Journal of Cellular Biochemistry 98:1308–1319 (2006)

Synergistic Regulation of Endothelial Tight Junctions by Antioxidant (Se) and Polyunsaturated Lipid (GLA) Via Claudin-5 Modulation

Tracey A. Martin,[1]* Tapas Das,[2] Robert E. Mansel,[1] and Wen G. Jiang[1]

[1]Department of Surgery, Metastasis & Angiogenesis Research Group, Wales College of Medicine, Cardiff University, Cardiff, UK
[2]Abbott Laboratories, RPD, Columbus, Ohio

Abstract Tight junctions (TJs) in endothelial cells act as cell–cell adhesion structures, governing paracellular permeability (PCP). Disruption can lead to leaky vascular bed and potentially to oedema and swelling of tissues, the aetiology of mastalgia. These changes may also cause vascular spread of cancer cells. This study aimed to determine whether the function of TJs in endothelial cells can be strengthened by gamma linolenic acid (GLA), selenium (Se) and iodine (I) in the presence of 17beta-estradiol (17β-estradiol), which causes leakage of endothelial cells by disruption of TJs in endothelium. GLA, I, and Se individually increased transendothelial resistance. The combination of all three agents also had a significant effect on TER. Addition of GLA/Se/I reduced PCP of the endothelial cells. Treatment with GLA/Se/I reversed the effect of 17β-estradiol in reducing TER and increasing PCP. Immunofluorescence revealed that after treatment with Se/I/GLA over 24 h there was increasing relocation to endothelial cell–cell junctions of the TJ proteins Claudin-5, Occludin, and ZO-1. Interestingly, this relocation was particularly evident with treatments containing I when probing with Claudin-5 and those containing Se for Occludin. There was a small increase in overall protein levels when examined by Western blotting after treatment with GLA/Se/I when probed with Claudin-5 and Occludin. We report that GLA, I, and Se alone, or in combination are able to strengthen the function of TJs in human endothelial cells, by way of regulating the distribution of Claudin-5, Occludin, and ZO-1. Interestingly, this combination was also able to completely reverse the effect of 17β-estradiol in these cells. J. Cell. Biochem. 98: 1308–1319, 2006.

Key words: tight junction; endothelium; synergy; selenium/iodine/gamma linoleic acid

Epithelial and endothelial cell sheets establish compositionally distinct fluid compartments [Tsukita and Furuse, 1999]. For these to function as barriers maintaining the distinct internal environment of each compartment, the paracellular pathway between adjacent cells in the sheet must be tightly sealed to prevent the diffusion of solutes; the apical and basolateral membrane domains must be differentiated to allow active transport across the sheet [Tsukita and Furuse, 1999]. Tight junctions (TJs) are directly involved in paracellular sealing and in membrane domain differentiation. TJs are found at the most apical region of the cell junction complex where they occlude the extracellular space [Wong and Gumbiner, 1997]. The TJ is thus crucial for endothelium and epithelium to generate chemical and electrical gradients across the cell monolayer that is necessary for vectorial transport processes such as absorption and secretion.

ZO-1 is a peripheral membrane protein of the zonula occludens family and is localized in the immediate vicinity of the plasma membrane of TJs in both epithelial and endothelial cells [Furuse et al., 1998]. Members of this family (ZO-1, ZO-2, and ZO-3) have similarity to the product of lethal discs large-l (dlg), on of the tumor suppressor molecules in Drosophila. Both ZO-2 and -1 bind to ZO-3. ZO proteins are involved in the organization of epithelial and endothelial intercellular junctions, binding to

Grant sponsor: CR-UK; Grant number: C7603/A3065; Grant sponsor: Cancer Research Wales; Grant number: 120504WGJ.

*Correspondence to: Dr. Tracey A. Martin, Metastasis & Angiogenesis Research Group, University Department of Surgery, Wales College of Medicine, Cardiff University, Heath Park, Cardiff, CF14 4XN, UK. E-mail: Martinta1@cf.ac.uk

Received 27 September 2005; Accepted 18 January 2006
DOI 10.1002/jcb.20860

the cytoplasmic carboxyl termini of junctional transmembrane proteins, linking them to the actin cytoskeleton. They are characterized by several conserved modules, including three PDZ domains, one SH3 domain, and a guanylate kinase-like domain, elements that indicate that ZO's may serve multiple purposes.

Occludin, a 60–65 kDa integral membrane protein is localized at TJs. It bears four transmembrane domains in its N-terminal half, with both the N- and C-termini located in the cytoplasm; the C-terminal (approximately 150 amino acids) binding to ZO-1 [Furuse et al., 1998; Tsukita and Furuse, 1999]. Medina et al. [2000] previously demonstrated that the extracellular loops of Occludin are adhesive, indicating the possibility that they contribute to localizing Occludin at the TJ. The authors show that the second extracellular domain is required for stable assembly of Occludin in the TJ, and that Occludin influences the structural organization of the paracellular barrier.

Claudins comprise a multigene family consisting of more than 20 members (of around 22 kDa) and heterogeneous Claudin species (and also Occludin) are co-polymerized to form individual TJ strands as heteropolymers [Hamazaki et al., 2002]. Between adjacent cells Claudin molecules adhere to each other in both homotypic and heterotypic manners. Claudins generally have a valine residue at their COOH termini, suggesting that they strongly attract PDZ-containing proteins, such as ZO-1, -2, and -3. Claudins (with Occludin) constitute the major transmembrane proteins of TJs [Hoevel et al., 2002]. Claudin-5 is a protein component of many endothelial TJs, including those at the blood-brain barrier [Wen et al., 2004] and it has been shown that expression of Claudin-5 selectively decreased the permeability of the blood-brain barrier to ions. New insights into the functions of Claudin-5 at the molecular level in TJs may account for some aspects of the blood-brain barrier's selective permeability.

The C-terminal cytoplasmic domains of Occludin and the Claudins bind to the ZO family cytoplasmic proteins underlying the TJ strands. ZO proteins belong to the membrane-associated guanylate kinase superfamily and their function is to link the TJ to the cellular cytoskeleton. ZO-1 forms a bridge between the C-terminal sequences of Occludin and beta-actin. The second extracellular loop of Occludin is required for Occludin localization at the TJ [Medina et al., 2000] and at least one of the transmembrane domains is involved in PCP [Balda et al., 2000]. Such results suggest a more complex mechanism of the assembly and regulation of barrier-forming strands involving multiple interactions of different domains of Occludin with itself, other cytoplasmic or transmembrane proteins, resulting in a selective transport system for the paracellular pathway [Mankertz et al., 2002].

We have previously shown that treatment of human endothelial cells (HECV) with GLA, increased transendothelial cell resistance (TER) and reduced the PCP to large molecules. The effects were seen without any changes in the viability of the endothelial cells. Occludin, which plays a major role in TJs was upregulated by this fatty acid as revealed by both Western blotting and immunofluorescence [Jiang et al., 1998]. Moreover, GLA has been demonstrated to increase TER and the expression of Occludin mRNA in brain capillary endothelial cells [Yamagata et al., 2003], with induction of increased TJ assembly.

We have also shown that 17beta-estradiol (17β-estradiol) can induce concentration- and time-related biphasic effects on TJ functions expression of Occludin in endothelial cells and that this perturbation of TJ functions may have implications in the aetiology of mastalgia [Ye et al., 2003].

Selenium (Se) is an essential dietary nutrient for mammals, being required for a number of enzymes in metabolism and has been found to exert a chemopreventative activity in cancer [Ip and Ganther, 1991; Kowaltowski et al., 1998] with increased Se level associated with decreased cancer incidence, but the mechanism of effect being unclear [Gupta et al., 1994]. It suggested that Se is able to inhibit neoangiogenesis [Jiang et al., 1999; Lu, 2001] and is anti-angiogenic in malignant breast tumors possibly due to reducing VEGF production [Streicher et al., 2004]. Se has been shown to be able to affect the expression of cell adhesion molecules (ICAM-1, VCAM-1) crucial in the inflammatory process [Jahnova et al., 2002].

Iodine (I) is an essential component of the thyroid hormones thyroxine and triiodothyronine and has been thought to be used almost exclusively by the thyroid. Iodinated contrast media (ICM) are extensively employed for diagnostic imaging and should be biologically inert [Fanning et al., 2002]. However, there are

reports indicating that these agents are able to alter cell function.

This current study examined how the effect that GLA has on endothelial TJs may be increased. GLA was used in varying concentrations with/without the addition of Se and/or I, together with any effect Se and I may have independently. We also investigated if GLA/Se/I alone or in combination could inhibit the effect of 17β-estradiol on HECV.

MATERIALS AND METHODS

Reagents and Antibodies

Anti-Occludin, ZO-1, Claudin-5, and anti-actin antibodies were purchased from PharMingen International. Peroxidase-conjugated anti-mouse, anti-rabbit IgG for Western blotting were from Sigma-Aldrich Ltd (Poole, UK). Fluorescein isothiocyanate (FITC) conjugated Dextran (40 kDa) was obtained from Molecular Probe Inc. (Eugene, OR). Carbonate filter inserts with pore size of 0.4 μm (for 24-well plates) were from Becton Dickinson Labware (Oxford, UK). GLA, sodium selenate (Se) and iodate (I) were purchased from Sigma-Aldrich Ltd and 17β-estradiol from Sigma.

Cell Line

HECV, Human umbilical vein endothelial cell line was obtained from ICLC (Genova, Italy). The cell line was routinely maintained in Dulbecco's Modified Eagle's medium (DMEM; Sigma Ltd) supplemented with 10% foetal calf serum (FCS), penicillin, and streptomycin (Sigma-Aldrich Ltd).

Transendothelial Resistance (TER)

TER was measured with an EVOM voltohmmeter (EVOL, World Precision Instruments, Aston, Herts, UK), equipped with a pair of STX-2 chopstick electrodes (WPI, Sarasota, FL). Briefly, HECV cells were seeded into the 0.4 μm pore size insert (upper chamber) and allowed to reach full confluence, after which fresh medium was replaced for further experiments. Inserts without cells, inserts with cells in medium and inserts with cells with GLA/Se/I were tested for a period of 24 h. Electrodes were placed at the upper and lower chambers and resistance measured with the voltohmmeter.

Transendothelial Cell Permeability

This was determined using fluorescently labeled dextran FITC-Dextran 40, molecular weight being 40 kDa [Jiang et al., 1998]. HECV were prepared and treated as in the TER study, but with the addition of Dextran 40 to the upper chamber. Medium from the lower chamber was collected for intervals up to 24 h after addition of GLA/Se/I. The relative fluorescence from these collections was read on a multichannel fluorescence reader (Denly, Sussex, UK).

Immunofluorescent Staining of Human Endothelial Cells (HECV)

For immunofluorescence staining, cells were grown in 16-well chamber slides (LAB-TEK) (30,000 cells/well) in the presence or absence of GLA/Se/I and incubated in a 37°C/5% incubator for a set period of time (0–24 h). After incubation, the culture medium was aspirated, the wells rinsed with balanced salt solution (BSS) buffer and the cells fixed in methanol for 20 min at −20°C. After fixation the cells were washed twice using BSS buffer and permeabilized by the addition of 200 μl of 0.1% Triton X-100 (Sigma) detergent in phosphate buffered solution (PBS) for 5 min at room temperature. Cells were rinsed twice with BSS buffer and 200 μl of blocking buffer (10% horse serum in TBS) was added to each well and the chamber slide incubated for 40 min at room temperature on a bench rocker. The wells were washed once with wash buffer (3% horse serum in TBS buffer containing 0.1% Tween 20) and 100 μl of primary antibodies prepared in wash buffer was added to the appropriate wells. The chamber slide was incubated on the rocker for a further 60 min at room temperature. Wells were washed twice with TBS buffer (with 0.1% Tween 20) and cells were incubated in 100 μl of secondary antibodies (FITC conjugate) (diluted in the same manner as the primary antibodies) for 50 min. The chamber slide was wrapped in foil to prevent light reaching the conjugate. Finally, the wells were rinsed twice with wash buffer, once in BSS buffer mounted with FluorSave (Calbiochem-Novabiochem Ltd, Nottingham, UK) reagent and visualized using an Olympus BX51 microscope with a Hamamatsu Orca ER digital camera at X 100 using oil immersion lens.

SDS–PAGE and Western Blotting

Total cell lysates were prepared as follows: Cells were pelleted and lysed in HCMF buffer plus 0.5% SDS, 0.5% Triton X-100, 2 mM CaCl2, 100 μg/ml phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml aprotinin and, 10 mM

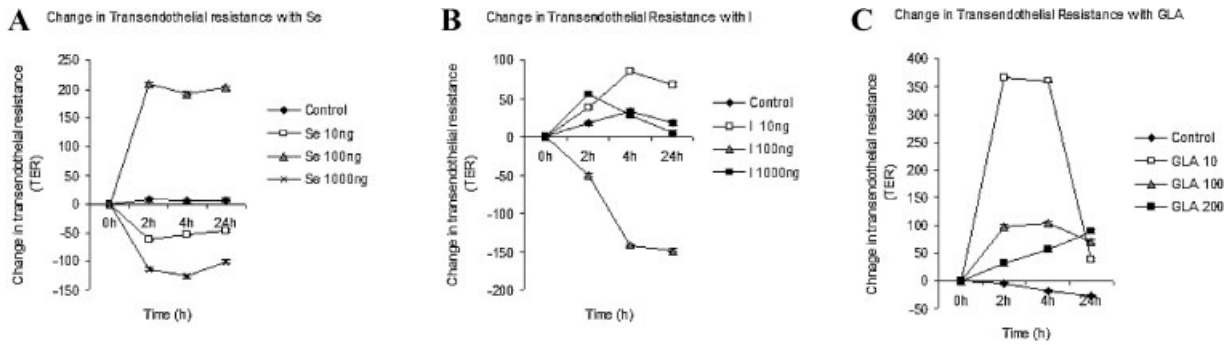

Fig. 1. Change in transendothelial resistance (Ω) of human endothelial cells (HECV) treated with varying concentrations of (**A**) Se, (**B**) I, and (**C**) GLA over 24 h incubation.

sodium orthovanadate for 40 min, sample buffer was added and the protein boiled at 100°C for 5 min before clarification at 13,000*g* for 10 min. Equal amounts of protein from each cell sample (controls and those treated with GLA/I/Se) were added onto an 8% polyacrylamide gel. Following electrophoresis, proteins were blotted onto nitrocellulose sheets and blocked in 10% horse serum for 60 min before probing with specific primary antibodies, following with peroxidase-conjugated secondary antibody (1:2,000). Protein bands were visualized with Supersignal West Dura Extended Duration Substrate chemi-luminescent system (Perbio Science UK Ltd, Cramlington, UK) and detected using a CCD UVIprochemi system (UVItec Ltd, Cambridge, UK).

Statistical Analysis

Statistical analysis was performed by MINITAB version 12 (Minitab Inc. State College, PA) using *t*-tests.

RESULTS

Effect of Se, I, GLA and Combinations on TER and PCP in Endothelial Cells

From the functional studies carried out we can conclude that all three compounds were able to increase junctional function to some extent in HECV. Each substance was tested to ascertain a concentration of maximum effect on increased TJ function as assessed using changes in transendothelial resistance (TER). Se was most consistently effective at 100 ng/ml (control: Increase in resistance (Ω) by 24 h 7.33 ± 2; Se at 100 ng/ml 202.667 ± 3), Fig. 1A; I at 10 ng/ml (control: Increase in resistance (Ω) by 24 h 19.66 ± 2; I at 10 ng/ml 69 ± 4), Fig. 1B; GLA at 100 μm (control: Increase in resistance (Ω) by 24 h (26.33 ± 2; GLA at 100 μm 71 ± 3), Fig. 1C ($P < 0.05$). Alone, Se showed the most effect on increasing TER over 24 h (Fig. 2A).

Using these concentrations, combinations were then used for further experiments. Again, when using TER, all the combinations increased TJ function over 0–24 h, with the combination of all three compounds being particularly effective (control at 24 h 31.33 ± 2 vs. Se and I and GLA 76.6 ± 3, $P < 0.04$) over the 24 h, each combination exhibited a peak in effect by 4 h, Fig. 2B.

Addition of 17β-estradiol (the most effective, water soluble form estrogen commercially available) was used to ascertain the effect of Se, I, and GLA on 17β-estradiol-induced TER in endothelial cells. 17β-estradiol significantly reduced TER over 24 h (Fig. 2C). Individually, GLA was most effective at negating any effect of 17β-estradiol (at 25×10^{-9} M), although both Se and I showed some small effect (at 24 h: Control -164.66 ± 4; Se 49.33 ± 3; I 40.33 ± 4; GLA 114.33 ± 3, $P < 0.05$) Fig. 2D. Combinations of these were then used to assess their effect against 17β-estradiol induced changes at 25×10^{-9} M. Combinations of Se, I, and GLA all reversed this effect, particularly the combination of all three (with 17β-estradiol at 24 h -164.66 ± 4 vs. 17β-estradiol with Se, I, and GLA in combination 6 ± 2, $P = 0.01$), Fig. 2E. We have previously shown that 17β-estradiol reduced TJ function in endothelial cells—these results indicate that these compounds are able (in combination) to reverse the effect of 17β-estradiol in this cell type.

An additional test was also performed, namely change in PCP another assessment of TJ function. When evaluating the effect of these

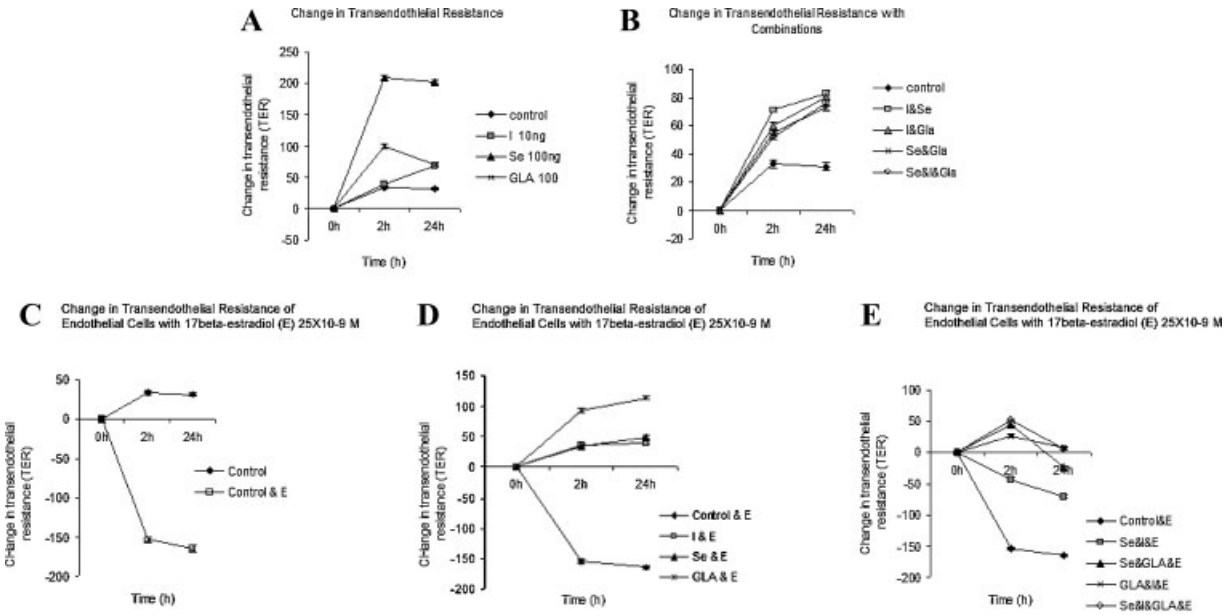

Fig. 2. Change in transendothelial resistance (TER) of HECV treated with Se, I, or GLA (**A**); combinations of Se, I, and GLA (**B**); and the effect of 17beta-estradiol (17β-estradiol) on TER, reversed by Se, I, or GLA (**C**) and negated by combinations of Se, I, and GLA (**D**).

compounds using PCP, there was a less dramatic effect: Although an effective decrease in permeability in endothelial cells with the compounds individually (Fig. 3A). Permeability increases over time due to changes in the structure of the endothelial cells layer. Addition of all three substances reduced this permeability effect over 24 h (at 24 h control 135.66 ± 3 rfu; Se -29.66 ± 4; I -121 ± 3; GLA 41 ± 2). Combinations of Se, I, and GLA were also effective at reducing PCP, with a combination of I and Se being particularly effective over 24 h (control 135.66 ± 3 vs. I and Se -381 ± 5, $P = 0.01$), Fig. 3B.

Interestingly, the addition of 17β-estradiol did not increase PCP of the endothelial cells when compared to no 17β-estradiol control although addition of Se, I, and GLA alone did reduced permeability (Fig. 3C); Addition of combinations of Se, I, and/or GLA did show some effect at reducing permeability over 24 h with addition of 17β-estradiol (Fig. 3D).

Immunofluorescent Location of Tight Junction Molecules

We assessed the integrity of TJs after treatment with the Se/I/GLA alone or in combination over 24 h treatment, and carried out immunofluorescence to evaluate the staining pattern and intensity of the three TJ proteins; ZO-1 (a plaque/peripheral protein responsible for maintenance of TJ structure and function), Occludin (a transmembrane protein that confers cell–cell adhesiveness and TJ functioning) and Claudin-5 (thought to be responsible for most of the cell–cell integrity of the TJ formation). Over the 24 h incubation, it was evident that the intensity of staining was increased for all three molecules, Claudin-5, Occludin, and ZO-1 in the HECV (Figs. 4–6 respectively, representative of four replicate experiments), which remained to some extent for up to 24 h.

Claudin-5 intensity increased upon treatment by all combinations, and by each substance individually (Fig. 4). This was especially evident in those treatments containing I, especially in combination with Se, where the effect was strongly observable by 0.5 h treatment. Similarly, Occludin also showed increased intensity with all treatments (Fig. 5), with distinct relocation from the cytoplasm to the cell periphery in those containing GLA. Overall intensity was increased with the addition of Se, and Se with GLA.

ZO-1 staining did not show such an obvious increase in intensity; indeed, there was no change at all on treatment with I alone (Fig. 6). Between 1 and 2 h treatment, there was some increase in intensity for the other treatments. The most interesting was the

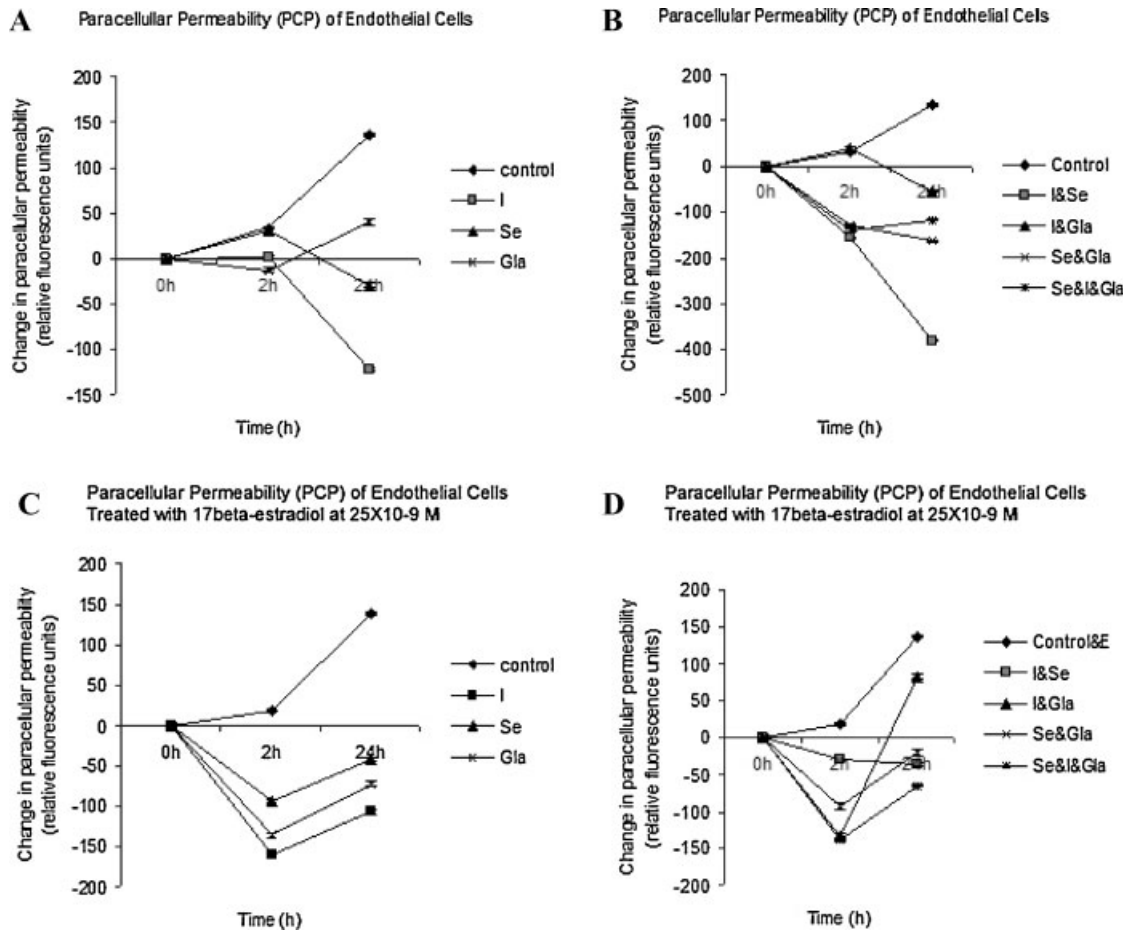

Fig. 3. Change in paracellular permeability (PCP) of HECV treated with Se, I, or GLA (**A**); combinations of Se, I, and GLA (**B**); and the effect of 17β-estradiol on PCP, reversed by Se, I, or GLA (**C**) and negated by combinations of Se, I, and GLA (**D**).

marked increase in junctional staining after addition of Se, I, and GLA together, after only 0.5 h incubation.

Western Blotting of Tight Junction Molecules

Western blotting was carried out to assess whether the changes in intensity of the TJ molecules were simply due to re-localization or increased protein expression after treatment with the combined Se, I, and GLA over 24 h. As can be seen from the blots presented (Fig. 7A) there was some increase in Claudin-5 expression over the 24 h period of treatment with GLA/Se/I. There was also a small increase in expression of Occludin (Fig. 7B). Curiously, an apparent reduction in Occludin was observed on treatment with Se or Se/I by 4 h, which was not observed from immunofluorescence and may indicate a change in phosphorylation status of Occludin. Actin was also probed to eliminate the possibility of loading error (Fig. 7C).

DISCUSSION

This study has shown for the first time that I and Se, alone or in combination can alter the function of TJs in HECV, particularly in conjunction with GLA, which we have previously shown to increase TJ integrity. The combination of Se, I, and GLA was also able to negate the effect 17β-estradiol had on TJ function. It was apparent from immunocytochemical staining, that these increases in TJ function were affected by the relocation of the important TJ proteins Claudin-5, Occludin, and ZO-1 to the cell–cell junction of these cells. This suggests that these substances may have an impact in the regulation of permeability of vessels, and thus a mechanism whereby tissue oedema might be controlled.

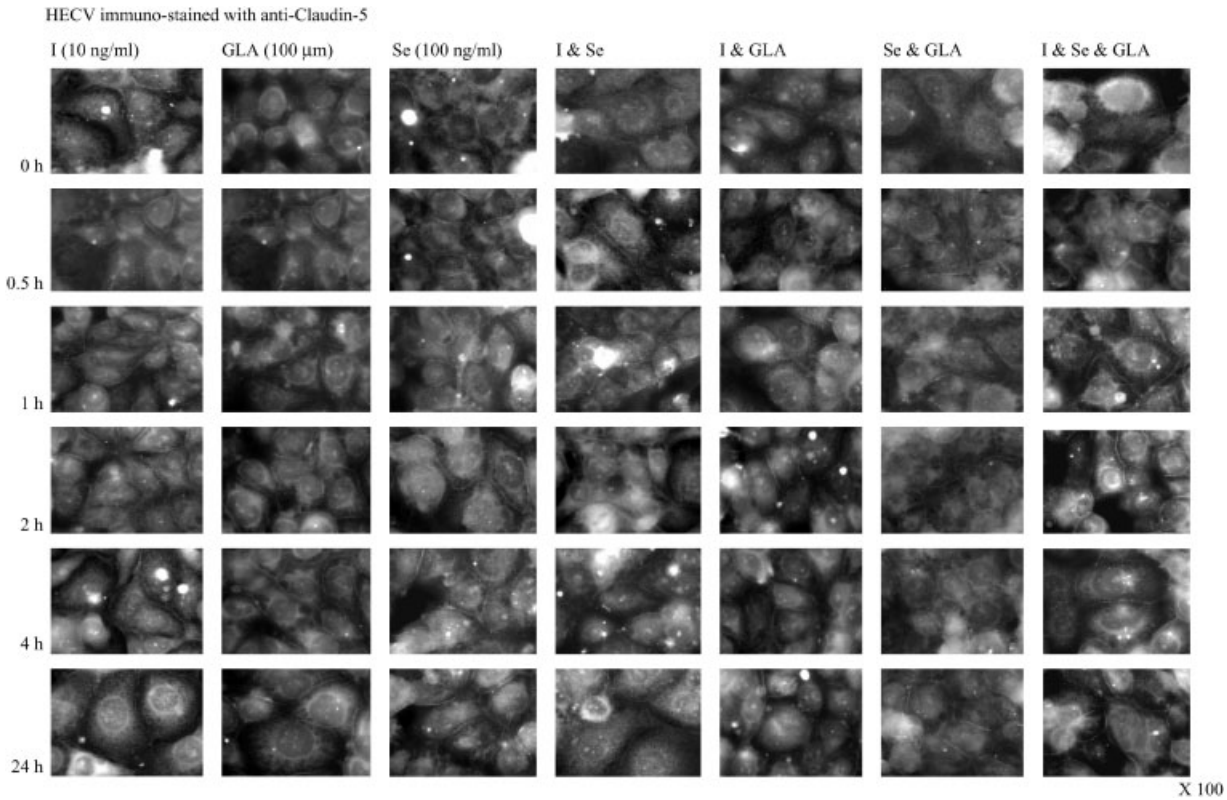

Fig. 4. Effect of I, Se, and GLA on relocation of Claudin-5 in HECV treated over 24 h (X100).

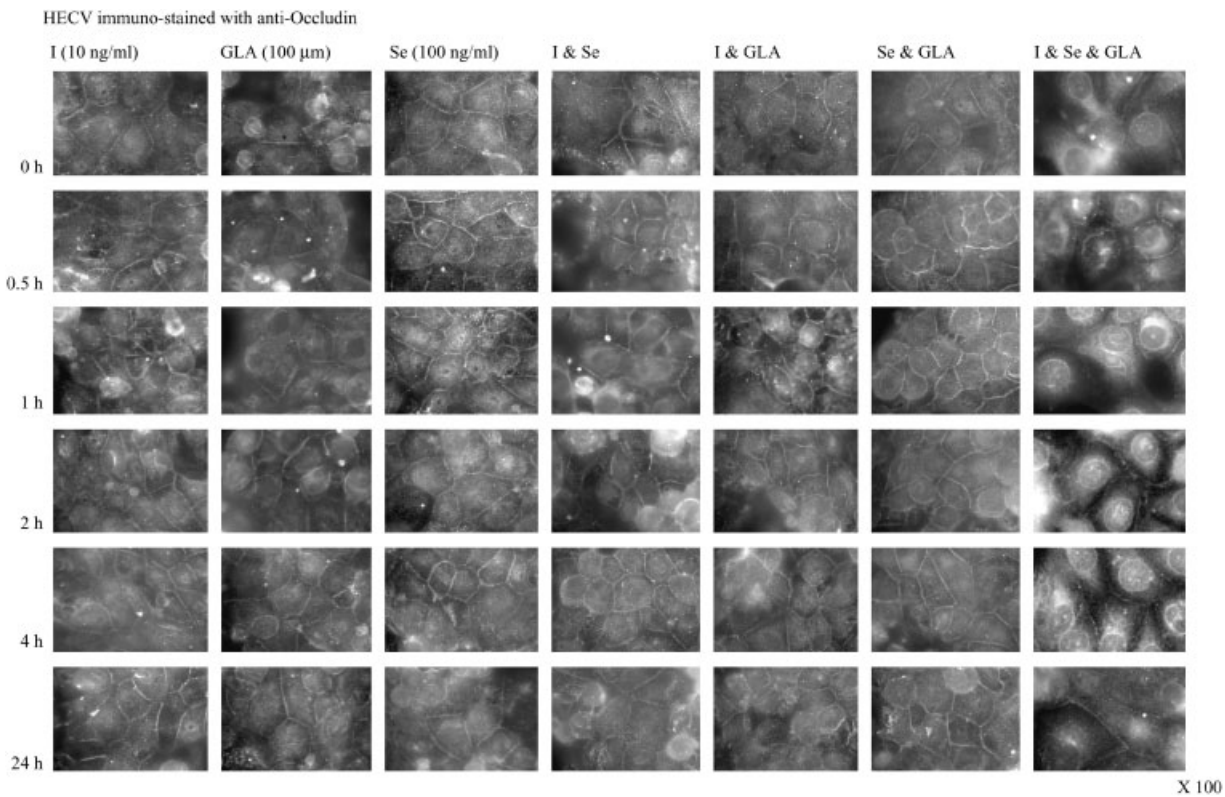

Fig. 5. Effect of I, Se, and GLA on relocation of Occludin in HECV treated over 24 h (X100).

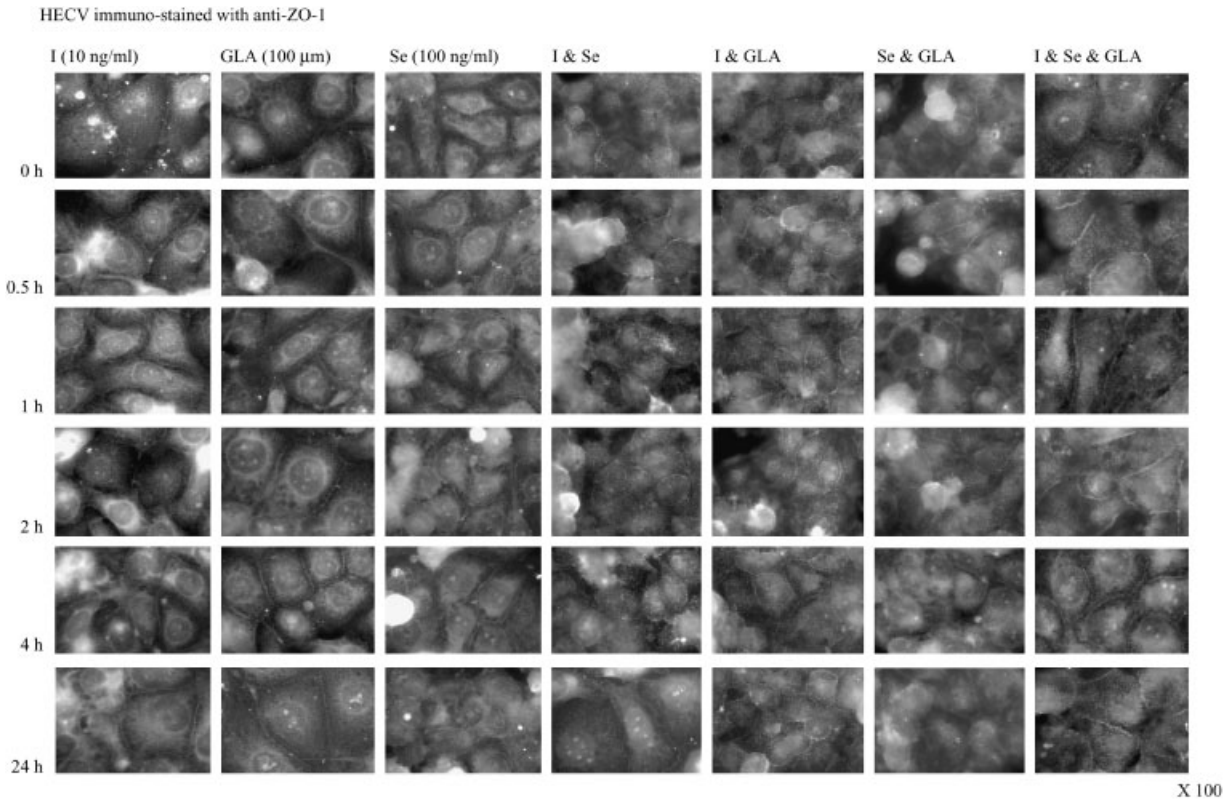

Fig. 6. Effect of I, Se, and GLA on relocation of ZO-1 in HECV treated over 24 h (X100).

The TJ is the most apical element of the junctional complex in epithelial and endothelial cells. This complex includes the TJ, adherens junction, desmosomes, and gap junctions. The TJ forms a barrier to paracellular movement of substances, keeping the apical and basolateral fluid compartments on opposite sides of the epithelial cell layer distinct. The TJ also acts in the maintenance of apical versus basolateral plasma membrane compositional asymmetry by restricting the movement of lipids and integral membrane proteins within the plane of the membrane [Wittchen et al., 1999].

TJs are thought to be directly involved in barrier function and fence functions in epithelial and endothelial cells by sealing them to generate the primary barrier against the diffusion of solutes through the paracellular pathway and by acting as a boundary between the apical and basolateral plasma membrane domains to create and maintain cell polarity, respectively [Furuse et al., 1998]. TJs appear as discrete sites of apparent fusion involving the outer leaflet of plasma membrane of adjacent cells (in ultra thin section EM's). They appear as a set of continuous unastomosing intramembrane particle strands in the protoplasmic face with compulsory grooves in the extracellular face (by freeze-fracture EM).

The TJs of different tissues have different permeability properties to ions and molecules [Wong, 1997]. These differences in permeability properties appear to be important for the respective physiological function of each tissue. Due to the location of the TJs they are involved in a wide variety of pathological conditions where the physiological regulation of passage of ions, molecules and inflammatory cells may be affected [Sawada et al., 2003]. These conditions include vascular system barrier function disturbance (oedema, multiple sclerosis, metastasis, and cytokinemia) gastrointestinal tract diseases (Crohn's, colitis, gastritis, and Coeliac disease) as well as jaundice, asthma, viral infections etc. [Sawada et al., 2003]. During such various dynamic processes physiological processes such as leukocyte transmigration across endothelium, the TJs are tightly controlled. The permeability barrier is temporarily disrupted, but subsequently resealed, relatively

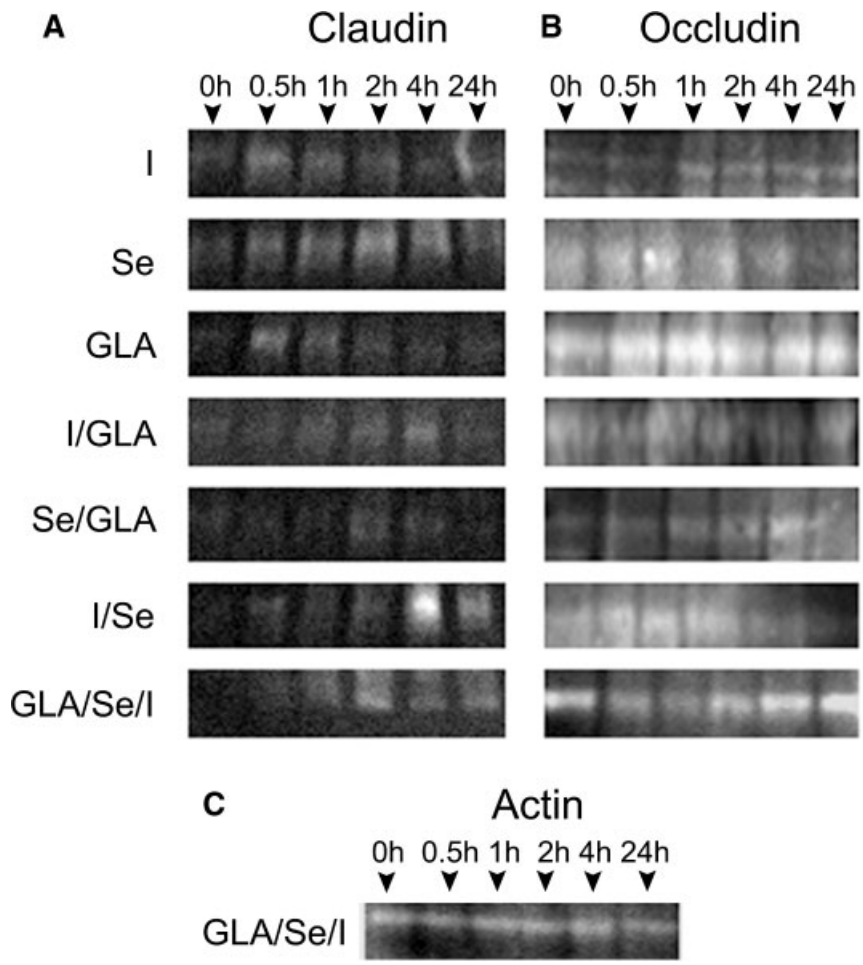

Fig. 7. Effect of I, Se, and GLA on expression of (**A**) Caludin-5 and (**B**) ZO-1 in HECV treated over 24 h. The combined effect of GLA/Se/I produced the most marked increase in expression of both tight junction proteins. **C**: Shows the protein signal for actin, which remained unchanged.

quickly (usually within 1 h). This resealing is accomplished by assembly of pre-existing elements, rather than resynthesis [Wong, 1997].

Claudins comprise a multigene family consisting of more than 20 members (approximately 22 kDa) structurally related, with four transmembrane domains, but no sequence similarity to Occludin [Morita et al., 1999; Tsukita and Furuse, 1999]. Heterogeneous Claudin species (and also Occludin) are copolymerized to form individual TJ strands as heteropolymers [Hamazaki et al., 2002]. Between adjacent cells Claudin molecules adhere to each other in both homotypic and heterotypic manners. Claudins bind to ZO-1 [Morita et al., 1999] and constitute the major transmembrane proteins of TJs with Occludin [Hoevel et al., 2002]. Claudin-5 is a protein component of many endothelial TJs, including those at the blood-brain barrier [Wen et al., 2004]. In this study, we have shown that Claudin-5 is recruited to the TJ in HECV following the application of I and Se, after 0.5–1 h incubation. This is concurrent with increased TJ functions, as assessed by transendothelial resistance and PCP.

Occludin, a 60–65 kDa integral membrane protein is localized at TJs where the C-terminal binds to ZO-1 [Furuse et al., 1998; Tsukita and Furuse, 1999]. The extracellular surface of Occludin was found to be directly involved in cell–cell adhesion and the ability to confer adhesiveness correlated with the ability to colocalise with ZO-1 [Van Itallie and Anderson, 1997]. Wachtel et al. [1999] have suggested that the regulation of Occludin expression could contribute to the control of PCP in endothelial cells. This current study has demonstrated that

during the increased TJ functionality of HECV treated with Se, I, and GLA, Occludin exhibits an increased location from the cytoplasm to the junctional area of these cells. This was particularly evident in those cells treated with Se, alone and when combined with GLA. We have previously demonstrated that GLA is able to regulate the expression of Occludin in HECV [Jiang et al., 1998; Martin et al., 2000] and it has been shown that GLA increases TER and expression of Occludin in brain capillary endothelial cells [Yamagata et al., 2003]. Moreover, we have also previously demonstrated that 17β-estradiol is able to reduce TJ function via reduced Occludin expression in HECV, supporting the evidence presented here, that application of GLA, I, and Se can reduce the effects of this hormone on endothelial cells, an important indication in the etiology of mastalgia.

The zonula occludens family are peripheral membrane proteins that are localized in the immediate vicinity of the plasma membrane of TJs in epithelial and endothelial cells [Furuse et al., 1998] where they are part of the organization of epithelial and endothelial TJs, linking the transmembrane proteins to the actin cytoskeleton, and hence nuclear regulation. In non-epithelial cells, ZO-1 works as a cross-linker between cadherin/catenin complex and the actin-based cytoskeleton through direct interaction with alpha-catenin and actin filaments at its N- and C-terminal halves respectively, and that ZO-1 is also a functional component of the cadherin-based cell adhesion system [Itoh et al., 1997]. ZO-1, -2, and -3 constitute the undercoat structure of the TJ, together with other peripheral proteins such as cingulin, 7H6 antigen and symplekin [Itoh et al., 1997]. Contrary to the increased localization of Claudin-5 and Occludin observed in this study, after the application of I, ZO-1 did not show increased localization to the TJ. There was some increase in staining intensity with Se and GLA after 1–2 h incubation. However, the combination of all three substances did result in a faster increase in intensity, that is, after 0.5 h incubation.

TJs are being increasingly associated with a number of pathological conditions, and as such, studies on TJs have multiplied over recent years. However, the majority of these studies are conducted as investigations into the mode and means of cancer and metastatic spread, concentrating on agents that are able to disrupt and dismantle TJs. Few studies have investigated agents that are able to increase the "strength" of TJs and thus prevent "leakiness" of this vital structure. This is the first study to suggest possible combinations of agents that may have this strengthening effect. It has long been known that GLA has a positive effect on cell–cell adhesion, but we have for the first time shown that this effect can be increased with the addition of I and Se, two dietary supplements. Evidence has existed to suggest that Se may possess cancer-preventative activity via inhibition of angiogenesis [Jiang et al., 1999; Lu and Jiang, 2001], particularly in breast cancer, where low levels of Se are linked to induction of pro-angiogenic growth factors such as VEGF [Streicher et al., 2004]. Interestingly, Se has also been shown to regulate permeability of mitochondrial pores [Shilo et al., 2003] and that Se deficiency in rats leads to increased permeability of the heart and eye [Demirel-Yilmaz et al., 1998]. Such evidence, together with our observations may indicate that part of this anti-angiogenic effect may be contributed by increased "tightness" of TJs, thus leading to loss of proliferation and tubulogenesis of endothelial cells. Se has also been demonstrated to be able to modulate adhesion molecule expression during the inflammatory process [Jahnova et al., 2002].

Increased tissue permeability is a common characteristic of a number of diseases such as pulmonary oedema, inflammatory bowel disease, several kidney diseases, diabetic retinopathy, and tumors [Harhaj et al., 2002]. 17β-estradiol is known to promote permeability of cervical cells, playing a role in cervical and vaginal secretion [Gorodeski, 2000]. Nguyen et al. [2001] looked at the hormonal regulation of TJs in mouse mammary epithelium. Closure of the TJs of the mammary epithelium has been shown to accompany the onset of copious milk secretion (lactogenesis) in both goats and humans. Progesterone withdrawal was a trigger for TJ closure within 4 h. The hormonal requirements for TJ closure were similar to those required to promote lactogenesis. It appears that regulation of TJs could have a direct impact on the permeability of blood vessels within mammary tissue; it could be construed that this may be an important regulatory means of oedema in the breast during the menstrual cycle, consequently effecting changes in the breast associated with mastalgia.

It has been proposed that the digestion of nutrients high in I and Se (having a synergistic effect with I) contributes to lower incidences of both benign and malignant breast disease [Cann et al., 2000]. It has been shown that I deficiency enhances mammary tissue sensitivity to 17β-estradiol in rats [Eskin et al., 1995], and that in humans, I containing desiccated thyroid or thyroxine were effective in reducing mastalgia [Daro et al., 1964; Estes, 1981]. Moreover, I supplementation in women with benign breast disease and breast pain led to a beneficial effect [Vishniakova and Murav'eva, 1966; Ghent et al., 1993].

In conclusion, the current study has provided evidence that I and Se, two elements known to influence endothelial cells, work together with GLA and enhance the function of TJs in HECV. These two compounds also increase the action of GLA, which we have previously shown to increase TJ integrity. Furthermore, the combination of three compounds is able to negate the 'leaky' effect of 17β-estradiol on TJs. The primary mechanism underlying the effects is by relocation of the important TJ proteins Claudin-5, Occludin, and ZO-1 to the cell–cell junction of these cells. The study has therefore provided mechanistic insight into the biological and physiological impact of the compounds, and in particular their combination on cell junctional functions.

REFERENCES

Balda MS, Flores-Maldonado C, Cereijido M, Matter K. 2000. Multiple domains of occludin are involved in the regulation of paracellular permeability. J Cell Biochem 78(1):85–96.

Cann SA, van Netten JP, van Netten C. 2000. Hypothesis: Iodine, selenium and the development of breast cancer. Cancer Causes Control 11:121–127.

Daro AF, Gollin HA, Samos FH. 1964. The effect of thyroid on cystic mastitis. J Int Coll Surg 41:58–59.

Demirel-Yilmaz E, Dincer D, Yilmaz G, Turan B. 1998. The effect of selenium and vitamin E on microvascular permeability of rat organs. Biol Trace Elem Res 64(1–3): 161–168.

Eskin BA, Grotkowski CE, Connolly CP, Ghent WR. 1995. Different tissue responses for iodine and iodide in rat thyroid and mammary glands. Biol Trace Elem Res 49(1):9–19.

Estes NC. 1981. Mastodynia due to fibrocystic disease of the breast controlled with thyroid hormone. Am J Surg 142(6):764–766.

Fanning NF, Manning BJ, Buckley J, Redmond HP. 2002. Iodinated contrast media induce neutrophil apoptosis through a mitochondrial and caspase mediated pathway. Br J Radiol 75(899):861–873.

Furuse M, Fujita K, Hiiragi T, Fujimoto K, Tsukita S. 1998. Claudin-1 and -2: Novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. J Cell Biol 141(7):1539–1550.

Ghent WR, Eskin BA, Low DA, Hill LP. 1993. Iodine replacement in fibrocystic disease of the breast. Can J Surg 36(5):453–460.

Gorodeski GI. 2000. Effects of menopause and 17beta-estradiol on cervical epithelial permeability. J Clin Endocrinol Metab 85(7):2584–2595.

Gupta S, Narang R, Krishnaswami K, Yadav S. 1994. Plasma selenium level in cancer patients. Indian J Cancer 31(3):192–197.

Hamazaki Y, Itoh M, Sasaki H, Furuse M, Tsukita S. 2002. Multi-PDZ domain protein 1 (MUPP1) is concentrated at tight junctions through its possible interaction with claudin-1 and junctional adhesion molecule. J Biol Chem 277(1):455–461.

Harhaj NS, Barber AJ, Antonetti DA. 2002. Platelet-derived growth factor mediates tight junction redistribution and increases permeability in MDCK cells. J Cell Physiol 193(3):349–364.

Hoevel T, Macek R, Mundigl O, Swisshelm K, Kubbies M. 2002. Expression and targeting of the tight junction protein CLDN1 in CLDN1-negative human breast tumor cells. J Cell Physiol 191(1):60–68.

Ip C, Ganther HE. 1991. Combination of blocking agents and suppressing agents in cancer prevention. Carcinogenesis 12(2):365–367.

Itoh M, Nagafuchi A, Moroi S, Tsukita S. 1997. Involvement of ZO-1 in cadherin-based cell adhesion through its direct binding to alpha catenin and actin filaments. J Cell Biol 138(1):181–192.

Jahnova E, Horvathova M, Gazdik F, Weissova S. 2002. Effects of selenium supplementation on expression of adhesion molecules in corticoid-dependent asthmatics. Bratisl Lek Listy 103(1):12–16.

Jiang WG, Bryce RP, Horrobin DF, Mansel RE. 1998. Regulation of tight junction permeability and occludin expression by polyunsaturated fatty acids. Biochem Biophys Res Commun 244(2):414–420.

Jiang C, Jiang W, Ip C, Ganther H, Lu J. 1999. Selenium-induced inhibition of angiogenesis in mammary cancer at chemopreventive levels of intake. Mol Carcinog 26(4):213–225.

Kowaltowski AJ, Netto LE, Vercesi AE. 1998. The thiol-specific antioxidant enzyme prevents mitochondrial permeability transition. Evidence for the participation of reactive oxygen species in this mechanism. J Biol Chem 273(21):12766–12769.

Lu J. 2001. Apoptosis and angiogenesis in cancer prevention by selenium. Adv Exp Med Biol 492:131–145.

Lu J, Jiang C. 2001. Antiangiogenic activity of selenium in cancer chemoprevention: Metabolite-specific effects. Nutr Cancer 40(1):64–73.

Mankertz J, Waller JS, Hillenbrand B, Tavalali S, Florian P, Schoneberg T, Fromm M, Schulzke JD. 2002. Gene expression of the tight junction protein occludin includes differential splicing and alternative promoter usage. Biochem Biophys Res Commun 298(5): 657–666.

Martin TA, Matsumoto K, Nakamura T, Mansel RE, Jiang WG. 2000. A combination of NK4, a HGF/SF variant and gamma linolenic acid (GLA) increases inhibition of

HGF/SF stimulated human vascular endothelial angiogenesis. Breast Cancer Res Treat 64(1):133.

Medina R, Rahner C, Mitic LL, Anderson JM, Van Itallie CM. 2000. Occludin localization at the tight junction requires the second extracellular loop. J Membr Biol 178(3):235–247.

Morita K, Furuse M, Fujimoto K, Tsukita S. 1999. Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. Proc Natl Acad Sci USA 96(2):511–516.

Nguyen DA, Parlow AF, Neville MC. 2001. Hormonal regulation of tight junction closure in the mouse mammary epithelium during the transition from pregnancy to lactation. J Endocrinol 170(2):347–356.

Sawada N, Murata M, Kikuchi K, Osanai M, Tobioka H, Kojima T, Chiba H. 2003. Tight junctions and human diseases. Med Electron Microsc 36(3):147–156.

Shilo S, Aronis A, Komarnitsky R, Tirosh O. 2003. Selenite sensitizes mitochondrial permeability transition pore opening in vitro and in vivo: A possible mechanism for chemo-protection. Biochem J 370(Pt 1): 283–290.

Streicher KL, Sylte MJ, Johnson SE, Sordillo LM. 2004. Thioredoxin reductase regulates angiogenesis by increasing endothelial cell-derived vascular endothelial growth factor. Nutr Cancer 50(2):221–231.

Tsukita S, Furuse M. 1999. Occludin and claudins in tight-junction strands: Leading or supporting players? Trends Cell Biol 9(7):268–273.

Van Itallie CM, Anderson JM. 1997. Occludin confers adhesiveness when expressed in fibroblasts. J Cell Sci 110(Pt 9):1113–1121.

Vishniakova VV, Murav'eva NI. 1966. On the treatment of dyshormonal hyperplasia of mammary glands. Vestn Akad Med Nauk SSSR 21(9):19–22.

Wachtel M, Frei K, Ehler E, Fontana A, Winterhalter K, Gloor SM. 1999. Occludin proteolysis and increased permeability in endothelial cells through tyrosine phosphatase inhibition. J Cell Sci 112(Pt 23):4347–4356.

Wen H, Watry DD, Marcondes MC, Fox HS. 2004. Selective decrease in paracellular conductance of tight junctions: Role of the first extracellular domain of claudin-5. Mol Cell Biol 24(19):8408–8417.

Wittchen ES, Haskins J, Stevenson BR. 1999. Protein interactions at the tight junction. Actin has multiple binding partners, and ZO-1 forms independent complexes with ZO-2 and ZO-3. J Biol Chem 274(49):35179–35185.

Wong V. 1997. Phosphorylation of occludin correlates with occludin localization and function at the tight junction. Am J Physiol 273(6 Pt 1):C1859–C1867.

Wong V, Gumbiner BM. 1997. A synthetic peptide corresponding to the extracellular domain of occludin perturbs the tight junction permeability barrier. J Cell Biol 136(2):399–409.

Yamagata K, Tagami M, Takenaga F, Yamori Y, Nara Y, Itoh S. 2003. Polyunsaturated fatty acids induce tight junctions to form in brain capillary endothelial cells. Neuroscience 116(3):649–656.

Ye L, Martin TA, Parr C, Harrison GM, Mansel RE, Jiang WG. 2003. Biphasic effects of 17-beta-estradiol on expression of occludin and transendothelial resistance and paracellular permeability in human vascular endothelial cells. J Cell Physiol 196(2):362–369.