Expression of Endoplasmic Reticulum Stress-Related Factors in the Retinas of Diabetic Rats

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Recent reports show that ER stress plays an important role in diabetic retinopathy (DR), but ER stress is a complicated process involving a network of signaling pathways and hundreds of factors. What factors involved in DR are not yet understood. We selected 89 ER stress factors from more than 200. A rat diabetes model was established by intraperitoneal injection of streptozotocin (STZ). The expression of 89 ER stress-related factors was found in the retinas of diabetic rats, at both 1- and 3-months after development of diabetes, by quantitative real-time polymerase chain reaction arrays. There were significant changes in expression levels of 13 and 12 ER stress-related factors in the diabetic rat retinas in the first and third month after the development of diabetes. Based on the array results, homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 (HERP), and synoviolin (HRD1) were studied further by immunofluorescence and Western blot. Immunofluorescence and Western blot analyses showed that the expression of HERP was reduced in the retinas of diabetic rats in first and third month. The expression of HRD1 did not change significantly in the retinas of diabetic rats in the first month but was reduced in the third month.

1. Introduction

Diabetic retinopathy (DR) is one of the severe complications of diabetes leading to loss of vision. Although the pathogenic mechanism of DR has been investigated for many years and a number of theories have been proposed [1, 2], the mechanism of DR remains unknown and needs further exploration.

Some diabetic patients are susceptible to DR, while others are quite resistant or develop minimal pathological changes [3]. It may be supposed that such DR-resistant patients are protected genetically. The existence of a DR-resistant gene was proposed, and a comparative study was performed of the gene expression between susceptible and resistant DR patients [4]. It was found that many endoplasmic reticulum (ER) stress-related factors are highly expressed in non-DR diabetic patients.

In our earlier work, we found that P58IPK/DNAJC3, an ER stress-related factor, binds to the ER transmembrane protein PERK (protein kinase RNA-activated- (PKR-) like ER kinase), which is normally activated by the ER stress/unfolded protein response. By binding to PERK, P58IPK thereby inhibits its phosphorylation of the α-subunit of eukaryotic translation initiation factor 2 (eIF-2α) and thus compromises eIF2/eIF2S3’s mediator role in the translation of mRNA [5]. In this way, P58IPK inhibits ER stress in the endothelial cells of human retinal vessels. P58IPK also downregulates the expression of vascular endothelial growth factor (VEGF), which is associated with regulation of the pathology of DR [6]. VEGF plays a key role in DR [7, 8] and is regulated at the transcriptional level by the unfolded protein response pathway [9]. Recent reports also show that ER stress plays an important role in DR [10, 11]. Li et al. [12] demonstrated that multiple ER stress markers, including 78 kDa glucose-regulated protein (GRP78), phosphoinositol-requiring transmembrane kinase (IRE)1α, and phosphor-eIF2α were significantly upregulated in the retinas of animal models of type 1 diabetes and oxygen-induced retinopathy. Our recent work suggests that early progression of DR may be mediated by ER stress, but probably does not involve changes in activating transcription.
factor (ATF)4 or GRP78 [13]. Together, these studies suggest that although ER stress is involved in the development of DR, its specific pathogenesis is not yet understood.

ER stress is a complicated process involving a network of signaling pathways and hundreds of factors that function by triggering the PERK, IRE1 and ATF6 signaling pathways [14–16]. In order to delve into the effects of these ER stress-related factors on DR, we classified them into 11 categories according to function (Figure 1, Table 3), based on Jonikas et al. [17]. We selected 89 ER stress factors from more than 200, based on our work and that of others (Table 4) [13, 17–21]. These factors contain the 11 categories of ER stress. Expression of these factors in the retinas of diabetic rats was determined by quantitative real-time PCR (Q-PCR) arrays to find the specific factors and the ER stress signaling pathways that may play a key role in the pathogenesis of DR.

2. Methods

2.1. Diabetic Rat Model. Two-month-old male Sprague Dawley rats weighing 150 to 200 g were obtained from the animal center of Huazhong University of Science and Technology. Care, use, and treatment of animals were approved by the laboratory animal center of Huazhong University of Science and Technology. Rats were randomly divided into diabetic and control groups (n = 30 per group). The diabetic model was created by intraperitoneal injection of a single dose of streptozotocin (STZ; 65 mg/kg in 0.01 M citrate buffer, pH 4.5) [22]. Nondiabetic rats (the control group) were injected with citrate buffer only. Fasting plasma glucose was examined 3 d after STZ injection, and diabetes was confirmed by a value ≥16.7 mmol/L using Touch Glucometer (Boehringer Mannheim Diagnostics, Indianapolis, IN). Our previous work [13] and that of others [23] have established that in the STZ-induced diabetes model, diabetic retinopathy develops within one month of the development of diabetes. Accordingly, one and three months after the STZ injection, the retinas were separated from the eyes of both the diabetic and control groups. RNA was extracted and assessed using Q-PCR arrays, with 9 rats in each group.

2.2. Quantitative Real-Time RNA Polymerase Chain Reaction (Q-PCR) Arrays. The mRNA levels of 96 factors (89 ER stress-related factors and 7 quality control factors) were measured using Q-PCR arrays. Total RNA was extracted from rat retinal tissue using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer’s instructions. RNA was treated with DNase (Invitrogen, Carlsbad, USA) and purified using Rneasy MinElute Clean-up Kit (Qiagen, Hilden, Germany). The cDNA was then synthesized using a SuperScript III kit (Invitrogen, Carlsbad, USA). Removing the plate seal from the PCR Array (SABioscience, Frederick, USA) and adding the cocktails to the PCR Array, Q-PCR was performed by using the Hot Star polymerase kit (Qiagen, Venlo, The Netherlands) with SYBR Green technology (ABI, Tampa, FL). PCR reaction buffer was added to a 384-well PCR array plate which was then tightly sealed with an optical adhesive cover. The thermocycling program consisted of 95°C for 10 min, then 40 cycles at 95°C for 15 s, and 60°C for one minute, then compared the differential expression of gene between the two groups.

2.3. Immunofluorescence. Immunofluorescence was performed on 5 μm frozen sections. Briefly, retinal sections were incubated with a rabbit anti-HERP (Santa Cruz Biotechnology, Santa Cruz, Calif) or anti-Hrd1 (Biosynthesis Biotechnology, Beijing, China) antibody (1:200) at 4°C overnight. This was followed by the secondary antibody, fluorescein-conjugated goat antirabbit IgG (Antigene, Wu Han, China), for one hour. The slides were visualized and photographed under a fluorescence microscope (Olympus, Hamburg, Germany).

2.4. Western Blot. Total protein was extracted from rat retinal tissue in 300 μL lysis buffer (50 mM Tris pH 7.5, 0.5 M NaCl, 1% NP-40, 1% sodium deoxycholate monohydrate, 2 mM EDTA, and 0.1% SDS). After centrifugation at 1000 x g for 3 min, protein extracts were diluted with sample buffer (126 mM Tris HCl pH 6.8, containing 20% glycerol, 4% SDS, 0.005% bromophenol blue, and 5% 2-mercaptoethanol) at a 1:1 ratio and boiled for 3 minutes. The samples were fractionated according to size on a 12.5% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane (Millipore, Billerica, Mass), and probed with polyclonal anti-HERP (Santa Cruz Biotechnology, Santa Cruz, Calif) or polyclonal anti-Hrd1 (Biosynthesis Biotechnology, Beijing, China) antibodies. A secondary antibody, goat antirabbit IgG (Biosynthesis Biotechnology, Beijing, China) diluted 1:1000, was applied, and the chemiluminescent signal was detected. The same membrane was reused to detect β-actin (the internal control) by incubating it with mouse antihuman β-actin antibody (Gene, Hong Kong, China). Bands observed on the photography films were analyzed by automatic image analysis. The integrated optical density of each protein band was normalized to that of the corresponding β-actin band from the same sample.

2.5. Rat Retinal Capillary Endothelial Cell (RRCEC) Culture. RRCECs cultured in vitro were prepared as previously described [24]. Two-month-old male Sprague Dawley rats weighing 150–200 g (n = 60) were obtained from the animal center of Huazhong University of Science and Technology. After anesthesia, the eyes were removed, and the retinas were harvested and homogenized by two gentle up-and-down strokes in a 15 mL homogenizer (Dounce; Belloco Glass, Vineland, NJ). The homogenate was filtered through an 88 μm sieve. The retentate was digested in 0.066% collagenase for 45 min at 37°C. The homogenate was centrifuged (1000 x g for 10 min), and the pellet was resuspended in endothelial basal growth medium (Invitrogen-Gibco, Grand Island, NY), supplemented with 20% fetal bovine serum, 50 U/mL endothelial cell growth factor (Sigma-Aldrich, St. Louis, Mo), and 1% insulin-transferrin-selenium. RRCECs were cultured in fibronectin-coated dishes and incubated at 37°C in a humidified atmosphere containing 5% CO2.

Cultured endothelial cells were characterized by evaluating expression of factor VIII antigen (von Willebrand factor) and determining unchanged morphology under culture
Table 1: Q-PCR arrays showed that the expression of the ER stress factor had significant differences in the first and the third month in diabetic rat retina: The ER stress factor of differential gene expression in the first month.

| Symbol | Gene name | The average ratio of gene expression | t-test P value |
|--------|-----------|--------------------------------------|---------------|
| CCT4   | Cctd      | 9.50E−02 5.90E−02 | 0.0134 |
| DNAJB9 | Erdj4     | 4.10E−02 6.20E−02 | 0.0125 |
| DNAJC3 | PSIPK     | 1.70E−02 3.00E−02 | 0.0173 |
| Casp12 | Casp12    | 3.20E−04 1.30E−03 | 0.002 |
| ERP44  | Pdia10    | 2.10E−02 2.50E−02 | 0.0337 |
| GANAB  | GluII     | 8.10E−02 1.40E−01 | 0.0457 |
| HERPUD1| Herp      | 1.50E−01 2.60E−01 | 0.0006 |
| HSPA1L | Hsp70-3   | 6.30E−04 1.00E−03 | 0.0183 |
| HSPA2  | Hsp70     | 7.30E−03 1.30E−02 | 0.0183 |

Table 2: Q-PCR arrays showed that the expression of the ER stress factor had significant differences in the first and the third month in diabetic rat retina: The ER stress factor of differential gene expression in the third month.

| Symbol | Gene name | The average ratio of gene expression | t-test P value |
|--------|-----------|--------------------------------------|---------------|
| ATF4   | CREB-2    | 8.80E−01 1.60E+00 | 0.0178 |
| DNAJB9 | Erdj4     | 4.10E−02 5.40E−02 | 0.0106 |
| ERO1L  | Ero1      | 9.50E−03 1.20E−02 | 0.0492 |
| TRB3   | Trib3     | 5.80E−03 6.10E−02 | 0.0024 |
| HERPUD1| Herp      | 7.20E−02 2.80E−01 | 0.0008 |
| HTRA2  | PARK13    | 2.20E−02 4.00E−02 | 0.0064 |
| PPIA   | CYPA      | 4.30E−01 6.50E−01 | 0.0238 |
| SREBF1 | SREBP1    | 2.10E−02 2.80E−02 | 0.0187 |
| SYVN1/Hrd1 | HRD1    | 7.90E−02 1.20E−01 | 0.0067 |
| UFD1L  | UFD1      | 1.60E−01 8.00E−02 | 0.0463 |
| UGCGL1 | HUGT1     | 9.80E−03 2.10E−02 | 0.0833 |
| USP14  | TGT       | 7.30E−02 4.80E−02 | 0.0405 |

Table 3: Q-PCR arrays showed that the expression of the ER stress factor had significant differences in the first and the third month in diabetic rat retina: the ER stress factor of significant differences belongs to different ER stress signaling pathways.

| Signaling pathway              | First month                  | Third month                  |
|--------------------------------|------------------------------|------------------------------|
| Unfolded protein binding       | Cctd, ERdj4, Hsp70-3         | ERdj4, OMI/PARK13, CYPA, HUGT1|
| ER protein folding quality control | GluII, Pdia10               | HUGT1                        |
| Regulation of cholesterol Metabolism | —                           | SREBP1                       |
| Regulation of translation      | —                            | —                            |
| ERAD                           | Herp, NUC, Os9, ADO15        | Herp, OMI/PARK13, Hrd1       |
| Ubiquitination                 | Herp                         | Herp, UFD1, TGT              |
| Transcription factors          | —                            | ATF4, SREBP1                 |
| Protein folding                | Cctd, ERdj4, APG-1, Pdia10   | ERdj4, Ero1, CYPA            |
| Protein disulfide isomerization | Pdia10                      | SREBP1                       |
| Heat shock proteins            | ERdj4, PSIPK, Hsp70-3        | ERdj4                        |
| Apoptosis                      | JNK/JNK1, Casp12             | OMI/PARK13, NIPK/Trib3       |
Table 4: Q-PCR array gene table. We selected 89 ER stress-related factors, and other 6 genes as a quality control a total; of 96 genes were detected in Q-PCR arrays.

| A01  | Rn.107561 | XM_341644 | AMFR | Autocrine motility factor receptor | AMFR |
| A02  | Rn.161941 | NM_001108183 | ARMET | Arginine rich, mutated in early-stage tumors | ARMET |
| A03  | Rn.2423 | NM_024403 | ATF4 | Activating transcription factor 4 (tax-responsive enhancer element B67) | CREB-2/CREB2 |
| A04  | Rn.222130 | NM_001107196 | ATF6 | Activating transcription factor 6 | ATF6A |
| A05  | Rn.18179 | NM_001002809 | ATF6B | Activating transcription factor 6 beta | CREB-RP/CREBL1 |
| A06  | Rn.42932 | NM_021702 | ATXN3 | Ataxin 3 | AT3/ATX3 |
| A07  | Rn.10668 | NM_017059 | BAX | BCL2-associated X protein | BCL2LA |
| A08  | Rn.974 | NM_022399 | CALR | Calreticulin | CRT/RO |
| A09  | Rn.1762 | NM_172008.2 | CANX | Calnexin | CNX/IP90 |
| A10  | Rn.97889 | NM_182814.2 | CCT4 | Chaperonin containing TCP1, subunit 4 (delta) | CCT-Delta/Cctd |
| A11  | Rn.62267 | NM_001106603.1 | CCT7 | Chaperonin containing TCP1, subunit 7 (eta) | CCT-Eta/Cet7 |
| A12  | Rn.6479 | NM_001106603.4 | CEBPB | CCAAT/enhancer binding protein (C/EBP), beta | C/EBP-beta |
| B01  | Rn.104043 | NM_001013092.1 | CREB3 | CAMP responsive element binding protein 3 | LUMAN/LZIP |
| B02  | Rn.20059 | NM_001012115.1 | CREB3L3 | CAMP responsive element binding protein 3-like 3 | CREB-H/CREBH |
| B03  | Rn.11183 | NM_001109986 | DDIT3 | DNA-damage-inducible transcript 3 | CEBPZ/CHOP |
| B04  | Rn.110990 | NM_001014202.1 | DERL1 | Der1-like domain family, member 1 | DER-1/DER1 |
| B05  | Rn.11209 | NM_031627 | CHOP | Rattus norvegicus nuclear receptor subfamily 1, group H, member 3 | LXRalpha/Nr1h3 |
| B06  | Rn.40780 | NM_001109541 | DNAJB2 | DnaJ (Hsp40) homolog, subfamily B, member 2 | HSJ1/HSPF3 |
| B07  | Rn.29778 | NM_012699 | DNAJB9 | DnaJ (Hsp40) homolog, subfamily B, member 9 | DKFZp564F1862/ErnJ4 |
| B08  | Rn.8642 | NM_001106486 | DNAJC10 | DnaJ (Hsp40) homolog, subfamily C, member 10 | DKFZp434J1813/ErnJ5 |
| B09  | Rn.162234 | NM_022232 | DNAJC3 | DnaJ (Hsp40) homolog, subfamily C, member 3 | HP58/5P8 |
| B10  | Rn.91398 | NM_001013196 | DNAJC4 | DnaJ (Hsp40) homolog, subfamily C, member 4 | DANJC4/HSPF2 |
| B11  | Rn.107459 | NM_001033909 | Elf2 | E74-like factor 2 | Elf2 |
| B12  | Rn.81078 | NM_130422 | Casp12 | Caspase 12 | Casp12 |
| C01  | Rn.198593 | NM_001109339 | eIF2A | Eukaryotic translation initiation factor 2A, 65 kDa | CDA02/EIF-2A |
| C02  | Rn.24897 | NM_031599 | EIF2AK3 | Eukaryotic translation initiation factor 2-alpha kinase 3 | DKFZp781H1925/HRI |
| C03  | Rn.19198 | NM_001037208 | CRELD2 | Cysteine-rich with EGF-like domains 2 | CrelD2 |
| C04  | Rn.218563 | XM_344959.3 | ERN2 | Endoplasmic reticulum to nucleus signaling 2 | Ern2 |
| C05  | Rn.64648 | NM_138528 | ERO1L | ERO1-like (S. cerevisiae) | Ero1 |
| C06  | Rn.22325 | NM_144755 | TRB3 | Tribbles homolog 3 | NIPK/Trib3 |
| C07  | Rn.2459 | NM_001008317 | ERP44 | Thioredoxin domain containing 4 (endoplasmic reticulum) | PDIA10/TXNDC4 |
| C08  | Rn.57325 | NM_138917 | FBXO6 | F-box protein 6 | FBG2/FBS2 |
| C09  | Rn.99241 | NM_001106334 | GANAB | Glucosidase, alpha; neutral AB | G2AN/GluII |
| C10  | Rn.23744 | NM_001145840 | GANC | Glucosidase, alpha; neutral C | MGC138256 |
| C11  | Rn.4028 | NM_053523 | HERPUD1 | Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 | Sup |
| C12  | Rn.1950 | NM_212504 | HSPA1B | Heat shock 70 kDa protein 1B | HSP70-1B/HSP70-2/Hsp72 |
| D01  | Rn.187184 | NM_212546 | HSPA1L | Heat shock protein 1-like | Hsp70-3/MGC112562/MGC114222 |
| D02 Rn.211303   | NM_021863 | HSPA2 | Heat shock protein 2          | Hsp70/Hst70/MGC93458 |
| D03 Rn.163092   | NM_153629 | HSPA4 | Heat shock protein 4          | Hsp110/ Hsp70/irp94 |
| D04 Rn.144829   | NM_001106428 | HSPA4L | Heat shock protein 4-like | APG-1; MGC187594; OSP94 |
| D05 Rn.11088    | NM_013083 | HSPA5 | Heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa) | BIP/GRP78 |
| D06 Rn.37805    | NM_001011901 | HSPH1 | Heat shock 105 kDa/110 kDa protein 1 | DKFZp686M05240/HSP105 |
| D07 Rn.107325   | NM_001106599 | HTRA2 | Htra serine peptidase 2 | OMI/PARK13 |
| D08 Rn.163330   | NM_001107321 | HTRA4 | Htra serine peptidase 4 | FLJ90724 |
| D09 Rn.772      | NM_022392 | INSIG1 | Insulin-induced gene 1 | CL-6 |
| D10 Rn.16736    | NM_178091 | INSIG2 | Insulin-induced gene 2 | MGC26273 |
| D11 Rn.9911     | NM_012806 | MAPK10 | Mitogen-activated protein kinase 10 | JNK/JNK3A |
| D12 Rn.4090     | XM_001056513 | MAPK8 | Mitogen-activated protein kinase 8 | JNK/JNK1 |
| E01 Rn.9910     | NM_017322 | MAPK9 | Mitogen-activated protein kinase 9 | JNK-55/JNK2 |
| E02 Rn.2362     | NM_053569 | MBTPS1 | Membrane-bound transcription factor | PCSK8/SIP |
| E03 Rn.212224   | NM_001035007 | MBTPS2 | Membrane-bound transcription factor | S2P |
| E04 Rn.144645   | NM_080577 | NPLC4 | Nuclear protein localization 4 homolog (S. cerevisiae) | NPL4 |
| E05 Rn.1492     | NM_053463 | NUCB1 | Nucleobindin 1 | DKFZp686A15286/NUC |
| E06 Rn.1579     | NM_001007265 | OS9 | Osteosarcoma amplified 9, endoplasmic reticulum associated protein | OS-9 |
| E07 Rn.11527    | NM_017319 | PIA3 | Protein disulfide isomerase family A, member 3 | ER60/EpSP7 |
| E08 Rn.7627     | NM_001109476 | PFN2 | Prefoldin subunit 2 | PF2 |
| E09 Rn.3401     | NM_001106794 | PFN5 | Prefoldin subunit 5 | MM-1/MM1 |
| E10 Rn.1463     | NM_017101 | PPIA | Peptidylprolyl isomerase A (cyclophilin A) | CYP3/CYPF |
| E11 Rn.2232     | NM_133546 | PPP1R15A | Protein phosphatase 1, regulatory (inhibitor) subunit 15A | GADD34 |
| E12 Rn.104417   | NM_001106806 | PRKCSH | Protein kinase C substrate 80K-H | AGE-2/G19P1 |
| F01 Rn.209127   | NM_001127545 | RNF139 | Ring finger protein 139 | HRCA1/RCA1 |
| F02 Rn.209127   | NM_006913 | RNF5 | Ring finger protein 5 | RING5/R5A1 |
| F03 Rn.4224     | NM_013067 | RP1 | Ribophorin I | DKFZp686B16177/OST1 |
| F04 Rn.99548    | NM_001109699 | SCAP | SREBP chaperone | KIAA0199 |
| F05 Rn.98327    | NM_001034129 | SEC62 | SEC62 homolog (S. cerevisiae) | Tdrp1/HTP1 |
| F06 Rn.24233    | NM_001107637 | SEC63 | SEC63 homolog (S. cerevisiae) | ErbD2/PRO2507 |
| F07 Rn.20802    | NM_177933 | SEL1 | Sel-1 suppressor of lin-12-like (C. elegans) | IBD2/PRO1063 |
| F08 Rn.4197     | NM_173120 | SELS | Selenoprotein S | AD-015/AD015 |
| F09 Rn.2119     | NM_030835 | SERP1 | Stress-associated endoplasmic reticulum protein 1 | RAMP4 |
| F10 Rn.103851   | NM_199376 | SIL1 | SIL1 homolog, endoplasmic reticulum chaperone (S. cerevisiae) | BAP/MSS |
| F11 Rn.221929   | XM_001075680 | SREBF1 | Sterol regulatory element binding transcription factor 1 | SREBP-1c/SREBP1 |
| F12 Rn.41063    | NM_001033694 | SREBF2 | Sterol regulatory element binding transcription factor 2 | SREBP2/bHLHd2 |
| G01 Rn.162486   | NM_001100739 | SYV1 | Synovial apoptosis inhibitor 1, synoviolin | HRD1 |
| G02 Rn.7102     | NM_012670 | TCP1 | T-complex 1 | CCT-alpha/CCT1 |
| G03 Rn.20041    | NM_153303 | TOR1A | Torsin family 1, member A (torsin A) | DQ2/DYT1 |
| G04 Rn.139603   | NM_001106380 | UBE2G2 | Ubiquitin-conjugating enzyme E2G 2 (UBC7 homolog, yeast) | UBC7 |
| G05 Rn.106299   | NM_001007655 | UBE2J2 | Ubiquitin-conjugating enzyme E2, J2 (UBC6 homolog, yeast) | NCUBE2/PRO2121 |
| G06 Rn.2022     | NM_001012025 | UBXN4 | UBX domain protein 4 | UBXD2/UBXD1 |
| G07 Rn.11946    | NM_053418 | UFD1L | Ubiquitin fusion degradation 1-like (yeast) | UFD1 |
Table 4: Continued.

| Gene ID  | Rn.162227 | NM_133596 | UGCGL1 | UDP-glucose ceramide glucosyltransferase-like 1 | HUGT1 |
|---------|-----------|-----------|--------|-----------------------------------------------|-------|
| Gene ID  | Rn.107678 | NM_019381 | BI-1   | Transmembrane BAX inhibitor motif containing 6 | Tmbim6 |
| Gene ID  | Rn.11790  | NM_001008301 | USP14 | Ubiquitin-specific peptidase 14 (tRNA-guanine transglycosylase) | TGT |
| Gene ID  | Rn.98891  | NM_053864 | VCP    | Valosin-containing protein | IBMPFD/TERA |
| Gene ID  | Rn.101044 | NM_001004210 | XBP1  | X-box binding protein 1 | TREB5/XBP2 |
| Gene ID  | Rn.973    | NM_001007604 | Rplp1  | Ribosomal protein, large, P1 | MGC72935 |
| Gene ID  | Rn.47     | NM_012583 | Hprt   | Hypoxanthine guanine phosphoribosyl transferase | Hgprtase/Hprt1 |
| Gene ID  | Rn.922111 | NM_173340 | Rpl13a | Ribosomal protein L13A | Rpl13a |
| Gene ID  | Rn.107896 | NM_017025 | Ldha   | Lactate dehydrogenase A | Ldh1 |
| Gene ID  | Rn.94978  | NM_031144 | Actb   | Actin, beta | Actx |
| Gene ID  | N/A       | U26919    | RGDC   | Rat genomic DNA contamination | RGDC |
| Gene ID  | N/A       | SA_00104  | RTC    | Reverse Transcription Control | RTC |
| Gene ID  | N/A       | SA_00104  | RTC    | Reverse transcription control | RTC |
| Gene ID  | N/A       | SA_00104  | RTC    | Reverse transcription control | RTC |
| Gene ID  | N/A       | SA_00103  | PPC    | Positive PCR control | PPC |
| Gene ID  | N/A       | SA_00103  | PPC    | Positive PCR control | PPC |
| Gene ID  | N/A       | SA_00103  | PPC    | Positive PCR control | PPC |

Figure 1: Assessment of the expression of ER stress-related factors in diabetic retinas in the first and third months after the development of diabetes by Q-PCR arrays. (a) the histogram of the expression of different genes in 11 signaling pathways related to ER stress after the first month; (b) the histogram of the expression of different genes in 11 signaling pathways related to ER stress after the third month. Unfolded Protein Binding: UPB, ER Protein Folding Quality Control: ERPFQC, Regulation of Cholesterol Metabolism: RCM, ER-associated degradation: ERAD, Ubiquitination: Ub, Transcription Factors: TF, Protein Folding: PF, Protein Disulfide Isomerization: PDI, Heat Shock Proteins: HSP, Apoptosis: Ap°.

2.6. Cell Immunofluorescence. The RRCECs were grown in 24-well plates in human endothelial serum-free material basal growth medium containing 8.3 mM glucose. Upon attaining 80%, confluency cells were treated with medium containing 25 mM glucose for 2 d. Cells were then fixed by light microscopy. The expression of acetyl-LDL (Ac-LDL) receptors in endothelial cells was measured by adding fluorescence-labeled AC-LDL (Biomedical Technologies, Palatine, IL). Only cells from passages 3 to 7 were used in the experiments.
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![Image](image.png)

**Figure 2**: Western blot and immunofluorescence detected the expression of HERP and HRD1 in the first month after the development of diabetes: (a) Western blot detection of the expression of HERP and HRD1 in the first month. The expression of HERP in the diabetic group was less than that of the control group ($P = 0.004$); Hrd1 expression was similar in both groups ($P = 0.338$). (b) Immunofluorescence detection of the expression of HERP and HRD1 in the first month. The expression of HERP in the diabetic group was less than that of the control group ($P = 0.008$) Hrd1 expression was similar in both groups ($P = 0.572$).

with 4% formaldehyde for 15 min and permeabilized in 0.1% Triton X-100 for 10 min. Cells were incubated with primary antibody at 4°C overnight followed by secondary antibody for one hour. The slides were visualized and photographed under a fluorescence microscope (Olympus, Hamburg, Germany).

2.7. Statistical Analysis. Normally distributed data were compared using Student’s independent samples t-test or one-way ANOVA where appropriate. When a significant difference was detected between groups, multiple comparisons of means were performed using the Bonferroni procedure, with type-I error rate at a maximum of 0.017 (0.05/3) adjustment. Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) 15.0 software (SPSS, Chicago, IL). Data were presented as the mean $\pm$ standard deviation (SD). A probability ($P$) value $< 0.05$ was considered statistically significant.

3. Results

3.1. Q-PCR Arrays. We detected 89 ER stress-related genes and found that the mRNA levels of 13 genes in the diabetic rats changed significantly during the first month (Table 1). We found that in the third month the levels of expression of 12 genes were changed significantly in these diabetic rats (Table 2). The changes in the expression levels of genes corresponded to 8 and 10 categories of signal pathways in the first and third months, respectively (Figure 1 and Table 3). The mRNA expressions of *Erdj4* and *HERP* were lower both in the first and third months.
3.2. Expression of HERP and HRD1 in the Retinas of Diabetic Rats. We detected HERP and Hrd1 protein expression levels in the retinas of diabetic rats by Western blot and immunofluorescence in the first and the third months of diabetes development. The Western blot suggested that the HERP expression decreased significantly in the first month ($P = 0.004$) and third month ($P = 0.012$) compared with the nondiabetic control group. No significant change in the expression level of Hrd1 was observed in the first month ($P = 0.338$), while it decreased significantly in the third month ($P = 0.001$; Figures 2 and 3).

The results of immunofluorescence were consistent with the Western blot. The protein level of HERP decreased significantly at both the first and third months ($P = 0.008$ and 0.007, resp.; Figures 2 and 3). There was no significant change in the expression of retinal HRD1 in the first month, while it decreased significantly in the third month ($P = 0.572$ and 0.003, resp. Figures 2 and 3).

3.3. Expression of HERP and HRD1 in RRCECs in the Presence of High-Glucose Concentration. The expression levels of HERP and Hrd1 in RRCECs in vitro in the presence of high glucose concentration were decreased significantly compared to the control group ($P = 0.013$ and 0.024, resp.; Figure 4).

4. Discussion

The STZ-induced rat diabetes model is an established animal model for studying DR. Although we did not verify the development of DR in this study, our previous studies and the publication from another group have demonstrated that DR develops within one month of STZ-induced diabetes.
Figure 4: Immunofluorescence detection of the expression of HERP and HRD1 in RRCECs. The expression of HERP in the high glucose group was decreased compared to the control group, $P = 0.013$. The expression of HRD1 in the high glucose group was also decreased compared to the control group, $P = 0.024$.

[13, 22, 23]. Our results indicate that of 89 ER stress genes, the expression of 12 genes in the retinas of diabetic rats was downregulated by the third month of diabetes development, and the expression of CCT4 increased within the first month. We did not observe any change in the expression of AFT4 or GFP78 at either time point in our study, which is consistent with our earlier results [13].

The expression of genes belonging to 8 different categories of ER stress factors was altered in the first month, while those of 10 categories were changed by the third month, suggesting that with increasing time more categories of ER stress factors were involved in the pathogenic process of DR. The expression of a number of related factors of the ERAD signaling pathways was downregulated, indicating that the ERAD signaling pathway may play an important role in DR. The ERAD system is an important pathway of protein degradation in the ER [25, 26] and plays important physiological roles. The ER is the location of protein synthesis, and secretion [27, 28] and has strict quality control mechanisms which allow secretion of correctly folded protein into the cytoplasm. The wrongly folded protein will be degraded through ERAD. ERAD therefore is a quality control system of the ER.

Recent studies found that HRD1 plays a central role in the ERAD-luminal pathway [29] and that HERP coordinates and regulates HRD1-mediated ubiquitylation [28], so we selected HRD1 and HERP from the ERAD pathway for further study. HERP expression was downregulated significantly in the retinas of diabetic rats in the first and third months. HERP is a membrane-bound, ubiquitin-like protein that is located in the ER. It forms a complex with ubiquitinated proteins and with the 26S proteasome [30–33]. HERP functions to degrade wrongly folded nonglycosylated proteins by forming a protein-enzyme complex with Derlin-1, HRD1, and p97 [34]. In our study, HRD1 expression in the retinas of diabetic rats remained unchanged in the first month, while it decreased in the third month. HRD1 is an E3 ubiquitin ligase and a key factor of ERAD [35–37]. ERAD has three pathways in yeast [38]: ERAD-L, ERAD-M, and ERAD-C. Both ERAD-L and ERAD-M are the key enzymes of HRD1. In the mammalian ERAD, HRD1 plays a very broad role in the ubiquitination process of abnormal proteins in the ER. The ubiquitin ligase HRD1 is mainly involved in the degradation of glycosylation proteins [39–41].

The decreased expression of HERP and HRD1 at both the mRNA and protein levels could lead to a decrease in function of ERAD’s ability to remove wrongly folded proteins in the cell. Misfolded protein accumulation in the ER induces ER stress and activates signaling pathways, including PERK,
ATF6, and IRE1 [15]. Persistent ER stress leads to cell death and induction of inflammation [42–45]. An inflammatory milieu is instrumental in breaking down the blood-retinal barrier in DR [46, 47].

In conclusion, we have shown by in vivo and in vitro experiments that an elevated concentration of glucose leads to downregulation of the ERAD signaling pathway. Such downregulation may result in local inflammation and DR.

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