Nitric oxide participates in IFN-γ-induced HUVECs hyperpermeability

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Short title:

NO and IFN-γ-Induced HUVECs Hyperpermeability

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Summary

The endothelial barrier function is tightly controlled by a broad range of signaling cascades including nitric oxide-cyclic guanosine monophosphate (NO-cGMP) pathway. It has been proposed that disturbances in NO and cGMP production could interfere with proper endothelial barrier function. In this study, we assessed the effect of interferon-gamma (IFN-γ), a pro-inflammatory cytokine, on NO and cGMP levels and examined the mechanisms by which NO and cGMP regulate the IFN-γ-mediated HUVECs hyperpermeability. The flux of fluorescein isothiocyanate-labeled dextran across cell monolayers was used to study the permeability of endothelial cells. Here, we found that IFN-γ significantly attenuated basal NO concentration and increased NO levels supplied by a NO donor, sodium nitroprusside (SNP). Besides, application of IFN-γ also significantly attenuated basal cGMP concentration and increased cGMP production donated by a cell permeable cGMP analogue, 8-bromo-cyclic GMP (8-Br-cGMP). In addition, the exposure of the cell monolayer to IFN-γ significantly increased HUVECs basal permeability. However, L-NAME pretreatment did not suppress IFN-γ-induced HUVECs hyperpermeability. L-NAME pretreatment followed by SNP or SNP pretreatment partially reduced IFN-γ-induced HUVECs hyperpermeability. Pretreatment with a guanylate cyclase inhibitor, 6-anilino-5,8-quinolinedione (LY83583), led to a further increase in IFN-γ-induced HUVECs hyperpermeability. The findings suggest that the mechanism underlying IFN-γ-induced increased HUVECs permeability is partly related to the inhibition of the NO production.

Body text

Vascular wall is composed of a single layer of endothelial cells which plays a critical role in the maintenance of vascular homeostasis, regulation of blood flow, thrombosis, leukocyte trafficking and barrier function (Rajendran *et al.* 2013). Nitric oxide/cyclic guanosine monophosphate (NO/cGMP) signaling cascade has been reported to regulate endothelial cell permeability (Francis *et al.* 2010). A growing body of evidence suggests that depletion of NO bioavailability impairs normal endothelium function. Importantly, endothelial dysfunction is a hallmark for various life-threatening diseases such as cancer, diabetes mellitus and cardiovascular diseases (Rajendran *et al.* 2013).

IFN-γ, released by natural killer cells, T1 helper cells and cytotoxic T cells, is a known pro-inflammatory cytokine. In pathological conditions, IFN-γ potentiates the progression of vascular inflammatory diseases such as atherosclerosis (McLaren *et al.* 2009). IFN-γ-induced impairment of barrier function has previously been reported but the underlying mechanism remains poorly understood. The endothelial permeability is regulated by multiple signaling cascades. A previous study reported by Wong *et al*. (2004) has shown that NO and cGMP modulate the increased permeability of brain mircovascular endothelial cells caused by IFN-γ (Wong *et al.* 2004). Moreover, epithelial hyperpermeability stimulated by IFN-γ is also associated with activation of phosphatidylinositol 3'-kinase and nuclear factor-κB (Boivin *et al.* 2009). To date, the mechanism by which IFN-γ affects the basal production of NO and cGMP in HUVECs and whether these changes lead to IFN-γ-induced HUVECs hyperpermeability remain unknown. Therefore, the aim of this study was to explore the role of NO-cGMP signaling pathway in the regulation of IFN-γ-induced increased HUVECs permeability.

HUVECs (Life technologies, CA, USA) were cultured in 25 cm^2 tissue culture flasks and

nourished in endothelial cell media (ScienCell, CA, USA). The cells were maintained at 37°C and 5% CO_2 atmosphere. NO production was evaluated by using a colorimetric-based Griess assay. HUVECs at a density of 2 x 10^5 cells per well were seeded onto 24-well plates. HUVECs were exposed to media, sodium nitroprusside (SNP) (Calbiochem, CA, USA), IFN-γ (eBioscience, CA, USA) or pretreated with SNP followed by IFN-γ. The supernatant was mixed with an equal volume of Griess reagent (Merck and Co., Inc, NJ, USA). The absorbance was measured at a wavelength of 548 nm using a microplate reader (Infinite M200, TECAN, Männedorf, Switzerland). The nitrite levels were calculated from the nitrite standard curve (linear range 0-100 μM).

Endothelial cGMP levels were quantified using a Cyclic GMP XP™ assay kit (Cell Signaling Technology, MA, USA). HUVECs at a cell density of 2 x 10^5 cells per well were seeded onto 24-well plates. Then, the cells were incubated with either media, IFN-γ, 8-Br-cGMP (Biovision Inc., CA, USA) or pretreated with 8-Br-cGMP followed by IFN-γ. After the indicated treatment times, the cells were lysed on ice for 5 min. Then, 50 μl of HRP-linked cGMP was mixed with 50 μl of sample and added onto a 96-well plate pre-coated with anti-cGMP antibody and incubated at RT for 3 hrs. Then, the well plate was washed and TMB substrate was added to allow for color development. The color intensities were measured by a microplate reader at 450 nm. The color intensities were inversely proportional to cGMP levels. The cGMP levels in the samples were calculated from the cGMP standard curve (0-166.7 nM).

In vitro vascular permeability assay (Milipore, MA, USA) was performed as previously described (Ng *et al.* 2015). Briefly, HUVECs were seeded on collagen-coated inserts at a concentration of 2 x 10^5 cells per insert and the bottom wells were filled with 500 μl of ECM. The cells were incubated for 72 hrs to allow the formation of intact endothelial cell monolayers.

Then, the cells were treated with various treatments according to the experimental design. After the indicated treatment times, the inserts were transferred onto a new 24-well plate in which the plate wells were filled with basal ECM. Then, the solution in each insert was replaced with 150 μl of FITC-dextran. Lastly, the passage of FITC-dextran across the endothelial cell monolayers was measured using a fluorescence microplate reader at an excitation/emission wavelength of 485 nm/530 nm. The results were expressed as a percentage compared to control. All the results were expressed as the mean ± standard error of mean and analyzed using the Student's t test. The difference between each group was considered significant when $p<0.05$.

In Figure 1A, NO levels were significantly suppressed by IFN-γ from 0.1579 ± 0.0145 μM to 0.06491 ± 0.02182 μM. To examine whether IFN-γ could suppress the exogenous NO in HUVECs, the effect of IFN-γ on the levels of NO in SNP-treated HUVECs was measured. SNP, at a concentration of 1mM, increased NO levels compared with basal (2.568 ± 0.0983 μM). Incubation of HUVECs with IFN-γ significantly suppressed SNP-induced increased NO levels (2.030 ± 0.0571 μM). These results indicate that IFN-γ suppresses both the basal NO and exogenous NO levels.

Next, Figure 1B showed that stimulation of IFN-γ led to suppression of baseline cGMP levels in a HUVECs monolayer, from 18.260 ± 1.376 nM to 4.178 ± 1.041 nM. To examine whether IFN-γ could suppress the exogenous cGMP in HUVECs, the effect of IFN-γ on the levels of cGMP in 8-Br-cGMP-treated HUVEC was measured. 8-bromo-cGMP, at a concentration of 1mM, caused a significant increase in cGMP concentration compared to basal levels (127.6 ±15.48 nM). Incubation of HUVECs with IFN-γ significantly suppressed 8-Br-cGMP-induced increased cGMP levels (44.63 ± 7.399 nM). These data suggest that IFN-γ inhibits both the basal cGMP and exogenous cGMP levels.

To study the potential role of NO and cGMP in the regulation of HUVECs hyperpermeability elicited by IFN-γ, HUVECs were pre-treated with a NO donor (SNP) and a guanylate cyclase inhibitor (LY83583) and the cell permeability was measured (Figure 2). The singly effects of SNP and LY83583 on the basal permeability of HUVECs were measured. SNP, at a concentration of 1mM, significantly decreased HUVECs permeability compared with basal levels (79.94 ± 2.193 % of control); whereas LY83583, at a concentration of 10 μM, increased basal permeability to 178.5 ± 9.803 % of control. Exposure of the monolayer to IFN-γ significantly increased HUVECs permeability to 532.2 ± 37.34 % of control, compared with basal. However, HUVECs pre-incubated with SNP showed a partial decrease in IFN-γ-induced increased permeability (328.6 ± 16.51 % of control). This indicates that NO is partially involved in the regulation of IFN-γ-induced increased permeability.

Pretreatment of LY followed by IFN-γ stimulation produced a further increase in permeability (782.2 ± 34.57 % of control) as compared to IFN-γ group. Notably, LY alone slightly increased basal permeability to a percentage of 178.5 ± 9.8% (Figure 2). This implies that basal cGMP levels are required to maintain the basal permeability. Following the slightly increased permeability caused by LY, the subsequent further increase in the cell permeability caused by IFN-γ suggests that there could be another target molecule upstream of cGMP that could directly affect the IFN-γ-induced cell permeability as well. This indicates that cGMP might not be involved in the regulation of IFN-γ-induced HUVECs permeability. Therefore, the further increase in HUVECs permeability in LY+IFN-γ might result from other upstream signaling molecules such as NO, in addition to the effect of LY alone. As cGMP is the downstream target of NO, we speculate that the reduction of cGMP levels observed upon induction of IFN-γ might result from the IFN-γ-mediated reduced NO levels, in which NO is partially regulating the IFN-

γ-induced HUVECs hyperpermeability.

To further confirm that NO is a key regulator in modulating the IFN-γ-mediated increased permeability, HUVECs were pre-treated with an eNOS inhibitor (L-NAME) and the cell permeability was measured. As shown in Figure 3, stimulation of IFN-γ induced HUVECs hyperpermeability to 584.3 ± 44.29 % of control. However, pretreatment of HUVECs with L-NAME did not have a significant effect on increased permeability induced by IFN-γ. This indicates that eNOS might not take part in the regulation of IFN-γ-induced HUVECs hyperpermeability. Therefore, the reduced NO levels caused by IFN-γ is not a consequence of NOS inhibition, but rather, IFN-γ may act through other mechanisms such as ROS generation to reduce NO bioavailability and that this might subsequently causes an increase in cell permeability. However, further study is vital to investigate this possible mechanism.

Importantly, one extra group was added in which the HUVECs were pre-treated with L-NAME to inhibit NO production and a subsequent NO replenishment by SNP before IFN-γ induction (Figure 3). In this group, the endothelial hyperpermeability induced by IFN-γ was significantly reduced to 317.2 ± 64.96% of control, which was comparable to SNP+IFN-γ group (Figure 2). These data imply that replenishment of NO partially suppressed the IFN-γ-induced increased permeability. The data further confirm that NO partially regulates the IFN-γ-mediated increased HUVECs permeability.

A previous study demonstrated that NO production triggered by IFN-γ is greatly influenced by the doses used (Morikawa *et al.* 2000). Relatively low concentrations of IFN-γ (0.1 and 1 ng/ml) reduced NO production in murine aortic endothelial cell line via down-regulation of eNOS. In contrast, IFN-γ induced a significant increase in NO levels by increasing iNOS expression at relatively high concentrations (50 and 100 ng/ml). Interestingly, an

intermediate dose of IFN-γ (10 ng/ml) did not alter the basal NO production in HUVECs (Morikawa *et al.* 2000). In apposition, we showed that 10 ng/ml of IFN-γ significantly reduced the NO production in HUVECs. The discrepancy between these findings may be due to different types of vascular beds used; and this explanation is supported by previous findings showing that the mechanism of NO production is largely dependent on the origin of vascular beds (Geiger *et al.* 1997, Sugiyama *et al.* 2003).

Interestingly, IFN-γ has been reported to induce ROS production in retinal pigment epithelium and hepatocytes (Watanabe *et al.* 2003, Yang *et al.* 2007). Increased ROS generation may lead to a decrease in NO bioavailability (Forstermann 2010). Besides, *in vivo* study using 10 ng/ml of IFN-γ has shown that IFN-γ induces oxidative stress and motoneuron death in rat spinal cord embryonic explants (Mir *et al.* 2009). Therefore, it is possible that 10 ng/ml of IFN-γ used in our experimental setting could also induce oxidative stress and lead to impairment of NO bioavailability. However, further investigation is needed to clarify the possibility.

Previous study has shown that the plasma levels of IFN-γ are higher in patients with atherosclerosis and hyperlipoproteinemia IIb compared to healthy subjects, 44.4 ± 5.3 pg/ml and 19.4 ± 2.1 pg/ml, respectively (Madej *et al.* 1998). Besides, the plasma concentrations of IFN-γ in peripheral atherosclerosis obliterans patients and in healthy subjects are 327 ± 70.0 pg/ml and 181 ± 43.0 pg/ml, respectively (Correa *et al.* 2011). Furthermore, the serum levels of IFN-γ in children with food allergy and in healthy children are 633.75 pg/ml and 180 pg/ml, respectively (Hofman 1995). Collectively, the circulating levels of IFN-γ under physiological and/or disease states are varied among the studies; this is likely due to differences in several factors such as the types of disease, severity and age.

There is a great controversy over the role of NO and cGMP in the modulation of

endothelial barrier function as both the barrier protective and barrier disruptive effects of NO and cGMP have previously been demonstrated. *In vitro* study using NO donors, such as SNP and DETA NONOate, has reported that NO treatment increases human brain microvascular endothelial cell resistance, evidenced by a reduction in permeability. Besides, SNP treatment also reverses the increased permeability induced by LPS, IL-1β and IFN-γ (Wong *et al.* 2004). In agreement with their findings, we found that HUVECs stimulated with SNP showed a significant reduction in both the basal permeability and IFN-γ-induced increased permeability.

In contrast, NO has also been found to strengthen endothelial permeability as the application of spermine NONOate has been shown to augment the H_2O_2-induced endothelial hyperpermeability (Okayama *et al.* 1997). Besides, upregulation of NO plays a vital role in the IFN-γ-mediated increased permeability of Caco-2BBe intestinal epithelial cells (Unno *et al.* 1995). Furthermore, induction of iNOS expression is important for IFN-γ-induced Caco cells hyperpermeability (Chavez *et al.* 1999, Unno *et al.* 1999). On the contrary, other researchers have reported that induction of iNOS does not correlate with IFN-γ-induced hyperpermeability of Caco cells (Satake *et al.* 2001). These controversial data indicate that the role of NO in regulating the cell permeability in the presence of IFN-γ remains debatable. Interestingly, vascular hyperpermeability induced by TNF-α is further augmented by IFN-γ in a dose-dependent fashion but the response is inhibited by a depletion of neutrophils with a monoclonal antibody (Abe *et al.* 1990).

A previous study has demonstrated that treatment of HUVECs with 8-bromo-cGMP and 8-PCPT-cGMP at a concentration of 1 mM decreases both the basal permeability and thrombin-induced endothelial hyperpermeability (Draijer *et al.* 1995). Besides, incubation of HUVECs with 1mM of 8-bromo-cGMP for 1 h has been shown to increase basal permeability (Varma *et al.*

2002). Here, we showed that inhibition of cGMP production in HUVECs resulted in increased basal permeability and also augmented the IFN-γ-induced increased permeability. Consistent with our finding, a previous study has reported that application of a guanylyl cyclase inhibitor (ODQ) in brain microvessel endothelial cells reduces endothelial monolayer resistance (Wong *et al.* 2004).

In conclusion, IFN-γ significantly suppressed NO and cGMP levels in HUVECs. The underlying mechanism of IFN-γ-induced increased HUVECs permeability is partly associated with the reduction of NO the production.

Conflict of Interest

There is no conflict of interest.

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References

Abe, Y., S. Sekiya, T. Yamasita and F. Sendo. Vascular hyperpermeability induced by tumor necrosis factor and its augmentation by IL-1 and IFN-gamma is inhibited by selective depletion of neutrophils with a monoclonal antibody. *J Immunol* **145**: 2902-2907, 1990.

Boivin, M. A., P. K. Roy, A. Bradley, J. C. Kennedy, T. Rihani and T. Y. Ma. Mechanism of interferon-gamma-induced increase in T84 intestinal epithelial tight junction. *J Interferon Cytokine Res* **29**: 45-54, 2009.

Chavez, A. M., M. J. Menconi, R. A. Hodin and M. P. Fink. Cytokine-induced intestinal epithelial hyperpermeability: role of nitric oxide. *Crit Care Med* **27**: 2246-2251, 1999.

Correa, C. R., L. A. Dias-Melicio, S. A. Calvi, S. Lastoria and A. M. Soares. Activation of monocytes and cytokine production in patients with peripheral atherosclerosis obliterans.

J Inflamm (Lond) **8**: 23, 2011.

Draijer, R., D. E. Atsma, A. van der Laarse and V. W. van Hinsbergh. cGMP and nitric oxide modulate thrombin-induced endothelial permeability. Regulation via different pathways in human aortic and umbilical vein endothelial cells. *Circ Res* **76**: 199-208, 1995.

Forstermann, U. Nitric oxide and oxidative stress in vascular disease. *Pflugers Arch* **459**: 923-939, 2010.

Francis, S. H., J. L. Busch, J. D. Corbin and D. Sibley. cGMP-dependent protein kinases and cGMP phosphodiesterases in nitric oxide and cGMP action. *Pharmacol Rev* **62**: 525-563, 2010.

Geiger, M., A. Stone, S. N. Mason, K. T. Oldham and K. S. Guice. Differential nitric oxide production by microvascular and macrovascular endothelial cells. *Am J Physiol* **273**: L275-281, 1997.

Hofman, T. IL-4 and IFN-gamma level in blood serum of children with food allergy. *Rocz Akad Med Bialymst* **40**: 462-467, 1995.

Madej, A., B. Okopien, J. Kowalski, M. Zielinski, J. Wysocki, B. Szygula, Z. Kalina and Z. S. Herman. Effects of fenofibrate on plasma cytokine concentrations in patients with atherosclerosis and hyperlipoproteinemia IIb. *Int J Clin Pharmacol Ther* **36**: 345-349, 1998.

McLaren, J. E. and D. P. Ramji. Interferon gamma: a master regulator of atherosclerosis. *Cytokine Growth Factor Rev* **20**: 125-135, 2009.

Mir, M., V. J. Asensio, L. Tolosa, M. Gou-Fabregas, R. M. Soler, J. Llado and G. Olmos. Tumor necrosis factor alpha and interferon gamma cooperatively induce oxidative stress and motoneuron death in rat spinal cord embryonic explants. *Neuroscience* **162**: 959-971, 2009.

Morikawa, A., N. Koide, Y. Kato, T. Sugiyama, D. Chakravortty, T. Yoshida and T. Yokochi. Augmentation of nitric oxide production by gamma interferon in a mouse vascular endothelial cell line and its modulation by tumor necrosis factor alpha and lipopolysaccharide. *Infect Immun* **68**: 6209-6214, 2000.

Ng, C. T., L. Y. Fong, M. R. Sulaiman, M. A. Moklas, Y. K. Yong, M. N. Hakim and Z. Ahmad. Interferon-Gamma Increases Endothelial Permeability by Causing Activation of p38 MAP Kinase and Actin Cytoskeleton Alteration. *J Interferon Cytokine Res* **35**: 513-522, 2015.

Okayama, N., C. G. Kevil, L. Correia, D. Jourd'heuil, M. Itoh, M. B. Grisham and J. S. Alexander. Nitric oxide enhances hydrogen peroxide-mediated endothelial permeability in vitro. *Am J Physiol* **273**: C1581-1587, 1997.

Rajendran, P., T. Rengarajan, J. Thangavel, Y. Nishigaki, D. Sakthisekaran, G. Sethi and I. Nishigaki. The vascular endothelium and human diseases. *Int J Biol Sci* **9**: 1057-1069, 2013.

Satake, M., H. Watanabe, Y. Miyamoto and M. Shimizu. Induction of nitric oxide synthase and subsequent production of nitric oxide not involved in interferon-gamma-induced hyperpermeability of Caco-2 intestinal epithelial monolayers. *Biosci Biotechnol Biochem* **65**: 428-430, 2001.

Sugiyama, T., M. Fujita, N. Koide, A. Morikawa, K. Takahashi, T. Yoshida, H. Mori and T. Yokochi. Differences in the mechanism of nitric oxide production between mouse vascular endothelial cells and macrophages. *J Endotoxin Res* **9**: 108-112, 2003.

Unno, N., R. A. Hodin and M. P. Fink. Acidic conditions exacerbate interferon-gamma-induced

intestinal epithelial hyperpermeability: role of peroxynitrous acid. *Crit Care Med* **27**: 1429-1436, 1999.

Unno, N., M. J. Menconi, M. Smith and M. P. Fink. Nitric oxide mediates interferon-gamma-induced hyperpermeability in cultured human intestinal epithelial monolayers. *Crit Care Med* **23**: 1170-1176, 1995.

Varma, S., J. W. Breslin, B. K. Lal, P. J. Pappas, R. W. Hobson, 2nd and W. N. Duran. p42/44MAPK regulates baseline permeability and cGMP-induced hyperpermeability in endothelial cells. *Microvasc Res* **63**: 172-178, 2002.

Watanabe, Y., O. Suzuki, T. Haruyama and T. Akaike. Interferon-gamma induces reactive oxygen species and endoplasmic reticulum stress at the hepatic apoptosis. *J Cell Biochem* **89**: 244-253, 2003.

Wong, D., K. Dorovini-Zis and S. R. Vincent. Cytokines, nitric oxide, and cGMP modulate the permeability of an in vitro model of the human blood-brain barrier. *Exp Neurol* **190**: 446-455, 2004.

Yang, D., S. G. Elner, Z. M. Bian, G. O. Till, H. R. Petty and V. M. Elner. Pro-inflammatory cytokines increase reactive oxygen species through mitochondria and NADPH oxidase in cultured RPE cells. *Exp Eye Res* **85**: 462-472, 2007.

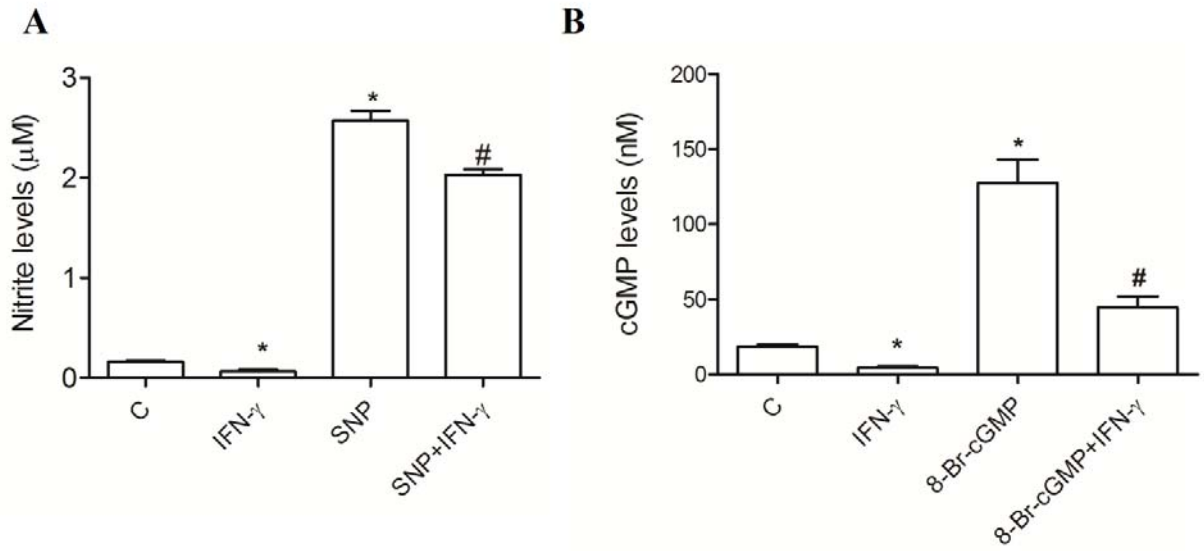

Figure 1. Effect of IFN-γ on NO and cGMP levels in HUVECs. **A,** HUVECs were treated with media (control), IFN-γ (10 ng/ml, 8 h), SNP (1 mM, 5 mins) or SNP pretreatment followed by IFN-γ. **B,** HUVECs were incubated with media (control), IFN-γ (10 ng/ml, 8 h), 8-Br-cGMP (1 mM, 1 h) or 8-Br-cGMP pretreatment followed by IFN-γ. The data were expressed as the mean ±

SEM from three independent experiments. Each experiment was performed in triplicate. * P<0.05 significantly different from control. # P<0.05 significantly different from IFN-γ-induced group. SNP, sodium nitroprusside.

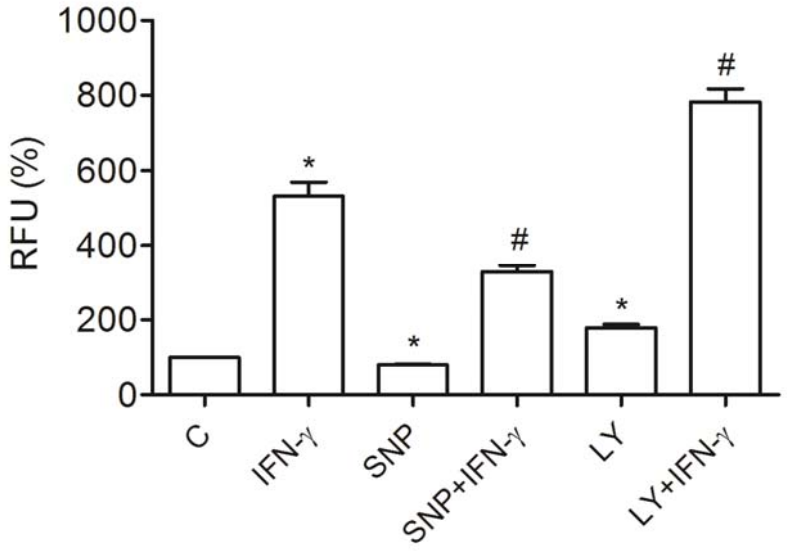

Figure 2. Evaluation of the role of NO and cGMP in IFN-γ-stimulated increased HUVECs permeability. HUVECs were challenged with media (control), IFN-γ (10 ng/ml, 8 h), SNP (1 mM, 5 mins), LY83583 (10 μM, 1 h), SNP pretreatment followed by IFN-γ or LY83583 pretreatment followed by IFN-γ. The data were expressed as the mean ± SEM from at least three independent experiments. Each experiment was performed in triplicates. * P<0.05 significantly different from control. # P<0.05 significantly different from IFN-γ-induced group. SNP, sodium nitroprusside; RFU, relative fluorescence unit.

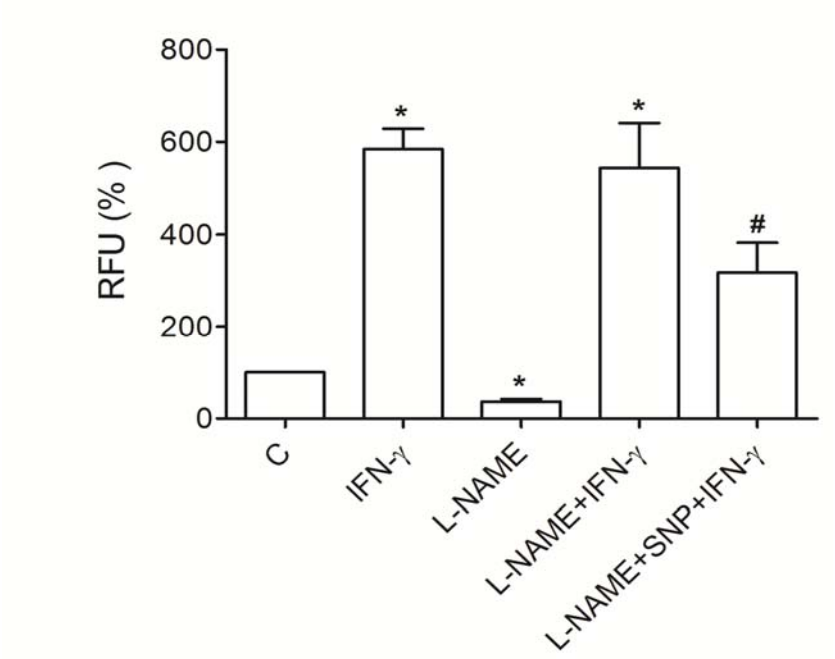

Figure 3. Effect of NO inhibition in IFN-γ-stimulated increased HUVECs permeability. HUVECs were challenged with media (control), IFN-γ (10 ng/ml, 8 h), L-NAME (200 μM, 1 h), L-NAME pretreatment followed by IFN-γ or L-NAME pretreatment followed by SNP (1 mM, 5 mins) and subsequently challenged with IFN-γ. The data were expressed as the mean ± SEM from at least three independent experiments. Each experiment was performed in triplicates. * $P<0.05$ significantly different from control. # $P<0.05$ significantly different from IFN-γ-induced group. SNP, sodium nitroprusside; RFU, relative fluorescence unit.