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Sepsis progression to multiple organ dysfunction in carotid chemo/baro-denervated rats treated with lipopolysaccharide

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ABSTRACT

Sepsis progresses to multiple organ dysfunction (MOD) due to the uncontrolled release of inflammatory mediators. Carotid chemo/baro-receptors could play a protective role during sepsis. In anesthetized male rats, we measured cardiorespiratory variables and plasma TNF-α, glucocorticoids, epinephrine, and MOD marker levels 90 min after lipopolysaccharide (LPS) administration in control (SHAM surgery) and bilateral carotid chemo/baro-denervated (BCN) rats. BCN prior to LPS blunted the tachypneic response and enhanced tachycardia and hypotension. BCN-LPS rats also showed blunted plasma glucocorticoid responses, boosted epinephrine and TNF-α responses, and earlier MOD onset with a lower survival time compared with SHAM-LPS rats. Consequently, the complete absence of carotid chemo/baro-sensory function modified the neural, endocrine and inflammatory responses to sepsis. Thus, carotid chemo/baro-receptors play a protective role in sepsis.

1. Introduction

Sepsis syndrome is closely associated with many pathological processes such as systemic inflammation, coagulopathies, hemodynamic abnormalities, and multiple organ dysfunction syndrome (MODS) (Riedemann et al., 2003). Sepsis syndrome includes systemic inflammatory response syndrome (SIRS) and its consequences (severe sepsis and septic shock). The progression of MODS associated with systemic inflammation is mainly caused by an uncontrolled release of pro-inflammatory mediators, which damage parenchymatous organs. Additionally, sepsis activates and/or depresses numerous other systems within the body, including neural, hormonal, and metabolic pathways (Carre and Singer, 2008; Deutschman and Tracey, 2014; Singer et al., 2004). Thus, systemic inflammation initiates the disruption of communication between different organ systems, and, subsequently, MODS reflects a progressive uncoupling that might become irremediable.

Despite many efforts and significant advances in maintaining therapies, sepsis syndrome and MODS are the main causes of death in critical care patients (Martin et al., 2003). This is mainly due to the absence of a truly effective therapy (Riedemann et al., 2003), along with the increasing projected incidence in the United States of 1.5% per annum and average costs per case of US$22,100 (Angus et al., 2001). Thus, the knowledge of the immunometabolic and neurophysiological mechanisms and the pathophysiology of sepsis progression to organ dysfunction and death would help us to improve current therapies and to identify new pharmacological therapeutic targets.

The nervous system, acting through the autonomic nervous system, coordinates the fine-tuning of cardiorespiratory interplay to maintain cellular bioenergetics and appropriate oxygen delivery to the tissues (Abboud and Thames, 1983; Eyzaguirre et al., 1983). Autonomic

Abbreviations: MODS, multiple organ dysfunction syndrome; CB, carotid body; LPS, lipopolysaccharide; IP, intraperitoneally; BCN, bilateral carotid neurotomy; P_S, systolic blood pressure; f_H, instantaneous heart frequency; V_T, tidal volume; f_R, instantaneous respiratory frequency; V_E, minute ventilatory volume; ECG, electrocardiogram; TNF-α, tumor necrosis factor-alpha; IL, interleukin; ELISA, enzyme-linked immunosorbent assay; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TBIL, total bilirubin; GGT, gamma-glutamyl transferase; CRE, creatinine; BUN, blood urea nitrogen; CK, creatine kinase; LDH, lactic dehydrogenase; ALP, alkaline phosphatase; AMY, amylase; GLU, glucose; LAC, lactic acid; NTS, *nucleus tractus solitarii*.

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(sympathetic–parasympathetic) balance is maintained by several reflex arcs such as arterial baroreflexes (Kirchheim, 1976), central chemoreflexes, peripheral arterial chemoreflexes, and pulmonary stretch reflexes (Liljestrand, 1958). Therefore, the interactions among these reflexes are of special clinical interest because the overactivity of a single reflex, which pathophysiologically occurs in several disorders, can lead to the suppression of opposite reflex responses (Schmidt et al., 2001).

Increasing evidence obtained by us and other researchers has shown that a particular neural reflection, carotid body (CB) reflexes, not only serves as a chemoreceptor for respiratory reflex responses, as traditionally accepted, but also as a sensor for the immune status (Fan et al., 2009; Fernandez et al., 2011; Reyes et al., 2012; Wang et al., 2002, 2006; Zapata et al., 2011; Zhang et al., 2007) and as a modulator of autonomic balance, tending to coordinate the cardiorespiratory interplay (Del Rio et al., 2011, 2012) devoted to maintaining oxygen homeostasis in different pathologies. On the other hand, we found that the CB develops acute inflammation induced by local and systemic lipopolysaccharide (LPS) administration. Acute CB inflammation manifests itself as diminished chemosensory activity, ventilatory chemoreflexes and the ventilatory chemosensory drive (Fernandez et al., 2008). Shi et al. (2007) also found that the survival time in sinoaortic-denervated rats is significantly reduced when compared with sham-operated animals in a model of sepsis evoked by cecal ligation and puncture (Shi et al., 2007). The objective of this study was to assess the role played by carotid chemo/baro-receptors in the progression from sepsis to multiple organ dysfunction in LPS-induced septic rats. Consequently, we propose that carotid chemo/baro-receptors play a protective role during sepsis syndrome and MODS.

2. Materials and methods

2.1. Animals and surgical procedures

Young (5-week-old) male Sprague–Dawley rats, weighing from 90 to 130 g, were used. Experimental protocols were approved by the Commission of Bioethics and Biosafety of the Universidad Andres Bello. The animals were anesthetized with 60 mg/kg sodium pentobarbitone administered intraperitoneally (IP) (kindly provided by Dr. Patricio Zapata), with supplementary doses (12 mg kg^{-1} h^{-1}) given IP to maintain a light level of surgical anesthesia (stage 3, plane 2), and were placed in a supine position. The animals breathed spontaneously throughout the experiment. The body temperature, assessed with a rectal thermistor probe, was maintained at approximately 37.0 ± 0.1 °C, by placing a regulated heating pad under the rat.

The animals (total n = 50) were separated into four groups: control groups with a sham surgical intervention that omitted the neurotomy, i.e., with intact carotid/sinus nerves (SHAM), treated IP with either saline (0.9% NaCl) (SHAM-saline, n = 8) or 15 mg/kg lipopolysaccharide (LPS, from *Escherichia coli* serotype 0127:B8; L3129, Sigma-Aldrich Corp., USA) (given in 100 μL saline/100 g body weight) (SHAM-LPS, n = 12); and experimental groups submitted to bilateral carotid neurotomy (BCN), also treated IP with either saline (BCN-saline, n = 9) or 15 mg/kg LPS (BCN-LPS, n = 21). To gain access to the carotid regions on both sides, a ventral midline incision of the neck was performed. Carotid/sinus nerves were sectioned at their entrance to the carotid bodies (in BCN animals). BCN was confirmed by testing the ventilatory chemosensory drive, i.e., the suppression of the decrease in ventilation provoked by breathing 100% O_2 (Fernandez et al., 2003).

2.2. Cardiorespiratory recordings

To confirm the effectiveness of the LPS treatment (i.e., the induction of severe sepsis), the systolic blood pressure (P_S), instantaneous heart frequency (f_H), tidal volume (V_T), and instantaneous respiratory frequency (f_R) were recorded before surgery (either SHAM or BCN) and for up to 90 min after saline or LPS treatment (usually administered 15 min after simulated or effective surgery) with a physiological recording acquisition system.

The heart frequency was derived through a tachograph fed by the ECG signal obtained at Einthoven's lead II. P_S was measured using a pressure tail cuff for a non-invasive blood pressure recording system for rats (ML125/R) coupled to a MLT125/R pulse transducer (AD Instruments, Castle Hill, Australia). Transient introductions (1 min) of the rat head into a plastic mask connected to a respiratory flow head (MLT1L, AD Instruments) were conducted to measure the ventilatory flow ($\delta V/\delta t$), which was converted into V_T through a volumetric differential pressure transducer. Ventilatory signals were derived through a tachograph to determine f_R. All transducers were connected to a PowerLab® 8/30 (AD Instruments), and physiological variables were instantaneously displayed through the Chart® software (AD Instruments). In addition, raw signals were stored and subsequently analyzed.

The reported physiological values were obtained by averaging raw data recorded 2 min before surgery (Basal), 2 min before saline or LPS injections (post-surgery, either SHAM or BCN) or 2 min after the mentioned time point. At the end of the experiments, animals that did not die as a result of the treatment were euthanized by an overdose of pentobarbitone.

2.3. Blood samples and plasma measurements

At the end of the experiments (90 min after saline or LPS administration), cardiac puncture was performed for blood extraction into lithium heparin-containing tubes. The blood was immediately centrifuged at 5000 rpm for 10 min at 4 °C to separate the plasma, which was then used to measure tumor necrosis factor-alpha (TNF-α), corticosterone, cortisol, epinephrine, and different markers of multiple organ dysfunction (MOD).

Plasma TNF-α was measured by an enzyme-linked immunosorbent assay (ELISA), according to the protocol described elsewhere (Christodoulides et al., 2000), using a monoclonal anti-rat TNF-α capture antibody (R&D Systems Inc., MN, USA), matched biotinylated anti-rat TNF-α detecting antibody (R&D Systems, Inc.) and recombinant rat TNF-α standard (R&D Systems Inc.). The reaction was revealed with streptavidin-conjugated alkaline phosphatase and *p*-nitrophenyl phosphate, and the absorbance was determined at 405 nm. Plasma corticosterone and cortisol were measured with competitive EIA kits (Cayman Chemical Company, MI, USA) according to the manufacturers' instructions. Plasma epinephrine was also measured using a commercially available competitive ELISA kit (ALPCO Immunoassay, NH, USA).

2.4. Blood biochemical analyses

For evaluating organ functions, we measured plasma levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TBIL), gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP) for the liver; creatinine (CRE) and blood urea nitrogen (BUN) for the kidney; creatine kinase (CK) and lactic dehydrogenase (LDH) for the heart and other organs (such as muscle); amylase (AMY) for the exocrine pancreas; and glucose (GLU) and lactic acid (LAC) for metabolic function. MOD markers were measured by the Piccolo Xpress Chemistry Analyzer (General Chemistry, 13 panel) (Abaxis, CA, USA), the iSTAT System (CG4+ cartridge) (Abbott Laboratories, IL, USA) or by commercial kits (Valtek Diagnostics, Santiago, Chile), according to the manufacturers' instructions.

2.5. Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM) or as the mean ± 95% confidence interval (CI) for the relative risk. For the cardiorespiratory recordings, significant differences were assessed by a one-way ANOVA followed by Dunnett's post-test to compare

with basal recordings and by a two-way ANOVA followed by the Bonferroni post-test to compare with the saline or SHAM recordings (see the figure legends for detailed explanations). Kaplan–Meier curves and the log-rank and Gehan–Breslow–Wilcoxon tests were used to estimate the survival rates. Contingency analyses with Fisher's exact test were used to assess the relative risk of death. Plasma measurements were performed with Kruskall–Wallis ANOVA followed by the Dunn's post-test. $p < 0.05$ was considered significant. The statistical analysis and graphs were completed using GraphPad Prism v6.0 (GraphPad Software Inc., CA, USA).

3. Results

3.1. Cardiorespiratory changes after IP administration of LPS

To demonstrate that under our experimental conditions, 15 mg/kg LPS was sufficient to evoke cardiorespiratory changes — complying with the accepted criteria for the diagnosis of severe sepsis in humans (Levy et al., 2003a, 2003b), as characterized by hypotension, tachycardia and tachypnea — we recorded the heart rate (f_H), systolic blood pressure (P_S), tidal volume (V_T) and respiratory frequency (f_R) before surgery (basal), immediately after surgery and before saline or LPS injection (post-surgery, either SHAM or BCN), and for up to 90 min after the IP administration of saline or LPS.

The basal minute ventilatory volume (V_E: V_T multiplied by f_R) in each rat was uniform in amplitude and rate. Additionally, the basal f_H and P_S were not significantly different in the four experimental groups. Neither simulated surgery (SHAM) nor bilateral carotid/sinus neurotomy (BCN) significantly modified the basal values (Table 1, post-surgery). Subsequent saline administration failed to evoke any significant change in either the basal or the post-surgical ventilatory parameters in both the SHAM-saline and BCN-saline groups (Fig. 1). LPS administration in the SHAM-operated rats increased f_R and V_E, with no significant changes in V_T (Fig. 1A, C, E). After BCN, LPS administration significantly reduced f_R and V_E, with no significant changes in V_T (Fig. 1B, D, E). Thus, LPS-induced tachypnea in the SHAM animals was suppressed after BCN.

Saline administration failed to evoke any significant change in both f_H and P_S (Fig. 2), whereas LPS significantly reduced P_S within 60 to 90 min in SHAM rats (Fig. 2A) and within 30 to 90 min in BCN rats (Fig. 2B). Notably, the fall in blood pressure was more abrupt in BCN-LPS than in SHAM-LPS rats ($p < 0.05$, at 50, 60 and 70 min after LPS, two-way ANOVA and the Bonferroni post-test), but both conditions reached a nadir at *ca.* 60 mm Hg, with an overall reduction in the systolic blood pressure of greater than 40 mm Hg from the baseline. By contrast, f_H was significantly increased within 50 to 90 min after LPS administration in both SHAM and BCN rats (Fig. 2C, D). However, tachycardia was more pronounced in the BCN rats starting 70 min after endotoxin administration ($p < 0.05$, two-way ANOVA and the Bonferroni post-test). Thus, the cardiorespiratory changes observed in the LPS-treated rats resembled severe sepsis in humans.

Table 1
Cardiorespiratory variables recorded before (basal) and after (post-surgery) simulated bilateral carotid/sinus nerve section (SHAM) or after bilateral carotid neurotomy (BCN) in rats that were subsequently treated IP with saline or 15 mg/kg LPS.

	SHAM		BCN	
	Basal	Post-Surgery	Basal	Post-surgery
Saline	(n = 8)		(n = 7)	
V_T (mL)	1.09 ± 0.09	1.05 ± 0.08	0.97 ± 0.05	0.97 ± 0.04
f_R (min^{-1})	78.2 ± 1.7	80.9 ± 2.1	76.4 ± 1.6	78.4 ± 2.0
V_E (mL/min)	85.2 ± 4.1	85.0 ± 4.2	74.2 ± 3.8	75.7 ± 3.5
Ps (mm Hg)	103.7 ± 2.9	100.0 ± 2.8	109.2 ± 3.5	102.9 ± 4.1
f_H (min^{-1})	359.7 ± 14.9	365.0 ± 15.9	392.3 ± 11.6	396.0 ± 11.7
LPS	(n = 7)		(n = 8)	
V_T (mL)	1.11 ± 0.05	1.10 ± 0.08	0.98 ± 0.06	0.95 ± 0.03
f_R (min^{-1})	73.2 ± 2.1	75.0 ± 1.9	74.4 ± 2.4	72.0 ± 3.4
V_E (mL/min)	81.2 ± 4.5	82.5 ± 5.1	73.2 ± 2.4	68.7 ± 3.7
Ps (mm Hg)	94.2 ± 4.1	98.0 ± 4.2	110.1 ± 3.8§	106.8 ± 3.3
f_H (min^{-1})	386.7 ± 13.3	392.0 ± 15.1	394.4 ± 7.6	397.3 ± 7.8

Values are expressed as the Means ± SEMs. Significant differences: §$p < 0.05$ vs. basal SHAM-LPS. Two-way ANOVA followed by Bonferroni's post-test.

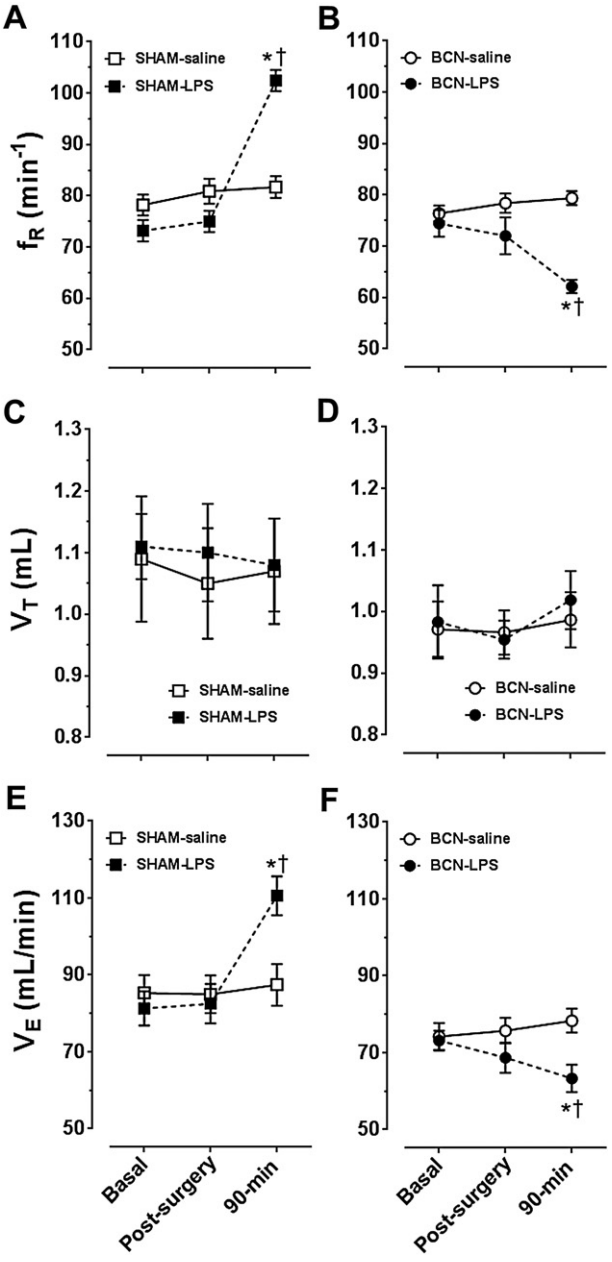

Fig. 1. LPS-induced ventilatory changes in carotid chemo/baro-denervated rats. Ventilatory variables in rats treated IP with saline or 15 mg/kg LPS in the control condition (SHAM surgery; leaving the carotid/sinus nerves intact) or after carotid chemo/baro-denervation (BCN; bilateral carotid neurotomy). f_R, respiratory frequency; V_T, tidal volume; V_E, minute ventilatory volume ($V_T * f_R$). Values are expressed as the mean ± SEM. †, $p < 0.05$ vs. basal, assessed by ANOVA and Dunnett's post-test; *, $p < 0.05$ vs. saline, assessed by two-way ANOVA and the Bonferroni post-test. n = 7–8.

3.2. Bilateral carotid/sinus neurotomy modifies the neuroendocrine response to sepsis

Glucocorticoids and epinephrine are known as endogenous anti-inflammatory mediators during sepsis. LPS administration to the SHAM rats significantly increased the plasma levels of corticosterone, cortisol and epinephrine (Fig. 3A, B, C). Bilateral carotid/sinus neurotomy did

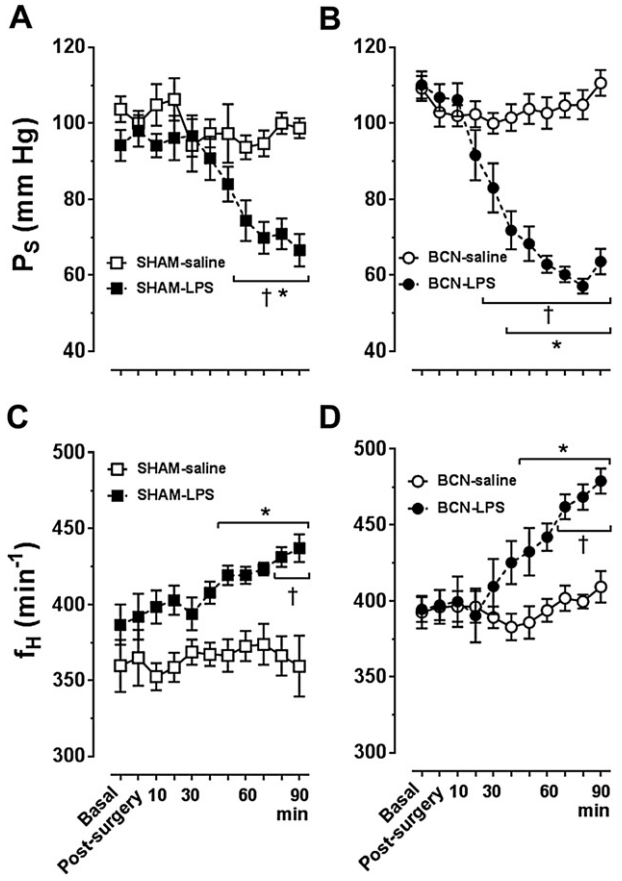

Fig. 2. LPS-induced cardiovascular changes in carotid chemo/baro-denervated rats. Cardiovascular variables in rats treated IP with saline or 15 mg/kg LPS in the control condition (SHAM surgery; leaving the carotid/sinus nerves intact) or after carotid chemo/baro-denervation (BCN; bilateral carotid neurotomy). P_S, systolic blood pressure; f_H, heart rate. Values are expressed as the mean ± SEM. †, $p < 0.05$ vs. basal, assessed by ANOVA and Dunnett's post-test; *, $p < 0.05$ vs. saline, assessed by two-way ANOVA and the Bonferroni post-test. n = 7–8.

not affect the plasma levels of the neuroendocrine mediators mentioned above. BCN prior to endotoxin administration suppressed the LPS-induced increase in both corticosterone (Fig. 3A) and cortisol (Fig. 3B) and boosted the epinephrine response by 5-fold, from 65.4 ± 17.8 pg/dL (SHAM-LPS) to 336.1 ± 75.6 pg/dL (BCN-LPS) (Fig. 3C).

3.3. Bilateral carotid/sinus neurotomy increases plasma TNF-α during sepsis

Because LPS administration to rats increased plasma TNF-α levels, reaching a peak between 60 and 90 min post-injection, we measured this pro-inflammatory cytokine in our experimental model. Effectively, LPS administration in the SHAM rats (SHAM-LPS) evoked a two-fold increase in plasma TNF-α levels compared with the saline control (SHAM-saline) (Fig. 4). Whereas BCN did not affect the plasma levels of TNF-α in saline-treated rats (BCN-saline), the TNF-α response to LPS was boosted in the neurotomized rats (BCN-LPS) (Fig. 4).

3.4. Bilateral carotid/sinus neurotomy increases tissue damage

Tumor necrosis factor-α is a potent cytotoxic cytokine that causes tissue damage. As indicated in the previous results, the plasma levels of TNF-α were higher in the BCN rats than in the SHAM animals. Therefore, by measuring the plasma markers of tissue damage, we analyzed whether BCN could increase organ dysfunction in LPS-treated rats. Ninety minutes after LPS administration, the SHAM animals exhibited increased CK, LDH and LAC levels and reduced glycemia, with no significant changes in ALP, AMY, ALT, GGT, TBIL, or CRE, compared with the SHAM-saline animals, suggesting heart deterioration or muscle damage (Table 2) and hypoperfusion. Saline administration to chemo/baro-denervated rats (BCN-saline) did not modify the measured plasma markers. LPS administration after BCN evoked dramatic increases in CK, LDH, ALP, AMY, ALT, GGT, TBIL and LAC levels compared with those of SHAM-saline, SHAM-LPS and BCN-saline rats (Table 2). In addition, more pronounced hypoglycemia was induced compared with the SHAM-saline group. We also measured plasma AST and BUN levels, but no statistically significant differences were found between the four groups.

3.5. Bilateral carotid/sinus neurotomy increases the relative risk of death during sepsis syndrome

Contingency analysis revealed that BCN increases the risk of death in LPS-treated rats. None of the eight saline-treated SHAM rats died after the 90-min recording (saline administration at time = 0). Ninety minutes after LPS administration in the SHAM rats (at time = 0), 2 out

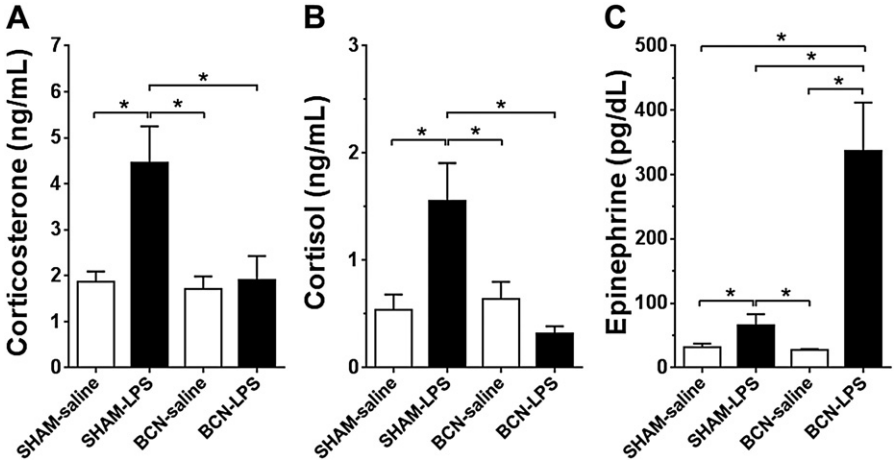

Fig. 3. Neuroendocrine modulators of systemic inflammation in carotid chemo/baro-denervated rats. Plasma levels of corticosterone (A), cortisol (B) and epinephrine (C) in the rats 90 min after IP treatment with saline or 15 mg/kg LPS in the control condition (SHAM) or after carotid chemo/baro-denervation (BCN). Values are expressed as the mean ± SEM.*, $p < 0.05$, assessed by Kruskal–Wallis ANOVA and Dunn's post-test. n = 7–8.

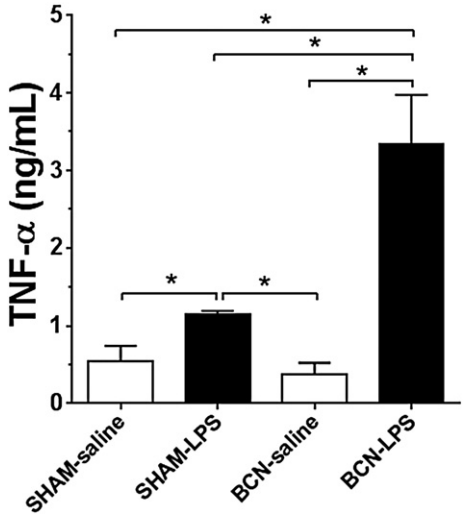

Fig. 4. Carotid chemo/baro-denervation increases the TNF-α response in septic rats. Plasma levels of TNF-α in rats 90 min after IP treatment with saline or 15 mg/kg LPS in the control condition (SHAM) or after carotid chemo/baro-denervation (BCN). Values are expressed as the mean ± SEM.*$p < 0.05$, assessed by Kruskal–Wallis ANOVA and Dunn's post-test. n = 7–8.

of 12 animals died ($p = 0.4947$, Fisher's exact test, SHAM-LPS vs. SHAM-saline). In the saline-treated BCN rats, 2 out of 9 rats died ($p = 0.4706$, Fisher's exact test, BCN-saline vs. SHAM-saline). LPS administration to the BCN rats increased the risk of death, and 13 out of 21 rats died after 90 min post-endotoxin administration ($p = 0.0033$, BCN-LPS vs. SHAM-saline; $p = 0.0272$, BCN-LPS vs. SHAM-LPS, Fisher's exact test) (Fig. 5A). Comparison of the survival curves to the SHAM-saline group revealed that, despite the deaths registered at the late time points, neither the SHAM-LPS (survival proportion 83%) nor the BCN-saline (survival proportion 78%) groups differed from the SHAM-saline group (survival proportion 100%). By contrast, LPS administration to the BCN rats (BCN-LPS group) increased the risk of death at early time points (Gehan–Breslow–Wilcoxon test, $p = 0.0084$), and this higher risk was maintained at the late time points (survival proportion 40%) ($p = 0.0067$, BCN-LPS vs. SHAM-saline; $p = 0.0095$, BCN-LPS vs. SHAM-LPS; $p = 0.0325$, BCN-LPS vs. BCN-saline; log-rank Mantel–Cox test) (Fig. 5B).

Table 2
Plasma markers of multiple organ dysfunction in rats, 90 min after the IP administration of saline or 15 mg/kg LPS in the control condition (SHAM surgery; leaving intact carotid/sinus nerves) or after carotid chemo/baro-denervation (BCN; bilateral carotid neurotomy).

	SHAM-saline	SHAM-LPS	BCN-saline	BCN-LPS
CK (UI/L)	124.1 ± 23.9	251.3 ± 33.6[a]	145.3 ± 29.4	544.6 ± 84.2[b]
LDH (UI/L)	53.5 ± 3.8	99.0 ± 7.5[a]	59.7 ± 3.4	154.7 ± 20.0[b]
AMY (UI/L)	446.5 ± 41.1	540.5 ± 35.3	504.8 ± 44.3	771.7 ± 74.5[b]
AST [GOT] (UI/L)	44.0 ± 5.6	48.5 ± 7.1	46.7 ± 4.0	44.5 ± 1.5
ALT [GPT] (UI/L)	12.9 ± 1.6	11.7 ± 1.6	11.6 ± 1.1	19.4 ± 1.7[b]
GGT (UI/L)	<5[c]	<5[c]	<5[c]	6.1 ± 0.5
ALP (UI/L)	273.1 ± 29.7	293.2 ± 15.8	251.7 ± 12.2	448.0 ± 33.5[b]
TBIL (mg/dL)	0.29 ± 0.04	0.38 ± 0.03	0.25 ± 0.04	0.71 ± 0.05[b]
CRE (mg/dL)	0.66 ± 0.05	0.61 ± 0.05	0.60 ± 0.05	0.98 ± 0.04[b]
BUN (mg/dL)	21.3 ± 1.5	19.4 ± 3.4	20.4 ± 1.4	17.9 ± 1.7
LACT (mM)	1.09 ± 0.16	1.82 ± 0.18[a]	1.05 ± 0.31	7.04 ± 1.05[b]
GLU (mg/dL)	150.5 ± 9.1	106.8 ± 2.5[a]	161.6 ± 8.4	75.7 ± 6.0[b]

Values are expressed as the mean ± SEM. $p < 0.05$, Kruskal–Wallis ANOVA, Dunn's post-test. n = 6–11. CK, creatine kinase; LDH, lactic dehydrogenase; ALP, alkaline phosphatase; AMY, amylase; AST, aspartate aminotransferase, ALT, alanine aminotransferase; CRE, creatinine; BUN, blood urea nitrogen; NGAL, neutrophil gelatinase-associated lipocaline; GGT, gamma-glutamyl transferase; TBIL, total bilirubin; LAC, lactic acid; GLU, glucose,
[a] vs. SHAM-saline.
[b] vs. SHAM-saline, SHAM-LPS, BCN-saline.
[c] Lower detection limit of 5 UI/L.

4. Discussion

The results reported here suggest that the carotid chemo/baro-receptors, i.e., primarily the carotid body (CB), modulate the systemic inflammatory response during septic shock because endotoxemic rats subjected to bilateral carotid neurotomy exhibited altered cardiorespiratory variables, plasma TNF-α and immunomodulator levels, increased multiple organ damage and, accordingly, decreased survival rates compared with septic animals with intact carotid/sinus nerves. This is the first study to demonstrate the protective role played by carotid chemo/baro-receptors during sepsis syndrome progression.

Systemic inflammation associated with sepsis involves pathological processes such as inflammation, tachypnea, fever, leukopenia, hemodynamic abnormalities and hypotension, tachycardia, multiple organ dysfunction (MOD), and death (Riedemann et al., 2003). In agreement with this finding, LPS administration to rats decreased the systolic blood pressure and increased the heart rate, respiratory frequency, and minute ventilatory volume. Thus, our animal model of systemic inflammation complies with the accepted criteria for the diagnosis of severe sepsis in humans (Bone et al., 1992, 2009; Levy et al., 2003a).

Carotid chemo/baro-denervation suppresses the ventilatory response to LPS. In fact, BCN turns the tachypneic response into bradypnea in septic rats. Bradypnea could be due to either LPS- or TNF-α-induced stimulation of vagally and non-vagally innervated chemoreceptors (presumably thoracic and abdominal), the activation of which might elicit ventilatory reflexes (Cardenas and Zapata, 1983). On the other hand, the stimulation of capsaicin-sensitive vagal lung afferents mediates respiratory reflexes evoked by reactive oxygen species (ROS) in the lungs of anesthetized rats. ROS inhalation (aerosolized H_2O_2) evokes bradypnea (Ruan et al., 2003, 2005). In lung microvessels, soluble TNF-α increases mitochondrial Ca^{2+}, leading to the release of ROS (Dada and Sznajder, 2011). Additionally, increased pro-inflammatory cytokines activate neutrophils that are sequestered in the pulmonary circulation, inducing the release of ROS (Chabot et al., 1998).

Endotoxemic rats with intact carotid/sinus innervation showed a 30-mm Hg decrease in the systolic blood pressure and an increased heart rate (*ca.* $+80\ min^{-1}$). Hypotension and tachycardia are also used to diagnose severe sepsis in humans (Bone et al., 1992, 2009; Levy et al., 2003a). The presence of hypotension and tachycardia is consistent with previously published data (Lin et al., 1999; Vayssettes-Courchay et al., 2005). After BCN, the LPS-induced decrease in P_S was steeper than in the SHAM control group, but the maximal fall in PS at the end of the recordings (at 90 min) was not significantly different between the SHAM-LPS and BCN-LPS groups. Additionally, the LPS-induced increase in f_H was higher in the BCN rats than in the SHAM rats. Tang et al. (1998) also obtained similar results, with the caveat that their rats had undergone bilateral carotid and aortic denervation (Tang et al., 1998).

It is known that IP administration of LPS stimulates vagal primary afferent fibers (Goehler et al., 1999, 2000), which, in turn, activate central nervous system (CNS) neurons (Borsody and Weiss, 2005; Mascarucci et al., 1998). However, despite cytokine receptor expression in the vagal afferent fibers (Goehler et al., 1997), interleukin (IL)-1β and TNF-α had no significant effect on the frequency of action potentials recorded in single fibers from isolated superfused rat glomus cells (GCs) obtained from vagal paraganglia (Mac Grory et al., 2010). In addition, neither the basal nor the hypoxic discharge rate of the vagal paraganglia was modulated by IL-1β, TNF-α or LPS, suggesting that these structures are not the afferent limb of the 'immune reflex' (O'Connor et al., 2012).

In previous years, we proposed that the CB might also serve as a peripheral sensor for the presence of immunogenic agents in the blood, owing to its rich vascularization and abundant chemosensory innervations. Apart from the presence of the functional LPS canonical receptor Toll-like receptor (TLR)-4 (Fernandez et al., 2011) and TNF-α receptors (Fernandez et al., 2008, 2011), it is known that GCs from the rat CB express both IL-1 receptor type I (Wang et al., 2002) and IL-6 receptor α (Wang et al., 2006). In vitro-cultured GCs respond to IL-1β with

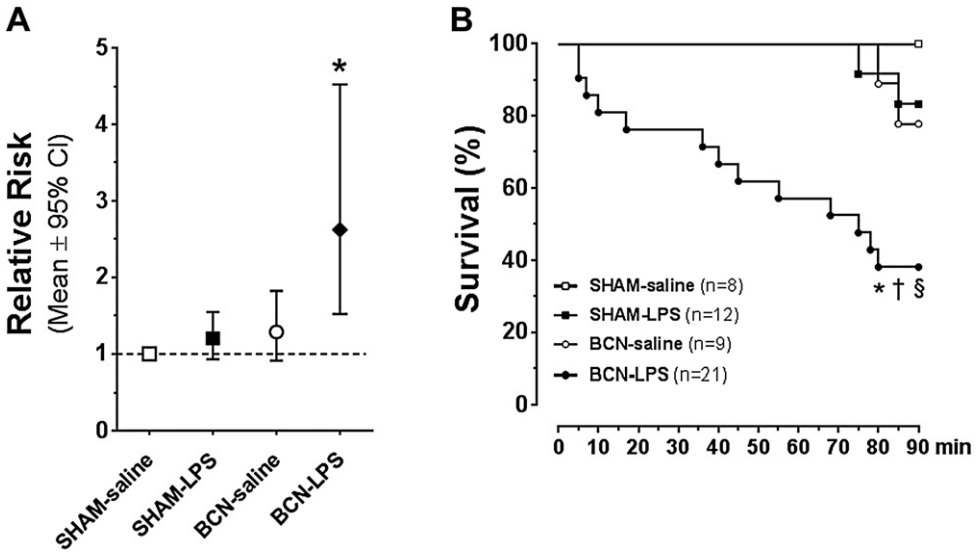

Fig. 5. Carotid chemo/baro-denervation increases mortality in septic rats. Contingency analyses (A) and survival (Kaplan–Meier) curves (B) in rats 90 min after IP treatment with saline or 15 mg/kg LPS in the control condition (SHAM) or after carotid chemo/baro-denervation (BCN). In (A), values are expressed as the mean ± 95% CI. *, $p = 0.0033$ Fisher's exact test vs. SHAM-saline. In (B), *, $p = 0.0067$ vs. SHAM-saline; †, $p = 0.0095$ vs. SHAM-LPS; §, $p = 0.0325$ vs. BCN-saline, log-rank (Mantel–Cox) test.

depolarization and a transient rise in $[Ca^{2+}]_i$. Furthermore, IL-1β significantly increases carotid/sinus nerve chemosensory discharge in anesthetized rats (Shu et al., 2007), although the extracellular administration of IL-6 induces a rise in $[Ca^{2+}]_i$ and catecholamine release from in vitro-cultured GCs (Fan et al., 2009).

We have reported a significant and maintained increase in basal chemosensory discharge after IV LPS infusion in cats (Fernandez et al., 2008); however, we have not assessed in situ TNF-α administration. On the other hand, neither IL-1β nor IL-6 expression in the CB during sepsis has been reported, but systemic pro-inflammatory cytokines could reach the CB due to its extensive vascularization. Thus, the augmented basal CB chemosensory activity could be due to either IL-1β or IL-6 stimulation. In fact, IL-1β appears to mimic the responses of the CB to hypoxia (i.e., evokes GC $[Ca^{2+}]_i$ oscillations and increases normal CB chemosensory activity) (Shu et al., 2007) and might, therefore, act in an telecrine manner to enhance the peripheral chemoreceptor drive during systemic inflammation.

The fact that pro-inflammatory cytokines and their receptors are functionally expressed in the carotid body suggests that inflammatory mediators might have different functional roles in the activation of chemosensory neurons, even in the absence of sepsis syndrome Consequently, pro-inflammatory cytokines might be recognized by membrane receptors located in the GC, modifying chemosensory activity and reaching the *nucleus tractus solitarii* (NTS), which, in turn, stimulate or inhibit specific components of the systemic inflammatory response.

Although the disease manifestations (sickness syndrome: fever, anorexia, sleepiness, hyperalgesia and activation of the NTS, *locus coeruleus* and hypothalamus) observed in rodents in response to IP injections of LPS or IL-1β are prevented by subdiaphragmatic section of the vagus nerves (reviewed by Fernandez and Acuna-Castillo (2012)), LPS-induced c-Fos activation of NTS neurons persists after cervical bivagotomy (Hermann et al., 2001). Thus, prominent manifestations of endotoxemia are apparently caused by incoming neural signals from different peripheral receptors, i.e., carotid bodies (Reyes et al., 2012).

Previous reports have shown an uncontrolled release of TNF-α during sepsis syndrome. TNF-α is the first cytokine to appear in the circulation (van der Poll et al., 1997). In rats, this cytokine reaches its maximum level at 90 min post-LPS administration, given either IP or IV (Asari et al., 1996; Waage, 1987). Consistent with the above-mentioned findings, our results demonstrated that LPS increased plasma TNF-α levels. These results are relevant because in LPS-treated BCN rats (BCN-LPS), the plasma levels of TNF-α were significantly higher than those found in the SHAM-LPS rats. Shi et al. (2007) also found greater plasma TNF-α, norepinephrine and epinephrine levels in sinoaortic-denervated rats compared with sham-operated rats in a model of sepsis evoked by cecal ligation and puncture (Shi et al., 2007).

The plasma levels of TNF-α are markedly increased in the BCN-LPS rats, presumably because its endogenous regulators in the plasma are also altered. Physiologically, adrenal glucocorticoids, which inhibit the expression of pro-inflammatory mediators, counteract the pro-inflammatory response (Irwin and Cole, 2011). The results reported here show that LPS administration to SHAM animals increases plasma glucocorticoids, as has been described in human patients with sepsis (Melby and Spink, 1958). The increase in glucocorticoids was abolished in carotid chemo/baro-denervated rats, which is an extremely new finding because the activation of anti-inflammatory mechanisms is only suppressed by interrupting this CNS activation pathway. It must be noted that CB stimulation provokes a wide array of cardiopulmonary and autonomic reflexes, as well as endocrine responses (e.g., plasma release of catecholamines and cortisol) (Fitzgerald, 2014; Raff et al., 1982). This finding is explained, at least in part, in our previous work, in which we demonstrated that bilateral carotid neurotomy suppresses the activation of NTS neurons (assessed by c-Fos immunoreactivity) (Reyes et al., 2012).

During sepsis, catecholamines are released due to neural reflexes involving the sympathetic nervous system (Hahn et al., 1995). The results reported here show an increase in the plasma concentration of epinephrine in the SHAM-LPS rats, which might be interpreted as an immunosuppressive response via activation of the β_2-adrenergic receptor (Monastra and Secchi, 1993; Severn et al., 1992). In LPS-treated BCN rats, plasma epinephrine was increased by 5-fold compared with SHAM-LPS rats. The increased plasma levels of epinephrine found in the BCN-LPS group were similar to those found in septic patients immediately before death (Baue et al., 1984).

It is traditionally accepted that TNF-α is the main effector of damage to parenchymatous organs, which ultimately causes MOD (Tracey et al., 1986). In our experimental design, BCN-LPS rats had higher plasma levels of TNF-α than control animals (SHAM-saline), so we expected to find greater organ damage. Usually, plasma levels of CK, LDH, ALP, AMY, BUN, CRE, AST, ALT, GGT, TBIL, LAC and glucose change as a consequence of organ failure (Yang et al., 2007). The present results demonstrate that LPS-treated chemo-barodenervated rats had greater plasma biochemical marker levels than LPS-treated SHAM animals did. Increased

CK and LDH in endotoxemic rats suggested heart (or muscle) damage. In the BCN-LPS rats, plasma enzyme activities were significantly increased. AMY was not altered during endotoxemia (SHAM-saline) but was significantly increased in the BCN-LPS rats, suggesting additional pancreatic damage.

By examining changes in the kidney function, we observed that BUN was not modified under any conditions but that CRE was significantly increased in the BCN-LPS rats. The BUN to CRE ratio allowed us to propose that acute kidney injury was induced in BCN-LPS rats. Hepatic damage was also found. Although we did not observe significant changes in AST levels, the plasma levels of ALP, ALT and TBIL were increased in BCN-LPS rats. In addition to tissue damage, we also found metabolic dysfunction. Plasma glucose levels were decreased in endotoxemic rats, and BCN augmented the decrease in plasma glucose levels. Hypoglycemia is a metabolic disorder associated with sepsis (Tsai et al., 2011) that provides a sharper increase in the mortality rate (Igaki et al., 2003; Maitra et al., 2000). By contrast, the increased lactate levels in BCN-LPS rats suggest decreased lactate clearance and increased mortality (Rivers et al., 2012).

Whether tissue damage is evoked by higher TNF-α levels in BCN-LPS rats must be elucidated. It is known that endotoxemic rats treated with anti-TNF-α capture antibodies exhibit significantly improved survival (Beutler et al., 1985; Remick et al., 2000; Riedemann et al., 2003; Tracey et al., 1986, 1987). However, pre-clinical testing in humans has demonstrated no effect on the survival of patients with sepsis (Reinhart and Karzai, 2001).

Treatments for sepsis and septic shock involve the administration of antibiotics, intravenous fluids, vasopressors and/or inotropes, corticosteroids, and recombinant human activated protein C (Barochia et al., 2010). Interestingly, adrenergic agents such as epinephrine are used to increase the blood pressure (Levy, 2005). However, despite higher epinephrine levels in the BCN-LPS rats, the blood pressure decrease was not prevented, possibly due to the following different factors: i) refractory vasodilation (vasoplegic syndrome) (Shanmugam, 2005); ii) the partial absence of baroreflexes. (It must be noted that under our experimental conditions, aortic baroreflexes, which are predominant in rats (Pickering et al., 2008), are still intact); iii) the absence of the glucocorticoid response, which can also be responsible for cardiovascular dysfunction (Szabo et al., 1993); iv) pentobarbitone-induced CNS depression (Macdonald and McLean, 1982); or v) a combination of the above-mentioned factors. Additionally, in BCN-LPS rats, increased epinephrine levels could induce hyperlactatemia but could not restore the glucose levels to normal (Levy, 2005). This condition might be worse because the glucocorticoid levels are not increased. Thus, the suppression of carotid chemosensory function during sepsis syndrome plays an adverse role in physiological cardiorespiratory and organ functions of the animals.

Recently, Rodriguez-Gonzalez et al. found that in a "clinically relevant animal model of sepsis," hyperoxia was associated with the following alterations: i) higher plasma levels of IL-6, IL-10 and TNF-α; ii) a greater number of infected biological samples; and iii) increased mortality. They concluded that "oxygen therapy greatly influences the progression and clinical manifestation of multiple system organ dysfunction in experimental sepsis. If these results are extrapolated to humans, they suggest that oxygen therapy should be carefully managed in septic patients to minimize its deleterious effects" (Rodriguez-Gonzalez et al., 2014). Notably, hyperoxia reduces CB chemosensory activity (Dejours, 1957; Fernandez et al., 2003); consequently, in addition to the adverse effects of cecal ligation and puncture-induced ROS, the withdrawal of carotid chemo/baro-sensory function induced by hyperoxia modifies the systemic inflammatory response during sepsis syndrome through a network of neural, humoral and cytokine responses.

Finally, when comparing the mortality of LPS-treated BCN rats vs. SHAM animals, a higher percentage of death was observed (60% vs. 15%, respectively). There is a direct association between the numbers of damaged tissues and metabolic disorders and death.

Despite many efforts and significant advances in maintenance therapies, sepsis syndrome and MOD are the main causes of death in critical care patients, mainly due to the absence of an effective therapy (Riedemann et al., 2003). This work is certainly a step forward in understanding the progression of sepsis, but further studies would be of value to achieve an effective treatment that lowers the mortality of sepsis.

In addition, the present results imply that the carotid body serves not only as a chemoreceptor for respiratory reflex responses, as traditionally accepted, but also as a sensor for the immune status, a modulator of autonomic balance, tending to coordinate the cardiorespiratory interplay devoted to maintaining oxygen homeostasis in different pathologies, and a protective factor during sepsis and MOD. The withdrawal of CB chemosensory activity induced by ventilation with pure oxygen decreases central sympathetic outflow (Kara et al., 2003). Chemoreflexes are important modulators of sympathetic activation (Abboud and Thames, 1983; Alanis et al., 1968; Montarolo et al., 1976). Thus, tonic activation of carotid chemoreceptors during sepsis might also contribute to high levels of sympathetic immunomodulatory activity (Kara et al., 2003).

Additionally, hypoxic hypoxia (the natural stimulus of CB chemoreceptors) increases the adrenal cortisol secretion rate in anesthetized, paralyzed, ventilated and maintained normocapnic mongrel dogs (Raff et al., 1982). Thus, the CB influences adrenal cortisol secretion. Interestingly, as mentioned above, bilateral carotid/sinus neurotomy attenuated the LPS-induced glucocorticoid response in septic rats.

Consequently, as a therapeutic target, the stimulation of CB chemoreceptors could modify the inflammatory response during sepsis syndrome by inducing sympathetic activation and glucocorticoid secretion.

Conflict of interest

All authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jneuroim.2014.12.002.

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