

Nitric Oxide Participates in 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 the increased NO levels supplied by a NO donor, sodium nitroprusside (SNP). Besides, application of IFN- γ also significantly attenuated both the basal cGMP concentration and the increased cGMP production donated by a cell permeable cGMP analogue, 8-bromo-cyclic GMP (8-Br-cGMP). In addition, 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 NO production.

Key words

Interferon-gamma • Permeability • Human umbilical vein endothelial cells • Nitric oxide • Cyclic guanosine monophosphate

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Vascular wall is composed of a single layer of endothelial cells which plays critical role in the maintenance of vascular homeostasis, regulation of blood flow, thrombosis, leukocyte trafficking and barrier function (Rajendran *et al.* 2013). The 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 endothelial 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 and Ramji 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 microvascular 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- κ B (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 could 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 cm² tissue culture flasks and nourished in endothelial cell media (ScienCell, CA, USA). The cells were maintained at 37 °C and 5 % CO₂ atmosphere. NO production was evaluated using a colorimetric-based Griess assay. HUVECs at a density of 2 x 10⁵ 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 μ M).

Endothelial cGMP levels were quantified using a Cyclic GMP XPTM assay kit (Cell Signaling Technology, MA, USA). HUVECs at a cell density of 2 x 10⁵ 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 μ l of HRP-linked cGMP was mixed with 50 μ l of sample and added onto a 96-well plate pre-coated with anti-cGMP antibody and incubated at RT for 3 h. 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 10⁵ cells per insert and the bottom wells were filled with 500 μ l of ECM. The cells were incubated for 72 h 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 μ l 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 \pm 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 \pm 0.0145 μ M to 0.06491 \pm 0.02182 μ M. 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 1 mM, increased NO levels compared with basal (2.568 \pm 0.0983 μ M). Incubation of HUVECs with IFN- γ significantly suppressed SNP-induced increased NO levels (2.030 \pm 0.0571 μ M). 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 HUVECs, from 18.260 \pm 1.376 nM to 4.178 \pm 1.041 nM. To examine whether IFN- γ could suppress 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 1 mM, caused a significant increase in cGMP concentration compared to basal levels (127.6 \pm 15.48 nM). Incubation of HUVECs with IFN- γ significantly suppressed 8-Br-cGMP-induced increased cGMP levels (44.63 \pm 7.399 nM). These data suggest that IFN- γ inhibits both the basal cGMP and exogenous cGMP levels.

To study the potential roles 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 (Fig. 2). The singly effects of SNP and LY83583 on the basal permeability of HUVECs

were measured. SNP, at a concentration of 1 mM, significantly decreased HUVECs permeability compared with basal levels (79.94 ± 2.193 % of control); whereas LY83583, at a concentration of 10 μ M, 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.

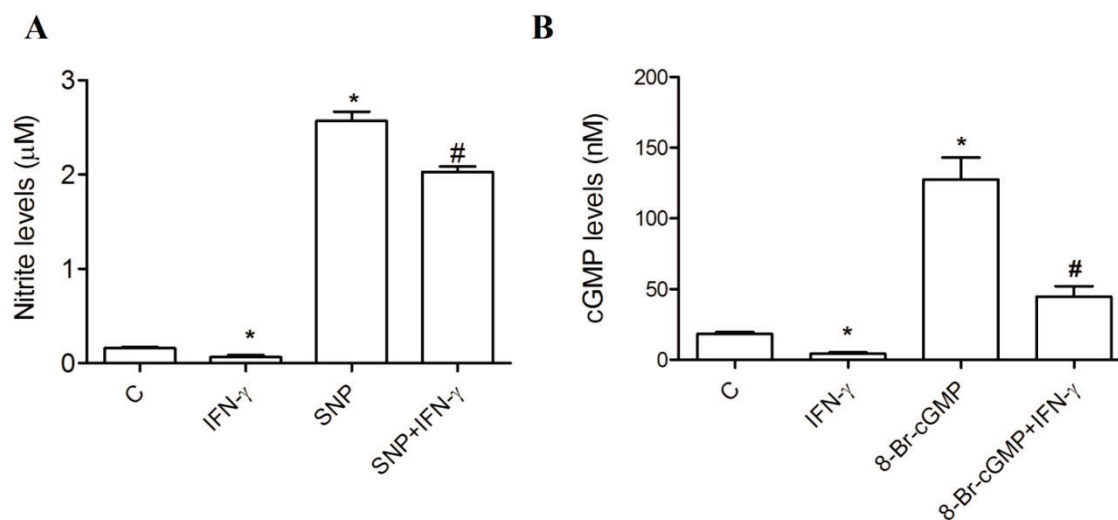


Fig. 1. Effect of IFN- γ on NO and cGMP levels in HUVECs. **A**) HUVECs were treated with media (control), IFN- γ (10 ng/ml, 8 h), SNP (1 mM, 5 min) or SNP pretreatment followed by IFN- γ . **B**) HUVECs were incubated with media (control), IFN- γ (10 ng/ml, 8 h), 8-Br-cGMP (1 mM, 1 h) or 8-Br-cGMP pretreatment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

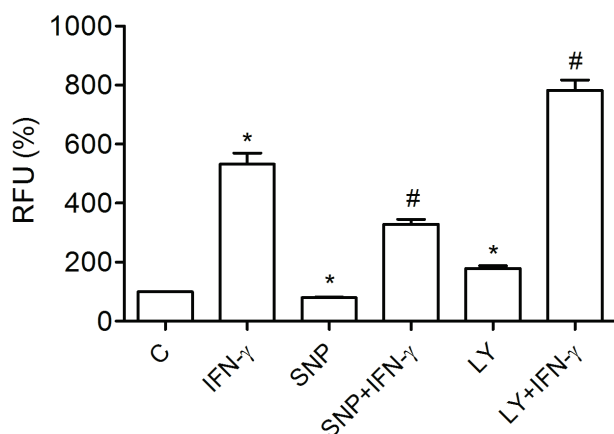


Fig. 2. Evaluation of the role of NO and cGMP in IFN- γ -stimulated increased HUVECs permeability. HUVECs were challenged with media (control), IFN- γ (10 ng/ml, 8 h), SNP (1 mM, 5 min), LY83583 (10 μ M, 1 h), SNP pretreatment followed by IFN- γ or LY83583 pretreatment followed by IFN- γ . The data were expressed as the mean \pm 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.

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 % (Fig. 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.

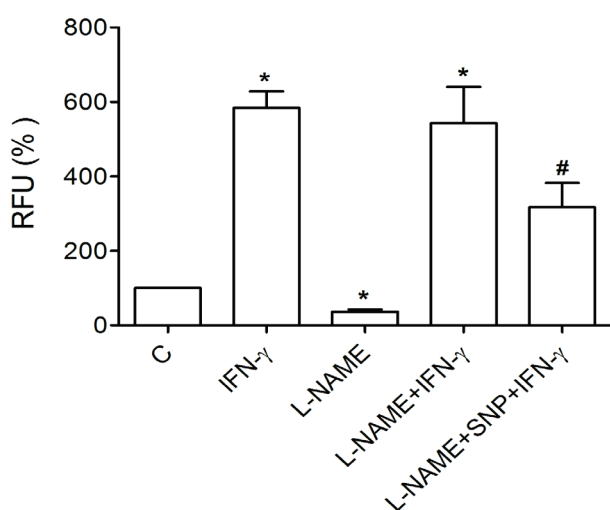


Fig. 3. Effect of NO inhibition in IFN- γ -stimulated increased HUVECs permeability. HUVECs were challenged with media (control), IFN- γ (10 ng/ml, 8 h), L-NAME (200 μ M, 1 h), L-NAME pretreatment followed by IFN- γ or L-NAME pretreatment followed by SNP (1 mM, 5 min) and subsequently challenged with IFN- γ . The data were expressed as the mean \pm 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.

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 (Fig. 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 (Fig. 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 addition, 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.

A previous study has shown that the plasma levels of IFN- γ were 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 were 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 were 633.75 pg/ml and 180 pg/ml, respectively (Hofman 1995). Collectively, the circulating levels of IFN- γ under physiological and/or disease states vary among these 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 roles 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-1 β 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 to augments the H₂O₂-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 decreased both the basal permeability and thrombin-induced endothelial hyperpermeability (Draijer *et al.* 1995). Besides, incubation of HUVECs with 1 mM 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 production.

Conflict of Interest

There is no conflict of interest.

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