Biomedical Research 2008; 19 (2): 117-124

Quantification of Zonula Occludens-1 *mRNA* expression in cultured bovine retinal endothelial cells, using SYBR green real-time PCR

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Keywords: Diabetes; Bovine retinal endothelial cells; Zonula Occludens-1; Real time reverse transcription polymerase chain reaction

Accepted Aprl 17 2008

Abstract

A simple and rapid real-time reverse transcription-PCR (RT-PCR) technique using SYBR green fluorescence was developed to quantify Zonula Occludens-1 (ZO-1) mRNA expression in histamine-treated cultured bovine retinal microvessel endothelial cells. There was no difference between the quantity of ZO-1 mRNA expression in histamine-treated (10^{-7}M to 10^{-9} M) and untreated bovine retinal endothelial cells at high magnesium levels. Immunohistochemistry staining and western blot assay unequivocally demonstrated the approximately similar levels of ZO-1 protein in both histamine-treated and non-treated endothelial cells at high magnesium concentrations.

Introduction

Zonula Occludens-1, a serine phosphorylated protein with approximately 225 kDa, is the main peripheral membrane protein associated with tight junctions [1]. There are two isoforms of ZO-1, namely, ZO-1 a^+ and ZO-1 a^- that differ by the presence or absence of an 80 amino acid region [2]. ZO-1 has been suggested to play a crucial role in the regulation of microvascular permeability and development of retinopathy in diabetic patients partly due to histamine and other conditions prevalent in diabetes [3, 4]. Since evidence suggests that high magnesium concentrations may be beneficial for endothelial function, we were particularly interested in ZO-1 expression by endothelial cells at high magnesium concentrations.

It this study we developed a simple and precise method to quantify ZO-1. Gardner et al used the western blot to quantify ZO-1 in histamine-treated bovine retinal capillary endothelial cells [3]. Takeuchi et al. used the conventional reverse transcriptase PCR to study the ZO-1 mRNA expression in histamine-treated cultured human nasal epithelial cells [5]. In the present study, we developed the real-time PCR method which is fast, accurate and sensitive to study the expression of ZO-1 at high concentrations of magnesium ions. To the best of our knowledge, this study is the first attempt to quantify the ZO-1 mRNA expression using real-time PCR.

Materials and Methods

Bovine Retinal Microvessel Endothelial Cells (BREC)

The BREC line was obtained from VEC Technologies (New York) and was cultured according to the protocol suggested by the manufacturers. Briefly, the cells were cultured in fibronectin (50 μg/ml)-coated flasks and was supplemented with MCDB-131 complete medium [6]. BREC from the 4^{th} passage were used throughout our study. Confluent BREC were treated with different concentrations of histamine after tenfold dilutions of a stock solution of 0.1M (Sigma Chemical Company, St. Louis).

The above cells were incubated at 37°C for 5 hours with 5% CO_2. The treated and untreated cells were then washed with PBS (0.15M) and scraped prior to the isolation of total RNA and immunohistochemical staining.

Total RNA and mRNA Isolation

Total RNA was isolated from the cells pellet using the High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim) according to the instruction in the manual. In brief, washed cells were spun, resuspended in PBS and lysed with lysis-binding buffer. Cells were subsequently treated with DNase to remove contaminated genomic DNA and the RNA was eluted with the elution buffer. Quality and quantity of the extracted RNA were assessed by measuring A_{260}/A_{280} ratio and the value at A_{260} nm, respectively. We did not use the RiboGreen assay to determine the concentration of the RNA since the extracted RNA was more than 100 ng/µl [7]. The ZO-1 mRNA isolation was performed using the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Mannheim) according to the instruction manual. DNase treatment was performed to avoid the co-amplification of genomic DNA. Random hexamers were used to isolate the mRNA since it primes throughout the whole length of all the RNAs and at the same time allows the reverse transcription of the RNAs that do not carry a poly (A) tail. A total of two microliter of cDNA were used for real-time RT-PCR throughout the study.

Real-Time Quantitative RT-PCR

We performed real-time quantitative RT-PCR in a LightCycler® 2.0 (Roche Diagnostics, Mannheim), using Failsafe Real-time PCR Capillary Premix Selection kit according to the instructions in the manual (EPICENTRE, Madison). Primers used for the PCR are listed in Table 1b. The primers were designed using the Primer 3 software [8] and FastPCR [9]. For the Primer 3 software, the default parameters of the program were applied, except

Table 1a. PCR conditions used for real-time PCR

Program	Temperature/temperature transition rate (°C/s)	Incubation Time
Seek temperature	Set to temperature correspond to annealing temperature of the primers	-
Initial denaturation	95°C/20.0	60 sec
	35 cycles of:	
Denaturation	95°C/20.0	1 sec
Annealing	59°C for ZO-1;61°C for beta-actin/20.0	5 sec
Extension	72°C/2.0	13 sec

Melting curve analysis at 65°C– 95°C with 0.1°C/s temperature transition rate
Cooling at 40°C for 30 sec at 20.0°C/s

Table 1b: Primer sequences used in real time PCR

Gene (expected size of amplicon)	Accession Number	Primer Sequences (5'-3')
ZO-1 (211 bp)	AJ313183	Forward: **cga cca gat cct cag ggt aa** (Corresponds to nucleotide position 40-59 of bovine ZO-1 mRNA) Reverse: **tcc ata ggg aga ttc ctt ctc a** (Corresponds to nucleotide position 250-229 of bovine ZO-1 mRNA)
Beta-actin (120 bp)	AY141970	Forward: **cac cac acc ttc tac aac gag c** (Corresponds to nucleotide position 298-319 of bovine beta-actin mRNA) Reverse: **ggt ctc gaa cat gat ctg ggt c** (Corresponds to nucleotide position 417-396 of bovine beta-actin mRNA)
18S rRNA (108 bp)	AF176811	Forward: **aaa cgg cta cca cat cca ag** (Corresponds to nucleotide position 58-77 of bovine 18S rRNA mRNA) Reverse:**tac agg gcc tcg aaa gag tc** (Corresponds to nucleotide position 165-146 of bovine 18S rRNA mRNA)

for the following changes: Product size for ZO-1=200-250 bp; beta-actin and 18S rRNA =100-150 bp. Primer T_m=58-62°C with a max T_m difference of 2.0°C, max self and 3' self complementary= 6.0 and max poly-X=3. For the FastPCR software, the highest efficiency ranking of the primers, particularly reverse primers were manually chosen for the same amplicon size as designed using Primer3 software. The amplicon lengths were kept between 100-300 bp to ensure highest PCR efficiency [10] and reproducibility [11].

A preliminary run was performed to determine the optimal premix. The PCR reaction was carried out in a 20.0 µl final volume. The real-time PCR conditions were as follows;

The size of the PCR product was confirmed by agarose gel electrophoresis (2% w/v). Melting curve analysis was performed at the end of each run to confirm the specificity of amplification [12]. The final concentrations of the primers used were 0.625 µM for ZO-1 and 0.3125 µM for beta-actin and 18SrRNA for both RT-PCR. RT- (samples containing RNA which was not reverse transcribed) was included to assess the amplification of contaminating genomic DNA [10].

The acquisition mode was single at the end of each extension and the measurement of the fluorescence was done at 530 nm.

Standard Curve Construction and Calculation

The amplified PCR products were purified using GENEALL PCR Purification Kit (General-Biosystem, Seoul) sequenced and confirmed its identity against the available sequences. A ten-fold serial dilutions of a purified DNA were used to construct a standard curve as previously described [13, 14]. The dilutions were made to cover the range of Cp (Cross-Point or Ct, threshold cycle at which the fluorescence rises appreciably above the background noise) that included the Cp of target mRNA in the experiment to maximize the accuracy [11]. The mRNA levels were expressed in relative copy numbers normalized against beta-actin mRNA. The $\Delta\Delta C_t$ was determined using the method originally described by Stordeur [14]. The normalized ratio of ZO-1 in comparison to beta-actin was determined by the LightCycler software (Version 3.5).

ZO-1 Immunohistochemistry (IHC)

Scraped BREC cells were spun, washed with PBS and fixed in 10% buffered formalin for 3 days. The fixed cells were subsequently dehydrated with graded ethanols, cleared with xylene and embedded in paraffin. Five µm paraffin sections were cut and stained for ZO-1 protein by a standard Avidin-Biotin-peroxidase method. Prior to staining, the sections were treated with different concentrations of proteinase K at varying incubation times to determine the optimal conditions. The above method was compared with the heat-induced epitope retrieval method (microwave). The primary antibody was a rabbit polyclonal anti-ZO-1 (Zymed, San Franscisco), which was used at a dilution of 1:100-1:200 (v/v) and incubated at 37°C for 2 hours or 4°C, 18 hours. This antibody is known to react with both isoforms of ZO-1. Biotinylated, swine anti-rabbit was used as secondary antibody. The immunohistochemistry reaction was developed with 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St Louis) as chromogen. The sections were counterstained in Harris's hematoxylin, dehydrated through graded ethanols and mounted in DPX (BDH, London). Negative controls were performed by replacing the primary antibody with PBS and by omitting the secondary antibody.

Results

Preparation of total RNA

Total RNA was prepared from the histamine-treated and untreated BREC. All the extracted RNA samples were quantified using a spectrophotometer. The mean concentration was 686.6 ± 140.0 µg/ml and the mean A_{260}/A_{280} ratio was 1.84± 0.04 (mean ± SD).

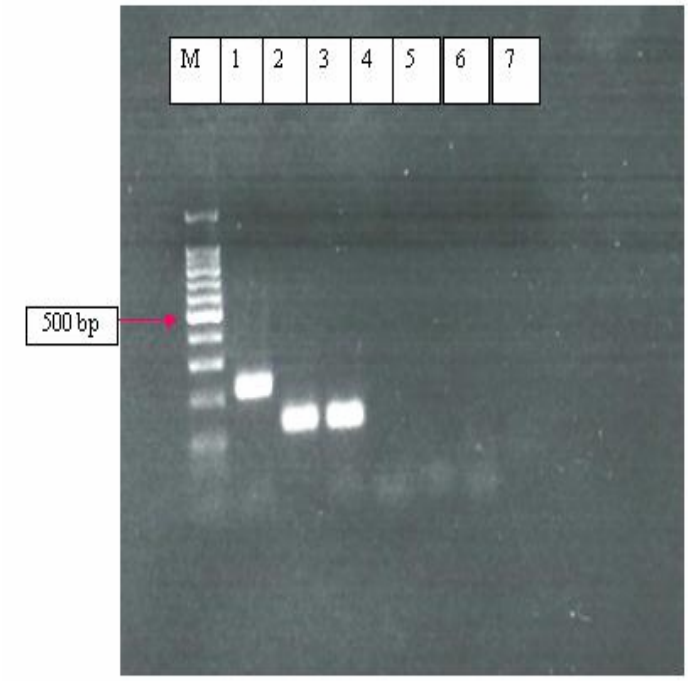

Fig. 1: *Agarose gel electrophoresis (2%, w/v) of bovine ZO-1 (lane 1, 211 bp), beta actin (lane 2, 120 bp), and 18S rRNA (lane 3, 108 bp). M is a 100 bp size marker; lanes 4, 5 and 6 are the negative controls of ZO-1, beta actin and 18S rRNA, respectively. Lane 7 is a reagent control.*

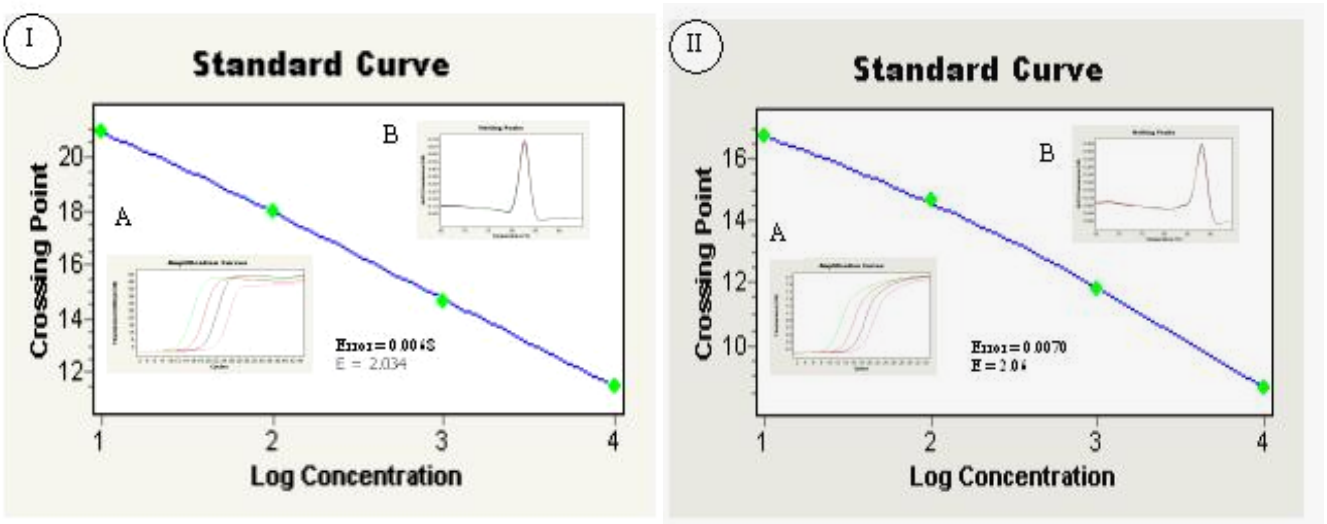

Fig. 2: *Standard curves of ZO-1 (I) and beta-actin (II). Inserts in each standard curve are the amplification curves (A) and melting peak (B) of each transcript. The linearity (1-eror) and PCR efficiency (E) as calculated by the LightCycler software was shown.*

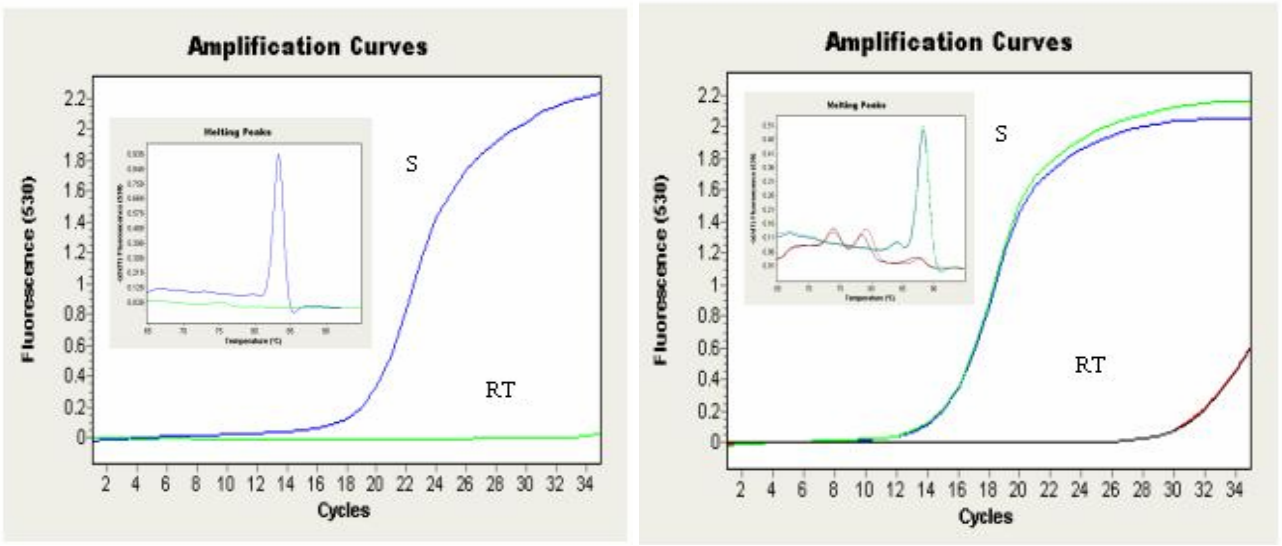

Fig. 3: *Amplification curves of ZO-1 (A) and beta-actin (B) in sample (S) and control sample without reversed transcribed (RT-) for the assessment of genomic DNA contamination. Inserts are the melting curve analysis of the amplifications.*

Primer design and specificity

Primer for ZO-1, beta-actin and 18S rRNA was designed using Primer3 and FastPCR software, and analyzed with free primer analysis software, NetPrimer (www. premier-biosoft.com/ netprimer/netprlaunch/ netprlaunch). The ZO-1 primers designed by Primer3 gave better results than that by FastPCR. However, the primers for beta-actin designed by FastPCR produced less primer-dimers than that designed by Primer3. Primers for 18S rRNA were equally good using both software (unpublished observations).

The designed primers were blasted with nucleotide BLAST (blastn, NCBI) [15] which resulted in highest score (bits) and lowest E value, indicating specificity of the primers. The available bovine mRNA partial sequence of ZO-1 share 93% similarity with that of human and located upstream of the 240 bp spilicing region (motif-α) of the ZO-1 mRNA transcript (unpublished data). Hence, the designed ZO-1 primers were unable to differentiate both isoforms.

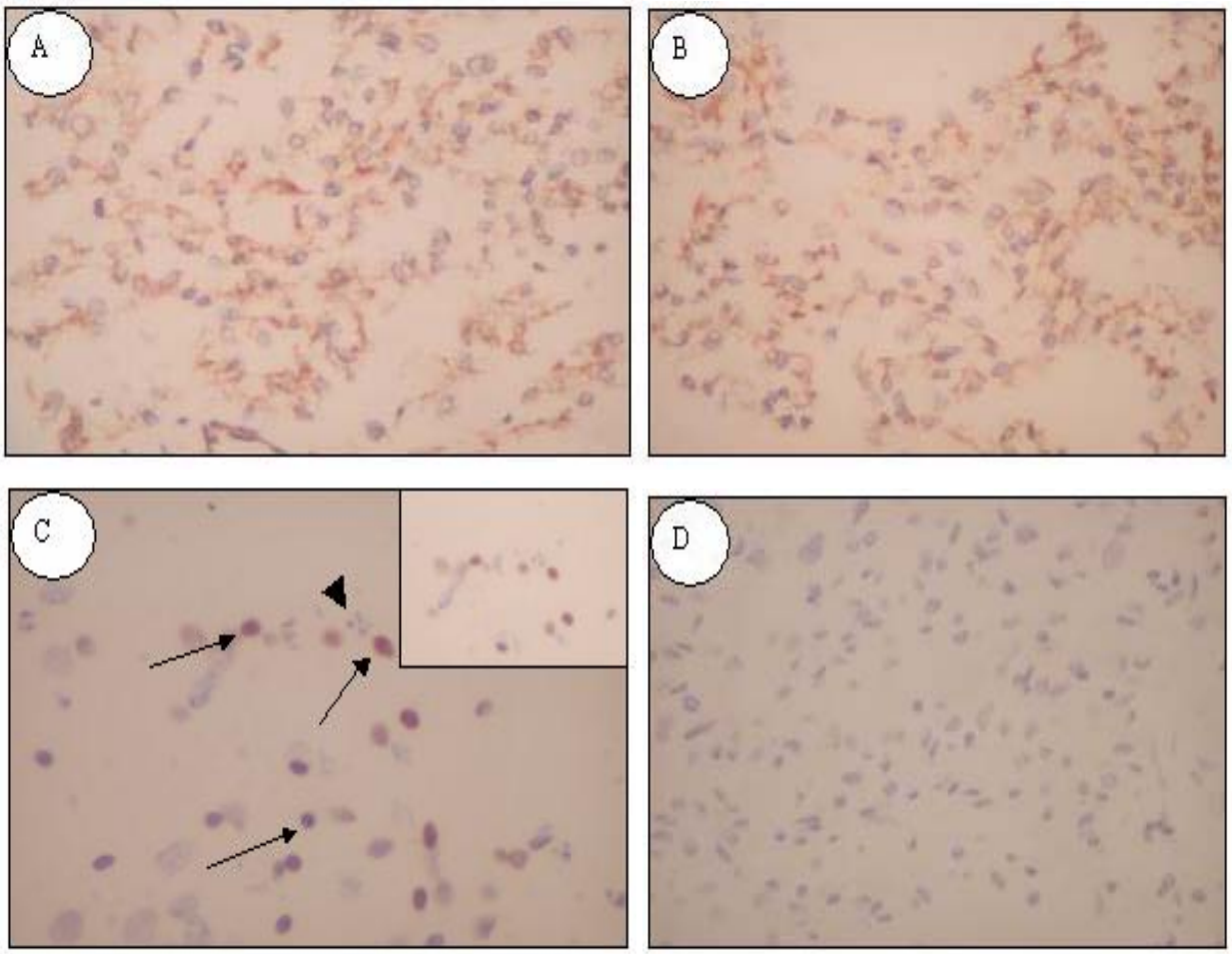

Fig. 4: *Immunohistochemistry staining of ZO-1 in (A)normal, (B)histamine-treated at 10^{-7}M, in (C)histamine-treated bovine retinal microvessel endothelial cells. (C)Occasionally nuclear localization of ZO-1 could be seen, especially at the free edge of cells (arrows) and in the proximity of apoptotic-like cells (arrowhead). Insert showed the higher magnification. No immunoreactivity of ZO-1 was seen in control negative (D).*

Ideally, the primers for the transcript should anneal with different exons or on an intron-exon junction to avoid the co-amplification of genomic DNA. Since the complete genome of the investigated transcripts was not available it was not possible to design such primers. DNase treatment was then performed, as recommended in the manual to prevent the genomic DNA contamination. The specificity of the PCR products was determined using gel electrophoresis, which resulted in a single product with the expected lengths (ZO-1, 211 bp; beta-actin, 120 bp and 18S rRNA, 108 bp) (figure 1). Melting curve analysis of the samples also resulted in a mean melting temperature, Tm = 82.73 ± 0.10 for ZO-1 and Tm = 88.31 ± 0.06 for beta-actin (mean ± SD). In our study, 18S rRNA was highly expressed (Cp= ~ 4-5) and could not be determined by the LightCycler software. Since the histamine had no effect on the beta actin (see later), it was used for normalization while quantifying ZO-1 for the subsequent analysis.

Real-time PCR amplification efficiencies and linearity

The PCR efficiency (E) of ZO-1 and beta-actin were 2.03 and 2.06, respectively with high Pearson correlation coefficient, R^2 (1-error) = 0.993 for both transcripts as calculated by the LightCycler software Version 3.5(figure 2).

Real-time quantitative RT-PCR

Table 2 showed the results obtained by real-time PCR. No change in ZO-1 mRNA expression was apparent since the fold of change (normalized ratio) was less than 2-folds. In order for the differences to be significant, at least two-fold changes in normalized ratio in mRNA expression (up-regulation) or 0.5 (down-regulation) must be taken into consideration [16].

Table 2: Relative copy numbers of ZO-1 normalized against beta-actin, was calculated using the formulae described by Stordeur et al. [14], for the different concentrations of histamine treatment. The normalizedratio was calculated by the LightCycler Software (Version, 3.5) with untreated BREC as a calibrator.

Treatment	Relative copy numbers of ZO-1 (%) *(Stordeur et al. 2002)*	Normalized ratio *(LightCycler Software, Version 3.5)*
10^{-7}M His	104.17	0.96
10^{-8}M His	107.78	1.11
10^{-9}M His	110.00	1.12

Validation with Housekeeping Gene

Housekeeping gene allows normalization by standardizing the differing amounts of RNA, variation in nucleic acid recovery, pipetting errors, RNA degradation, and variation in cDNA synthesis. Ideally, housekeeping gene should remain invariant throughout the biological treatment and should have the similar expression level as the investigated RNA. Increasing number of reports have shown the effect of various treatments on the housekeeping genes [11,18-20]. Therefore, the validation of housekeeping gene is crucial to the accuracy of data. In our study, beta-actin and 18SrRNA were chosen as the housekeeping genes and the effect of histamine on them were examined. There was no difference between the histamine-treated and in the untreated BREC since the cross point values between them were similar ($C_{p\ \text{beta-actin}}$ = 13.64 ± 0.13, mean ± SD, n=5). It was not possible to calculate the Cp values of 18S rRNA by the software because of the high expression values (Cp~4-5). The Cp values were similar across the histamine treatment and in normal BREC, indicating no effect of histamine to the 18S rRNA expression (unpublished observations). For the subsequent studies, we used only beta-actin as housekeeping gene to normalize the ZO-1 since it is invariant throughout the histamine treatment and its Cp value was closer to ZO-1.

Immunohistochemistry of ZO-1 Protein

In order to determine the correlation between the ZO-1 mRNA expression and ZO-1 protein levels, we performed the immunohistochemical staining of ZO-1 protein in the paraffin sections. ZO-1 immunoperoxidase staining was treated with different concentrations of Proteinase K at varying incubation times to obtain maximum staining. We also microwaved the slides at a high temperature at different times and compared the staining with that of Proteinase K. A combination of microwave and Proteinase k were used in another set of slides. The optimal staining was obtained by Proteinase K treatment at 3 μg/ml (final concentration) for 15 minute at 37°C. Incubation step of ZO-1 primary antibody (dilution 1:100-1:200 v/v) either at 4°C for 18 hours or at 37°C for 2 hours gave the same results. Combination of Microwave followed by Proteinase K had no advantages (unpublished data). The ZO-1 immunoreactivity was mainly seen in apical and apicolateral of the membrane of fibroblast-like BREC cells, in the cytoplasm and occasionally in the nucleus (arrows in figure 3). The latter was seen exclusively at the free-edge of an island of cells and in the proximity of apoptotic-like bodies. The same nuclear accumulation of ZO-1 has been described by Gottardi et al. [21]. Interestingly, the fragmented nuclei of the apoptotic-like bodies also showed the immunoreactivity of ZO-1 (insert, figure 4 C). The significant of this finding is unknown. It is crucial that the cell density is high to obtain optimal staining and also to be aware that the staining could be different because of the different levels of exposure to the antigen. It was difficult to quantitate the staining since staining was not confined to one cell, and because the pattern of staining could be in diffused-type due to different planar sectioning. Although there could be some differences in BREC treated with different concentrations of histamine, nevertheless, no apparent difference was noted between normal and histamine-treated BREC in term of staining intensity and area of positivity at the concentration of magnesium used (Figure 4).

Discussion

In the present study, we developed a real-time RT-PCR method to study the ZO-1 mRNA expression in histamine-treated bovine retinal microvascular endothelial cells. Quantification of ZO-1 mRNA expression by conventional reverse transcriptase PCR has been described by Takeuchi et al. [5]. However, the real-time approach offers several advantages to study the gene expression compared to the conventional reverse transcriptase PCR. The method is simple, fast and does not require post-PCR manipulation.

To validate the results from real-time quantitative RT-PCR, we did an immunohistochemical staining on the cultured cells. The ZO-1 protein levels were the same for the different concentrations of histamine, indicating neither up-regulation nor down-regulation of ZO-1 mRNA expression. Some reports suggested that the protein levels correlated with the mRNA expression, while others were unable to prove the correlation [22-24]. In our study the results obtained from the immunohistochemical staining were in good agreement with that of mRNA expression.
Gardner et al. developed an in vitro system with retinal microvascular endothelial cells that mimicked the human environment of the eye [3]. They used standard Modified Eagle's medium that contained 0.07mM of magnesium. Takeuchi et al. used 0.7 mM Mg^{2+} in their study [5]. In our study, however, we used the complete medium MCDB-131 which contains 10mM magnesium [6]. The concentration of magnesium in our medium was more than that used by Gardner et al. and Takeuchi et al [5]. Magnesium has been reported to attenuate histamine-induced bronchoconstriction in asthmatic patients [25]. Magnesium is also known to be a natural suppressor of histamine action. We have demonstrated that at high magnesium concentrations histamine has little effect on the expression of ZO-1.

In conclusion, we have developed a simple and fast quantitative real-time RT-PCR for ZO-1 mRNA quantification. This technique should be of particular interest for those quantifying the ZO-1 mRNA and could provide an insight into the role of ZO-1 in the regulation of microvascular permeability.

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