Effect of Lipoxin A₄ on Lipopolysaccharide-Induced Endothelial Hyperpermeability

Huayan Pang¹, Pan Yi², Ping Wu², Zhuoya Liu², Zhongjie Liu¹, Jianming Gong¹, Hua Hao², Lei Cai², Duyun Ye², and Yinping Huang¹,*

¹Department of Obstetrics and Gynecology, First Affiliated Hospital of Wenzhou Medical College, Wenzhou, Zhejiang, China; ²Department of Pathophysiology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, China

E-mail: yphuangp@126.com

Received December 20, 2010; Revised April 4, 2011; Accepted April 7, 2011; Published May 5, 2011

Excessive oxidative stress, decreased antioxidant capacity, and enhanced cellular calcium levels are initial factors that cause endothelial cell (EC) hyperpermeability, which represents a crucial event in the pathogenesis of pre-eclampsia. Lipoxin A₄ (LXA₄) strongly attenuated lipopolysaccharide (LPS)-induced hyperpermeability through maintaining the normal expression of VE-cadherin and β-catenin. This effect was mainly mediated by a specific LXA₄ receptor. LXA₄ could also obviously inhibit LPS-induced elevation of the cellular calcium level and up-regulation of the transient receptor potential family C 1, an important calcium channel in ECs. At the same time, LXA₄ strongly blocked LPS-triggered reactive oxidative species production, while it promoted the expression of the NF-E2 related factor 2 (Nrf2) protein. Our findings demonstrate that LXA₄ could prevent the EC hyperpermeability induced by LPS in human umbilical vein endothelial cells (HUVECs), under which the possible mechanism is through Nrf2 as well as Ca²⁺-sensitive pathways.

KEYWORDS: lipoxin, endothelial permeability, pre-eclampsia, VE-cadherin, Nrf2, oxidative stress

INTRODUCTION

Pre-eclampsia (PE), a pregnancy-specific syndrome characterized by the onset of hypertension, proteinuria, and edema, has been reported in approximately 8% of all pregnancies[1]. Although the pathophysiology of PE still remains undefined, placental oxidative stress is regarded as a key event[1,2,3]. It is widely accepted that in pregnant women with PE, excessive generation of reactive oxidative species (ROS) in the placenta contributes to widespread maternal vascular endothelial cell (EC) hyperpermeability[2].

Lipoxins (LXs) are the first identified lipid family serving as endogenous “braking signals” in inflammation that possesses a wide spectrum of anti-inflammatory and proresolution bioactions[4]. Recently, we observed that PE patients presented much lower LX levels in systemic circulation (data not shown). In addition, we previously confirmed that LXs had an antioxidant effect both in
lipopolysaccharide (LPS)-stimulated macrophages[5,6] and in carbon tetrachloride–induced liver injury[7]. Combining these above results, we speculate that deficient LX production might conduce to persistent inflammatory and oxidative conditions, which in turn cause EC hyperpermeability, but the detailed mechanism is still unknown.

Therefore, the current study aimed to elucidate the role of lipoxin A₄ (LXA₄), a major endogenous LX, on preventing LPS-induced endothelial barrier disruption in human umbilical vein endothelial cells (HUVECs), which is a commonly applied cell model for studying systemic vascular function[8]. To further explore the possible mechanism, cellular calcium and NF-E2 related factor 2 (Nrf2), a crucial transcription factor in regulating cellular antioxidant response and phase II enzymes[9], were also investigated.

**MATERIALS AND METHODS**

- **Materials** — LXA₄ was from Cayman Chemical (USA) and was stored at −80°C until diluted in serum-free culture medium immediately before use. Commercially obtained LXA₄ was diluted in ethanol, of which the final concentration in the cell culture medium was 0.0036% or lower. Endothelial cell growth medium-2 (EGM-2) and fetal bovine serum (FBS) were purchased from Gibco (Australia). LPS, bovine serum albumin (BSA), and Fura-2 acetoxyethyl ester (Fura-2/AM) were from Sigma Aldrich (USA). 2',7'Dichlorofluorescin diacetate (DCFH-DA) was from Molecular Probes (USA). Antibodies to VE-cadherin, β-catenin, Nrf-2, and β-actin were from Santa Cruz (USA). TRIzol Reagent, Moloney murine leukemia virus (M-MLV) reverse transcriptase, and Amplex Red Hydrogen Peroxide/Peroxidase Assay kit were from Invitrogen Life Technologies (USA). Horseradish peroxidase (HRP) or fluorescein isothiocyanate (FITC) conjugated secondary antibodies were from Pierce (USA).

- **Primary culture of HUVECs and treatments** — Human umbilical cords were obtained from women with normal pregnancies immediately after delivery from the First Affiliated Hospital of Wenzhou Medical College, which received permission from the local Ethics Committee. Primary HUVECs were prepared from umbilical veins and cultured as published previously[10]. Cells at passage 3–5 were cultured in EGM and 20% FBS at 37°C with 5% CO₂. Cells were serum starved for 24 h before experiments, and then treated with 10 µg/ml LPS with or without 100 nM LXA₄ for 12 h unless stated otherwise.

- **Measurement of endothelial permeability** — HUVECs (1 × 10⁵/cm²) were plated on 1% gelatin-coated transwell inserts in 12-well plates until confluent. Endothelial permeability was determined with permeability coefficient of albumin (Pa) according to published method[11].

- **Morphological observation** — HUVECs were observed under an inverted microscope after regular hematoxylin and eosin (H&E) staining.

- **Immunofluorescence assay** — Immunofluorescence assay was performed to detect VE-cadherin according to our published method[12].

- **Western-blotting analysis** — Western blotting was performed to detect VE-cadherin, β-catenin, and Nrf-2 expression as described previously[6].

- **Reverse transcription – polymerase chain reaction (RT-PCR)** — Total RNA extraction, reverse transcription, and PCR were conducted according to the manuscript. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as internal control. Sequences of primers and reactions conditions are shown in the Supplementary material.

- **Measurement of cytosolic free calcium concentration ([Ca²⁺]ᵢ)** — After cells were treated with LPS with or without LXA₄ for 6 h, [Ca²⁺]ᵢ was measured with Fura-2 according to our previous report[13]. The intensities of fluorescence due to excitation at 340 (F340) and 380
(F380) nm were measured after background subtraction, and F340/F380 was used to represent changes in [Ca$^{2+}$].

- **Intracellular ROS measurement** — Intracellular ROS levels were determined by measuring the oxidative conversion of DCFH-DA to fluorescent compound DCFH[14]. The results were expressed as the percentage of fluorescence intensity in control cells.
- **Measurement of H$_2$O$_2$ production by HUVECs** — The assay is based on the detection of H$_2$O$_2$ that reacts with Amplex Red in the presence of HRP with a 1:1 stoichiometry producing resorufin[15]. Amplex Red (50 μM) and HRP (5 U/2 ml) were added to cells exposed to LPS or LXA$_4$ for indicated periods of time. Fluorescence was detected at 37°C in a fluorescence spectrophotometer 30, 60, or 90 min after incubation. The excitation and emission wavelengths were 550 and 585 nm, respectively. Calibration signals were generated using known amounts of H$_2$O$_2$.
- **Statistical analysis** — Statistical analysis was performed using SPSS 13.0 for Windows. Data were presented as mean ±SEM and analyzed by Student’s t test. Values of $p < 0.05$ indicated a statistically significant difference.

**RESULTS**

**LXA$_4$ Prevented LPS-Induced Hyperpermeability of HUVECs**

After primary HUVECs were successfully isolated and cultured, which was confirmed by CD31 and von Willebrand factor (vWF) positive staining as shown in Supplementary Fig. 1, vascular permeability was measured. As presented in Fig. 1, exposure to LPS for 12 h markedly increased the Pa value to ~183% that of control cells, while coadministration of LXA$_4$ attenuated this LPS-induced hyperpermeability ($p < 0.05$). It was also observed that 50–200 nM LXA$_4$ showed no dose-dependent manner. Therefore, in the following experiments, only 100 nM LXA$_4$ was applied.

Since the bioactions of LXA$_4$ were mainly elicited through the LXA$_4$ receptor (ALXR/FPRL-1), which had already been confirmed to exist in HUVECs[16], then 10 μM Boc-2 (an effective antagonist of FPRL-1) was used to explore the effect of FPRL-1. Results showed that when Boc-2 was administrated 30 min in advance, it effectively blocked the influence of LXA$_4$ on LPS-induced Pa enhancement ($p < 0.01$; shown in Fig.1).

**LXA$_4$ Maintained the Normal Morphology of HUVECs**

The morphology of ECs could also reflect the cell contractile state and vascular permeability, thus it was observed with routine H&E staining. As shown in Fig. 2, cells in control groups showed a cobblestone-like morphology, with tight cell-cell contact. When treated with LPS for 12 h, HUVECs presented a thinner and flatter, spindle-shaped morphology. This morphological change was concomitant with the increased albumin permeability shown in Fig. 1. Meanwhile, shapes of cells cotreated with LXA$_4$ were well preserved and the cell-cell conjunction was recuperated. LXA$_4$ alone showed no effect on the morphology of HUVECs.

**LXA$_4$ Maintained the Expression of VE-Cadherin and β-Catenin in LPS-Stimulated HUVECs**

As normal expression of VE-cadherin and β-catenin is required for the maintenance of proper endothelial adhesion junctions (AJs) and vascular permeability[17], we further determined the role of these two proteins in the protective effect of LXA$_4$ on EC permeability. As shown in Fig. 3, both VE-cadherin mRNA
FIGURE 1. Effect of LXA₄ on endothelial permeability in LPS-stimulated HUVECs. LPS with or without LXA₄ were administrated for 12 h in the upper chamber. Boc-2 was applied 30 min prior to LXA₄. Pa value was applied to indicate the endothelial permeability. Data are expressed as percentage of control cells; #p < 0.01 compared to control cells; *p < 0.05 compared to LPS-stimulated cells; **p < 0.01 compared to LXA₄ + LPS-treated cells. All experiments were carried out in triplicate and for three independent times.

FIGURE 2. LXA₄ maintained the normal morphology of HUVECs. Representative images were from one of three separate experiments that yielded similar results.

and protein could be detectable in all of the four groups, with obviously the lowest level in cells treated with LPS (p < 0.05 vs. control cells or cells treated with both LPS and LXA₄). The Western blotting result was further confirmed by immunofluorescence assay with FITC-conjugated antibody to VE-cadherin. HUVECs in the control group demonstrated tight apposition between cells and strong membrane fluorescence. When LPS was administered, large intercellular gaps could be observed. At the same time, VE-cadherin staining was much lower. In cells cotreated with LXA₄, LPS-induced changes mentioned above, like gap formation and down-regulation of VE-cadherin, were much less apparent (Fig. 4). LXA₄ alone had no significant effect on VE-cadherin expression.

It was presented in Fig. 3 that LXA₄ blocked LPS-induced β-catenin protein down-regulation (p < 0.05), while it showed no statistical effect on β-catenin protein expression in control cells.
FIGURE 3. The expression of VE-cadherin and β-catenin in HUVECs under different conditions. (A) Representative RT-PCR for VE-cadherin is shown. GAPDH served as internal control. Bar graphs indicate means ± SEM from at least three independent experiments. (B) Representative Western blots for VE-cadherin and β-catenin are shown. Beta-actin served as internal control. Bar graphs indicate means ± SEM from at least three independent experiments; #p < 0.05 compared to control cells; *p < 0.05 compared to cells treated by LPS alone.

FIGURE 4. VE-cadherin protein expression detected by immunofluorescence. Representative images were from one of three separate experiments that yielded similar results.

**LXA₄ Inhibited LPS-Induced [Ca²⁺]ᵢ Elevation and TRPC1 Expression**

Calcium is critical for the maintenance of cell-cell junctions[18]; thus, we next measured [Ca²⁺]ᵢ in HUVECs to explore the possible mechanism under which LXA₄ could maintain VE-cadherin and β-catenin expression. As shown in Fig. 5A, treatment of HUVECs with LPS for 6 h induced the increase of [Ca²⁺]ᵢ for more than 50% that of control cells (p < 0.01). However, it was statistically lower in cells cotreated with LXA₄ (p < 0.01).

Transient receptor potential protein family C 1 (TRPC1) acts as a store-operated calcium entry channel (SOC) in the cell membrane, which regulates not only cellular calcium concentration, but also vascular permeability[19]. As shown in Fig. 5B, its mRNA expression increased obviously in cells treated with LPS, while in cells treated simultaneously by LPS and LXA₄, this abnormal up-regulation was inhibited significantly (p < 0.05).
FIGURE 5. [Ca\textsuperscript{2+}]i and TRPC1 expression in HUVECs under different conditions. (A) [Ca\textsuperscript{2+}]i was measured with Fura-2 after cells were treated with LPS with or without LXA\textsubscript{4} for 6 h. Data are presented as ratio F340 to F380. All values are means ± SEM from at least three independent experiments. (B) Representative RT-PCR for TRPC1 is shown. Bar graphs indicate means ± SEM from at least three independent experiments; ##p < 0.01 compared to control cells; **p < 0.01 compared to cells treated by LPS alone; *p < 0.05 compared to cells treated by LPS alone.

**LXA\textsubscript{4} Blocked LPS-Induced ROS Generation in HUVECs**

We further explored the effect of LXA\textsubscript{4} on cellular oxidative stress. Cells treated with LPS for 12 h showed ~1.5-fold increase of fluorescence intensity, indicating a significantly higher endogenous ROS formation (Fig. 6A). Cells cotreated with LXA\textsubscript{4} had significantly lower intensity compared with that in cells treated by LPS alone (p < 0.05).

The release of H\textsubscript{2}O\textsubscript{2} from HUVECs is shown in Fig. 6B. At all three time points, H\textsubscript{2}O\textsubscript{2} levels in the LPS group were much higher than those in the control group (p < 0.05) or the LPS + LXA\textsubscript{4} group (p < 0.05).
The effect of LXA₄ on LPS-induced endothelial hyperpermeability

**FIGURE 6.** Effect of LXA₄ on cellular oxidative stress condition. (A) Intracellular ROS was evaluated with fluorometric assay employing DCFH-DA. (B) Extracellular H₂O₂ was measured with Amplex Red assay kit. #p < 0.05 compared to control cells. *p < 0.05 compared to cells treated by LPS alone.

**Effect of LXA₄ on the Antioxidant Response in HUVECs**

In order to elucidate how LXA₄ inhibited ROS generation in LPS-stimulated HUVECs, Nrf2 protein level was measured. As shown in Fig. 7, it was down-regulated in LPS-treated cells, while up-regulated in cells cotreated with LXA₄ (p < 0.05).

**DISCUSSION**

Ever since it was found that the disappearance of Evans blue dye from plasma was faster in patients with PE[20,21], increased capillary endothelial permeability was considered as a central event in the pathogenesis of PE[22]. Despite numerous studies pointing to the potent anti-inflammatory activities of LXs, there is a paucity of research examining their direct influence on the umbilical vessel. The purpose of this study was to determine the effect of LXA₄ on modulating permeability of HUVECs and the possible mechanism.
First, hyperpermeability of HUVECs was induced by administration of LPS, a commonly applied model in vascular leakage research[23]. Then it was found that LXA₄ strongly attenuated LPS-induced hyperpermeability, confirmed by both albumin permeability assay and morphological observation. ALX₄R is a well-known specific receptor of LXA₄ on a variety of cell types, including HUVECs[16]. In addition, Gronert et al. confirmed that LXs could bind with vascular endothelial-derived LTD₄ receptor[24]. Furthermore, bioactions of LXs might also be mediated by some other distinct receptors[25]. In order to explore the signaling mechanisms responsible for the protective effect of LXA₄, the antagonist of ALXR was applied. Results indicated that LXA₄ inhibited LPS-induced hyperpermeability in HUVECs mainly through ALXR.

Cell permeability is regulated in part by the dynamic opening and closing of cell-cell AJs. In ECs, AJs are mainly composed of VE-cadherin, an endothelium-specific member of the cadherin family, which is linked through its cytoplasmic tail to the AJ proteins p120, β-catenin, and plakoglobin[17]. In the current study, LXA₄ obviously reversed down-regulation of both VE-cadherin and β-catenin caused by LPS. This might help to explain the prevention of LXA₄ on LPS-induced hyperpermeability. Although Serhan’s group previously found that LXs inhibited the neutrophil-mediated increase of vascular permeability in the mouse ear[26,27,28], and Ereso et al. recently also confirmed that LXA₄ attenuates microvascular fluid leakage during inflammation in rats[29], our study presented for the first time that LXA₄ had directly protective effect on AJs.

Regulation of endothelial permeability is a complex process that often depends on SOC[30]. Recent studies have identified TRPC1, a member of TRP family, as the essential component of the SOC in ECs[31]. The rise in [Ca²⁺]ᵢ through TRPC1 has been established as the initial pivotal signal that precedes EC cytoskeletal reorganization and the disassembly of VE-cadherin[18,31,32]. The fact that LXA₄ obviously attenuated the expression of TRPC1 in cultured HUVECs helped us to explain how it blocked the elevation of [Ca²⁺]ᵢ in LPS-stimulated cells. However, the details of the cellular signals on regulating
TRPC1-induced Ca\textsuperscript{2+} influx and how these signals control endothelial permeability still need to be clarified. TRPC1 is also considered to be a link between cellular Ca\textsuperscript{2+} overload and excessive production of ROS[33]. The latter could also affect EC permeability through the regulation of VE-cadherin and β-catenin[34].

In the endeavor to explore the mechanism involved in redox disturbance of PE women, the ischemic placenta is widely regarded as a key resource of ROS[1,2,3], but it should not be ignored that activated leukocytes, neutrophils in circulation, and ECs are also likely contributors to ROS accumulation[2,3,35,36,37]. These cells could be activated by factors derived from PE placentae, such as inflammatory and vasobioactive mediators, as well as ROS[38,39]. For example, when HUVECs were stimulated with plasma from women with PE, they has increased oxidative ability[40]. Our group previously presented the inhibitory effect of LXA\textsubscript{4} on ROS production in several disease models[5,6,7]. Nascimento-Silva et al. further reported that an aspirin-triggered LXA\textsubscript{4} analog suppressed ROS generation in ECs[41]. In the current study, LXA\textsubscript{4} strongly blocked LPS-triggered ROS production in HUVECs.

In addition to increased pro-oxidant activity, there is also evidence for a decreased antioxidant protective capacity in women with PE[42]. Nrf2 is a crucial transcription factor in regulating cellular antioxidant response. When cells are exposed to high levels of ROS, Nrf2 translocates into the nucleus and sequentially results in up-regulation of series downstream phase II enzymes, such as NAD(P)H:quinone oxidoreductase (NQO1) and heme oxygenase 1( HO-1), which have emerged as important mediators of antioxidant and cytoprotective action[43]. Combined with the data we recently published[44], we demonstrated that LXA\textsubscript{4} could trigger nuclear translocation of Nrf2 and promote the expression of Nrf2, NQO1, and HO-1. It has illuminated a possible mechanism of its antioxidant effect on HUVECs.

In summary, the present study indicates for the first time that LXA\textsubscript{4}, at physiological concentrations, could prevent the LPS-induced EC hyperpermeability through maintaining the expression of VE-cadherin and β-catenin. It might involve modulation of LXA\textsubscript{4} on Ca\textsuperscript{2+} and redox homeostasis. Although there are still deep gaps to fill, especially about the underlying mechanism, our data might also contribute to the potential therapeutic value of LXs for PE and other vascular endothelial hyperpermeability-associated diseases.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (No. 81070510), the Natural Science Foundation of Wenzhou City, China (No. Y20090129), and the Doctoral Fund of the Ministry of Education of China (No. 200803430002).

REFERENCES

1. Gilbert, J.S., Ryan, M.J., LaMarca, B.B., Sedeek, M., Murphy, S.R., and Granger, J.P.(2008) Pathophysiology of hypertension during preclampsia: linking placental ischemia with endothelial dysfunction. Am. J. Physiol. Heart Circ. Physiol. 294, H541–550.
2. Gupta, S., Agarwal, A., and Sharma, R.K. (2005) The role of placental oxidative stress and lipid peroxidation in preclampsia. Obstet. Gynecol. Surv. 60, 807–816.
3. Siddiqui, I.A., Jaleel, A., Tamimi, W., and Al Kadri, H.M. (2010) Role of oxidative stress in the pathogenesis of preeclampsia. Arch. Gynecol. Obstet. 282, 469–474.
4. Romano, M. (2010) Lipoxin and aspirin-triggered lipoxins. TheScientificWorldJOURNAL 10, 1048–1064.
5. Jin, S.W., Zhang, L., Lian, Q.Q., Yao, S.L., Wu, P., Zhou, X.Y., Xiong, W., and Ye, D.Y. (2006) Close functional coupling between Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} channels and reactive oxygen species production in murine macrophages. Mediators Inflamm. 2006, 36192.
6. Zhou, X.Y., Wu, P., Zhang, L., Xiong, W., Li, Y.S., Feng, Y.M., and Ye, D.Y. (2007) Effects of lipoxin A(4) on lipopolysaccharide induced proliferation and reactive oxygen species production in RAW264.7 macrophages through modulation of G-CSF secretion. *Inflamm. Res.* **56**, 324–333.

7. Zhang, L., Wan, J., Li, H., Wu, P., Jin, S., Zhou, X., Yuan, P., Xiong, W., Li, Y., and Ye, D. (2007) Protective effects of BML-111, a lipoxin A(4) receptor agonist, on carbon tetrachloride-induced liver injury in mice. *Hepatol. Res.* **37**, 948–956.

8. Kim, W., Moon, S.O., Lee, S., Sung, M.J., Kim, S.H., and Park, S.K. (2003) Adrenomedullin reduces VEGF-induced endothelial adhesion molecules and adhesiveness through a phosphatidylinositol 3'-kinase pathway. *Arterioscler. Thromb. Vasc. Biol.* **23**, 1377–1383.

9. Li, W. and Kong, A.N. (2009) Molecular mechanisms of Nrf2-mediated antioxidant response. *Mol. Carcinog.* **48**, 91–104.

10. Larrivee, B. and Karsan, A. (2005) Isolation and culture of primary endothelial cells. *Methods Mol. Biol.* **290**, 315–329.

11. Tinsley, J.H., Wu, M.H., Ma, W., Taullman, A.C., and Yuan, S.Y. (1999) Activated neutrophils induce hyperpermeability and phosphorylation of adherens junction proteins in coronary venular endothelial cells. *J. Biol. Chem.* **274**, 24930–24934.

12. Chen, Y., Hao, H., He, S., Cai, L., Li, Y., Hu, S., Ye, D., Hoidal, J., Wu, P., and Chen, X. (2010) Lipoxin A4 and its analogue suppress the tumor growth of transplanted H22 in mice: the role of antiangiogenesis. *Mol. Cancer Ther.* **9**, 2164–2174.

13. Li, Y.S., Wu, P., Zhou, X.Y., Chen, J.G., Cai, L., Wang, F., Xu, L.M., Zhang, X.L., Chen, Y., Liu, S.J., Huang, Y.P., and Ye, D.Y. (2008) Formyl-peptide receptor like 1: a potent mediator of the Ca2+ release-activated Ca2+ current ICRAC. *Arch. Biochem. Biophys.* **478**, 110–118.

14. Li, K.G., Chen, J.T., Bai, S.S., Wen, X., Song, S.Y., Yu, Q., Li, J., and Wang, Y.Q. (2009) Intracellular oxidative stress and cadmium ions release induce cytotoxicity of unmodified cadmium sulfide quantum dots. *Toxicol. In Vitro* **23**, 1007–1013.

15. Ismail, S., et al. (2009) NOX4 mediates hypoxia-induced proliferation of human pulmonary artery smooth muscle cells: the role of autocrine production of transforming growth factor-β1 and insulin-like growth factor binding protein-3. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **296**(3), L489–99.

16. Baker, N., O’Meara, S.J., Scannell, M., Madera, P., and Godson, C. (2009) Lipoxin a4: anti-inflammatory and anti-angiogenic impact on endothelial cells. *J. Immunol.* **182**, 3819–3826.

17. Dejana, E., Orsenigo, F., and Lampugnani, M.G. (2008) The role of adherens junctions and VE-cadherin in the control of vascular permeability. *J. Cell Sci.* **121**, 2115–2122.

18. Sandoval, R., Malik, A.B., Minshall, R.D., Kouklis, P., Ellis, C.A., and Tiruppathi, C. (2001) Ca(2+) signalling and PKCα activate increased endothelial permeability by disassembly of VE-cadherin junctions. *J. Physiol.* **533**, 433–445.

19. Parekh, A.B. and Putney, J.W., Jr. (2005) Store-operated calcium channels. *Physiol. Rev.* **85**, 757–810.

20. Campbell, D.M. and Campbell, A.J. (1983) Evans Blue disappearance rate in normal and pre-eclamptic pregnancy. *Clin. Exp. Hypertens.* **B 2**, 163–169.

21. Brown, M.A., Zammit, V.C., and Lowe, S.A. (1989) Capillary permeability and extracellular fluid volumes in pregnancy-induced hypertension: *Clin. Sci. (Lond.)* **77**, 599–604.

22. Wang, Y., Gu, Y., Granger, D.N., Roberts, J.M., and Alexander, J.S. (2002) Endothelial junctional protein redistribution and increased monolayer permeability in human umbilical vein endothelial cells isolated during preeclampsia. *Am. J. Obstet. Gynecol.* **186**, 214–220.

23. Xing, J. and Birukova, A.A. (2010) ANP attenuates inflammatory signaling and Rho pathway of lung endothelial permeability induced by LPS and TNFalpha. *Microvasc. Res.* **79**, 56–62.

24. Gronert, K., Martinsson-Niskanen, T., Ravasi, S., Chiang, N., and Serhan, C.N. (2001) Selectivity of recombinant human leukotriene D(4), leukotriene B(4), and lipoxin A(4) receptors with aspirin-triggered 15-epi-LXA(4) and regulation of vascular and inflammatory responses. *Am. J. Pathol.* **158**, 3–9.

25. McMahon, B., Stenson, C., McPhillips, F., Fanning, A., Brady, H.R., and Godson, C. (2000) Lipoxin A4 antagonizes the mitogenic effects of leukotriene D4 in human renal mesangial cells. Differential activation of MAP kinases through distinct receptors. *J. Biol. Chem.* **275**, 27566–27575.

26. Takano, T., Chish, C.B., Gronert, K., Petasis, N., and Serhan, C.N. (1998) Neutrophil-mediated changes in vascular permeability are inhibited by topical application of aspirin-triggered 15-epi-lipoxin A4 and novel lipoxin B4 stable analogues. *J. Clin. Invest.* **101**, 819–826.

27. Serhan, C.N., Takano, T., Chiang, N., Gronert, K., and Clark, A. (2000) Formation of endogenous "antiinflammatory" lipid mediators by transcellular biosynthesis. Lipoxins and aspirin-triggered lipoxins inhibit neutrophil recruitment and vascular permeability. *Am. J. Respir. Crit. Care Med.* **161**, S95–S101.

28. Serhan, C.N., Takano, T., Chish, C.B., Gronert, K., and Petasis, N. (1999) Aspirin-triggered 15-epi-lipoxin A4 and novel lipoxin B4 stable analogs inhibit neutrophil-mediated changes in vascular permeability. *Adv. Exp. Med. Biol.* **469**, 287–293.

29. Ereso, A.Q., Cureton, E.L., Cripps, M.W., Sadjadi, J., Dua, M.M., Curran, B., and Victorino, G.P. (2009) Lipoxin a(4) attenuates microvascular fluid leak during inflammation. *J. Surg. Res.* **156**, 183–188.
30. Townsley, M.I., King, J.A., and Alvarez, D.F. (2006) Ca2+ channels and pulmonary endothelial permeability: insights from study of intact lung and chronic pulmonary hypertension. Microcirculation 13, 725–739.
31. Tiruppathi, C., Ahmmed, G.U., Vogel, S.M., and Malik, A.B. (2006) Ca2+ signaling, TRP channels, and endothelial permeability. Microcirculation 13, 693–708.
32. Vandenbroucke, E., Mehta, D., Minshall, R., and Malik, A.B. (2008) Regulation of endothelial junctional permeability. Ann. N. Y. Acad. Sci. 1123, 134–145.
33. Groschner, K., Rosker, C., and Lukas, M. (2004) Role of TRP channels in oxidative stress. Novartis Found. Symp. 258, 222–230; discussion 231–235, 263–266.
34. Usatyuk, P.V., Parinandi, N.L., and Natarajan, V. (2006) Redox regulation of 4-hydroxy-2-nonenal-mediated endothelial barrier dysfunction by focal adhesion, adherens, and tight junction proteins. J. Biol. Chem. 281, 35554–35566.
35. Holthe, M.R., Staff, A.C., Berge, L.N., and Lyberg, T. (2004) Leukocyte adhesion molecules and reactive oxygen species in preeclampsia. Obstet. Gynecol. 103, 913–922.
36. von Dadelszen, P., Hurst, G., and Redman, C.W. (1999) Supernatants from co-cultured endothelial cells and syncytiotrophoblast microvillous membranes activate peripheral blood leukocytes in vitro. Hum. Reprod. 14, 919–924.
37. Shah, T.J. and Walsh, S.W. (2007) Activation of NF-kappaB and expression of COX-2 in association with neutrophil infiltration in systemic vascular tissue of women with preeclampsia. Am. J. Obstet. Gynecol. 196, e41–48.
38. Wang, Y., Gu, Y., Philibert, L., and Lucas, M.J. (2001) Neutrophil activation induced by placental factors in normal and pre-eclamptic pregnancies in vitro. Placenta 22, 560–565.
39. Gu, Y., Lewis, D.F., Alexander, J.S., and Wang, Y. (2009) Placenta-derived chymotrypsin-like protease (CLP) disturbs endothelial junctional structure in preeclampsia. Reprod. Sci. 16, 479–488.
40. Sankaralingam, S., Xu, Y., Sawamura, T., and Davidge, S.T. (2009) Increased lectin-like oxidized low-density lipoprotein receptor-1 expression in the maternal vasculature of women with preeclampsia: role for peroxynitrite. Hypertension 53, 270–277.
41. Nascimento-Silva, V., Arruda, M.A., Barja-Fidalgo, C., and Fierro, I.M. (2007) Aspirin-triggered lipoxin A4 blocks reactive oxygen species generation in endothelial cells: a novel antioxidative mechanism. Thromb. Haemost. 97, 88–98.
42. LaMarca, B.D., Gilbert, J., and Granger, J.P. (2008) Recent progress toward the understanding of the pathophysiology of hypertension during preeclampsia. Hypertension 51, 982–988.
43. Osburn, W.O., Wakabayashi, N., Misra, V., Nilles, T., Biswal, S., Trush, M.A., and Kensler, T.W. (2006) Nrf2 regulates an adaptive response protecting against oxidative damage following diquat-mediated formation of superoxide anion. Arch. Biochem. Biophys. 454, 7–15.
44. Liu, Z.J., Huang, Y.P., Yi, P., Pang, H.Y., Gong, J.M., Huang, Y.J., Zhou, J., Wu, P., Ye, D.Y., and Hao, H. (2010) Effect of lipoxin A4(4) on lipopolysaccharide-induced oxidant stress in human umbilical vein endothelial cells. Zhonghua Fu Chan Ke Za Zhi 45, 848–853.

This article should be cited as follows:

Pang, H., Yi, P., Wu, P., Liu, Z., Liu, Z., Gong, J., Hao, H., Cai, L., Ye, D., and Huang, Y. (2011) Effect of lipoxin A4 on lipopolysaccharide-induced endothelial hyperpermeability. TheScientificWorldJournal 11, 1056–1067. DOI 10.1100/tsw.2011.98.
SUPPLEMENT

Sequences of Primers and Reactions Conditions for RT-PCR

- VE-cadherin:
  5'- TGT TCA CGC ATC GGT TGT -3', 5’- GAA AGC GTC CTG GTA GTC G -3’
- Nrf2:
  5’- ATT GCC TGT AAG TCC TGG TCA -3’, 5’- ACT GCT CTT TGG ACA TCA TTT CG -3’
- TRPC1:
  5’-AAT GTC GTG GTT GTG ATT GTG C -3’, 5’-GTC TTT GGT GAG GGA ATG ATG T-3’
- GAPDH:
  5’-ACC AGC CCC AGC AAG AGC ACA AG-3’, 5’-TTC AAG GGG TCT ACA TGG CAA CTG-3’

Amplification protocols included initial melt at 95°C for 5 min, melt at 94°C for 30 sec, anneal for 45 sec, and extend at 72°C for 30 sec, circle 35. Anneal temperature is 54°C for Nrf2 and TRPC1, while 57°C for VE-cadherin and GAPDH.

SUPPLEMENTARY FIGURE 1. Identification of primary HUVECs. Primary HUVECs were stained with antihuman CD31 (A) or von Willebrand factor (vWF) (B). Immunofluorescence assay showed that ≥95% cells isolated and cultured were positive. Rat lung fibroblasts stained with antirat vWF served as negative control (C).