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HARVESTING, IDENTIFICATION AND BARRIER FUNCTION OF HUMAN LUNG MICROVASCULAR ENDOTHELIAL CELLS

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Abstract

Endothelial barrier dysfunction is an important contributor to the pathogenesis of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). Even though approaches that target the prevention and repair of endothelial barrier dysfunction are clearly needed, our understanding of the molecular regulation of pulmonary microvascular endothelial permeability remains incomplete. Cultured pulmonary microvascular endothelial cells represent an attractive paradigm for the study of barrier function. Here, we describe a method for the harvest, identification and culture of human lung microvascular endothelial cells (HLMVEC). HLMVEC thus obtained, grow as a monolayer, exhibit contact inhibition and have the typical cobblestone appearance. They express endothelial proteins, such as von Willebrand Factor and endothelial nitric oxide synthase and take up acetylated LDL. Furthermore, HLMVEC respond predictably and with superior sensitivity to the barrier disruptive effects of Gram positive and Gram negative bacterial products, thrombin, vascular endothelial growth factor and microtubule disrupting agents. These HLMVEC present an in-house-derived alternative to commercially available human cells for the study of mechanisms contributing to ALI and ARDS.

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INTRODUCTION

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) represent a continuum of progressive respiratory failure in the absence of left heart failure. ARDS patients represent a subset of ALI patients, distinguished by a greater severity. In ALI/ARDS, the integrity of the capillary barrier is compromised, leading to increased vascular permeability and alveolar flooding. Gram negative sepsis (indirect injury) is by far the most common cause of ALI (Hudson *et al.* 1995). Sepsis represents the systemic inflammatory response to infection[3], (Jacobi 2002). Lungs are among the most frequently affected organs in severe sepsis leading to ALI and ARDS (Martin *et al.* 2003). The incidence of sepsis has increased by 8.7% from 1979–2000 (Martin *et al.* 2003) and mortality ranges from 30–50% (Rangel-Frausto *et al.* 1995; Angus *et al.* 2000; Annane *et al.* 2000). Clinical trials targeting inflammatory mediators have shown no survival benefit (Fisher *et al.* 1994; McCloskey *et al.* 1994; Abraham *et al.* 1998; Dhainaut *et al.* 1998; Fink 1998; Abraham *et al.* 2001) and other strategies have failed to reduce morbidity associated with severe sepsis except for the survival benefit with the use of recombinant activated protein C (Bernard *et al.* 2001).

Even though approaches that target the prevention and repair of endothelial barrier dysfunction are clearly needed, our understanding of the molecular regulation of pulmonary microvascular endothelial permeability remains incomplete. Cultured pulmonary microvascular endothelial cells represent an attractive paradigm for the study of barrier function. However animal-derived endothelial cells do not necessarily reflect the complex biology of human endothelial cells. Moreover, human endothelial cells, while available commercially, are expensive and, frequently of inconsistent quality. Here we present a method for the harvesting, identification and culture of human lung microvascular endothelial cells. We further provide data to suggest that these cells exhibit a strong baseline barrier function and respond predictable to common edemagenic agents.

MATERIALS AND METHODS

1. Materials

Tosyl activated Dynabeads and Prolong Gold were from Invitrogen; eNOS antibody was from Becton Dickinson; vWF antibody was from Sigma; goat anti-mouse IgG and Cy3 goat anti- rabbit IgG were from Jackson Laboratories. Fetal bovine serum (FBS) was from Hyclone. All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Eight-well arrays were from Applied Biophysics (Albany, NY).

2. Harvest, culture and identification of human lung microvascular endothelial cells. (HLMVEC)

Peripheral lung specimens from patients that were undergoing lobectomy or pneumonectomy at the Medical College of Georgia Hospital were obtained. The overwhelming majority of these patients were undergoing curative surgery for lung cancer. All tissue was from anatomic resection specimens obtained away from the primary tumor, where tumor margins are not an issue. We did not use pulmonary wedge resection specimens because of the small amount of tissue that would be available. Patients with

known underlying lung disease such as tuberculosis, pulmonary fibrosis or interstitial lung disease and patients with known systemic diseases such as HIV were not enrolled. All specimens were obtained from subjects 70 years of age or younger. We have observed that the age of the donor is a major determinant of the quality of the endothelial cell isolation. Specimens of varying size (5–15g) were placed on ice in a sterile 50ml tube containing culture medium and were transported to the laboratory for processing within one hour of resection. Isolation and culture of HLMVEC was performed by a modification of the methods of Hewett and Murray (Hewett & Murray 1993) and Burg et al. (Burg *et al.* 2002). Briefly, isolation of HLMVEC was performed as follows: sub-pleural lung tissue was cut into small fragments with scissors. After removal of debris and erythrocytes through a 40μm nylon net, the tissue collected in the net was treated with dispase (1 U/ml at 4°C for 18h). After filtration through a 100μm nylon net, the filtrate was treated in a volume of 15ml M199, 15% FBS, 1mg dispase/ml at 37°C for 1h, followed by a further 100μm net filtration. The cell clumps within the filtrate were repeatedly resuspended in M199 and filtered through a 40μm net, followed by centrifugation for 10min at 1000rpm and re-suspension in M199 with 20% FBS. The positive selection of HLMVEC was achieved by interacting the cell suspension with magnetic beads (Tosyl activated Dynabeads) coated with *Ulex europaeus I*, according to the method of Jackson et al (Jackson *et al.* 1990). Cells were cultured in M199 supplemented with 20% FBS, 100 Units/ml heparin, 150μg/ml ECGF, 1μg/ml hydrocortisone, 292mg/l L-glutamine, and 110mg/l sodium pyruvate. The cells thus collected were identified as HLMVEC by their 1) growth as a contact-inhibited monolayer; 2) exhibition of cobblestone-like appearance;3) uptake of 1,1_-dioctadecyl-1,3,3_,3_-tetramethyl-indocarbocyanine-acetylated low-density lipoprotein (Dil-Ac-LDL), 4) expression of endothelial nitric oxide synthase (eNOS or NOS3) and 5) expression of von Willebrand factor (vWF), as described below. Cells were sub-cultured 1:3 using standard techniques.

For purposes of identification, cells were grown on glass coverslips and incubated in Dil-Ac-LDL (10μg/ml medium) for 4h at 37°C. Cells were washed three times with PBS, fixed in 4% neutral buffered formalin for 15min, washed three times with PBS, and mounted with Prolong Gold on microscope slides. For immunostaining, cells were grown on coverslips, washed three times in PBS, fixed in acetone:methanol (1:1) at −20° C for 10min, washed three times in PBS and blocked for 1h in 1% BSA in PBS. Cells were then incubated overnight at 4° C in primary antibody at 1:1000 dilution in blocking buffer for eNOS and 1:200 dilution in blocking buffer for vWF. Cells were then washed three times in blocking buffer and then incubated in secondary antibody for 1h at room temperature: either 1:1000 Cy3 conjugated goat anti-mouse IgG antibody for eNOS or 1:1000 Cy3 goat anti- rabbit IgG antibody for vWF. Cells were washed three times in PBS and mounted with ProLong Gold on microscope slides. Dil-Ac-LDL uptake and eNOS and vWF expression were observed using an Axio Observer D1 microscope (Zeiss) with rhodamine filter.

3. Measurements of transendothelial resistance (TER) across endothelial monolayers

Cells were grown in special wells (0.8 cm^2) at seeding density of 1.25×10^5 cells/cm^2, as we have previously reported (Antonov *et al.* 2008; Chatterjee *et al.* 2008). Electrical resistance was continuously monitored during the course of treatments with an Applied Biophysics

Electric Cell-Substrate Impedance Sensing System (Applied Biophysics, Albany, NY). The system can monitor 16 wells simultaneously and includes real time calculation and presentation of transendothelial resistance. At the bottom of each well there is a gold-film electrode. When cells cover the electrode, the impedance changes, because the cells block the current flow. The main contribution to impedance is due to narrow spaces beneath the cells and the intercellular junctions. As the cell alters its morphology or moves about, these passages vary causing changes in the electrical impedance. From changes in impedance, the barrier function of the cell can be determined (Tiruppathi *et al.* 1992).

4. Data analysis

All TER measurements were performed in triplicate or quadruplicate and each experiment was repeated at least three times. Values are presented as means ± SEM from one representative experiment. Differences among groups were examined by the two way ANOVA for repeated measures, followed by the Neuman-Keuls test, and were considered significant at $P<0.05$.

RESULTS

Identification of human lung microvascular endothelial cells

The harvesting procedures outlined in Methods consistently produced endothelial cells free of other cellular contamination. We call these cells microvascular because we utilized sub-pleural lung segments with no visible large vessels, as well as subjected the chopped tissue to two successive 100μm filtrations. Furthermore, these cells are endothelial cells for several reasons. As shown in Figure 1, *left upper panel*, they are of small size, form a contact-inhibited monolayer, exhibit the characteristic cobblestone appearance of endothelial cells and are highly homogeneous. These cells also possess properties and express proteins, which are characteristic of vascular endothelial cells. Thus, as shown in Figure 1, *upper right panel*, they take up acetylated LDL (Ac-LDL), while the two lower panels of Figure 1 demonstrate that these cells express widely two proteins constitutively found in endothelial cells: von Willebrand factor (vWF) and endothelial (type 3) nitric oxide synthase (eNOS). The HLMVEC depicted in Figure 1 are of passage 3; similar results were observed in HLMVEC at passages 4–10. We have not studied HLMVEC beyond passage 10.

Barrier properties of human lung microvascular endothelial cells

Twenty-four to forty-eight hours after being seeded on 8-well arrays, in preparation for measurements of transendothelial resistance (TER), HLMVEC exhibited a tight monolayer with excellent resistance (~1000MΩ). To demonstrate the barrier properties of the HLMVEC, we tested six substances known to increase pulmonary endothelial permeability (a Gram negative and two Gram positive bacterial products, two receptor-acting agents and one cytoskeleton disrupting agent).

Figure 2 shows that lipopolysaccharide (LPS), a product of the wall of the Gram negative bacteria, *E.coli*, produced a time and concentration-dependent decrease in HLMVEC TER. LPS decreased TER at concentrations ranging from 1 to 10 EU (endotoxin units)/ml. At the

lowest concentration tested, 1 EU/ml, LPS reduced TER as early as one hour after addition and reached a nadir of 45% decrease in TER by 3–4h.

Figure 3 shows that the Gram positive bacterial products, listeriolysin (LLO; top panel) and pneumolysin (PLY; bottom panel) also decreased the TER of HLMVEC in a time- and concentration-dependent manner, but with different profiles. Whereas LLO produced a 20–40% inhibition in TER in concentrations of 250–500 ng/ml, PLY caused a 40–70% inhibition at much lower concentrations of 62.5–250 ng/ml; additionally, the effect of PLY was much quicker than that of LLO: the nadir of the PLY-induced decrease in TER occurred at about 40min, whereas for LLO it was at around 12–14 hours.

To investigate the response of HLMVEC to two well-characterized receptor-acting edemagenic agents, we studied time- and concentration- dependent responses of HLMVEC to changes in TER caused by thrombin (Figure 4) and VEGF (Figure 5). Both thrombin and VEGF induced a profound and both time- and concentration-dependent decrease in TER, as expected. Thrombin, at concentrations between 0.0625 and 1 U/ml, produced a rapid 5–15% decrease in TER, within a few minutes, that – at the two higher concentrations- partly recovered, but remained depressed for at least three hours. Conversely, VEGF, at concentrations between 10–50 ng/ml produced a slow, but sustained 10–30% decrease in TER, which remained depressed for at least 11 hours.

Microtubules are an important cytoskeletal regulator of paracellular endothelial permeability. We exposed HLMVEC to the microtubule depolymerizing agent, nocodazole (Figure 6). At concentrations between 0.05 and 2μM, nocodazole caused a quick, 5–35% decrease in TER that was sustained for at least 30min.

DISCUSSION

The maintenance of a barrier between blood and tissue is a very important function of vascular endothelium. This is especially true for pulmonary microvascular endothelium, because failure to maintain a healthy barrier may result in impaired gas exchange, reduced blood oxygenation, acid-base disturbances, acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS). Over the past three decades, cultured endothelial cells have been used frequently as a model for the study of mechanisms regulating endothelial barrier integrity. Most of these studies have utilized bovine and other animal endothelial cells or human umbilical vein endothelial cells, although recently a number of investigators have utilized commercially available human pulmonary arterial endothelial cells and a few of them, have employed human pulmonary microvascular endothelial cells. Because commercially available endothelial cells are both costly and of inconsistent quality, we developed a method for the routine and large scale in-house harvesting and culture of human lung microvascular endothelial cells (HLMVEC). This method produces consistently excellent quality HLMVEC, as shown under phase contrast microscopy (Figure 1). Importantly, they express proteins, characteristic of endothelial cells. Thus, HLMVEC strongly express both vWF and eNOS, two proteins that are nearly exclusively expressed by endothelial cells. vWF, a glycoprotein involved in hemostasis, is contained within the endothelial perinuclear Weibel-Palade bodies (Jaffe *et al.* 1974; Wagner *et al.* 1982).

Outside of platelets, endothelial cells are the only cells expressing vWF; since the cells shown in Figure 1 are not platelets, their extensive expression of vWF strongly suggest that they are endothelial cells with undetectable non-endothelial contamination. Similarly, even though, both platelets and alveolar epithelial cells express endothelial NOS (Muruganandam & Mutus 1994; Vyas-Read *et al.* 2007), the simultaneous expression of both eNOS and vWF in our cells confirms their identity as endothelial cells.

With this approach, it is not possible –nor intended- to separate arterial from venous microvascular cells. It is reasonable to expect that these cultures contain a mixture of both. Similarly, we cannot be certain of the exact vessel size of origin of these cells, even though no specimen contained visible-size vessels.

To investigate the barrier function of HLMVEC, we tested a number of permeability-inducing agents. The Gram negative bacterial product, LPS, elicited a strong increase in HLMVEC permeability. This agrees with numerous literature reports. For example, in BPAEC, LPS decreases transendothelial electrical resistance through a mechanism sensitive to hsp90 inhibitors (Antonov *et al.* 2008), (Chatterjee *et al.* 2007). An important means of LPS-induced hyperpermeability involves the stimulation of endothelial contractile mechanisms and inhibition of Rho kinase effectively prevents endothelial contraction induced by LPS and reduces edema formation during septic inflammation (Essler *et al.* 2000; Tasaka *et al.* 2005). In general, Rho A is considered to be important for the increase of endothelial permeability in response to inflammatory stimuli, such as thrombin, tumor necrosis factor-[alpha], and LPS (Essler *et al.* 1998; van Nieuw Amerongen *et al.* 2000; Wojciak-Stothard & Ridley 2002; Baumer *et al.* 2008). LPS-induced lung edema is also blocked by sphingosine-1-phosphate, a known activator of Rac 1 (Peng *et al.* 2004), and by cAMP, which, in part, stabilizes endothelial barrier function also via activation of Rac 1 (Wojciak-Stothard *et al.* 2001; Birukova *et al.* 2007; Birukova *et al.* 2008). Endothelial barrier properties are known to be strictly dependent on the integrity of endothelial adherens and tight junctions (Bazzoni & Dejana 2004). LPS also increases paracellular permeability of human lung microvascular EC through tyrosine phosphorylation of VE-cadherin, p120 catenin, and [gamma]-catenin (Gong *et al.* 2008), as well as through inhibition of NADPH oxidase activity (Chen *et al.* 2008). In these studies, the overwhelming majority of human lung endothelial cells (arterial or microvascular) were from commercial sources (Chen *et al.* 2008; Gong *et al.* 2008; Tiruppathi *et al.* 2008). Our HLMVEC compare favorably and with increased sensitivity to those commercially available. Thus, Tiruppathi et al (Tiruppathi *et al.* 2008) reported that LPS concentrations as high as 4μg/ml (~12,000U/ml) did not alter the resting TER in commercially obtained HLMVEC, whereas at 1 U/ml, LPS produced a 45% decrease in TER in our own HLMVEC. Similarly, in our lab, the TER response of our HLMVEC to 1U/ml LPS is equivalent to the TER response of our in-house harvested bovine pulmonary arterial endothelial cells (BPAE) to 1000EU/ml (Chatterjee *et al.* 2008).

Our findings that both pneumolysin (PLY), the main virulence factor of *Streptococcus pneumoniae* and listeriolysin (LLO), the main virulence factor of *Listeria monocytogenes*, profoundly decrease TER in HLMVEC agree with published studies. The family of cholesterol-dependent pore-forming toxins, to which PLY and LLO belong, produce a rapid increase in intracellular Ca^{2+} and diacylglycerol levels (Repp H 2002) and have been

implicated in severe pulmonary hyper-permeability (Ananthraman A 1983), (Witzenrath M 2006). The interaction of *Listeria monocytogenes* with endothelial cells represents a crucial step in the pathogenesis of listeriosis. Incubation of human umbilical vein endothelial cells (HUVEC) with wild-type *L. monocytogenes* provokes a strong, immediate NO synthesis, attributable to LLO and can be reproduced by purified LLO (Rose F 2001). In addition, incubation of HUVEC with LLO is a potent stimulus for sustained up-regulation of pro-inflammatory cytokines (IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor) (Rose F 2001). The LLO-induced transmembrane Ca^{2+} flux in endothelial cells leads to the activation of phospholipase, generation of diacylglycerol, ceramide, and NF-κB, which may contribute to the pathogenic sequelae in severe listerial infection and sepsis. Recently, intravascular PLO dose-dependent increased pulmonary vascular resistance and lung microvascular permeability. In these studies, PLY was mainly detected in pulmonary arterial endothelial cells. PLY also increased permeability of HUVEC monolayers (Witzenrath M 2006). Furthermore, in neuroblastoma cells, PLY induced cholesterol- and Rho and Rac GTPase-dependent actin remodeling leading to the formation of actin stress fibers, filopodia, and lamellipodia (Iliev AI 2007). It is not clear why PLY exhibited a much faster time course in the decrease of TER in HLMVEC than LLO (1h vs. 12h). We have not yet investigated whether similar differences exist in the time course of calcium influx and stimulation of the previously described pro-inflammatory pathways between LLO and PLY in HLMVEC.

In agreement with literature reports, our HLMVEC responded to thrombin by decreasing TER in a concentration-dependent manner. Thrombin induces barrier dysfunction of pulmonary endothelial monolayer and this is associated with dramatic cytoskeletal reorganization, activation of actomyosin contraction, and gap formation (Birukova *et al.* 2004b). Thrombin-induced actin reorganization in BPAEC requires activation of both myosin light chain kinase (MLCK) and protein kinase C (PKC) (Zhao & Davis 1996). The thrombin-induced endothelial hyper-permeability occurs in conjunction with calcium mobilization as well as PKC activation (Lum *et al.* 1993), (Lum *et al.* 1992). Like LPS, thrombin‘s effect on endothelial cell cytoskeletal rearrangement and TER is inhibited by hsp90 inhibitors (Antonov *et al.* 2008). Also like LPS, the thrombin-induced barrier dysfunction requires RhoA (Vouret-Craviari *et al.* 1998; Hippenstiel *et al.* 2000; Woo & Kim 2002), as well as the induction of superoxide (Holland *et al.* 1998; Li *et al.* 2002; Pandian *et al.* 2005). These effects of thrombin are mediated via G-proteins, which couple the thrombin receptor to several key physiological responses.

VEGF increases permeability by at least two different pathways: one involving Raf-1, MEK, and ERK-1/2; and the other involving NOS. PKC, which increases permeability via increased NO production (Huang & Yuan 1997), is a mediator of VEGF-induced ERK-1/2 phosphorylation and hyperpermeability (Breslin *et al.* 2003). *In vitro* studies demonstrate that VEGF causes an increase in protein permeability across primary cultures of bovine macro- and microvascular lung endothelial cell monolayers and that this is associated with VE- and E-cadherin phosphorylation and the formation of actin stress fibers. Activation of the stress protein response prevents the VEGF-mediated changes in protein permeability across EC monolayers and reduces the phosphorylation of VE-and E-cadherin, as well as the

formation of actin stress fibers (Godzich *et al.* 2006). The VEGF increase in endothelial cell permeability is prevented and reversed by hsp90 inhibitors (Antonov *et al.* 2008). The sensitivity of HLMVEC to VEGF was comparable to that of BPAEC: in both cell types 50ng/ml VEGF elicited ~30% decrease in TER. (Antonov *et al.* 2008)

The endothelial cytoskeleton plays a critical role in the regulation of endothelial barrier function (Dudek & Garcia 2001). Disassembly of microtubules by various agents, including nocodazole, results in a hyper-permeable endothelial monolayer (Verin *et al.* 2001; Birukova *et al.* 2004a; Birukova *et al.* 2005). We confirmed this observation in our HLMVEC. Nocodazole, produced a concentration-dependent decrease in HLMVEC TER, that was comparable to that previously observed in BPAE (Antonov *et al.* 2008).

In summary, we present a method for in-house harvesting, identification and culture of human lung microvascular endothelial cells and provide evidence that these cells exhibit predictable barrier functions. Altered pulmonary microvascular endothelial barrier function is a hallmark of ALI and ARDS, where mortality remains virtually unchanged in over 40 years. Whenever available, human tissue is a superior alternative to animal tissue for experimental studies; in addition in-house harvested human endothelial cells can provide a more economical and better quality alternative to those available commercially.

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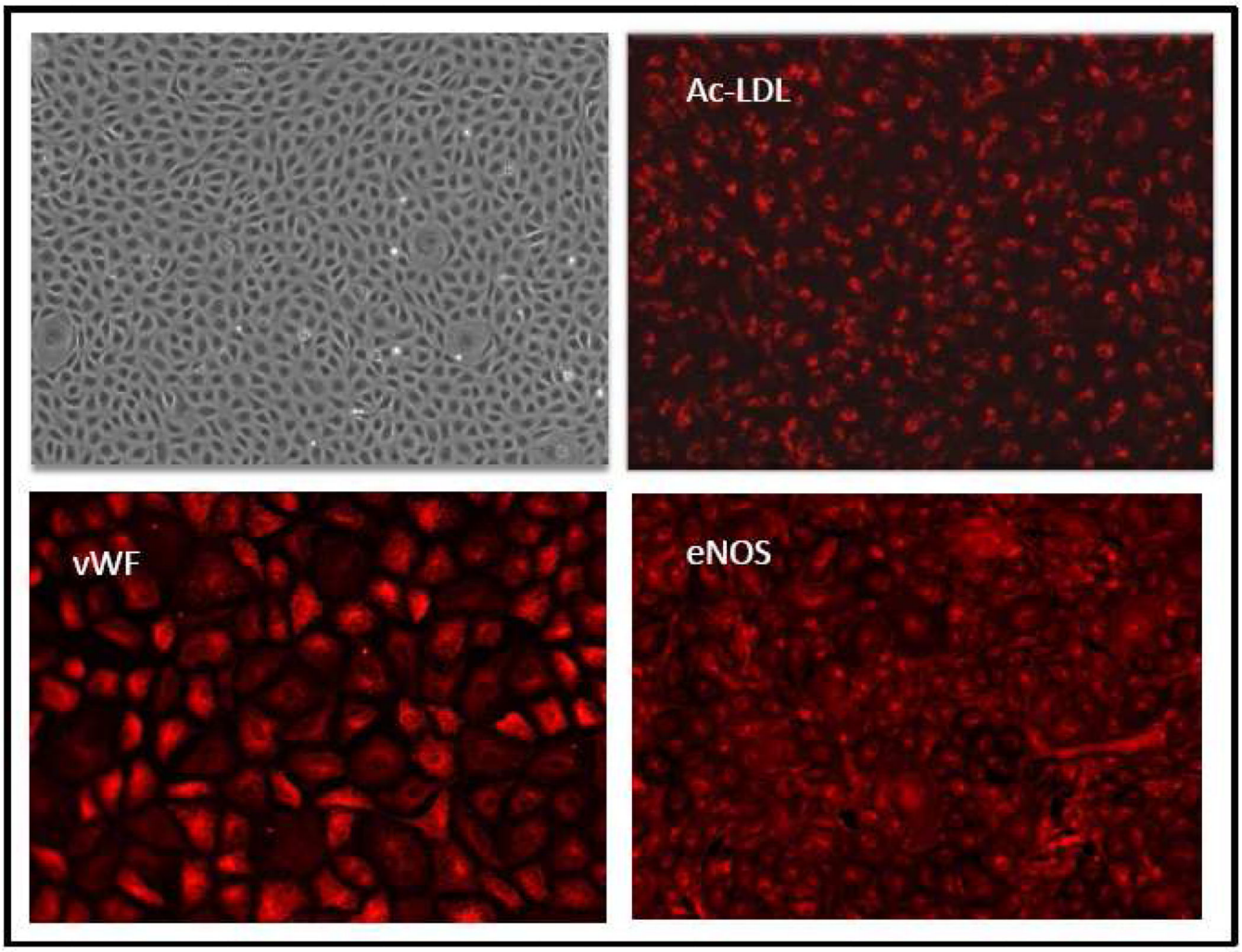

FIGURE 1.

top left panel: phase contrast micrograph of HLMVEC (passage 3); the other three panels demonstrate well established characteristics of all endothelial cells, i.e., uptake of acetylated LDL (Ac-LDL: top right panel), expression of von Willebrand factor (vWF; bottom l;eft panel) and expression of endothelial (or type 3) nitric oxide synthase (eNOS; bottom right panel). See *Materials and Methods* for details.

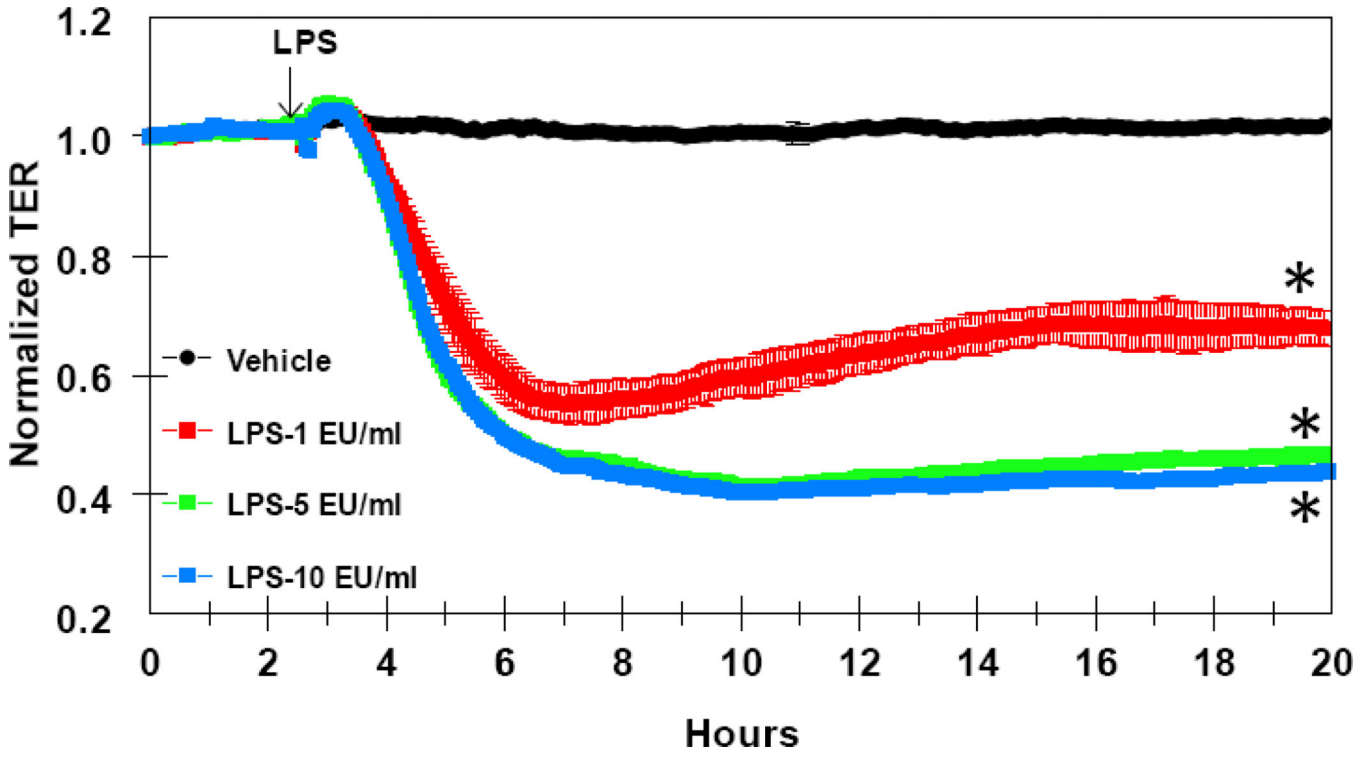

FIGURE 2.

time and concentration-dependent decrease in HLMVEC transendothelial resistance (TER) by LPS. Eight-well arrays were inoculated with HLMVEC (100,000); 48h later, confluent monolayers were observed exhibiting resistance of ~900MΩ. LPS or vehicle were added (15μl) at the time indicated by the arrow and TER values were continuously recorded at 20sec intervals over the next 18h. Data shown are means ±SEM of four replicates. *:P<0.05 from Vehicle group.

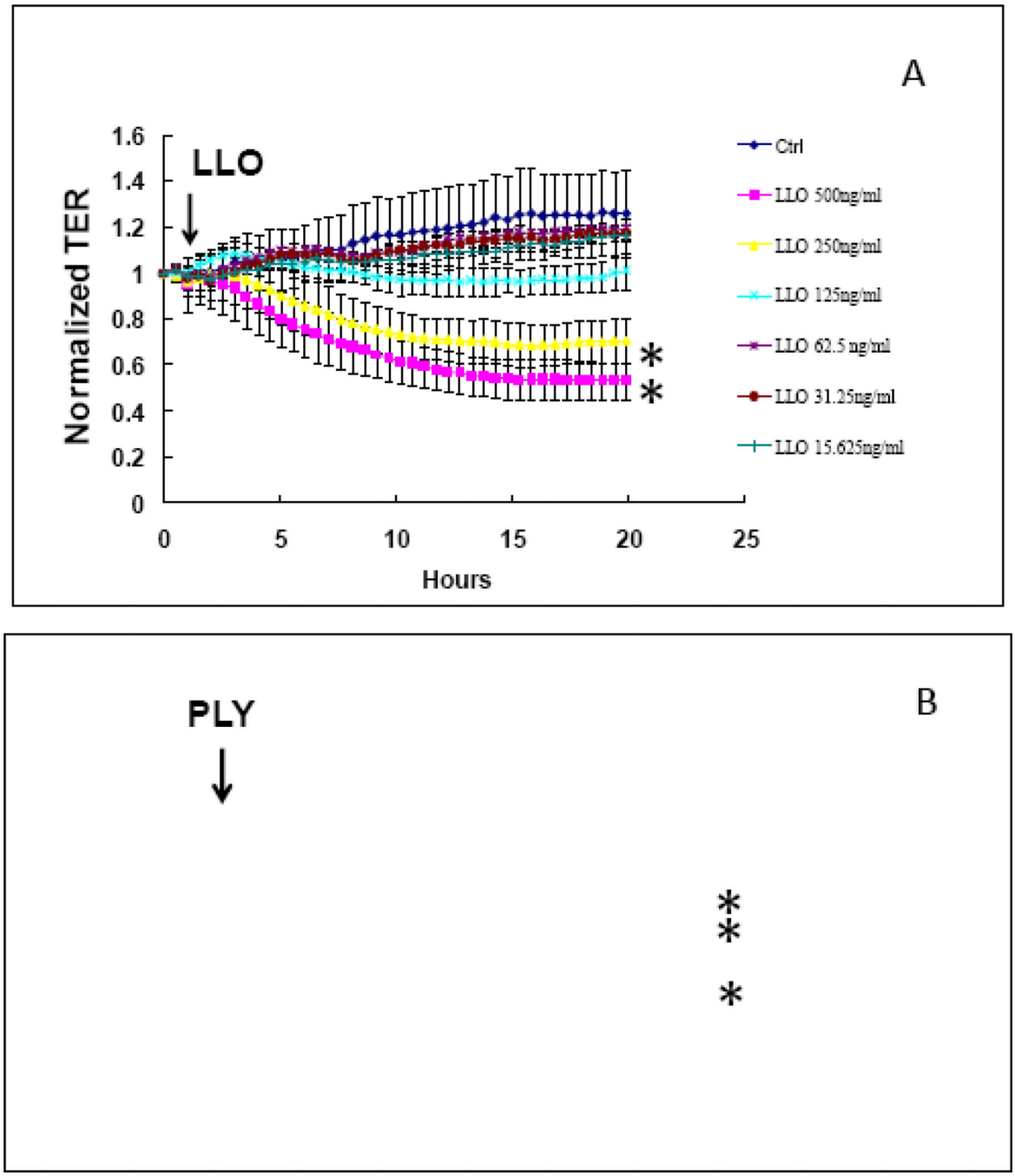

FIGURE 3.

time and concentration-dependent decrease in HLMVEC transendothelial resistance (TER) by the Gram positive bacteria products lysteriolysin (LLO; A) and pneumolysin (PLY; B). Eight-well arrays were inoculated with HLMVEC (100,000); 48h later, confluent monolayers were observed exhibiting resistance of ~900MΩ. LPS or vehicle were added (15μl) at the time indicated by the arrow and TER values were continuously recorded at 20sec intervals over the next 18h (A) or 150min (B). Data shown are means ±SEM of four replicates. *:P<0.05 from corresponding Vehicle group.

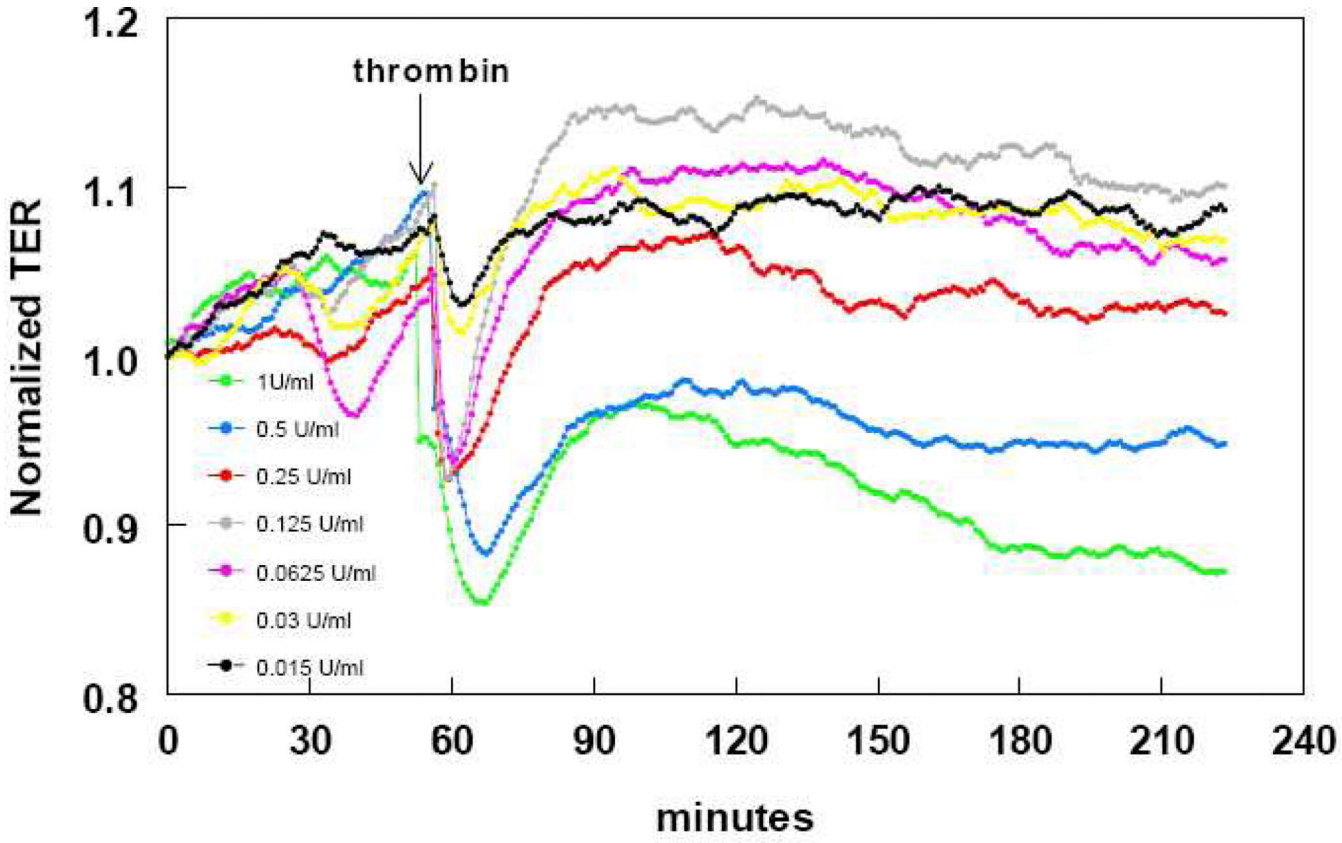

FIGURE 4.

time and concentration-dependent decrease in HLMVEC transendothelial resistance (TER) by thrombin. Eight-well arrays were inoculated with HLMVEC (100,000); 48h later, confluent monolayers were observed exhibiting resistance of ~900MΩ. Thrombin or vehicle was added (15μl) at the time indicated by the arrow and TER values were continuously recorded at 20sec intervals over the next 160min. Data shown are representative of three separate experiments with comparable results.

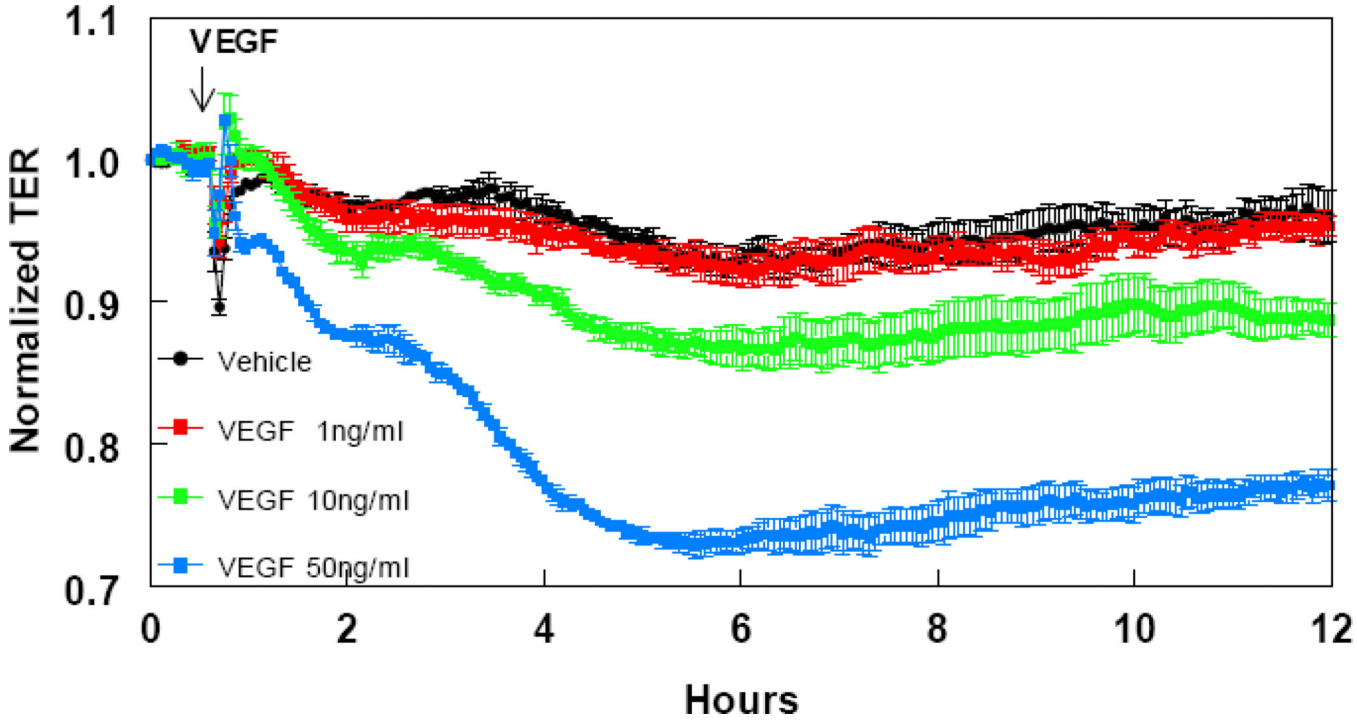

FIGURE 5.

time and concentration-dependent decrease in HLMVEC transendothelial resistance (TER) by vascular endothelial growth factor (VEGF). Eight-well arrays were inoculated with HLMVEC (100,000); 48h later, confluent monolayers were observed exhibiting resistance of ~900MΩ. VEGF or vehicle was added (15μl) at the time indicated by the arrow and TER values were continuously recorded at 20sec intervals over the next 11h. Data shown are means ±SEM of four replicates. *:P<0.05 from Vehicle group.

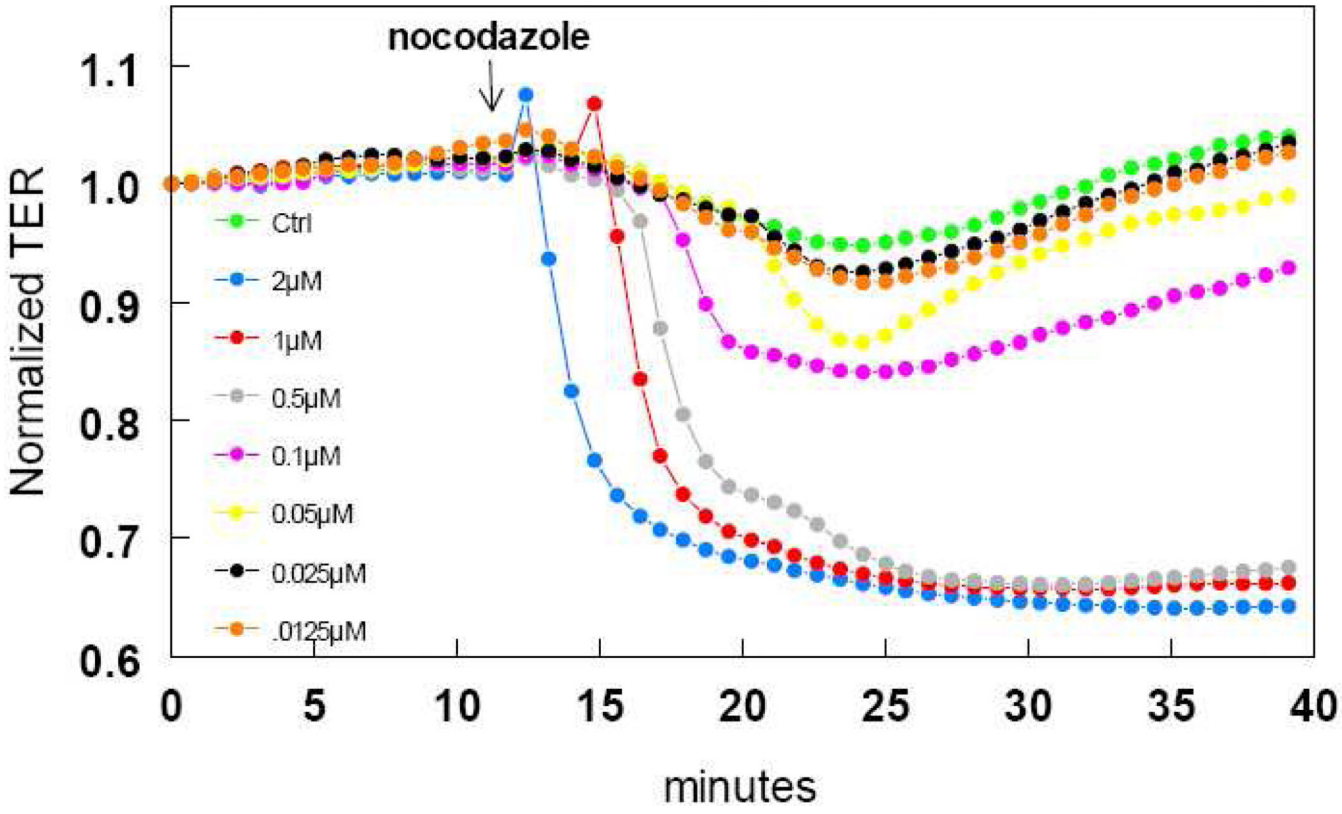

FIGURE 6.

time and concentration-dependent decrease in HLMVEC transendothelial resistance (TER) by the microtubule depolymerizing agent, nocodazole. Eight-well arrays were inoculated with HLMVEC (100,000); 48h later, confluent monolayers were observed exhibiting resistance of ~900MΩ. Nocodazole or vehicle was added (15μl) at the time indicated by the arrow and TER values were continuously recorded at 20sec intervals over the next 30min. Data shown are representative of three separate experiments with comparable results.