  | 0.005   |
| 71          | P14046                   | α₁-inhibitor 3 | 371          | 16                  | 18.8        | −2.21  | 0.019   |
| 79          | P14046                   | α₁-inhibitor 3 | 90           | 5                   | 6.3         | −1.98  | 0.007   |
| 127         | P14046                   | α₁-inhibitor 3 | 74           | 3                   | 6.1         | −7.65  | 2.9E-07 |
| 183         | P14046                   | α₁-inhibitor 3 | 649          | 27                  | 26          | −7.32  | 6.0E-06 |
| 214         | P14046                   | α₁-inhibitor 3 | 589          | 22                  | 24.3        | −5.45  | 1.9E-04 |
| 218         | P08932                   | α₁-inhibitor 3 | 68           | 2                   | 4.5         | −4.07  | 3.8E-04 |
| 220         | P14046                   | α₁-inhibitor 3 | 445          | 16                  | 18.3        | −3.47  | 2.2E-04 |
| 222         | P14046                   | α₁-inhibitor 3 | 586          | 26                  | 23.8        | −2.49  | 0.003   |
| 232         | P14046                   | α₁-inhibitor 3 | 79           | 4                   | 6.7         | −5.00  | 0.004   |
| 239         | P14046                   | α₁-inhibitor 3 | 99           | 3                   | 6.2         | −3.96  | 0.001   |
| 252         | P14046                   | α₁-inhibitor 3 | 70           | 2                   | 5           | −3.52  | 0.002   |
| 253         | P14046                   | α₁-inhibitor 3 | 635          | 21                  | 19.5        | −9.06  | 0.017   |
| 298         | P14046                   | α₁-inhibitor 3 | 559          | 24                  | 19.8        | −4.48  | 0.029   |
| 308         | P14046                   | α₁-inhibitor 3 | 65           | 3                   | 4.8         | −6.16  | 0.028   |
| 319         | P14046                   | α₁-inhibitor 3 | 52           | 1                   | 2.5         | −5.59  | 0.006   |
| 334         | P14046                   | α₁-inhibitor 3 | 130          | 4                   | 8.9         | −8.45  | 0.001   |
| 348         | P14046                   | α₁-inhibitor 3 | 135          | 5                   | 6.8         | −11.16 | 0.001   |
| 372         | P14046                   | α₁-inhibitor 3 | 352          | 19                  | 14.2        | −10.27 | 0.001   |
| 388         | P14046                   | α₁-inhibitor 3 | 630          | 26                  | 25          | −8.27  | 0.001   |
| 393         | P14046                   | α₁-inhibitor 3 | 546          | 25                  | 22.5        | −8.45  | 4.8E-04 |
| 394         | P14046                   | α₁-inhibitor 3 | 499          | 23                  | 19          | −6.64  | 0.001   |
| 395         | P14046                   | α₁-inhibitor 3 | 569          | 24                  | 21.5        | −2.22  | 0.024   |
| 396         | P14046                   | α₁-inhibitor 3 | 587          | 26                  | 22.7        | −3.04  | 0.002   |
| 606         | P14046                   | α₁-inhibitor 3 | 154          | 4                   | 9.5         | −3.16  | 0.002   |
| 607         | P14046                   | α₁-inhibitor 3 | 51           | 2                   | 6.8         | 3.17   | 0.032   |
| 612         | P14046                   | α₁-inhibitor 3 | 134          | 6                   | 7.8         | 5.14   | 0.008   |
| 931         | P14046                   | α₁-inhibitor 3 | 127          | 4                   | 8           | −3.45  | 0.006   |
| 1,034       | P14046                   | α₁-inhibitor 3 | 417          | 14                  | 10          | −5.5   | 1.4E-04 |
| 49          | Q63041                   | α₁-macroglobulin | 81           | 2                   | 4.5         | −2.88  | 0.010   |
| 347         | Q63041                   | α₁-macroglobulin | 66           | 2                   | 4           | −3.73  | 0.002   |
| 1,165       | P24090                   | α₂-H5-glycoprotein | 68          | 2                   | 22.4        | 2.32   | 0.035   |
| 1,178       | P24090                   | α₂-H5-glycoprotein | 189         | 5                   | 28.1        | 2.63   | 0.010   |
| 1,200       | P24090                   | α₂-H5-glycoprotein | 96          | 3                   | 14.8        | 2.29   | 7.7E-05 |
| 1,690       | P02650                   | Apolipoprotein E | 446         | 14                  | 45.8        | 2.59   | 0.036   |
| 1,707       | P02650                   | Apolipoprotein E | 224         | 8                   | 27.9        | 2.42   | 0.010   |
| 1,726       | P02650                   | Apolipoprotein E | 433         | 16                  | 39.4        | 2.49   | 0.029   |
| 1,732       | P02650                   | Apolipoprotein E | 505         | 17                  | 37.5        | 2.33   | 2.7E-04 |
| 1,460       | P08649                   | Complement component 4 | 124       | 3                   | 5.6         | −2.65  | 0.039   |
| 1,723       | D3ZH2                  | Microtubule-actin | cross-linking factor 1 | 42 | 1 | 0.7 | 1.83 | 0.013 |
| 56          | Q03626                   | Murinoglobulin-1 | 596         | 21                  | 26          | −6.11  | 3.2E-04 |
| 72          | Q03626                   | Murinoglobulin-1 | 113         | 3                   | 3.7         | −2.19  | 0.009   |
| 74          | Q03626                   | Murinoglobulin-1 | 94           | 2                   | 3.8         | −2.56  | 0.001   |
SERPINA3N) that are differentially expressed in the prediabetic state. The changes of CRP and SERPINA3 expression were also confirmed to be increased by a longitudinal study on LEA rats and a study on type 2 diabetes mellitus patients (Table S4; Figure 1; Table 3).

The LEA rat was established as a novel, non-obese rat strain with spontaneous diabetes and derived from the Long-Evans strain. This strain is characterized by the gradual progression with spontaneous diabetes and derived from the Long-Evans rat strain. The LEA rat was the first report to show that serum SERPINA3 level was increased in type 2 diabetes mellitus patients compared with control patients, which implies that the increased secretion of SERPINA3 might occur in the liver (Figure S5). However, the increase of serum SERPINA3 levels might be caused by the decreased reuptake of SERPINA3 by the liver, as the liver has been shown to be the major recycler of kallikrein complexes from the circulation. SERPINA3N is known as a granzyme B inhibitor, and elevated expression and activity of SERPINA3N accelerate wound healing in diabetes. Recently, circulating levels of serine protease granzyme B and insulin receptor α-subunit cleaved by proteases were found to be elevated in type 2 diabetes mellitus patients. Expression of SERPINA3N might be compensatorily induced, and the increased expression of SERPINA3 inhibits Wnt/β-catenin signaling, thereby contributing to impaired insulin secretion in the pancreas under the diabetic state. Further functional studies are required to clarify the involvement of SERPINA3 in the onset of type 2 diabetes mellitus. We cannot totally exclude the possibility that the elevation of SERPINA3 in serum might reflect the obesity-related phenotype, as the weight gain after 8 weeks was more prominent in LEA rats than that in BN rats (Table S1). However, in a study on type 2 diabetes mellitus patients, SERPINA3 level was not correlated
Table 2 | List of differentially expressed serum proteins between 16-week-old Long-Evans Agouti diabetic rats and age-matched Brown Norway control rats as identified by liquid chromatography-coupled with tandem mass spectrometry after 2-D fluorescence difference gel electrophoresis analysis

| Spot number | Uniprot accession number | Protein name | Mascot score | Significant peptides | Coverage (%) | LEA/BN² | P-value |
|-------------|--------------------------|--------------|--------------|----------------------|-------------|---------|---------|
| 32          | P14046                   | α-1-inhibitor 3 | 626          | 26                   | 24.6        | -8.05   | 9.7E-05 |
| 45          | P14046                   | α-1-inhibitor 3 | 398          | 12                   | 17.1        | -5.45   | 0.004   |
| 47          | P14046                   | α-1-inhibitor 3 | 744          | 29                   | 27.4        | -6.69   | 1.8E-04 |
| 57          | P14046                   | α-1-inhibitor 3 | 521          | 23                   | 30.7        | -6.95   | 5.4E-06 |
| 71          | P14046                   | α-1-inhibitor 3 | 371          | 16                   | 18.8        | -1.87   | 3.4E-04 |
| 183         | P14046                   | α-1-inhibitor 3 | 649          | 27                   | 26          | -5.73   | 5.9E-04 |
| 214         | P14046                   | α-1-inhibitor 3 | 589          | 22                   | 24.3        | -3.42   | 0.002   |
| 220         | P14046                   | α-1-inhibitor 3 | 445          | 16                   | 18.3        | -2.64   | 0.004   |
| 222         | P14046                   | α-1-inhibitor 3 | 586          | 26                   | 23.8        | -2.26   | 0.009   |
| 252         | P14046                   | α-1-inhibitor 3 | 635          | 21                   | 19.5        | -5.68   | 3.1E-04 |
| 319         | P14046                   | α-1-inhibitor 3 | 559          | 24                   | 19.8        | -4.23   | 0.014   |
| 348         | P14046                   | α-1-inhibitor 3 | 379          | 16                   | 18.2        | -11.75  | 3.7E-06 |
| 372         | P14046                   | α-1-inhibitor 3 | 352          | 19                   | 14.2        | -12.14  | 1.8E-06 |
| 388         | P14046                   | α-1-inhibitor 3 | 630          | 26                   | 25          | -10.38  | 6.5E-06 |
| 392         | P14046                   | α-1-inhibitor 3 | 481          | 19                   | 27          | -2.14   | 0.009   |
| 393         | P14046                   | α-1-inhibitor 3 | 546          | 25                   | 22.5        | -7.52   | 1.6E-04 |
| 394         | P14046                   | α-1-inhibitor 3 | 499          | 23                   | 19          | -5.52   | 3.7E-04 |
| 395         | P14046                   | α-1-inhibitor 3 | 569          | 24                   | 21.5        | -2.75   | 0.003   |
| 396         | P14046                   | α-1-inhibitor 3 | 587          | 26                   | 22.7        | -3.87   | 0.004   |
| 606         | P14046                   | α-1-inhibitor 3 | 154          | 4                    | 9.5         | -2.44   | 0.002   |
| 612         | P14046                   | α-1-inhibitor 3 | 134          | 6                    | 7.8         | 7.72    | 6.5E-04 |
| 931         | P14046                   | α-1-inhibitor 3 | 127          | 4                    | 8           | -2.9    | 0.002   |
| 1,034       | P14046                   | α-1-inhibitor 3 | 417          | 14                   | 10          | -3.69   | 5.9E-04 |
| 1,165       | P24090                   | α-2-HS-glycoprotein | 68           | 2                    | 22.4        | 2.86    | 0.021   |
| 1,178       | P24090                   | α-2-HS-glycoprotein | 189          | 5                    | 28.1        | 3.04    | 0.001   |
| 1,200       | P24090                   | α-2-HS-glycoprotein | 96           | 3                    | 14.8        | 1.86    | 0.001   |
| 1,636       | P02650                   | Apolipoprotein E | 149          | 1                    | 13          | 2.4     | 0.007   |
| 1,637       | P02650                   | Apolipoprotein E | 138          | 4                    | 20          | 2.67    | 0.011   |
| 1,707       | P02650                   | Apolipoprotein E | 224          | 8                    | 27.9        | 2.21    | 0.023   |
| 1,732       | P02650                   | Apolipoprotein E | 505          | 17                   | 37.5        | 2.12    | 2.2E-04 |
| 755         | P01026                   | Complement component 3 | 194         | 8                    | 9           | -2.41   | 0.002   |
| 1,491       | P01026                   | Complement component 3 | 366         | 14                   | 11          | 1.95    | 0.014   |
| 1,833       | P01026                   | Complement component 3 | 216         | 7                    | 7           | 2.47    | 9.0E-04 |
| 1,460       | P08649                   | Complement component 4 | 124         | 3                    | 5.6         | -4.34   | 0.004   |
| 1,859       | P48199                   | C-reactive protein | 54           | 2                    | 9           | 1.58    | 0.004   |
| 1,723       | D3ZH2V2                  | Microtubule-actin | 42           | 1                    | 0.7         | 2.35    | 1.1E-04 |
| 56          | Q03626                   | Murinoglobulin-1 | 596          | 21                   | 26          | -4.02   | 0.009   |
| 224         | Q03626                   | Murinoglobulin-1 | 695          | 24                   | 26.3        | -1.59   | 0.043   |
| 230         | Q03626                   | Murinoglobulin-1 | 340          | 14                   | 18.3        | -3.62   | 2.5E-04 |
| 1,072       | Q03626                   | Murinoglobulin-1 | 208          | 6                    | 13.4        | -3.31   | 0.004   |
| 599         | Q6 IE52                  | Murinoglobulin-2 | 286          | 7                    | 9.7         | -1.69   | 0.008   |
| 1,304       | Q6 IE52                  | Murinoglobulin-2 | 65           | 1                    | 3           | 1.95    | 0.003   |
| 954         | Q6P734                   | Plasma protease C1 inhibitor | 234   | 9                    | 36.9        | -2.36   | 0.049   |
| 1,164       | P05545                   | Serine protease inhibitor A3K | 639    | 20                   | 44          | 1.62    | 0.008   |
| 1,185       | P05545                   | Serine protease inhibitor A3K | 405    | 13                   | 38          | 2.51    | 0.005   |
| 1,198       | P05545                   | Serine protease inhibitor A3K | 173    | 5                    | 29.3        | 1.91    | 0.030   |
| 1,312       | P09006                   | Serine protease inhibitor A3N | 717    | 20                   | 45.2        | 2.32    | 3.3E-04 |

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Therefore, it is unlikely that obesity-induced SERPINA3 expression could largely affect the present results.

A limitation of this study was that the present measurements were mostly restricted to the detection of abundant to moderately abundant serum proteins. We used a 2D-DIGE gel-based method for serum profiling; however, 2D-DIGE analysis is not efficient for the discovery of low abundance proteins because of the difficulty of resolving hydrophobic and very high or low molecular weight proteins. In recent years, several quantitative proteomic approaches, such as gel-free proteomics, have been developed. These advanced quantitative proteomic approaches, alternative fractionation strategies and instrumental improvements might hold the key to finding lower abundance serum proteins.

In the present study, in order to examine the possible relationships of SERPINA3 to type 2 diabetes mellitus, we compared serum SERPINA3 levels in type 2 diabetes mellitus patients with non-diabetic participants. It would be interesting to examine the SERPINA3 levels in the population with normal body mass index. Therefore, it is unlikely that obesity-induced SERPINA3 expression could largely affect the present results.

Table 2 (Continued)

| Spot number | Uniprot accession number | Protein name | Mascot score | Significant peptides | Coverage (%) | LEA/BN | P-value |
|-------------|--------------------------|--------------|--------------|----------------------|-------------|--------|---------|
| 1,313       | P09006                   | Serine protease inhibitor A3N | 620          | 14                   | 52          | 2.18   | 5.0E-05 |
| 984         | P01048                   | T-Kininogen 1 | 274          | 8                    | 22.3        | -1.69  | 0.024   |
| 1,047       | P01048                   | T-Kininogen 1 | 122          | 9.1                  |             | -2.94  | 0.002   |

† Average fold-difference of replicate samples’ run on different gels from DeCyder analysis show the abundance ratio of Long-Evans Agouti (LEA) diabetic rats to Brown Norway (BN) control rats. Proteins displaying an average 1.5-fold increase or decrease where P < 0.05 and spots matched in all images are listed. Total LEA rats, n = 4. Total BN rats, n = 4.

Figure 1 | Multiple reaction monitoring analysis confirmed that eight peptides derived from five proteins were consistently increased or decreased in Long-Evans Agouti rats (closed bars) compared with Brown Norway rats (open bars) from 8 to 24 weeks-of-age. The sequences in parentheses below the protein name show the peptide sequences for quantitation of the target proteins. The area under the most intense peak was calculated and normalized to the input internal standard. The black and white bars represent the relative abundances in Long-Evans Agouti and Brown Norway rats, respectively (Long-Evans Agouti rats, n = 4; Brown Norway rats, n = 5). A1I3, α-1-inhibitor 3; APOE, apolipoprotein E; CRP, C-reactive protein; IS, internal standard; MUG1, murinoglobulin-1; SERPINA3N, serine protease inhibitor A3N. *P < 0.05, **P < 0.01 to the age-matched control.
and impaired glucose tolerance, and newly diagnosed type 2 diabetes mellitus, as the serum elevation of SERPINA3 was identified in the prediabetic period in LEA rats.

In summary, we identified differentially expressed proteins in LEA rats in comparison with control BN rats. At 8 and 16 weeks-of-age, LEA rats are still prediabetic, but their serum

Table 3 | Clinical characteristics of the control participants and type 2 diabetic patients

|                                | Control participants | Type 2 diabetic patients | P-value |
|--------------------------------|----------------------|--------------------------|---------|
| n (men/women)                  | 98 (52/46)           | 68 (37/31)               | 0.86    |
| Age (years)                    | 63.3±4.4             | 62.3±8.5                 | 0.33    |
| BMI (kg/m²)                    | 23.9±3.1             | 24.7±2.7                 | 0.10    |
| Duration of diabetes (years)   | –                    | 62.3±3.1                 | –       |
| Systolic blood pressure (mmHg) | 128.0±17.6           | 125.3±13.6               | 0.28    |
| Diastolic blood pressure (mmHg)| 78.7±10.5            | 74.5±10.4                | 0.01*   |
| HbA1c (%)                      | 5.7±0.3              | 7.1±1.2                  | 3.1E-14**|
| Total cholesterol (mg/dL)      | 225.3±38.3           | 190.2±32.3               | 5.7E-09**|
| HDL-C (mg/dL)                  | 62.8±17.5            | 51.7±12.3                | 4.4E-06**|
| Triglyceride (mg/dL)           | 95.0 (73.0–146.0)    | 128.5 (88.0–182.0)       | 3.0E-03**|
| Creatinine (mg/dL)             | 0.8±0.2              | 0.8±0.2                  | 0.61    |
| ALT (IU/L)                     | 239±89               | 315±29.1                 | 0.04*   |
| γGTP (IU/L)                    | 27.0 (20.0–39.0)     | 28.0 (19.0–53.0)         | 0.47    |
| eGFR (mL/min/1.73 m²)          | 70.3±11.8            | 74.4±18.5                | 0.11    |
| Fasting plasma glucose (mg/dL) | 92.9±8.6             | 139.1±43.0               | 7.1E-13**|
| Fasting Insulin (μU/mL)        | 3.3 (2.1–5.3)        | 4.7 (2.3–9.7)            | 6.2E-03**|
| HOMA-β                         | 43.8 (30.5–65.1)     | 24.9 (14.3–52.3)         | 1.5E-05**|
| HOMA-IR                        | 0.80 (0.53–1.32)     | 1.54 (0.79–2.82)         | 1.9E-05**|
| SERPINA3 (μg/mL)               | 157.9±24.1           | 169.0±38.1               | 0.04*   |

Data are shown as means±standard deviation or median (interquartile range). *P-value<0.05. **P-value<0.01. Both groups are matched for sex, age and body mass index (BMI): γGTP, γ-glutamyl transpeptidase; ALT, alanine aminotransferase; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; HOMA-β, homeostasis model assessment index of β cell function; HOMA-IR, homeostasis model assessment index of insulin resistance; SERPINA3, serine protease inhibitor A3.

Table 4 | Relationship between the serum serine protease inhibitor A3 levels and metabolic parameters

|                                | Pearson's analysis | Multivariate regression analysis |
|--------------------------------|-------------------|---------------------------------|
|                                | r                 | P-value                         | β       | t-value | P-value |
| Age (years)                    | 0.06              | 0.45                            | –       | –       | –       |
| BMI (kg/m²)                    | 0.25              | 0.88                            | –       | –       | –       |
| Systolic blood pressure (mmHg) | -0.08             | 0.32                            | –       | –       | –       |
| Diastolic blood pressure (mmHg)| -0.02             | 0.77                            | –       | –       | –       |
| HbA1c (%)                      | 0.13              | 0.10                            | 0.23    | 2.76    | 0.01*   |
| Total cholesterol (mg/dL)      | -0.02             | 0.80                            | –       | –       | –       |
| HDL-C (mg/dL)                  | -0.07             | 0.41                            | –       | –       | –       |
| Triglyceride (mg/dL)?          | -0.08             | 0.31                            | –       | –       | –       |
| Creatinine (mg/dL)             | 0.16              | 0.04*                           | –       | –       | –       |
| ALT (IU/L)                     | -0.11             | 0.15                            | –       | –       | –       |
| γGTP (IU/U)L                   | -0.14             | 0.07                            | –       | –       | –       |
| eGFR (mL/min/1.73 m²)          | -0.17             | 0.03*                           | -0.25   | -3.07   | 3.0E-03*|
| Fasting plasma glucose (mg/dL) | 0.02              | 0.79                            | –       | –       | –       |
| Fasting Insulin (μU/mL)        | -0.10             | 0.20                            | -0.17   | -2.16   | 3.2E-02*|
| HOMA-β                         | -0.03             | 0.70                            | –       | –       | –       |
| HOMA-IR                        | -0.10             | 0.22                            | –       | –       | –       |

†Data were log-transformed before the analysis. *P-value<0.05. γGTP, γ-glutamyl transpeptidase; ALT, alanine aminotransferase; BMI, body mass index; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; HOMA-β, homeostasis model assessment index of β cell function; HOMA-IR, homeostasis model assessment index of insulin resistance; SERPINA3, serine protease inhibitor A3.

In summary, we identified differentially expressed proteins in LEA rats in comparison with control BN rats. At 8 and 16 weeks-of-age, LEA rats are still prediabetic, but their serum
protein profiles have already been changed. Among them, SERPINA3 levels were also confirmed to be induced in type 2 diabetes mellitus patients. Disturbed expression of SERPINA3 might cause the development of type 2 diabetes mellitus, which could be used as a potential biomarker of type 2 diabetes mellitus, although further studies are required to validate this hypothesis.

ACKNOWLEDGMENTS
We thank Souko Nakagami for her assistance with the clinical study (Department of Diabetes, Endocrinology and Metabolism, Center Hospital of the NCGM), and the staff of the Laboratory of Animal Unit, Research Institute, NCGM, for their assistance with issues regarding animal care. This work was supported in part by Grants-in-Aid from the Japan Society for the Promotion of Science (KAKENHI, 23500870, 25461370, 26670455) for the study design, and the collection, analysis and interpretation of data. A depository of human serum used in the present study was partially supported by Grants-in-Aid from the Ministry of Health, Labor and Welfare of Japan (Research on Biological Markers for New Drug Development, H25-016), from the Japan Science and Technology Agency (Adaptable and Seamless Technology Transfer Program Through Target-Driven R&D, A-STEP), and from the National Center for Global Health and Medicine (26S104).

DISCLOSURE
The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Figure S1**  Two-dimensional fluorescence difference gel electrophoresis comparative analysis of serum samples from Long-Evans Agouti diabetic rats and Brown Norway control rats.

**Figure S2**  Typical extracted ion chromatogram overlay of the predetermined different transitions for the 16 proteins in multiple reaction monitoring assay.

**Figure S3**  Full-scan tandem mass spectrum of the peptides for each multiple reaction monitoring peak.

**Figure S4**  Overall workflow for discovery (2-D fluorescence difference gel electrophoresis) and verification (multiple reaction monitoring) experiments.

**Figure S5**  Tissue distribution of serine protease inhibitor A3N (SERPINA3N) messenger ribonucleic acid in 16-week-old Long-Evans Agouti (closed bars, \( n = 4 \)) and Brown Norway control rats (open bars, \( n = 6 \)). Values represent mean ± standard deviation. *\( P < 0.05 \) compared with the corresponding tissue in the Brown Norway control rats.

**Table S1**  Characteristics of the Long-Evans Agouti diabetic rats and Brown Norway control rats included in the 2-D fluorescence difference gel electrophoresis analysis

**Table S2**  Experimental design for 2-D fluorescence difference gel electrophoresis analysis

**Table S3**  Peptide sequences and Q1/Q3 transitions of the 15 proteins quantitated in the multiple reaction monitoring assay

**Table S4**  Multiple reaction monitoring validation of the differentially expressed proteins using an independent sample set (sera obtained from 8-, 16- and 24-week-old Long-Evans Agouti \( [n = 4/each\ group] \) and Brown Norway rats \( [n = 5/each\ group] \))