Hemorrhagic Shock: Nitric Oxide in Anesthetized and Non Anesthetized Rats

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SUMMARY

Background
We have previously demonstrated that hypovolemia induced by acute bleeding is accompanied by a dynamic, heterogenous and time-dependent activation of the cardiac nitric oxide synthase (NOS). This system might be involved in the hemodynamic anomalies observed after blood volume depletion.

Objective
To assess the role of the mitochondrial nitric oxide (NO) system in the adaptive response of the cardiovascular system in anesthetized and non anesthetized rats under hypovolemic shock.

Material and Methods
Animals were divided in four groups (n=7 animals per group): Group A, anesthetized control rats; group C, non anesthetized control rats; group AB, anesthetized rats subjected to bleeding (20% of blood volume), and group CB, non anesthetized rats subjected to bleeding. Oxygen consumption, functional activity of mitochondrial NOS (mtNOS) and mitochondrial production of NO were assessed.

Results
There were no significant differences in the values of respiratory parameters among the different study groups. Group AB had less functional activity of mtNOS compared to group A (12±2 and 19±1, respectively). This effect was even lower in non anesthetized animals subjected to bleeding (17±1 and 20±1, respectively). Mitochondrial production of NO decreased in anesthetized and non anesthetized animals with acute bleeding compared to controls.

Conclusions
Mitochondrial NO system might be involved in the adaptive response of the cardiovascular system under acute volume depletion, depending on the animal's degree of anesthesia.

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Key words > Hemorrhage - Mitochondria - Nitric Oxide - Hypotension

Abbreviations >

ANOVA	Analysis of variance	**NO**	Nitric oxide
HR	Heart rate	**NOS**	Nitric oxide synthase
L-NAME	N^G-Nitro-L-Arginine Methyl Ester	**MAP**	Mean arterial pressure
mtNOS	Mitochondrial nitric oxide synthase		

BACKGROUND

Hemorrhagic shock frequently leads to dysfunction of different vital organs with subsequent high morbidity and mortality rates. (1) The heart is one of the affected organs and also plays a key role in the adaptive response of the body in hypotension induced by acute bleeding. Cardiovascular adaptation during acute hypovolemia depends not only on the control of the autonomic nervous system but also on the magnitude and the velocity of bleeding and on the species studied. (2) Among other alterations, acute blood volume loss induces hemodynamic instability, reduced tissue perfusion and cellular hypoxia. (3) There is evidence that the production of nitric oxide (NO) increases during decompensated bleeding, contributing

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to vascular hyporeactivity and, probably, to abnormalities in the autonomic regulation of the heart. (4, 5) We have previously demonstrated that hypovolemic state induced by acute hemorrhage triggers a dynamic, heterogeneous and time-dependent activation of the cardiac nitric oxide synthase (NOS). The production of endothelial NO increases in the early phases (60 minutes) after blood loss. However, in the delayed phases (120 minutes) the inducible isoform predominates and constitutes the main source of production of NO in this phase. (6) In our laboratory, the inhibition of the NO system with L-NAME attenuates the acceleration of heart rate induced by acute hypovolemic state. (6) It is well-known that the effects of NO on the cardiovascular system are mediated by the reaction of NO with different targets that include hemoproteins, thiols and superoxide anions. Mitochondria possess several hemoproteins (cytochrome c oxidase), thiols (glutathione) and cysteine-containing proteins, and they are major cellular sources of superoxide anion. Consequently, mitochondria contribute to several of the biological functions of NO. (7) Mitochondrial NOS (mtNOS) activity may present abnormalities under different situations, such as adaptation to low oxygen bioavailability and hypoxia, adaptation to cold environments, and processes related to cell life and death. (8, 9) Yet, little is known about the role of mitochondrial NO in the adaptation to hemorrhagic shock. We wanted to evaluate if the production of mitochondrial NO modulates the efficiency of the cardiovascular adaptation to the hemodynamic imbalance induced by an acute hypovolemic state and if this response depends on the state of consciousness of the subject studied. In steady state, NO concentration might modulate mitochondrial bioenergetics and functional mitochondrial activity, regulating the cellular activity in the hypovolemic state. Taking this hypothesis into account, the goal of the present study was to evaluate the participation of mitochondrial NO in the adaptive response of the cardiovascular system in anesthetized and non anesthetized rats under hemorrhagic shock.

MATERIAL AND METHODS

The experiments were performed on male Sprague–Dawley rats (230 - 250 g). Animals were housed in humidity and temperature controlled environment, illuminated with a 12:12 hours light-dark cycle. They were fed with rat chow provided by Nutrimentos Purina, Argentina and water ad libitum until the day of the experiments. Animal care was in accordance with the 6344/96 regulation of the *Administración Nacional de Medicamentos Alimentos y Tecnología Médica* (ANMAT, National Drug Food and Medical Technology Administration), *Ministerio de Salud y Ambiente de la Nación*.

Non anesthetized animals during hemorrhage.

The animals were anesthetized with ether and kept under anesthesia throughout the surgery. The right and the left femoral arteries were cannulated to measure mean arterial pressure (MAP) and heart rate (HR), and for blood withdrawal, respectively. Animals were housed in metabolic cages 24 hours before initiating the experiment.

Anesthetized animals during hemorrhage

All rats were anesthetized with urethane (1.0 g/kg, ip). Body temperature was monitored with a rectal thermometer and maintained between 36 and 38 °C throughout the experiment. A tracheotomy was performed using a polyethylene (PE-240) tubing to ensure appropriate pulmonary ventilation. Then, the right and the left femoral arteries were cannulated to measure MAP and HR, and for blood withdrawal, respectively. Mean arterial pressure was measured with a pressure transducer (Statham P23 ID, Gould Inst. Cleveland, OH) and recorded with a polygraph (Physiograph E&M Co, Houston, TX). Heart rate was determined from the pulse pressure signal by beat-to-beat conversion with a tachograph preamplifier (S77-26 tachometer, Coulbourn Inst., Allentown, PA). The Labtech Notebook program (Laboratory Tech., Wilmington, MD) was used for data acquisition. Mean arterial pressure and HR were continuously recorded. Hemorrhagic shock was induced by withdrawing 20% of total blood volume in 2 min period; the total amount of blood withdrawn was kept constant. Blood withdrawl (20% of blood volume) was calculated for each animal from the total blood volume corresponding to the body weight of each animal.

Experimental protocol.

Four experimental groups were used:

1. Anesthetized control rats (A): after a 15-minute stabilization period, basal values of MAP and HR were measured for 5 minutes. Thereafter, MAP and HR were continuously recorded for 120 minutes (n = 7).
2. Non anesthetized control rats (C): after a 15-minute stabilization period, basal values of MAP and HR were measured for 5 minutes. Thereafter, MAP and HR were continuously recorded for 120 minutes (n = 7).
3. Anesthetized rats subjected to bleeding (AB): After a 15-minute stabilization period, basal values of MAP and HR were measured for 5 minutes, and thereafter the animals were subjected to bleeding (withdrawal of 20% of blood volume). Then, MAP and HR were continuously recorded for 120 minutes (n = 7).
4. Non anesthetized rats subjected to bleeding (CB): after a 15-minute stabilization period, basal values of MAP and HR were measured for 5 minutes, and thereafter the animals were subjected to bleeding (20% of blood volume). Then, MAP and HR were continuously recorded for 120 minutes (n = 7).

Isolation of cardiac mitochondrial fraction: after the experimental time, the animals were killed by decapitation and the hearts were immediately removed and placed in a solution containing 0.23 M mannitol, 0.07 M sucrose, 1 mM EDTA and 10 mM Tris-HCl (pH 7.4) (MSTE). Tissues were homogenized in MSTE with a Teflon Potter-Elvehjem homogenizer (9 ml/g of heart). The homogenate was centrifuged at 700 g for 10 min; the supernatant was separated and centrifuged at 8000 g for 10 min. The mitochondrial fraction was washed and suspended in MSTE buffer. All the procedures were performed at a temperature of 0-2°C. Protein concentration in the mitochondrial fractions was determined using the Lowry assay. (10)

Mitochondrial respiration and functional activity of mtNOS: oxygen consumption was determined polarographically using a Clark-type oxygen electrode connected to a 1.5-ml chamber at 30°C in the following reaction medium 0.23

M mannitol, 0.07 M sucrose, 20 mM Tris-HCl,5 mM phosphate buffer , 1 mM EDTA (pH 7.4), with air-saturated oxygen (225 mM O_2), and 0.5-1.0 mg of mitochondrial protein/ml. Mitochondria were supplemented with 7mM succinate as substrate, in the absence (state 4) or in the presence (state 3) of 0.5 mM of 5 mM ADP. Oxygen uptake was expressed in ng-at O/min.mg protein. Respiratory control index was calculated as the ratio of the state 3 to state 4 mitochondrial oxygen consumption rates, described by Boveris et al. in 1999. (10) The mtNOS-regulated state 3 respiration, also termed mtNOS functional activity, was calculated as the difference in state 3 mitochondrial respiration between a condition of maximal intramitochondrial NO levels, with mitochondria added with 1 mM arginine and 0.5 μM CuZn-superoxide dismutase (SOD), and a condition of minimal intramitochondrial NO levels, with mitochondria added with 2 mM L-NAME and 10 μM oxyhemoglobin (HbO_2) (8)

Nitric oxide production: NO production was measured by spectrophotometric (model DU 7400 diode array spectrophotometer, Beckman) monitoring (at 577–591 nm) of the oxidation of HbO_2 to methemoglobin at 37°C. The reaction medium used to determine NO production consisted of 50 mM K_2HPO_4/KH_2PO_4 (pH 7.4), 1 mM L-arginine, 1mM $CaCl_2$, 0.1 mM NADPH, 10 mM dithiotreitol, 2 mM SOD, 0.1 mM catalase and 30 mM oxyhemoglobin. Control experiments adding 1 mM NG-methyl - L-arginine (L-NMMA) were performed and L-NMMA-sensitive hemoglobin oxidation was considered due to NO formation that was expressed as nmol NO/min.mg protein.

Statistical Analysis

Results are expressed as mean values ± standard error of the means (SEM). Analysis of variance (ANOVA) followed by Bonferroni test was used for multiple comparisons. A Student's *t* -test was used to compare matched and unmatched pairs between the two groups. A p value < 0.05 was considered statistically significant. The software GraphPad Prism 3.02 (Graph Pad Software, San Diego, CA, USA) was used for statistical analysis.

RESULTS

Changes in mean blood pressure and heart rate

Figure 1 (graph A and B) Illustrates the time course of MBP in groups AB and CB. Hemorrhage induced a decrease in MAP in anesthetized animals; with subsequent stabilization at about 50 ± 5 mm Hg (*p < 0.01 versus basal values) at 15 min. Hypotension was maintained throughout the whole experimental period. Pretreatment with L-NAME did not attenuate the immediate hypotension induced by hemorrhage in these animals, yet at 30 minutes MAP was not significantly different from basal values. In non anesthetized animals, the magnitude of immediate hypotension induced by bleeding was similar to that observed in anesthetized rats (25 ± 4 mm Hg at 2 min), and stabilized at about 100 mm Hg 5 minutes after hemorrhage. This value of MAP was maintained throughout the whole experimental period.

The time course of HR is illustrated in Figure 1, graphs C and D. In group AB, bleeding firstly produced the expected reflex tachycardia, and subsequently induced a decrease in HR followed by a gradual increase in the later phases (basal HR = 322 ± 6; HR at 60 min = 352 ± 7*; HR at 120 min = 382 ± 6*, *p < 0.01 versus basal values). Pretreatment with L-NAME neutralized the changes in HR induced by bleeding (basal HR = 328 ± 10, HR at 60 min = 334 ± 15, HR at 120 min = 335 ± 17). This reduction in HR was not observed in non anesthetized animals. In theses rats a progressive increase in HR was seen after hemorrhage with subsequent stabilization (45 min) until the end of the experimental period (basal HR = 353 ± 7, HR at 60 min = 396 ± 6*; HR at 120 min = 392 ± 4*, *p < 0.01 versus basal values).

Mitochondrial respiration: Table 1 shows that respiratory control index was similar among groups A, AB, C, and CB. Oxygen uptake in state 4 respiration was greater in group AB compared to group A and similar between groups CB and C. Oxygen uptake in state 3 respiration after hemorrhage showed a similar pattern of response to state 4 both in anesthetized and non anesthetized animals. Mitochondrial respiration and functional activity of mtNOS was lower in group AB compared to group A (12 ± 2 y 19 ± 1, respectively). This effect was even lower in non anesthetized animals compared to group C (17 ± 1 and 20 ± 1, respectively) (Table 1).

Biochemical activity of mtNOS: the production of NO by cardiac mitochondria was evaluated by determining mtNOS activity of the mitochondrial fraction in presence of the corresponding substrates and cofactors. Our results showed that the mitochondrial production of NO decreased in groups AB and CB (48% and 59%, respectively) compared to controls (Figure 2).

DISCUSSION

The cardiovascular adaptation to volume depletion induced by acute bleeding involves the mitochondrial nitric oxide system, and its effect on the energetic metabolism is different in anesthetized and non anesthetized animals.

Blood loss of 20% of blood volume induced a rapid and significant reduction in arterial pressure, both in anesthetized and non anesthetized animals (70% and 76%, respectively). However, only anesthetized animals maintained hypotension throughout the whole experimental time. These observations suggest that immediate hypotension does not depend on the state of consciousness. Nitric oxide system, neurohormonal factors (catecholamines, endothelins, vasopresin, renin-angiotensin system) and blood volume depletion would be responsible of the systemic cardiovascular response observed in this experimental model. Increased circulating levels of these factors have been reported associated with hemorrhagic shock (12-15)

Hypovolemic state produced different patterns of heart rate response in anesthetized and non anesthetized animals. After the expected immediate reflex tachycardia, acute hemorrhage induced a brief

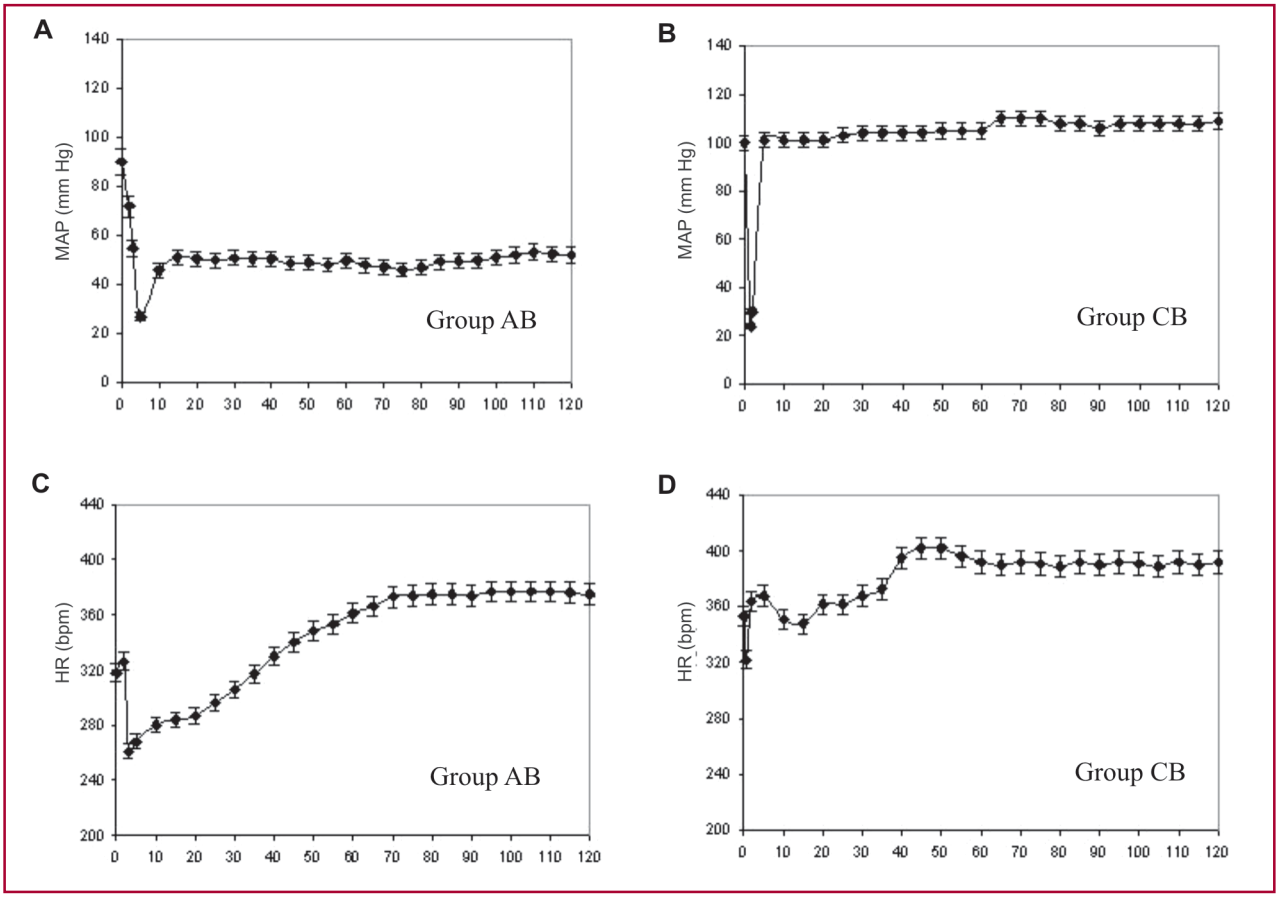

Fig. 1. Time course of mean arterial pressure (MAP) (**A** and **B**) heart rate (HR) (**C** and **D**). Anesthetized rats subjected to bleeding (AB) and non anesthetized rats subjected to bleeding (CB); *p < 0.01 versus basal values (n = 7 per group).

Succinate	Mitochondrial respiration (ng-át O/min.mg proteín)			
Origin	Group A	Group AH	Group C	Group CH
State 3	223 ± 10	251 ± 8#	225 ± 10	211 ± 9
State 4	111 ± 8	144 ± 8*#	109 ± 8	95 ± 5
Respiratory control index	2.03	1.75	2.06	2.22
L-arginine (a)	205 ± 14	232 ± 14	202 ± 16	213 ± 14
L-NMMA (b)	249 ± 26	263 ± 22	248 ± 31	250 ± 12
mtNOS activity (b – a / a x 100)	19	12	20	17

Control animals (anesthetized: group A; non anesthetized: group C) and animals subjected to bleeding (anesthetized: group AB; non anesthetized: groups CB). Values corresponding to mean ± SEM *p < 0.05 versus C; #p< 0.05 versus A (n = 7 per group).

Tabla 1. Mitochondrial oxygen uptake and mtNOS functional activity

bradycardia followed by a gradual and progressive increase at 60 and 120 minutes (14% and 18%, respectively) in anesthetized animals. However, in non anesthetized animals, the duration of bradycardia was shorter and was followed by a gradual increase in heart rate. The identification that NO is a messenger molecule that controls arterial pressure and heart rate constitutes a milestone in the comprehension of the action mechanism of NO in diverse physiological scenarios. The hypothesis that a toxic gas might exert important functions in cellular metabolism would have been difficult to accept 20 years ago. Nowadays, the role of NO as paracrine or endocrine regulator of myocardial function has raised interest. (16, 17) The evidence available shows that NO might modulate beta adrenergic responses by inhibiting the release of norepinephrine in the sympathetic nerves and facilitating the vagal tone. (18) Thus, NO would contribute to the autonomic control of heart rate and contractility in the early stages of hemorrhagic shock. Neverthe-

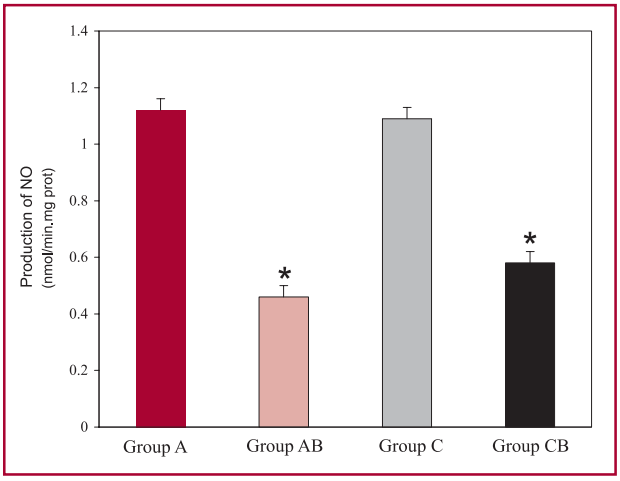

Fig. 2. Mitochondrial production of NO. Control animals (anesthetized: group A; non anesthetized: group C) and animals subjected to bleeding (anesthetized: group AB; non anesthetized: groups CB). Values expressed as mean ± SEM *p < 0.05 versus A or C (n = 7 per group).

less, this effect might be partially attenuated in non anesthetized animals by increased basal sympathetic tone.

The heart plays a key role in the adaptive response in hypotension induced by hypovolemic state. Hemorrhage is a stress stimulus for the cardiovascular system that decreases both preload and perfusion at the tissue level. (19) Table 1 shows high values of respiratory control index in the mitochondria of the isolated hearts in groups A, AB, C and CB, indicating that oxidative phosphorylation was correctly accomplished by the organelles. In addition, we have demonstrated that acute bleeding induced a reduction in the functional activity of mtNOS evaluated by O_2 uptake in both groups of animals. Yet, the magnitude of these changes was lower in non anesthetized animals. Our results also showed that the hypovolemic state induced by hemorrhage is accompanied by decreased production of mitochondrial NO in anesthetized and non anesthetized animals. Again, the magnitude of the changes observed in non anesthetized animals was smaller. These animals present higher concentrations of NO which might be related to greater activity of the sympathetic nervous system. This fact might explain the rapid recovery of blood pressure and the tachycardia seen in these animals after blood withdrawal.

RESUMEN

Shock hemorrágico: óxido nítrico en ratas anestesiadas y no anestesiadas

Antecedentes

En un trabajo previo mostramos que el estado hipovolémico inducido por una pérdida aguda de sangre se acompaña de una activación dinámica, heterogénea y dependiente del tiempo de la óxido nítrico sintetasa (NOS) cardíaca. Este sistema estaría involucrado en las alteraciones hemodinámicas que se observan luego de la depleción de volumen sanguíneo.

Objetivo

El objetivo del presente trabajo fue evaluar la participación del sistema del óxido nítrico (NO) mitocondrial en la respuesta adaptativa del sistema cardiovascular ante un *shock* hipovolémico en ratas anestesiadas y no anestesiadas.

Material y métodos

El estudio se llevó a cabo con cuatro grupos de animales (n = 7 por grupo): grupo A, ratas control anestesiadas; grupo C, ratas control no anestesiadas; grupo AH, ratas anestesiadas sometidas a una hemorragia (20% de la volemia) y grupo CH, ratas no anestesiadas sometidas a una hemorragia. Se evaluaron el consumo de oxígeno, la actividad funcional de la NOS mitocondrial (mtNOS) y la producción mitocondrial de NO.

Resultados

No se observaron diferencias significativas entre los valores de control respiratorio en los distintos grupos estudiados. La actividad funcional de la mtNOS fue menor en el grupo AH respecto del grupo A (12 ± 2 y 19 ± 1, respectivamente). Este efecto fue de menor magnitud cuando la hemorragia se provocó en animales no anestesiados (17 ± 1 y 20 ± 1, respectivamente). La producción mitocondrial de NO disminuyó en los grupos sometidos a una pérdida aguda de sangre, tanto no anestesiados como anestesiados, respecto de los animales controles.

Conclusiones

El sistema del NO mitocondrial estaría involucrado en la respuesta de adaptación del sistema cardiovascular frente a la depleción aguda de volumen. Esta participación dependería del grado de anestesia del animal.

Palabras clave > Hemorragia - Óxido nítrico - Mitocondria - Hipotensión

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