Neuropeptide Y
A Novel Angiogenic Factor From the Sympathetic Nerves and Endothelium

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Abstract—Sympathetic nerves have long been suspected of trophic activity, but the nature of their angiogenic factor has not been determined. Neuropeptide Y (NPY), a sympathetic cotransmitter, is the most abundant peptide in the heart and the brain. It is released during nerve activation and ischemia and causes vasoconstriction and smooth muscle cell proliferation. Here we report the first evidence that NPY is angiogenic. At low physiological concentrations, in vitro, it promotes vessel sprouting and adhesion, migration, proliferation, and capillary tube formation by human endothelial cells. In vivo, in a murine angiogenic assay, NPY is angiogenic and is as potent as a basic fibroblast growth factor. The NPY action is specific and is mediated by Y1 and Y2 receptors. The expression of both receptors is upregulated during cell growth; however, Y2 appears to be the main NPY angiogenic receptor. Its upregulation parallels the NPY-induced capillary tube formation on reconstituted basement membrane (Matrigel); the Y2 agonist mimics the tube-forming activity of NPY, whereas the Y2 antagonist blocks it. Endothelium contains not only NPY receptors but also peptide itself, its mRNA, and the “NPY-converting enzyme” dipeptidyl peptidase IV (both protein and mRNA), which terminates the Y1 activity of NPY and cleaves the Tyr1-Pro2 from NPY to form an angiogenic Y2 agonist, NPY3-36. Endothelium is thus not only the site of action of NPY but also the origin of the autocrine NPY system, which, together with the sympathetic nerves, may be important in angiogenesis during tissue development and repair. (Circ Res. 1998;83:187-195.)

Key Words: angiogenesis ■ chemotaxis ■ dipeptidyl peptidase IV ■ NPY receptor ■ endothelial cell

Angiogenesis, the process of new vessel formation or neovascularization, has aroused increasing interest over the last 25 years.1-5 Normal angiogenic activity is low in the adult organism but increases during injury and in diseases such as cancer, retinopathies, or arthritis, where it contributes to pathological changes. Conversely, in states of inadequate tissue perfusion such as myocardial or limb ischemia, enhanced angiogenesis is essential and beneficial. Inhibition or enhancement of angiogenesis may thus prove an attractive strategy for the treatment of several disorders. Although numerous growth factors stimulating vessel development are known (eg, bFGF or VEGF), the exact mechanisms controlling angiogenesis are poorly understood.5 The sympathetic nerves have long been known to have in vivo trophic effects on blood vessel growth. This process was believed to be mediated by catecholamines; at physiological plasma concentrations, however, catecholamines have weak growth-promoting effects on vascular cells.5-9 Moreover, the sympathetic nerves release not only norepinephrine but also other nonadrenergic neurotransmitters, such as NPY10 and purines.11 NPY is present in all sympathetic nerves innervating the cardiovascular system and is the most abundant peptide in the brain and the heart.10 Additionally, in rats but not in humans, NPY is also found extraneuronally in platelets10,12 and endothelium.13 Originally, NPY was known as a potent vasoconstrictor and a neuromodulator. Released by stress,10,14 exercise,15 and myocardial ischemia,16 NPY has been implicated in coronary heart disease, congestive heart failure, and hypertension.10 More recently, because of the potent ability of NPY to stimulate food intake, it is suspected to play a role in obesity and diabetes.17 Our latest findings indicate that NPY is also a mitogen for rat aortic vascular smooth muscle cells.7,18 These diverse functions, together with its highly evolutionarily preserved peptide structure and its high degree of identity among mammalian species, suggest an important physiological role for NPY. The hypothesis tested here is that NPY functions as a trophic/angiogenic factor.
Neuropeptide Y and Angiogenesis

The NPY system encompasses several NPY-related peptides, receptors, and processing enzymes.19 NPY activates six receptors, designated as Y1 to Y6, of which Y120 and, more recently, Y2,21 Y4,22 and Y517,23 have been cloned. NPY-induced vasoconstriction is mediated primarily by Y1 (the predominant vascular receptor), with little or no contribution of the Y2 receptor, the functions of which in blood vessels are less clear.19 In addition to NPY, PYY, a gastrointestinal peptide with 75% homology to NPY, activates all NPY-Y receptors except Y3.19 The endogenous products of NPY and PYY metabolism, such as NPY/PYY3-36, are selective Y2 agonists;19,24 Interestingly, NPY3-36 is produced from NPY by an endothelial serine protease, DPPIV,24 which itself has been implicated in endothelial-matrix interactions in cancer.25

In the present study we demonstrate for the first time that NPY is an angiogenic factor. We investigated the effect of NPY and related compounds in vitro, on capillary tube formation by HUVECs and on rat aortic ring capillary sprouting, as well as in vivo using the murine reconstituted basement membrane (Matrigel) assay. Additionally, we examined the receptor specificity of the angiogenic activity and the endothelial expression of NPY, its receptors, and the NPY-processing enzyme DPPIV.

Materials and Methods

Cell Culture

HUVECs were isolated from freshly delivered umbilical cords after incubation at 37°C for 20 minutes with collagenase type I enzyme solution and plated on gelatin-coated T75 flasks. After the first passage, cells were grown on noncoated Nunc flasks. The HUVEC media consisted of medium 199 (GIBCO-BRL) supplemented with 20% FCS (HyClone), 1000 U/dL penicillin/streptomycin, 5 mg/dL gentamicin, 2 mmol/L glutamine, 500 U/dL sodium heparin, 2.5 mg/dL amphotericin B (Biofluids), and 2 mg/dL ECGS (Collaborative Research Inc). Aliquots of cells were preserved frozen between passages 2 and 4. Biological experiments were performed with cells between passages 3 and 7.

Adhesion Assay

Subconfluent HUVECs preincubated with or without NPY (18 hours) were resuspended in the serum-free medium and plated (2×104 cells per well) on 96-well laminin-coated plates (5 mg per well; experiments were performed on two different cultures in triplicates). After 0 to 40 minutes, the adherent cells were fixed, stained (0.2% crystal violet/80% methanol), and quantified spectrophotometrically (A=560 nm). The method has been validated and extensively used in the laboratory of Dr H. Kleinman, as described in Reference 26.

Migration/Chemotraction

HUVECs in medium 199 were added to the upper wells (48-multwell chemotaxis Boyden microchamber) at 3 to 4×104 cells per well; the lower wells contained NPY or analogues diluted in medium 199 (n=4 in triplicates). After 2 hours at 37°C in 5% CO2, the membranes were fixed and stained, and the number of cells that migrated through to the lower surface of each membrane was counted. A negative control consisted of medium 199, and a positive control contained medium 199 supplemented with 20% FBS, ECGS, and heparin.27

DNA Synthesis

HUVECs plated onto 96-well dishes (104 cells per well) were growth-arrested in serum-free media supplemented with insulin, transferrin, and selenite for 24 hours and then treated for 24 hours with or without NPY or agonists in 10% FBS-DMEM (n=6); 0.5 µCi [3H]thymidine per well was added for the last 6 hours. Cells were harvested in a 96-well harvester (Tomtec) and counted in a Betaplate liquid scintillation counter (model 1205; Wallac Inc).

In Vitro Capillary Tube Formation on Matrigel

Cells were incubated (18 hours, 37°C) on Matrigel-coated 24-well plates26 at 4×104 cells per well in the 10% FBS-containing medium with NPY, its analogues, or the vehicle (n=4 in duplicates). Cells were fixed and stained (DiffQuick Fixative and Solution I, II) and the area of the tube network was quantified at X40 magnification with a Nikon microscope connected to an NIH image system.27

In Vivo Murine Model of Angiogenesis

Eight-week-old female C57BL mice were injected subcutaneously either with Matrigel alone or with Matrigel mixed with bFGF29 or NPY (n=4 in duplicates). After 14 days, Matrigel plugs were excised, fixed in 10% formaldehyde, and embedded in paraffin. Sections of the paraffin-embedded plugs were stained with Masson’s trichrome and photographed. Vessel ingrowth was quantified with the use of a Nikon microscope connected to an NIH image system.27 Results were expressed as mean area of tubes per square millimeter.

Rat Aortic Ring Assay

Rat aortic rings were prepared as previously described,31 with modifications. The thoracic and abdominal aorta was obtained from 100- to 150-g male Sprague-Dawley rats (Taconic, Germantown, NY). Excess perivascular tissue was removed, transverse sections (1 to 2 mm) were made, and the resulting aortic rings were then extensively washed in medium 199 (Mediatech Inc). The rings were then embedded in rat tail collagen 200 mg/dL, in Nunc eight-well chamber slides (Nalgene Nunc International) so that the lumen was parallel to the base of the slide. After the collagen I gelled (by adjustment of pH to neutral with NaOH), serum-free medium (endothelial basal medium supplemented with antibodies and e-aminocaproic acid, 30 mg/dL) was added to each well, and the rings were incubated at 37°C for 3 days. Once sprouts began to appear, NPY was added at concentrations of 0.002 to 2.2 nmol/L (n=6 per dose). VEGF (1 nmol/L) was used as a positive control. The rings were incubated for 3 days, photographed, and fixed and stained for image analysis and quantification with the use of an NIH image system. The ring assay was repeated three times.

Northern Blot Analysis of Y1, Y2, and NPY mRNA Expression in HUVECs

Subconfluent HUVECs were gently trypsinized (2 to 3 minutes of incubation with 0.025% versene-trypsin applied onto the cells and immediately removed), plated in their full growth medium on plastic- or Matrigel-coated T75 flasks, and allowed to adhere for 1 hour, at which point they were harvested for RNA (time zero); cells grown on plastic were used as a control for cells grown on Matrigel. Other cells were allowed to grow on plastic and on Matrigel for additional 1, 6, and 20 hours before being harvested for RNA for...
Northern blot analysis (n=3 to 4 per time point). Total RNA was purified from cells with the use of standard guanidinium-isothiocyanate and cesium chloride centrifugation. Ten micromegrams of total RNA was electrophoresed through a 1% agarose denaturing gel and transferred to Nytran membranes. The blots were UV-cross-linked (Stratalinker, Stratagene) and hybridized with 32P-labeled cDNA probes for human Y1 (provided by Dr D. Larhammar), Y2 (from Dr P. Rose), and NPY (from Dr S. Sabol) at 42°C overnight. After hybridization, the blots were washed in 2× SSC plus 0.1% SDS for 10 minutes and in 1× SSC plus 0.1% SDS for 10 minutes and then exposed to autoradiography film with the use of a Kodak intensifying screen. After hybridization with one of the receptor probes, the blots were stripped and rehybridized with another one, in random order. Results were expressed as relative densities of specific mRNAs normalized to the density of 28S rRNA. Changes in the expression of the Y1 and Y2 mRNAs were calculated as changes in density relative to the specific mRNA in control cells on plastic at zero time, with all the samples run on the same Northern blot.

RT-PCR of Human NPY, Y1, Y2, and DPPIV mRNAs in HUVECs

In separate experiments, 90% confluent cells were harvested for RNA to determine NPY, DPPIV, and NPY receptor expression by RT-PCR. First-strand cDNAs were synthesized from 1 to 2 μL total RNA by reverse transcriptase (Stratagene) with the use of receptor-specific oligonucleotide primers (Y1: forward, CTC TTG CTT ATG GRG ATG TGA; reverse, CTG GAA GTA TTT GTT CCT CAG GAA YCC A; Y2: forward, CCT ACT GCT CCA TCA TCT TGC; reverse, GTA GTC CCT GCT GTT CAT CCA GCC; DPPIV: forward, GTC TCT GAG GAC AAT CTC AAG CCG GAC AA; reverse, 5′-3′: TCT CAT TTC CCA TCA TCA CAT (G)). The cDNAs were amplified by PCR for 35 cycles (denaturing at 94°C for 1 minute, annealing at 50°C for 1 minute, and extension at 72°C for 1 minute and 30 seconds) with Taq DNA polymerase (Promega). The PCR internal control, 18S rRNA (488 bp from PCR), was coamplified with each cDNA with 18S rRNA primers for a total of 20 cycles (same as above; Ambion, Inc). PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining. The expected sizes of the PCR products are 850, 685, and 210 bp for Y1, Y2, DPPIV, and NPY, respectively.

Immunocytochemistry of DPPIV and NPY in HUVECs

HUVECs, grown at 104 cells per milliliter on coverslips, were fixed with 3% paraformaldehyde in PBS, permeabilized (0.5% Triton X-100 in PBS for 10 minutes), immunolabeled with primary antibodies—either a rabbit antiserum (gift from Dr Michalkiewicz, West Virginia University, Morgantown) displaying 100% cross-reactivity with NPY and 60% with NPY1,3,6 or a rat monoclonal antibody E26 against DPPIV (gift from Dr W.-T. Chen), both at 0.1 mg/dL—and stained with a fluorescein-conjugated secondary antibody. The specificity of the NPY immunostaining was demonstrated by absence of staining on slides in which (1) primary antiserum was competed out with the peptide by incubating the rabbit antiserum at 4°C for 24 hours with synthetic 10 nM NPY. Slides were evaluated with a Zeiss Photomicroscope III equipped with epifluorescence.

NPY Measurement in Cells and Media by ELISA

NPY immunoreactivity in media and cell extracts was measured by a sensitive direct sandwich ELISA with the use of monoclonal antibodies directed against the N-terminus and C-terminal amide of NPY; these antibodies were developed by Dr E. Grouzmann. Cells were harvested into 50 mmol/L Tris buffer containing 50 mmol/L EDTA and 0.08% Tween 20 and sonicated. After centrifugation, the supernatants (100 μL per well) were applied directly onto the ELISA plate. This ELISA detects 100% of the native NPY1–36 and 80% of NPY1–36. The level of detection of the assay is 0.25 pmol/L. The interassay and intra-assay coefficients of variation were 10% and 5%, respectively.

Statistical Analysis of Data

Data were analyzed by one-way ANOVA for repeated measures followed by a post hoc Dunnett’s t test when appropriate. Data are presented as mean±SEM for the indicated number of n and considered significant at P<0.05.

Drugs

Human NPY, NPY1–36, and Leu1-Pro7NPY were purchased from Peninsula Laboratories. BBP3226 and BBP3435AC were gifts from Dr Karl Thomae GmbH, Biberach, Germany, and T6-(NPY1–36), was a gift from Dr E. Grouzmann. Recombinant bFGF and VEGF were purchased from R and D Systems Inc.
Results

Effects of NPY on Attachment, Migration, and Proliferation In Vitro

HUVECs incubated with 10 nmol/L NPY attached at a greater (50% to 70%) rate to laminin than did vehicle-treated cells (P<0.05, n=2 in triplicates) (Figure 1A). NPY also increased the migration of HUVECs up to 4-fold compared with the control cells treated with medium 199 alone (ANOVA: df=11, F=3.1615, P=0.0072) (Figure 1B). The chemotactic effect of NPY was biphasic (Figure 1B), and the first peak of activity occurred at concentrations of 0.1 to 10 pmol/L (P<0.05, n=4 in triplicates), then declined at 1 nmol/L (no significant change from basal) and increased again at 100 nmol/L (P<0.01, n=4 in triplicates). In view of the fact that pathophysiological NPY concentrations range between 1 pmol/L and 10 nmol/L in various species,10 concentrations of NPY <0.01 pmol/L and >1 μmol/L were not tested. In stimulating chemotaxis, NPY was equipotent to bFGF (P<0.05 compared with basal) and as efficacious as the full HUVEC media (P<0.01) (Figure 1B). This bimodal effect of NPY was reproducible and occurred in each of the four primary HUVEC cultures; however, the concentrations at which NPY exerted its maximal and minimal effects varied, resulting in increased variability but not elimination of the bimodality.

In quiescent HUVECs, the addition of NPY to the 10% FBS (half of the regular serum concentration) dose-dependently (0.1 to 10 nmol/L) increased [3H]thymidine uptake up to 350% over that induced by the control media (P<0.05, n=6) (Figure 1C). The Y1 and Y2 receptor agonists, Leu31-Pro34NPY and NPY3-36, also exerted proliferative effects (P<0.05), but they tended to be less effective than NPY itself (Figure 1C).

Capillary Tube Formation In Vitro: The Role of Y1 and Y2 Receptors

Beginning with subnanomolar concentrations, NPY evoked a dose-dependent increase in the formation of capillary-like tubes by HUVECs on Matrigel (Figure 2A through 2C). The peak activity, a 150% increase over the effect of Matrigel alone, occurred at 1 nmol/L (Figure 2B and 2C), and activity declined to 50% at 100 nmol/L (not shown). The NPY effect was nearly twice that of the 20% FBS (P<0.05, n=4 in duplicates) (Figure 2C) and was greater than that evoked by the 4-fold increase in concentration of the ECGS in the half-full medium (Figure 3). NPY3-36 was fully active at
concentrations similar to those of NPY, while Leu31Pro34NPY was less efficacious. There was no additive effect of NPY3–36 and Leu31Pro34NPY applied together (Figure 2C).

BIBP3226 \([(R)-N_2-(diphenacyl)-N_\{(4\text{-}hydroxyphenyl)methyl\}-D-arginineamide]\), a nonpeptide antagonist selective for Y1 receptors, given at 100 nmol/L was ineffective in blocking the action of NPY (n=3 in duplicates) (Figure 3). However, BIBP3226 applied alone in media containing 10% FBS, as well as its enantiomer BIBP3435AC (100 nmol/L), which is inactive at Y1 receptors, displayed intrinsic stimulatory effects on tube formation, similar to that of NPY alone (Figure 3).

In contrast to the Y1 receptor blocker, 1 μmol/L T4 (NPY33–36), a peptidergic Y2-selective antagonist, had no intrinsic activity and eliminated the stimulatory effects on tube formation of both NPY3–36, the Y2 agonist, and NPY (P<0.05 compared with the effects of NPY and NPY3–36, respectively; n=3 in duplicates) (Figure 3). The effect of NPY on tube formation was also blocked by NPY-specific antiserum but not by normal rabbit serum (Figure 3).

Expression of Y1 and Y2 Receptor mRNA in HUVECs

Subconfluent HUVECs expressed low levels of Y1 and Y2 receptor mRNA by Northern blot analysis (Figure 4) and by RT-PCR (Figure 5). Matrigel upregulated both Y1 (P<0.05, n=3 to 4) (Figure 4A) and Y2 (P<0.05) (Figure 4B) mRNA compared with the receptor mRNA expression in cells attached to plastic at zero time. During cell growth on plastic, the expression of the Y1 mRNA increased significantly at 1 hour (P<0.05) (Figure 4A) and rose continuously until 20 hours after the cells had attached to plastic (Figure 4). The Y2 receptor mRNA expression, in contrast, did not change until 20 hours after cell attachment to plastic (P<0.05, n=4) (Figure 4). In HUVECs plated on Matrigel, both receptors were similarly upregulated within 1 to 6 hours after cell attachment.
attachment, but while the Y2 mRNA expression remained elevated for up to 20 hours, the Y1 mRNA decreased to basal levels in that time (Figure 4).

**In Vivo and Ex Vivo Angiogenic Activities of NPY**

In the in vivo mouse Matrigel model, 14 days after subcutaneous implantation of Matrigel mixed with compounds, the plugs containing either 30 ng/mL bFGF (2 mmol/L, positive control) (Figure 2F) or 0.1 to 1000 ng/mL NPY (0.022 to 220 mmol/L) (Figure 2G and 2H) appeared vascularized on macroscopic examination. Microscopically, they showed marked infiltration by cells forming capillaries compared with the control plugs containing vehicle (Figure 2E). The threshold effect of NPY was at 0.1 ng/mL (0.022 nmol/L) (Figure 2D), with maximal activity at 100 ng/mL (22 nmol/L) (Figure 2D and 2H). The maximal effect of NPY was a 10-fold increase in the area of cells infiltrating the Matrigel plugs (at 22 nmol/L NPY) compared with Matrigel alone \( (P < 0.01, n=4 \text{ in duplicates}) \) (Figure 2D). The effect of 2.2 nmol/L NPY was similar to that of equimolar amounts of bFGF (Figure 2D, 2F, and 2G).

The effect of NPY was also tested in another angiogenesis assay with the use of the ex vivo rat aortic ring capillary sprouting system. Transverse sections of rat aorta embedded in collagen and stimulated with NPY or VEGF were examined. NPY was able to induce sprouting from the rings at concentrations as low as 2 pmol/L (although the increase in sprouting did not reach statistical significance). At 0.022 nmol/L and 2.2 nmol/L (Figure 6), NPY stimulated numerous and very long capillary sprouts, 60% greater than control \( (P < 0.05, n=6) \) (Figure 6, middle right panel). This stimulation in angiogenesis was slightly reduced at 2.2 nmol/L (Figure 6, bottom left panel). The capillary sprouts induced by 0.22 nmol/L NPY were not significantly different from the sprouting observed with a half-maximal dose (1 nmol/L) of VEGF (Figure 6G and 6H). Thus, the data indicate that NPY can induce angiogenic sprouting at sub-nanomolar concentrations in the rat aortic ring.

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Figure 6. Rat aortic ring assay. Rat aortic ring capillary sprouting in response to 0, 0.002, 0.022, 0.22, and 2.2 nmol/L NPY and 1 nmol/L VEGF. Capillary sprouting occurred from the cut edge of the ring (left side of each panel). Quantification of mean sprout area per field, using NIH image morphometric analysis graph, showed that NPY (0.22 nmol/L) stimulated significant sprouting comparable to but somewhat lower than (but not statistically different than) that stimulated by VEGF (1 nmol/L). *\( P < 0.05 \) compared with control by one-way ANOVA followed by Dunnett’s t test; \( n = 3 \) to 6 per treatment. Note that NPY stimulated formation of particularly long sprouts.

Figure 7. Distribution of DPPIV (A) and NPY (B and C) in HUVECs. The specificity of the NPY immunostaining was ensured by the absence of staining on slides where primary antisera was replaced with normal rabbit serum, and a competition of antibody against NPY with peptide by incubating the rabbit antisera at 4°C for 24 hours with synthetic 10 μmol/L NPY (shown in panel C).
Colocalization of NPY and DPPIV in the Endothelium

HUVECs in culture showed intense immunostaining with an anti-DPPIV antibody localized to cell surfaces and intracellular membranes (Figure 7A). A similar distribution of immunostaining was found with NPY-specific antisera (Figure 7B). The specificity of the NPY immunostaining was ensured by the absence of staining on slides where primary antisera was replaced with normal rabbit serum and a competition of the anti-NPY serum by synthetic 10 μmol/L NPY during an incubation at 4°C for 24 hours (Figure 7C). In addition, measurements of NPY content in HUVEC extracts by ELISA revealed an abundance of NPY (24.8 ± 2.1 femtomoles/10⁶ cells).

In addition to possessing the NPY and DPPIV immunoreactivity, HUVECs grown on plastic expressed low levels of NPY mRNA and DPPIV mRNA by RT-PCR (Figure 4). Although expression of DPPIV mRNA was present in all cells tested, the expression of NPY mRNA was variable and present only in some cultures.

Discussion

NPY as an Angiogenic Factor

In the present study we have shown for the first time that NPY is angiogenic. In vitro NPY stimulated attachment, migration, DNA synthesis, and the formation of capillary tubes on Matrigel by human endothelial cells. The activity of NPY was specific, since it was blocked by an NPY antiserum in the capillary tube-forming assay. The potency of the peptide varied with different assays and individual primary HUVEC cultures. In the migration and aortic sprouting assays, NPY activity began with subpicomolar concentrations, thus being approximately equipotent to bFGF. The efficacy of NPY was nearly twice that of the 20% FBS and greater than that of the 4-fold increase of the ECGS in chemotaxis and capillary tube formation assays, and its efficacy was similar to that of an ED₃₀ dose of VEGF in the aortic sprouting assay.

NPY-Induced Chemotaxis in HUVECs

The chemotactic activity of NPY showed a bimodal pattern in all HUVEC cultures tested. The first significant increase in NPY-induced chemotaxis occurred at subpicomolar concentrations, then it returned to baseline at 0.1 nmol/L and increased again at 100 nmol/L. However, the maximum and minimum of the NPY dose-response curve differed by up to one order of magnitude between cultures. While not common, such bimodality of action has been reported with other systems, e.g., stimulation of protein kinase C activity by vasoactive intestinal peptide. The broad spectrum of the chemotactic activity of NPY resembles that of chemokines, which are known to stimulate migration over a wide range of concentrations. A bell-shaped chemotactic activity of NPY and PYY in murine peritoneal macrophages has also been observed by others over a more limited concentration range. In addition, bimodality was observed by us in the NPY-induced proliferation of vascular smooth muscle cells. The mechanisms of the bimodal effects of NPY in chemotaxis are not fully understood but may result from the involvement of multiple receptors. Preliminary data appear to indicate that the first peak is mediated by activation of either Y1 or Y2 receptors, which become desensitized at 0.1 to 1 nmol/L NPY, and the second peak by a Y5-like receptor activated by higher peptide concentrations.

The first peak of the chemotactic activity of NPY may be of importance for endothelial function and repair in basal conditions and in tissues that are not sympathetically innervated and have low NPY content (e.g., aorta), whereas the second peak may be relevant to high NPY states (e.g., in fetuses or pheochromocytoma) or tissues with high peptide content (e.g., brain or heart) (see below).

NPY-Induced Sprouting in Rat Aorta: Relevance of NPY to In Vivo Angiogenesis

NPY was also very potent in an angiogenic assay in which rat aortic rings were stimulated to form sprouting capillaries. This assay provides all the steps of angiogenesis including endothelial cell invasion, migration, proliferation, differentiation, and new vessel formation. Here, the NPY effect peaked at 0.2 nmol/L. These levels correspond to physiological plasma NPY levels found in rats during stress or platelet aggregation. In humans, circulating plasma NPY levels are 10 to 20 times lower than in rats because of a lack of NPY in human platelets, but they increase up to 10 nmol/L in conditions with sympathetic hyperactivity such as stress or pheochromocytoma and more so in newborns than in adults. Systemic or regional NPY release is particularly elevated in states associated with tissue ischemia, e.g., myocardial ischemia and exercise in hypoxic conditions.

Since the same conditions are also associated with neovascularization, the potential of NPY to stimulate chemotaxis and vessel formation at these plasma levels suggests that the peptide may be one of the contributing angiogenic factors in these states.

Role of Y1 and Y2 Receptors in NPY-Induced Capillary Formation on Matrigel

To determine which of the two major vascular receptors, Y1 and Y2, plays a role in these activities, several agonists and antagonists were studied. Most of the effects of NPY on cell proliferation and capillary tube formation were almost fully mimicked by NPY₃₋₃₆, the Y2 receptor agonist, but only partially by Leu₃₋₆⁵NPY, the Y1 receptor agonist. Taken together with the ability of the Y2 receptor selective antagonist to block both the NPY- and NPY₃₋₃₆-induced responses, these data suggest the primary role of this receptor in angiogenic activities of NPY. We found the Y2 receptor to be constitutively expressed on HUVECs in culture and to be upregulated during cell growth, particularly on Matrigel. The time course of the upregulation of the Y2 mRNA (6 to 20 hours) corresponded with the time of formation of capillary tubes by HUVECs on Matrigel. A similar time course of NPY receptor induction or upregulation has been observed in growing vascular smooth muscle cells and in neurons during epileptic seizures.

The role of the Y1 receptor in angiogenesis could not be determined. Despite the ability of the Leu₃₋₆⁵NPY/Y1 agonist to stimulate DNA synthesis and capillary tube forma-
tion (but less than NPY), the blockade of the Y1 receptor with a specific nonpeptide antagonist, BIBP3226, failed to inhibit the action of NPY. This provided a sharp contrast to the effectiveness of BIBP3226 in blocking the Y1 receptor–mediated effects of endogenous and exogenous NPY on vasoconstriction. Present data, however, are not conclusive since BIBP3226, and to a lesser degree its enantiomer BIBP3435AC, which is inactive at Y1 receptors, both exhibited a stimulatory effect on tube formation by themselves when added to 10% FBS–containing medium. Further studies using Y1 receptor antagonists without intrinsic angiogenic activity would be required to elucidate the contribution of this receptor to NPY-mediated angiogenesis.

Despite the proangiogenic activity of the Y1 agonist, there was no additive effect but rather a decrease in activity when both NPY3–36 and LeuY1-Pro2NY were applied together. The mechanism of this inhibition is not known; this could be due to either simultaneous activation of Y1 and Y2 receptors resulting in heterologous desensitization of the Y2 receptor or competition for some common signaling molecule in the receptor pathways. We hypothesize that a sequential activation of receptors is required for the full angiogenic effect of NPY. The early events of vessel formation (adhesion and migration) may be dependent on both the Y1 and Y2 receptors, which are then markedly upregulated, while the final angiogenic effect may result from increased expression and activation of the Y2 receptors in the later phases, which coincide with proliferation and capillary tube formation. Sequential receptor activation may have the advantage of preventing desensitization and allowing full receptor activities. This hypothesis is indirectly supported by the presence in the endothelium of a protease, DPPIV, for which NPY1–36 is the best-known substrate.

**Endothelial NPY System: Expression and Colocalization of NPY and DPPIV**

DPPIV terminates the Y1 activity of NPY by cleaving Tyr1-Pro2 and converting it to a potent Y2 agonist, NPY3–36; this protease can thus be considered an NPY “converting enzyme.” Previously, the presence of DPPIV has been reported in HUVECs and NPY in rat endothelium. We have confirmed these findings and also determined that cultured human endothelial cells contain DPPIV mRNA as well as possess their own NPY mRNA and peptide. Although the expression of NPY mRNA was low and variable (not all HUVECs possessed it), the NPY content in cells was very high (~20 fmoles/10^6 cells), suggesting that endothelial NPY may also be derived from internalization of the peptide released from the outside sources, i.e., the sympathetic nerves. The neuronal origin of the NPY-like immunoreactivity found in the rat endothelium was suggested by the observation that the endothelial peptide content increased after chronic sympathetic stimulation of vessels. In the present study both DPPIV and NPY exhibited similar distribution on cell surfaces and intracellular membranes in the endothelium, further suggesting that the enzyme may be involved in NPY processing. Whether endothelial NPY represents an intact or a processed peptide (NPY3–36) cannot be determined since our antiserum cross-reacts with both forms.

In summary, our data indicate that NPY is angiogenic, with activity comparable to that of bFGF and VEGF, two established angiogenic factors. NPY stimulates multiple steps of angiogenesis by activating at least two receptors. The Y1 receptors stimulate proliferation of endothelial cells and, although to a lesser degree than the Y2 receptors, capillary formation. Although the major source of circulating NPY in vivo is the sympathetic nerves, the endothelium possesses its own NPY autocrine system, which includes receptors, a protease, DPPIV, and the peptide itself. The Y2 receptor, the increased expression of which lasts into the later phases of cell differentiation on Matrigel, appears to be the primary NPY angiogenic receptor. The DPPIV may play an important role in shifting the activity of NPY away from the Y1 receptor–mediated events toward the Y2 receptor–mediated capillary formation. Since NPY is angiogenic at concentrations below those required for vasoconstriction, it seems that the primary function of the peptide is regulation of endothelial function and angiogenesis, not of vascular tone. Angiogenic and chemotactic activities of NPY may be provided in vivo by the sympathetic nerves and/or endothelium, and both sources may play a role in angiogenesis during development, ischemic vascular diseases, and cancer.

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Neuropeptide Y: A Novel Angiogenic Factor From the Sympathetic Nerves and Endothelium
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