Age-Related Changes of Cardiac Caveolins and Nitric Oxide Synthase Modulation During Hypovolemic State

NOELIA D. ARRECHE, LORENA I. SARATI, CARLA R. MARTÍNEZ, ANDREA L. FELLET, ANA M. BALASZCZUK

Received: 11/26/2010
Accepted: 02/16/2011

Address for reprints:
Farm. Noelia D. Arreche
Cátedra de Fisiología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires
Junín 956 - 7º Piso
(C1113AAD) CABA
Phone number:
4964-8279 extension 312
Fax number:
4964-8279 extension 309
e-mail: narreche@ffyb.uba.ar

SUMMARY

Background
Nitric Oxide (NO) is one of the factors activated by hypovolemic state. Caveolins might affect the production of NO with increasing age.

Objective
To investigate the interaction between caveolin-1 (cav-1) and endothelial nitric oxide synthase (eNOS) in the ventricles of young and adult rats subjected to acute bleeding.

Material and Methods
We used young (2 months old) and adult (12 months old) male Sprague-Dawley rats. The animals from each age group (young and adult) were divided into two experimental groups: Control: control group, and Hem: bleeding group (20% of blood volume). The animals were sacrificed and the left ventricle was resected.

Results
After bleeding, NOS activity increased by 21% and 45% in young rats and by 32% and 56% in adult rats. Western blot analysis revealed that NOS levels were lower (31%) in adult rats. Sixty minutes after bleeding, eNOS activity increased by 147% in young animals and by 66% in adults, with lower increase at 120 minutes. Colocalization showed a diffuse pattern of eNOS location associated with cav-1 in group Control of young animals. A dissociation pattern was observed in group Hem at 60 min which was partially restored at 120 min. The dissociation pattern of eNOS and cav-1 in group Control adult animals was greater compared to that of young rats. This characteristic was also seen after 60 min and 120 min.

Conclusions
The increased production of NO during hypovolemic state depends on the presence of cav-1 which plays a key role in the aging process.

Rev Argent Cardiol 2011;79:487-492.

Key words > Bleeding - Nitric Oxide - Left Ventricle

Abbreviations >

cav-1	Caveolin-1	**NO**	Nitric oxide
eNOS	Endothelial nitric oxide synthase	**NOS**	Nitric oxide synthase
HR	Heart rate	**MAP**	Mean arterial pressure

BACKGROUND
Acute bleeding induces a hypovolemic state characterized by an abnormal circulatory system with inadequate tissue perfusion and oxygenation. Cardiovascular adaptation during acute hypovolemia depends not only on the dynamic control of the autonomic nervous system but also on the magnitude and the velocity of bleeding and on the species studied. (1) Nitric oxide system is one of the multiple factors that are stimulated during acute depletion of volume and has been involved in the pathogenesis of hemorrhagic shock. (2, 3) Three isoforms of nitric oxide synthase (NOS) spatially confined in specific intracellular compartments in cardiac cells are encoded by three different genes. (4) We have previously demonstrated that endothelial NO synthase (eNOS) activation constitutes a protective mechanism of the cardiovascular system in the early stages of hemorrhagic shock. (5) Actually, eNOS is expressed in endothelial cells, endocardium, cardiac myocytes, monocytes and platelets. (6, 7) The sub-cellular localization of eNOS significantly influences

the biological role of the enzyme. Several studies have demonstrated that eNOS directly interacts with the structural proteins of caveolae, or caveolins (cav), which modulate signal transduction pathways, including the production of NO. (8, 9)

In addition, aging is a multifactorial process that takes place during the last stage of the vital cycle. (10) Advanced age is accompanied by biochemical, histological and morphological changes in the cardiovascular system which make the heart's ability to withstand injury and stress decline markedly. (11) Therefore, with normal aging, a variety of events, such as sickness, disease or injury, exposure to toxins, or physical activity levels could influence the levels of the different NOS isoforms and the production of NO. (12) In vivo experiments have demonstrated a greater expression of cav-1 in brain, heart, spleen and liver of adult rats. These results suggest that caveolins might play an important role in aging. (13) Considering these facts, the goal of this study was to investigate the interaction between cav-1 and eNOS in the ventricles of young and adult rats subjected to acute bleeding.

MATERIAL AND METHODS

We used young (2 months old) and adult (12 months old) male Sprague–Dawley rats. Animals were housed in a humidity and temperature controlled environment, illuminated with a 12:12 hours light-dark cycle. They were fed rat chow provided by Nutrimentos Purina, Argentina and tap 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), Argentinean Ministry of Health.

Animals

The 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 an appropriate pulmonary ventilation. Then, the right femoral artery was cannulated and connected to a pressure transducer (Statham P23 ID, Gould Inst. Cleveland, OH) to measure mean arterial pressure (MAP), which was recorded with a polygraph (Physiograph E&M Co, Houston, TX). Heart rate (HR) was determined from the pulsatile 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 in order to ensure that the hemodynamic parameters of all the animals from the different experimental groups were similar. Hypovolemic state was induced by withdrawing 20% of total blood volume in a 2-min period at a constant rate. Volume withdrawal was individually standardized on the basis of body weight so that 20% of the calculated total blood volume was extracted.

Experimental protocol

The animals from each age group (young and adult rats) were divided into two experimental groups:

1. Control rats (Control): After a 30-minute stabilization period, baseline values of MAP and HR were measured for 5 minutes. Thereafter, MAP and HR were recorded for 60 or 120 minutes (n = 14).

2. Hypovolemic rats (Hemo): After a 30-minute stabilization period, baseline values of MAP and HR were measured for 5 minutes, and thereafter the animals were subjected to bleeding (withdrawal of 20% of blood volume). Thereafter, MAP and HR were recorded for 60 or 120 minutes (n = 14).

NOS activity

NADPH diaphorase histochemistry was used to identify cells containing NOS (n = 5 per group). The young and adult animals from the groups Control and Hemo were killed by decapitation 60 and 120 minutes after bleeding, respectively. The left ventricle was then removed, fixed in 4% paraformaldehyde in 0.1M phosphate buffer pH 7.4. The tissues were cryopreserved in 15% sucrose and frozen thereafter. Tissue sections 15-μm-thin were cut on a Leitz cryostat and mounted on gelatin-coated glass slides and incubated at 37 °C for 60 minutes with a reaction mixture containing 1.2 mM NADPH and 0.24 mM nitro blue tetrazolium in phosphate buffer added with 0.3% Triton X-100. A Zeiss Axiophot microscope equipped with a digital camera was used for observation and photography. Image analysis of stained sections was carried out using a Kontron-Zeiss Vidas analyzer, and the optical density was determined using Scion Image software..

Western blot analysis

The young and adult animals from the groups Control and Hemo (n = 5 per group) were killed by decapitation 60 and 120 minutes after bleeding, and the right atrium and left ventricle were resected. The tissues were immediately transferred to a glass beaker containing homogenization buffer (50 mmol/L Tris, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1% triton, 1 mmol/L PMSF, 1 μmol/L pepstatin, 2 μmol/L leupeptine; Roche Diagnostics). The protein concentration was determined using the Lowry assay. Equal amounts of protein from each sample (45 μ g) were separated in 7.5% SDS-PAGE gel and transferred to a nitrocellulose membrane (Bio-Rad, Munchen, Germany), and the incubated with rabbit polyclonal anti-eNOS primary antibody (1:500 dilution) and a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5000 dilution). Samples were revealed by chemiluminescence using the ECL reagent (Amersham Pharmacia Biotechnology, Uppsala, Sweden) for 2–4 min. Quantification of the bands was performed by digital image analysis using a Hewlett-Packard scanner and Totallab analyzer software (Biodynamics, Seattle, WA).

Immunofluorescence

The interaction between cav-1 and e-NOS in the left ventricle was evaluated by co-immunoprecipitation analysis. (n = 4) Tissues of the young and adult animals from the groups Control and Hemo were fixed with 4% paraformaldehyde phosphate buffer (PBS) at 4 °C. The ventricles were fixed overnight in the same solution and were thoroughly washed. Then, they were cryopreserved in 30% sucrose and frozen at –80 ºC. Tissue sections 16-mm-thin were cut on a Leitz cryostat and mounted on gelatin-coated glass slides, washed in PBS and incubated in a blocking buffer (10% normal goat serum in PBS) for 60 minutes at room temperature. These sections were incubated overnight at 4 °C with rabbit

Table 2. Temporal course of mean blood pressure and heart rate

	MBP			HR	
	Baseline	Hemo	120 min	Baseline	120 min
Control group					
Young animals	84 ± 3	–	88 ± 2	325 ± 8	326 ± 12
Adult animals	87 ± 3	–	85 ± 5	316 ± 9	314 ± 10
Hemo group					
Young animals	90 ± 4	27 ± 2*	52 ± 3*	321 ± 9	382 ± 6*
Adult animals	93 ± 5	24 ± 3*	58 ± 4*	315 ± 6	391 ± 9*

* p < 0.01 vs. Baseline values. MBP: Mean blood pressure. HR: Heart rate.

monoclonal anti-cav-1 antibodies (1:500 dilution) and mousse polyclonal anti-eNOS antibodies (1:500 dilution). Sections were washed in PBS and then incubated for one hour at room temperature with fluorescence-conjugated secondary antibodies, fluorescein-conjugated goat anti-rabbit antibody (1:50 dilution) and rhodamine-conjugated goat anti-rabbit antibody (1:50 dilution). The sections were washed again with PBS and mounted with PBS/glycerol (3.1 vol/vol) before being examined by a fluorescent microscope (Olympus BX 51). Negative control sections incubated only with the primary antibody were free from immunostaining (data not shown). Colocalization of cav-1 and eNOS was determined by the superposition of green images (eNOS) and red images (cav-1) using QCapture Pro 6.0 software (QImaging Corporation, 2006), producing a yellow image in the area of colocalization (Merge). The white arrows show the colocalization area in the Merge microphotos.

Statistical analysis

Data are expressed as mean values ± standard error of the means (SEM). The paired Student t test was used to compare differences in the hemodynamic parameters. Analysis of variance (ANOVA) followed by Bonferroni test for multiple comparisons were used to analyze the other variables. The assumptions of normality, homogeneity of variances and independence were tested. A probability of 5% was considered statistically significant. The software GraphPad Prism 3.02 (Graph Pad Software, San Diego, CA, USA) was used for statistical analysis.

RESULTS

Hemodynamic parameters

Table 1 shows the course time of MAP and HR during the experimental period in all the groups of animals studied. The baseline hemodynamic parameters did not show significant differences in the four groups. Bleeding induced a significant decrease in MAP in young and adult animals with subsequent stabilization at about 50 ± 5 mm Hg (* p < 0.01 vs. baseline values) at 15 min. Hypotension was maintained throughout the whole experimental period. Bleeding firstly produced the expected reflex tachycardia, subsequently induced bradycardia for two minutes, followed by a gradual increase in HR after the end of the experimental period in both age groups studied.

NOS activity

We did not find any significant differences in the histochemical activity of NOS in the ventricles of adult animals and young animals of the Control group. After bleeding, NOS activity increased by 21% and 45% in young rats at 60 and 120 min, respectively and by 32% and 56%, respectively, in adult animals compared to controls (Figure 1).

Western blot analysis

Figure 2 illustrates Western blot analysis using anti-eNOS antibodies in the left ventricular tissue from all the groups of animals. Western blot analysis revealed that the expression of eNOS protein were significantly lower (31%) in adult rats compared to young rats. In young animals, bleeding increased the expression of eNOS protein at 60 min (147%) compared to control animals; this increase attenuated after 120 min. Adult

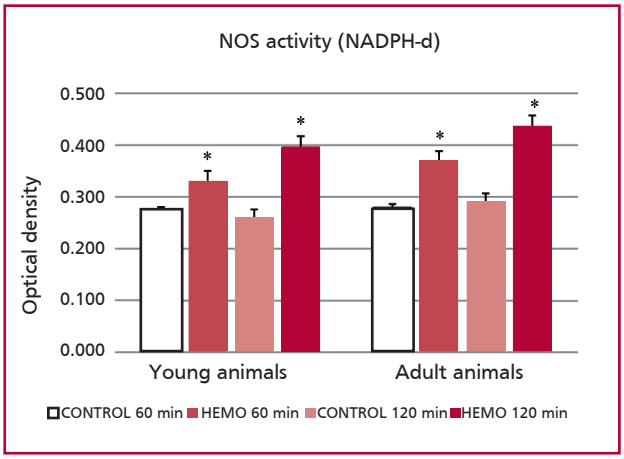

Fig. 1. NADPH diaphorase activity in the left ventricle of young and adult rats, 60 and 120 min after bleeding (withdrawal of 20% of blood volume). * p < 0.001 vs. Control group of the same age group.

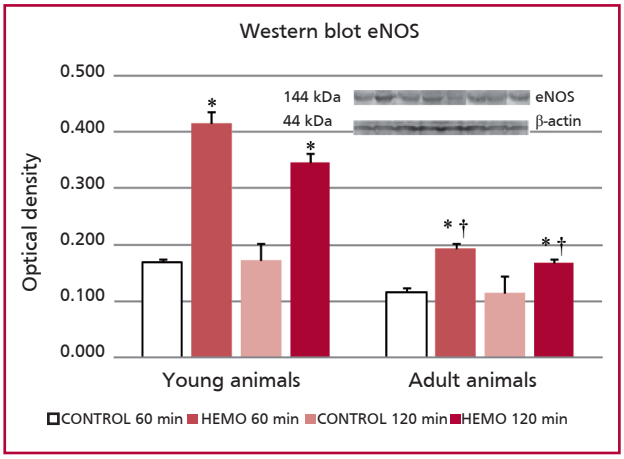

Fig. 2. Western blot analysis representing eNOS in the left ventricle of young and adult rats, 60 and 120 min after bleeding (withdrawal of 20% of blood volume). * p < 0.05 vs. Control group of the same age group. † p < 0.05 vs. young animals.

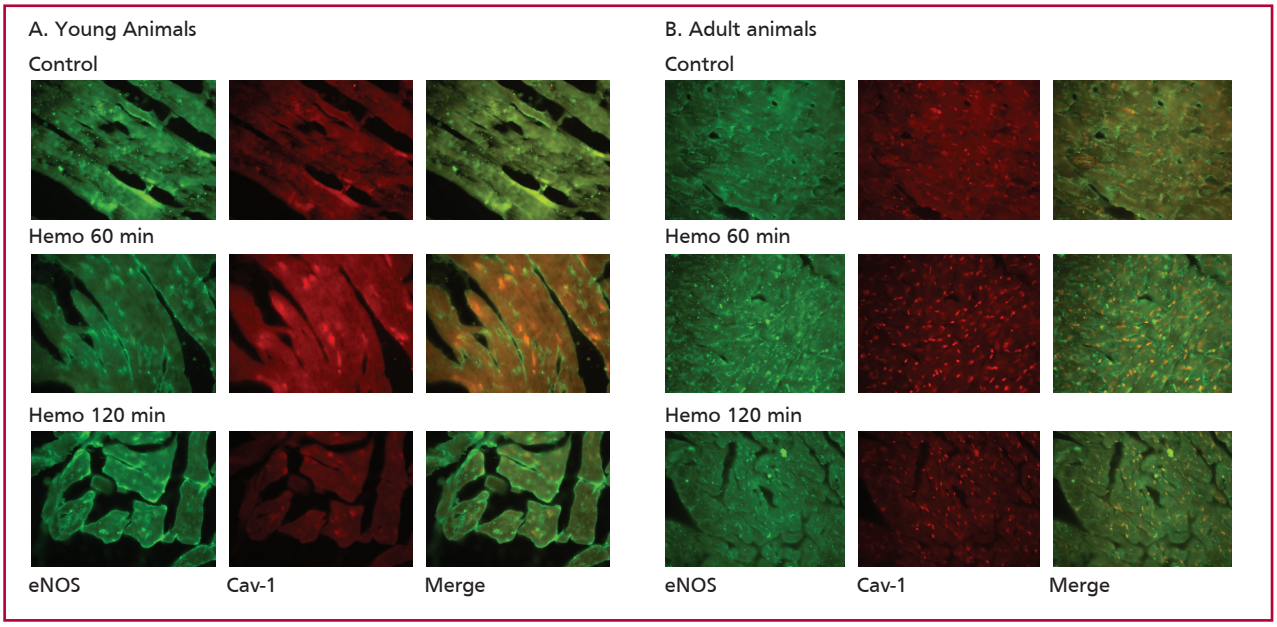

Fig. 3. A. Young animals. **B.** Adult animals. Colocalization of eNOS and cav-1 using indirect immunofluorescence in the left ventricle of young and adult rats (40×), 60 and 120 min after bleeding (withdrawal of 20% of blood volume). The arrows indicate the colocalization (Merge).

animals also showed increased expression of eNOS protein (66%) at 60 min which attenuated at 120 min.

Immunofluorescence

Colocalization analysis showed the presence of a diffuse pattern of eNOS localization associated with cav-1 in ventricular cells of young rats of the Control group. After 60 minutes, the animals subjected to acute bleeding (withdrawal of 20% of blood volume) presented a dissociation between cav-1 and eNOS that was partially restored after 120 min (Figure 3 A). This dissociation was greater in the adult controls compared to young controls. This characteristic was also seen after 60 and 120 min of acute bleeding (Figure 3 B).

DISCUSSION

Several physiological changes occur in response to the hypovolemic state produced by acute bleeding. The adaptive response to hypovolemia includes activation of the sympathetic nervous system and release of vasoactive hormones; however, the potential role of vasodilator mediators is not well understood. (3) The animals of the different groups presented similar baseline hemodynamic parameters. Blood loss of 20% of blood volume induced a rapid and significant reduction in arterial pressure (68%) in young and adult animals. Hypotension was maintained throughout the whole experimental period. After the expected immediate reflex tachycardia, the hypovolemic state induced a brief bradycardia followed by a gradual and progressive increase of the HR in the animals from both age groups. It has been demonstrated that NO acts presynaptically facilitating the vagal tone (14) and decreasing the HR after the initial reflex tachycardia in rats subjected to bleeding. In addition, our results show that the acute loss of 20% of blood volume induces an increase in the ventricular NOS activity at 60 and 120 min after blood withdrawal in both age groups. The main characteristics of aging in the cardiovascular system reflect anatomical and structural changes of blood vessel walls, myocardial relaxation, ventricular filling and response to catecholamines. (15) In addition, there is evidence demonstrating that NO system might modulate the functional changes of cardiovascular aging. (16, 17) Our results demonstrate that the increase in NOS activity correlated with increased expression of eNOS protein. However, eNOS expression in the ventricles of adult rats was significantly lower compared to young rats. Also, 60 and 120 min after acute bleeding, the increase in eNOS expression was lower in adult animals compared to young animals. Therefore, the hypovolemic state after acute bleeding activates NO system in the ventricles in the early and late stages. The origin and magnitude of this change might depend on the age of the individual studied. The importance of the physical association between the different NOS isoforms and a variety of regulatory and structural proteins has been demonstrated over the last years. (18) Different investigators demonstrated direct interaction of cav-1 inhibiting the activation of eNOS in vivo and in vitro studies. (19-21). Cav-1 has been postulated as an important regulator of the underlying processes of normal aging. The results obtained by Cho et al. (13) indicate that the recovery of

senescent cells might be achieved simply by lowering the caveolin level with a profound influence in the aging phenotype. In our study we have demonstrated that the association between cav-1 and eNOS activity in the left ventricle is heterogeneous and depends on the time after inducing bleeding. The dissociation is greater at 60 min of bleeding compared to the one observed at 120 min. Therefore, the increased eNOS activity seen after an hour of induced hypovolemia might be due to decreased eNOS inhibition by cav-1. Age would also contribute to the regulation of NO system by cav-1,as we observed a greater dissociation between these proteins in adult animals compared to young animals in both studied groups (Control and Hemo).

CONCLUSION

The increased production of NO during hypovolemic state induced by acute bleeding depends on the presence of cav-1 which plays a key role in the aging process.

RESUMEN

Caveolinas cardíacas y modulación de la óxido nítrico sintetasa durante el estado hipovolémico según avanza la edad

Introducción
El óxido nítrico (NO) es uno de los factores que se estimulan durante el estado hipovolémico. Las caveolinas afectarían la producción de NO en el corazón según avanza la edad.

Objetivo
Investigar la interacción entre la caveolina-1 (cav-1) y la óxido nítrico sintetasa endotelial (eNOS) en ventrículos de ratas jóvenes y adultas sometidas a hemorragia aguda.

Material y métodos
Se utilizaron ratas Sprague-Dawley machos jóvenes (2 meses de edad) y adultas (12 meses de edad). Se conformaron dos grupos experimentales de cada grupo etario (jóvenes y adultas): Control y Hemo (sangrado: 20% de la volemia). Los animales se sacrificaron para la extracción del ventrículo izquierdo.

Resultados
Luego del sangrado, la actividad de la NOS aumentó el 21% y el 45% en ratas jóvenes y el 32% y el 56% en adultas. El Western blot reveló que los niveles de eNOS fueron menores (31%) en ratas adultas. La hemorragia ocasionó un aumento del 147% de la eNOS a los 60 min en animales jóvenes y del 66% en adultos, atenuándose a los 120 min. La colocalización mostró un patrón difuso de localización de la eNOS asociado con cav-1 en animales del grupo Control jóvenes. En el grupo Hemo, a los 60 min se observó un patrón de disociación, el cual fue parcialmente restablecido a los 120 min. En los animales adultos del grupo Control se observó un patrón de localización de la eNOS y cav-1 con una disociación mayor que en las ratas jóvenes. Esta característica se observó también luego de los 60 y los 120 min.

Conclusión
El aumento de la producción de NO ante un estado hipovolémico se ve condicionado por la presencia de cav-1, la cual cumple un papel preponderante en el proceso del envejecimiento.

Palabras clave > Hemorragia - Óxido nítrico - Ventrículo izquierdo

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