The casein peptide NPWDQ enforces the intestinal tight junction partly by increasing occludin expression in Caco-2 cells

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; EMC, enzyme-modified cheese; JAM, junctional adhesion molecule; NPWDQ, Asn-Pro-Trp-Asp-Gln; TER, transepithelial resistance; TJ, tight junction; ZO-1, zonula occludens-1

Abstract

We have previously demonstrated that NPWDQ (Asn-Pro-Trp-Asp-Gln, amino acids 107–111 of αs_2-casein) inhibited allergen permeation, such as that demonstrated by ovalbumin, using Caco-2 cells as an *in vitro* human intestinal epithelial model and in mouse jejuna and ileac loops *ex vivo*. In the present study, the mechanism underlying this inhibitory activity was examined in Caco-2 cells. Transepithelial resistance value increased in response to the addition of increasing NPWDQ concentrations (10^{-6}–10^{-4} M), which suggested that this peptide enhanced epithelial barrier function. Next, changes in mRNA expression by the addition of NPWDQ (10^{-6} M) were analysed in Caco-2 cells using the microarray method. NPWDQ up-regulated the expression of the occludin gene in cells, but the level of genes of the claudin family and zonula occludens-1 was unchanged. Increased protein expression of occludin (but not claudin-1 nor zonula occludens-1 was also observed. Therefore, it was suggested that NPWDQ up-regulated the expression of occludin in particular and enforced the tight junction barrier. These data imply that a food-derived peptide can fine-tune the epithelial barrier.

Introduction

Allergic diseases are the most common of all immunologically mediated conditions and are increasing in prevalence in most developed countries. Food allergy has been thought to involve an excessive immune reaction against allergens permeating from the intestinal tract. Theoretically, the intestinal epithelium acts as a barrier restricting the permeation of macromolecules. However, a small proportion (10^{-3}–10^{-4}) of dietary proteins can cross the epithelium to access and activate effector cells, resulting in food allergy[1].

It is hypothesised that altered intestinal barrier function permits increased transport of dietary antigens across the intestinal barrier and exposure of dietary antigens to the mucosal immune system, leading to the development of a dietary antigen-specific response[2]. Consistent with this hypothesis, intestinal permeability in infants with food allergy, as assessed by the lactulose/mannitol ratio in urine, was significantly increased compared with that seen in healthy young children[3]. It is therefore conceivable that enhancing the barrier function of the intestinal epithelium would help prevent food allergy.

Our research team has established an *in vitro* system to evaluate allergen permeation using differentiated Caco-2 cells grown on a permeable filter[4]. Caco-2 cells, human adenocarcinoma cell line derived from a colon carcinoma, have been used as an *in vitro* model for various purposes. For example, we estimated the transepithelial transport of dietary components, such as the flavonoid hesperidin, in the Caco-2 cell monolayer[5]. Caco-2 cells differentiate under standard culture conditions to form confluent monolayers and acquire many features of absorptive intestinal cells during

culture. These cells spontaneously exhibit various enterocytic characteristics, including the formation of intercellular tight junctions (TJs).

Using this TJ model, we have reported that enzyme-modified cheese (EMC) inhibited the permeation of the representative food allergen ovalbumin (OVA). NPWDQ (Asn-Pro-Trp-Asp-Gln, amino acids 107–111 of αs_2-casein) was isolated from the hydrolysate and identified as one of the responsible peptides for this inhibitory activity [(4)]. Intestinal permeability of both fluorescein isothiocyanate-conjugated dextran and horseradish peroxidase decreased in Caco-2 cells by the addition of NPWDQ[(6)]. We also examined if NPWDQ inhibited allergen permeation *ex vivo* using rats with intestinal inflammation induced by subcutaneous injections of indomethacin. When OVA was injected into the jejuna and ileac loops of rats orally-administered with NPWDQ, it was found that NPWDQ administration effectively diminished OVA permeation from both loops compared with that in rats not administered NPWDQ[(6)]. From these results, it was assumed that NPWDQ would enhance epithelial barrier function.

The epithelium maintains barrier function by the formation of complex protein–protein networks that mechanically link adjacent cells and seal the intercellular space. The protein networks that connect epithelial cells form three adhesive complexes: TJs, adherens and desmosomes. These complexes consist of transmembrane proteins that interact extracellularly with adjacent cells and intracellularly with adaptor proteins that link to the cytoskeleton[(2)]. For example, TJs consist of several proteins, and among them zonula occludens-1 (ZO-1) was the first to be identified. It binds to transmembrane proteins, such as claudins and occludin, and links them to cytoskeletal actin. TJ proteins are regulated by phosphorylation by kinases, phosphatases and other signaling molecules.

In the present study, we investigated changes in the mRNA expression of genes related to TJ in Caco-2 cells by the addition of NPWDQ using the microarray method. Western blot analyses of three major TJ proteins (occludin, claudin-1 and ZO-1) were also performed. We demonstrated that NPWDQ enforced TJs, at least partly, by increasing occludin expression.

Materials and methods

Caco-2 cells

Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD, USA). In this study, cells were used between 45-50 passages. The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) with 10% featal bovine serum (FBS), 1% nonessential amino acids, and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamycin). DMEM, nonessential amino acids, penicillin, streptomycin, and gentamycin were all obtained from Invitrogen Corp. (Carlsbad, CA, USA). FBS was obtained from ICN Biomedicals, Inc. (Osaka, Japan).

Cells were cultured at 37°C under a humidified atmosphere of 5% CO_2. They were normally grown in 75 cm^2 tissue culture flasks to approximately 80% confluence, and seeded into a 12-well Transwell cell culture chamber (0·4 μm in pore size and 12 mm in diameter) (Corning Coster, Cambridge, MA, USA) at a density of 5 x 10^5 cells/cm^2. Each well was placed in a cluster plate with an outside medium (basolateral side, 1·5 ml) and an inside medium (apical side, 0·5 ml). The cell monolayers were fed

fresh medium every 24 hrs.

Transepithelial electrical resistance (TER) measurement

After 12-14 d of culture, TER was measured using a Millicell-ERS instrument with Ag/AgCl electrodes (Millipore, Bedford, MA, USA). Caco-2 cell monolayers were used whose TER values were 550-700 ohm cm^2. NPWDQ was synthesized according to the solid phase method using a peptide synthesizer and purified by reversed-phased HPLC by Takara Bio (Osaka, Japan). To examine the effect of NPWDQ on TER, 0·5 mL of the culture medium containing NPWDQ (0, 10^{-6}, 10^{-5} and 10^{-4}M) was added to the apical side and 1·5 mL of fresh culture medium without NPWDQ was added to the basolateral side. Cells were, then, incubated for 24 hrs, after which TER was measured.

Microarray analysis

After the above mentioned experiments, RNA was extracted from Caco-2 cells, with or without incubation with NPWDQ (10^{-6} M), using a TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA was extracted from six wells of the 12-well Transwell cell culture chamber and pooled.

cRNA was synthesised using an Illumina® RNA Amplification kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. In brief, total RNA (150 ng) from Caco-2 cells was reverse transcribed with T7-oligo (dT) primer to synthesise first and second strand cDNA, followed by clean-up. *In vitro* transcription was performed to synthesise biotin-labelled cRNA.

Labelled cRNA (1·5 μg) was hybridised to a BeadChip (Sentrix Human WG-6, Illumina) at 58°C for 18 h. Forty-eight thousand transcripts representing six whole-genome samples can be analysed on a single BeadChip. The hybridised BeadChip was washed and labelled with streptavidin-Cy3 (GE Healthcare Biosciences, Buckinghamshire, England) and scanned with a Illumina BeadStation 500GX-WG system (Illumina). Background-corrected values for each probe on the BeadChip array were extracted using BeadStudio (Illumina). These results were expressed as an arbitrary unit and analysed by GeneViewer (ver 1.0, Moritex, Tokyo, Japan).

Real-time RT-PCR

The level of occludin mRNA (n = 6) was measured with the real-time RT-PCR method using SYBR® green. The experiment was performed twice independently. Total RNA was treated with DNase (TURBO DNA-free™ kit, Applied Biosystems, Foster City, CA, USA). The RT reaction was conducted with Multiscribe™ reverse transcriptase using a high-capacity cDNA reverse transcription kit (Applied Biosystems) at 25°C for 10 min and at 37°C for 120 min. The reaction was terminated by heating at 85°C for 5 s followed by cooling at 4°C.

Real-time RT-PCR was performed with a 7300 Real-Time PCR system (Applied Biosystems) using Power SYBR® Green PCR Master Mix (Applied Biosystems). Primers were designed using the Primer Express® programme (Applied Biosystems). Their sequences are shown in Table 1. The reaction was performed at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 62°C for 1 min. The dissociation stage was analysed at 95°C for 15 s, followed by one cycle of 60°C for 15 s and 95°C for 15 s.

The fluorescence of the SYBR green dye was determined as a function of the PCR cycle number, giving the threshold cycle number at which amplification reached a significant threshold. Data were analysed by generating a standard curve from a dilution series and presented as fold change in gene expression, after normalisation of the β-actin gene.

SDS–PAGE and western blotting

The expressions of occludin, claudin-1, and zonula occludens-1 (ZO-1), were examined by the ordinary method (n=7). The experiment was performed twice independently. Briefly, Caco-2 cell monolayers were washed with ice-cold PBS thrice and lysed in the lysis buffer (25 mM Tris–HCl, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 1% SDS, at pH 7·4). The cell suspensions were sonicated for 30 min using a sonicator (USK-3, As One Corp., Osaka, Japan) and centrifuged at 14,000 *g* for 30 min at 4°C to yield a clear lysate. The proteins were separated using 12·5% gel for occludin and claudin-1 detection or 7·5% gel for ZO-1 detection, and then transferred onto blotting membranes (Immunobilon-P PVDF, Millipore). After overnight blocking with PBS/Tween 20 supplemented with 1% bovine serum albumin (BSA), the blots were incubated with primary (mouse anti-occludin, rabbit anti-claudin-1, or rabbit anti-ZO-1) and secondary antibodies (HRP-conjugated anti-mouse IgG or anti-rabbit IgG as appropriate) for 1 h each at room temperature. Anti-claudin-1, anti-occludin, and anti-ZO-1 antibodies were all obtained from ZYMED Lab (South San Francisco, CA, USA). HRP-conjugated anti-mouse IgG were obtained from Sigma-Aldrich (St. Louis, MO,USA). Anti-rabbit IgG was obtained from Kirkegaard & Perry Lab (Baltimore**,** MD,

USA). To confirm equal loading, the blots were reprobed with rat anti-α-tubulin antibody (Millipore). The proteins were visualized using chemiluminescence (ECL, GE Healthcare) and exposed to X-ray film (Fujifilm, Kanagawa, Japan). Band densities were read using Scion Image Software (Scion, Frederick, MD, USA), and normalized with α-tubulin. SDS-PAGE and western blotting were performed in duplicate.

Statistical analyses

All data are expressed as the mean ± S.D. Statistical analysis was performed using Tukey-Kramer method.

Results

Effect of NPWDQ on transepithelial electrical resistance (TER) of Caco-2 monolayers

The effect of the addition of NPWDQ on the integrity of Caco-2 monolayers was evaluated by the measurement of TER. TER value increased in response to the addition of increasing NPWDQ concentrations (10^{-6}–10^{-4} M), which suggested that this peptide enhanced epithelial barrier function (Fig. 1).

Microarray analysis of TJ-related molecules

DNA microarray analysis was performed to investigate the effect of the addition of

NPWDQ in detail. NPWDQ (10^{-6} M) was added to the apical side of Caco-2 cells, and cells were incubated for 24 h, after which RNA was extracted. NPWDQ and the longer peptide GPIVLNPWDQ (amino acids 102–111 of αs_2-casein) significantly inhibited OVA permeation in Caco-2 cells at a concentration of 10^{-6} M as shown previously[4], so this concentration of NPWDQ was used in this study.

Changes in 48,000 types of gene expression by NPWDQ were evaluated. To select genes with an abundant level of expression, those showing an expression more than 1000 (arbitrary fluorescence units), a total of 7,108 genes were chosen and further analysed. Among these genes, only two [*fos* (GenBank accession number NM_005252) and *egr1* (NM_001964)] were up-regulated by more than 2-fold, and three genes, *mgc14376* (NM_032895), *grb2* (NM_002086) and *dusp1* (NM_004417) were up-regulated by 1.5–2-fold; the fold changes for *fos*, *egr1*, *mgc14376*, *grb2* and *dusp1* were 2·93, 2·92, 1·54, 1·53 and 1·51, respectively. In addition, none of the genes was down-regulated by more than 1·5-fold (Table 2). Therefore, it was found that generally NPWDQ did not drastically affect mRNA expression in Caco-2 cells.

Differences in gene expression of major TJ-related molecules, occludin, claudins, ZO and junctional adhesion molecule (JAM) families, were analysed. As shown in Table 3, it was observed that occludin was up-regulated by 1·49-fold. On the other hand, gene expressions of claudin-1, -2, -3, -4, -7, -12, -14, -15, -19, -23, ZO family (ZO-1, -2 and -3) and JAM-1 were not remarkably changed (fold changes, 0·88–1·13). The genes of claudin-5, -6, -8, -9, -10, -11, -16, -17, -18, -20, -22, JAM-2 and -3 showed expressions at less than 100 arbitrary fluorescence units. From these data, it was clearly suggested that NPWDQ up-regulated mRNA expression of occludin.

Effect of NPWDQ on occludin expression

The effect of NPWDQ on the expression of the occludin gene was evaluated by real-time RT-PCR. As shown in Fig. 2, the addition of 10^{-6} M NPWDQ increased occludin mRNA expression by 1·3-fold. To examine this phenomenon more clearly, increasing concentrations of NPWDQ (10^{-5} and 10^{-4} M) were added to the apical side of Caco-2 cells, and mRNA expression of occludin was examined. As a result, NPWDQ (10^{-5} M) significantly ($p < 0·01$, 1·7-fold) increased occludin mRNA expression. The addition of 10^{-4} M NPWDQ also increased occludin mRNA expression, but the effect (1·4-fold) was less than that with 10^{-5} M NPWDQ.

The effect of NPWDQ (10^{-6} M) on the expression of major TJ-related proteins, occludin, claudin-1 and ZO-1, was evaluated by western blotting. NPWDQ significantly ($p < 0·05$) increased the level of occludin by 1·5-fold (Fig. 3). Protein expressions of claudin-1 and ZO-1 were not changed by NPWDQ, which was consistent with the microarray data (Table 3). These data suggested that NPWDQ especially increased the protein level of occludin and enforced the TJ barrier.

Discussion

The intestine is constantly exposed to food components as well as antigens, commensal microflora and pathogens. The epithelial TJ is therefore very important; several intestinal diseases have been reported to be associated with TJ dysfunction[7]. The relationship between food components and the intestinal TJ barrier has not been

completely elucidated, but information relating to glutamine[8], polyunsaturated fatty acids[9], zinc[10] and polyphenols[11] is available. As for the modulation of occludin expression, Jiang et al. [9] reported that occludin was up-regulated by γ-linolenic acid in the human vascular endothelial cell line ECV304. Following this, TER was increased, and paracellular permeability to large molecules was thereby reduced. To our knowledge, the present study is the first to show that occludin expression was up-regulated by a food-derived peptide.

Since we have previously reported that NPWDQ inhibited OVA permeation in Caco-2 cells 24 h after the addition of the peptide[4], we performed the DNA microarray analysis using RNA from Caco-2 cells that was incubated with the peptide for 24 h. Indeed, the level of occludin mRNA expression did not change before 8h-incubation (data not shown). However, it would be interesting to examine whether the expression of other TJ molecules would change in much early stage[11].

We demonstrated that NPWDQ up-regulated occludin expression in Caco-2 cells using the DNA microarray method followed by real-time RT-PCR and western blot analyses. Occludin is a transmembrane protein of TJ and has an important role in the assembly and maintenance of TJ[11-14]. For example, the interaction between the C-terminal region of occludin and ZO-1 is crucial for TJ assembly[13]. Therefore, up-regulation of occludin by NPWDQ might have great impact on TJ in Caco-2 cells, although occludin was the only molecule whose expression level was significantly changed by NPWDQ. Further studies are necessary to examine the effect of NPWDQ on the localization of other TJ proteins and on TJ structure. The effect of NPWDQ was the most prominent at a concentration of 10^{-5} M, and it reduced at 10^{-4} M (Fig. 2). One possible explanation for this phenomenon is that in Caco-2 cells a relatively high dose

of the peptide might be recognised as a signal for ingested nutrients and this might trigger a decrease in TER in order to absorb the peptide. Nevertheless, this data implies that a food-derived peptide can fine-tune the epithelial barrier by modulating the expression of TJ proteins such as occludin.

Is the effect of NPWDQ on occludin expression dependent on this peptide sequence? Or, are amino acids in the peptide sequence enough for the effect?

With respect to the activity of the constitutive amino acids (N, P, W, D and Q) towards the intestine, the protective effects of glutamine and tryptophan on the intestine have been characterised[15-18]. Glutamine supplementation was demonstrated to improve intestinal barrier function in highly stressed patients[15] and in piglets with endotoxin-related changes in ileal permeability[16]. As for tryptophan, Kobayashi and Watanabe[17] reported that its ethyl ester inhibited OVA permeation in the Caco-2 cell monolayer. Also, Kim et al.[18] recently reported that tryptophan exhibited a therapeutic effect in piglets suffering from dextran sodium sulfate-induced colitis. Therefore, it is possible that NPWDQ would be hydrolysed into amino acids and subsequently P and W would trigger the up-regulation of occludin. However, investigations are needed to ascertain if the existence of P and W residues is sufficient or if the specific amino acid sequence is indispensable for the up-regulation of occludin.

Nevertheless, it is highly probable that NPWDQ would act as a peptide form because proline-containing peptides are, in general, resistant to degradation by digestive enzymes[19]. In this case, the next question is whether NPWDQ would be absorbed into Caco-2 cells prior to its function inside cells or whether it is transported into the paracellular space to act from outside the cells. The underlying mechanisms of occludin up-regulation by NPWDQ are also unclear. It was reported that occludin was regulated

by collagen IV, one of the components of the extracellular matrix (ECM), in TJs of Sertoli cells[20]. Savettieri et al. [21] also reported that ECM and neurons modulate the expression of occludin in rat brain endothelial cells. If NPWDQ were to be transported paracellularly and reacted with ECM, it would regulate occludin expression by reacting with molecules such as collagens in ECM. From our microarray data, NPWDQ did not drastically affect mRNA expressions of collagen IV α3 and α5 (data not shown). Further studies are necessary to examine the effect of NPWDQ on ECM in intestinal cells.

As mentioned above, TJ dysfunction leads to intestinal disorders such as inflammatory bowel disease (IBD). For an *in vitro* model of IBD, cytokines (e.g. tumour necrosis factor (TNF-α)-stimulated Caco-2 cells are frequently used[22,23]. Upon stimulation, inflammatory cytokines, such as interleukin (IL)-8, are induced, and the TJ structure becomes loose. Several food components are under investigation for the treatment and/or prevention of IBD using this cell model. For example, histidine was reported to inhibit TNF-α-induced IL-8 secretion in Caco-2 cells[22]. Some types of lactobacilli also inhibited IL-8 production and restored the protein expression of ZO-1 in TNF-α-treated Caco-2 cells[23]. It would be interesting to examine if NPWDQ up-regulates occludin expression in such inflamed intestinal cells.

For practical intake of NPWDQ, food materials comprising this type of peptide are more acceptable to the industry than synthetic peptides. In this context, EMC seems to be favourable. We have found NPWDQ peptide from EMC that has an inhibitory activity towards allergen absorption in the intestine[4,6]. EMC is generally produced by the hydrolysis of cheese with commercial proteases and is currently used in the food industry. We hope that EMC will be used for the treatment of food allergy and intestinal disorders or for an intestinal barrier-promoting purpose in healthy people.

In conclusion, a casein-derived peptide NPWDQ, which has inhibitory effects against allergen permeation in the intestine, up-regulates occludin expression and enforces the TJ barrier. These data imply that a food-derived peptide can fine-tune the epithelial barrier.

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1

Table 1. Sequences of primers used for real-time PCR.

	Amplicon length (bp)	Oligonucleotide sequence		GenBank accession no.
occludin	77	forward	CCCATCTGACTATGTGGAAAGA	NM_002538
		reverse	AAAACCGCTTGTCATTCACTTTG	
β-actin	63	forward	TCATGAAGTGTGACGTGGACATC	NM_001101
		reverse	TGCATCCTGTCGGCAATG	

2

Table 2. Summary of changes in mRNA expressions in Caco-2 cells by the addition of NPWDQ as evaluated by DNA microarray.

change fold	gene numbers
> 1·5	5
1·2 - 1·5	122
0·8 - 1·2	6,850
0·67 - 0·8	131
< 0·67	0

NPWDQ (10^{-6} M) was added to the apical side, and cells were incubated for 24 h. RNA was extracted from Caco-2 cells with or without incubation with NPWDQ (n = 6 each) and pooled. Changes in 48,000 types of gene expression were evaluated. This table is the summary of 7,108 genes those showing an expression more than 1,000 (arbitrary fluorescence units).

1

Table 3. Changes in mRNA expressions of TJ-related molecules by the addition of NPWDQ.

gene	GenBank accession no.	NPWDQ		change fold
		(-)	(+)	
occludin	NM_002538	164±34 (36)	245±43 (31)	1·49
claudin-1	NM_021101	23,834±640 (34)	27,039±790 (32)	1·13
claudin-2	NM_020384	1,150±76 (35)	1,041±43 (44)	0·91
claudin-3	NM_001306	2,470±115 (31)	2,557±105 (41)	1·04
claudin-4	NM_001305	624±57 (30)	588±35 (45)	0·94
claudin-7	NM_001307	11,205±319 (24)	10,881±329 (42)	0·97
claudin-12	NM_012129	3,117±130 (31)	3,225±121 (48)	1·03
claudin-14	NM_012130	3,770±139 (34)	3,303±141 (37)	0·88
claudin-15	NM_014343	3,306±92 (43)	3,487±133 (37)	1·05
claudin-19	NM_148960	531±35 (51)	587±52 (39)	1·11
claudin-23	NM_194284	4,389±168 (30)	4,492±220 (23)	1·02
ZO-1	NM_003257	5,398±133 (63)	5,536±150 (45)	1·03
ZO-2	NM_201629	2,070±87 (37)	2,223±79 (41)	1·07
ZO-3	NM_014428	9,363±286 (39)	8,851±204 (48)	0·95
JAM-1	NM_016946	8,142±352 (43)	7834±264 (36)	0·96

2 The number in parentheses indicates the number of beads analysed. The expressions of
3 claudin-5, -6, -8, -9, -10, -11, -16, -17, -18, -20, and -22 and JAM-2 and -3 were less
4 than 100 (arbitrary fluorescence units).

Figure Captions

Fig. 1. Changes in TER by the addition of NPWDQ to Caco-2 cells.

NPWDQ (10^{-6}–10^{-4} M) was added to the apical side, and cells were incubated for 24 h, after which TER was measured (n = 6 each).

*, **, $p<0{\cdot}05$ and $p<0{\cdot}01$ vs samples without the addition of NPWDQ (-), respectively.

Fig. 2. Changes in mRNA expression of occludin by the addition of NPWDQ to Caco-2 cells.

RNA was extracted from Caco-2 cells after measurement of TER (Figure 1). The mRNA expression of occludin was evaluated by real-time RT-PCR (n = 6 each).

*, $p<0.05$ vs samples without the addition of NPWDQ (-).

Fig. 3. Changes in expressions of three tight junction proteins by the addition of NPWDQ to Caco-2 cells.

NPWDQ (10^{-6} M) was added to the apical side, and cells were incubated for 24 h. Protein was extracted from Caco-2 cells with or without incubation with NPWDQ (n = 7 each). Protein expressions were detected by western blotting and normalized with α-tubulin for the comparison.

(A) occludin (with a representative blotting pattern), (B) claudin-1, (C) ZO-1.

*, $p<0{\cdot}05$ vs samples without the addition of NPWDQ (-). NS, not significant.

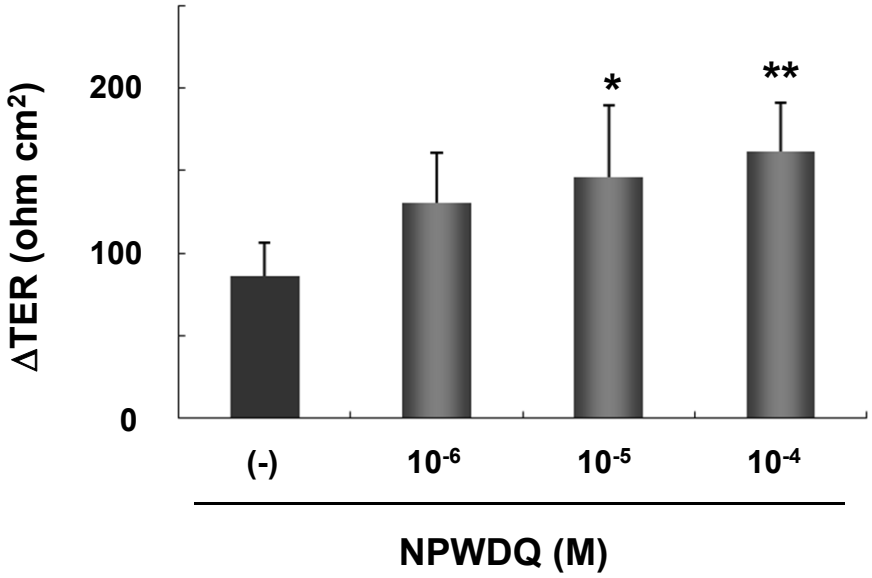

Figure 1

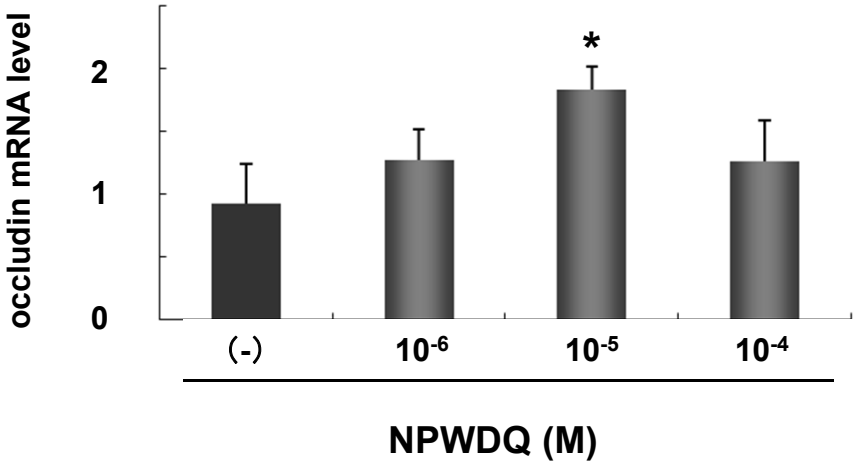

Figure 2

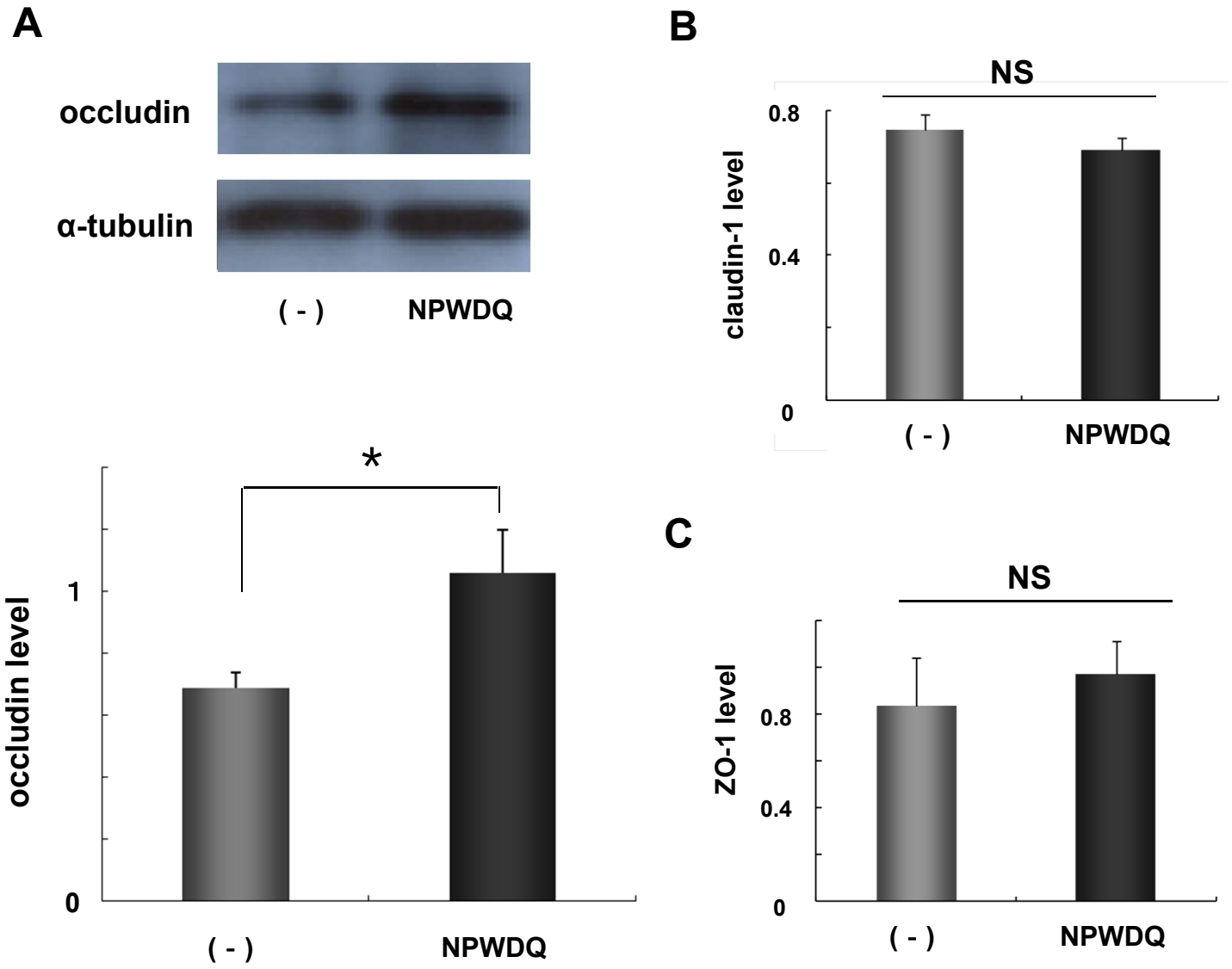
A
occludin
α-tubulin
(-)
NPWDQ
B
NS
0.8
0.4
0
claudin-1 level
(-)
NPWDQ
*
1
0
occludin level
(-)
NPWDQ
C
NS
0.8
0.4
0
ZO-1 level
(-)
NPWDQ

Figure 3