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Digoxin Immune Fab Protects Endothelial Cells from Ouabain-Induced Barrier Injury

Yuping Wang[1], **Ruping Fan**[1], **Yang Gu**[1], and **C. David Adair**[2]

[1]Department of Obstetrics and Gynecology, Louisiana State University Health Sciences Center (LSUHSC), Shreveport, LA, USA

[2]Department of Obstetrics and Gynecology, University of Tennessee, Chattanooga, TN, USA

Abstract

Problem—Endogenous digitalis-like factors (EDLF) inhibit sodium pump Na^+ / K^+ATPase activity, and maternal EDLF levels are elevated in preeclampsia (PE). This study determined whether digoxin immune Fab (DIF) could protect endothelial cells (ECs) from EDLF-induced endothelial barrier dysfunction.

Method of study—ECs were treated with escalating doses of ouabain (a known EDLF) in the presence or absence of DIF. EC barrier integrity was examined by junction protein VE-cadherin and occludin expressions. EC permeability was determined by horseradish-peroxidase (HRP) leakage and transendothelial electrical resistance (TEER).

Results—EC junction protein VE-cadherin distribution was disrupted in cells treated with ouabain. DIF, but not control IgG Fab fragment, blocked ouabain-induced decreases in VE-cadherin and occludin expressions and prevented ouabain-induced HRP leakage and TEER changes.

Conclusion—DIF protects ECs from ouabain-induced barrier injury, providing evidence of beneficial effects of DIF on EC function and supporting that Na^+ /K^+ATPase might be a therapeutic target to ameliorate endothelial dysfunction.

Keywords

Endogenous digitalis-like factors; endothelial barrier function; Na^+ / K^+ATPase; occludin; VE-cadherin

Introduction

Endogenous digitalis-like factors (EDLFs) inhibit sodium pump Na^+ /K^+ATPase activity,[1,2] which could affect Ca^{++} influx and result in vasoconstriction. During pregnancy, maternal EDLF levels are increased in women with preeclampsia compared with those of

Correspondence: Yuping Wang, MD, PhD, Department of Obstetrics and Gynecology, Louisiana State University Health Sciences Center, PO Box 33932, Shreveport, LA 71130, USA. ywang1@lsuhsc.edu.

Declaration

Yuping Wang, MD, PhD, is a member of Advisory Board of Glenveigh Medical LLC. C. David Adair, MD, is CSO of Glenveigh Medical LLC, Chattanooga, TN 37402.

normotensive pregnancies.[1–3] Although the reason for increased EDLFs in preeclampsia is not known, it is believed that elevated EDLF levels may contribute to increased vasoconstriction associated with maternal hypertension and reduced utero-placental blood flow in preeclampsia.[3–5] Studies have also shown that digoxin immune Fab (DIF), a polyclonal DIF, may be beneficial to preeclampsia. DIF has an antihypertensive effect to reduce maternal blood pressure and increase utero-placental blood flow.[4,5] It could restore Na^+ /K^+ATPase activity of red blood cells both in humans and in animals.[6] Recently, the DIF Efficacy Evaluation in Preeclampsia (DEEP) clinical trial also found that DIF could improve renal function in severe preeclamptic patients.[7]

Increased vascular permeability is an underlying pathophysiology in preeclampsia. Studies have revealed that marinobufagenin (MBG) could induce vascular leakage in an animal model and promote endothelial permeability *in vitro*.[8,9] MBG is an endogenous mammalian cardiotonic steroid that is involved in the inhibition of sodium pump Na^+ / K^+ATPase. Increased plasma levels of MBG were also reported in patients with preeclampsia.[10] These observations suggest that inhibition of Na^+ /K^+ATPase may promote endothelial permeability in preeclampsia. To test this, we designed an *in vitro* cell culture study and specifically tested whether ouabain, an EDLF, could disrupt endothelial junction structure and induce endothelial permeability and whether DIF could protect endothelial cells from ouabain-induced barrier injury.

Materials and methods

Chemicals, Reagents, and Supplies

Endothelial growth medium (EGM) was purchased from Lonza Walkersville, Inc. (Walkersville, MD, USA). DIF was provided by Glenveigh Medical LLC. (Chattanooga, TN, USA). Ouabain, penicillin and streptomycin, phosphate-buffered saline (PBS) and Hank's balanced salt solution, sodium phosphate buffer, horseradish peroxidase (HRP, VI-A type, 44,000 MW), guaiacol, hydrogen peroxide (H_2O_2), and β-actin antibody were from Sigma Chemical Co. (St. Louis, MO, USA); trypsin/EDTA from Cellgro, Mediatech Inc. (Manassas, VA, USA); fibronectin from Biomedical Technologies Inc (Stoughton, MA, USA); collagenase from Worthington Biochemical Corporation (Lakewood, NJ, USA); monoclonal anti-VE-cadherin antibody from Beckman Coulter Technology, Inc. (Fullerton, CA, USA); monoclonal anti-occludin antibody from Zymed Laboratories Inc. (South San Francisco, CA, USA); Cy3 donkey anti-mouse immunoglobulin (IgG) (H + L) fluorescent-labeled antibody, goat anti-human IgG, F(ab′)2 fragment and anti-sheep whole serum from Jackson Immunoresearch Laboratories Inc. (West Grove, PA, USA), and vectashield mounting medium from Vector Lab Inc. (Burlingame, CA, USA). All other chemicals were from Sigma unless otherwise noted.

Endothelial cell isolation and culture

Endothelial cells were isolated from human umbilical cord vein (HUVECs) of normotensive term placentas. Using umbilical cords as a source of endothelial cells was approved by the Institutional Review Board (IRB) for Human Research at Louisiana State University Health Sciences Center – Shreveport (LSUHSC-S), LA, USA. HUVECs were isolated as previously

described.[11,12] Briefly, umbilical cord vein endothelial cells were isolated by collagenase digestion. Isolated endothelial cells were seeded on to fibronectin-coated culture plates or flasks and incubated with EGM. Only first passage cells were used in experiments. Endothelial cells grown on glass cover slips were used for immunofluorescent staining of endothelial junction protein VE-cadherin; Cells grown on six-well plates or 25-cm^2 flasks were used for extraction of total cellular protein for VE-cadherin and occludin expression; cells seeded on cell culture inserts (8 μm pore size) in 24-well plates were used to examine endothelial permeability.

Immunofluorescent Staining

Endothelial VE-cadherin expression and distribution were determined by immunofluorescent staining as previously described.[13] Briefly, cells grown on glass cover slips were fixed with 95% ethanol and permeabilized with 0.5% Triton X-100 in 0.3 M glycine. For staining, fixed cells were incubated with 1% BSA in PBS to block non-specific binding for 1 hr and then probed with primary monoclonal antibody specific against VE-cadherin, then followed by Cy3-conjugated secondary antibody. After staining, cover slips were mounted on slides with vectashield mounting medium and examined by fluorescent microscope (Olympus IX71, Tokyo, Japan). Images were captured by a digital camera with PictureFrame computer software (Uptronics Inc., Sunnyvale, CA, USA) and recorded to a microscope-linked PC.

Protein Expression for VE-cadherin and Occludin

Endothelial expressions of VE-cadherin and occludin were determined by Western blot. Total cellular protein was extracted with ice-cold protein lysis buffer containing 50 mM Tris, 0.5% NP40, and 0.5% Triton X-100 with protease inhibitors of phenylmethylsulfonyl fluoride, dithiothreitol, leupeptin, and aprotinin. An aliquot of total cellular protein (10 μg of each sample) was subject to electrophoresis (Bio-Rad, Hercules, CA, USA) and then transferred to nitrocellulose membranes. The membranes were then probed with specific antibody against VE-cadherin and secondary antibody. The bound antibody was visualized with an enhanced chemiluminescent (ECL) detection kit (Amersham Corp, Arlington Heights, IL, USA). The membranes were stripped, blocked, and then re-probed with anti-occludin antibody and secondary antibody, followed by detection with an ECL detection kit and exposure to the films. β-actin expression was determined as an indicator of equal loading for each sample.

Endothelial Permeability Assays

Endothelial permeability was determined by two assays: (i) by measuring HRP leakage through cell culture inserts and (ii) by measuring trans-endothelial electrical resistance (TEER) as previously described.[14,15] For HRP assay, an aliquot of medium collected from the lower chamber was used for measuring HRP enzymatic activity. Briefly, 25 μL of the culture medium was added to 860 μL of a reaction buffer (50 mM NaH_2PO_4 with 5 mM guaiacol). Each sample was measured in duplicate. The reaction was initiated by adding 500 μL H_2O_2. After reaction, the samples were read by a spectrophotometer at 470 nm (Ultraspec 3000; Pharmacia Biotech, Cambridge, UK). Data was calculated as OD470nm sample-OD470nm blank and expressed as OD470nm for permeation of HRP across

transwell filters. For TEER assay, an Endohm™ EVOM endothelial ohmmeter (World Precision Instruments, Sarasota, FL, USA) was used. TEER was measured in duplicate per well at each time point. The average reading from duplicated measurements was used for data calculation. The reading obtained from a blank insert was subtracted to give the net resistance, which was multiplied by the membrane area to give the resistance in area-corrected units. The value for endothelial resistance was expressed as $\Omega \cdot cm^2$, taking into account the surface area of the filter (0.30 cm^2).

Statistical Analysis

Data obtained from HRP and TEER assays are expressed as means ± S.E. and analyzed by analysis of variance (ANOVA) with Student–Newman–Keuls test as a *post hoc* test. The computer software program StatView (Cary, NC, USA) was used. A probability level of $P < 0.05$ was considered statistically significant.

Results

Ouabain-induced Disruption of Endothelial Junction Structure

We first determined whether inhibition of sodium pump Na^+ /K^+ATPase could disrupt endothelial barrier structure. VE-cadherin was examined as an indicator of endothelial junction structure integrity. Ouabain, a plasma membrane sodium pump Na^+ / K^+ATPase inhibitor, was used as an EDLF. Confluent endothelial cells were treated with ouabain at different concentrations (100, 200, and 500 nM) for 6 hr. Cells were then fixed, and endothelial junction adhesion protein VE-cadherin expression and distribution were examined by immunofluorescent staining. We found that VE-cadherin distribution was interrupted and revealed as linear or discontinuous pattern compared with the untreated control cells, which exhibited a continuous and zigzag pattern along the cell border (cell contact region) (Fig. 1A). Altered VE-cadherin expression induced by ouabain was further examined by Western blot. We noticed that ouabain-induced disorganization of VE-cadherin distribution at cell junction and downregulation of VE-cadherin expression was in a dose- and time-dependent manner (Fig. 1B).

DIF Restored Ouabain-induced Altered VE-cadherin expression and Distribution in Endothelial Cells

Ouabain is a Na^+ /K^+ATPase inhibitor. If altered VE-cadherin expression is associated with Na^+ /K^+ATPase activity, then DIF could potentially protect endothelial cells from ouabain-induced altered VE-cadherin distribution and expression. To test this, confluent endothelial cells were pretreated with DIF (100 μg /mL) for 1 hr and then different concentrations of ouabain (100, 200, and 500 nM) were added to the cultures and cells were continuously incubated for 6 hr. VE-cadherin distribution was evaluated by immunofluorescent staining. Our results showed that pretreatment of endothelial cells with DIF could block ouabain-induced altered VE-cadherin distribution at endothelial junction (Fig. 2A).

The ouabain-induced harmful effect and DIF protective effect on VE-cadherin expression and distribution were further examined by Western blot. As shown in Fig. 2B, VE-cadherin expression was dose dependently reduced in cells treated with ouabain. In contrast, VE-

cadherin expression showed no difference when cells were pretreated with DIF. We also examined occludin expression. Occludin is a tight junction protein in endothelial cells. Similar to VE-cadherin, occludin expression was also dose dependently reduced in cells treated with ouabain and the ouabain-induced downregulation of occludin was no longer present in cells pretreated with DIF (Fig. 2B).

To further determine whether the barrier structural protective effect by DIF is specific via Na^+ /K^+ATPase, two control antibodies were used to pretreat endothelial cells, goat anti-human IgG, F(ab′)2 fragment, and anti-sheep whole serum (No sheep anti-human IgG, F(ab′)2 fragment is available). Our results showed that both anti-human IgG, F(ab′)2 fragment and anti-sheep whole serum had no effect to prevent ouabain-induced downregulation of VE-cadherin and occludin expressions in endothelial cells (Fig. 2C). These results indicate that the DIF-produced protective effect on endothelial junction molecules is specific *via* Na^+ /K^+ATPase.

DIF Protects Endothelial Cells from Ouabain-Induced Increased Endothelial Permeability

Ouabain-induced endothelial barrier injury was further evaluated by examination of endothelial permeability by measuring HRP leakage and TEER. In the permeability experiment, ouabain at concentration of 200 nM was used. Confluent cells grown in cell culture inserts were treated with ouabain with or without addition of DIF in culture. HRP leakage and TEER were measured at 2, 4, 6, 20, and 24 hr. Results are shown in Fig. 3a,b, respectively. Cells treated with ouabain showed a time-dependent increased in HRP leakage and decreased in TEER, which were significantly different from control cells and cells pretreated with DIF.

Discussion

In this study, we found that digoxin-like factor ouabain could disturb endothelial barrier structure and induce endothelial permeability as demonstrated by disruption and downregulation of endothelial junction adhesion protein VE-cadherin and tight junction protein occludin. Most significantly, we found that DIF, a polyclonal digoxin antibody fragment, could protect endothelial cells from ouabain-induced endothelial injury in cell junction structure and permeability. These results indicate that EDLFs exert harmful effects on endothelial barrier function.

VE-cadherin is a specific endothelial adhesion junction molecule in maintaining endothelial integrity. VE-cadherin plays a key role in the organization of lateral endothelial junctions, and the expression of VE-cadherin is required for preservation of the normal barrier properties of vascular endothelium.[16] Occludin is a transmembrane glycoprotein located at the tight junction. Tight junction is thought to play dual roles in endothelial function by sealing endothelial junctions to create the primary barriers to the diffusion of solutes through the paracellular pathway and by serving as a boundary between the apical- and baso-lateral plasma membrane domains to create and maintain cell polarity.[17,18] We previously reported that disrupted VE-cadherin and occludin expression and distribution at cell junction are associated with increased endothelial permeability in HUVECs from preeclampsia.[13] Maternal EDLF levels are increased in women with preeclampsia.[2,3] Thus, the finding of

disruption of endothelial junction molecules and increase in endothelial permeability induced by ouabain supports the notion that increased circulating EDLF levels could contribute to increased endothelial permeability in women with preeclampsia.

Na^+ /K^+ATPase is well known for its role as a maintainer of electrolyte and fluid balance in cells. The mechanism of action of ouabain involves binding to and inhibition of the plasma membrane Na^+ /K^+ATPase. Interestingly, recent studies demonstrate that Na^+ /K^+ATPase could also function as a signal transducer. Ouabain binding to Na^+ /K^+ATPase-induced signal transduction was found through regulation of protein tyrosine phosphorylation.[19] Ouabain could trigger several protein phosphorylation events including the activation of mitogen-activated protein kinase signal cascades, mitochondrial reactive oxygen species production as well as activation of phospholipase C and inositol triphosphate receptor (IP3R) in different intracellular compartments.[19] A study also shows that Na^+ /K^+ATPase binds Src and mediates Src signaling regulation. Src inhibitor that targets the Na^+ / K^+ATPase / Src receptor complex could antagonize ouabain-induced protein kinase cascades.[20] The association of Src and VE-cadherin was also reported.[21,22] ICAM-1-mediated, Src-dependent endothelial cadherin tyrosine phosphorylation is required for leukocyte transendothelial migration.[21] In the present study, we did not examine ouabain-induced signal response in endothelial cells associated with increased endothelial permeability, because activation of Src family kinases and the subsequent phosphorylation of VE-cadherin have been proposed as major regulatory steps leading to increase in vascular permeability in response to inflammatory mediators and growth factors.[21,22]

The most significant finding of our study is the inhibitory effect of DIF on ouabain-induced disruption of endothelial junction molecules and increased endothelial permeability. DIF is an antibody made from immunoglobulin fragments from sheep. To test the specificity of DIF protective effects on endothelial cells, two control antibodies were also used in our study. One is goat anti-human IgG, F(ab′)2 fragment, and the other is anti-sheep whole serum. Our results showed that both anti-human IgG, F(ab′)2 fragment and anti-sheep whole serum had no effect against ouabain-induced downregulation of VE-cadherin and occludin expression in endothelial cells, indicating that DIF-produced protective effects on endothelial junction molecules are specific. As Na^+ /K^+ATPase is the target of ouabain, our data also suggest that Na^+ /K^+ATPase activity is upstream of endothelial junction molecule signaling regulation in endothelial cells. It is very likely that normal functionality of cell membrane Na^+ / K^+ATPase, the molecular machinery of the cellular sodium pump, is a key regulator in controlling endothelial junction integrity. Therefore, protection of cell membrane Na^+ / K^+ATPase from toxic factor-induced injures such as EDLFs is critical for maintaining endothelial junction integrity. We speculate that Na^+ /K^+ATPase might be a therapeutic target to ameliorate endothelial dysfunction-associated disorders, such as preeclampsia.

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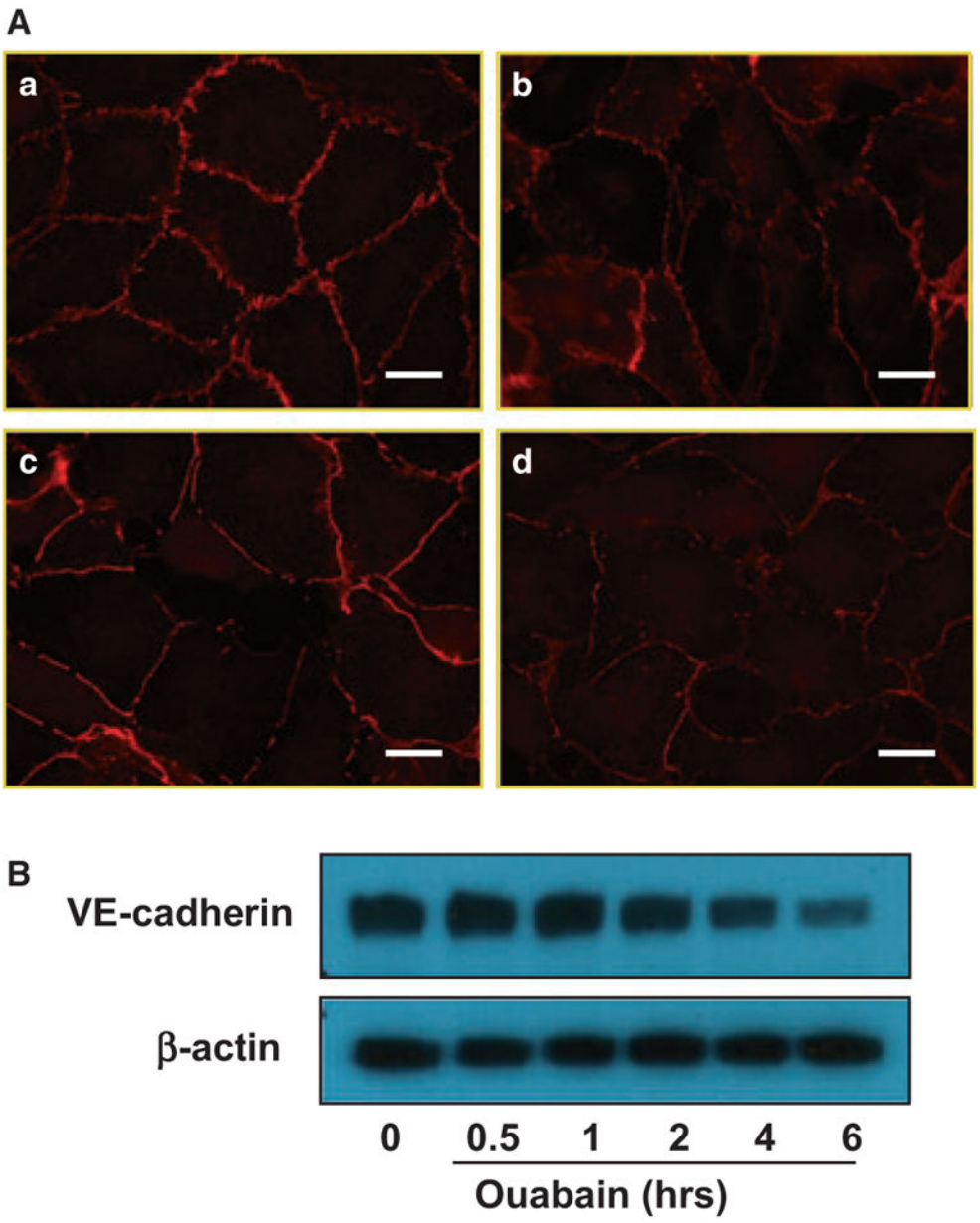

Fig. 1.

VE-cadherin expression and distribution in ECs treated with ouabian. (A) Immunofluorescent staining of VE-cadherin in ECs treated with ouabain. (a) Control cells; (b) ouabain 100 nM; (c) ouabain 200 nm; and (d) ouabain 500 nm, bar = 20 μm. (B) VE-cadherin expression by Western blot. ECs were treated with ouabain at 100 nm for 0, 0.5, 1, 2, 4, 6 hr. Images and blot are representative from three independent experiments.

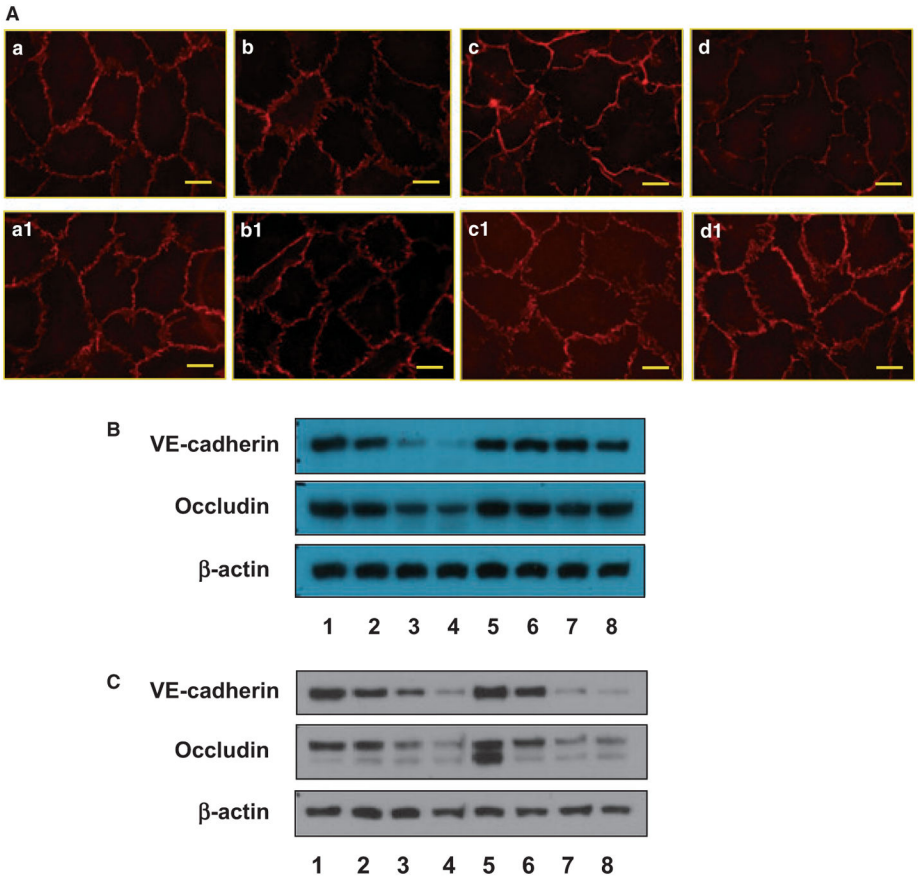

Fig. 2.

Effects of digoxin immune Fab (DIF) on ouabain-treated ECs. (A) Immunofluorescent staining of VE-cadherin. (a–d) Control cells, cells treated with ouabain at 100, 200, and 500 nM for 6 hr. (a1–d1) ECs treated with DIF 100 μg / mL only, DIF + ouabain 100 nm; DIF + ouabain 200 nm; and DIF + ouabain 500 nm, respectively. Bar = 20 μm. Representative images are from three independent experiments. (B) VE-cadherin and occludin expression by Western blot. Lanes 1–4 are control ECs, ECs treated with ouabain at 100, 200, and 500 nm for 6 hr; lanes 5–8 are cells treated with DIF alone, and DIF + ouabain at 100, 200, and 500 nm for 6 hr, respectively. Ouabain dose dependently downregulates VE-cadherin and occludin expression, which could be attenuated by pretreatment of ECs with DIF. Representative images are from three independent experiments. (C) VE-cadherin and occludin expression by Western blot. Lanes 1–4 are control ECs, ECs treated with ouabain at 100, 200, and 500 nm for 6 hr; lanes 5–8 are cells treated with goat IgG, F(ab′)2 fragment alone, and goat IgG, F(ab′)2 fragment + ouabain at 100, 200, and 500 nm for 6 hr, respectively. IgG, F(ab′)2 fragment had no effect on ouabain-induced downregulation of VE-cadherin and occludin expressions. Representative images are from three independent experiments.

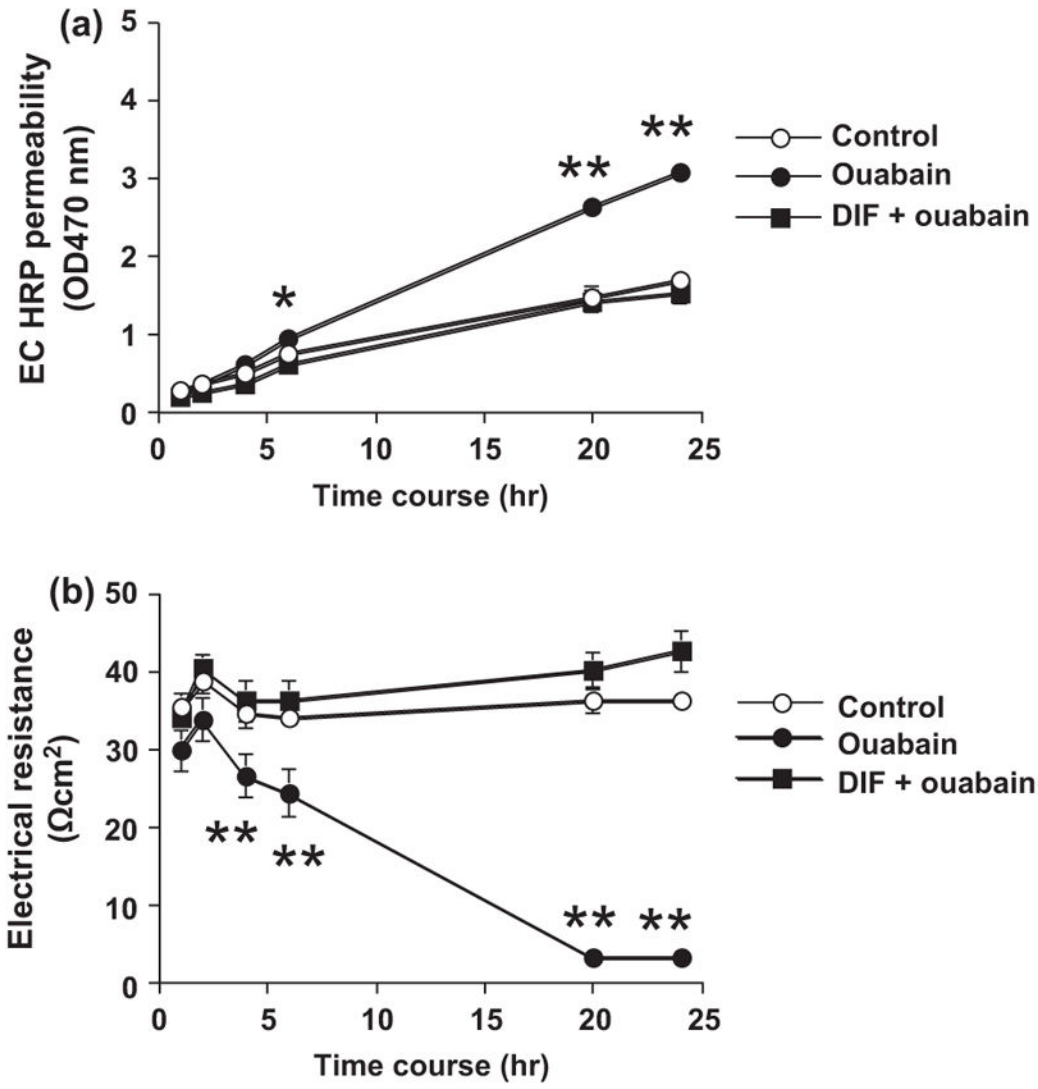

Fig. 3.

Endothelial permeability assays measured by horseradish-peroxidase (HRP) leakage and by transendothelial electrical resistance (TEER). (a) HRP permeability and (b) TEER. Data are means from four independent experiments and triplicates in each, $*P < 0.05$ and $**P < 0.01$ in ouabain 200 nM treated cells compared with control cells and cells pretreated with digoxin immune Fab 100 μg / mL + ouabain 200 nm at each time point, respectively.