Chemico-Biological Interactions 184 (2010) 313–318

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

In situ and real time muscle chemiluminescence determines singlet oxygen involvement in oxidative damage during endotoxemia

Virginia Vanasco, Pablo Evelson, Alberto Boveris, Silvia Alvarez*

Laboratory of Free Radical Biology, School of Pharmacy and Biochemistry, University of Buenos Aires, Junín 956, C1113AAD Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 25 August 2009
Received in revised form
14 December 2009
Accepted 5 January 2010
Available online 14 January 2010

Keywords:
Chemiluminescence
Singlet oxygen
Oxidative stress
Endotoxemia

ABSTRACT

Many studies have reported the occurrence of oxidative stress in different models of sepsis, but no measurements in real time and in non-invasive manner in an acute model of endotoxemia were done, being its mechanism still under debate. In the present work, we have used *in situ* surface chemiluminescence to evaluate the reactive oxygen species steady-state concentrations and to identify the main chemical species involved in this phenomenon. Experimental endotoxemia provoked a twofold increase in skeletal muscle chemiluminescence (control value: 31 ± 4 cps/cm^2). The use of cutoff filters and D_2O and biacetyl as specific enhancers, indicates that singlet oxygen is the main emitting species in this model. This result closely correlates with elevated TBARS levels, an index of oxidative damage to lipids. Increased NO production and NADPH oxidase activity may support the formation of $ONOO^-$, which in turn may originate $HO^\bullet$, an initiator of the lipid oxidation chain. In summary, our data show for the first time that $^1O_2$ is the main chemical and emitting species involved in the mechanism of oxidative stress present in an acute model of endotoxemia. This work provides new insights necessary to understand free radical mechanisms behind endotoxemic syndrome.

© 2010 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Sepsis and endotoxemia are described as a paradigm of acute whole body inflammation, with massive increases of nitric oxide (NO) and inflammatory cytokines in biological fluids, systemic damage to vascular endothelium, and impaired tissue and whole body respiration despite adequate oxygen supply [1,2]. Without timely and effective therapeutic intervention, this scenario evolves to multiple organ dysfunction and ultimately to death.

A series of studies have reported the occurrence of oxidative stress in different models of sepsis and endotoxemia in animals [3–5], a situation also reported in patients in which systemic inflammation was associated with plasma oxidative stress [6]. Increased production of reactive oxygen ($O_2^{\bullet-}$ and H_2O_2) and nitrogen (NO) species has been reported in experimental sepsis and endotoxemia [4,5]. The increased production of $O_2^{\bullet-}$ and NO leads to an increased formation rate of peroxynitrite ($ONOO^-$), the powerful oxidating and nitrating species. The causative series, $O_2^{\bullet-}$ and NO, $ONOO^-$, mitochondrial dysfunction and tissue dysfunction was addressed as an explanation of tissue injury in sepsis and endotoxemia [7,8].

In this present work, *in situ* chemiluminescence was used to evaluate the increased rate of lipid peroxidation and its time course and to identify the main species involved in organ surface photoemission. Interestingly, this methodology is non-invasive for the intact organ. In biological systems, it has been recognized that *in situ* chemiluminescence is a low-intensity emission in the visible range mainly due to the dimol emission of singlet oxygen and secondarily to the emission of excited carbonyls [9,10]. Both, singlet oxygen and excited carbonyls are by products formed in termination steps of the chain reaction of lipid peroxidation. The spontaneous chemiluminescence of organs *in situ* correlates with the development of oxidative stress and damage, and has been used in several models of lung, heart and liver toxicity [11,12].

The detection of increased photoemission indicates the increased formation of electronically excited species as a chemical phenomenon associated to cellular oxidative stress and damage [13]. In order to understand the molecular mechanism other approaches must be included in the analysis: (a) a correlation between the emission detected and the accumulation of oxidation products, (b) spectral analysis of maximal chemiluminescence which can accordingly be attributed to a particular excited species, and (c) an assessment of the feasibility of energy transfer to different specific acceptors (photoemission signal enhancers and quenchers) is important to identify the excited species involved.

The aim of this work was to evaluate, in real time, the molecular mechanisms involved in the oxidative damage present in rat skeletal muscle in an acute model of endotoxemia using chemilumines-

Abbreviations: SMM, submitochondrial membranes; CL, chemiluminescence; L-NMMA, N^G-methyl-L-arginine.

* Corresponding author. Tel.: +54 11 4508 3646; fax: +54 11 4508 3646.
E-mail address: salvarez@ffyb.uba.ar (S. Alvarez).

0009-2797/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.cbi.2010.01.011

cence as a non-invasive technique for the organ, and to identify the photoemissive species involved. To strengthen the analysis, a correlation with oxidative stress markers was performed. This study was conducted in hind limb adductor muscle, a tissue that is thought highly affected due to its high physiological oxygen extraction.

2. Materials and methods

2.1. Drugs and chemicals

Lipopolysaccharide (LPS, serotype 026:B6 from *Escherichia coli*) was from Sigma–Aldrich (St. Louis, MO). Other reagents, enzymes, and enzyme substrates were reagent grade and also purchased from Sigma–Aldrich.

2.2. Experimental design

Rats (Sprague–Dawley, female, 45 days old, 180 g) from the animal facility of the School of Pharmacy and Biochemistry of the University of Buenos Aires, were used. The animals were housed under standard conditions of light, temperature and humidity with unlimited access to water and food (pelleted rodent food). LPS was injected in a single dose of 10 mg/kg body weight. The treatments were performed 6 h before sacrifice or chemiluminescence assays. The two groups studied were (a) control group: animals were injected i.p. with saline solution and (b) LPS group: animals were injected i.p. with LPS (10 mg/kg). Animal treatment was carried out in accordance with the guidelines of the 6344/96 regulation of the Argentinean National Drug, Food and Medical Technology Administration (ANMAT).

2.3. Isolation of mitochondria

Rats were anesthetized [ketamine (50 mg/kg) plus xylazine (0.5 mg/kg)] and adductor muscle was immediately excised. The tissues were homogenized in a glass–Teflon homogenizer in a medium consisting of 0.23 M mannitol, 0.07 M sucrose, 10 mM Tris–HCl, and 1 mM EDTA, pH 7.4, at a ratio of 1 g/5 mL of medium. The homogenates were centrifuged at $700 \times g$ for 10 min to discard nuclei and cell debris, the sediment was discarded, and the supernatant was either used as homogenate fraction (for further assays) or centrifuged at $7000 \times g$ for 10 min to precipitate mitochondria [14]. The mitochondrial pellet was washed twice and resuspended in the same buffer; it consisted of mitochondria able to carry out oxidative phosphorylation. Purity of isolated mitochondria was assessed by determining lactate dehydrogenase activity; only mitochondria with less than 5% impurity were used [14]. Protein content was assayed with the Folin reagent using bovine serum albumin as standard.

2.4. In situ muscle and liver chemiluminescence

The whole animal was covered with aluminium foil, in which a window was cut allowing exposure of the organ (adductor muscle or liver) only. Determinations were performed with a Johnson Research Foundation photon counter. An EMI 9658 photomultiplier (responsive in the range 300–800 nm) cooled at −20 °C with an applied potential of −1.4 kV was used. The phototube output was connected to an amplifier-discriminator adjusted to a single photon counting which was in turn connected to both a frequency counter and a recorder. Efficient light collection and isolation from the organ surface were established by using a lucite rod as optical coupler placed in front of the exposed organ. Results were expressed as counts/second per unit of organ surface (cps/cm^2) [9]. Spectral analysis of organ photoemission was performed with cutoff Kodak Wratten type filters and the results corrected for the photomultiplier efficiency. As chemiluminescence enhancers, pure D_2O and biacetyl (10 mM) were used. Muscle was topicated with the enhancers and the CL was immediately measured.

2.5. Nitric oxide synthase activity

Nitric oxide production was determined in tissue homogenate by the oxidation of oxyhemoglobin to methemoglobin, followed spectrophotometrically at two wavelengths 577–591 nm ($\varepsilon = 11.2\ mM^{-1}\ cm^{-1}$) in a Beckman DU 7400 diode array spectrophotometer at 37 °C [15], in a reaction medium containing 50 mM phosphate buffer (pH 7.4), 0.1 mM $CaCl_2$, 0.2 mM L-arginine, 100 μM NADPH, 10 μM dithiothreitol, 4 μM Cu,Zn-SOD, 0.1 μM catalase, 0.2–0.5 mg protein/mL, and 20 μM oxyhemoglobin. Control measurements in the presence of 2 mM N^G-methyl-L-arginine (L-NMMA) were performed to consider only L-NMMA-sensitive hemoglobin oxidation, usually 90–95% and due to NO formation. Results were expressed as nmol NO/min per mg protein.

2.6. Oxygen consumption

A two-channel respirometer for high-resolution respirometry (Oroboros Oxygraph, Paar KG, Graz, Austria) was used. Briefly, muscle was cut into cubes of 1 mm^3 and oxygen consumption rates were measured in a reaction medium containing 118 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$ and 5.5 mM glucose at 30 °C. Results were expressed as ng-at O/min per g of tissue [16].

2.7. NADPH oxidase activity

NADPH oxidase activity was measured as NADPH-dependent superoxide production by tissue cubes, using the lucigenin-enhanced chemiluminescent method [17]. Briefly, muscle was cut into cubes of 1 mm^3 and superoxide production rate was measured in a reaction medium containing 99 mM NaCl, 4.7 mM KCl, 1 mM K_2HPO_4, 1.2 mM $MgSO_4$, 1.9 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM glucose and 10 mM HEPES (pH 7.4), 5 μM lucigenin and 10 μM NADPH at 37 °C [18]. A LKB Wallack 1209 Rackbeta liquid scintillation counter was used. Control measurements were performed in the presence of 1 μM SOD. Data were corrected for background activity and only SOD-sensitive signal was informed as NADPH-dependent superoxide production. Results were expressed as counts/minute per gram of tissue (cpm/g tissue).

2.8. Thiobarbituric acid-reactive substances (TBARS)

TBARS were determined using a fluorescence assay. An aliquot of skeletal muscle homogenate or mitochondrial fraction were added to 2 mL of 0.1N HCl, 0.3 mL 10% (w/v) phosphotungstic acid and 1 mL of 0.7% (w/v) 2-thiobarbituric acid. Butylated hydroxytoluene was added to a final concentration of 0.1% (w/v). The mixture was heated in boiling water for 60 min. TBARS were extracted in 5 mL of n-butanol. After a brief centrifugation, the fluorescence of the butanolic layer was measured in a Perkin Elmer LS 55 luminescence spectrometer at 515 nm (excitation) and 553 nm (emission). A calibration curve was prepared using 1,1,3,3-tetramethoxypropane as standard. Results were expressed as pmol of TBARS/mg of protein [19].

2.9. Determination of carbonyl content

The content of carbonyl groups in oxidatively modified proteins was measured in muscle homogenate and mitochondrial fraction by determining the amount of 2,4-Dinitrophenylhydrazone formed upon reaction with 2,4-Dinitrophenylhydrazine. The samples were

Table 1
D_2O and biacetyl effect on muscle CL of control and LPS-treated animals.[a].

Treatment	CL (cps/cm^2)	% of increase
Control	32 ± 4	
Control + D_2O	48 ± 5**	51
Control + biacetyl	41 ± 3**	27
LPS	61 ± 3	
LPS + D_2O	114 ± 8♦	86
LPS + biacetyl	86 ± 6♦♦	41

[a] The adductor muscle was exposed and analyzed as described in Section 2. Muscle was topicated with D_2O (pure) or biacetyl (10 mM in water) and the CL was immediately measured. Data are means ± SEM of five rats for each group.
** $p < 0.05$ with respect to control by ANOVA–Dunnet test.
♦ $p < 0.01$ with respect to LPS by ANOVA–Dunnet test.
♦♦ $p < 0.05$ with respect to LPS by ANOVA–Dunnet test.

homogenized in 0.5N $HClO_4$. The suspension was centrifuged at 600 × *g* for 10 min at 0–4 °C to remove nuclei and cell debris. The pellet was discarded and the supernatant was used. The samples were treated with 2 mM 2,4-Dinitrophenylhydrazine at room temperature for 1 h. Proteins were precipitated with 20% TCA and 10% TCA, washed with ethanol/ethyl acetate (1:1), and dissolved in 6 M guanidine hydrochloride (pH 2.5). Carbonyl content was calculated from the absorbance maximum of 2,4-Dinitrophenylhydrazone at 360 nm normalized to the absorbance at 350 nm, with an $\varepsilon_{390-350}$ of 22 mM^{-1} cm^{-1} [20]. Results were expressed in nmol of carbonyl group/mg of protein.

2.10. Statistics

Results were expressed as mean values ± SE, and represent the mean of 6–11 independent experiments. Student's *t*-test for unpaired data was used to analyze differences between mean values of two groups. Statistical significance was considered at $p < 0.05$. For Table 1 ANOVA followed by Dunnet test was used to analyze statistical significance, results were expressed as mean ± SEM with $n = 5$.

3. Results

3.1. In situ muscle and liver chemiluminescence in control and LPS-treated rats

In situ spontaneous hind limb adductor (leg muscle) and liver chemiluminescence were determined at the organ surface. Of note, surface CL accounts for the photoemission from a 0.05 mm thickness of the organ. Fig. 1A shows two independent and superimposed representative measurements of leg muscle chemiluminescence of control and LPS-treated animals. The lower trace corresponds to the dark current of the phototube and the upper traces are related to the emission that is recorded after opening the optical shutter and correspond to the muscle surface photoemission in control and LPS-treated animals. Experimental endotoxemia provoked a twofold increase in skeletal muscle chemiluminescence, as compared to the control value: 31 ± 4 cps/cm^2, while liver chemiluminescence remained unchanged (control value: 15 ± 1 cps/cm^2) (Fig. 1B).

Identification of the photoemissive species in the present experimental model was approached by physical (spectral distribution of light emission) and chemical (effect of enhancers of singlet oxygen and excited carbonyls chemiluminescence) analysis of the chemiluminescence. For the spectral analysis of the emitted light in LPS-treated rats, optical filters were placed into the light path. The spectral analysis of the skeletal muscle chemiluminescence (Fig. 2) showed a broad and intense emission band in the red region, between 660 and 800 nm, which is consistent with $^1O_2$ dimol emission at 634 and 703 nm, and a smaller emission band between 560 and 600 nm that may be consistent with the emission of dioxetane or bicarbonyl compounds. The effect of D_2O (an enhancer of $^1O_2$ dimol emission) and biacetyl (an enhancer of excited carbonyl emission by triplet–triplet transfer) is shown in Table 1. In the muscle of LPS-treated animals an 86% increase with D_2O and a 41% increase with biacetyl were obtained. These results clearly indicate that $^1O_2$ is the main species involved in photoemission and one of the oxidizing species generated in leg muscle during endotoxemia.

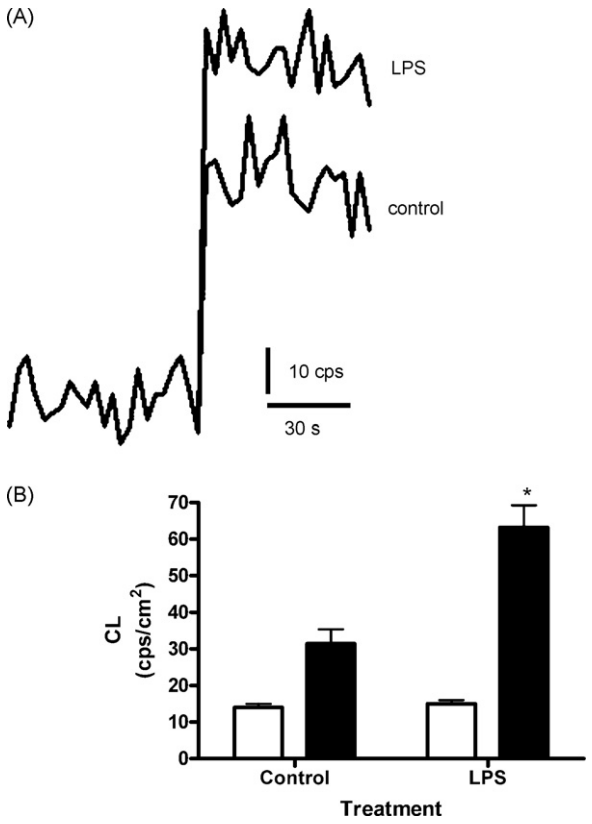

Fig. 1. (A) Chemiluminescence of the adductor muscle of control and LPS-treated rats. (B) Liver (white bars) and skeletal muscle (black bars) chemiluminiscence from endotoxemic rats. Experimental details in Section 2. *$p < 0.01$ with respect to control group, unpaired *t*-test. Data are means ± SE, $n = 11$.

3.2. Oxygen consumption and NADPH oxidase activity in tissue cubes

Experimental endotoxemia slightly increased (by 26%) tissue O_2 consumption (Table 2). NADPH oxidase seems to have a role under

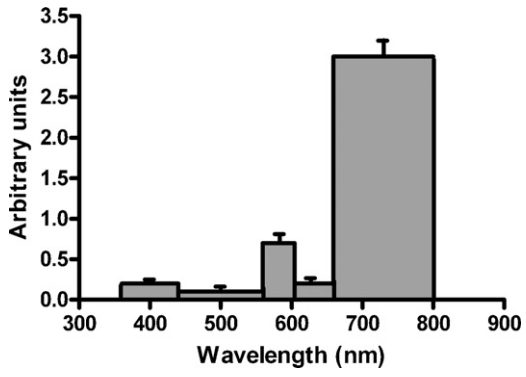

Fig. 2. Spectral analysis of adductor muscle chemiluminescence of LPS-treated rats. Experimental details in Section 2.

Table 2
Oxygen consumption and NADPH oxidase activity in tissue cubes, and NO production in homogenate of adductor muscle from control and LPS-treated animals.[a].

Treatment	O_2 consumption (ng-at O/min g tissue)	NADPH oxidase activity (10^5 cpm/g tissue)	NO production (nmol NO/min mg protein)
Control	1441 ± 84	14.9 ± 2.1	0.45 ± 0.02
LPS	1784 ± 93**	22.7 ± 2.5**	0.60 ± 0.08**

Data are means ± SE of six rats for each group.
[a] Oxygen consumption and NADPH oxidase activity were assessed in tissue cubes of 1 mm^3.
** $p < 0.05$ with respect to control, unpaired *t*-test.

Table 3
TBARS and protein carbonyl content in mitochondrial fraction and homogenate of adductor muscle from control and LPS-treated animals.[a].

Treatment	TBARS (pmol/mg protein)		Protein carbonyls (nmol/mg protein)	
	Mitochondria	Homogenate	Mitochondria	Homogenate
Control	77 ± 8	145 ± 10	1.25 ± 0.09	9.0 ± 0.8
LPS	112 ± 13**	187 ± 9**	1.54 ± 0.07**	7.7 ± 0.9

[a] Data are means ± SE of six rats for each group.
** $p < 0.05$ with respect to control, unpaired *t*-test.

inflammatory conditions as source of reactive oxygen species, primarily $O_2^{\bullet -}$. The activity of this enzyme, assayed as NADPH-dependent superoxide production, was increased 1.3 times with respect the control value [$(24.9 \pm 2.1)\,10^5$ cpm/g tissue] (Table 2).

3.3. Nitric oxide production in tissue homogenate

NO plays an important regulatory role on mitochondrial respiration and the inflammatory response and is likely involved the nitroxidative damage present in endotoxemic and septic syndromes. For that reason, we determined the NO production in homogenate from adductor muscle. The production of NO by muscle homogenate was found increased by 33% (control value: 0.45 ± 0.02 nmol NO/min mg protein). This result is shown in Table 2.

3.4. Phospholipid oxidation and protein carbonyl content in tissue homogenate and mitochondrial fraction

Phospholipid oxidation (measured as TBARS content) and protein carbonyl content are indications of oxidative damage to tissue lipids and proteins and are direct markers of oxidative stress and damage, and the results are shown in Table 3. The TBARS levels increased in muscle homogenate and mitochondria in endotoxemic animals. The increases were 30 and 45%, respectively. The protein carbonyl content was observed increased in muscle mitochondria by 23%. It is worth to note that CL measurement (*in vivo* assay) closely and positive correlates to phospholipid oxidation in homogenate and mitochondrial fraction (*in vitro* assay) (Fig. 3).

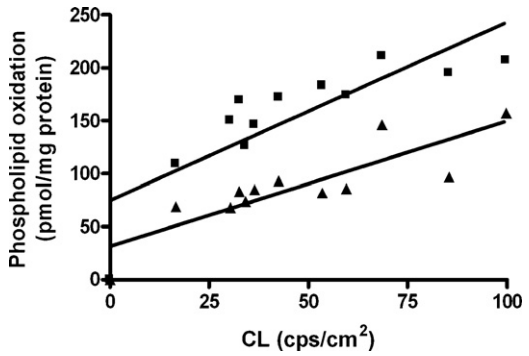

Fig. 3. Linear correlation between surface chemiluminescence and phospholipid oxidation assayed as TBARS content, from muscle of control and LPS-treated animals (■, homogenate: $p < 0.0008$, $r^2 = 0.70$; ▲, mitochondria: $p < 0.0004$, $r^2 = 0.73$).

4. Discussion

The present study shows that *in situ* surface organ chemiluminescence is a useful approach to determine the occurrence of oxidative stress and damage *in vivo*, and to identify in real time the chemical species involved. According to the classical definition by Sies [21], an increased content of oxidants (oxidative chemical species) leads to oxidative stress and oxidative damage. Considering that oxidative species are continuously produced in cells and tissues, an increased steady-state level in the cells of oxidizing free radicals ($HO^{\bullet}$, $ROO^{\bullet}$) and related species ($O_2^{\bullet -}$, H_2O_2, $^1O_2$) that substain the biochemical free-radical reaction chain, constitute the chemical basis for the biological condition of cellular oxidative stress. Acute endotoxemia increased the steady-state concentration of $^1O_2$ and excited carbonyls in muscle, as measured *in vivo* and in real time by *in situ* chemiluminescence. Contributing to oxidative stress, as assessed by organ chemiluminescence, is the production of NO and lipid peroxidation. It is worth to note that the identification, *in vivo* and in real time, of the chemical species involved in the mechanism of oxidative stress in muscle endotoxemia, has never been informed before.

Oxidative stress is a common event in the septic condition, and it is accepted that muscle is one of the first organs to be affected, probable due to a homeostatic response [1,22]. Organ chemiluminescence is a non-invasive and non-destructive assay that is suited to the study of oxidative stress and oxidative damage. It directly determines the *in vivo* steady-state level of $^1O_2$, with a steady-state level of 10^{-16} M $^1O_2$ corresponding to 20–30 cps/cm^2 [13]. This species is a product of the peroxyl-radical intermediates in the lipid peroxidation process according to a Russell mechanism (Eqs. (1) and (2)) [23]. Secondary peroxyl radicals yield alternatively $^1O_2$ or =CO* as products:

$$2 > CHOO^{\bullet} \rightarrow {}^1O_2 + > C{=}O + > CHOH \quad (1)$$

$$2 > CHOO^{\bullet} \rightarrow O_2 + > C{=}O* + > CHOH \quad (2)$$

Adductor muscle photoemission was found to be increased in LPS-treated animals, being this result in agreement with a previous observation [4] in an animal model of sepsis produced by cecal ligation and double perforation. To understand the molecular mechanism of the formation of excited species, identification of the wavelength of maximal photoemission must be determined and attributed to a particular excited species. The use of cutoff filters to obtain organ emission spectra is a complex procedure that is worth to analyze. The difference in photoemission between successive cutoff filters is corrected for filter transmittance and for the photomultiplier efficiency. The spectral analysis of the emission,

mainly in the range 650–800 nm, indicates $^1O_2$ as the main chemical species involved in the emission. This emission refers to the process of the $^1O_2$ dimol emission with molecules decaying to the ground state ($2[^1\Delta g] \rightarrow 2[^3\Sigma g^-]$ transition) [24,25]. A minor or secondary participation of excited carbonyls is also indicated by the 560–600 nm emission band, clearly distinct from adjacent emissions. The situation is similar to the 520–550 nm emission band observed in liver after neutrophile recruitment and activation [12]. This band seems associated to the inflammatory response and NO involvement.

As described in Section 1, it is advisable to use different approaches in the identification of the generated excited species. The use of specific enhancers or inhibitors is convenient for a second approach. For this reason, we used D_2O that specifically enhances $^1O_2$ signal by extending its lifetime [26] and biacetyl that specifically enhances excited carbonyls signal by an energy transfer mechanism [27]. The probes were found effective in enhancing $^1O_2$ dimol emission an carbonyl emission respectively, indicating the occurrence of the Russel reaction *in vivo*, as it was also recognized for *in vitro* lipid peroxidation by Di Mascio et al. [28]. The obtained results strengthen the conclusion that $^1O_2$ is the main chemical species involved in the oxidative stress mechanism with a secondary participation of CO*.

The correlation of photoemission and production of excited species or accumulation of stable molecular products is advisable to further understand the underlying mechanisms. For this reason, on the one hand, determination of oxygen consumption and NADPH oxidase activity in tissue cubes, and NO production in tissue homogenate were assays performed with the aim of clarifying the involvement of active species ($O_2^{\bullet -}$ and NO) in the initiation of processes leading to increased chemiluminescence observed in this model. On the other hand, carbonyl content (due to protein oxidation) and TBARS (due to phospholipid oxidation) were analyzed as index of accumulation of stable products due to oxidative stress. TBARS, a phospholipid oxidation indicator, were found increased in homogenates and in mitochondria, although protein carbonyl content was found increased only in mitochondria. The positive and linear correlation between electronically excited states formation and accumulation of phospholipid oxidation products (Fig. 3) adds to the biological meaning of this study. This observation is in agreement with the idea that $^1O_2$ and CO* are formed during the termination steps of the chain reaction of lipid peroxidation [11]. Other studies have informed a relationship between chemiluminescence and oxidative damage, in models of skin damage by exposure to UVA radiation [29], exposure to 85% oxygen [11], liver ischemia-reperfusion [12] and particulate air pollution inhalation [30].

NO plays an important regulatory role on mitochondrial respiration [31,32] and the inflammatory response [33], and is likely to be involved in the nitroxidative damage present in endotoxemia and sepsis. Tissue oxygen uptake was found increased in muscle. The activation of NADPH oxidase and the non-mitochondrial production of reactive oxygen species can explain this observation. Inflammatory processes (as endotoxemia and sepsis) include leukocyte recruitment and activation (respiratory burst) in tissues, thus of importance in this pathological processes as a mechanism of tissue injury. It has been extensively shown that NADPH oxidases are important sources of reactive oxygen species under inflammatory conditions [34] and that iNOS expression requires NADPH oxidase-dependent redox signaling [35]. Another mechanism is related to mitochondrial oxygen consumption and production of reactive oxygen species. Increased tissue oxygen consumption may be originated by an elevated mitochondrial in state 3 (active respiration) in order to respond to high ATP demand, despite inhibition of mitochondrial respiration due to increased NO production [8]. The increased formation of $O_2^{\bullet -}$ and NO [35,36] may account for the formation of H_2O_2 and $ONOO^-$. Peroxynitrite is a powerful oxidant that can yield the highly reactive hydroxyl radical ($HO^{\bullet}$) after homolysis by reaction with Fe^{3+}, initiating free radical chain reactions (as lipid peroxidation) [37]. Also, due to high levels produced, NO diffuses to mitochondria were it can exert inhibitory effects on the complexes of the respiratory chain (mainly complexes IV and I), and ultimately produces mitochondrial dysfunction [5,38].

In summary, our data show for the first time that $^1O_2$ is involved in the mechanism of oxidative stress and damage in acute endotoxemia. The usefulness of organ chemiluminescence is further supported by its positive correlation with NO production and TBARS content. This work provides new insights to the understanding of the molecular mechanisms involving free radicals and excited species in endotoxemia.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was supported by research grants B030 from the University of Buenos Aires, PICT 20494 from Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT), and PIP 6320 from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). The authors are extremely grateful to Dr. Jorge Daniel Rasmussen for his generous support.

References

[1] M.R. Pinsky, Sepsis: a pro- and anti-inflammatory disequilibrium syndrome, in: C. Ronco, R. Bellomo, G. La Greca (Eds.), Contributions to Nephrology, vol. 132, Karger, Basel, 2001, pp. 354–366.
[2] C. Szabó, Pathophysiological roles of nitric oxide in inflammation, in: L.J. Ignarro (Ed.), Nitric oxide: Biology and Pathobiology, Academic Press, San Diego, 2000, pp. 841–872.
[3] E. Crimi, V. Sica, A.S. Slutsky, H. Zhang, S. Williams-Ignarro, L.J. Ignarro, C. Napoli, Role of oxidative stress in experimental sepsis and multisystem organ dysfunction, Free Radic. Res. 7 (2006) 665–672.
[4] S. Llesuy, P. Evelson, B. Gonzalez-Flecha, J. Peralta, M.C. Carreras, J.J. Poderoso, A. Boveris, Oxidative stress in muscle and liver of rats with septic syndrome, Free Radic. Biol. Med. 16 (1994) 445–451.
[5] S. Alvarez, A. Boveris, Mitochondrial nitric oxide metabolism in rat muscle during endotoxemia, Free Radic. Biol. Med. 35 (2004) 1472–1478.
[6] J.M. Alonso de Vega, J. Diaz, E. Serrano, L.F. Carbonell, Oxidative stress in critically ill patients with systemic inflammatory response syndrome, Crit. Care Med. 8 (2002) 1782–1786.
[7] J. Boczkowski, C.L. Lisdero, S. Lanone, A. Samb, M.C. Carreras, A. Boveris, M. Aubier, J.J. Poderoso, Endogenous peroxynitrite mediates mitochondrial dysfunction in rat diaphragm during endotoxemia, FASEB J. 13 (1999) 1637–1646.
[8] V. Vanasco, M.C. Cimolai, P. Evelson, S. Alvarez, The oxidative stress and the mitochondrial dysfunction caused by endotoxemia are prevented by α-lipoic acid, Free Radic. Res. 42 (2008) 815–823.
[9] A. Boveris, E. Cadenas, R. Reiter, M. Filipowski, Y. Nakase, B. Chance, Organ chemiluminescence: noninvasive assay for oxidative radical reactions, Proc. Natl Acad. Sci. U.S.A. 77 (1980) 347–351.
[10] E. Cadenas, H. Sies, Low-level chemiluminescence as an indicator of singlet molecular oxygen in biological systems, Methods Enzymol. 105 (1984) 221–231.
[11] P. Evelson, B. Gonzalez-Flecha, Time course and quantitative analysis of the adaptive responses to 85% oxygen in the rat lung and heart, Biochim. Biophys. Acta 1523 (2000) 209–216.
[12] J.C. Cutrin, A. Boveris, B. Zingaro, G. Corvetti, G. Poli, In situ determination by surface chemiluminescence of temporal relationships between evolving warm ischemia-reperfusion injury in rat liver and phagocyte activation and recruitment, Hepatology 31 (2000) 622–632.
[13] E. Cadenas, C. Giulivi, F. Ursini, A. Boveris, Electronically excited state formation, in: C. Tyson, J. Frazier (Eds.), Methods in Toxicology, Academic Press, San Diego, 1994, pp. 384–399.
[14] E. Cadenas, A. Boveris, Enhancement of hydrogen peroxide formation by protophores and ionophores in antimycin-supplemented mitochondria, Biochem. J. 188 (1980) 31–37.
[15] A. Boveris, S. Lores-Arnaiz, J. Bustamante, S. Alvarez, L. Valdez, A.D. Boveris, A. Navarro, Pharmacological regulation of mitochondrial nitric oxide synthase, Methods Enzymol. 359 (2002) 328–339.

[16] J.J. Poderoso, S. Fernandez, M.C. Carreras, D. Tchercanski, C. Acevedo, M. Rubio, J. Peralta, A. Boveris, Liver oxygen uptake dependence and mitochondrial function in septic rats, Circ. Shock 44 (1994) 175–182.
[17] Y. Li, H. Zhu, R. Nistala, P. Kuppusamy, V. Roubaud, J.L. Zweier, M.A. Trush, Validation of lucigenin (bis-N-methylacridinium) as a chemilumigenic probe for detecting superoxide anion radical production by enzymatic and cellular systems, J. Biol. Chem. 273 (1998) 2015–2023.
[18] H.J. Wang, Y.X. Pan, W.Z. Wang, I.H. Zucker, W. Wang, NADPH oxidase-derived reactive oxygen species in skeletal muscle modulates the exercise pressor reflex, J. Appl. Physiol. 107 (2009) 450–459.
[19] C.G. Fraga, B.E. Leibovitz, A.L. Tappel, Lipid peroxidation measured as thiobarbituric acid-reactive substances in tissues slices characterization and comparison with homogenates and microsomes, Free Radic. Biol. Med. 4 (1988) 155–161.
[20] R.L. Levine, J.A. Williams, E.R. Stadtman, E. Shacter, Carbonyl assays for determination of oxidatively modified proteins, Methods Enzymol. 233 (1994) 346–357.
[21] H. Sies, Oxidative stress: introductory remarks, in: H. Sies (Ed.), Oxidative Stress, Academic Press, San Diego, 1985, pp. 1–7.
[22] S. Alvarez, P. Evelson, M.C. Cimolai, Oxygen and nitric oxide metabolism in sepsis, in: S. Alvarez, P. Evelson, A. Boveris (Eds.), Free Radical Pathophysiology, Transworld Research Network, Kerala, 2008, pp. 223–236.
[23] G.A. Russell, Deuterium-isotope effects in the autoxidation of arakyl hydrocarbons: mechanism of the interaction of peroxy radicals, J. Am. Chem. Soc. 79 (1957) 3871–3877.
[24] E. Cadenas, H. Sies, Low-level chemiluminescence of as an indicator of singlet molecular oxygen in biological systems, Methods Enzymol. 105 (1984) 221–231.
[25] E. Cadenas, A. Boveris, B. Chance, Low-level chemiluminescence of biological systems, in: W. Pryor (Ed.), Free Radicals in Biology, Academic Press, New York, 1984, pp. 211–242.
[26] C. Schweitzer, R. Schmidt, Physical mechanisms of generation and deactivation of singlet oxygen, Chem. Rev. 103 (2003) 1685–1757.
[27] I. Nantes, A. Faljoni-Alario, A. Vercesi, K. Santos, E. Bechara, Liposome effect on the cytochrome c catalyzed peroxidation of carbonyl substrates to triplet species, Free Radic. Biol. Med. 25 (1998) 546–553.
[28] P. Di Mascio, L.H. Catalani, E.J. Bechara, Are dioxetanes chemiluminescent intermediates in lipoperoxidation? Free Radic. Biol. Med. 12 (1992) 471–478.
[29] P. Evelson, C. Ordoñez, S. Llesuy, A. Boveris, Oxidative stress and *in vivo* chemiluminescence in mouse skin exposed to UVA radiation, J. Photochem. Photobiol. B: Biol. 38 (1997) 215–219.
[30] S. Gurgueira, J. Lawrence, B. Coull, G. Krishna Murthy, B. Gonzalez-Flecha, Rapid increases in the steady-state concentration of reactive oxygen species in the lungs and heart after particulate air pollution inhalation, Environ. Health Perspect. 110 (2002) 749–755.
[31] A. Boveris, L.E. Costa, E. Cadenas, J.J. Poderoso, Regulation of mitochondrial respiration by adenosine diphosphate, oxygen and nitric oxide synthase, Methods Enzymol. 301 (1999) 188–198.
[32] D.L. Boveris, A. Boveris, Oxygen delivery to the tissues and mitochondrial respiration, Front. Biosci. 12 (2007) 1014–1023.
[33] S. Alvarez, P. Evelson, Nitric oxide and oxygen metabolism in inflammatory conditions: sepsis and exposition to polluted ambients, Front. Biosci. 12 (2007) 964–974.
[34] R. Brandes, G. Koddemberg, W. Gwinner, D. Kim, H. Kruse, R. Busse, A. Mugge, Role of increased production of superoxide anions by NADPH oxidase and xanthine oxidase in prolonged endotoxemia, Hypertension 33 (1999) 1243–1249.
[35] F. Wu, K. Tyml, J.X. Wilson, iNOS expression requires NADPH oxidase-dependent redox signaling in microvascular endothelial cells, Cell. Physiol. 217 (2008) 207–214.
[36] K. Stadler, M. Bonini, S. Dallas, J. Jiang, R. Radi, R. Mason, M. Kadiiska, Involvement of inducible nitric oxide synthase in hydroxyl radical-mediated lipid peroxidation in streptozotocin-induced diabetes, Free Radic. Biol. Med. 45 (2008) 866–874.
[37] J.S. Gujral, J.A. Hinson, A. Farhood, H. Jaeschke, NADPH oxidase-derived oxidant stress is critical for neutrophil cytotoxicity during endotoxemia, Am. J. Physiol.: Gastroenterol. Liver Physiol. 287 (2004) 243–252.
[38] D. Brealey, M. Brand, I. Hargreaves, S. Heales, J. Land, R. Smolenski, N.A. Davies, C.E. Cooper, M. Singer, Association between mitochondrial dysfunction and severity and outcome of septic shock, Lancet 360 (2002) 219–223.