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Calcitonin gene related peptide and *N*-procalcitonin modulate CD11b upregulation in lipopolysaccharide activated monocytes and neutrophils

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Abstract *Objective:* Circulating levels of calcitonin gene related peptide (CGRP) and calcitonin precursors, including procalcitonin (PCT) and its free aminopeptide *N*-procalcitonin (N-PCT), have been found dramatically increased in septic patients. PCT is known to attenuate the chemotaxis of monocytes in response to chemoattractants. This study examined whether CGRP and N-PCT modulate the LPS-induced expression of CD11b, which is one of the major integrins involved in monocyte and neutrophil chemotaxis during a response to microbial infections. *Design and setting:* In vitro cell culture study in the immunology laboratory of a university hospital. *Participants:* Healthy volunteers. *Measurements and results:* We assessed the effects of N-PCT and CGRP on CD11b expression on monocytes and neutrophils after LPS (2 ng/ml) or fMLP (10^{-8} M) challenges. We used a human whole blood model, and measurements were made by flow cytometry. Both peptides in a dose-dependent manner decreased the LPS- and fMLP-induced rise in CD11b in monocytes and neutrophils. As these peptides are thought to act by raising cAMP, we also mimicked their effects with the use of rolipram and forskolin and found similar results. *Conclusions:* These findings are in line with recent studies demonstrating anti-inflammatory properties for this family of peptides. CGRP and calcitonin precursors may function as factors suppressing the propagation of inflammation through the inhibition of several processes involved during a response to a bacterial stimulus.

Keywords Sepsis · Chemotaxis · Procalcitonin · N-Procalcitonin · Calcitonin gene related peptide · CD11b

Introduction

Despite major advances in critical care medicine and many clinical trials mortality from sepsis and septic shock remains dramatically high [1]. It is now well established that the initial systemic inflammation of this syndrome is rapidly counterbalanced by an anti-inflammatory response which in some patients provokes an excessive immunoparalysis [2, 3]. This would partly explain the failure of trials using anti-inflammatory drugs since inhibiting the immune response may have been the contrary of what patients needed [4, 5]. Immunoparalysis is characterized mainly by a functional monocytic deactivation, a shift of T lymphocytes toward a type 2 profile, a rise in apoptotic processes, and a failure of neutrophil chemotactic function [2, 3, 4, 6].

Another difficulty in this context is to distinguish sepsis from other noninfectious conditions on the basis of nonspecific clinical signs (fever, tachycardia, hypotension) and then to classify patients presenting very heterogeneous causes, courses of diseases, and stages of immune responses [7]. Therefore there is still a need for improved biological markers of sepsis and for a better characterization of immune status. Circulating levels of

calcitonin gene related peptide (CGRP) and calcitonin precursors, including procalcitonin (PCT) and its free aminopeptide *N*-procalcitonin (N-PCT), have been found dramatically increased in response to microbial infections, and in many cases these rises are correlated with severity and mortality [8, 9, 10, 11, 12, 13, 14). Hence they have been proposed as reliable markers of sepsis. Of these peptides PCT has been the most studied in clinical situations, and there is now a growing body of works supporting its use [7, 15]. Nevertheless, much remains to be learned and widespread adoption of PCT in monitoring septic patients would be premature since we are lacking fundamental data about PCT, and because well-designed multicenter studies have yet not been conducted [16]. Increased PCT has also been reported independently of any bacterial stimulus in clinical situations in which tumor necrosis factor-α (TNF) is massively released [16, 17]. On the basis of these observations some authors have demonstrated that the elevated serum levels of PCT are directly induced by TNF [18, 19].

Consequently we hypothesized that calcitonin precursors regulate the TNF release. This point has been partly demonstrated by showing that CGRP, PCT, and N-PCT (which seems to be the fragment responsible for the biological effect of PCT) significantly decrease lipopolysaccharide (LPS) induced TNF production in a human model [20]. Taken together these data indicate that in terms of time course and modulating TNF-release property, these peptides may participate as anti-inflammatory mediators in sepsis-induced immunoparalysis. Although PCT and calcitonin may under certain conditions be monocyte chemoattractant, it has also been reported in the same study that these peptides deactivate migration of monocytes in response to chemoattractants [21]. Considering that altered leukocyte chemotaxis is an important feature of immunoparalysis, the objective of the current study was to determine whether CGRP and N-PCT modulate the upregulation of the CD11b integrin, which constitutes a critical step in monocyte and neutrophil chemotaxis during a response to microbial infections.

Methods

Whole blood model

Blood samples from healthy volunteers were collected into endotoxin-free heparin tubes (Chromogenix, Mölndal, Sweden). Whole blood was diluted 1/2 in RPMI 1640 (Life Technologies, Paisley, UK) supplemented with 2 mM glutamine. We used LPS (2 ng/ml) and *N*-formyl-methionyl-phenylalanine (fMLP; 10^{-8} M) as activation inducers, both products from Sigma (St Louis, Mo, USA). Blood was incubated in ultra low attachment Costar plates (Corning, N.Y., USA). CGRP (10^{-6} to 10^{-9} M, Sigma), N-PCT (10^{-6} to 10^{-9} M, Bachem, Bubendorf, Switzerland), rolipram (10^{-5} M, phosphodiesterase-4 inhibitor, Sigma), and forskolin (10^{-5} M, adenyl cyclase activator, Sigma) were added 5 min prior to the addition of LPS or fMLP. Blood was incubated at 37°C for either 15 min (fMLP stimulation) or 30 min (LPS stimulation). In all cases the reaction was terminated by the addition of ice-cold phosphate-buffered solution (Gibco BRL, Grand Island, N.Y., USA) and centrifugation. Cells were then incubated for 30 min at 4°C in a dark chamber with various labeled antibodies. Finally, samples were lysed and fixed using the Q-Prep system (Beckman-Coulter, Hialeah, Fla., USA).

Antibodies

The following monoclonal antibodies were purchased from Immunotech (Marseille, France): phycoerythrin-cyanin-5 conjugated anti-human CD45, fluorescein isothiocyanate conjugated anti-human CD14, phycoerythrin conjugated anti-human CD11b and used according to manufacturer's recommendations.

Flow cytometry

Samples were analyzed on a Coulter EPICS XL flow cytometer (System II software, Beckman-Coulter) which was calibrated daily with Flow-Check fluorospheres (Beckman-Coulter). After debris was excluded by means of a leukogate [22] we initially defined a region with side scatter characteristics and CD45 expression which comprised monocytes along with some lymphocytes. Monocytes were gated out from other cells on the basis of labeling with fluorescein isothiocyanate–anti-CD14 [23]. Neutrophils were commonly isolated on a sideward/forward scatter dot plot. We then focused only on these gated populations in which we examined CD11b expression. The results were measured as the means of fluorescence intensities related to the entire populations of monocytes or neutrophils and expressed as the percentage of the effect induced by either LPS or fMLP or as the percentage of the maximal inhibitory effect of N-PCT (dose-response experiments).

Statistical study

Due to the sample size the nonparametric Wilcoxon paired test (for nonnormalized data) was used to assess the effect of peptides.

Results

Flow cytometry allows simultaneous measurement of neutrophils and monocytes while at the same time minimizing the artifactual activation that occurs during purification. We first determined a 90-min time-course of LPS-induced changes in the CD11b expression (data not shown). On the basis of these data we decided to work at 30 min after LPS addition since this time point exhibited the maximal rise in monocytic CD11b (60% increase above control value) as well as a significant increase in neutrophils. CGRP and N-PCT (at 10^{-6} and 10^{-7} M) had no direct effect on CD11b expression. However, they decreased in a concentration-dependent manner the LPS-induced CD11b upregulation (Fig. 1). Maximal inhibitory effects were obtained with peptide concentrations of 10^{-6} M (Table 1). To confirm that the previous results were not restricted to the response induced by LPS we also determined the effects of peptides after stimulation by fMLP which is a typical chemoattractant. We first determined that 10^{-8} M fMLP was the lowest concentration

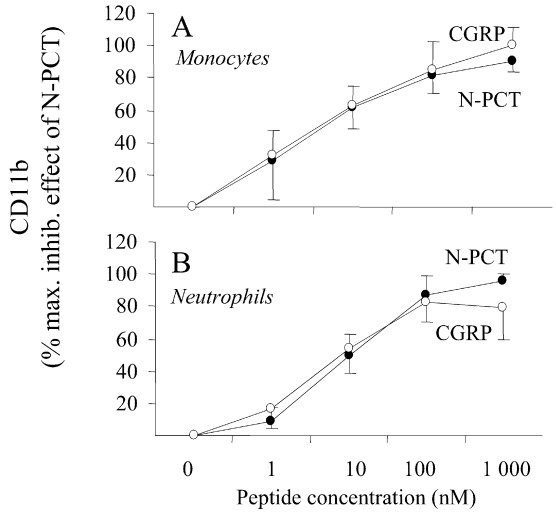

Fig. 1 Effects of CGRP and N-PCT on CD11b elevation in monocytes (**A**) and neutrophils (**B**) after an exposure to LPS. Cells were incubated for 30 min with LPS (2 ng/ml) following preincubation for 5 min with either CGRP or N-PCT. CD11b expression (means of fluorescence intensities) is shown as mean ±SE and expressed as percentages of the maximal inhibitory effect of N-PCT (n=6)

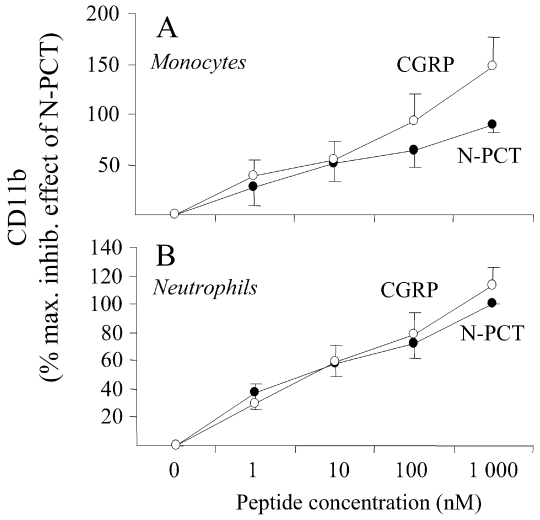

Fig. 2 Effects of CGRP and N-PCT on CD11b elevation in monocytes (**A**) and neutrophils (**B**) after an exposure to fMLP. Cells were incubated for 15 min with fMLP (10^{-8} M) following preincubation for 5 min with either CGRP or N-PCT. CD11b expression (means of fluorescence intensities) is shown as mean ±SE and expressed as percentages of the maximal inhibitory effect of N-PCT (n=6)

Table 1 Maximal effects of calcitonin gene-related peptide and N-procalcitonin on CD11b upregulation. Results are expressed as percentages of controls (means ±SE) of the maximal response after LPS or fMLP stimulation. Experiments were performed on whole blood from six different donors. In three additional experiments we checked the inhibitory effects of cAMP inducers (rolipram and forskolin) on CD11b elevation in neutrophils after stimulation by fMLP (*nd* not done)

	Monocytes	Neutrophils
LPS (2 ng/ml)	100	100
LPS + N-PCT (10^{-6} M)	70±11*	43±10**
LPS + CGRP (10^{-6} M)	61±11**	46±17**
fMLP (10^{-8} M)	100	100
fMLP + N-PCT (10^{-6} M)	65±20*	80±6*
fMLP + CGRP (10^{-6} M)	57±19*	80±5*
fMLP (10^{-8} M)	nd	100
fMLP + rolipram (10^{-5} M)	nd	69±7
fMLP + forskolin (10^{-5} M)	nd	58±6
fMLP + rolipram + forskolin (10^{-5} M)	nd	27±6

*p<0.05, **p<0.02 (Wilcoxon paired test on nonnormalized data)

that allowed a maximal increase in the expression of CD11b on both monocytes and neutrophils (data not shown). Our results indicated that both N-PCT and CGRP also decreased in a concentration-dependent manner the elevation of CD11b expression on monocytes and neutrophils after fMLP challenge (Fig. 2). However, in neutrophils their maximal effects were less pronounced than their inhibition on the LPS-induced CD11b increase (Table 1). It is noteworthy that whatever the magnitude of the peptide effects (i.e., on either LPS or fMLP stimulation, either on neutrophils or monocytes), the median effective concentrations (EC_{50}) were similar, ranging between 10^{-9} and 10^{-8} M (Figs. 1, 2). Such concentrations are equivalent to those described in patients with septic shock (as example for PCT, 10^{-8} M: 130 µg/l).

As CGRP and PCT are thought to act by raising adenosine 3′-5′-cyclic monophosphate (cAMP) levels [21, 24], we finally investigated whether cAMP inducers such as the phosphodiesterase-4 inhibitor rolipram or the adenylyl cyclase stimulator forskolin mimic the effects of peptides on neutrophil CD11b response. Indeed, in three additional experiments these two compounds impaired CD11b upregulation in response to fMLP (Table 1).

Discussion

The calcitonin gene peptide superfamily consists of CGRP, calcitonin, adrenomedullin, and amylin. Together, these peptides have similarities: bioactive precursors, overlapping biological actions, common structural features that are essential for their effects, and cross-reactivity between receptors [25]. Furthermore, CGRP and calcitonin are tissue-specific alternative transcripts of the same gene, *Calc-1* [26]. Normally CGRP is a 37 amino acid neuropeptide produced in the nervous system in a tissue-specific manner, while calcitonin is specifically generated by thyroid C-cells [26]. In contrast, under septic conditions this tissue-selective expression pattern seems to be overridden, and mRNA of calcitonin precur-

sors and CGRP are widely expressed throughout the body [27, 28, 29]. In such a systemic inflammatory context little is known about the effects of these peptides. However, there is a growing body of evidence supporting the fact that CGRP is a potent anti-inflammatory mediator. Briefly, CGRP is thought to inhibit type 1 cytokines, for example, interleukin 12 [30] and interferon γ [31], and to enhance interleukin 10 production [31], one of the most immunosuppressive cytokines. Targeted expression of CGRP to pancreatic β cells prevents diabetes (type 1) in nonobese diabetic mice [32]. CGRP also decreases LPS-induced TNF production in rats [33] as well as in humans [20]. As there are several reports that circulating levels of CGRP and calcitonin precursors are increased in sepsis [8, 9, 10, 11, 12, 13, 14], it is tempting to hypothesize an involvement of these peptides in sepsis-induced immunoparalysis.

In the current study we asked whether CGRP and N-PCT modulate CD11b elevation in response to known chemoattractants. We showed that both peptides have no direct effect but modulate with the same potency LPS- or fMLP-induced CD11b up-regulation on neutrophils and monocytes. These effects seemed to be concentration dependent, which suggests a receptor-dependent mechanism in their actions. Furthermore, EC_{50} values ($<10^{-8}$ M) were similar to those reported in patients with septic shock. Due to the CGRP-receptor intracellular pathway, known for inducing cAMP [24], our results on CD11b expression appear quite consistent with the demonstration that increase in cAMP levels impairs granulocyte CD11b response. The phosphodiesterase-4 inhibitor rolipram has been shown to inhibit cell migration and CD11b expression in response to different stimuli [34, 35, 36]. Stimulation of adenylate cyclase with forskolin [36] or isoproterenol [34] or addition of dibutyril cAMP [34] had similar effects. Indeed, we have been able to modulate CD11b elevation by use of rolipram or forskolin in our model. These findings are in line with the results of recent study by Wiedermann et al. [21] which demonstrated that PCT stimulates cAMP production in monocytes dose-dependently and thus decreases their migration toward typical attractants such as fMLP or RANTES. Consequently regulation of CD11b increase could be one of the mechanisms by which PCT attenuates monocyte chemotaxis. Interestingly, the study by Saito et al. [37] also supports this view by showing that adrenomedullin (which belongs to the calcitonin gene peptide superfamily) suppresses fMLP-induced up-regulation of CD11b on human neutrophils. This inhibition was partly abolished by a CGRP receptor antagonist or by the addition of an adenylate cyclase inhibitor. The consequences of the up-regulation of CD11b expression on monocytes and neutrophils are numerous. Increased CD11b expression is an early event implicated directly in mediating migration of cells into the area of inflammation as well as cell to cell communication and thus in playing a significant role in host defense and inflammatory response. In addition, patients lacking CD11b present with recurrent and life-threatening bacterial infections [38], and the failure of neutrophil chemotactic function is suggested to be correlated with a poor prognosis in sepsis-induced immunoparalysis [6].

In addition to the results presented in the current study and those of Wiedermann et al. [21] mentioned above, several other works have also described anti-inflammatory properties of PCT. It is thought to diminish the LPS-induced TNF release in various models [20, 39, 40], and to decrease LPS-induced interleukin 12 and interferon γ release while increasing interleukin 10 production in a human whole blood model [41]. PCT modulates inducible nitric oxide synthase gene expression and nitric oxide synthesis [42]. Addition of PCT leads in vitro to inhibition of arachidonic acid-induced prostaglandin and thromboxane synthesis [39]. Finally, Whang and colleagues [43] have shown in a model of septic hamsters that PCT slightly decreases interleukin 1 release at the time of peak secretion. These different effects might explain why neutralization of PCT increased survival in an animal model of sepsis [44]. Given the time-courses after endotoxin, TNF or OKT3 antibodies injections [16, 17, 18, 19, 45] where PCT peaks followed (or were induced by) high TNF levels, PCT is likely released in response to an elevation in inflammatory cytokines. In addition, its synthesis after endotoxin administration in humans is modulated when soluble TNF receptors are given before LPS injection [46]. In accordance, no study has shown a direct effect of PCT in control conditions, suggesting that PCT is only a secondary mediator that requires a primed inflammatory context to exert its effects.

Two articles investigating the role of calcitonin precursors were published after submission of the present study. In contradiction to their previous study, Hoffmann et al. [47] reported that PCT amplified inducible nitric oxide synthase when added 3 h after a bacterial challenge, raising the question of the timing of PCT administration in such in vitro experiments. Interestingly, Kaneider et al. [48] reported that pretreatment of $CD14^+$ mononuclear cells with PCT, calcitonin, and katacalcin deactivated their chemotactic response to fMLP by a mechanism involving a rise in cAMP. On the other hand, they have also shown that N-PCT had no effect in their model, either on chemotaxis or on cAMP production. Consequently the effect of N-PCT in our model warrants further investigation to determine its mechanism of action since the latter was shown to be cAMP independent in their experiments.

Collectively, these data suggest that CGRP and calcitonin precursors function as modulators of inflammation by their action on several processes involved during a response to a bacterial stimulus. Although PCT is surely

not *the* mediator of immunoparalysis, especially in comparison to the properties of interleukin 10 or TGF-β, it should be reinvestigated in the light of all these recent findings. In particular, a correlation between high PCT levels and the severity of immunoparalysis, as measured by monocytic HLA-DR expression [4], must be established. Indeed, PCT could bring a dual information in monitoring septic patients since it is both a good marker of sepsis as well as a potential marker of pro-/anti-inflammatory disorders.

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