EFFECT OF VASOACTIVE INTESTINAL PEPTIDE (VIP) ON CYTOKINE LEVELS AND HAEMOSTATIC AND BIOCHEMICAL PARAMETERS IN A RAT ENDOTOXAEMIC MODEL

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Abstract

The presented study was planned to determine whether vasoactive intestinal peptide (VIP) could prevent cytokine haemostatic, haematological, and biochemical disturbances in LPS-treated rats. Adult male Wistar rats (weight range: 200–250 g) were used. The study included four groups: group 1 served as a control group (C); animals in group 2 were given intravenously 1.6 mg/100 g of LPS (*E. coli*, serotype 0.111:B4); in group 3, rats were injected intraperitoneally with 25 ng/kg of VIP; in group 4, the same doses of VIP and LPS were injected simultaneously. Blood samples were collected 6 h after treatments. In endotoxaemic rats, platelet count, fibrinogen, and antithrombin levels were decreased, the activated partial thromboplastin time and prothrombin time were prolonged, and leucopenia, as well as significant changes in differential leukocyte percentage were demonstrated. In addition, LPS caused statistically significant increases in plasma TNF- α , IL-6, and IL-10 levels, and AST, ALT, creatinine, cholesterol, triglyceride concentrations. However, it caused a statistically significant decrease in total protein and albumin levels when compared to control group. The results showed that during endotoxaemia, VIP had moderately therapeutic effect as an antiinflammatory agent, suppressing TNF- α and IL-6, and stimulating IL-10; however, it was not effective against the adverse effect of LPS on investigated haematological and biochemical parameters.

Key words: rat, endotoxin, vasoactive intestinal peptide, cytokine, disseminated intravascular coagulation.

Septic shock is an increasingly serious health problem in animals, despite the potent antimicrobial treatments and intensive supportive care in the last decade. Severe Gram-negative bacterial infections such as some enteric diseases, septicaemia, metritis, mastitis, and pneumonia can cause the development of endotoxic shock, a condition characterised by fever, tachycardia, tachypnea, hypotension, disseminated intravascular coagulation (DIC), multiple organ failure, and even death (15). Endotoxaemia is a serious problem worldwide with high mortality rates (3). Endotoxin, a lipopolysaccharide component of the outer membrane of Gram-negative bacteria, is involved in the pathogenesis of endotoxic shock, coagulopathy, and plays a pivotal role in the production of the proinflammatory cytokines such as TNF- α , IL-1, and IL-6 from monocytes, macrophages, and endothelium (29). Release of these cytokines leads to activation of leukocytes, coagulation, and complement system. These processes initiate the haemodynamic derangement and organ dysfunction often seen during severe sepsis and septic shock (19).

Over the last few years, various pharmacological agents such as recombinant active protein C, the selective iNOS inhibitor, vagus nerve stimulator, 1,25-dihydroxy-vitamin D3, resveratrol, and

platelet-activating factor antagonist have gained increasing importance in endotoxaemia studies (3, 21, 28). Vasoactive intestinal peptide (VIP) is a neuropeptide, which exhibits a broad anti-inflammatory activity, inhibiting cytokine production, phagocytosis, respiratory burst, neutrophils, and T cell proliferation, chemokine-dependent leukocyte recruitment, and production of reactive oxygen species such as superoxide radical and hydrogen peroxide (12, 25). VIP administration protects against lethal endotoxaemia and increases the survival rate in models of sepsis (5, 25). VIP is secreted following endotoxin stimulation to counterbalance the generation of pro-inflammatory cytokines, but becomes ineffective under conditions of excessive cytokine production (5).

VIP has been studied for its pharmacological actions, but its effect on the haematological, haemostatic, and biochemical profile is yet unclear. The administration of exogenous VIP during septic shock might control the pro-inflammatory cytokine network, and haematological and biochemical derangements. The present study therefore was undertaken to determine whether VIP could prevent cytokine, haemostatic, haematological, and biochemical disturbances in LPS-treated rats.

Material and Methods

Forty healthy adult male Wistar rats, weighing 200–250 g, were randomly divided to four equal groups. The animals were acclimatised at a constant temperature of 20°C for 3 d before the study and were allowed a standard laboratory diet and water *ad libitum*. All rats were in excellent physical condition prior to the experiment. The experimental studies were approved by the local ethics committee. The study included four equal groups: group 1, control group (C) was treated intravenously only with 0.9% saline (0.2 ml); animals in group 2 were given 1.6 mg/100 g of LPS (*E. coli*, serotype 0.111:B4, Sigma) in the tail vein, LPS was dissolved in physiological saline immediately before use; in group 3, rats were injected intraperitoneally with 25 ng/kg of VIP (Sigma V6130), freshly dissolved in PBS; in group 4, the same doses of VIP and LPS were injected simultaneously. Six hours after LPS injections, blood samples were collected from the heart using 3.8% sodium citrate (1v/9v) as the anticoagulant. Plasma was prepared by centrifugation at 3,000 g for 20 min at 4°C, and either analysed immediately or stored frozen at -70°C until use. Additionally, the blood was centrifuged at 5,000 g for 5 min using EDTA anticoagulant at 4°C to prepare plasma for biochemical analysis. The samples were stored at -20°C until analysis.

To determine activated partial thromboplastin time (APTT), 25 µl of plasma was mixed with 25 µl of APTT EA liquid kit (Dialab, Austria) and preincubated for 5 min at 37°C. After adding 25 µl of CaCl₂ (0.020 Mol/L) into the mixture, APTT was determined with coagulometer (DIACLOT C1, Dialab, Austria). To measure prothrombin time (PT), 25 µl of plasma was preincubated for 1 min at 37°C, and the coagulation time

was measured after adding 50 µl of thromboplastin solution (Dialab, Austria). Plasma fibrinogen was quantified by determining the clotting time with the coagulometer using a commercial kit (Bovine Thrombin, Dialab, Austria). Antithrombin (AT) activity was measured with STA compact analyser (Diagnostica Stago, France) following the manufacturer's protocol using AT test kits (Stachrom AT, Diagnostica Stago, France).

The leukocyte and platelet counts were determined by a haemocytometer method using Türk and Rees-Ecker solution, respectively. Selected blood smears were stained with May-Grünwald-Giemsa technique and then the percentage values of different leukocytes were determined.

For biochemical analyses, plasma concentrations of cholesterol, triglycerides, creatinine, glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea, total protein (TP), albumin (Alb) were determined by a computer process-controlled multiparametric autoanalyser (Tokyo Boeki, TMS 1024) using commercial kits (Sprinreact SA, Spain).

Cytokine concentrations were determined by ELISA, using commercial kits that are selective for rat cytokines (BioSource International Immunoassay kit, Nivelles, Belgium). Plasma TNF-α, IL-6, and IL-10 levels were quantified following manufacturer's directions, and expressed as picogram (pg) per milliliter.

All values were expressed as mean ± SEM. The results were analysed by Duncan test (SPSS for Windows, release 10.0). In all cases, probability of error of less than 0.05 was selected as the criterion for the statistical significance.

Table 1
Effects of vasoactive intestinal peptide on cytokine levels, and haemostatic and haematological parameters in endotoxaemic rats (mean ±SE)

Parameters	Control (n=10)	LPS (n=10)	VIP (n=10)	VIP+LPS (n=10)
TNF-α (pg/mL)	BDL	856±95a	BDL	630±86b
IL-6 (pg/mL)	BDL	339±43a	BDL	207±35b
IL-10 (pg/mL)	BDL	234±47b	BDL	335±48a
ATIII (%)	97.3±2.4a	68.2±4.2b	96.1±2.1a	69.7±3.2b
APTT (s)	21.6±0.9b	40.3±1.6a	23.2±1.7b	37.3±1.9a
PT (s)	16.4±0.5b	27.2±2.3a	17.5±0.5b	24.3±1.1a
Fibrinogen (mg/dl)	256±20.8a	123±11.2b	234±14.9a	135±14.0b
PLT (x10 ⁹ /L)	694±48.5a	173±24.7b	628±44.0a	211±21.4b
Leukocyte (mm ³)	5,916±535a	1,912±271c	6,665±604a	3,930±381b
Neutrophil (%)	20.2±2.0c	69.1±3.8a	28.2±3.4c	48.5±3.4b
Lymphocyte (%)	70.6±2.9a	25.5±2.5c	63.4±2.9a	45.6±3.7b

a, b, c - differences in the same row are statistically significant when the values are marked with different letters (P<0.05). LPS - lipopolysaccharide, VIP - vasoactive intestinal peptide, BDL - below the detection limit.

Table 2
Effects of vasoactive intestinal peptide on biochemical parameters in endotoxaemic rats (mean±SE)

Parameters	Control (n=10)	LPS (n=10)	VIP (n=10)	VIP+LPS (n=10)
AST (U/L)	56.8± 3.8b	190.3± 17.0a	54.2± 4.2b	177.8± 15.9a
ALT (U/L)	24.9± 2.7b	68.1± 5.9a	23.1± 2.8b	54.4±7.3a
Creatinine (mg/dL)	0.28± 0.06b	0.61±0.09a	0.34±0.06b	0.65±0.09a
Urea (mg/dL)	18.9±2.9	14.4±2.1	20.1±2.3	15.6±1.6
Glucose (mg/dL)	95.8±8.2b	76.3±6.2b	120.3±7.8a	81.3±8.6b
T. protein (g/dL)	5.71±0.14a	3.92±0.13b	5.91±0.25a	4.35±0.17b
Albumin (g/dL)	3.02±0.17a	2.11±0.12b	3.08±0.19a	2.51±0.14b
Triglyceride (mg/dL)	114±7.7b	189±11.2a	127±9.4b	166±6.3a
Cholesterol (mg/dL)	47±3.1b	73±6.1a	46±4.7b	59±6.4ab

Denotations are explained in the footnote to Table 1.

Results

The effects of VIP on cytokine levels, and haemostatic, haematological, and biochemical parameters are presented in Tables 1 and 2.

Discussion

In experimental studies on laboratory animals, the intravenous administration of endotoxin or proinflammatory cytokines are used to model the clinical features associated with sepsis (19). TNF- α and IL-6 are important mediators that contribute to pathophysiological changes associated with sepsis (14). VIP causes down-regulation of proinflammatory mediators such as TNF, IFN, IL-6, IL-12, and NO and up-regulation of IL-10 production (6).

In earlier studies VIP diminished or prevented shock, organ dysfunction and mortality without adverse effects in endotoxaemic animal models (25). VIP is present in various lymphoid organs, and immune cells express specific receptors for VIP (5). In addition, elevated plasma levels of VIP were determined in septic shock patients and in some endotoxic animal models (23). Tuncel *et al.* (25) reported that prevention of mast cell reactivity by an agent such as VIP could be a potential therapeutic strategy in controlling sepsis. In addition, VIP inhibits cell responses to chemokines in monocytes and CD4- T cells by *trans*-deactivating chemokine receptors, a mechanism involving chemokine receptor phosphorylation (13).

In the present investigation, plasma TNF- α and IL-6 were undetectable in control group (C), while in group 2, a marked elevation of plasma TNF- α and IL-6 levels were noted 6 h after LPS administration ($P<0.05$). VIP treatment (LPS+VIP, group 4) decreased plasma concentrations of proinflammatory TNF- α and IL-6 in a

statistically significant manner, when compared with LPS group 2 ($P<0.05$) (Table 1). Mathiak *et al.* (20) have reported that LPS-induced IL-6 has the highest plasma concentration peak around 4–6 h. Immunomodulatory effects of VIP on TNF- α and IL-6 were clearly shown (6, 12). Delgado *et al.* (5) described inhibition of TNF- α and IL-6 production by VIP in LPS-stimulated cells. They also found in their *in vitro* and *in vivo* studies that VIP significantly reduced TNF- α and IL-6 mRNA accumulation in cells. In agreement with these reports, our study showed that when rats were challenged with LPS, a single dose of VIP (25 ng/kg) administered simultaneously with the challenge, markedly inhibited the levels of circulating TNF- α , and IL-6.

IL-10 is a potent and pleiotropic anti-inflammatory cytokine produced by lymphocytes and macrophages. It inhibits the synthesis of proinflammatory cytokines, such as IL-6 and TNF- α , by T helper type 1 cells, macrophages, and polymorphonuclear cells, and reduces T-cell activation *in vitro* and *in vivo* (8, 18). We determined a notable difference between the control group and LPS-stimulated group. VIP induced a significant up-regulation of IL-10. In the present study, the plasma level of the anti-inflammatory IL-10 was under the detectable level in the control group, but the cytokine-inductor LPS caused a marked increase in plasma IL-10 concentration. In the absence of endotoxin, VIP alone did not stimulate IL-6, TNF- α , and IL-10 cytokine releases. Delgado *et al.* (6) observed that VIP can only amplify the endotoxin-generated signal and suggests that the *in vivo* action of VIP may be restricted to cells which are actively involved in responding to LPS. Similarly to the effect on other cytokines, such as IL-6 and TNF- α , the stimulation of IL-10 requires intact VIP molecules. Our findings are consistent with the previous data. They found a modest but consistent stimulation of IL-10 in

LPS-stimulated PBMC cultures, or in mice challenged with LPS. (2, 5, 6).

In our study, APTT and PT were significantly prolonged in endotoxaemic rats 6 h after LPS administration. The injection of endotoxin caused a significant decrease in the fibrinogen level 6 h after the injection (Table 1). The LPS injection caused also a considerable decrease in the platelet count. Thrombocytopenia in experimental endotoxaemia is a constant feature and is thought to be a consequence of platelet aggregation in the lungs and other capillary beds, and of shortened platelet survival (4, 30). In endotoxaemic rats, the prolongation in APTT and PT is due to the consumption of several procoagulant clotting factors such as fibrinogen and factors II, V, VII, IX, and X. An impaired fibrinogen synthesis, resulting from the damage of hepatic tissue, caused increased consumption during DIC, loss into the extra vascular spaces, and massive haemorrhage, at the same time decreasing plasma fibrinogen concentration in endotoxaemia, as shown in the presented investigation (17, 21). In our study, haemostatic disturbances associated with endotoxin-induced DIC were not suppressed as required by treatment with VIP. Some experimental studies have reported that AT is a good predictor of mortality in sepsis patients (9, 27). In the present study, the observed decrease in plasma AT activity in LPS group is consistent with the results of previous *in vitro* and *in vivo* investigations (4, 30). However, the simultaneous administration of VIP and LPS in our endotoxaemic DIC model did not prevent the decrease in plasma AT activity. It is possible that plasma AT activity decreased due to consumption during coagulation, resulting in the inactivation by proteolytic enzymes (*e.g.*, neutrophil elastase), and extravascular leakage of this protease inhibitor as a consequence of capillary leakage (19).

The endotoxin-induced leukopenia is mainly mediated by TNF- α . This decrease is related to an increased adherence of neutrophils to endothelial cells. These adherent neutrophils cannot be detected anymore in the circulation (26). Neutropenia is followed by significant neutrophilia over the next several hours due to increased levels of activated complement products, G-CSF, and proinflammatory cytokines TNF- α , IL-1, and IL-6 (11). This may be caused by severe consumption of fibrinogen during coagulation and inactivation by proteolytic enzymes. The drastic diminution seen in the WBC count and the percentage of lymphocytes, together with an increase in the percentage of neutrophils after LPS administration was partially prevented by VIP (Table 1). This may be ascribed to inhibiting effect of VIP on leukocyte migration (13).

In harmony with earlier findings, LPS caused statistically significant increase in plasma AST, ALT, creatinine, cholesterol, and triglyceride concentrations (Table 2), however, it caused statistically significant decrease in total protein and albumin levels, compared to control group (1, 21, 32). In the absence of LPS (group 3), VIP administration did not change biochemical parameters except glucose, which increased significantly in the VIP-treated rats. It may be due to glycogenolysis produced by VIP through Ca and a

cAMP-dependent mechanism (24). LPS increased hepatic and renal damage markers and changed some lipid values, but VIP did not have any protective effect on the liver, kidneys, and lipid metabolism of rats, as judged from biochemical profile in this endotoxaemia model. LPS causes liver damage and loss of organ integrity, with subsequently results in the increase in AST and ALT levels in plasma (3, 16). We determined that the endotoxin reduced the blood urea, along with TP and albumin level 6 h after treatment. Al-Dughaym (1) reported that in endotoxaemia, the decrease of these metabolites may be attributed to hypovolaemia due to increased capillary permeability and reduced liver synthesis, or by the decrease in intestinal absorption. Several authors showed that in case of the glucose levels the initial rise to endotoxin is followed by hypoglycaemia (7, 1). Observation of an increase in serum cholesterol and triglycerides in endotoxaemia in previous studies confirms our present findings (10, 31). In endotoxaemia, tumour necrosis factor level is increased, and this increase may suppress lipolytic enzymes, which convert serum VLDL and triglyceride rich proteins to LDL and HDL. This suppression may raise serum levels of cholesterol, triglyceride, and VLDL (10). These increases were not suppressed by the administration of VIP.

In conclusion, at the administered dose and route, VIP has moderately therapeutic potential as an anti-inflammatory agent, suppressing TNF and IL6, and stimulating IL10; however, it has no useful effect as required by treatment with VIP on haematological and biochemical parameters. The effects of VIP may be dependent on various factors such as dose, administration route, and animal species. Further studies focusing on these issues are necessary to clarify the effects of VIP on haemostatic, haematological, and biochemical derangement observed in endotoxaemia.

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