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NITRIC OXIDE PRODUCTION BY LYMPHATIC ENDOTHELIAL CELLS IN VITRO

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SUMMARY: The present study demonstrates that confluent monolayer cultures of lymphatic endothelial cells produce and secrete NO. Immunofluorescent studies showed that eNOS activity can be stimulated with Ca ionophore to enhance the production of NO. Cells exposed to LPS and various cytokines stimulated the production of iNOS which showed the greatest increase in activity at 4 hrs and declined at 18 and 24 hrs. These studies provide evidence that, within the lymphatic vascular lumen, nitric oxide may be produced by the lymphatic endothelium which interact with various vasoactive substances to regulate lymphatic vascular tone. In addition, the production of NO by LEC may be important in the regulation of lymphatic vascular tone in order to more readily accommodate sudden fluctuations in lymph flow and pressure that normally occur during the process of lymph formation and propulsion.

Lymphatic vessels play a major role in the maintenance of homeostasis by removing fluids and plasma proteins that constantly permeate the blood capillary wall. Although the walls of lymphatic vessels are much thinner than the accompanying arteries and veins, lymphatics can rapidly dilate to accommodate the removal of large increases in connective tissue fluid (1). These vessels can also become grossly distended to form tortuous tubes in inflammatory and edematous conditions. Because of their unique structural arrangement within the connective tissue, it has been generally thought that the control of lymphatic vascular tone is largely dependent on the activities of the surrounding

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muscles and body movements (2). Recent studies have shown that nitric oxide (NO), plays an important role in blood vascular hemodynamics (3). Nitric oxide is formed in vascular endothelial cells from the guanidino nitrogen of L-arginine via a Ca^{++} dependent process that is mediated by NO synthase (4,5). NO is a labile and highly reactive compound which diffuses into the vascular wall and activates soluble guanylate cyclase in vascular smooth muscle cells to form cyclic GMP which causes vasorelaxation (3,6,7). A number of other cell types are also capable of NO production including macrophages, neurons and some tumor cells (5,8-11). However, little is known of the mechanisms which regulate lymphatic vascular tone. Likewise very little is known regarding mechanisms that control the selective dilatation and constriction of these vessels to accommodate increased rates of lymph flow and pressure within the lymphatic vascular lumen during inflammation.

In the present study we show that NO is produced in monolayers of lymphatic endothelial cells (LEC) in culture. In addition, we show that NO production by LEC is enhanced when cells are treated with various agonists and cytokines.

MATERIALS AND METHODS

Cell Culture. Lymphatic endothelial cells (LEC) were isolated from sheep mesenteric lymphatic vessels as previously described (12). The endothelial nature of LEC was characterized by their flattened cobblestone appearance, and by Immunofluorescent staining for factor VIII-related antigens (12). In all experiments LEC were used between passages 4 through 6.

Nitrite Assay: To determine the production of NO, LEC cultures were grown in 24 well plates. At confluence cells were washed in serum free medium, cultured for various time periods and nitrite concentration in the medium measured. Studies were carried out to assess the effect of various agents on NO production. These included NOS inhibitors (L-N-aminoarginine,(NNA), L-N-G-Nitroarginine methyl ester (L-NAME), E.coli lipopolysaccharide (LPS), Ca ionophore A23187, histamine, and tumor necrosis factor, alpha (TNFα). The agents were added in LEC serum free culture medium and nitrite accumulation, a stable degradation product of NO was measured after various incubation periods using the Griess Assay (13) adapted to a 96 well microtiter

plate reader (14). Nitrite concentrations were determined by comparison with standard solutions of sodium nitrite prepared in serum free culture medium. The product of the reaction was read at 540 nM on a Bio-Rad multiplate Reader. The NO absorbances were based on the average of three wells per solution and NO concentrations were calculated from a standard curve obtained with sodium nitrite.

Statistics: All experiments were carried out in triplicate, and repeated at least three times with similar results. The values were expressed as mean ± s.e. Student's unpaired t-test was used to determine the significant difference between means and a value of $P< 0.05$ was taken as significant.

Immunocytochemistry: To localize nitric oxide synthases, cells were grown on glass cover slips and processed for immunofluorescent studies (12) using rabbit polyclonal antibodies to eNOS and iNOS as the primary antibody and goat anti rabbit IgG conjugated to FITC as the secondary antibody (Affinity Bioreagents, Neshanic Station, NJ). For negative controls the primary antibody was replaced with preimmune rabbit serum. Cells were analyzed with a Zeiss Axiovert 10 Reflected Fluorescent Microscope.

RESULTS

Basal levels of nitrite produced by LEC in culture ranged from 7 to 7.5 uM between 4 to 18 hours. Treatment of LEC with NNA and L-NAME caused a reduction in nitrite production (Fig. 1). The treatment of LEC cultures with Ca ionophore greatly enhanced the production of nitrite within 4 hours and the accumulation of nitrite in the culture medium remained high after 18 hours (Fig. 1). This enhanced nitrite production following Ca treatment was inhibited in the presence of NNA (data not shown). Histamine caused a dose dependent increase in nitrite production (Fig. 2). The production of nitrite in LEC cultures following treatment with histamine was also time dependent with the first detectable accumulation occurring between 4 to 6 hours. This increase in nitrite was sustained for up to 24 hours (data not shown).

The effect of LPS on the production of nitrites by LEC in culture is shown in Fig. 3. The exposure of LEC to various concentrations of LPS (0.1, 0.5, 1, and 100 ng/ml) for 4 hours resulted in an enhanced production of nitrite in the culture medium with cells exposed to 0.5 ng/ml LPS giving the great-

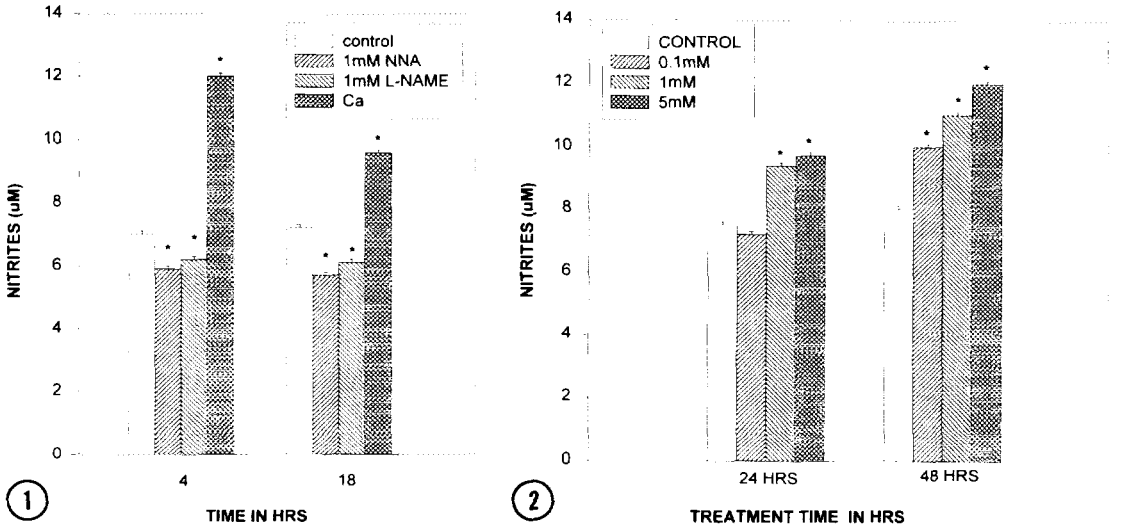

Figure 1. Effects of inhibitors [L-NNA (1mM), L-NAME (1mM)] and Ca ionophore (5.6 uM) stimulation on the production of NO in cultured LEC. Control levels of NO from LEC grown in serum free medium was 7.2 µM after 18 hrs. Treatment with NNA and L-NAME gave reduced nitrite levels after 18 hrs. Treatment with Ca ionophore A23187 greatly enhanced nitrite production after 4 hrs. Vertical bars in all bar graphs represent the mean ± S.E. (n=3) from representative experiments. *P<.02 between control and treatment with inhibitors or Ca.

Figure 2. This bar graph shows an enhanced dose response of NO production that is stimulated with various concentrations of histamine after 24 and 48 hrs. The effects of the 0.1 mM treatment was seen only after 48 hrs. *P<.05 between control and histamine treated cells.

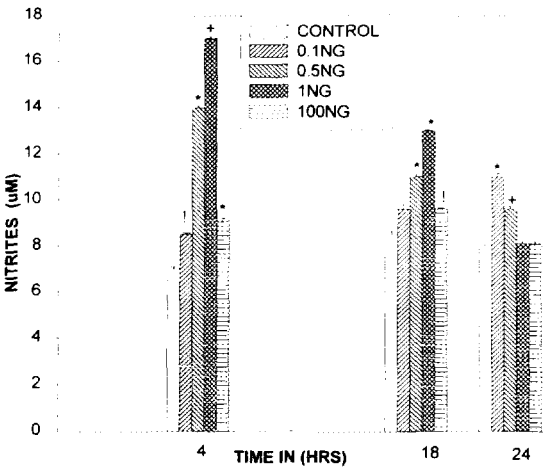

Figure 3. LEC cultures were incubated with varying concentrations of LPS as indicated in the graph and culture media sampled at 4,18 and 24 hours to measure nitrite accumulations. The greatest enhancement of NO production is observed at 4 hrs with the 0.5 ng treatment. Cells treated with 1 and 100 ng LPS showed no effect at 24 hrs. *P<.001, +P<.002, !P<.02 when compared to the control value.

est accumulation of nitrite (Fig. 3). This enhanced accumulation of nitrite in the culture medium was maintained after 18 hours (Fig. 3).

Exposure of LEC to TNFα for 4 hours caused an increased accumulation of nitrite (12 μM) in the culture medium (Fig. 4). By 18 hours the level of nitrite in the culture medium decreased to 9.9 μM and by 24 hours nitrite levels had returned to normal (Fig. 4). We also treated LEC with a combination of endotoxin and cytokine (TNFα /LPS). This combined treatment produced a high level of nitrite at 4 hours which was sustained after 24 hours (Fig. 4).

To determine the localization of NO synthase in LEC monolayer cultures, immunocytochemistry was carried out using rabbit polyclonal antibodies to eNOS as shown in Fig.5A. LEC grown in serum free medium exhibited a staining reaction for eNOS activity within the nucleus and within the cytoplasm. The treatment of LEC monolayers with Ca ionophore for 4 hours showed an enhanced

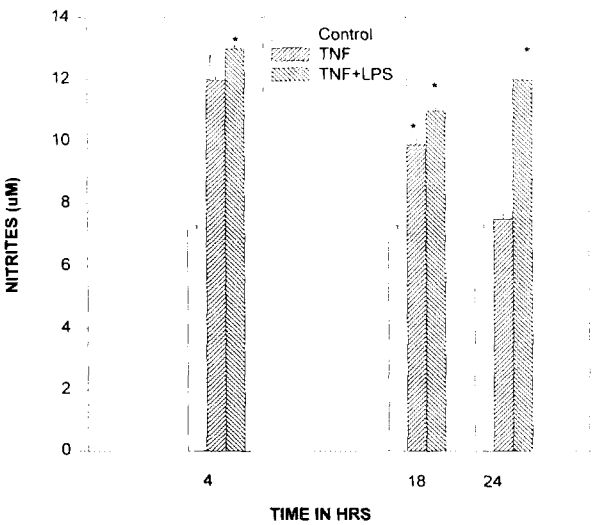

Figure 4. With TNFα alone, the greatest enhancement in nitrite occurs at 4 hrs and sharply declines at 24 hrs. However, the coincubation with TNFα and LPS showed a sustained enhanced stimulation of nitrite accumulation in the culture media over a 24-hr period. *P<.001, !P<.02 when compared to the control value.

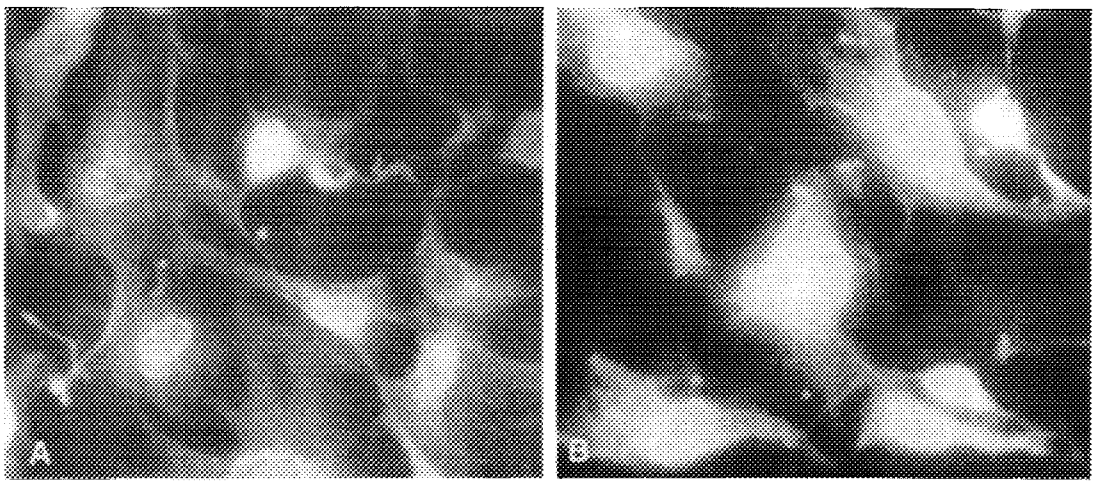

Figure 5. Immunofluorescent localization of eNOS. In A, cells from LEC cultures grown in serum free medium incubated with antibody to eNOS show staining reaction in the nucleus and cytoplasm. In B, cultures were stimulated with 5.6μM A23187 for 4 hrs and then processed for immunofluorescent analysis, using antibody to eNOS. The treatment with Ca produced an enhanced staining reaction in the nucleus and the cytoplasm.

production of eNOS activity throughout the cytoplasm and within the nucleus (Fig.5B). This increase in eNOS activity correlates with the enhanced accumulation of nitrites in conditioned medium analyzed from cultures of 4 to 6 hours following stimulation with Ca ionophore as shown in Fig. 1.

To localize the inducible isoform of nitric oxide synthase (iNOS) in the lymphatic endothelium, cultures were exposed to LPS for 4 hours and then processed for immunofluorescence using rabbit polyclonal anti iNOS antibody. An intense staining reaction for iNOS activity was exhibited in the nucleus and throughout the cytoplasm (Fig. 6).

DISCUSSION

For the blood vascular endothelium the level of the constitutive isoform of NOS is sufficient to produce NO for the physiological regulation of vascular tone and the maintenance of blood pressure homeostasis (15). The basal levels of NO synthe-

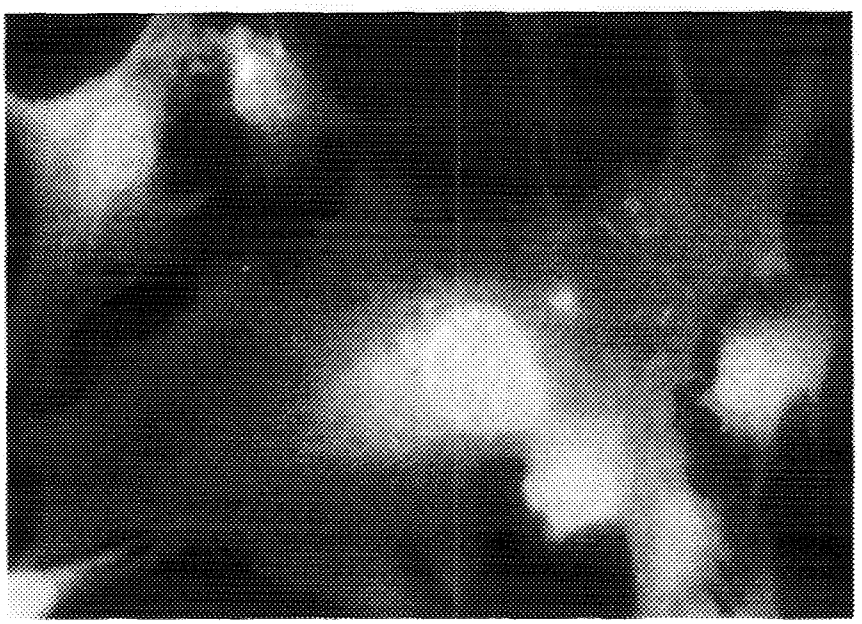

Figure 6. Immunofluorescent localization of iNOS. LEC cultures were treated with LPS for 4 hrs and then processed for Immunofluorescent microscopy using rabbit polyclonal anti iNOS antibody. All cells exhibit an intense staining reaction for the anti iNOS antibody in the nucleus and cytoplasm.

sized by blood vascular endothelial cells activate soluble guanylate cyclase in the underlying vascular smooth muscle cells causing a decrease in intracellular Ca^{++} and a relaxation with a consequent vasodilatation of the vascular wall (6). Serving as a vital component of the cardiovascular system, lymphatic vessels provide a one-way drainage system for the rapid and constant removal of connective tissue fluids and plasma proteins that permeate the blood capillary vascular wall (16). However, the mechanism for the regulation of lymphatic vascular tone in the propulsion of lymph has not been defined. In the present study we show that lymphatic endothelial cells in culture express a constitutive isoform of NOS, as demonstrated by the accumulation of nitrites in conditioned medium of LEC cultures and the localization of eNOS in these cells by immunofluorescent methods. The demonstration of NO secretion by LEC provides evidence

for the production of an EDRF within the lymphatic vessels for the regulation of lymphatic vascular tone, in the propulsion and maintenance of lymph flow.

The increased accumulation of NO in culture medium of LEC treated with endotoxins and cytokines also demonstrates the production of an inducible form of the enzyme for NOS in the lymphatic endothelium. This finding is in agreement with previous studies which showed that iNOS could be induced in the blood vascular endothelium, smooth muscle cells, macrophages and a number of other cells types, after stimulation with cytokines and endotoxins (17-21). The present study indicates that TNFα alone was effective in inducing an increased production of NO in LEC cultures that was maximum at 4 hours and declined to levels below control values by 24 hours. Recent studies have also shown that TNFα alone can induce NO synthesis in bovine aortic endothelium, with a maximal release of NO between 16 and 24 hours (22). The delay in NO production in aortic endothelium may be due to differences in cell types and will require additional studies. The present study also showed that increased levels of NO production by LEC could be sustained for up to 24 hours following exposure to TNFα in combination with LPS. The treatment of blood vascular endothelial cells with LPS in combination with interferon-γ has also been shown to produce a sustained release of NO (14,17). The effects of cytokines and endotoxins in the production of an enhanced secretion of NO that is sustained over an extended period in the lymphatic endothelium would also explain why lymphatic vessels in vivo remain dilated for an extended period following an inflammatory response produced by bacterial toxins (16). Located within the connective tissue areas lymphatic vessels provide a first line of defense against foreign substances. In addition to providing pathways and channels for

the removal and subsequent transport of these invading substances, the lymphatic endothelium is also capable of responding to the stimulatory effect of endotoxins and cytokines with the production of an inducible form of the enzyme for NOS. This results in an increased accumulation of NO which in turn would produce a sustained relaxation and dilation of the lymphatic vessels in order to facilitate the removal of an excess permeation of fluids and plasma proteins across the blood vascular capillary wall. The production of NO by the constitutive isoform of NOS in LEC suggest that physiological levels of NO are produced by the lymphatic endothelium to interact with various vasoactive substances in regulating lymphatic vascular tone.

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