Analysis of the role of the PAC1 receptor in neutrophil recruitment, acute-phase response, and nitric oxide production in septic shock

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Abstract: Infections caused by Gram-negative bacteria constitute one of the major causes of septic shock, which results from the inability of the immune system to limit bacterial spread during the ongoing infection. In the last decade, it has been demonstrated that vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activing polypeptide (PACAP) are two endogenous immunopeptides, which together with three G protein-coupled receptors (VPAC1, VPAC2, and PAC1) exert a significant, therapeutic effect attenuating the deleterious consequences of septic shock by balancing pro- and anti-inflammatory factors. We have recently shown PAC1 receptor involvement in vivo as an anti-inflammatory receptor, at least in part, by attenuating lipopolysaccharide-induced production of proinflammatory interleukin-6. The present study deepens in the protective role of PAC1 receptor in septic shock, elucidating its involvement in the modulation of neutrophil recruitment and in the expression of different molecular sensors such as intercellular adhesion molecule-1, vascular cell adhesion molecule-1, fibrinogen, serum amyloid A, and nitric oxide as important, systemic players of the development of septic shock. Our results, using a mice deficient in PAC1 and a PAC1 antagonist, show that VIP and PACAP as well as the PAC1 receptor are involved in neutrophil recruitment in different target organs, in adhesion molecules expression, and in coagulation-related molecule fibrinogen synthesis. Thus, this study provides some important insights with respect to the involvement of PAC1 into the complexities of sepsis and represents an advantage for the design of more specific drugs complementing standard intensive care therapy in severe sepsis, confirming VIP and PACAP as candidates for multitarget therapy of septic shock. *J. Leukoc. Biol.* 77: 729–738; 2005.

Key Words: *endotoxin · inflammation · immunopeptides*

INTRODUCTION

Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) are two immunopeptides synthesized by immune cells, especially under inflammatory and antigen stimulation conditions. Their functions on innate and acquired immunity are exerted through binding to the family of G protein-coupled receptors, VIP receptors type 1 and 2 (VPAC1 and VPAC2, respectively), and PACAP receptor (PAC1), which stimulate primarily the adenylate cyclase system. To date, eight variants produced by alternative splicing of the transcript have been described for PAC1, seven of them coupled to the activation of adenylate cyclase and inositol/phosphate/phospholipase C (PLC) systems, and the eight variants that stimulate an L-type calcium channel. Recently, a novel splice variant in the C-terminal domain of the frog PAC1 receptor has been described [1]. Application of the anti-inflammatory properties of VIP and PACAP has demonstrated that these immunopeptides exert significant therapeutic effects in murine experimental models of rheumatoid arthritis, Crohn's disease, and septic shock [2–6].

Infections caused by Gram-negative bacteria constitute one of the major causes of sepsis or septic shock, which results from the inability of the immune system to limit bacterial spread during the ongoing infection. Although normally helping to eradicate pathogens from a local infection of peripheral tissues, inflammation during sepsis develops into a systemic syndrome with multiple manifestations such as hypotension, tissue injury, increased vascular permeability, disseminated intravascular coagulation, and ultimately, multiorgan failure and shock [7].

It is well known that the extent of leukocyte recruitment and the levels of inflammatory mediators produced by the migrating leukocytes determine the intensity of an inflammatory reaction. Among all cell adhesion molecules, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), expressed on endothelial cells, play a major role in migration of leukocytes to sites of inflammation [8]. ICAM-1 is

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shed by the cell and detected in plasma as soluble ICAM-1 (sICAM-1), being increased in many pathological conditions [9–11].

Disseminated intravascular coagulation is a disorder that affects the function of the clotting system and is frequently associated with septic shock. One of the factors involved is fibrinogen, a complex dimeric protein with each subunit composed of three nonidentical polypeptide chains (α, β, and γ), which is secreted into the blood following its synthesis in liver cells. A single gene encodes each polypeptide. Increased expression of this protein in pathophysiological conditions leads to the perturbation in coagulation regulation, resulting in multiple organ dysfunction [12].

The acute-phase (AP) serum amyloid A (SAA) proteins are multifunctional apolipoproteins, which are involved in cholesterol transport and metabolism and in modulating numerous immunological responses during inflammation and the AP response to infection [13].

Another molecule frequently used as a marker of inflammation is nitric oxide (NO), an unstable, free radical gas generated by the family of NO synthases (NOS), which is important in cell signaling, vasodilatation, and cell-mediated immunity. Excessive generation of NO has been proposed to be a major factor involved in the pathologic vasodilatation of septic shock [14, 15].

The complexities of the septic cascade continue to emerge, and new targets are identified to improve survival. Interference with adhesion molecules, inflammatory agents, and coagulation abnormalities as a whole may offer hopeful, therapeutic possibilities.

Reports have shown that VIP and PACAP attenuate the deleterious consequences of septic shock by balancing pro- and anti-inflammatory factors [5, 16]. Moreover, we have recently demonstrated the PAC1 receptor involvement in vivo as an anti-inflammatory receptor, at least in part, by attenuating lipopolysaccharide (LPS)-induced production of proinflammatory interleukin (IL)-6 [17], which appears to be the main cytokine regulating the expression of the majority of the AP protein genes, important deleterious components of septic shock.

The aim of the present study is to deepen in the protective role of the PAC1 receptor in an experimental model of lethal endotoxemia, elucidating its involvement in the modulation of neutrophil recruitment and in the expression of different molecular sensors such as ICAM-1, VCAM-1, fibrinogen, SAA, and NO as important, systemic players of the development of septic shock. Our results show that VIP and PACAP and the PAC1 receptor are involved in the neutrophil recruitment in different target organs, in adhesion molecules expression, as well as in coagulation-related molecule fibrinogen synthesis, confirming these peptides as candidates for multitarget therapy of septic shock.

MATERIALS AND METHODS

Experimental animals

Adult male mice deficient in PAC1 ($PAC1^{-/-}$) were obtained by gene targeting as described [18]. $PAC1^{-/-}$ mice were compared with wild-type (C57BL/6×129 Sv) counterparts, which served as controls. Across all of the experimentation, mice were kept in the animal house in a temperature-controlled room with a 12-h light/dark cycle; free access to standard laboratory chow and water was allowed. Procedures involving animals were conducted in compliance with international laws and policies (European Economic Community Council Directives 86/6091).

Experimental endotoxemia

Adult (8–10 weeks of age) wild-type and $PAC1^{-/-}$ mice were injected intraperitoneally (i.p.) with 1 mg LPS (from *Salmonella enteridis*; Sigma Chemical Co., St. Louis, MO). A 5-nmol dose of VIP or PACAP-38 (Neosystem, Strasbourg, France) was administered i.p. concurrently with LPS injection. Control animals received only medium RPMI 1640. Mice receiving LPS (1 mg) alone or mice injected with LPS concurrently with VIP or PACAP were killed at various time-points. Blood was extracted by cardiac puncture. When indicated, wild-type mice were i.p.-injected with a 40-nmol dose of PACAP(6–38; Neosystem) simultaneously with VIP/PACAP and LPS as described above. Blood samples were allowed to clot for 1 h at room temperature, and serum was obtained and kept frozen until sICAM-1, SAA, and NO measurement.

Measurement of myeloperoxidase (MPO) activity

Liver and large intestine were removed at 24 h, washed with phosphate-buffered saline (PBS), and cut in small pieces. For MPO determination, 50 mg/ml tissues were homogenized in 50 nmol/l phosphate buffer, pH 6.0, with 0.5% hexadecyltrimethylammonium bromide using a Polytron (Kinematica, Littau/Luzerne, Switzerland). Samples were frozen and thawed three times and centrifuged for 20 min at 30,000 *g*, and then supernatants were stored at –20°C until assay.

MPO was determined in 96-well plates using a modification of the method described by Bradley et al. [19]. Briefly, 6.67 μl sample was added to 193.33 μl assay buffer (phosphate buffer 50 mM, pH 6.0, containing 0.167 mg/ml o-dianisidine, Sigma Chemical Co., and 0.0005% H_2O_2). Absorbance at 450 nm ($A_{450\ nm}$) was measured in a microtiter reader at 15 min.

Histopathology

Liver and large intestine were collected 24 h after treatment, fixed in Bouin's solution, and embedded in paraffin; 6-μm sections were stained with Masson's haemalum/picroindigocarmine using standard techniques. Two independent observers performed semiquantitative scoring of leukocyte infiltration in liver and lamina propria from large intestine sections in different microscopic fields and slides.

sICAM-1 assay

Serum concentrations of sICAM-1 were determined by the enzyme-linked immunosorbent assay (ELISA) technique using a commercially available kit (Amersham Pharmacia Biotech, Little Chalfont, UK), as described by the manufacturer, and $A_{450\ nm}$ was measured.

RNA extraction

A tissue tearer was used to homogenize mice livers and intestines, and total RNA was extracted with the Ultraspec RNA reagent (Biotecx, Houston, TX) as recommended by the manufacturer. RNA was resuspended in diethylpyrocarbonate water and quantitated at 260/280 nm.

Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Quantitative PCR analysis was performed using the SYBR® Green PCR master mix and RT-PCR kit (Applied Biosystems, Foster City, CA), as suggested by the manufacturer. Briefly, reactions were performed in 20 μl with 50 ng RNA, 10 μl 2× SYBR Green PCR master mix, 5 U MultiScribe RT, 8 U RNase inhibitor, and 0.1 μM primers. The sequences of primers used were: mouse β-actin sense 5′-AGAGGGAAATCGTGCGTGAC-3′, antisense 5′-CAATAGTGATGACCTGGCCGT-3′; mouse ICAM-1 sense 5′-CATCCCAGAGAAGCCTTCCTG-3′, antisense 5′-TCAGCCACTGAGTCTCCAAGC-3′; mouse VCAM sense 5′-AACGACCTTCATCCCCACC-3′, antisense 5′-TCTGCCTCTGTTTGGGTTCAG-3′; mouse fibrinogen sense 5′-ATGGAATACT-

GCCGCACTCC-3′, antisense 5′-TCGGATGTCTCACCTCCCTT-3′. The GenBank accession numbers of the nucleotides for the PCR products are: β-actin, NM007393; ICAM-1, NM010493; VCAM-1, NM011693; and β-fibrinogen, BC031715. The amplification conditions were 30 min at 48°C, 10 min at 95°C, 40 cycles of denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min.

For relative quantitation, we used a method that compared the amount of target normalized to an endogenous reference. The formula used was $2^{-\Delta\Delta Ct}$, representing the n-fold differential expression of a specific gene in a treated sample compared with the control sample, where Ct is the mean of threshold cycle (cycle at which the amplification of the PCR product is detected initially); ΔCt is the difference in the Ct values for the target gene and the reference gene β-actin (in each sample assayed); and ΔΔCt represents the difference between the ΔCt from the control and each datum. Before using this method, we performed a validation experiment comparing the standard curve of the reference and the target to demonstrate that efficiencies were approximately equal [20]. The correct size of the amplified products was checked by electrophoresis.

Measurement of SAA

Serum samples were collected for the detection of SAA levels by a murine SAA ELISA kit (Tridelta Development, Ltd., Ireland) 6 h after endotoxemia induction, according to the manufacturer's recommendations, and $A_{450\ nm}$ was measured.

Determination of NO

The amount of NO formed was estimated from the accumulation of the stable NO metabolite nitrite by the Griess assay [21]. Equal volumes of serum samples (90 μl) and Griess reagents [90 μl of 1% sulfanilamide/0.1% *N*-(naphthyl)ethyl-enediamide dihydrochloride in 2.5% H_3PO_4] were mixed, and the $A_{550\ nm}$ was measured. The amount of nitrite was calculated from a $NaNO_2$ standard curve.

Measurement of cyclic adenosine monophosphate (cAMP) accumulation

cAMP levels were determined by means of an enzyme immunoassay kit from Cayman Chemical (Ann Arbor, MI). Liver and large intestine were removed 3 h after endotoxemia induction, washed with PBS, cut in small pieces, and homogenized in 5% trichloroacetic acid. After centrifugation, cAMP levels in the supernatants were measured according to manufacturers. Protein concentration was determined by Bradford's method using bovine serum albumin (BSA) as a standard. Results were expressed in pmol cAMP/μl/μg protein and showed as percent of control values.

Quantitative measurement of diacylglyecerol (DAG) levels

Liver and large intestine were removed 3 h after endotoxemia induction, washed with PBS, cut in small pieces, and homogenized in 50 nmol/l phosphate buffer, pH 7.6, using a Polytron (Kinematica). The lipids were then extracted by the method described by Bligh and Dyer [22]. Following centrifugation, the lower chloroform phase was analyzed for 1,2 DAG. Each sample was added to a sample reaction buffer as described by Preiss et al. [23], containing *Escherichia coli* DAG kinase, which phosphorylates DAG to phosphatidic acid (PA) with the addition of [γ^{32}-P]adenosine 5′-triphosphate solution. The chloroform phase was evaporated in a vacuum. Each of the dried samples was re-dissolved in 100 μl chloroform/methanol (2:1, v/v) and streaked onto silica gel thin-layer chromatography plates, which were placed in a running solvent (chloroform/acetone/methanol/acetic acid/H_2O; 40:15:13:12:8), and the radioactive spots corresponding to PA were visualized with autoradiography, then scrapped, and quantified by liquid scintillation counting. The levels of DAG were determined by using a standard curve (from 0 to 1000 pmol/l). Protein concentration was determined by Bradford's method using BSA as a standard. Results were expressed in pmol DAG/mg protein and showed as percent of control values.

Statistical analysis

All values are expressed as the mean ± SD. Comparison between groups was made using the Student's *t*-test and ANOVA test with $P < 0.05$ as the significance level.

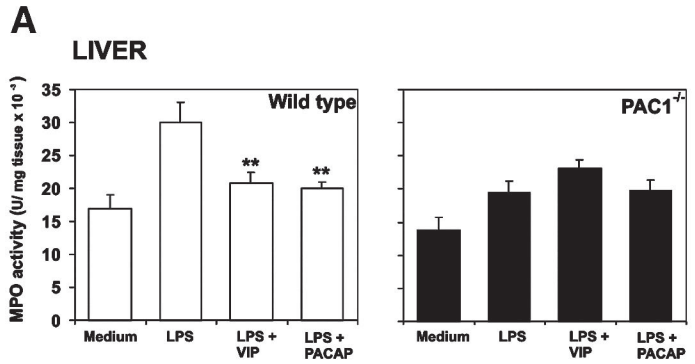
RESULTS

Involvement of the PAC1 receptor in the VIP/PACAP leukocyte recruitment inhibition

In acute inflammatory response, the initial phase comprises recruitment of blood leukocytes to the site of infection resulting in their sequestration, which often leads to tissue dysfunction and damage. In an attempt to determine the role of the PAC1 receptor on leukocyte infiltration during endotoxemia, mice defective in PAC1 expression (knockout of PAC1, $PAC1^{-/-}$) and wild-type mice (C57BL/6×129 Sv) were injected i.p. with 1 mg, a lethal dose, of LPS. First, as a quantitative measure of the neutrophil infiltration, we evaluated the effect of VIP/PACAP on MPO activity in liver and intestinal extracts 24 h after LPS administration. MPO activity values were significantly increased in wild-type and $PAC1^{-/-}$ mice, although this increase was lesser in $PAC1^{-/-}$ mice, especially in intestine extracts. Treatment with 5 nmol VIP or PACAP administered i.p. significantly inhibited MPO activity in wild-type mice. In contrast, both peptides failed to inhibit MPO activity in $PAC1^{-/-}$ mice (**Fig. 1A**).

Data obtained from MPO activity assay have been confirmed by means of light microscopy analysis, by examining histopathological alterations associated with the neutrophil sequestration during endotoxic shock. The histopathological analysis showed massive infiltration of leukocytes into liver and intestinal tract from wild-type and $PAC1^{-/-}$ LPS-treated mice (Fig. 1B). Administration of VIP improved these signs in wild-type mice, restoring the histological appearance compared with control animals injected with medium alone. However, this immunopeptide failed to inhibit leukocyte recruitment in $PAC1^{-/-}$ mice (Fig. 1B). Similar results were obtained for the animals treated with PACAP (results not shown). Histological score of leukocyte infiltration in liver and intestine sections from wild-type mice was significantly decreased after VIP treatment, as compared with sections from LPS-treated mice. No differences were observed in $PAC1^{-/-}$-treated mice (Fig. 1C). The degree of infiltrating leukocyte by semiquantitative scoring was correlated with data obtained by MPO activity assay.

It is well known that transmigration of leukocytes involves a sequential, multistep adhesion cascade between leukocyte and endothelial cell adhesion molecules, including ICAM-1 and VCAM-1. The first approach of our studies was to investigate whether VIP and PACAP could affect sICAM levels in this model, which indirectly reflects the amount of adhesion molecules produced. As **Figure 2A** shows 6 h after LPS injection, serum sICAM-1 levels were significantly increased in wild-type and $PAC1^{-/-}$ endotoxemic mice compared with the untreated levels. Treatment with VIP and PACAP induced a significant decrease in sICAM-1 serum levels only in wild-type mice, whereas no differences were observed in $PAC1^{-/-}$ mice. Next, we determined whether VIP/PACAP also affect adhesion molecule transcripts. To this extent and as the highest levels of MPO activity were found in intestinal extract, total RNA was prepared from intestine samples obtained 3 h after endo-



C

Histological scoring of leukocyte infiltration

	Liver		Intestine	
	Wild type	PAC1 -/-	Wild type	PAC1 -/-
Control	8±0.8	10±1.4	15±4.2	13±4.9
LPS	30±10	19±2.8	31±3.1	24±4.6
LPS+VIP	13±0.9*	15±3	17±3.6**	20±7

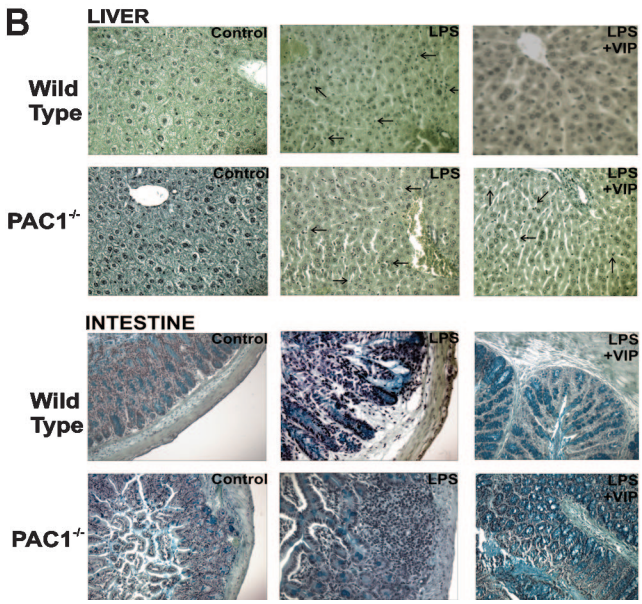


Fig. 1. PAC1 receptor mediates the VIP/PACAP inhibitory effect on MPO activity. Microscopical analysis. Wild-type (C57BL/6×129 Sv) and PAC1$^{-/-}$ adult mice were injected i.p. with 1 mg LPS or with LPS plus VIP or PACAP-38 (5 nmol per mouse). Mice treated with RPMI-1640 medium alone were used as controls. (A) Liver and large intestine were removed at 24 h and homogenized to obtain protein extracts. MPO activity was determined as described in Materials and Methods. Results are the mean ± SD from eight mice per group. **, $P < 0.01$, and ***, $P < 0.001$, versus LPS-treated animals. (B) Liver and large intestine sections were obtained after 24 h of treatment and were stained with Masson's haemalum and picroindigocarmine (original magnification, 100×). Arrows indicate infiltrated polymorphonuclear cells. (C) Histological scoring of leukocyte infiltration was performed by two independent observers, counting the number of leukocytes per 100 hepatocytes or epithelial cells from intestinal mucosa in four fields. Results represent mean ± SD of two experiments, each with three animals per group. *$P < 0.05$ and **$P < 0.01$ versus LPS-treated animals.

toxemia induction and subjected to real-time PCR analysis. Our results indicated that ICAM-1 and VCAM-1 expression was significantly up-regulated but more notably in wild-type than in PAC1$^{-/-}$ mice, as it happened for MPO activity, suggesting that alterations in the pattern of the immune cell traffic to inflammation sites could be affected by the endogenous VIP/PACAP/PAC1 system (Fig. 2, B and C). VIP and PACAP down-regulate ICAM-1 and VCAM-1 expression induced by endotoxin in wild-type mice. Neither VIP nor PACAP affected the increased ICAM-1 and VCAM-1 expression in PAC1$^{-/-}$ mice treated with LPS (Fig. 2, B and C).

Together, these data demonstrate that the protective action of VIP and PACAP on neutrophil infiltration is mainly mediated through the PAC1 receptor.

The AP proteins, β-fibrinogen, and SAA are differentially regulated by VIP/PACAP in wild-type and PAC1$^{-/-}$ mice

The AP of the inflammatory response involves a large increase in the hepatic production of a number of serum proteins including the coagulation factor fibrinogen and the family of SAA. Continuous, high expression of both AP proteins may have damaging consequences. To evaluate the involvement of the PAC1 receptor on the AP response, we tested the effects of VIP and PACAP on β-fibrinogen expression and SAA levels. Total RNA was isolated from liver of treated mice after 4 h of endotoxemia induction and subjected to real-time PCR analysis. The mRNA levels of β-fibrinogen increased after the induction of endotoxemia in wild-type and PAC1$^{-/-}$ mice.

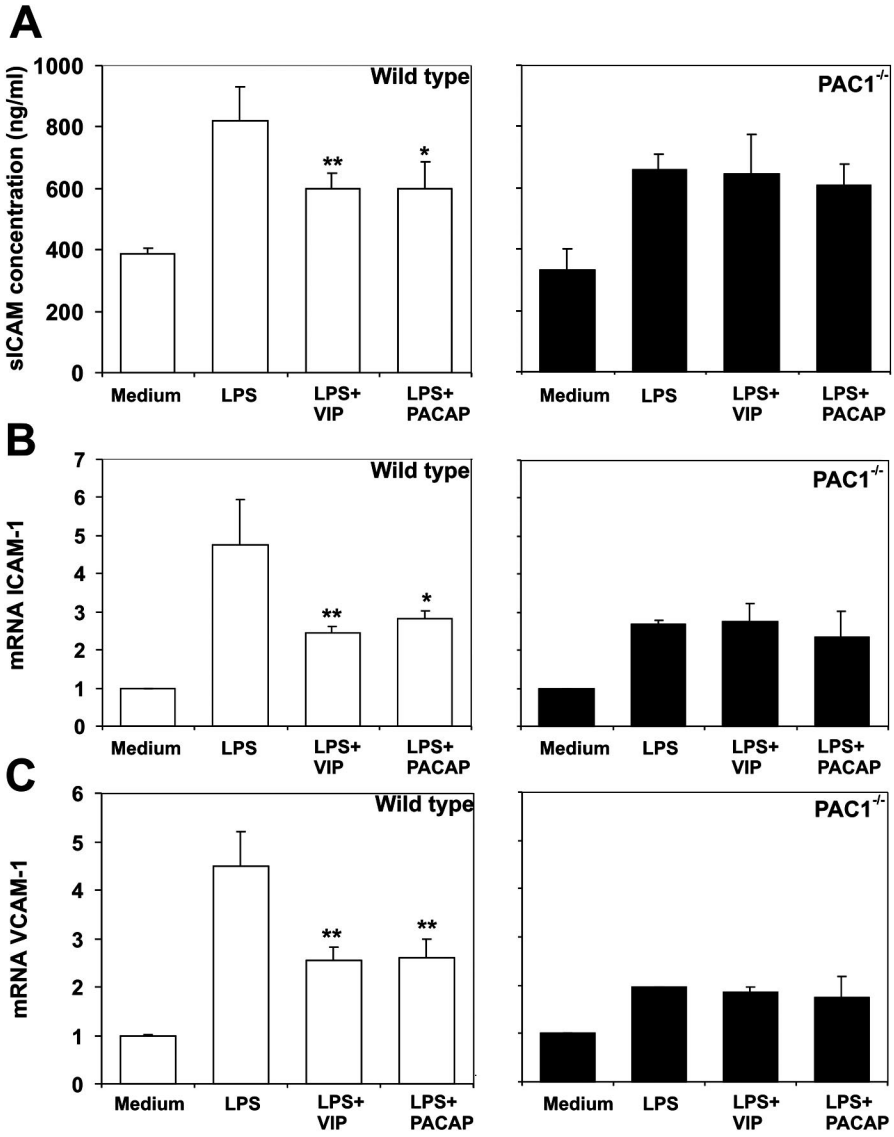


Fig. 2. PAC1 participates in the VIP/PACAP down-regulation of sICAM-1, ICAM-1, and VCAM-1 mRNA expression. Induction of endotoxemia and treatment with VIP and PACAP were performed as described in the legend to Figure 1. (A) Sera were collected at 6 h, and sICAM-1 levels were measured by ELISA. Values are means ± SD of three experiments, each with four animals per group. *, $P < 0.05$, and **, $P < 0.01$, versus LPS-treated animals. (B and C) After 3 h of induction of endotoxemia, total RNA from intestine was extracted, and ICAM-1 (B) and VCAM-1 (C) mRNA expression was measured by quantitative real-time RT-PCR and corrected by mRNA expression of β-actin for each sample, and arbitrary units were calculated with respect to control expression being 1 ($2^{-\Delta\Delta Ct}$). Results represent mean ± SD of two experiments, each with three animals per group. *, $P < 0.05$, and **, $P < 0.01$, versus LPS-treated animals.

VIP/PACAP treatment resulted in a significant down-regulation of β-fibrinogen expression in wild-type. In contrast, both peptides failed to reduce LPS-induced β-fibrinogen mRNA expression in PAC1$^{-/-}$ mice (**Fig. 3A**).

Next, plasma samples were obtained 6 h after injection of LPS alone or LPS plus VIP, or PACAP and SAA levels were evaluated by ELISA. As Figure 3B shows, SAA levels increased during endotoxemia more than 100-fold compared with the untreated animals. Treatment with VIP and PACAP induced a marked decrease in SAA levels in wild-type and PAC1$^{-/-}$ mice. These results indicate that the inhibition of VIP/PACAP on endotoxin-induced β-fibrinogen expression is mainly mediated through PAC1, whereas both peptides inhibit SAA production in a PAC1-independent way.

The VIP/PACAP inhibition on NO production is not mediated through the PAC1 receptor

Although NO synthesis exerts a critical role in host defense, elevated levels lead to the hypotension, which accompanies septic shock. Thus, we investigated the effect of VIP/PACAP on NO production in PAC1$^{-/-}$ and wild-type mice during lethal endotoxemia. Serum was collected at 6 h, and NO production was detected measuring nitrite levels. Serum nitrite levels were increased following LPS administration in wild-type and PAC1$^{-/-}$ mice. However, PAC1$^{-/-}$ mice receiving VIP or PACAP showed a significant reduction in serum NO levels (**Fig. 4**), suggesting that PAC1 receptor is not involved in the VIP/PACAP inhibition of NO production.

PAC1-mediated inhibition of MPO activity and ICAM-1, VCAM-1, and fibrinogen expression are reversed by PACAP(6–38)

We investigated whether the inhibitory effects of VIP/PACAP mediated through PAC1 could be reproduced using PACAP(6–38), a PAC1 antagonist with specificity for PAC1 and VPAC2 receptors [24]. Antagonist effects were rated based on MPO activity and ICAM-1 and VCAM-1 expression as neutrophil infiltration markers as well as on β-fibrinogen expression in wild-type mice treated with LPS and VIP or PACAP. As shown in **Figure 5**, in the presence of PACAP(6–38), both peptides failed to inhibit all markers assayed during LPS-induced endotoxemia. These results confirm the findings obtained with

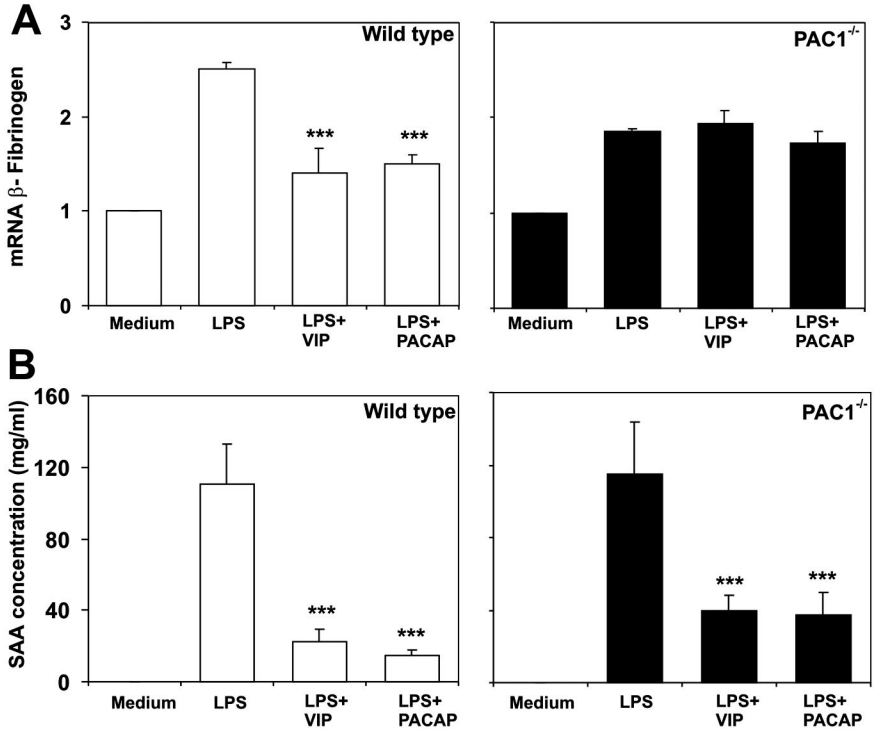


Fig. 3. PAC1 is involved in the VIP/PACAP down-regulation of endotoxin-induced β-fibrinogen expression but not in SAA levels. Mice were treated as described in the legend to Figure 1. (A) After 4 h, total RNA from liver was extracted, β-fibrinogen mRNA expression was determined by quantitative real-time RT-PCR and corrected by mRNA expression of β-actin for each sample, and arbitrary units were calculated with respect to control expression being 1 ($2^{-\Delta\Delta Ct}$). Results represent mean ± SD of two experiments, each with three animals per group. ***, $P < 0.001$, versus LPS-treated animals. (B) Sera were obtained at 6 h, and SAA levels were measured by ELISA. Values are means ± SD of three experiments, each with four animals per group. ***, $P < 0.01$, versus LPS-treated animals.

PAC1-deficient mice and strengthen PAC1 as the main receptor involved.

Analysis of intracellular signal pathways involved in the inhibitory effects of VIP and PACAP

In contrast to VPAC1 and VPAC2, which are coupled primarily to the adenylate cyclase system, the PAC1 receptor is coupled to the adenylate cyclase and inositol/phosphate/PLC cascades [25]. To study the mechanisms underlying the modulatory effects of VIP and PACAP, we measured, as a first approach, the levels of cAMP and 1,2 DAG in liver and large intestine extracts from wild-type and PAC1$^{-/-}$ mice 3 h after treatment. VIP and PACAP induced a significant increase in cAMP levels in wild-type and PAC1$^{-/-}$ endotoxemic mice (**Table 1**). Regarding DAG production, although an increase in DAG levels after LPS stimulation in several in vitro systems has been described [26, 27], under our conditions, we have not detected such increase. On the contrary, we have observed reduced DAG levels in intestine after LPS treatment. The in vivo model, the time-course production of DAG, or the intrinsic complexity of the extract samples could explain this discrepancy. Although VIP and PACAP treatment caused a significant increase in wild-type mice, however, both peptides did not modify the PAC1$^{-/-}$ DAG levels (**Table 2**). This lack of DAG stimulation by VIP and PACAP underlines the absence of the PAC1 receptor in PAC1$^{-/-}$ mice. This fact together with DAG up-regulation in wild-type suggest an important involvement of the inositol/phosphate/PLC cascade in the beneficial effects of both peptides.

DISCUSSION

The present study addressed the important impact of PAC1 deficiency during experimental endotoxic shock in a murine model. The lack of functional PAC1 prevented the beneficial effects of VIP and PACAP on recruitment of neutrophils into the inflamed liver and intestine and overexpression of coagulation factor β-fibrinogen, which was corroborated using the PAC1 antagonist PACAP(6–38).

The early stages of sepsis are characterized by inflammatory cytokine up-regulation, leading to activation of immune effec-

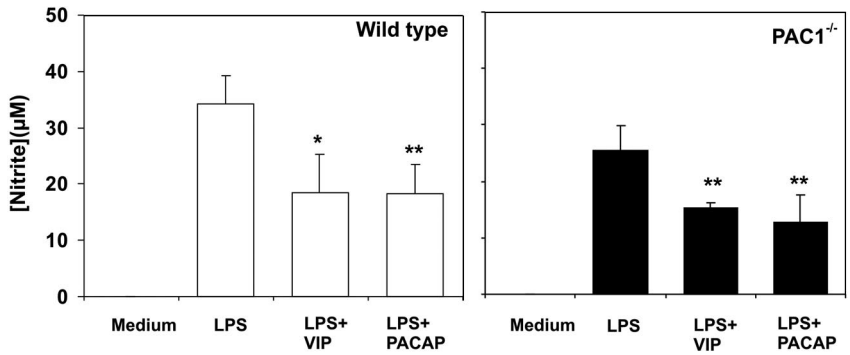


Fig. 4. Inhibition of NO production by VIP/PACAP is not mediated through PAC1. Mice were treated as described in the legend to Figure 1. Serum samples were collected at 6 h and assayed for nitrite production as described in Materials and Methods. Data are the mean ± SD of nine animals per group. *, $P < 0.05$, and **, $P < 0.01$, versus LPS-treated animals.

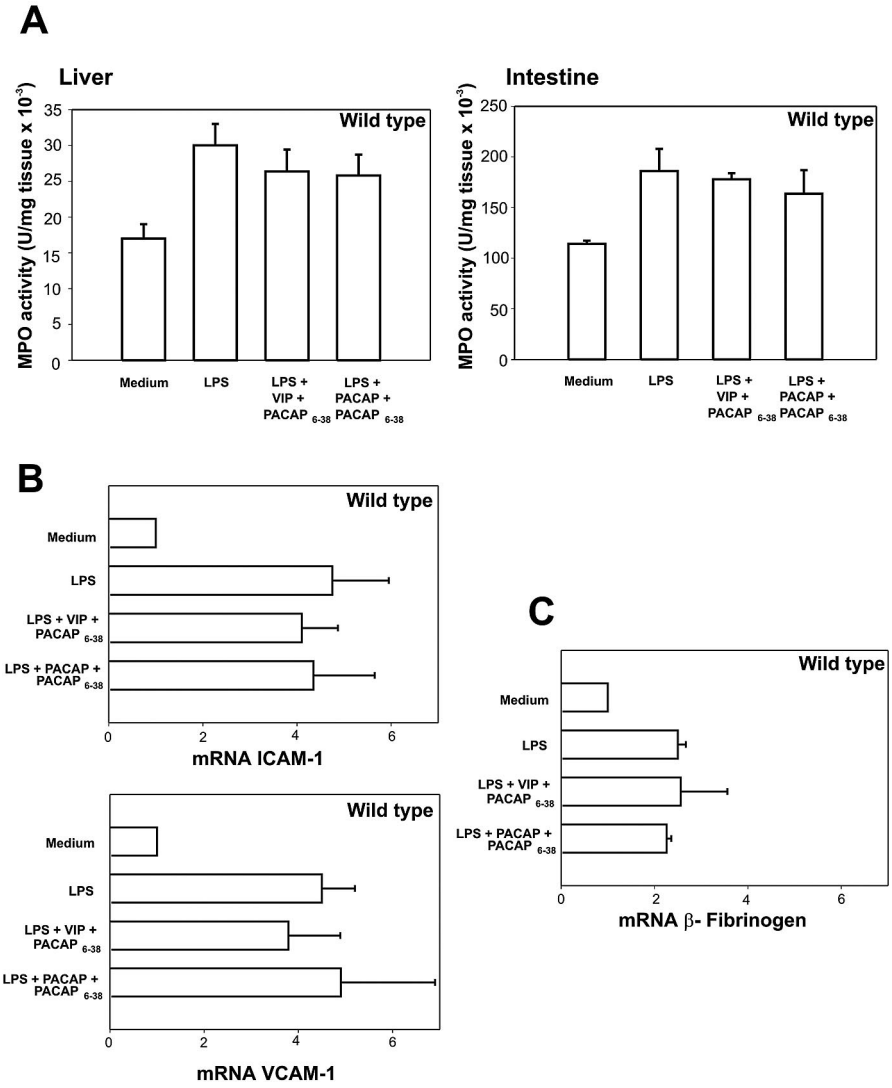


Fig. 5. The inhibitory effects of VIP and PACAP on MPO activity and ICAM-1, VCAM-1, and β-fibrinogen are reversed by PACAP(6–38). Wild-type mice were injected i.p. with 1 mg LPS or with LPS plus PACAP(6–38; 40 nmol per mouse) in the presence of VIP or PACAP-38 (5 nmol per mouse). Mice treated only with RPMI-1640 medium were used as controls. (A) Liver and large intestine were removed at 24 h and homogenized to obtain protein extracts. MPO activity was determined as described in Materials and Methods. (B) After 3 h of induction of endotoxemia, total RNA from liver and intestine was extracted, and β-fibrinogen, ICAM-1, and VCAM-1 mRNA expression was measured by quantitative real-time RT-PCR as described in Figures 2 and 3. Results are the mean ± SD from four mice per group performed in duplicate.

tor cells and vascular endothelium. Activated neutrophils bind to vascular endothelial cells expressing adhesion molecules, migrate outside the vessel, and infiltrate tissue. The adherent infiltrating neutrophils are activated to release proteases such as elastase and free radicals from granulocytes, leading to organ damage [28].

We have previously demonstrated an inhibitory effect of VIP/PACAP on chemokine production in different experimental models, which is mediated mainly through the VPAC1 receptor [2–4]. However, data on adhesion molecules are scarce. A recent report showed that VIP abated the inflammatory lesion of bronchial epithelial cells by means of a down-regulatory effect on ICAM-1 transcription and nuclear factor-κB activation [29].

Upon inflammatory stimuli, such as cytokines or LPS, the expression of ICAM-1 and VCAM-1 is strongly up-regulated [8, 30]. Different authors have reported that concentrations of circulating vascular endothelial adhesion molecules are in-

TABLE 1. Effect of VIP and PACAP on cAMP Levels

	Liver		Intestine	
	Wild-type	PAC1$^{-/-}$	Wild-type	PAC1$^{-/-}$
Control	100	100	100	100
LPS	170.8 ± 14.4	123 ± 19	183 ± 15.8	169 ± 85
LPS + VIP	330 ± 73.6*	207 ± 46**	238 ± 9.4*	286 ± 27**
LPS + PACAP	399 ± 21.7*	161 ± 12**	234 ± 10.7*	261 ± 16*

LPS-administered mice were treated i.p. with VIP or PACAP (5 nmol). After 3 h, liver and large intestine were homogenized, and cAMP levels were determined as described in Materials and Methods. Data are expressed as pmol cAMP/μl/μg protein (% control). Each result is the mean ± SD from three mice per group performed in duplicate.

* $P < 0.001$ and ** $P < 0.05$ versus LPS-treated animals.

TABLE 2. Effect of VIP and PACAP on DAG Levels

	Liver		Intestine	
	Wild-type	PAC1$^{-/-}$	Wild-type	PAC1$^{-/-}$
Control	100	100	100	100
LPS	88.6 ± 37.0	143 ± 43.8	15.4 ± 2.7	47.5 ± 27.8
LPS + VIP	284 ± 71.7*	85.7 ± 47.1	177 ± 89.4*	44.2 ± 49.2
LPS + PACAP	474 ± 171*	93.7 ± 58.4	135 ± 47.8*	48.0 ± 16.6

LPS-administered mice were treated i.p. with VIP or PACAP (5 nmol). After 3 h, liver and large intestine were homogenized, and DAG levels were determined as described in Materials and Methods. Data are expressed as pmol DAG/mg protein (% control). Each result is the mean ± SD from three mice per group performed in duplicate, and.

* $P < 0.001$ versus LPS-treated animals.

creased in sepsis and systemic inflammatory response syndrome and that this seems to be, in some way, involved in the evolution of the pathology [10, 11, 31].

Some molecular and histological data shown in this report prove that VIP/PACAP are involved in the inhibition of neutrophil infiltration modulating sICAM-1 production and ICAM-1 and VCAM-1 expression.

Previous studies with PAC1$^{-/-}$ mice in the same experimental model have revealed an important anti-inflammatory role for this receptor, protecting mice from lethal endotoxemia through the inhibition of IL-6 production [17], which acts at different stages of inflammation, being one of the main cytokines involved in the development of sepsis.

Elevated IL-6 concentrations have been described in numerous clinical disorders, where it appears to orchestrate a variety of inflammatory responses. Examination of IL-6-deficient mice has recently suggested that soluble IL-6R/IL-6 complex plays a significant role in amplifying leukocyte recruitment at sites of inflammation in several models [32–36]. This effect is mediated, at least in part, by augmenting the local production of chemokines and modulating the expression of endothelial ICAM-1 and VCAM-1 to bind neutrophils [32, 33]. It has been described as a region mediating IL-6 and interferon-γ (IFN-γ) responsiveness in the ICAM-1 promoter. Thus IL-6/IFN-γ activate nuclear factors in the regulation of ICAM-1 gene expression [37].

Although in some endothelial cells, it has been described as a high density of a functional VIP receptor, it has not been detected the presence and expression of PAC1 to date [38, 39]. Therefore, the effect of VIP and PACAP on neutrophil recruitment and ICAM-1 and VCAM-1 expression could be at least partly explained by their inhibitory effect on IL-6 production mediated through PAC1.

A wide range of proinflammatory mediators induces the hepatic AP response, including IL-6 and tumor necrosis factor α (TNF-α), which modify the profile of circulating plasma proteins. Induced expression of fibrinogen during inflammation is considered as a major cardiovascular risk factor, as it is involved in blood clot formation. In this study, we report for the first time the effect of VIP and PACAP on fibrinogen expression. Our data show that VIP/PACAP treatment down-regulates endotoxin-induced β-fibrinogen expression only in wild-type mice, supporting the involvement of PAC1 in this protective effect. Plasma levels of three fibrinogen polypeptides rise coordinately in response to many inflammatory conditions. In cultured liver cells, addition of IL-6 can mimic this response [40]. According to functional analysis of the promoter of the γ-chain of human fibrinogen [41], it contains an IL-6 response element. In line with this, the inhibitory effect of VIP and PACAP on fibrinogen expression was correlated with the down-regulation of IL-6 production [17], suggesting that PAC1 deficiency leads to suppression of the negative control mechanism that allows the modulation of fibrinogen induction during endotoxemia, to protect mice from multiple organ failure, at least partially via the inhibited production of IL-6. However, we do not rule out that to a minor extent, both peptides may exert this effect directly through their action on hepatocytes, as although VPAC1 is the major receptor expressed in liver, a selective PACAP receptor corresponding to PAC1 is found in liver membranes [42, 43].

SAA is the archetypal vertebrate major AP protein. During the first stages of inflammation, these apolipoproteins have a crucial role in inflammation; however, its maintained overproduction may have negative clinical consequences [13], and therefore, effective temporal control is desirable. We have recently demonstrated that treatment with VIP drastically reduced SAA levels in the murine models of Crohn's disease [4] and rheumatoid arthritis (manuscript in preparation), mainly through VPAC1. In agreement with these previous data, the present report confirms these results in a murine model of endotoxic shock, showing that SAA levels were significantly reduced in the serum of wild-type and PAC1-deficient mice after treatment with VIP and PACAP. SAA can be induced by IL-1β or TNF-α, whereas IL-6 has little effect by itself, although it can synergize with them [44, 45]. Furthermore, there appears to be a significant correlation between TNF-α levels and SAA concentrations in patients with sepsis [46]. In this sense, we have previously demonstrated that both peptides inhibit in vivo TNF-α production in a PAC1-independent way [17].

Following exposure to bacteria, macrophages, neutrophils, endothelial, and smooth muscle cells express the inducible isoform of NOS [47, 48], which generates large quantities of NO over extended periods. Overaccumulated NO-derived nitrate anion may contribute to endotoxic shock and multiple organ dysfunctions during endotoxemia [49]. It has been shown that VIP/PACAP inhibit NO production by peritoneal macrophages stimulated by LPS, suggesting, by agonist studies, that VPAC1 is the major mediator [50]. In this study, the results obtained with PAC1$^{-/-}$ mice support and confirm that the

inhibitory effect of VIP/PACAP on NO production is not mediated by PAC1.

Finally, it has been described that VIP/PACAP exert most of their actions on the immune system by increasing intracellular cAMP levels, including the inhibition of NO production [50–53]. In the present study, VIP and PACAP treatment increased cAMP levels in wild-type and PAC1-deficient mice, suggesting that both peptides could mediate some of the anti-inflammatory effects by activating adenylyl cyclase and protein kinase A (PKA). Furthermore, previous reports from our laboratory indicated that VIP/PACAP affect macrophage functions and LPS-induced IL-6 production through the PKC system [54–56]. The fact that both peptides increase DAG levels only in wild-type mice is a consequence of PAC1 absence in knockout mice, as this receptor is also coupled to the inositol/phosphate/PLC system. Besides, it suggests that in the present study, many functions modulated by VIP and PACAP, to protect mice from endotoxic shock, could be exerted through the activation of PKC in a PAC1-dependent way.

In conclusion, this study provides some important insights regarding the involvement of PAC1 into the complexities of sepsis and represents an advantage for the design of more specific drugs complementing standard intensive care therapy in severe sepsis. Significant advances have been made in our understanding of the septic cascade; however, significant morbidity and mortality continue. As it appears that systemic inflammation of sepsis requires more than a blockade of a single mediator, new therapies blocking several inflammatory components should be an interesting focus of attention. We are beginning to appreciate the potential, protective role that PAC1 may have in this pathology. As a result, futures studies must address this issue to elucidate the molecular signaling mechanisms of PAC1 to protect from septic shock.

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REFERENCES

1. Alexandre, D., Vaudry, H., Grumolato, J., Turquier, V., Fournier, A., Jegou, S., Anouar, Y. (2002) Novel splice variants of type I pituitary adenylate cyclase-activating polypeptide receptor in frog exhibit altered adenylate cyclase stimulation and differential relative abundance. *Endocrinology* **143,** 2680–2692.
2. Delgado, M., Abad, C., Martinez, C., Leceta, J., Gomariz, R. P. (2001) Vasoactive intestinal peptide prevents experimental arthritis by downregulating both autoimmune and inflammatory components of the disease. *Nat. Med.* **7,** 563–568.
3. Abad, C., Martinez, C., Leceta, J., Gomariz, R. P., Delgado, M. (2001) Pituitary adenylate cyclase-activating polypeptide inhibits collagen-induced arthritis: an experimental immunomodulatory therapy. *J. Immunol.* **167,** 3182–3189.
4. Abad, C., Martinez, C., Juarranz, M. G., Arranz, A., Leceta, J., Delgado, M., Gomariz, R. P. (2003) Therapeutic effects of vasoactive intestinal peptide in the trinitrobenzene sulfonic acid mice model of Crohn's disease. *Gastroenterology* **124,** 961–971.
5. Delgado, M., Martinez, C., Pozo, D., Calvo, J. R., Leceta, J., Ganea, D., Gomariz, R. P. (1999) Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) protect mice from letal endotoxemia through the inhibition of TNF-α and IL-6. *J. Immunol.* **162,** 1200–1205.
6. Gomariz, R. P., Martinez, C., Abad, C., Leceta, J., Delgado, M. (2001) Immunology of VIP: a review and therapeutical perspectives. *Curr. Pharm. Des.* **7,** 89–111.
7. Riedemann, N. C., Guo, R. F., Ward, P. A. (2003) The enigma of sepsis. *J. Clin. Invest.* **112,** 460–467.
8. Henninger, D. D., Panes, J., Eppihimer, M., Russell, J., Gerritsen, M., Anderson, D. C., Granger, D. N. (1997) Cytokine-induced VCAM-1 and ICAM-1 expression in different organs of the mouse. *J. Immunol.* **158,** 1825–1834.
9. Cowley, H. C., Heney, D., Gearing, A. J. H., Hemingway, I., Webster, N. R. (1994) Increased circulating adhesion molecule concentrations in patients with the systemic inflammatory response syndrome: a prospective cohort study. *Crit. Care Med.* **22,** 651–657.
10. Nakae, H., Endo, S., Yamada, Y., Inada, K. (2000) Bound and soluble adhesion molecule and cytokine levels in patients with severe burns. *Burns* **26,** 139–144.
11. Nakae, H., Endo, S., Inada, K., Takakuwa, T., Kasai, T. (1996) Changes in adhesion molecule levels in sepsis. *Res. Commun. Mol. Pathol. Pharmacol.* **91,** 329–338.
12. Heinrich, P. C., Castell, J. V., Andus, T. (1990) Interleukin-6 and acute phase response. *Biochem. J.* **265,** 621–636.
13. Uhlar, C. M., Whitehead, A. S. (1999) Serum amyloid A, the major vertebrate acute-phase reactant. *Eur. J. Biochem.* **265,** 501–523.
14. Kilbourn, R., Traber, D. L., Szabo, C. (1997) Nitric oxide and shock. *Dis. Mon.* **43,** 277–348.
15. Thiemermann, C. (1997) Nitric oxide and septic shock. *Gen. Pharmacol.* **29,** 159–166.
16. Delgado, M., Abad, C., Martinez, C., Juarranz, M. G., Arranz, A., Gomariz, R. P., Leceta, J. (2002) Vasoactive intestinal peptide in the immune system: potential therapeutic role in inflammatory and autoimmune diseases. *J. Mol. Med.* **80,** 16–24.
17. Martinez, C., Abad, C., Delgado, M., Arranz, A., Juarranz, M. G., Rodríguez-Henche, N., Brabet, P., Leceta, J., Gomariz, R. P. (2002) Anti-inflammatory role in septic shock of pituitary adenylate cyclase-activating polypeptide receptor. *Proc. Natl. Acad. Sci. USA* **99,** 1053–1058.
18. Jamen, F., Persson, K., Bertrand, G., Rodriguez-Henche, N., Puech, R., Bockaert, J., Ahren, B., Brabet, P. (2000) PAC1 receptor-deficient mice display impaired insulinotropic response to glucose and reduced glucose tolerance. *J. Clin. Invest.* **105,** 1307–1315.
19. Bradley, P. P., Priebat, D. A., Christensen, R. D., Rothstein, G. (1982) Cellular and extracellular myeloperoxidase in pyogenic inflammation. *J. Invest. Dermatol.* **78,** 206–209.
20. Bustin, S. A. (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reactions assays. *J. Mol. Endocrinol.* **25,** 169–193.
21. Green, L. C., Wagner, D. A., Glogowski, J. S., Skipper, P. L., Wishnok, J. S., Tannenbaum, S. R. (1982) Analysis of nitrate, nitrite and [15-N]nitrite in biological fluids. *Anal. Biochem.* **126,** 131–138.
22. Bligh, E. G., Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37,** 911–917.
23. Preiss, E., Loomis, C. R., Bell, R. M., Niedel, J. E. (1987) Quantitative measurement of sn-1,2 diacylglicerol. *Methods Enzymol.* **141,** 294–300.
24. Gourlet, P., Vandermeers, A., Vandermeers-Piret, M. C., Rathe, J., De Neef, P., Christophe, J. (1995) Fragments of pituitary adenylate cyclase activating polypeptide discriminate between type I and II recombinant receptors. *Eur. J. Pharmacol.* **287,** 7–11.
25. Rawlings, S. R. (1994) PACAP, PACAP receptors, and intracellular signalling. *Mol. Cell. Endocrinol.* **101,** C5–C9.
26. Zhang, F., Zhao, G., Dong, Z. (2001) Phosphatidylcholine-specific phospholipase C and D in stimulation of RAW264.7 mouse macrophage-like cells by lipopolysaccharide. *Int. Immunopharmacol.* **1,** 1375–1384.
27. Lee, S. C., Han, J. S., Seo, J. K., Cha, Y. N. (2003) Modulation of cyclooxygenase-2 expression by phosphatidylcholine specific phospholipase C and D in macrophages stimulated with lipopolysaccharide. *Mol. Cells* **15,** 320–326.
28. Nuytinck, J. K. S., Goris, R. J. A., Redl, H., Schlag, S. M., Zimmerman, G. A. (1986) Posttraumatic complications and inflammatory mediators. *Arch. Surg.* **121,** 886–890.

29. Tan, Y. R., Qin, X. Q., Guan, C. X., Zhang, C. Q., Luo, Z. Q., Sun, X. H. (2003) Regulatory peptides modulate ICAM-1 gene expression and NF-κB activity in bronchial epithelial cells. *Sheng Li Xue Bao* **55,** 121–127.
30. Sellati, T. J., Burns, M. J., Ficazzola, M. A., Furie, M. B. (1995) *Borrelia burgdorferi* upregulates expression of adhesion molecules on endothelial cells and promotes transendothelial migration of neutrophils in vitro. *Infect. Immun.* **63,** 4439–4447.
31. Endo, S., Inada, K., Kasai, T., Takakuma, T., Yamada, Y., Koike, S., Wakabayashi, G., Niimi, M., Taniguchi, S., Yoshida, M. (1995-96) Levels of soluble adhesion molecules and cytokines in patients with septic multiple organ failure. *J. Inflamm.* **46,** 212–219.
32. Romano, M., Sironi, M., Toniatti, C., Polentarutti, N., Fruscella, P., Ghezzi, P., Faggioni, R., Luini, W., Van Hinsberg, V., Sozzani, S., Bussolino, F., Poli, V., Ciliberto, G., Mantovani, A. (1997) Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. *Immunity* **6,** 315–325.
33. Modur, V., Li, Y., Zimmerman, G. A., Prescott, S. M., Mc, M., Intyre, T. M. (1997) Retrograde inflammatory signaling from neutrophils to endothelial cells by soluble interleukin-6 receptor α. *J. Clin. Invest.* **100,** 2752–2756.
34. Fenton, R. R., Molesworth-Kenyon, S., Oakes, J. E., Lausch, R. N. (2002) Linkage of IL-6 with neutrophil chemoattractant expression in virus-induced ocular inflammation. *Invest. Ophthalmol. Vis. Sci.* **43,** 737–743.
35. Biswas, P., Delfanti, F., Bernasconi, S., Mengozzi, M., Cota, M., Polentarutti, N., Mantovani, A., Lazzarin, A., Sozzani, S., Poli, G. (1998) Interleukin-6 induces monocyte chemotactic protein-1 in peripheral blood mononuclear cells and in the U937 cell line. *Blood* **91,** 258–265.
36. Rijneveld, A. W., Van den Dobbelsteen, G. P., Florquin, S., Standiford, T. J., Speelman, P., Van Alphen, L., Van der Poll, T. (2002) Roles of interleukin-6 and macrophage inflammatory protein-2 in pneumolysin-induced lung inflammation in mice. *J. Infect. Dis.* **185,** 123–126.
37. Caldenhoven, E., Coffer, P., Yuan, J., Van de Stolpe, A., Horn, F., Kruijer, W., Van der Saag, P. T. (1994) Stimulation of the human intercellular adhesion molecule-1 promoter by interleukin-6 and interferon-γ involves binding of distinct factors to a palindromic response element. *J. Biol. Chem.* **269,** 21146–21154.
38. Pasyk, E. A., Cipris, S., Daniel, E. E. (1996) A G protein, not cyclic AMP, mediates effects of VIP on the inwardly rectifying K^+ channels in endothelial cells. *J. Pharmacol. Exp. Ther.* **276,** 690–696.
39. Groneberg, D. A., Hartmann, P., Dinh, Q. T., Fischer, A. (2001) Expression and distribution of vasoactive intestinal polypeptide receptor VPAC(2) mRNA in human airways. *Lab. Invest.* **81,** 749–755.
40. Geiger, T., Andus, T., Klapproth, J., Hirano, T., Kishimoto, T., Heinrich, P. C. (1988) Induction of rat acute-phase proteins by interleukin 6 in vivo. *Eur. J. Immunol.* **18,** 717–721.
41. Ray, A. (2000) A SAF binding site in the promoter region of human γ-fibrinogen gene functions as an IL-6 response element. *J. Immunol.* **165,** 3411–3417.
42. Guijarro, L. G., Rodriguez-Henche, N., Garcia-Lopez, E., Nogerales, F., Dapena, M. A., Juarranz, M. G., Prieto, J. C. (1995) Receptors for pituitary adenylate cyclase-activating peptide in human liver. *J. Clin. Endocrinol. Metab.* **80,** 2451–2457.
43. Robberecht, P., Gourlet, P., Cauvin, A., Buscail, L., De Neef, P., Arimura, A., Christophe, J. (1991) PACAP and VIP receptors in rat liver membranes. *Am. J. Physiol.* **260,** G97–G102.
44. Jensen, L. E., Whitehead, A. S. (1998) Regulation of serum amyloid A protein expression during the acute-phase response. *Biochem. J.* **334,** 489–503.
45. Steel, D. M., Whitehead, A. S. (1994) The major acute phase reactants: C-reactive protein, serum amyloid P component and serum amyloid A protein. *Immunol. Today* **15,** 81–88.
46. Fratelli, M., Zinetti, M., Fantuzzi, G., Spina, C., Napoletano, G., Donatiello, G., Ravagnan, R., Sipa, J. D., Casey, C. A., Ghezzi, P. (1997) Time course of circulating acute phase proteins and cytokines in septic patients. *Amyloid.* **4,** 33–97.
47. Morris, S. M., Billiar, T. R. (1994) New insights into the regulation of inducible nitric oxide synthesis. *Am. J. Physiol.* **266,** E829–E839.
48. Nathan, C. (1992) Nitric oxide as a secretory product of mammalian cells. *FASEB J.* **6,** 3051–3064.
49. Titheradge, M. A. (1999) Nitric oxide in septic shock. *Biochim. Biophys. Acta* **1411,** 437–455.
50. Delgado, M., Muñoz-Elias, E. J., Gomariz, R. P., Ganea, D. (1999) 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. *J. Immunol.* **162,** 4685–4696.
51. Delgado, M., Muñoz-Elias, E. J., Kan, Y., Gozes, I., Fridkin, M., Brenneman, D. E., Gomariz, R. P., Ganea, D. (1998) Vasoactive intestinal peptide and pituitary adenylate cyclase activating polypeptide inhibit TNFα transcriptional activation by regulating NF-κB and CREB/c-Jun. *J. Biol. Chem.* **273,** 31427–31437.
52. Ganea, D., Sun, L. (1993) VIP downregulates the expression of IL-2 but not of IFNγ from stimulated murine T lymphocytes. *J. Neuroimmunol.* **47,** 147–158.
53. Tatsuno, I., Gottschall, P. E., Arimura, A. (1991) Inhibition of mitogen-stimulated proliferation of murine splenocytes by a novel neuropeptide, pituitary adenylate cyclase-activating polypeptide: a comparative study with vasoactive intestinal peptide. *Endocrinology* **128,** 728–734.
54. De la Fuente, M., Delgado, M., Del Río, M., Martínez, C., Hernanz, A., Gomariz, R. P. (1993) Stimulation by vasoactive intestinal peptide (VIP) of phagocytic function of rat macrophages. Protein kinase C involvement. *Regul. Pept.* **48,** 345–353.
55. Delgado, M., Garrido, E., De la Fuente, M., Gomariz, R. P. (1996) Pituitary adenylate cyclase-activating polypeptide (PACAP38) stimulates rat peritoneal macrophages functions. *Peptides* **17,** 1097–1105.
56. Martínez, C., Delgado, M., Pozo, D., Leceta, J., Calvo, J. R., Ganea, D., Gomariz, R. P. (1998) Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide modulate endotoxin-induced IL-6 production by murine peritoneal macrophages. *J. Leukoc. Biol.* **63,** 591–601.