Shock, Publish Ahead of Print

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In trauma patients, the occurrence of early-onset nosocomial infections is associated with increased plasma concentrations of chromogranin A

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Abstract

In previously healthy persons suffering from acute illnesses, nosocomial infections are frequent. Their prevalence suggests the existence of as yet unknown conditions that may promote care-related infection. This study assessed whether 1) the measurement of plasma chromogranin A, a stress-related protein involved in innate defence, is related to nosocomial infection risk and 2) whether any chromogranin-A-derived fragment included in vasostatin-I displays immunosuppressive activities related to AP-1 or NF-kappa B down regulation. At the clinical level, trauma patients and healthy controls were recruited to beeligible. Clinical histories were recorded, and standard biological tests (including plasma chromogranin A) were performed. For 9 randomly chosen patients and 16 controls the time-dependent concentrations of chromogranin A was assessed twice a-day over 66 h. The data show that trauma patients present a higher value of chromogranin A concentration during 66h incomparison with healthy controls. In addition, patients maintaining this significant increase in CGA readily develop nosocomial infections. We therefore studied the effects of chromogranin A-derived peptides on monocytes, focusing on transcription factors which play a central role in inflammation. *In vitro* assay demonstrated that a chromogranin A-derived fragment (CGA_{47-70}) displays a significant inhibition of NF-kappa B and AP-1 transcriptional activities in these cells. In conclusion, the occurrence of nosocomial infection in trauma patients is associated with significantly increased plasma chromogranin A concentrations. Down regulation of the two transcription factors by CGA_{47-70} might induce early acquired immune defect after a serious medical stress.

Keywords: chromogranin A; immunosuppression; infection; Monocyte; NF-kB; trauma; vasostatin-I.

Introduction

Nosocomial infections (NI), also often called care-related infections, still pose a major problem as recently confirmed in a large investigation(1). Their prevalence is now around 5% in hospitalized patients, and up to 19% in critically ill patients. In the absence of either proper hygiene or immune suppressive drug administration, clinicians readily accept immune paralysis as a cause of their occurrence (2-4). NI are also thought to be associated with transient immune cellular defects(5)whose improvement is expected to ameliorate the outcome of the initial disease. The high prevalence of NI in previously healthy persons undergoing a major medical stress suggests the existence of multifactorial mechanisms able to trigger common innate immune defects that favour the occurrence of care-related infections. Multiple trauma provides an attractive model to study the time-dependent immune consequences of an acute and severe disease (early after its onset)for two reasons: 1) the exact beginning of the stress is usually defined; 2) trauma frequently occurs in healthy young persons, which excludes confounding factors such as associated diseases or treatments. As in other severe injuries, the immune response to multiple trauma includes not only a severe hyper inflammatory response called systemic immune inflammatory response syndrome (SIRS) but also immune paralysis often leading to NI (1-5).

Taking into account that: 1) some transient innate defence dysfunction may be related to the innate immune system(6 ,7) and 2) severity of the shock could be a factor involved in the occurrence of NI, we focused our attention on chromogranin A in the plasma (pCGA)(8). CGA is a protein shown to connect the stress-activated adrenal medulla and immunity (9), and pCGA is a predictor of severity in critically ill patients (9). More recently, pCGA was evaluated after burn trauma showing that high concentration predicts organ dysfunction (10). CGA is enclosed in storage vesicles of chromaffin cells from the adrenal medulla and released with catecholamines upon splanchnic stimulation *in vitro* and *in vivo*(11). This acidic protein is also present in neutrophils(12) and under the action of proteases, it is

converted in numerous peptides with physiologically relevant functions (13, 14). VS-I (CGA_{1-76}), the major natural CGA-derived peptide (13) has already been recognized as a biomarker of severity in acutely ill patients (15).

In this study we postulated that CGA processing would play a role in driving some depression in monocytes'responses following trauma-related injury. We used both the clinical setting of trauma in humans and an *in vitro* model of NF-kappa B and AP-1 responses in genes up regulation in monocytes. Our data demonstrate a previously unidentified role of CGA in the occurrence of nosocomial infection through one of its derived peptide on immune cells. These finding support the notion that preventing the proteolysis of CGA may attenuate the immune depression following trauma.

Materials and Methods

1. CGA assessment in plasma from trauma patients

This study was approved by our institutional review board for human experimentation. Over a period of one year, trauma victims requiring critical care were prospectively screened and included in this study if formal consent for participation could be obtained from the patient or next of kin. Exclusion criteria were: age under 18 and known reason for increased CGA release (*i.e,* steroid treatment, neuroendocrine tumor,…), independently of acute stress. Mortality was defined as death occurring before Day 28 after admission. Diagnosis of NI was based on classical criteria: the early-onset NI used in our study were infections detected after 48 h of hospital admission in patients without previous contact with healthcare services and not related with disease incubation that could have started before admission (16).

Organ failure scores were assessed during the first days after admission (SAPS II, ISS, SOFA, delta Sofa max). Healthy controls were staff.

Preparation of blood samples

Blood samples were collected as early as possible within 6 h of admission by vascular puncture into plasma-separator tubes (Becton Dickinson, France), immersed in ice or stored at 4°C until transported to the laboratory. Plasma was separated by centrifugation at 1500 g for 10 min at room temperature and stored in 200 µL aliquots at –20°C until analysis. In 9 randomly chosen patients, one tube was further collected every 12 h after the first sampling for 3 days to study the kinetic of the plasma CGA concentration.

Laboratory assays

The CGA assay is a sandwich ELISA (Cisbio Bioassays, France) with 2 monoclonal antibodies against human CGA amino acid sequences 145-197 and 198-245 (17). Procalcitonin concentration was measured on the Kryptor system (Brahms Diagnostic) according to the assay manufacturer's recommendations; C-reactive protein was measured by immune turbidimetry, creatinine using an enzymatic method (Siemens ADVIA, Paris, France) and lactate in whole blood using a lactate oxydaseamperometric method (Roche, Cobas b221, Germany).

2. In vitro studies on monocytes

Cell culture

The human monocytic THP-1 cell line (ATCC) was maintained at 37°C, under 5% CO_2, in RPMI supplemented with 10% foetal calf serum and 2 mM L-glutamine.

Preparation of synthetic rhodaminated peptides

Rhodaminated synthetic peptides Rho-CGA_{47-70} and Rho-CGA_{7-40} were prepared on an Applied Biosystem 433A peptide synthesizer (Foster City, USA), using the stepwise solid-phase approach with 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. Thereafter, the synthetic peptides were purified by RP-HPLC on a Macherey Nagel Nucleosil RP 300-7C18 column (10x250 mm; particle size 7 µm and pore size 100 nm). Rhodamine fluorophore 5(-)-carboxytetramethyl rhodamine was conjugated with

peptides at the N-terminal end as previously described (18). Synthetic peptides were analysed by mass spectrometry and automated Edman sequencing on an Applied Sequencing System (Applied Biosystems, Foster City, USA). Maldi-Tof mass measurements were carried out on an Ultraflex^{TM} TOF/TOF (Bruker Daltonics, USA).

Florescence microscopy

THP-1 cells were seeded at 1x10^6 cells/mL and treated with 20 µM of Rho-CGA$_{47-70}$ or Rho-CGA$_{7-40}$ for 5 min or 15 min. Cells were then washed twice with cold PBS. Using a cytospincytocentrifuge (CytoSpin 4 Cytocentrifuge, Thermo Fisher), 2x10^5 cells were deposited onto a glass slide and fixed for 20 min at room temperature with 4% paraformaldehyde. Finally, coverslips were mounted onto the glass slides using Vectashield® Mounting Medium with DAPI (Vector Laboratories). Images were acquired using a fluorescence microscope (Nikon Eclipse TE200) at 60X magnification.

Luciferase assays

THP-1 were transiently transfected with a pNF-kappa B-Luc (a generous gift from Prof. Carine Van Lint, Université Libre de Bruxelles, Belgium) or pAP1-Luc reporter plasmids (Stratagene) using Lipofectamine™ LTX (Invitrogen). Twelve h post-transfection cells were treated with 20 µM of CGA$_{47-66}$ for 1 h, 6 h or 24 h. Cells were then washed twice, lysed and luciferase activities were measured (Dual Luciferase® Reporter Assay System, Promega). The assay was normalized to total protein content (Bradford assay).The first control,with a relative luciferase activity of 1, corresponds to transfection by pNF-kappa B-Luc or pAP1-Luc reporter plasmids before treatment with peptide. Negative control corresponds to the treatment with CGA$_{7-40}$.

3. Statistics

Variables were expressed as medians (interquartile range [25; 75]) or means +/- SD as indicated. Differences between continuous variables were tested using the Mann-Whitney test; differences between categorical variables were tested using Fisher's exact test. Univariate analyses were performed

using a non-parametric analysis of variance by the Kruskal Wallis test, followed by Scheffé's method analysis. When necessary, Anova for repeated non-parametric measures was used. Receiver-operating curves (ROCs) were constructed for biological biomarkers at the best cut-off level to predict NI occurrence, and the areas under the curve (AUC) were assessed and then compared using the Z statistic with correction for the correlation introduced by studying the same sample. The threshold of statistical significance was $p<0.05$.

Results

1. In vivo assessments

Samples were obtained from 31 patients among 40 screened within 6 h from admission (Figure 1). Nine patients were excluded because (i) they chronically took proton pump inhibitors (n=4), (ii) no consent was obtained (n= 2) or (iii) they died within 2 days (n= 3). The baseline characteristics of the study population are presented in Table I. None of the patients had previous significant medical history. The patients suffered predominantly from blunt trauma (n= 28) due to motor vehicle accidents, a fall from a great height or gunshot (n=3). The length of stay in ICU ranged from 2 to 40 days (mean 13+/-11 days). The entirecohort required mechanical ventilation and circulatory support with norepinephrine. None needed renal support with haemodialysis over the first 3 days. Surgical intervention under general anaesthesia was required immediately after admission in 22/31 patients, and 100% received blood transfusion in the first 24 h (mean transfusion requirement of 8 +/-15 units). Ten patients died within 28 days (mortality among study population 32%) either from intracranial hypertension (n=7) or from multiple organ failure subsequent to haemorrhagic shock (n=3). After Day 2, 11/31 patients (35%) developed NI. Among these patients, 9 were randomly chosen for a longitudinal survey of pCGA concentration over 66 h after admission (Figure 2) and demographic and clinical characteristics (Table II).

Plasma release of CGA after trauma

After the initial injury, the first concentration of CGA was significantly increased compared with healthy controls (112·3 [88·73; 144·3] ng/ml *vs* 19·5 [2·3; 36·6] ng/ml, $p<0·0001$) (Table I). As indicated in Figure 2, in a subset of 9 patients undergoing serial sampling, from its initial peak, the concentration of CGA showed a progressive decrease but remained significantly higher than the control values throughout the survey ($p<0·001$).

Nosocomial infection occurrence according to plasma CGA concentration at admission

Admission values of CGA were found to be significantly higher in those patients who developed NI (NI+) (111·1 [87·28; 149·0] ng/ml vs 63·08 [33·67; 204·7] ng/ml in patients without infections (NI-), p=0·003 (Figure 3). Using ROC curves, CGA concentration at admission predicted the occurrence of NI with a sensitivity of 100% and a specificity of 70% at the level of 67·25 ng/ml in the whole study population (n=31) (data not shown). AUC for this prediction was 0·837 (95% CI: 0·67-0·94), which was significantly greater than AUC for C reactive protein (0·557, 95% CI: 0·377-0·726, p=0·04) but no different from the AUC for procalcitonin (0·705, 95% CI: 0·524-0·848, p= 0·243). At the same CGA concentration, the positive likelihood ratio of the prediction was 3·33, whereas the negative likelihood ratio was zero.

2. In vitro tests

Vasostatin-I (VS-I; CGA_{1-76}) (Figure 4) corresponds to the predominant natural CGA-derived fragment (13) and is highly conserved during evolution (12). In a previous study, we reported that significant amounts of VS-I are detected on admission in critically ill patients and that a plasma concentration above 3.97 ng/ml is associated with poor outcome (15). Since VS-I has never been reported as interacting with immune cells, we decided to test whether VS-I derived fragments have the ability to impact on inflammation. The first peptide we tested corresponds to $CGA_{7-40,}$ (Figure 4)which, as has

previously been reported, binds biological membranes with the disulfide bridge (19). The second peptide we tested is $CGA_{47-70,}$ including chromofungin (CGA_{47-66}) which has already been described as a cell-penetrating peptide (18, 20) (Figure 4).

This *in vitro* study includes 2 parts (1) the confocal microscopy of the interaction of the 2 rhodaminated peptides with THP1 cells and (2) the effects of CGA_{47-70} against NF-kappa B and AP-1 activities.

Confocal microscopy

We investigated the ability of Rho-CGA_{47-70} and Rho-CGA_{7-40} to penetrate the outer membrane of THP-1 cells, using a human monocytic cell line as a model. The cellular internalization was visualized *via* confocal microscopy after 5 min and 15 min of treatment. Figure 5A indicates that CGA_{47-70} was significantly internalized by cells, in line with its positive charges. The fluorescence was predominantly detected at 5 min and 15 min at the nuclear level. We note an increase of the nuclear fluorescence after 15 min as compared with after 5 min (Figure 5A). Fluorescence was also detected at the perinuclear region with a lower level. In contrast, fluorescence of Rho-CGA_{7-40} could not be observed within cells- (Figure 5B) showing a specific behaviour forRho-CGA_{47-70}.

CGA_{47-66}inhibits the pro-inflammatory transcription factors NF-kappa B and AP-1

We carried out luciferase assays and THP-1 cells were then transfected with the NF-kappa B-Luc or AP-1-Luc reporter construct and incubated with CGA_{47-70} for 1 h, 6 h and 24 h. Surprisingly, CGA_{47-66} was able to inhibit both NF-kappa B and AP-1-mediated transcription in a time-dependent manner (Figure 6, columns 2–4 and columns 6-8, respectively). To be more precise, it was capable of inhibiting 95% of NF-kappa B activity and 70% of AP-1 activity at 24 h post-treatment (Figure 6, column 4 and 8, respectively). As NF-kappa B and AP-1 play a critical role in amplifying and perpetuating the inflammatory process by controlling the expression of numerous inflammatory genes (21), these results collectively reveal an anti-inflammatory potential for CGA_{47-66}.

Discussion

In our study population of multiple trauma patients, CGA levels were higher at admission and throughout the first 66 h after injury. Also, the plasma concentrations of CGA were significantly higher when care-related infection occurred. The stress induced by a life-threatening disease is thought to be responsible for the release of CGA within the plasma (9, for review); herein, the early damage control by surgery may also have partially contributed to such an increase (22). These data raise the question of a possible mechanism through which CGA and its endogenous fragments may contribute to a possible immunosuppression and the development of infection in trauma patients that are at risk of NI (4). Among the endogenous fragments, VS-I (CGA_{1-76}) is predominantly produced and CGA_{47-66} is able to penetrate into immune cells (20). One recent report demonstrates that orally given VS-I protects mice against inflammatory colitis by reducing the cytokine-induced increase of permeability of intestinal epithelial cells and by promoting healing of injured cells (23). Production of CGA_{47-66} containing fragments may occur within the plasma or at cell surfaces by enzymes that are up regulated after a life-challenging trigger. As a consequence of acute illness, several proteolytic enzymes are activated and up-regulated in a time-dependent manner in injured tissue and in circulating mononuclear cells when full-blown SIRS is occurring (24): peptidases, ADAMTS proteins and matrix metallo proteases(25-27). In addition some strains of *Staphylococcus aureus* release a glutamyl endopeptidase, able to break a bond after a glutamic residue and may therefore contribute to the production of several fragments such as CGA_{47-60} and CGA_{47-70} (18). Interestingly, numerous patients of ours developed care-related lung infection due to *S. aureus,* which may induce the production of CGA-derived fragments able to trigger or to increase an immune defect.

To further characterize such an immune defect we report that CGA_{47-70}, enters readily the monocytes, whereas its control peptide (CGA_{7-40}) does not. CGA_{47-66} is a cell-penetrating peptide, as shown previously by our group (18, 20) and *in vitro* we showed that these cells exposed only to the CGA_{47-70}

peptide display a rapid onset but long-lasting decrease of both NF-kappa B and AP-1 activities (Figure 6). In contrast, another VS-I-derived peptide, (CGA_{7-40}), was not able to modify the level of activity of these two transcriptional factors. These results suggest a subsequent down-regulation of their target genes (28), which are critical for the fine regulation of the balance between pro- and anti-inflammatory factors. In a recent paper it has also been reported that a cell-penetrating domain of human beta-defensin 3 (hBD3-3) displays anti-inflammatory activity (29). Comparison of the sequences of CGA_{47-70} andhBD3-3 is reported in Figure Supp. 1, Supplemental Digital Content 1, http://links.lww.com/SHK/A655. The authors demonstrated that hBD3-3 downregulated nuclear factor kappa B-dependent inflammation by suppressing the degradation of phosphorylated-I kappa B alpha and by down regulating active nuclear factor kappa B p65. As far as our peptide is concerned, CGA_{47-66} has been shown to develop anti-calcineurin activities (20) resembling those of cyclosporin and FK 506 (30), two immuno-suppressive drugs.

Our study has limitations: 1) we have used a small and specific population (ie: multiple trauma patients) and 2) different stresses may induce CGA processing in relation with the previous medical history of patients.

In conclusion, we speculate that *in vivo* the occurrence of nosocomial infections might be related with the VS-I processing into smaller fragments but the relevance of action of CGA_{47-66} needs further investigation of its role *in vivo*. These data support the need to rethink the concept of some forms of endogenous healthcare-associated infections: some of them may not be iatrogenic, but a consequence of immune deficiency due to severity of initial injury.

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Legends for figures

Figure 1: Flow chart of the study

Please note that only 31 out of 34 screened could be studied for nosocomial infection (NI) because 3 out of the 34 patients did not survive more than 48H.

NI^+: patients that developed care-related infection

NI^-: patients that did not develop care-related infection

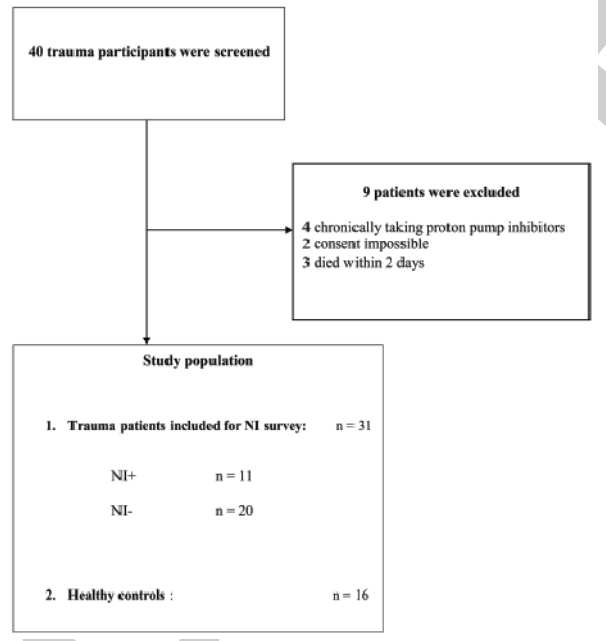

Figure 2: Admission CGA in trauma patients and follow up over 66 h

Data are medians +/- [IQ 25; 75] in randomly chosen patients (n = 9) and controls. Note that the time-dependent changes of CGA never decrease beyond control values during the survey. H corresponds to admission time. * $p < 0{\cdot}001$

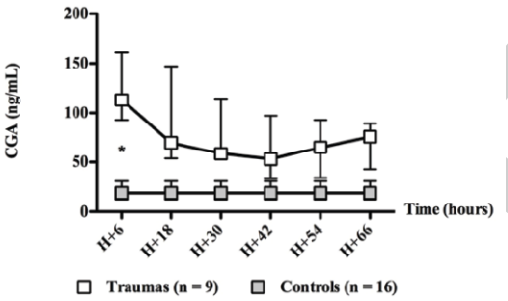

Figure 3: Admission CGA values according to the secondary development of nosocomial infection (NI+) or its absence (NI-)

Data are medians +/- IQ [25; 75] in patients with (n=11, NI+) or without (n=20, NI-). * $p = 0{\cdot}003$

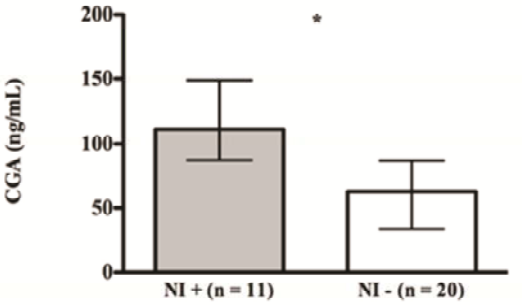

Figure 4: Sequence of vasostatin-I (CGA1-76) and the derived peptides CGA_{7-40} and CGA_{47-70} (UniProtKB, P10645).

Black arrows indicate where corresponding enzymes cleave CGA_{47-70} from vasostatin-I.

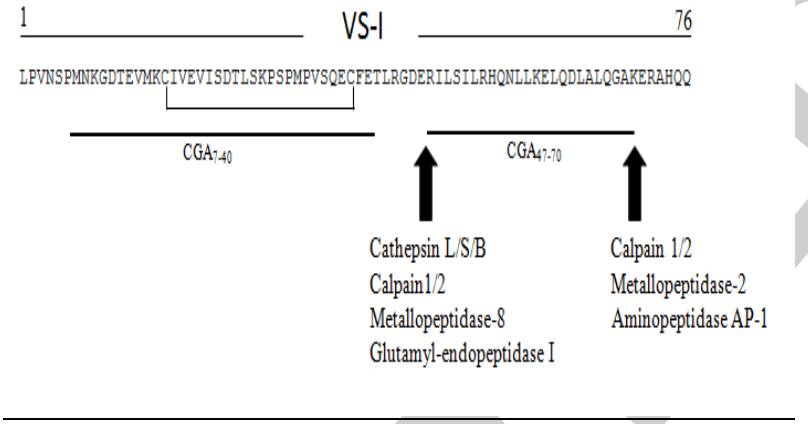

Figure 5: Intracellular translocation of CGA_{47-70} *in vitro* into HTP-1 cells**.**

Micrographs of THP1 cells incubated with Rho-CGA_{47-70} **(A)** or Rho-CGA_{7-40}**(B)** for 5 min or 15 min. THP-1 nuclei were stained with DAPI. Images depicting cellular peptide localization were obtained by fluorescence microscopy. Scale bars equal 10µm.

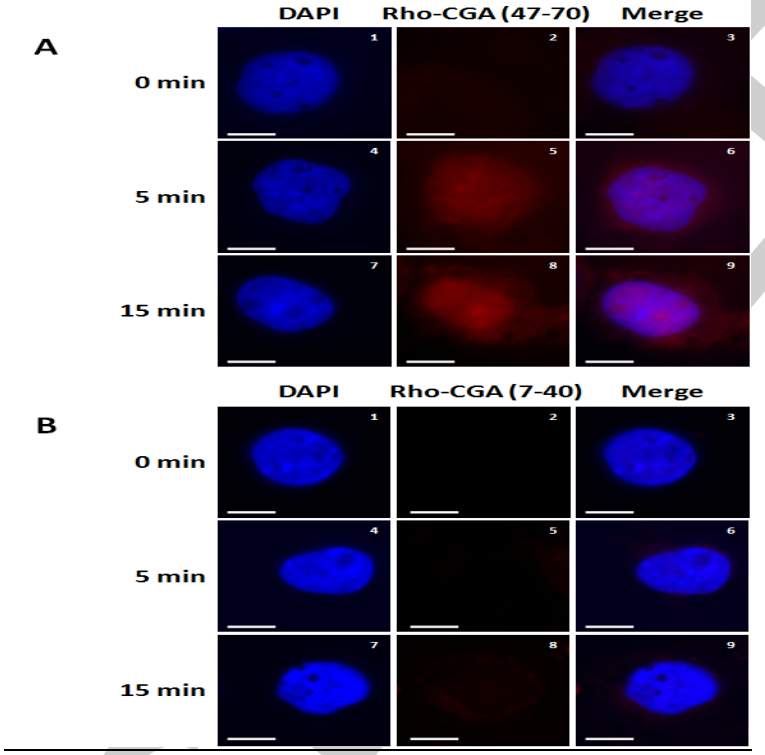

Figure 6: CGA_{47-66} inhibits both nuclear factor-kappa B (NF-kappa B) and Activator protein 1 (AP-1)-mediated transcription

The graph shows mean values ± SD of a representative experiment done in triplicate. * $p< 0{\cdot}05$.

Controls correspond to tranfection by luciferase NF-Kappa B or AP-1 before treatment with peptide.

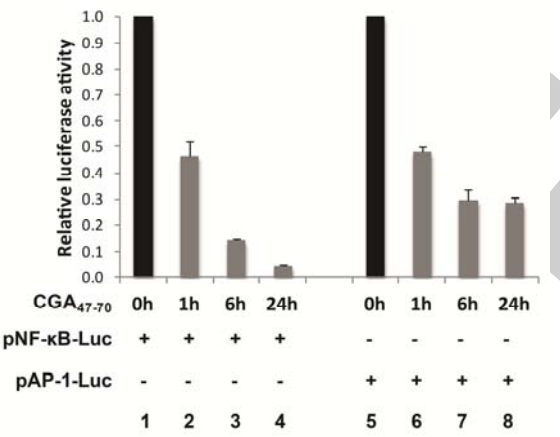

Table 1:Demographic, clinical and biological characteristics of the patients on admission

Data are medians interquartile range [25; 75], or numbers. Δ SOFA max = maximal change in the SOFA from Day 1 during ICU stay.
Comparisons were assessed between patients with (NI+) or without (NI-) nosocomial infection.
*p=0·05; **p<0·02; ****p<0·002; *****p<0·001#p<0·04
Please note that only 31 patients among the 34 screened fulfilled the criteria for NI occurrence (ie: survival>48h). CGA values for healthy controls are as follows: 19·5 [2·3; 36·6] ng/ml.

	All (n= 31)	NI^+ (n = 11/31)	NI^- (n = 20/31)
Demography:			
Age (years)	37·0 [20·75; 60·5]	36·5 [20·75;	39·0 [20·5; 62·0]
Gender (M, F)	(25; 9)	56·25]	(15; 7)
Survival (S, NS)	(25; 9)	(10; 2)	(16; 6)
Length of ICU/hospital stay (days)	8·0 [3·75; 21·25]	(9; 3) 18·5 [16·0; 27·5]	4·0 [2·75; 9·5]*****
Scores:			
Glasgow Coma Score	12·5 [3·0; 15]	7·5 [3; 15]	13·0 [5·0; 15]
Injury Severity Score	27·0 [21·75;	28 [21·75; 37·0]	27·0 [21·5; 30·5]
Simplified Acute	34·25]	43·5 [39·25;	41·0 [29·25; 63·5]
Physiology Score II	42·5 [32·25;	62·75]	6·0 [3·75; 10·0]**
Sequential Organ Failure	61·75]	11·0 [8·25; 13·0]	0 [0; 2·0]
Assessment (SOFA)	9·0 [5·0; 11·0	1·0 [0; 2·0]	5·0 [3·75; 6·5]
Δ SOFA max	0 [0; 2]	7·0 [4·25; 10·0]	2·5 [0; 6·75]
Multiple Organ	5 [4; 8·25]	4·5 [2·0; 14·75]	
Dysfunction Score	3·5 [0; 9]		
Red blood cells transfusion (units)			
Biology:			
Haemoglobin (g/100 ml)	9·9 [8·77; 12·8]	9·4 [8·65; 12·78]	10·3 [8·77; 12·83]
White blood cells counts	10·4 [7·42; 12·9]	8·65 [5·17; 14·12]	10·7 [8·77; 12·9]
(Giga/l)	78·58 [51·51;	112·3 [88·73;	64·6 [34·6;
Chromogranin A (ng/ml)	116·2]	144·3]	89·0]****
Chromogranin A /	2·3 [1·38; 3·71]	3·57 [2·58; 4·69]	1·775 [1·15;
albumin ratio	21·55 [0; 40]	20·3 [2·5; 61·75]	2·38]*****
C-reactive protein (mg/l)	0·945 [0.25; 2·06]	1·83 [0·52; 5·56]	21·55 [0; 33·75]
Procalcitonin (µg/l)	2·28 [0·99; 3·20]	2·56 [1·55; 6·13]	0·59 [0·16; 1·35]*
Lactate (mmol/l)	69·5 [59·5; 88·5]	79·5 [63·25;	1·59 [0·86; 2·93]
Creatinine (µmol/l)		93·75]	68·0 [50·75; 83·25]

Data are medians interquartile range [25; 75], or numbers. Δ SOFA max = maximal change in the SOFA from Day 1 during ICU stay.

Comparisons were assessed between patients with (NI+) or without (NI-) nosocomial infection.
*p=0·05; **p<0·02; ****p<0·002; *****p<0·001, #p<0·04
Please note that only 31 patients among the 34 screened fulfilled the criteria for NI occurrence (ie: survival>48h). CGA values for healthy controls are as follows: 19·5 [2·3; 36·6] ng/ml.

Table 2: Demographic and clinical characteristics of the 9 patients included for the time-dependent assessment of CGA *in vivo*

Patients	NI	Age (Years)	Gender	Focus of infection	Species involved	Time from admission to infection (days)
1	Y	20	M	Lung	MR *S. aureus*	4
2	Y	20	F	Urinary tract	*P. mirabilis*	7
3	Y	59	F	Lung	MS *S. aureus*	4
4	Y	48	M	Lung	*H. influenza*	9
5	Y	54	M	Lung	MS *S. aureus*	4
6	N	61	M	None	None	0
7	N	41	F	None	None	0
8	N	44	F	None	None	0
9	N	57	M	None	None	0

NI: nosocomial infection
M: male; F: female
MR: Methicillin resistant
MS: Methicillin sensitive
Y: yes; N: no