GENERAL PATHOLOGY AND PATHOLOGICAL PHYSIOLOGY

Enhanced Expression of Sarcoplasmic Reticulum Ca^{2+}-ATPase Gene Plays a Role in Protective Effects of Nitric Oxide

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 128, No. 10, pp. 375-379, October, 1999
Original article submitted April 27, 1999

This study verifies the hypothesis that NO-dependent regulation of sarcoplasmic reticulum Ca^{2+}-ATPase gene (SERCA) plays an important role in preventing Ca^{2+} overload after exposure to damaging factors. The data confirmed NO-dependent activation of SERCA expression and important role of this mechanism in the reduction of calcium overload.

Key Words: *nitric oxide; Ca^{2+}-ATPase; expression; reperfusion; physical exercise*

Nitric oxide (NO) is a signal molecule with a high regulatory potential due to its interaction with intracellular signaling systems and, in particular, with the Ca^{2+} transport system. This interaction provides the regulation of vascular tone, neurotransmission, macrophage activation, *etc.* [6] and includes NO-dependent activation of guanylate cyclase [6], nitrosylation of sarcoplasmic reticulum Ca-ATPase (SERCA) [14], Ca^{2+}-dependent activation of constitutive NO synthase (cNOS) [6] *etc.* These mechanisms are activated within several milliseconds, without involving the genetic apparatus. In addition, the synthesis of NO can be controlled by Ca^{2+} at the transcriptional level. It was found that low intracellular Ca^{2+} concentration down-regulates expression of the inducible NO synthase gene (iNOS) in macrophages [10], while high Ca^{2+} concentration stimulates the expression of both iNOS [10] and cNOS [12].

Interestingly, the enhanced expression of the SERCA gene in response to repeated stress [3] or physical exercise [4] is accompanied by intense NO production [2]. We assumed that NO regulates the expression of SERCA gene, thus participating in the transcriptional control over cell Ca^{2+} concentration.

SERCA is one of the enzymes which prevent intracellular Ca^{2+} overload. Therefore, NO-dependent regulation of SERCA genes can play a role in the prevention of Ca^{2+} overload induced by damaging factors.

In the present study we verified this suggestion by assessing the effect of NO donor on the expression of SERCA genes in the myocardium and skeletal muscles and by comparing these effects with changes in myocardial and muscle resistance to Ca^{2+} overload caused by reperfusion of isolated heart and severe physical exercises, respectively.

MATERIALS AND METHODS

Experiments were carried out on male Wistar rats weighing 240-270 g.

Dinitrosyl iron complex (DNIC) was used as a donor of NO [13]. Tissue and organ concentrations of donor-derived NO were assessed by electron paramagnetic resonance (EPR) [13]. DNIC was injected into the caudal vein in a dose of 200 μg/kg. The rats were

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0007-4888/99/0010-0981$22.00

decapitated 2, 4, 12, and 24 h postinjection and the content of DNIC in the blood, heart, and skeletal muscles was determined by EPR.

To analyze the expression of SERCA gene, total RNA fractions were isolated from the myocardium and skeletal muscles by phenol-guanidine isothiocyanate extraction [11]. Muscle tissue was powdered in liquid nitrogen, homogenized in buffer A containing 5 mM guanidine isothiocyanate, 1% SDS, 50 mM EDTA, 50 mM Tris-HCl (pH 7.5), and 0.2% mercaptoethanol at a 1:7 tissue:buffer ratio. RNA was precipitated with 96% distilled ethanol (0.3:1 v/v) and centrifuged at 14,000 rpm for 12 min in an Eppendorf centrifuge. The supernatant was discarded, while the sediment was dissolved in buffer A ($^1/_{10}$ to $^1/_5$ of the initial buffer volume) and washed 5 times with deproteinizing phenol:chloroform:isoamyl alcohol (25:24:1) mixture with 100 mM Tris-HCl (pH 7.5), 4 mM EDTA, and 0.2% mercaptoethanol. Aqueous and organic phases were separated by centrifugation, the aqueous phase was taken out, and the procedure was repeated. RNA was isolated with 96% ethanol as described above. Samples were washed 2 times with 80% ethanol, centrifuged, and RNA was dissolved in deionized water. The quality of RNA was tested by electrophoresis in 1% agarose glyoxal-denaturating gel. RNA was precipitated with ethanol and stored at -70°C until further study of SERGA gene expression (SERCA1 in skeletal muscles and SERCA2 in the myocardium) by Northern blot hybridization technique. The expression of SERCA2 and SERCA1 was measured with the corresponding deoxyribonucleotide probes: 5'-CAG ATT CAC CTG TAA GAA TCG ACT GGT CAA CTC TCA G-3' (724-760), GenBank accession number X 15635 [8] and 5'-GAG AAG CTC ATC TAG CCC GAT GAC TGG AAG TGA G-3' (3084-3117), GenBank accession number M99223 [15]. Quantitative changes in expression were assessed by the ratio between the SERCA mRNA signal and the signals of marker genes ("housekeeping genes"), i. e. the most abundant genes conservatively expressed in different tissues. Four protein genes were used as markers: S4 ribosomal protein (RPS4Q) (5'-GTG TAA TTC GAT GGA CAG CAA AGC GAC CCT TGG TG-3' (333-368), GenBank accession number: X14210); S9 ribosomal protein (RPS9Q) (5'-CAC CTG CTT GAG GAC CCT GAT GTG ACG TTG GC-3' (418-449), GenBank accession number: X66370), cytoskeletal β-actin (5'-GTC-GAC GAC GAG CGC AGC GAT ATC GTC ATC CAT-3' (1247-1279), GenBank accession number: V01217; J00691); and glyceraldehyde-3-phosphate dehydrogenase (5'-CAC GGA AGG CCA TGC CAG TGA GCT TCC CGT-3' (734-763), (739-768), Cat. #5840-1, Clontech, USA, GenBank accession numbers: M17701, X02231, X00972). Oligodeoxyribonucleotide probe sequences were chosen according to the algorithm developed at the Laboratory of Gene Engineering, Russian Cardiology Research-and-Production Complex. The probes were labeled with ^{32}P (specific activity no less than 10^7 cpm/pmol). The adequacy of hybridization signals was controlled by applying to the same gel 1) rat brain poly-$(A)^-$ RNA; 2) rat brain poly-$(A)^+$ RNA to identify the signals of marker genes; 3) total RNA from skeletal muscle to differentiate the two SERCA isoforms; and 4) DNA and RNA calibration mixture. Hybridization was performed in a Hybide furnace. The expression of SERCA genes at the mRNA level was assessed by the intensity of radioautographs on a Forma X-ray film measured using Alpha Imaging System apparatus (Alpha Innotech. Inc).

Calcium overload of the myocardium was modeled by postischemic reperfusion of Langendorff isolated hearts. Reperfusion was started after 20-min complete coronary occlusion and monitored for 20 min. Heart injury was evaluated by arrhythmia severity and the recovery of contraction amplitudes during reperfusion (technique was described in detail [9]).

Calcium overload of skeletal muscles was modeled by severe physical exercise [7] — forced swimming with a load (3% of body weight). Resistance to strenuous exercise was assessed by the duration of swimming (in min) until the animal submerged for more than 30 sec.

The data were expressed as $M \pm m$. Significance of the differences was assessed by Student's t test.

RESULTS

Two hours after injection of 200 μg/kg DNIC in the caudal vein we observed a significant increase in the content of NO bound in DNIC in the blood (587±67), heart (50±5), and skeletal muscles (41±4). After 4, 12, and 24 h the tissue level of NO decreased to zero. Thus, DNIC induced a transient increase in the content of NO which peaked 2 h postinjection.

The effect of DNIC on the expression of SERCA gene was assessed by the ratio of SERCA mRNA to marker gene mRNA signals. Both in the myocardium and skeletal muscles, the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene expression underwent the smallest changes (Fig. 1).

Two hours postinjection, myocardial SERCA2 expression increased by 20% compared to the control and G3PDH. In skeletal muscles, expression of SERCA1 peaked 6 h postinjection (156% of the baseline, Fig. 2).

Thus, NO donor transiently enhanced expression of SERCA genes encoding the key enzymes of the Ca^{2+} transport system in the myocardium and skeletal muscles.

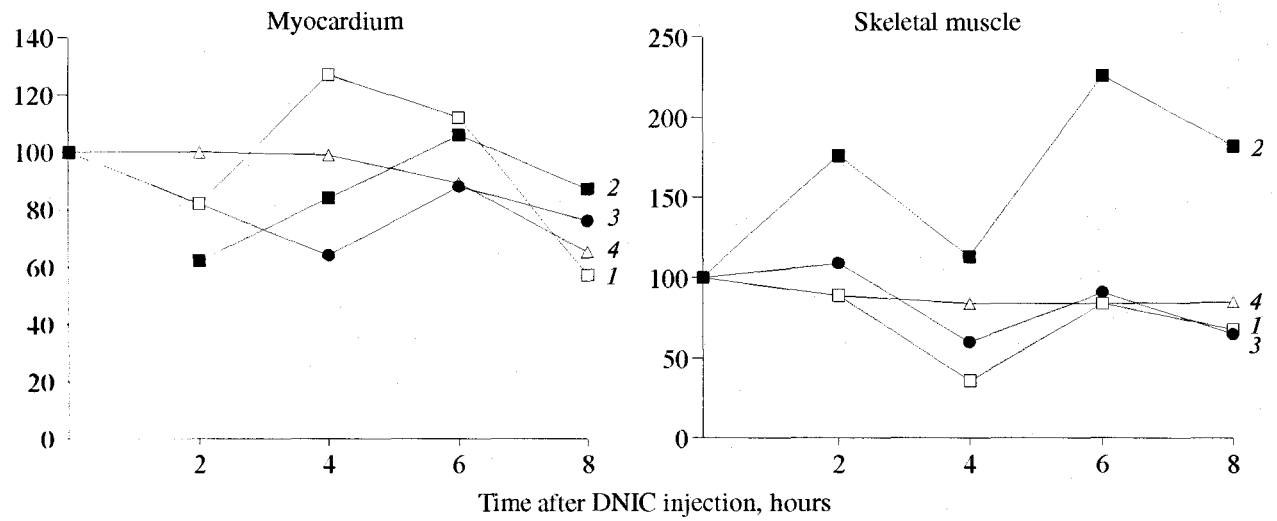

Fig. 1. Effect of dinitrosyl iron complex (DNIC) on expression of marker genes in the myocardium and skeletal muscles. Here and in Fig. 2: Ordinate: intensity of radiosignals, % of baseline (before DNIC injection). *1)* S4 ribosomal protein; *2)* S9 ribosomal protein; *3)* cytoskeletal β-actin; *4)* glyceraldehyde-3-phosphate dehydrogenase.

During reperfusion, the amplitude of heart contractions in the control group was restored to 8% of the baseline (Fig. 3, *a*). Fibrillations and cardiac arrest were observed in 70% cases (Fig. 3, *b*). Pretreatment with DNIC significantly improved heart resistance to reperfusion injury. Maximum resistance was observed 12 h postinjection. In this group reperfusion caused no cardiac arrests (Fig. 3, *b*), and the contraction amplitude returned to about 58%, which exceeded the control values 7.2-fold (Fig. 3, *a*).

The maximum duration of forced swimming (1.5-fold surpassing the control) was observed 24 h postinjection.

Thus, elevation of NO concentration improved organ and body resistance to damaging effect of Ca^{2+} overload during postischemic reperfusion or strenuous exercise.

Physiological concentrations of Ca^{2+} and NO in cells are controlled by a variety of autoregulatory mechanisms. In particular, Ca^{2+} -induced activation of

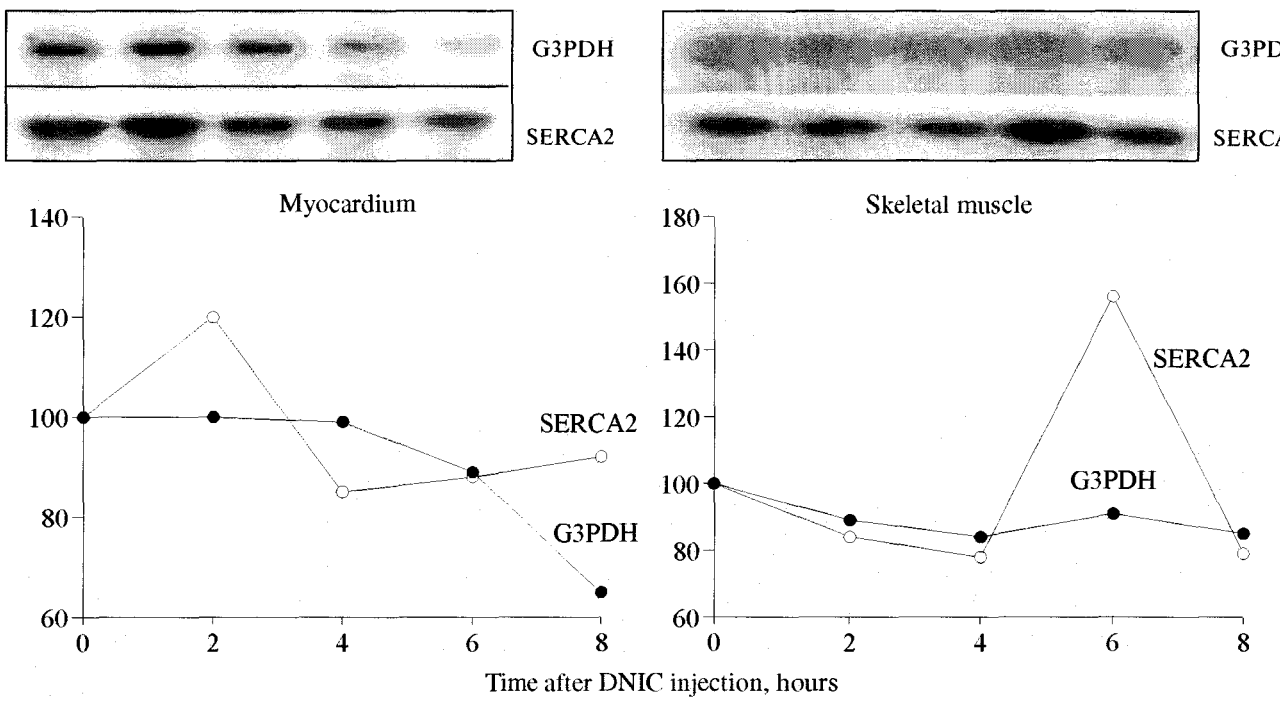

Fig. 2. Effect of dinitrosyl iron complex (DNIC) on SERCA expression in the myocardium and skeletal muscles. Inserts: scans of autoradiograms. G3PDH: glyceraldehyde-3-phosphate dehydrogenase.

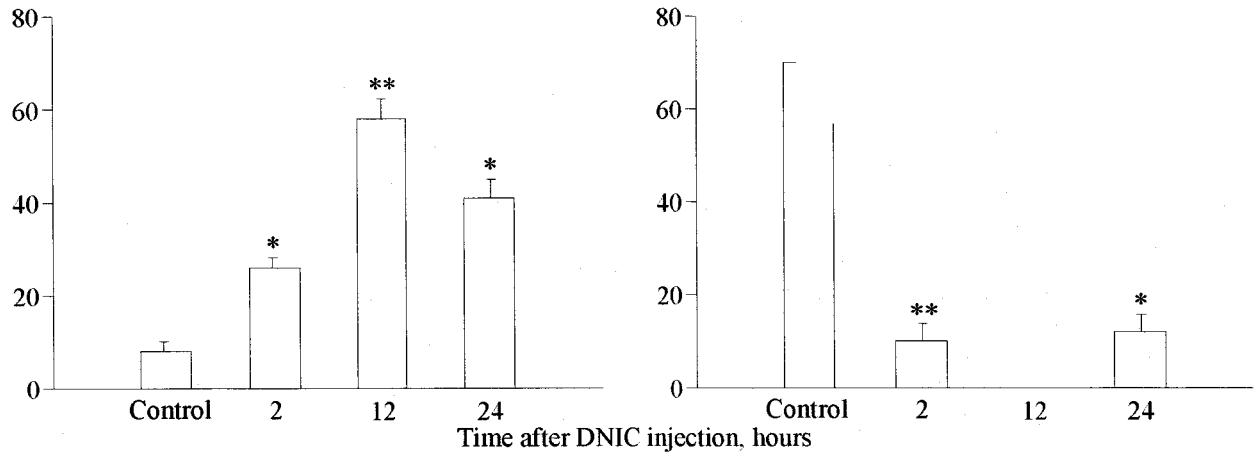

Fig. 3. Time-dependence of the cardioprotective effect of dinitrosyl iron complex (DNIC). Ordinates: *a)* recovery of contraction amplitudes during reperfusion, % of preischemic level; *b)* cardiac arrest, %. *$p<0.05$, **$p<0.01$ in comparison with the control.

SERCA can prevent calcium overload, while NO-induced inhibition of NOS [5] prevents NO hyperproduction. Our findings suggest a cross-regulation of Ca^{2+} and NO concentrations at the transcriptional level: NO activates the expression of SERCA genes which, induces accumulation of SERCA molecules, and promotes uptake of Ca^{2+} by the sarcoplasmic reticulum. The effect of Ca^{2+} on the expression of iNOS gene was shown previously [10,12].

Physiologically, the cross-control of SERCA and NOS gene expression improves reliability of intracellular regulation of Ca^{2+} and NO concentrations. This additional control becomes especially important when autoregulatory mechanisms are disturbed. For instance, reperfusion heart injury is associated with membrane damage by free radicals and excessive Ca^{2+} entry. The initial increase in intracellular Ca^{2+} concentration activates SERCA, i.e. triggers the autoregulatory mechanism. However, in deep Ca^{2+} overload, Ca^{2+} and free radicals damage SERCA [1] and disturb this autoregulatory mechanism. Under these conditions preliminary accumulation of SERCA molecules can reduce Ca^{2+} overload.

We found that the NO donor DNIC enhanced SERCA gene expression and protected isolated hearts from reperfusion injury induced by Ca^{2+} overload. DNIC exerted similar effects on skeletal muscles under conditions of strenuous exercise.

The dynamics of DNIC content in organs, SERCA expression (Fig. 2), and heart and body resistance (Fig. 3) showed that transient elevation of tissue and blood NO and the increase in SERCA gene expression occurred simultaneously within the first 2-6 h postinjection, while the protective effect of DNIC manifested itself much later, after 12-24 h. It probably means that the long-term protective effect of DNIC is associated with secondary NO-activated mechanisms, rather than with the direct effects of NO. NO-dependent activation of the SERCA gene expression can be one of these mechanisms.

This study was supported by the Russian Foundation for Basic Research (grants No. 97-04-48370, 97-04-48371), INTAS-OPEN CALL 1997 (grant No. 524), and The Netherlands Organization for Scientific Research (grant No. 047.006.006).

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