Reduced inotropic heart response in selenium-deficient mice relates with inducible nitric oxide synthase

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Gomez, Ricardo M., Orville A. Levander, and Leonor Sterin-Borda. Reduced inotropic heart response in selenium-deficient mice relates with inducible nitric oxide synthase. Am J Physiol Heart Circ Physiol 284: H442–H448, 2003; 10.1152/ajpheart.00560.2002.—Atria from mice fed a selenium-deficient (Se−) diet have a diminished β-adrenergic-receptor-inotropic cardiac response compared with atria from mice fed the same diet supplemented with 0.2 mg/kg Se as sodium selenite (Se+). This diminished response could be reversed by feeding Se− mice the Se+ diet for 1 wk or by pretreatment with nitric oxide synthase (NOS) inhibitors such as Nω-monomethyl-L-arginine or aminoguanidine. Elevated serum concentrations of nitrite/nitrate as well as a threefold increase in the atrial NOS activity were seen in the Se− versus Se+ mice. Western blotting and indirect immunofluorescence indicated an enhanced expression of inducible NOS in hearts from Se− mice. Increased expression and activity of NOS and increased nitrite/nitrate levels from Se− mice correlated with an impaired response to β-adrenergic receptor inotropic cardiac stimulation. Elevated nitric oxide levels may account for some of the pathophysiological effects of Se deficiency on the heart.

antioxidants; cardiomyopathy; isoproterenol; Keshan disease

SELENIUM (Se) is an essential micronutrient with antioxidant functions because it is incorporated as selenocysteine into proteins that protect against oxidative stress (44). Low dietary Se intake has been associated with pathologies frequently involving muscle in animals of agricultural value as well as in some human diseases (11). Of particular interest is the link established between Se deficiency and an endemic human disease (11). Of particular interest is the link established between Se deficiency and an endemic human disease, dilated cardiomyopathy (DCM) known as Keshan disease, which is found in areas of China with Se-poor soils (17). Although Se deficiency accounted for most features of the disease, some infectious agents were later suggested as etiological cofactors (43). Other epidemiological studies in Europe also have linked low serum Se levels to cardiac disease (37, 38) and Se supplementation has a protective effect during myocardial ischemia (33). The causal mechanisms by which Se deficiency contributes to heart disease are not clearly understood, although it is generally agreed that increased free radical damage may play an important role (9, 31).

Nitric oxide (NO) is a short-lived molecular free radical synthesized from L-arginine by the catalytic reaction of NO synthases (NOS). The mammalian NOS isoforms include two constitutively expressed enzymes, the neuronal NOS and the endothelial NOS (eNOS), as well as the inducible isoform (iNOS). Both constitutive NOS (cNOS) isoforms are regulated predominantly at the posttranslational level, whereas iNOS appears to be regulated primarily by the rate of transcription (5, 42). NO produced by either cNOS or iNOS influences normal cardiac function and may play an important role in the pathophysiology of certain disease states associated with cardiac dysfunction (20).

In this study, we demonstrate that atria taken from mice fed a Se-deficient diet (Se−) have a diminished isoproterenol (Iso) or norepinephrine (NE)-induced β-adrenergic receptor inotropic cardiac response compared with atria from Se-adequate (Se+) mice. This diminished response could be reversed either by feeding the Se− mice the Se+ diet for 1 wk or by prior treatment with NOS inhibitors. To our knowledge, this is the first report that dietary Se deficiency causes an NO-related depression of mouse atrial contractility stimulated by an adrenergic agent. These results could have important implications for the possible role of Se in preventing certain heart diseases.

METHODS

Animals. Three-week-old outbred F1 mice of either gender were randomly divided into two groups and fed either a Se− or a Se-supplemented diet for 4 wk before the experiment. They were housed in stainless steel, wire-bottomed cages at a density of 3–5 per cage and maintained at an ambient air temperature of 22 ± 1°C and a 12:12-h light-dark cycle. All mice were given water and diets ad libitum. All procedures used in the experiments were approved by the Ethics Committee of the Faculty of Odontology, University of Buenos Aires.

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Diets. The composition of the diets has been previously described (16). Briefly, the basal Se– diet consisted of (in percent) 30 torula yeast, 4 tocopherol-striped lard, 1 tocopherol-striped corn oil, 3.5 AIN–76 salt mix without Se, 1 AIN–76A vitamin mix without vitamin E, 0.3 dl-methionine, 0.152 choline dihydrogen citrate, and 60 sucrose. Vitamin E (35 mg/kg) was added as all natural-a-tocopherol acetate. For Se+ diets, Se was added to the basal diet at 0.2 mg/kg as sodium selenite. Before experiments, sera from mice were analyzed for Se concentration to confirm Se status, as previously described (29).

Atrial preparation for contractility. The mice were euthanized by cervical dislocation. The atria were carefully dissected from the ventricles, attached to a glass holder, and immersed in a tissue bath containing Krebs–Ringer bicarbonate (KRB) solution gassed with 5% CO2 in O2 and maintained at pH 7.4 and 30°C. KRB solution was composed as described previously (40). A preload tension of 350 g was applied to the atria, and tissues were allowed to equilibrate for 30 min. The initial control values for contractile tension of the isolated atria were recorded using a force transducer coupled to an ink writer (41). The preparations were gassed with a 5% CO2 and 95% O2 mixture and were excited with a bipolar electrode and a stimulator (model SK4, Grass). The stimuli had a duration of 2 ms and the voltage was 10% above threshold. Inotropic effects were assessed by recording the maximum rate of isometric force development over time (dF/dt) during electrical stimulation at a fixed frequency of 300 beats/min. Control values (100%) refer to the dF/dt before the addition of drugs. The absolute value for dF/dt at the end of the equilibration period (30 min) was 4.3 ± 1.2 g/s. Cumulative dose-response curves of ISO or NE were done on atria from Se– and Se+ mice according to a method previously described (46). A maximal effect was achieved within 5 min after each dose. When blockers were used, the atria were incubated previously for 30 min before dose-response curves of the agonists were done. In contrast, mice administered 2-amino-4-methylpyridine (AP) in vivo were injected subcutaneously at a daily dose of 0.3 mg·kg-1·day-1 for 3 days before they were euthanized. Controls were mice injected with PBS with the use of the same protocol.

Determination of NOS activity. NOS activity was measured in atria by production of [U-14C]citrulline from [U-14C]arginine according to the procedure described originally for brain slices (8) modified for isolated rat atria (41). Briefly, after 20 min of preincubation in KRB solution, the atria were transferred to 5 ml of prewarmed KRB equilibrated at −70°C until use. Samples were boiled for 5 min and electrophoresed on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Millipore HATF 2020) at 4°C. Equal loading and transfer of proteins between lanes was verified by Ponceau red staining before immunodetection. Blots were then probed consecutively with a rabbit anti-iNOS antibody (Cayman), a biotinylated anti-rabbit-antiserum (Vector), and a streptavidin peroxidase (Vector), intercalated with several washes. Blots were developed using 50 ml of PBS with 20 mg of 3,3′-diaminobenzidine tetrahydrochloride (Sigma) and 15 μl of hydrogen peroxide (30%).

Nitrite/nitrate analysis. Serum samples from Se+ and Se– mice were collected and stored at −70°C until analysis. After sample deproteinization, nitrite/nitrate levels were measured with a commercial kit (Calbiochem). Briefly, nitrate was converted to nitrite by incubation with nitrate reductase in the presence of NADPH. Lactate dehydrogenase was then used to destroy excess NADPH. Equal volumes of sample and Griess reagent were incubated at room temperature. After 10 min, absorbance was read at 550 nm. The nitrite concentration was determined by using sodium nitrate as a standard.

Indirect immunofluorescence. Heart samples from Se– and Se+ mice were quick frozen in isopentane that had been chilled in liquid nitrogen, and 8-μm cryostat sections were prepared, air dried for 10 min, and stored at −70°C until use. To reduce background, slides were incubated for 20 min with a blocking solution (1 M PBS with 10% goat serum and 0.5% casein). A 1:100 dilution of a rabbit anti-iNOS antibody (Cayman) was applied to the sections for 2 h at room temperature, washed for 15 min with PBS, and then incubated for 30 min with a FITC-conjugated goat anti-rabbit IgG (Vector). After several washes, the sections were photographed with a Nikon photomicroscope equipped with epiluminescence. As a negative control of the reaction, the first antiserum was omitted.

Drugs. ISO, NE, AP, and L-NMMA were purchased from Sigma. Stock solutions were freshly prepared in the corresponding buffers. The drugs were diluted in the bath to achieve the final concentration stated in the text.

Statistical analyses. The data in the dose-response curves (Figs. 1 and 2) were analyzed with the use of a nonlinear mixed ANOVA logistic regression model (Proc NLN, SAS Institute; Cary, NC). The data in Fig. 3, A and B, were analyzed by a two-way and one-way ANOVA, respectively. Differences were considered statistically significant if P < 0.05.

Fig. 1. Decreased contractility in force over time (dF/dt) of atria from selenium-deficient (Se–) mice (●) in the presence of increasing concentrations of isoprenaline (ISO) (A) or norepinephrine (NE) (B) compared with the response of selenium-adequate (Se+) mice (●) or Se– mice fed the Se– diet (Se–/–) for 1 wk before the experiment (○). The tissues were equilibrated for 30 min before the addition of agonist. Values are expressed as the percent change compared with the basal value before the addition of agonist and represent means ± SE (n = 6). Parameters composing the logistic regression models for the Se– diet group were significantly different from those composing the models for the Se+ or Se–/– groups.
RESULTS

Serum Se concentrations were significantly \((P < 0.05)\) lower in mice fed the Se\(^{-}\) diet versus those fed the Se\(^{+}\) diet \((27 \pm 14 \text{ vs. } 411 \pm 37 \text{ ng/ml, respectively; } n = 5)\), but there were no significant differences in body weight between treatment groups \(\text{(data not shown)}\).

To determine whether dietary Se had any influence on the mechanical response to \(\beta\)-agonists, the effects of Iso and NE on \(dF/dt\) of atria taken from Se\(^{-}\) and Se\(^{+}\) mice were examined. The basal absolute \(dF/dt\) values \((\mu\text{g/s})\) of atria from Se\(^{-}\) and Se\(^{+}\) mice \(\text{(before the addition of the } \beta\text{-agonists)}\) were \(4.3 \pm 1.2 \text{ for Se}\(^{-}\) and \(4.0 \pm 1.1 \text{ for Se}^{+} \) \((n = 6)\). Figure 1 shows the effect of increasing concentrations of Iso \(\text{(Fig. 1A)}\) and NE \(\text{(Fig. 1B)}\) on the contractility of atria from Se\(^{-}\) and Se\(^{+}\) mice. Both Iso and NE induced a concentration-dependent increase in atrial \(dF/dt\), but in Se\(^{-}\) atria the agonistic dose-response curve was shifted to the right and the potency of the agonist was decreased. When Se\(^{-}\) mice were fed the Se\(^{+}\) diet for 1 wk before the contractility assay, the diminished \(dF/dt\) responses were abolished and reached values similar to those of the Se\(^{+}\) mice \(\text{(Fig. 1, A and B)}\). This observation strengthened the causal relationship between the diminished \(dF/dt\) response of isolated atria to \(\beta\)-agonists and the Se\(^{-}\) diet.

To determine whether endogenous NO participated in the decreased response of Se\(^{-}\) atria to \(\beta\)-agonists, tissue was treated with different NOS inhibitors. The inhibition of all NOS isoenzymes with L-NMMA \(\text{(}5 \times 10^{-6} \text{ M)}\) increased the efficacy of Iso and shifted its dose-response curve to the left so that the \(dF/dt\) values of Se\(^{-}\) atria were similar to those obtained with Se\(^{+}\) atria, treated with L-NMMA or not \(\text{(Fig. 2A)}\). Similarly, in vivo treatment of Se\(^{-}\) atria with AP, an inhibitor of the iNOS isozyme, improved the positive inotropic effect of Iso so that \(dF/dt\) values similar to those seen with Se\(^{+}\) atria were obtained \(\text{(Fig. 2B)}\). At the concentrations used, all inhibitors had no effect per se on basal contractility.

These results encouraged us to determine whether higher NO levels were involved in the diminished response of Se\(^{-}\) atria to \(\beta\)-agonists. In this regard, we observed a higher NOS activity in the atria of Se\(^{-}\) mice when compared with atria of Se\(^{+}\) mice \(\text{\text{(Fig. 3A)}}\). The Se\(^{-}\) mice, which had been switched to a Se\(^{+}\) diet for 1 wk before the experiment \(\text{(Se}\^{+}/-)\), showed a NOS activity level similar to that of the Se\(^{+}\) mice that had been fed Se\(^{+}\) diet throughout the 4-wk feeding period, thereby demonstrating the relationship between the activity of NOS and the type of diet. Administration of AP decreased atrial NOS activity from Se\(^{-}\) mice to levels seen in atria from Se\(^{+}\) and Se\(^{+}/\) mice, which remained unchanged by AP treatment. In contrast, L-NMMA \(\text{(}5 \times 10^{-4} \text{ M)}\) inhibited 95% of the NOS activity in all three mouse groups \(\text{(Fig. 3A)}\). It is important to emphasize that in contractility experiments, L-NMMA was used at \(5 \times 10^{-6} \text{ M}\), a concentration known to inhibit basal NOS activity by 50% without modifying basal \(dF/dt\) \(\text{(41)}\). Significantly higher concentrations of nitrite/nitrate were found in the serum from Se\(^{-}\) mice when compared with those from Se\(^{+}\) mice \(\text{(Fig. 3B)}\). Again, Se\(^{-}\) mice, which had been fed a Se\(^{+}\) diet for 1 wk previously, to the serum nitrite/nitrate determination \(\text{(Se}\^{+}/-\text{ mice)}\), showed lower levels similar to those from the Se\(^{+}\) mice.

To establish whether the elevated levels of NO associated with the diminished response to \(\beta\)-agonists observed in atria from Se\(^{-}\) mice could be generated by the iNOS, we looked for expression of the enzyme by both indirect immunofluorescence and Western blot procedures. We observed a low background fluorescence signal in Se\(^{+}\) hearts when tested for iNOS expression \(\text{(Fig. 4A)}\). The staining is clearly enhanced in Se\(^{-}\) hearts but retained its apparent cytoplasmic localization \(\text{(Fig. 4B)}\). Expression of iNOS in heart tissue samples was confirmed by Western blot \(\text{(Fig. 5)}\).
DISCUSSION

In the present study, we have shown that atria taken from mice fed a Se⁻ diet for 4 wk have a diminished β-adrenoceptor inotropic cardiac response (dF/dt) to agonist stimulation (Iso or NE) when compared with atria from mice fed the same diet supplemented with Se (Se⁺). These data strongly relate to the diet consumed because feeding the Se⁻ diet to the Se⁺ mice for only 1 wk before the experiment resulted in dF/dt values similar to those observed in mice fed the Se⁺ diet throughout the entire 4-wk feeding period. The diminished dF/dt observed in this study can be partially explained by the enhanced NOS activity detected in atria of Se⁺ mice, together with increased serum nitrite/nitrate levels. Furthermore, the dF/dt curves of atria from Se⁺ mice resembled those obtained with atria from Se⁻ mice when the experiment was conducted in the presence of l-NMMA, a nonselective NOS blocker (27). Finally, it appears that the source of NO is a cardiac iNOS, as shown by its selective expression and also by the fact that the dF/dt response of Se⁻ atria was similar to that of Se⁺ atria when the specific iNOS inhibitor AP (13) was used.

The diminished β-adrenoceptor inotropic cardiac response to agonist stimulation (dF/dt) observed in this study can be explained by the enhanced NOS activity detected in the Se⁻ hearts and the known effects of NO on myocardial function (28). NO stimulates myocardial soluble guanylate cyclase to produce cGMP, which affects two major target proteins. A small increase in cGMP levels predominantly inhibits phosphodiesterase III, whereas high cGMP levels activate cGMP-dependent protein kinase. Accordingly, submicromolar NO concentrations improve myocardial contraction, whereas submillimolar NO concentrations decrease contractility. However, the inotropic effects of submicromolar NO are small and probably of minor importance for myocardial contractility. In contrast, submillimolar NO concentrations produce direct inhibitory effects of NO on ATP synthesis and voltage-gated calcium channels. Cardiodepressive actions of endogenous submillimolar NO concentrations may play a role in certain forms of heart failure because NO reduces the calcium affinity of the contractile apparatus, contributing to a negative inotropic effect, an abbreviation of contraction, and an enhancement of diastolic relaxation (20). Another possibility to explain the inhibitory effect of NO on agonist-stimulated cardiac contractility could be a direct interaction between NO and catecholamines because Klatt et al. (26) have presented evidence that NO can cause an oxidative inactivation and degradation of Iso in adipocyte cultures (26). However, there is no conclusive evidence that this process plays any role in vivo (26).

In pioneering studies that used rats, Se deficiency was associated with abnormal electrocardiograms (15) and decreased basal myocardial contractility (18). These data were questioned later because similar results could not be obtained unless a combined deficiency of Se and vitamin E were employed (36). It is of interest that some of these authors referred to a reduced tolerance for oxygen radicals as well as some ultrastructural alterations in isolated Langendorff-perfused hearts from Se deficient but vitamin E adequate rats (47). In this study, no changes in basal atria contractility were found due to Se deficiency. With the use of electrically stimulated papillary muscle isolated from rats fed a Se⁻ and vitamin E-deficient diet, Turan et al. (45) demonstrated that Iso-induced facilitation of muscle contraction was smaller than in control animals although no differences were detected in the absence of Iso (i.e., basal contractions). With the use of the same model, some underlying mechanisms were clarified recently when a reduced adenylate cyclase activity as well as a decreased β-adrenoceptor density (~30%) were observed, suggesting an interference with the β-adrenoceptor-adenylate cyclase coupling (39). Although Sayal et al. (39) used an experimental model different from ours, it could nonetheless be speculated that some of the mechanisms invoked by them might...
also be operative in our study, in addition to the involvement of NO.

The observation of iNOS expression in mouse hearts, including those of control Se− mice, is in agreement with other studies. It has been proposed that the three known NOS isoforms are present in the cardiomyocytes, but have distinct heterogeneous basal expression gradients, subcellular localization patterns, and different functions (7). Low basal expression of the iNOS has been detected in ventricular myocytes of the ferret heart at the cytoplasmic level, a localization conserved even after its upregulation (7). With regard to cNOS, neuronal NOS has been recently identified in isolated cardiac mitochondria with a proposed modulatory role for NO in oxidative phosphorylation (23), as well as in the sarcoplasmic reticulum, where it has been associated with Ca2+ release and enhanced contractility (4). eNOS localizes to caveolae (22), where compartmentalization with β-adrenergic receptors and L-type Ca2+ channels allows NO to inhibit β-adrenergic-induced inotropy (21). In addition, eNOS apparently colocalizes with the extracellular membrane bound form of superoxide dismutase, thereby implying a functional relationship in the modulation of the contractile apparatus (7). Considering that NO and/or iNOS inducers have been postulated to modulate intracellular NO by a direct effect on cNOS activity (10), it will be of future interest to investigate how such changes may affect cardiac physiology, particularly the role of nutritional Se status and its relationship with NO at a molecular level, in maintaining heart health.

It should be noted that others have found that Se deficiency may decrease serum nitrite/nitrate levels. For example, serum nitrite/nitrate content was found lower in male weanling C57/HeJ mice fed a Se− diet for 5 wk than in Se+ controls (A.D. Smith, personal communication). Similar results were found in Wistar rats (35). Clearly, more studies are needed to clarify this point.

The mechanism by which Se deficiency influences iNOS cardiac expression is unknown. Of interest in this context is the report that treatment of nuclear extracts of lipopolysaccharide-activated human T cells with relatively high concentrations of selenium inhibited nuclear factor-κB (NF-κB) binding and decreased NO production (24). However, as pointed out by the authors, this study should be interpreted with caution because equivalent mechanisms may not be operating in Se deficiency and in Se excess. Moreover, because of its high chemical reactivity, the metabolism of selenite added in vitro may not be the same as that consumed in the diet. One possible molecular mechanism whereby dietary Se deficiency might increase cardiac NO output is via an activation of NF-κB. The low level of antioxidant selenoproteins in the Se− mice would result in an elevated oxidative stress and an increased generation of reactive oxygen species, which in turn would lead to an activation of NF-κB because this transcription factor is reactive oxygen species sensitive. The activation of NF-κB would then cause the upregulation of multiple genes, including those involved in increasing the expression of several cytokines, chemokines and iNOS (2, 3). The recent findings of Prabhu et al. (34) assume importance in this connection because these workers were able to demonstrate a NF-κB mediated upregulation of iNOS expression in the RAW 264.7 macrophage cell line during Se deficiency. In support of those results, macrophages from glutathione peroxidase (GPX) knockout mice produced more NO than those from wild-type mice (14). In addition, it has been shown that GPX can prevent apoptosis and that NO can inhibit GPX, thereby altering the balance between oxidants and antioxidants affecting cellular homeostasis (1). Considering all these data together, it is possible that the NO concentration may be elevated in a variety of tissues and may account for the necrotic degeneration, involving primarily the heart muscle, but also the liver, peripheral muscle, kidneys, pancreas, and testes observed in murine selenium and vitamin E deficiency (12).

How can the results presented here relate to human cardiac pathophysiology? The only well-established link between Se deficiency and human heart disease is Keshan disease which usually presents in the form of a DCM. It has been postulated that viral infection could be a significant cofactor in the etiology of Keshan disease on the basis of three lines of evidence (30). First, coxsackie and other picornaviruses were found in the heart tissue of affected patients. Second, Se− mice suffered a more severe myocarditis than Se+− mice when inoculated with such viruses. Finally, an amyocarditic strain of coxsackie virus became myocarditic after replication in Se− mice. An abnormal immune response after a viral-induced myocarditis has long been postulated as a major pathogenic mechanism in sporadic DCM.

The data presented here suggest an independent mechanism that could also be involved because elevated NO production mediated by iNOS per se may contribute to the myocardial impairment and elevated apoptosis (25) associated with conditions such as myocarditis and DCM, among others. An increased expression and colocalization of tumor necrosis factor and iNOS was observed in the myocardium of individuals with DCM (19). It is of interest because tumor necrosis factor production by cardiac myocytes is known to contribute to contractile dysfunction by several mechanisms, including NO-induced myofilament desensitization (32). A major question that needs to be clarified is the extent of Se deficiency that is required before an increased iNOS expression is observed in the heart. That is, is there a minimum threshold or degree of deficiency that is needed before any increase in iNOS expression is seen or is the elevation in iNOS expression a continuous function of Se deficiency? Whether or not such a triggering threshold exists could have a great impact on the role of selenium in human cardiac health.

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REFERENCES


