Neuropeptide Y Upregulates the Adhesiveness of Human Endothelial Cells for Leukocytes

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The nervous system and the autonomic system in particular have been associated with stress-induced changes in host resistance to infections and inflammatory reactions. Since a key step in initiation of inflammation is adhesion of leukocytes to the endothelium, we hypothesized that neuron-derived factors might be involved in this process. Neuropeptide Y (NPY), a 36–amino acid neuropeptide that is colocated and released with norepinephrine from sympathetic nerves, has already been implicated in inflammatory reactions via modulation of histamine release from mast cells. This study was undertaken to examine the potential role of NPY in proinflammatory processes via modulation of endothelium-leukocyte interaction. NPY (0.01–10 μM) increased the adhesion of 51Cr-labeled human neutrophils or the human monocytic U937 cell line to human umbilical vein endothelial cells in a dose- and time-dependent manner. The stimulation of human umbilical vein endothelial cell adhesiveness occurred as early as 30 minutes and lasted over 48 hours. The increase of leukocyte adhesion to human umbilical vein endothelial cells by NPY was not inhibited by protein synthesis inhibitor cycloheximide, nor was it associated with expression of intercellular adhesion molecule-1 on human umbilical vein endothelial cells; in contrast, strong expression of intercellular adhesion molecule-1 was induced by tumor necrosis factor α and lipopolysaccharide endotoxin. These data suggest that neuron-derived factors such as NPY may serve as modulators of not only the neuromuscular unit but also the interaction of endothelial cells with leukocytes. In this capacity, the sympathetic nervous system might play an important role in the regulation of proaggregatory and hemostatic activity of microvessels. (Circulation Research 1991;68:314–318)

The sympathetic nervous system has been shown to innervate immune organs such as the spleen, lymph nodes, bone marrow, and thymus. The function of the sympathetic nervous system in these organs was related to both vascular cells (microcirculatory regulation) and parenchymal cells (e.g., B cells, T cells, and macrophages). Although the sympathetic nervous system has also been implicated in stress-related modulation of immune and inflammatory states, the neurotransmitters associated with those functions are still obscure. The presence of adrenoreceptors on inflammatory cells such as macrophages and cytotoxic T lymphocytes supports a role for norepinephrine and epinephrine in modulation of cell-mediated immune and inflammatory reactions. However, no clear evidence has been raised so far to suggest a physiological role of sympathetic nerve-derived amines in inflammation.

Sympathetic nerves possess, along with their primary neurotransmitter, norepinephrine, a 36–amino acid peptide termed neuropeptide Y (tyrosine) or NPY. NPY was identified in perivascular noradrenergic nerve endings of peripheral organs and is believed to be colocated and released from the same large norepinephrine vesicles in some nerve endings. The modulatory function of NPY in relation to the vascular system and primarily to adrenergic neurotransmission and α-adrenergic receptor function has also been suggested. Furthermore, NPY may modulate inflammatory cell function, since it releases histamine from mast cells.

Since the premonitory step of any inflammatory event involves adhesion of leukocytes to the endothelium, we hypothesized that neuron-derived factor(s) such as NPY might modulate leukocyte adhesion to endothelium. This hypothesis has been examined in an in vitro system of adherence of human neutrophils (polymorphonuclear leukocytes [PMNs]) or U937, a human monocytic cell line, to human umbilical vein endothelial cells (HUVECs).

Materials and Methods

Materials

HUVEC and U937 cells were purchased from American Type Culture Collection, Rockville, Md. Fetal bovine serum (FBS) was obtained from HyClone Laboratories, Logan, Utah. Endothelial cell
growth factor was purchased from Boehringer Mannheim Corp., Indianapolis. Heparin was obtained from Elkins-Sinn Inc., Cherry Hill, N.J. F12 media, Dulbecco's modified Eagle's medium (DMEM), and gentamycin were from GIBCO Laboratories, Grand Island, N.Y. NPY was purchased from Sigma Chemical Co., St. Louis. Mouse monoclonal anti-intercellular adhesion molecule-1 (ICAM-1) antibody was from AMAC Inc., Westbrook, Me. Na$_2^{35}$CrO$_4$ ($^{35}$Cr) and $^{125}$I-labeled rabbit anti-mouse antibody (immunoglobulin G fraction) were obtained from Du Pont NEN Research Products, Wilmington, Del. Lipopolysaccharide (LPS) from Salmonella typhosa 0901 was purchased from Difco Laboratories, Detroit. Human recombinant tumor necrosis factor α (TNF$_a$; specific activity=2×10$^7$ units/mg, LPS content=0.04 ng/mg) was kindly supplied by the Departments of Molecular Genetics and Cell Biology, SmithKline Beecham Pharmaceuticals, King of Prussia, Pa. All other agents were purchased from common commercial sources.

**HUVEC Culture**

HUVECs (passage 14, population doublings=31) were grown in T-150 flasks containing 50 ml F12 media supplemented with 20% FBS, 10 ng/ml endothelial cell growth factor, 20,000 units/ml heparin, and 50 mg/ml gentamycin (F12 complete) at 37°C under constant humidity (93%) and 5% CO$_2$-95% air. The cells were fed with F12 complete on the third day and passed every 6 or 7 days. Cells between population doublings 33–40 were used for experiments. The cells were harvested by brief trypsinization and suspended in F12 media containing 20% FBS. The cells were then seeded (1×10$^4$ cells/well) into 24-well culture plates (Nunc, Inc., Naperville, Ill.) and incubated until confluence.

**Preparation of Chromium-Labeled PMNs and U937 Cells**

Human PMNs were purified from freshly drawn heparinized blood by sequential Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataquay, N.J.) gradient separation, dextran sedimentation, and hypotonic lyses of erythrocytes. Isolated PMNs were more than 95% pure as assessed by Wright's stained cytocentrifuge preparations and more than 99% viable as assessed by trypan blue exclusion. U937 cells were grown in a spinner flask (Bellco Biotechnology, Vineland, N.J.) containing DMEM–20% FBS. The U937 cells were subcultured every 3 or 4 days and maintained in cell densities between 0.5×10$^5$ and 1.0×10$^6$ cells/ml.

PMNs or U937 cells were incubated in 1–2 ml F12–20% FBS with 300 μCi $^{51}$Cr for 90 minutes. Excess chromium was removed by three cycles of centrifugation and washing with 50 ml Dulbecco's phosphate-buffered saline solution. Cells were resuspended in F12–20% FBS and used within 20 minutes.

**Adhesion Assay**

HUVEC monolayers were washed once with 1 ml F12 medium and preincubated with NPY or TNF$_a$ in F12–20% FBS for the indicated time. After the removal of incubation medium, the monolayer was washed once, and fresh medium was added before addition of radiolabeled U937 cells or PMNs (0.5×10$^5$ to 1.0×10$^6$ cells/well).

After a 30-minute incubation at 37°C, nonadherent leukocytes were aspirated, and monolayers were washed three times with F12 media. The amount of leukocytes that adhered to HUVEC monolayers was determined by rapid filtration through GF/B glass fiber filters (Whatman Inc., Clifton, N.J.) under reduced pressure using a cell harvester (model M-24S, Brandel, Gaithersburg, Md.). The filters were washed 5 or 6 times with 2 ml each of cold Dulbecco's phosphate-buffered saline and counted for radioactivity (gamma counter model 5500, Beckman Instruments, Inc., Fullerton, Calif.).

**Determination of ICAM-1 Expression**

HUVEC monolayers were exposed to NPY, LPS, or TNF$_a$ for 48 hours. Media were removed, and the monolayers were washed three times with F12 media before treating with 5% normal rabbit serum in F12 media for 20 minutes. The monolayer was washed and incubated with mouse monoclonal anti–ICAM-1 antibody (2 μg/well in 0.3 ml) for 1 hour, followed by three washings with F12 media and one washing with 0.1% normal rabbit serum. $^{125}$I-labeled rabbit anti-mouse antibody was then added. After a 1-hour incubation, media were removed, and cells were washed three times with F12 media. The cells were solubilized with NaOH (1N) overnight, and radioactivity was measured.

**Statistical Analysis of Data**

Results are expressed as mean±SEM for the indicated number of experiments done in triplicate. Significance ($p<0.05$) was determined using analysis of variance or the Kruskal-Wallis test (when appropriate), followed by the Mann-Whitney U test.

**Results**

**Effect of NPY and TNF$_a$ on U937 Cells and PMN Adherence to HUVECs**

TNF$_a$ produced a dose-dependent (Figure 1A) and time-dependent (Figure 1B) increase in U937 cell adherence to HUVECs, confirming previous studies and indicating the responsiveness of our adhesion system. NPY produced a dose-dependent (Figure 1A) and time-dependent (Figure 1B) increase in PMN or U937 cell adherence to HUVECs. However, the kinetics of stimulation of HUVEC adhesiveness by NPY was remarkably different from that elicited by TNF$_a$. One hour of NPY incubation with HUVECs resulted in 80% of maximal activity, whereas a 1-hour incubation with TNF$_a$ did not significantly stimulate the adhesiveness of HUVECs...
for U937 cells (Figure 1B, insert). Additionally, maximal U937 cell adherence to HUVECs exposed to TNF, was higher and was sustained over 48 hours, whereas maximal adherence produced by NPY was somewhat lower and tended to decline after 24 hours. Adherence of PMNs to HUVECs was less pronounced than the adherence of U937 cells at lower (0.03–1.0 μM) concentrations of NPY.

ICAM-1 Expression by TNF, and NPY

Radioimmunoassay of the ICAM-1 molecules in TNF, or NPY-treated cells revealed strong expression of ICAM-1 molecules on TNF, and LPS-treated HUVECs (Figure 2A). This was dose dependent (see Figure 2B, insert, for TNF, used as example) and is in accord with previous reports.15,16 However, no expression of ICAM-1 molecules was found in HUVECs treated with NPY concentrations, which caused adhesion of U937 cells to HUVECs.

Discussion

This study clearly demonstrates that NPY alters the adherence properties of HUVECs for both human PMNs and U937 cells. The activation of adhesion by NYP was mediated through HUVECs since NPY was removed (by washing) before the addition of PMNs or U937 cells. In addition, NPY-pretreated U937 (followed by the removal of NPY by washing) did not increase the adhesion to HUVECs compared with nontreated U937 cells (data not shown). Significant induction of HUVEC adhesiveness occurred as early as 30 minutes of HUVEC incubation with NPY and persisted for over 48 hours in continuous presence of NPY. The kinetics of the NPY effect on HUVEC adherence thus precedes the adherence produced by TNF, yet is lesser in magnitude and also tends to revert to baseline earlier. This latter phenomenon might represent degradation of NPY by proteases in the medium or by HUVEC-associated proteases, but we have no data so far to support this possibility.

The early onset of NPY-induced adhesion suggests that ICAM-1 might not be the primary molecule associated with this event since expression of ICAM-1 by LPS or monokines such as TNF, is dependent on de novo protein synthesis and needs...
more than 2 hours to show any significant degree of expression.\textsuperscript{17} In contrast, NPY-stimulated adhesion was not inhibited by the protein synthesis inhibitor cycloheximide at concentrations (5–10 \textmu M) that

suppressed TNF\textsubscript{\textalpha }-stimulated adhesion (data not shown). We further confirmed this assumption by direct assay of ICAM-1 cell surface protein using a highly specific radioimmunoassay; indeed, unlike the strong expression of ICAM-1 elicited by exposure of HUVECs to TNF\textsubscript{\textalpha } or LPS, NPY-treated HUVECs failed to show evidence for the presence of ICAM-1 beyond the background level, at the time of enhanced leukocyte adhesion. The lack of NPY effect on ICAM-1 expression in HUVECs after 48 hours of NPY treatment indicates that NPY is not acting through the induction of endothelial cytokines (e.g., TNF\textsubscript{\textalpha }, interleukin-1\textsubscript{\textalpha }, interleukin-1\textbeta ) or through LPS. Furthermore, polymyxin B (20 \textmu g/ml) did not suppress the NPY effect (data not shown), suggesting that a possible “priming” effect by minute amounts of LPS (as a contaminant) could also be excluded. In studying the involvement of arachidonate metabolite(s) in the NPY upregulation of adhesion, we found that nontoxic concentrations of indomethacin (10 \textmu M) and nordihydroguaiaretic acid (50 \textmu M) did not affect the NPY stimulation of HUVEC adhesiveness (data not shown).

Since NPY-induced HUVEC adhesiveness did not involve de novo protein synthesis, ELAM-1 or VCAM-1 involvement in NPY stimulation of adhesion is also unlikely. Although the precise nature of the adhesion molecule associated with NPY-induced adhesion is still obscure, it is possible that GMP-140\textsuperscript{18,19} mediated the NPY-induced leukocyte adhesion to the endothelium. This possibility is supported by demonstrations that GMP-140 expressions are earlier than those of ICAM-1.\textsuperscript{16,19} However, GMP-140 expression (by thrombin or histamine) is transient, reaching a peak at 3 minutes and declining to basal levels by 20 minutes.\textsuperscript{19} In contrast, NPY-induced adhesion is significantly longer (>48 hours), suggesting that NPY may involve another (novel?) adhesion molecule other than GMP-140. Since we were unable to obtain the specific neutralizing antibodies to different adhesion molecules, whether the NPY-induced adhesion of leukocytes to the endothelium is mediated by a yet unknown intercellular adhesion molecule(s) remains to be elucidated.

Alternatively, rapid (<30 minutes) induction of adhesion of leukocytes to HUVECs by agonists such as leukotriene C\textsubscript{4}, histamine, or thrombin was shown to involve, in part, the production of platelet-activating factor, the potent phospholipid autacoid.\textsuperscript{20} However, the possibility that platelet-activating factor mediated, in part, the NPY-induced adherence awaits further investigation.

In summary, this study provides for the first time evidence in support of a role for neuron-derived factor(s) such as NPY in modulating endothelium-leukocyte interaction, an event fundamental to bacterial, viral, and cell migration across microvessels. Such data may facilitate our understanding of the relation between stress situations via activation of the autonomic nervous system, which might affect host resistance to immune and inflammatory stimuli.
References


Key Words • neuropeptide • neuroimmunology • leukocyte adhesion • inflammation
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