Neuropeptide Y enhances permeability across a rat aortic endothelial cell monolayer

Yong-Shan Nan,1 Guo-Gang Feng,2 Yoshihiro Hotta,2 Kimitoshi Nishiwaki, Yasuhiro Shimada,1 Atsuko Ishikawa,2 Nakako Kurimoto,3 Tatsuro Shigei,2 and Naohisa Ishikawa2

1Department of Anesthesiology, Nagoya University School of Medicine, Nagoya 480-1195; and Departments of Pharmacology and Anesthesiology, Aichi Medical University School of Medicine, Aichi 480-1195, Japan

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Nan, Yong-Shan, Guo-Gang Feng, Yoshihiro Hotta, Kimitoshi Nishiwaki, Yasuhiro Shimada, Atsuko Ishikawa, Nakako Kurimoto, Tatsuro Shigei, and Naohisa Ishikawa. Neuropeptide Y enhances permeability across a rat aortic endothelial cell monolayer. Am J Physiol Heart Circ Physiol 286: H1027–H1033, 2004. First published October 23, 2003; 10.1152/ajpheart.00630.2003.—Previously, in vivo studies showed that neuropeptide Y (NPY) elevates vascular permeability in isolated lung perfusion preparations, possibly through binding to the NPY Y3 receptor. The present study used monolayers in a double-chamber culture method under conditions of normoxia (5% CO2-20% O2-75% N2) or hypoxia (5% CO2-5% O2-90% N2) to test the hypothesis that NPY directly affects rat aortic endothelial cells (RAECs). RAECs were cultured on the base of the upper chamber, into which FITC-labeled albumin was introduced, and permeation into the lower chamber was measured. The RAEC monolayer was treated with 10−8–3 × 10−7 M NPY for 2 h in normoxia or hypoxia. In hypoxia, NPY concentration dependently increased the permeability of the RAEC monolayer, whereas in normoxia no significant change was observed. Peptide YY, NPY Y1, and NPY Y2 receptor agonists and NPY receptor antagonist exerted no significant effects under hypoxic conditions. NPY-(18–36), an NPY Y3 receptor antagonist, elicited an inhibitory action on the NPY-induced increase in monolayer permeability. Furthermore, neither N-monomethyl-L-arginine, a nitric oxide synthase inhibitor, the bradykinin B2 receptor antagonist and NPY Y1 receptor antagonist exerted no significant effects under hypoxic conditions. NPY-(18–36), an NPY Y3 receptor antagonist, elicited an inhibitory action on the NPY-induced increase in monolayer permeability. The results suggest that the NPY-induced increase in permeability across the RAEC monolayer is closely related to low O2 tension, possibly mediated by direct action on the NPY Y3 receptor expressed on the endothelial cell membrane. Furthermore, this NPY-induced increase is not likely due to nitric oxide, bradykinin, or vascular endothelial growth factor.

peptide YY; hypoxia

NEUROPEPTIDE Y (NPY) is a 36-amino acid neurotransmitter that is widely distributed throughout the central and peripheral nervous system (9, 39). In the latter, NPY acts as a co neurotransmitter with norepinephrine at the sympathetic nerve terminals, being released when the sympathetic nerves are highly excited, as in the case of strong stress. In addition to central actions in control of food intake (2), cerebrocortical excitability (42), and integration of emotional behavior (13), NPY has been implicated in peripheral functions in the cardiovascular system (36) and neurogenic pulmonary edema (14, 35).

Among the six different NPY receptors, Y1–Y6, only the NPY Y3 receptor has not been cloned (20, 25). The NPY Y3 receptor is pharmacologically characterized by its inability to be activated by peptide YY (PYY) (25), thus essentially differing from the other five receptor subtypes. In the course of studies on mechanisms of neurogenic pulmonary edema, Hirabayashi et al. (14) used isolated lung perfusion preparations to demonstrate that NPY dose dependently increased capillary filtration coefficients in the rat pulmonary vascular system. They further established that the elevation of vascular permeability was mediated by the NPY Y3 receptor (14). The Y3 receptor is very sparsely distributed, and few reports have appeared concerning its functions, for example, in the rat cardiopulmonary system (11) and in bovine chromaffin cells (41). Noll et al. (32) reported that NPY reduced macromolecule permeability across coronary endothelial monolayers through modulation of cAMP-dependent signal transduction but did not identify the NPY receptor subtype. There were discrepancies between the permeability results of Noll et al. and those of Hirabayashi et al., but their methods were different: cultured endothelial cells were used in the former, and an in vivo preparation was used in the latter. Usually, with isolated lung perfusion preparations, the lungs are inflated and maintained without respiratory movement and, therefore, might be under hypoxic conditions. In contrast, the experiments reported by Noll et al. were performed under normoxic conditions.

The endothelial cell layer forms a permeability barrier between circulating blood and the underlying vascular tissue, restricting fluid and material flow across the vascular wall. It is well known that the permeability of the endothelial barrier is increased in hypoxia, for example, in human microvascular endothelial cells and rat blood-brain barrier endothelial cells (33, 34). Hypoxia may release some mediators such as vascular endothelial growth factor (VEGF) (29) and nitric oxide (NO) (34), both of which increase vascular permeability. In human umbilical vein endothelial cells (HUVECs) (29), these effects did not appear until 24–48 h after exposure to 5% O2 hypoxia. These vascular responses to hypoxia contribute to acute respiratory distress syndrome (27) and pulmonary embolism and ischemia-reperfusion injury (4). Previous reports demonstrated that acute hypoxia or ischemia-reperfusion caused release of circulating NPY (40), possibly from sympathetic nerves or other extraneuronal cells such as platelets (28) and endothelial elements (21), increasing NPY concentration in the plasma of sheep fetus (7). In this context, we hypothesize that hypoxia-
induced elevation of vascular permeability may also be affected by another possible permeability mediator, NPY. Before examining such a hypothesis, in the present study, using a monolayer of rat aortic endothelial cells (RAECs), we evaluated the effects of NPY on large molecule permeability in normoxia and hypoxia. Furthermore, the study was undertaken to clarify a possible NPY receptor subtype participating in the permeability response of RAEC monolayers to NPY and to examine whether permeability mediators such as VEGF, NO, bradykinin, and prostaglandins are associated with NPY activity.

METHODS

Throughout the experiments, all animals were handled in accordance with the guidelines for animal experimentation set by the Japanese Association for Laboratory Animal Science, and the protocol used in the present study was approved by the Animal Care Committee of Aichi Medical University.

Isolation and culture of endothelial cells. RAECs were isolated from male Wistar rats (150–200 g, 7–9 wk old) and cultured according to the methods of Suh et al. (38). Briefly, rats were anesthetized with ketamine (50 mg/kg body wt im) and pentobarbital sodium (25 mg/kg body wt ip), and their aortas were removed and placed in phosphate-buffered saline (PBS, without Ca²⁺ or Mg²⁺). The vessels were cleaned, opened longitudinally, cut into two or three small pieces, and placed with their intimal side down on Matrigel-coated plates in growth medium (GM). GM contained 10% fetal calf serum, 75 µg/ml endothelial cell growth supplement, 10 U/ml heparin, 100 U/ml penicillin-streptomycin, 1% l-glutamine, and 100 µM MEM nonessential amino acids in DMEM. After 4–7 days, the pieces were removed and the cells were harvested. When assessed with trypan blue, viability of the primary cultured cells was >90%.

Establishment of endothelial cell monolayers for permeability assays. The incubation culture plates were composed of two chambers. The base of the upper chamber was a sleeve with a 3-µm pore size (Chemotaxicell, Kurabo, Osaka, Japan). We used 24-well microplates for the lower chamber. Before use, the upper chamber plate was coated with 50 µl of 50 µg/ml collagen IV and left to dry overnight in a laminar airflow cabinet. The chambers were then sterilized by rinsing with 70% ethanol and allowed to dry. Trypsin-EDTA was used to detach RAECs from the culture plates, and the cells were washed once with fresh GM and seeded at a density of 2 × 10⁵ cells/well in 200 µl of GM. They were incubated at 37°C in 5% CO₂-95% air for 4 days, during which time GM (300 µl) was changed every day.

Measurement of endothelial permeability. Endothelial monolayer permeability was assessed as the filtration velocity of FITC-labeled albumin from the upper to the lower chamber, as previously described (23). Into the upper chamber, 300 µl of 1% FITC-labeled albumin (Osaka, Japan); Chemotaxicell from Kurabo (Osaka, Japan); 96- and 384-well culture plates from Corning (Corning, NY); NPY, PYY, [Leu³¹,Pro³⁴]-NPY-(13–36) from Bachem (Basel, Switzerland); FK-3657 from Fujisawa Pharmaceutical (Osaka, Japan); tyrphostin SU-1498 from LC Laboratories (Boston, MA); and cAMP fluorescence polarization Biotrak immunoassay kit according to the manufacturer’s instructions. Brieﬂy, cells (50 µl) were incubated on 96-well microplates at a density of 10⁴ cells/ml overnight at 37°C in 5% CO₂-95% air. After administration of NPY into the wells, the cells were maintained for 2 h in normoxia or hypoxia. Thereafter, cells were lysed with 50 µl of cell lysis buffer and treated with 50 µl of rabbit anti-cAMP serum and then with Cy3B-cAMP conjugate. The antibody-bound Cy3B-cAMP conjugate elicits polarization, which may be measurable with the fluorescent spectrophotometer at an excitation wavelength of 535 nm and an emission wavelength of 590 nm. The content of cAMP in the cultured cells competing with Cy3B-cAMP conjugate in binding to the antibody was obtained with a standard curve.

Measurement of intracellular Ca²⁺ concentration in RAECs. RAECs were placed on 96-well culture plates coated with poly-l-lysine at a density of ~10⁴ cells/well and incubated in 5% CO₂-95% air for 18 h. The cells were washed three times with Tyrode solution before being loaded with 50 µl of 20 µM fluo 3-AM at 25°C for 1 h, and then excess dye was removed by three washes with Tyrode solution. Thereafter, baseline fluorescence (F₀) with 50 µl of Tyrode solution was measured in each well. Then, 50 µl of 3 × 10⁻⁷ M NPY, 10⁻⁶ M PYY, or 50 µl of Tyrode solution (as control) were applied to the cells. We calculated ΔF as the difference between F₀ and measured fluorescence. F_max and F_min were calculated by subtracting F₀ from signals obtained by addition of 20 µl of 1% NP-40 and 15 µl of 0.1 M EGTA to the medium 10 min before the measurement. The Ca²⁺ concentration for each well was calculated according to the following formula: 

\[ C_{a}^{2+} (nM) = K_d \times (\Delta F - F_{min})/F_{max} - \Delta F, \]

where the dissociation constant (K_d) was 390 nM for fluo 3-AM.

Materials. Matrigel-coated plates were purchased from Becton-Dickinson (Bedford, MA); DMEM, fetal calf serum, trypsin-EDTA, penicillin-streptomycin, l-glutamine, and MEM nonessential amino acid solution from Gibco Life Technologies (Eggenstein, Germany); NPY-(18–36), endothelial cell growth serum, trypan blue, collagen I, indomethacin, and FITC-labeled albumin from Sigma (St. Louis, MO); l-NMMA, heparin, BSA, fluo 3-AM, and NP-40 from Wako (Osaka, Japan); Chemotaxicell from Kurabo (Osaka, Japan); 96- and 24-well cell culture plates and poly-l-lysine from Becton-Dickinson Biosciences (Franklin Lakes, NJ); NPY, PYY, [Leu³¹,Pro³⁴]-NPY-(13–36), BIBP-3226, and NPY-(13–36) from Bachem (Basel, Switzerland); FK-3657 from Fujisawa Pharmaceutical (Osaka, Japan); tyrphostin SU-1498 from LC Laboratories (Boston, MA); and cAMP fluorescence polarization Biotrak immunoassay kit from Amersham Biosciences (Buckingham, Germany). Tyrode solution was composed of 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 0.2 mM NaH₂PO₄, 12 mM NaHCO₃, and 5.5 mM glucose. To make the stock solution of BIBP-3226, we dissolved BIBP-3226 in DMSO and diluted this solution with 0.1% PBS. A preliminary study showed that 0.1% DMSO did not affect RAEC monolayer permeability.

Statistical analysis. Differences between means were examined for significance with analysis of variance, unless indicated in paired t-tests. Statistical significance was evaluated by Scheffé’s method (37) at a level of 0.05, with values expressed as means ± SE.

RESULTS

Effects of NPY on RAEC monolayer permeability. In normoxia, FITC-labeled albumin concentration in the lower chamber, permeating across the RAEC monolayer, increased time
Neuropeptide Y on Endothelial Permeability

Dependently, even without NPY, from 63.2 ± 11.4 mg/l just before injection of FITC-labeled albumin into the upper chamber (0 h) to 2,843.8 ± 63.9 mg/l at 6 h (Fig. 1A). When NPY was injected into the upper chamber at $3 \times 10^{-7}$ M, FITC-labeled albumin concentration increased time dependently, with no significant differences in the mean values with and without NPY.

At 2 h after induction of hypoxia, FITC-labeled albumin concentration in the lower chamber also increased time dependently, even without NPY (Fig. 1B). When NPY was injected into the upper chamber at the highest concentration of $3 \times 10^{-7}$ M, FITC-labeled albumin concentration 2 and 4 h after injection of FITC-labeled albumin into the upper chamber was 1,976.9 ± 220.1 and 3,073.0 ± 115.2 mg/l, respectively. Both values were significantly greater than the respective values obtained without NPY at 2 and 4 h ($P < 0.01$ for each). The mean values obtained with $10^{-7}$ M NPY, 1,846.0 ± 262.0 mg/l at 2 h ($P < 0.05$), and 2,810.6 ± 100.9 mg/l ($P < 0.01$) at 4 h, were also significantly greater than the values obtained without NPY.

In subsequent studies of monolayer permeability, FITC-labeled albumin was measured after 2 h.

**Effects of bradykinin and histamine on RAEC monolayer permeability.** Bradykinin and histamine elevated FITC-labeled albumin concentration in the lower chamber in normoxia and hypoxia (Fig. 2). In normoxia, $10^{-5}$ M bradykinin significantly increased albumin concentration from 406.3 ± 120.5 to 1,595.1 ± 313.1 mg/l ($P < 0.05$) and $10^{-5}$ M histamine increased albumin concentration to 1,588.4 ± 172.0 mg/l ($P < 0.05$). Bradykinin and histamine were administered into the upper chamber after 2 or 24 h of hypoxia. FITC-labeled albumin concentration in the lower chamber in the presence of $10^{-5}$ M bradykinin was 1,863.4 ± 135.7 and 1,874.2 ± 109.0 mg/l after 2 and 24 h of hypoxia, respectively; both values are significantly greater than those obtained in the absence of bradykinin ($P < 0.01$). There were no significant differences in FITC-labeled albumin concentrations between 2 and 24 h of hypoxia. FITC-labeled albumin concentration in the presence of $10^{-5}$ M histamine was 1,390.9 ± 152.5 and 2,188.7 ± 164.1 mg/l after 2 and 24 h of hypoxia, respectively ($P < 0.05$); both values are significantly greater than those obtained in the absence of histamine ($P < 0.05$ for the former and $P < 0.01$ for the latter).

**Effects of concentration-response curve for NPY on RAEC monolayer permeability.** NPY concentration dependently increased the FITC-labeled albumin concentration in the lower chamber medium (Fig. 3). FITC-labeled albumin concentration in the presence of $10^{-7}$ and $3 \times 10^{-7}$ M NPY after 2 h of hypoxia was 1,846.0 ± 262.0 and 1,976.9 ± 220.1 mg/l, respectively; both values are significantly greater than those

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**Fig. 1.** Time trends for change of FITC-labeled albumin concentration in the lower chamber with permeation through the rat aortic endothelial cell (RAEC) monolayer with and without neuropeptide Y (NPY) in normoxia (A) and hypoxia (B). FITC-labeled albumin was added to the upper chamber at 0 h, and absorbance values were measured every 2 h. FITC-labeled albumin concentration leveled off 6 h after treatment with different concentrations of NPY, which was administered into the upper chamber at 0 h. Values are means ± SE; $n = 4$. *$P < 0.05$; **$P < 0.01$ vs. no drug.

**Fig. 2.** Effects of bradykinin (Brk) and histamine (His) on FITC-labeled albumin concentration in the lower chamber with permeation through RAEC monolayer. Bradykinin or histamine solution, at $10^{-4}$ M, was administered into the incubation medium 2 (2h) or 24 h (24h) after induction of hypoxia or normoxia (20%). Values are means ± SE; $n = 4$. *$P < 0.05$; **$P < 0.01$ vs. no drug. ¶$P < 0.05$ vs. His(2h).

**Fig. 3.** Concentration-response curves for effects of NPY on RAEC monolayer permeability. Absorbance of FITC-labeled albumin in the lower chamber medium was measured 2 h after addition of FITC-labeled albumin into the upper chamber. Values are means ± SE; $n = 4$. *$P < 0.05$; **$P < 0.01$ vs. normoxia; ¶$P < 0.05$ vs. 24 h in hypoxia.
obtained in normoxia ($P < 0.01$ for each). FITC-labeled albumin concentration in the presence of $10^{-7}$ and $3 \times 10^{-7}$ M NPY after 24 h of hypoxia was 1,147.3 ± 204.2 and 1,298.7 ± 120.5 mg/L, respectively; both values are significantly greater than those obtained in normoxia ($P < 0.05$ for each). In addition, we compared the concentration-response curves obtained at 2 and 24 h. In normoxia, no marked difference between 2 and 24 h was obtained at any concentration of NPY. In hypoxia, FITC-labeled albumin concentration in the presence of $10^{-7}$ and $3 \times 10^{-7}$ M NPY was significantly lower at 24 h than at 2 h ($P < 0.05$ for each).

NPY did not affect RAEC monolayer permeability in normoxia, whereas bradykinin and histamine elevated FITC-labeled albumin concentration, even in normoxia. Furthermore, NPY decreased FITC-labeled albumin concentration from 2 to 24 h after induction of hypoxia; histamine increased FITC-labeled albumin concentration, and bradykinin exerted no marked difference.

**Effects of NPY on intracellular cAMP content.** Intracellular cAMP levels were obtained after administration of NPY in normoxia and hypoxia (Fig. 4). NPY concentration dependence diminished the intracellular cAMP contents significantly in normoxia ($P < 0.05$) and hypoxia ($P < 0.001$). At $10^{-7}$ and $3 \times 10^{-7}$ M NPY, cAMP levels were significantly smaller in hypoxia than in normoxia ($P < 0.05$ and $P < 0.01$, respectively).

**Effects of NPY receptor agonists on RAEC monolayer permeability.** To ascertain the effects of NPY receptor agonists on RAEC monolayer permeability, $10^{-6}$ M PYY, $10^{-5}$ M [Leu$^{2},$Pro$^{34}]$-NPY-(13–36) (an NPY Y1 receptor agonist), or $10^{-5}$ M NPY-(13–36) (an NPY Y2 receptor agonist) was applied to RAEC monolayers 2 h after induction of hypoxia. FITC-labeled albumin concentration was 569.2 ± 93.6, 647.6 ± 125.1, and 604.8 ± 72.5 mg/L for PYY, [Leu$^{31},$Pro$^{34}]$-NPY-(13–36), and NPY-(13–36), respectively; these values are not different from the control value (749.4 ± 67.3 mg/L).

**Effects of NPY-(18–36), BIBP-3226, and L-NMMA on the NPY-induced increase in RAEC monolayer permeability.** RAEC monolayers were pretreated with $10^{-3}$ M NPY-(18–36) (an NPY Y3 receptor agonist), $3 \times 10^{-5}$ M BIBP-3226 (an NPY Y1 receptor antagonist), or $10^{-3}$ M L-NMMA 10 min before administration of $3 \times 10^{-7}$ M NPY into the upper chamber 2 h after induction of hypoxia. NPY-(18–36) prevented the response to NPY ($P < 0.01$), whereas BIBP-3226 and L-NMMA exerted no inhibitory action on the NPY-induced increase in FITC-labeled albumin concentration (Fig. 5).

**Effects of FK-3657 or indomethacin on the NPY-induced increase in RAEC monolayer permeability.** RAEC monolayers were pretreated with $10^{-3}$ M FK-3657 (a bradykinin B2 receptor antagonist) 10 min before administration of $3 \times 10^{-7}$ M NPY into the upper chamber 2 h after induction of hypoxia. FK-3657 exerted no inhibitory action on the NPY-induced increase in FITC-labeled albumin concentration but blocked the bradykinin-induced increase in FITC-labeled albumin concentration ($P < 0.01$; Fig. 6). Pretreatment with $10^{-6}$ M indomethacin elicited no inhibitory action on the NPY-induced increase in albumin concentration in the lower chamber.

**Effects of tyrphostin SU-1498 on the NPY-induced increase in RAEC monolayer permeability.** RAEC monolayers were pretreated with $10^{-6}$ M tyrphostin SU-1498 (a VEGF receptor-coupled tyrosine kinase inhibitor) 10 min before administration of $3 \times 10^{-7}$ M NPY into the upper chamber 2 h after induction of hypoxia. SU-1498 exerted no inhibitory action on the

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Fig. 4. Effect of NPY on cAMP contents of RAECs. Intracellular cAMP was measured after cells were incubated in normoxia or hypoxia for 2 h in the presence of NPY. Values are means ± SE; $n = 4$. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. $10^{-8}$ M NPY; $\uparrow P < 0.05$; $\uparrow\uparrow P < 0.01$ vs. normoxia.

Fig. 5. Effects of NPY Y1 receptor antagonist [NPY-(18–36)], NPY Y1 receptor antagonist [BIBP-3226 (BIBP)], and nitric oxide synthase inhibitor [N-nitro-L-arginine (L-NMMA)] on NPY-induced increase in RAEC monolayer permeability in hypoxia. Absorbance of FITC-labeled albumin in the lower chamber medium was measured 2 h after administration of FITC-labeled albumin into the upper chamber in hypoxia (5% O2). Cells were pretreated with $10^{-3}$ M NPY-(18–36), $3 \times 10^{-5}$ M BIBP-3226, or $10^{-3}$ M L-NMMA 10 min before treatment with $3 \times 10^{-7}$ M NPY. Values are means ± SE; $n = 4$. **$P < 0.01$ vs. no drug; $\uparrow\uparrow P < 0.01$ vs. NPY.

Fig. 6. Effects of FK-3657 (FK) and indomethacin (Indo) on NPY- or bradykinin-induced increase in RAEC monolayer permeability. Absorbance of FITC-labeled albumin in the lower chamber medium was measured 2 h after addition of FITC-labeled albumin into the upper chamber in hypoxia. Cells were pretreated with $10^{-3}$ M FK-3657, a bradykinin B2 receptor antagonist, 10 min before administration of $3 \times 10^{-7}$ M NPY into the upper chamber 2 h after induction of hypoxia. SU-1498 exerted no inhibitory action on the
NPY-induced increase in FITC-labeled albumin concentration but blocked the VEGF-induced increase in FITC-labeled albumin concentration (P < 0.01; Fig. 7).

Intracellular Ca²⁺ concentration after administration of NPY or PYY. Intracellular Ca²⁺ concentration before administration of NPY or PYY in hypoxia was 167.1 ± 34.0 nM. At 5 min after treatment with 3 × 10⁻⁷ M NPY and 10⁻⁶ M PYY, Ca²⁺ concentration was elevated to 420.9 ± 61.9 and 452.3 ± 101.5 nM, respectively (Fig. 8). These values were significantly greater than those obtained at 5 min without drugs (P < 0.01 for each). Within 2 h, the concentration tended to decrease toward the original level.

Discussion

The results obtained in the present study demonstrated that NPY concentration dependently increases large molecule permeability across an RAEC monolayer under conditions of hypoxia. The increase in RAEC monolayer permeability caused by NPY was eventually consistent with the results reported by Hirabayashi et al. (14), who studied the capillary filtration coefficient, presumably under hypoxic conditions. However, the permeability was not significantly increased under normoxic conditions, suggesting that the RAEC monolayer permeability response to NPY is closely related to O₂ content. Noll et al. (32), who showed a decrease in permeability using coronary arterial endothelial cells, performed experiments under aerated (~20% O₂) conditions. The discrepancy in the permeability responses to NPY may be partly explained in terms of O₂ tension.

The concentration-dependent decline in intracellular cAMP in RAECs treated with NPY in hypoxia corresponded to the increase in permeability. It has been reported that cAMP modulates endothelial monolayer permeability. Most investigators (26) suggested that an increase in cAMP inhibits the increase in vascular permeability. However, Noll et al. (32), using rat coronary endothelial cells in normoxia, reported that NPY decreased cAMP content and monolayer permeability, suggesting that some microvascular endothelial cells, especially the coronary arterial cells, differ from cells from most other vascular beds in the relation between vascular permeability and intracellular cAMP content. Other investigators (18, 44) also demonstrated differences in responses to some agonists between micro- and macrovascular endothelial cells. Furthermore, in RAECs, the effect of NPY on cAMP content was much smaller in normoxia than in hypoxia. As shown previously (6), hypoxia may diminish the cAMP content in endothelial cells; therefore, it is likely that the increase in RAEC monolayer permeability caused by NPY may be enhanced in hypoxia by the decrease in cAMP content.

Because NPY is present in the sympathetic nerves, it may have multiple physiological actions in the brain and peripheral nervous tissues. Sakakibara et al. (35) showed that electrical sympathetic nerve stimulation increased vascular permeability in isolated rat lung perfusion preparations, suggesting that NPY might mediate the response. NPY could, in part, by increasing vascular permeability in hypoxia, contribute to development of neurogenic pulmonary edema. In addition, NPY has been shown to be released by nerve injury, stress, and exercise (19, 24, 45). Thoresen et al. (40) and Fletcher et al. (7) demonstrated an increase in NPY serum concentration in hypoxia. In their reports, NPY serum concentration was 10⁻¹¹–10⁻¹⁰ M, which is much lower than the effective concentration range of NPY obtained in the present study. Such a discrepancy in NPY concentration may be due to a dilution effect of serum and barrier effects of the vascular wall. A possible role of NPY in sympathetic nerve-related diseases, e.g., cardiovascular diseases such as coronary diseases and hypertension, and obesity/diabetes (2), may result in an increase in vascular endothelial permeability.

The NPY family of peptides includes endogenous substances such as PYY and the pancreatic polypeptide (2), both of which are synthesized in endocrine cells. NPY is primarily produced in neurons, together with norepinephrine in postganglionic sympathetic perivascular and myocardial neurons, and plays a central role in regulation of cardiovascular function (10). In the present study, PYY had no effect on RAEC monolayer permeability in normoxia or hypoxia. Furthermore, NPY Y₁ and Y₂ receptor agonists and NPY Y₁ receptor antagonist elicited no remarkable effects, whereas NPY-(18–36), a weak NPY Y₃ receptor antagonist and Y₁/Y₂ receptor agonist (1), prevented the NPY-induced increase in RAEC monolayer permeability. These results suggest that NPY-induced hyperpermeability across the RAEC monolayer may be...
mediated by the NPY Y3 receptor, because it is not activated by PYY and blocked by NPY-(18–36). In addition to its localization in vascular endothelial cells, the NPY Y3 receptor has been identified in rat superior cervical ganglia sympathetic neurons, rat nucleus tractus solitarii, rat cardiac myocytes, and rat distal colon (1, 5, 8, 11, 31).

Several intracellular or intercellular substrates, such as Ca2+, NO, bradykinin, and VEGF, have been shown to affect vascular endothelial permeability. In the present study, however, neither L-NMMA, FK-3657, nor tyrphostin SU-1498 blocked the NPY-induced elevation of RAEC monolayer permeability. Therefore, the NPY-induced increase in RAEC monolayer permeability did not appear to be attributable to NO. If a twofold or greater increase in Ca2+ concentration in response to NPY (Fig. 8) activates endothelial NO synthase activity (22), the amount of NO released may not be enough to elevate monolayer permeability. Similarly, the lack of effect of tyrphostin SU-1498, which blocked the VEGF-induced increase in permeability, indicated no role for VEGF. In addition, bradykinin may not participate in the NPY-induced increase in RAEC monolayer permeability because of lack of influence with FK-3657, a bradykinin B2 receptor antagonist that was developed for blocking the nasal congestion and bronchospasm in cases of allergic airway diseases (15). Even if bradykinin causes NO release from endothelial cells via the B2 receptor (3), bradykinin seemed not to mediate the permeating action of NPY. Furthermore, indomethacin elicited no apparent inhibitory action on the NPY-induced elevation of monolayer permeability, suggesting that prostaglandins, products of cyclooxygenase, may not be involved in the action of NPY.

Hypoxia increases endothelial cell monolayer permeability through VEGF and NO, but these effects do not appear until a long time after exposure to 5% O2 hypoxia. The amount of VEGF produced by HUVECs (29) began to increase from 24 h after incubation with 5% O2 hypoxia and leveled off at 48 h. In the present study, the NPY-induced increase in permeability was greater at 2 h than at 24 h after hypoxia. The effects of bradykinin and histamine were quite different: the increase in permeability was smaller at 2 h than at 24 h after hypoxia. Furthermore, tyrphostin SU-1498 had no apparent effect on the NPY-induced increase in RAEC monolayer permeability at 2 h (Fig. 7) and 24 h (unpublished observation). These results indicate that VEGF may not contribute to the NPY-induced elevation of permeability.

NPY and PYY elevated the intracellular Ca2+ concentration within 5 min, whereas monolayer permeability was increased by NPY, but not by PYY. Several studies demonstrated that endothelial cytosolic Ca2+ and endothelial protein kinase C (PKC) are important regulators of endothelial permeability (23). Hypoxia, reportedly, increases human microvascular endothelial permeability through elevation of the intracellular Ca2+ concentration, activating NO synthase (12, 16, 22, 30), cGMP-dependent protein kinase, and PKC, which may control cadherin-occludin-cytoskeleton binding (33). Recently, Namiki et al. (29) showed that 5% O2 increased VEGF concentration in the culture medium of HUVECs, and VEGF increases microvascular permeability by a signaling cascade involving an increase in intracellular Ca2+ concentration (33), NO synthesis, cGMP-dependent protein kinase/PKC activation (43), and activation of extracellular signal-regulated kinase (17). In this context, the results obtained in the present study suggest that the increased permeability with NPY may not simply be explained by enhancement of a hypoxia-induced mechanism, i.e., an increase in intracellular Ca2+ concentration.

In conclusion, NPY, a sympathetic neurotransmitter, evokes an increase in vascular permeability in hypoxia via the NPY Y3 receptor. Because L-NMMA, FK-3657, indomethacin, and tyrphostin SU-1498 failed to inhibit such an increase in permeability, NPY seemed to elicit a direct action on endothelial cells, providing a research technique for evaluating the physiological function of the NPY Y3 receptor and specific antagonists relevant to its cloning.

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