Vasoactive Intestinal Peptide and Pituitary Adenylate Cyclase-Activating Polypeptide Prevent Inducible Nitric Oxide Synthase Transcription in Macrophages by Inhibiting NF-κB and IFN Regulatory Factor 1 Activation

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Vasoactive Intestinal Peptide and Pituitary Adenylate Cyclase-Activating Polypeptide Prevent Inducible Nitric Oxide Synthase Transcription in Macrophages by Inhibiting NF-κB and IFN Regulatory Factor 1 Activation

Mario Delgado,*† Ernesto J. Munoz-Elias,* Rosa P. Gomariz,† and Doina Ganea2*

High-output nitric oxide (NO) production from activated macrophages, resulting from the induction of inducible NO synthase (iNOS) expression, represents a major mechanism for macrophage cytotoxicity against pathogens. However, despite its beneficial role in host defense, sustained high-output NO production was also implicated in a variety of acute inflammatory diseases and autoimmune diseases. Therefore, the down-regulation of iNOS expression during an inflammatory process plays a significant physiological role. This study examines the role of two immunomodulatory neuropeptides, the vasoactive intestinal peptide (VIP) and the pituitary adenylate cyclase-activating polypeptide (PACAP), on NO production by LPS-, IFN-γ-, and LPS/IFN-γ-stimulated peritoneal macrophages and the Raw 264.7 cell line. Both VIP and PACAP inhibit NO production in a dose- and time-dependent manner by reducing iNOS expression at protein and mRNA level. VPAC1, the type 1 VIP receptor, which is constitutively expressed in macrophages, and to a lesser degree VPAC2, the type 2 VIP receptor, which is induced upon macrophage activation, mediate the effect of VIP/PACAP. VIP/PACAP inhibit iNOS expression and activity both in vivo and in vitro. Two transduction pathways appear to be involved, a cAMP-dependent pathway that preferentially inhibits IFN regulatory factor-1 transactivation and a cAMP-independent pathway that blocks NF-κB binding to the iNOS promoter. The down-regulation of iNOS expression, together with previously reported inhibitory effects on the production of the proinflammatory cytokines IL-6, TNF-α, and IL-12, and the stimulation of the anti-inflammatory IL-10, define VIP and PACAP as “macrophage deactivating factors” with significant physiological relevance. The Journal of Immunology, 1999, 162: 4685–4696.

Nitric oxide (NO)3 is an unstable free radical gas that mediates many physiological and toxic functions, such as macrophage cytotoxicity, neurotransmission, neurotoxicity, and regulation of blood pressure (1). The nitric oxide synthase (NOS) family of enzymes that catalyze the conversion of L-arginine to NO and L-citrulline can be classified into two major groups: constitutive NOS and inducible NOS (iNOS). Neuronal NOS and endothelial NOS are in general constitutively expressed and dependent on high intracellular Ca2+ levels (2). Macrophages express a transcriptionally inducible form of NOS (iNOS), independent of elevated Ca2+ and undetectable unless the cells are activated (2). Typically, a synergistic combination of stimuli is required for maximal induction of iNOS mRNA. For murine macrophages, IFN-γ and LPS represent one of the most potent combinations of synergizing stimuli (3). Once synthesized, iNOS is responsible for prolonged, high-output production of NO. Induced NO production is one of the principal mechanisms of macrophage cytotoxicity for tumor cells, bacteria, protozoa, helminthes, and fungi (1, 4). In general, expression of iNOS follows a generalized or localized inflammatory response resulting from infection or tissue injury. Despite its beneficial role in host defense, sustained NO production can be deleterious to the host, and NO synthesis induced by cytokines and/or inflammatory stimuli has been implicated in experimental arthritis, inflammatory bowel disease, hypotension associated with septic shock, and other types of tissue injury (5–8). Therefore, the selective inhibition of expression of this enzyme represents an important therapeutic goal.

Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) are two multifunctional neuropeptides whose primary immunomodulatory function is anti-inflammatory in nature. VIP and PACAP have been shown to attenuate reperfusion injury following ischemia of brain and lung (8–10), to inhibit T cell proliferation and cytokine production (reviewed in Ref. 11), and to inhibit several macrophage functions, such as phagocytosis, respiratory burst, chemotaxis (reviewed in Ref. 12), and LPS-induced IL-6 and TNF-α production (13–16). Furthermore, we have recently demonstrated that VIP and PACAP protect mice from lethal endotoxemia, presumably by reducing the levels of endogenous TNF-α and IL-6 (17).

The functional relationship between VIP and NO is rather complicated. VIP and NO can be colocalized and coreleased from some neurons and can regulate each other’s release in some tissues (reviewed in Ref. 8). In some physiological outcomes, such as the relaxation of smooth muscle, VIP and NO cooperate, whereas in...
others, such as the inflammatory response, they play opposite roles, with NO increasing and VIP defending against tissue and cell injury. The opposite role played by VIP and NO in an inflammatory process raises the possibility that VIP may regulate NO production or activity. Indeed, in injury models that involve neuronal NOS, VIP does not inhibit NO synthesis, but prevents its toxic action (18, 19).

To further clarify the role played by VIP and PACAP in the attenuation of the inflammatory response, in this study we examine the in vitro and in vivo effects of both neuropeptides on NO production and iNOS transcription in activated peritoneal macrophages. We investigate the molecular mechanisms involved, including the specific receptors, the intracellular signal pathways, and the nuclear transactivating factors that mediate the effect of VIP/PACAP on NO synthesis.

Materials and Methods

Reagents

Synthetic VIP, PACAP38, VIP1–12, and VIP10–28 were purchased from Novabiochem (Laufelfingen, Switzerland). The type 1 VIP receptor (VPAC1) antagonist [Ac-His3, d-Phe7, K15, R16, L27] VIP (3–7–GRF (8–27) and the VPAC1 agonist [K15, R16, L27] VIP (1–7–GRF (8–27) were kindly donated by Dr. Patrick Robberecht (Universite Libre de Bruxelles, Brussels, Belgium). The VPAC2 agonist Ro 25-1553 Ac-[Glu]-Lys5,Ac-[Glu]-Lys2,Ac-[Asp]-Leu9,Lys5,Ac-[Glu]-Thr21-25) was a generous gift from Drs. Ann Welton and David R. Bolin (Hoffmann-La Roche, Nutley, NJ). The synthetic PACAP receptor (PAC1) agonist maxadilan was a generous gift from Dr. Ethan A. Lerner (Massachusetts General Hospital, Charlestown, MA). The PAC1 antagonist PACAP6-38, secretin, and glucagon were obtained from Peninsula Laboratories (Belmont, CA). Oligonucleotides were synthesized by the Oligonucleotide Synthesis Service of Rutgers University (Newark, NJ). Marine recombinant IFN-γ was purchased from PharMingen (San Diego, CA). LPS (from Escherichia coli 055:B5), calf thymus DNA, glycerol, EGTA, and DTT were purchased from Sigma (St. Louis, MO).

Cell cultures

Mouse peritoneal macrophages were elicited by i.p. injection of 2 ml of 4% Brewer’s thioglycollate medium (Difco, Detroit, MI) into male BALB/c mice (aged 6–10 wk). Peritoneal exudate cells were obtained 72 h after injection by peritoneal lavage with ice-cold RPMI 1640 medium. Peritoneal exudate cells, containing lymphocytes and macrophages, were washed twice and resuspended in ice-cold RPMI 1640 medium supplemented with 2% heat-inactivated FCS (Life Technologies, Grand Island, NY) and treated with 10 mM HEPES buffer, 1 mM pyruvate, 0.1 M nonessential amino acids, 2 mM glutamine, 50 mM 2-ME, 100 U/ml penicillin, and 10 μg/ml streptomycin (RPMI 1640 complete medium). Cells were seeded in 96-well microtiter plates (Coming Glass, Corning, NY) at 8 × 104 cells/well in a final volume of 200 μl. The cells were incubated at 37°C for 2 h to allow adherence to plastic, and nonadherent cells were removed by repeated washing with RPMI 1640 medium. At least 96% of the adherent cells were macrophages as judged by morphologic and phagocytic criteria.

To study the effect of VIP and PACAP38 on NO production, mouse peritoneal macrophages from 12 to 10 weeks old were distributed to 96-well microtiter plates (Corning Glass, Corning, NY) at 8 × 104 cells/well in 2 ml of complete medium supplemented with 2 mM L-arginine, 2 mM NADPH, 5 mM tetrahydrobiopterin, 2 mM DTT, 5 μM pepstatin, and 10 μM leupeptin, 1 mM l-cysteine, 1 mM β-mercaptoethanol, 25% glycerol, and 10 mM HEPES, pH 7.9, containing 0.4 M NaCl, 1 mM EDTA, 5 μM L-arginine, and 0.1% N-[naphthyl]ethyl-enediamine dihydrochloride in 2.5% H2O2 were mixed, and the absorbance was measured at 550 nm. The amount of nitrate was calculated from a NaNO3 standard curve.

Determination of NO

The amount of NO formed was estimated from the accumulation of the stable NO metabolite nitrite by the Griess assay (20). Equal volumes of cell-free supernatants (90 μl containing 0.4 M NaCl, 1 mM EDTA, 15 U/ml L-lactic dehydrogenase and 83 mM sodium pyruvate) were applied to 1-ml Dowex AG50W-X8 (Na+ form) columns as previously described (23). The columns were eluted with three volumes of distilled water, and the radioactivity in the flow-through fractions (containing almost exclusively [3H]nitrite) was quantitated by scintillation spectroscopy. All measurements were made in duplicate, corrected for the number of counts per 4 × 106 cells, and the data are presented as recovery of radioiodinated nitrite.
DNA extraction and Northern blot analysis
Northern blot analysis was performed according to standard methods. Macrophage monolayers (2 × 10^6 cells/ml) were stimulated with LPS (0.5 μg/ml) and IFN-γ (200 U/ml) in the absence or presence of VIP and PACAP (10−8 M) for different time periods at 37°C. Total RNA was extracted by the acid guanidinium-phenol-chloroform method, electrophoresed on 1.2% agarose-formaldehyde gels, transferred to S&S Nytran membranes (Schleicher and Schuell, Keene, NJ), and cross-linked to the nylon membrane using UV light.

The probes for murine iNOS and β-actin were generated by RT-PCR as previously described (25, 26). Oligonucleotides were end-labeled with [γ-32P]ATP by using T4 polynucleotide kinase. The RNA-containing membranes were prehybridized for 16 h at 42°C and then hybridized at 42°C for 16 h with the appropriate probes. The membranes were washed twice in 2× SSC containing 0.1% SDS at room temperature (20 min each time), once at 37°C for 20 min, and once in 0.1× SSC containing 0.1% SDS at 50°C (20 min). The prehybridization and hybridization buffers were purchased from 5 Prime (Boulder, CO). The membranes were exposed to x-ray films (Kodak, Rochester, NY), and analyzed by densitometric analysis.

In vivo quantitation of NO production and iNOS activity and expression
Male mice (6–10 wk old) were injected with a single dose of LPS (100 μg/mouse) i.p. in the presence or absence of different amounts of VIP or PACAP (0.5–10 nmol/mouse). At different time points (2–8 h), blood was removed through cardiac puncture, and peritoneal exudate was obtained as described above. The peritoneal suspensions were centrifuged for 5 min at 1800 × g, and cell-free supernatants were harvested. Serum and peritoneal cell numbers were quantitated by using a commercially available ELISA kit (Amersham).

Electrophoretic mobility shift assay (EMSA)
Nuclear extracts were prepared by the microextraction procedure of Schreiber et al. (27) with slight modifications. Raw 264.7 cells were plated at a density of 10^4 cells in six-well plates, stimulated, washed twice with ice-cold PBS/0.1% BSA, and scraped off the dishes. The cell pellets were homogenized with 0.4 ml of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM NaN3). After 15 min on ice, Nonidet P-40 was added to a final 0.5% concentration, the tubes were gently vortexed for 15 s, and nuclei were sedimented and separated from cytosol by centrifugation at 12,000 × g for 40 s. Pelleted nuclei were washed once with 0.2 ml of ice-cold buffer A, and the soluble nuclear proteins were released by adding 0.1 ml of buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 25% glycerol, 1 mM DTT, 0.5 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM Na3VO4). After incubation for 30 min on ice, followed by centrifugation for 10 min at 14,000 rpm at 4°C, the supernatants containing the nuclear-clear proteins were harvested, the protein concentration was determined by the Bradford method, and aliquots were stored at −80°C for later use in EMSA.

Oligonucleotides corresponding to the κB (nucleotides −92 to −65) and IRF-1 (nucleotides −933 to −906) motifs of the iNOS promoter were synthesized (28, 29): 5′-CCAACTGCGACCTCTCCCTTGGGACA-3′ and 5′-AACATTTGACATATATGTTCAAAGT-3′ (IRF-1). The oligonucleotides were annealed after incubation for 5 min at 85°C in 10 mM Tris-HCl, pH 8.0, 5 mM NaCl, 10 mM MgCl2, and 1 mM DTT. Aliquots of 50 ng of the double-stranded oligonucleotides were end-labeled with [γ-32P]ATP by using T4 polynucleotide kinase. For EMSA with macrophage nuclear extracts, 20,000–50,000 cpm of double-stranded oligonucleotides, corresponding to approximately 0.5 ng, were used for each reaction. The binding reaction mixtures (15 μl) were set up containing 0.5–1 ng DNA probe, 5 μg nuclear extract, 2 μg poly(dI-dC):poly(dI-dC), and binding buffer (50 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 5% glycerol, and 10 mM Tris-HCl pH 7.5). The mixtures were incubated on ice for 15 min before adding the probe, followed by another 20 min at room temperature. Samples were loaded onto 4% nondenaturing polyacrylamide gels and electrophoresed in TGE buffer (50 mM Tris-HCl, pH 7.5, 0.38 M glycerine, and 2 mM EDTA) at 100 V, followed by transfer to Whatman paper, drying under vacuum at 80°C, and autoradiography. In competition and Ab supershift experiments, the nuclear extracts were incubated for 15 min at room temperature with the specific Ab (1 μg) or competing cold oligonucleotide (50-fold excess) before the addition of the labeled probe.

Statistical analysis
All values are expressed as the mean ± SD of the number of experiments. Each sample was assayed in duplicate. Comparisons between groups were made using the Student’s t test followed by Scheffe’s F test, with p < 0.05 as the minimum significant level.

Results
VIP and PACAP inhibit NO production
To determine the effect of VIP and PACAP on NO production, we first determined the amounts of NO following treatment of thioglycollate-elicited peritoneal macrophages with different stimuli. Macrophages were stimulated with LPS, IFN-γ, or LPS plus IFN-γ. Supernatants harvested 24 h later were assayed for the generation of nitrite, an accumulated oxidative product of NO. Unstimulated macrophages produce very low amounts of NO (Fig. 1A). Detectable levels of NO were present in macrophage cultures stimulated with all stimuli used, with the highest NO production observed upon stimulation with LPS plus IFN-γ (Fig. 1A). VIP and PACAP inhibited NO production by ~95% in macrophages stimulated with LPS, and by ~70% and 50% in macrophages activated with IFN-γ or LPS plus IFN-γ, respectively (Fig. 1A). In addition, VIP and PACAP inhibited in a dose- and time-dependent manner the NO production by LPS- and LPS/IFN-γ-stimulated cells (Fig. 1, B and C). The dose-response curves were similar for VIP and PACAP, showing maximal effects at 10−8 M and an IC50 (i.e., the concentration of neuropeptide producing 50% of maximal inhibition) of ~0.50 nM (Fig. 1) for both stimuli. The time curves indicate that the NO generation was significantly inhibited by VIP and PACAP as early as 16 h, with the maximum inhibitory effect after 24 h of culture (Fig. 1). Moreover, the reduction of NO generation was maintained throughout the 72-h incubation period (data not shown), indicating that VIP/PACAP do not delay, but rather reduce NO release.

The inhibitory effects were not the result of a decreased number of peritoneal macrophages, as neither VIP nor PACAP affected cell numbers or the viability of stimulated macrophages after 36 h of culture (viabilities were in the range of 89–97% with or without neuropeptides).

Optimal conditions were selected to further study the inhibitory effect of VIP and PACAP on NO production, i.e., macrophages were stimulated with LPS or LPS plus IFN-γ in the presence of 10−8 M neuropeptide for 24 h.

VPAC1, and to a lesser degree VPAC2, mediate the inhibition of NO production by VIP and PACAP
Next we investigated whether the inhibitory effect of VIP/PACAP could be related to occupancy of specific receptors. First, we compared the effect of VIP/PACAP to that of secretin, glucagon, and the VIP and PACAP fragments VIP1–12, VIP10–28, and PACAP6–38. NO production was not affected by secretin and glucagon (Fig. 2A). The two VIP fragments and PACAP6–38 failed to inhibit NO generation, suggesting that intact VIP and PACAP molecules are required for their inhibitory activity (Fig. 2A).

The immunological actions of VIP and PACAP are exerted through a family of receptors consisting of VPAC1, VPAC2, and PAC1 (30). Peritoneal macrophages express VPAC1 and PAC1.
mRNA constitutively, and VPAC2 mRNA is inducible upon LPS stimulation (31, 32, 71). Similar results were obtained for the Raw 264.7 macrophage cell line (33). To determine which of the VIP/PACAP receptors are involved in the inhibition of NO production, we used specific receptor agonists and antagonists. We investigated the effect of a VPAC1 agonist (34), a VPAC2 agonist (Ro 25-1553) (35), and of maxadilan, a specific PAC1 agonist (36), on the LPS/IFN-γ-induced NO production. VPAC1, and to a lesser degree VPAC2 agonists, but not the PAC1 agonist, inhibit NO release (Fig. 2B). The VPAC1 agonist exhibits a potency similar to that of VIP/PACAP (60% inhibition), whereas Ro 25-1553 was much less efficient (17–24% inhibition) (Fig. 2B). In addition, we investigated the ability of PACAP 6–38, an antagonist specific for PAC1 and, to a lesser degree for VPAC2 (37), and of a specific VPAC1 antagonist (38), to reverse the inhibitory effects of VIP and PACAP. Increasing concentrations of the antagonists (10^{-8} to 10^{-6} M) were added simultaneously with a fixed concentration of VIP or PACAP (10^{-8} M). The inhibitory effects of VIP and PACAP were reversed by the VPAC1 antagonist in a dose-dependent manner (Fig. 2C). In contrast, PACAP_6–38 did not reverse the inhibitory effect of VIP or PACAP (Fig. 2D). Neither the VPAC1 antagonist nor PACAP_6–38 significantly affected NO levels (Fig. 2, C and D). Furthermore, the simultaneous addition of VIP or PACAP and the VPAC1 agonist did not result in an additive effect on NO generation (Fig. 2E). Together these results confirm the specificity of the VIP and PACAP inhibitory activity, and suggest that both neuropeptides exert their action primarily through binding to VPAC1. Because VPAC2 appears to be expressed relatively late following LPS stimulation (12 and 24 h) (see Footnote 4), the lower efficiency of the VPAC2 agonist may result from a low number of VPAC2 receptors early during culture. Effects of delayed addition of VIP and PACAP after macrophage activation
In the experiments described so far, VIP and PACAP were added to cells at the same time as LPS. To find the level of VIP/PACAP-induced blocking in NO generation, we next investigated the effect
of exposing macrophages to VIP or PACAP after LPS or IFN-γ stimulation. We stimulated peritoneal macrophages with LPS (500 ng/ml) or with IFN-γ (200 U/ml) and added 10⁻⁶ M VIP or PACAP at different times after (from 0 to 3 h) the initiation of the cultures. Supernatants were collected 24 h later and assayed for nitrite production. The dotted line represents control values from cultures incubated with LPS (15.24 ± 1.11 μM) or IFN-γ (17.23 ± 1.54 μM) alone. Each result is the mean ± SD of four experiments performed in duplicate.

**FIGURE 2.** Inhibition of NO production by VIP and PACAP is specific. A and B, Comparative effects of VIP, PACAP38, VIP-related peptides, VIP and PACAP fragments, and VIP and PACAP agonists on NO production. Peritoneal macrophages were stimulated with LPS (500 ng/ml) and IFN-γ (200 U/ml) in the presence or absence of secretin, glucagon, VIP₁₂₋₇, PACAP₆₋₃₈, and PACAP₆₋₃₈ (10⁻⁷ M) (A) or maxadilan (a PAC1 agonist), Ro 25-1553 (a VPAC2 agonist), and [Ac-His₁,D-Phe₂,K₁₅,R₁₆,L₂₇]VIP (1–27)GRF (8–27) (a VPAC1 agonist) (B). Supernatants were collected 24 h later and assayed for nitrite production. Each result is the mean ± SD of five experiments performed in duplicate. * p < 0.001 with respect to control cultures treated with LPS/IFN-γ alone. C and D, Effect of PAC1 and VPAC1 antagonists on the inhibitory effect of VIP and PACAP. Peritoneal macrophages were stimulated with LPS (500 ng/ml) and IFN-γ (200 U/ml), and treated simultaneously with VIP or PACAP (10⁻⁶ M) and different concentrations of a VPAC1 antagonist, [Ac-His₁,D-Phe₂,K₁₅,R₁₆,L₂₇]VIP (3–27)GRF (8–27) (C), or a PAC1/VPAC2 antagonist (PACAP₆₋₃₈) (D). Supernatants were collected 24 h later and assayed for nitrite production. Nitrite production by LPS/IFN-γ-stimulated cells was, respectively, 30.15 ± 1.23 μM for 10⁻⁶ M VPAC1 antagonist and 31.33 ± 2.48 μM for 10⁻⁶ M PACAP₆₋₃₈. The dotted line represents control values from cultures incubated with LPS/IFN-γ alone (30.17 ± 2.41 μM). Each result is the mean ± SD of four experiments performed in duplicate. * p < 0.001 compared with samples treated with neuropeptides and without antagonists. E, Effect of the VPAC1 agonist on NO production by VIP/PACAP. Macrophages were stimulated with LPS (500 ng/ml) and IFN-γ (200 U/ml) and treated with the VPAC1 agonist (100 nM) in the presence or absence of VIP or PACAP (10⁻⁶ M). Supernatants were collected 24 h later and assayed for nitrite production. Percentage of inhibition was calculated by comparison with controls containing LPS/IFN-γ alone. Results are the mean ± SD of four experiments performed in duplicate.

**FIGURE 3.** Effects of delayed addition of VIP and PACAP on NO production. Peritoneal macrophages were stimulated with LPS (500 ng/ml) (A) or IFN-γ (200 U/ml) (B) at time 0. VIP or PACAP (10⁻⁸ M) were added at different times after the initiation of the cultures. Supernatants were collected 24 h after the initiation of the cultures and assayed for nitrite production. The dotted line represents control values from cultures incubated with LPS (15.24 ± 1.11 μM) or IFN-γ (17.23 ± 1.54 μM) alone. Each result is the mean ± SD of four experiments performed in duplicate.

**Intracellular pathways involved in the inhibition of NO production by VIP and PACAP**

Because all three types of VIP/PACAP receptors induce cAMP in various cell types, and cAMP-inducing agents inhibit NO production (39), we measured first the amounts of cAMP generated in LPS/IFN-γ-stimulated peritoneal macrophages after incubation with VIP or PACAP. VIP and PACAP increased, in a dose-dependent and time-dependent manner, the levels of intracellular cAMP (Fig. 4, A and B). However, the effect of the two neuropeptides was lower than that of forskolin (Fig. 4A).

To determine whether intracellular cAMP is involved in the inhibitory effect of VIP/PACAP on NO production, we investigated the effects of calphostin C (a protein kinase C inhibitor), H89 (a protein kinase A (PKA) inhibitor), and forskolin and PGE₂ (two cAMP-inducing agents). Forskolin and PGE₂ inhibit NO production in LPS/IFN-γ-stimulated macrophages, although they show less of an effect at lower concentrations (10⁻⁸ and 10⁻⁷ M) as compared with VIP and PACAP (Fig. 5A). In addition, the involvement of cAMP is supported by the results obtained with the two protein kinase inhibitors. In contrast to calphostin C, H89 partially reverses the inhibitory effect of VIP/PACAP (Fig. 5B). These results suggest that the inhibitory effect of VIP/PACAP is mediated, at least partially, through increases in intracellular cAMP.

**VIP and PACAP inhibit iNOS protein expression and iNOS activity**

Concentrations of VIP and PACAP shown to efficiently suppress the release of NO also reduce the expression of iNOS protein in LPS/IFN-γ-stimulated macrophages (Fig. 6A). The reduction in iNOS is comparable in total peritoneal macrophage lysates, membrane and cytosol preparations (data not shown). The reduction in iNOS protein expression was paralleled by a decrease in iNOS...
Although the iNOS promoter contains a complex array of trans-
duction in the Raw cells similar to peritoneal macrophages. In-
deed, VIP and PACAP inhibit, in a dose- and time-dependent man-
ner, the NO production in LPS/IFN-γ-stimulated Raw cells (Fig.
8A), with very similar kinetics to those observed in peritoneal
macrophages. In addition, the two neuropeptides significantly reduce
both iNOS activity and expression in Raw cells (Fig. 8A). The effects
of VIP and PACAP on NF-κB and IRF-1 were studied by
EMSA. Stimulation of Raw cells with LPS plus IFN-γ led to a
time-dependent increase in both NF-κB and IRF-1 binding com-
pared with unstimulated cells; treatment with VIP and PACAP
significantly inhibited the binding (Fig. 8, B and C, upper panels).
The specificity of the NF-κB and IRF-1 binding was evident by the
complete displacement of the NF-κB and IRF-1/DNA binding
complexes in the presence of a 50-fold excess of unlabeled ho-
omologous oligonucleotides in the competition reactions (Fig.
8B, upper panels). In contrast, a 50-fold excess of unlabeled
nonhomologous oligonucleotides had no effects on this DNA bind-
ing activity (Fig. 8B, upper panels). Ab supershift experi-
ments were performed to determine the composition of the NF-κB
and IRF-1-binding factors. Addition of either monospecific anti-
p50 or anti-p65 Abs to the binding reaction resulted in a marked
reduction in the intensity of the NF-κB band and led to the ap-
pearance of slow migrating bands, indicating that the NF-κB-bind-
ing complex is composed primarily of p50/p65 heterodimers (Fig.
8B, lower panel). Furthermore, the IRF-1/DNA complexes were
supershifted by an anti-IRF-1 Ab in both LPS/IFN-γ- and VIP/
PACAP-treated cells (Fig. 8C, lower panel).
Involvement of VPAC1 and cAMP in the effects of VIP on κB and IRF-1 binding

Because the inhibitory effect of VIP on NO production is mediated primarily through VPAC1 and cAMP, we determined the effect of the VPAC1 antagonist and of the PKA inhibitor H89 on the changes induced by VIP in κB and IRF-1 binding complexes. The inhibitory activity of VIP on LPS/IFN-γ-mediated NF-κB binding was completely reversed by the VPAC1 antagonist (Fig. 9A, second panel, lane 3), but not by H89 (Fig. 9A, second panel, lane 4). However, both the VPAC1 antagonist and H89 reversed the VIP inhibition of IRF-1 binding (Fig. 9A, first panel, lanes 3 and 4). These results suggest that the inhibition of NF-κB and IRF-1 binding by VIP are mediated through VPAC1, but only the inhibition of the IRF-1 binding complex is entirely cAMP-dependent. This is supported by the fact that forskolin (a cAMP inducer) does not affect NF-κB binding (Fig. 9A, second panel, lane 5) but inhibits IRF-1 binding similar to VIP (Fig. 9A, first panel, lane 5 compared with lane 1).

To determine whether VIP reduces the levels of nuclear NF-κB and IRF-1 proteins, we performed Western blots (Fig. 9B). High levels of p50, p65, and IRF-1 were present in nuclear extracts obtained from Raw 264.7 cells stimulated with LPS/IFN-γ (Fig. 9B, lane 1). In cells treated with VIP, the p50 levels remained constant, but both p65 and IRF-1 became undetectable (Fig. 9B, lane 2). The VPAC1 antagonist restored both p65 and IRF-1 levels (Fig. 9B, lane 3), whereas the PKA inhibitor H89 restored only the IRF-1 levels (Fig. 9B, lane 4). These results indicate that the reductions in the levels of p65 and IRF-1 nuclear proteins are mediated through the VPAC1 receptor and confirm the involvement of cAMP in the inhibitory effect of VIP on IRF-1, but not on NF-κB.

VIP and PACAP inhibit NO production and iNOS expression in endotoxic mice

TNF-α, IL-1, IL-6, IFN-γ, IL-12, and more recently NO have been shown to play pivotal roles in LPS-induced endotoxic shock (5, 6, 41–43). Indeed, inhibition of the LPS-induced cascade of proinflammatory cytokines is the primary mechanism through which anti-inflammatory immunomodulators such as IL-10, IL-11, and IL-13 confer protection against the lethal effects of LPS administration (44–48). Based on the ability of VIP and PACAP to downregulate cytokine production by LPS-activated macrophages, we reasoned that the in vivo protective effect of VIP/PACAP on endotoxin-induced lethal septic shock (17) might be mediated by a similar mechanism. To evaluate the effects of VIP and PACAP administration on NO production during lethal endotoxemia, mice were injected i.p. with a LD50 of LPS (100 μg) concurrently with either medium or different amounts of VIP or PACAP (0.5–10 nmol). Serum and extracellular fluid (peritoneal lavage) were collected at different time points, and NO production was approximated from nitrite levels. Serum and peritoneal nitrite levels peaked 4 h following LPS administration, and levels were still relatively high at 8 h (Fig. 10A). In contrast, mice receiving VIP or PACAP in combination with LPS showed a significant reduction in serum and peritoneal NO levels (Fig. 10A). The inhibitory effect was dose-dependent, with a maximum for 5–10 nmol VIP/PACAP.
FIGURE 8. VIP and PACAP inhibit the binding of NF-κB and IRF-1 to the iNOS promoter. A, VIP and PACAP inhibit NO production, iNOS activity and iNOS mRNA expression in LPS/IFN-γ-stimulated Raw 264.7 macrophages. Raw 264.7 cells were stimulated with LPS (500 ng/ml) and IFN-γ (200 U/ml) in the absence or presence of \(10^{-8}\) M VIP or PACAP (first, third, and fourth panels), or of a concentration range of either VIP or PACAP (second panel). Supernatants collected at different times (first and third panels), or 24 h (second panel) were assayed for nitrite production (first and second panels) and iNOS activity (third panel). Control cultures were incubated with LPS/IFN-γ alone. Each result is the mean ± SD of five separate experiments performed in duplicate. * \(p < 0.001\) with respect to control cultures with LPS alone. Fourth panel, Expression of iNOS mRNA expression was analyzed by Northern blot analysis at 12 h after LPS stimulation. Cells incubated with medium alone were used as basal iNOS mRNA level controls (lane 1). One representative experiment of three is shown.

B, VIP and PACAP inhibit NF-κB binding to the iNOS promoter. Upper panel, Nuclear extracts were prepared from Raw 264.7 cells incubated for 2, 4, and 8 h with LPS (500 ng/ml) and IFN-γ (200 U/ml) in the presence or absence of VIP or PACAP (\(10^{-8}\) M). NF-κB binding was assessed by EMSA using a radiolabeled oligonucleotide containing the murine NF-κB site of the iNOS promoter. Specificity was conducted by the addition of 50-fold excess of unlabeled homologous (NF-κB) or nonhomologous (IRF-1) oligonucleotides to nuclear extracts (Comp). Lower panel, Identification of the proteins bound to the NF-κB site using supershift analysis. Nuclear extracts (8 h incubation) were incubated with polyclonal Abs against p65 or p50 for 20 min before adding the probe. Similar results were observed in three independent experiments.

C, VIP and PACAP inhibit IRF-1 binding to the iNOS promoter. Upper panel, Nuclear extracts were prepared from Raw 264.7 cells incubated for 2, 4, and 8 h with LPS (500 ng/ml) and IFN-γ (200 U/ml) in the presence or absence of VIP or PACAP (\(10^{-8}\) M). IRF-1 binding was assessed by EMSA using a radiolabeled oligonucleotide containing the murine IRF-1 site of the iNOS promoter. Specificity was conducted by the addition of 50-fold excess of unlabeled homologous (IRF-1) or nonhomologous (NF-κB) oligonucleotides to nuclear extracts (Comp). Lower panel, Identification of the proteins bound to the IRF-1 site using supershift analysis. Nuclear extracts (8 h incubation) were incubated with polyclonal Abs against IRF-1 for 20 min before adding the probe. Arrow indicates the supershifted IRF-1-specific band. Similar results were observed in three independent experiments.
Despite its beneficial role in host defense, excessive NO production has been implicated in the pathogenesis of several types of tissue injury, including ischemia-reperfusion injury of myocardium and intestine, oxidant lung injury, N-methyl-
ß-aspartate glutamate neurotoxicity, neuronal death after cerebral ischemia, endothoxemia and en-
dotoxic shock, and cytokine-induced hypotension (reviewed in Ref. 8). Because VIP appears to prevent some of the types of tissue injury that NO promotes (50), we asked whether VIP and PACAP could interfere with the production of NO, especially during injury and in-
fammation. Because NO synthesis may result from either the consti-
tutive nNOS and eNOS (neuronal and endothelial) or iNOS (macro-
phage) activity, the effects of VIP/PACAP may be different depending on the type of NOS. Indeed, previous reports indicated that VIP in-
hibits NO toxicity, but not synthesis in an acute lung injury model, which is dependent on nNOS (8, 18, 19). Also, a recent report indi-
cates that VIP inhibits iNOS expression in stomach, spleen, and rectum, whereas augmenting nNOS in brain (51). In the present study, we investigated the effects of VIP and the structurally related neu-
ropetide PACAP on NO production and on the expression of iNOS (protein and mRNA) in murine macrophages activated with different stimuli. Both neuropeptides inhibit, in a similar way, NO production by peritoneal macrophages stimulated with LPS and/or IFN-ß. The inhibitory effect is dose-dependent within a wide range of neuropep-
tide concentrations (10⁻⁷ to 10⁻¹¹ M), with the maximum effect be-
ing observed at 10⁻⁸ M. This is the dose range at which VIP and PACAP module several immunological functions (11, 12).

Similar to the effect on cytokines such as IL-2, IL-6, IL-10, IL-12, and TNF-ß (13, 16, 52, 53; Footnote 4), the inhibition of NO production requires intact VIP/PACAP molecules. This is in agreement with previous reports showing that both C- and N-terminal truncations of VIP lead to significant losses in biological activity (54, 55). Peritoneal macrophages and the Raw 264.7 mac-
rophage cell line have been shown to constitutively express VPAC1 and PAC1 mRNA and VPAC2 mRNA following LPS-
stimulation (31–33; Footnote 4). Our agonist studies suggest that VPAC1 is the major mediator of the VIP/PACAP inhibitory effect on NO generation. The VPAC2 agonist Ro 25-1553 was much less efficient; however, because VPAC2 is expressed only later in ac-
tivated macrophages, the lack of effectiveness for the Ro com-
pound may be due to a lack of appropriate receptors during the early culture period. The role of VPAC1 as the major player in mediating the effect of VIP/PACAP on NO production is also sup-
ported by the fact that a VPAC1 antagonist, but not PACAP₆–₃₈ , an antagonist specific for PAC1 and to a lesser degree for VPAC2 (37), reverses the inhibitory effect of VIP/PACAP. Also, the VPAC1 antagonist blocked the effect of VIP/PACAP on IRF-1 and NFκB binding to the iNOS promoter, supporting the involvement of the VPAC1 in the inhibition of iNOS expression.

The VPAC1 is coupled primarily to the adenylate cyclase sys-
tem (56), and NO production is indeed inhibited by agents that increase intracellular cAMP levels (39). In the present study, VIP and PACAP induced intracellular cAMP in a dose-dependent man-
ner, and forskolin and PGE₂, two strict cAMP-inducing agents, inhibited NO generation. In addition, H89, a potent and selective PKA inhibitor, reversed the inhibitory effect of VIP/PACAP, sup-
porting the involvement of the cAMP/PKA pathway. However, because reversal was incomplete, a second cAMP-independent pathway may participate in the transduction of the VIP/PACAP signal. Similar observations were previously made for the inhibi-
tory effect of VIP/PACAP on TNF-ß and IL-12 production in macro-
phages (33; Footnote 4) and on IL-2 and IL-10 production in lymphocytes (our unpublished observations). The existence of a second cAMP-independent pathway is also supported by the fact that at concentrations that are physiologically relevant for VIP (10⁻⁹ M), the peptide induced less cAMP than forskolin, while acting as a more potent NO inhibitor. The nature of this second transduction pathway remains to be determined.

The iNOS activity is regulated at various levels, such as tran-
scriptional, posttranscriptional, translational, and posttranslational. However, iNOS transcription appears to be the primary regulatory site (57). The present study indicates that the inhibitory effect of VIP and PACAP on NO production occurs through the reduction in iNOS protein and mRNA levels. The precise molecular mech-
anisms that account for the VIP/PACAP inhibition of iNOS ex-
pression are largely unknown, and it remains to be established whether the reduction in steady-state iNOS mRNA levels results from a decrease in de novo transcriptional rate, message stabil-
ization, or both. However, some evidence points to a direct effect of
VIP/PACAP on the de novo transcription as the most likely possibility. Delayed treatment with VIP/PACAP did not affect NO production, suggesting that VIP and PACAP inhibit an early event in iNOS expression. The promoter of the iNOS gene contains two major discrete regions that synergize upon binding of transcriptional factors: a NF-κB binding site, activated mainly by LPS, and an IRF-1 binding site, for IFN-γ-induced transcriptional factors. As VIP and PACAP inhibit NO production induced by LPS and IFN-γ, the two neuropeptides might regulate the activation of both transactivating sites. The present study indicates that VIP and PACAP inhibit NF-κB (p50/p65) binding and nuclear p65 protein levels in LPS/IFN-γ-stimulated Raw 264.7 cells. We have recently reported a similar effect for the κB site specific for the TNF-α promoter (33). It remains to be determined whether the VIP/PACAP reduction in NF-κB binding and nuclear p65 levels are due to an increase in IκB protein levels, a decrease in IκB degradation, or inhibition in IκB phosphorylation, as described for other anti-inflammatory agents, such as IL-11, IL-10, TGF-β1, glucocorticoids, and antioxidants (44, 60–63).

In addition, treatment of the Raw 264.7 cells with LPS plus IFN-γ resulted in a marked IRF-1 binding that was strongly inhibited by VIP or PACAP. Similar to p65, the levels of nuclear IRF-1 protein were reduced by VIP. Although the precise mechanism underlying this effect remains to be determined, this finding points to a VIP/PACAP-sensitive step in the activation of IRF-1, which unlike NF-κB is not constitutively present in the cytosol in an inactive form, but synthesized de novo following exposure to IFN-γ (64). In macrophages, the IRF-1 gene responds to IFN-γ, through binding of the IFN-gamma activation factor complex generated by the Jak1/2-STAT1 pathway (reviewed in Ref. 65). Could VIP and PACAP affect the Jak-STAT signaling? There is indeed evidence that cAMP-elevating agents inhibit the activation of the
STAT1 pathway (66–68). We investigated the relationship between the cAMP/PKA pathway and the IRF-1 binding activity. Both VIP and forskolin inhibit IRF-1 binding, and H89 reverses the effect of VIP on IRF-1 binding. The inhibition of IRF-1 binding could be due to a reduction in IRF-1 expression, most probably by down-regulating STAT1 activation. In contrast to IRF-1, increases in CAMP do not appear to directly affect NF-κB binding. Forskolin does not affect NF-κB binding, and H89 does not reverse the inhibitory effect of VIP. Similar results were obtained for the NF-κB binding to the TNF-α promoter in LPS-stimulated macrophages (33). We propose that, similar to TNF-α transcription, the NFκB transactivation event required for iNOS expression is mediated through the cAMP-independent transduction pathway.

Production of NO by iNOS is beneficial for the protection against bacteria, fungi, parasites, viruses, and tumor cells (1, 4), but its overproduction can be harmful in endotoxemia, neurologic disorders, rheumatoid arthritis, and autoimmune diseases (5–7). Inhibitors of all NOS isoforms, which have been used to treat some of these diseases, led to significant harmful side-effects, such as hepatic injury, and even increased mortality (69, 70). Also, the clinical use of the NOS inhibitors has been hampered by the hypertensive effect resulting from the nonspecific inhibition of the constitutive NOS isoforms. Therefore, a specific iNOS inhibitor, with little or no effect on nNOS, holds significant therapeutic potential. The iNOS specificity of VIP initially documented by Bandypadhyay et al. in rat (51) has to be confirmed in other species, and the molecular mechanisms allowing the differential regulation of iNOS and nNOS have to be clarified. However, based on their ability to down-regulate iNOS expression and NO production in endotoxemic mice, as described in this study, VIP/PACAP are attractive candidates for the development of treatments for septic shock and other acute inflammatory diseases, as well as for autoimmune diseases. In fact, VIP and PACAP protect mice from endotoxic shock even when administered post-LPS (17). Unlike neutralizing Abs and receptor antagonists directed against a single cytokine, VIP and PACAP reduce the production of a wide spectrum of proinflammatory mediators. In vitro and in vivo studies indicate that VIP and PACAP directly interact with macrophages to suppress the LPS-induced production of TNF-α, IL-6, IFN-γ, IL-12, and NO (13, 16, 33).

In conclusion, we have shown that the binding of VIP and PACAP, primarily to VPAC1, inhibits iNOS expression at a transcriptional level in LPS/IFN-γ-stimulated macrophages through two intracellular pathways: a cAMP-dependent pathway that preferentially inhibits IRF-1 transactivation and a cAMP-independent pathway that blocks NF-κB binding to the iNOS promoter. The inhibition of iNOS transcription by VIP/PACAP may have therapeutic potential, because excessive NO production has been implicated in the tissue injuries characteristic for several inflammatory and autoimmune diseases.

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References


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