Dietary Selenium Influences Calcium Release and Activation of MLCK in Uterine Smooth Muscle of Rats

Mengyao Guo · Tingting Lv · Fangning Liu · Haiyang Yan · Teng Wei · Hua Cai · Wulin Tian · Naisheng Zhang · Zhe Wang · Guanghong Xie

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Abstract We sought to elucidate the effects of different concentrations of dietary selenium on calcium ion release, MLCK levels, and muscle contraction in the uterine smooth muscle of rats. The selenium (Se) content of blood and of uterine smooth muscle tissues was detected by fluorescence spectrophotometry. Ca²⁺ content was measured by atomic absorption spectroscopy. Calmodulin (CaM) and MLCK RNA and protein levels were analyzed by quantitative real-time polymerase chain reaction and Western blot, respectively. Dietary Se intake increased the Se levels in the blood and in uterine smooth muscle tissues and increased the Ca²⁺ concentration in uterine smooth muscle tissues. The addition of Se also promoted CaM expression and enhanced MLCK activation in uterine smooth muscle tissues. In conclusion, Ca²⁺, CaM, and MLCK were regulated by Se in uterine smooth muscle; Se plays a major role in regulating smooth muscle contraction in the uterus.

Keywords Selenium · Ca²⁺ · CaM · MLCK · Uterine smooth muscle

Introduction

The element selenium (Se) is recognized as an essential nutrient [1]. In 1973, the biochemical and biological roles of Se were further revealed when it was established as an essential component of the active site of the selenoenzyme GPx [2, 3]. Se, which is closely associated with health and disease, is a nutritionally essential trace element [4]. It has been reported that Se plays an important role in chemoprevention [5, 6], neurobiology [7], aging [8], immune function [9, 10], muscle metabolism [11], reproduction [12], redox reactions [9], and many other aspects of health [13, 14].

Previous research has indicated that the activity of Ca²⁺ release channels was modulated by Se in the sarcoplasm [15]. In addition, during PC12 cell differentiation, Se could reinforce the elevation of cytosolic Ca²⁺ concentration and stimulate the release of catecholamines [16]. Ca²⁺ can function as an intracellular information carrier, binding to calmodulin (CaM) for signal translation [17]. Smooth muscle MLCK plays a regulatory role in the contraction of smooth muscle by phosphorylating 20 kDa myosin light chain (MLC20) in a Ca²⁺/CaM-dependent manner.

Many muscle diseases are associated with Se, but the relationship between Se, Ca²⁺/CaM, and MLCK has not been reported. We therefore examined the concentration of Ca²⁺, the expression of CaM, and the activity of MCLK in the uterine smooth muscle tissue of rats fed with different quantities of Se and probed the effects of Se on Ca²⁺/CaM and MLCK. The function of Se in uterine contraction was also explored.

Materials and Methods

Animals

In total, 90 adult female Wistar rats (6–8 weeks old, weighing 160–175 g) were used and were provided by the Center of...
Experimental Animals of Baiqien Medical College, Jilin University, China. The experimental procedures were conducted according to the US NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Jilin University.

Experimental Groups and Administration

The rats were randomly divided into three groups (30 rats per group) as follows:

1. Low level group (LG) rats were fed a Se-deficient granulated diet (containing 0.02 mg/kg Se, to simulate the Se deficiency that exists in Jilin Province, China).
2. Normal level group (NG) rats were fed a 0.3-mg/kg Se-supplemented granulated diet.
3. High level group (HG) rats were fed a 1.0-mg/kg Se-supplemented granulated diet.

Each group was separated into three mini-groups (ten rats each) that were fed for 20, 40, or 60 days. The animals were maintained under standard husbandry conditions of light (12 h) and dark (12 h) at a temperature of 25±1 °C with food and drinking water ad libitum. After euthanasia with sodium pentobarbital, the plasma and uterine smooth muscle tissues were quickly collected. The tissues were rinsed with ice-cold sterile deionized water, frozen immediately in liquid nitrogen, and stored at -80 °C.

Detection of the Se Contents of Blood and Uterine Smooth Muscle Tissues

The Se contents of blood and uterine smooth muscle were estimated using the method described by Hasunuma et al. [18]. This assay is based on the principle that the Se within samples is converted to selenous acid by acid digestion. The reaction between selenous acid and aromatic -diamines, such as 2,3-diamino-naphthalene, leads to the formation of 4,5-benzopiazselenol, which displays a brilliant lime-green fluorescence when excited at 366 nm in cyclohexane. The fluorescence of extracted cyclohexane was measured with a fluorescence spectrophotometer with an excitation wavelength of 366 nm and an emission wavelength of 520 nm. The Se content in the samples was calculated based on a standard curve.

Assay for Ca\(^{2+}\) Content

Different volumes of a Ca\(^{2+}\) standard stock solution were placed in 50 mL measuring flasks and diluted with water to prepare 0.5, 1.0, 2.0, 5.0, and 10.0 mg/L standards. Specimens were placed into an oven at 72 °C for 3 days, and 18 mg of this dried material was then accurately weighed and added to a 50-mL beaker. Three milliliters of HNO\(_3\) and 2 mL of HClO\(_4\) were added. After being left overnight covered by a watch glass, the specimens were decomposed at a low temperature and heated until almost dry. The contents of specimens were then soaked, transferred, and fixed by 10 mL deionized water and analyzed by atomic absorption spectroscopy. The Ca\(^{2+}\) content in the samples was calculated from a standard curve.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from the uterine smooth muscle samples (50 mg tissue) using the TRIzol reagent according to the manufacturer’s instructions (Invitrogen, China). The concentration and purity of the total RNA were determined spectrophotometrically at 260/280 nm. First-strand cDNA was synthesized from 5 μg of total RNA using oligo (d)T primers and Superscript II reverse transcriptase according to the manufacturer’s instructions (Invitrogen, USA). The synthesized cDNA was diluted 1:4 with sterile water and stored at -80 °C.

Primer Premier software (PREMIER Biosoft International, USA) was used to design specific primers for CaM, MLCK, and β-actin based on known sequences (Table 1). Quantitative real-time PCR was performed on an ABI PRISM 7500 Detection System (Applied Biosysyms, USA). Reactions were performed in a 25-μL reaction mixture containing 12 μL of 2× SYBR Green I PCR Master Mix (TaKaRa, China), 2 μL of diluted cDNA, 0.5 μL of each primer (10 μM), 0.8 μL of 50× ROX reference Dye II, and 9.2 μL of PCR-grade water. The PCR protocol for CaM, MLCK, and β-actin consisted of 95 °C for 30 s followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 60 °C for 30 s. A melting curve analysis showed only one peak for each PCR product. The amplification efficiency for each gene was determined using the DART-PCR program [19]. The mRNA relative abundance was calculated according to the method of Pfaffl [20], accounting for gene-specific efficiencies, and was normalized to the mean expression of β-actin. The results (fold changes) were expressed as 2^(-ΔΔCt), in which ΔΔCt=(Ct\(_{\text{CaM/MLCK}}\)-Ct\(_{\beta\text{-actin}}\))−(Ct\(_{\text{CaM/MLCK}}\)-Ct\(_{\beta\text{-actin}}\)), where Ct\(_{\text{CaM/MLCK}}\) and Ct\(_{\beta\text{-actin}}\) are the cycle thresholds for CaM, MLCK, and β-actin genes, respectively, t is the treatment group, and c is the control group.

Western Blot Analysis

Uterine smooth muscle tissues were homogenized, and total protein was extracted according to the manufacturer’s protocols; protein concentrations were determined using the BCA protein assay kit. Samples with equal amounts of protein (50 μg) were separated on 10 % SDS polyacrylamide gels, transferred to a polyvinylidene difluoride
membrane, and blocked in 5% skim milk in TTBS for 2 h at room temperature. The membranes were then incubated overnight at 4°C with primary antibodies (1:1,000 dilution). After washing the membrane with TTBS, incubation with a 1:5,000 dilution (v/v) of secondary antibody was conducted for 1 h at room temperature. Protein expression was detected with an enhanced chemiluminescence detection system, and the membrane was exposed to an X-ray film. The β-actin signal was used as a loading control.

MLCK Activity Assays

The Ca²⁺/CaM-dependent activity of MLCK was measured by the rate of [γ-³²P]ATP incorporation into myosin light chain substrate according to the method of Ren et al. [21]. MLCK activity was assayed in a 50-μl reaction containing 3.25 g/L substrate, 5 mmol/L DTT, 0.1 mmol/L CaCl₂, 0.5 mmol/L KCl, 0.1 mmol/L BSA, 50 mmol/L HEPES (pH=7.0), and 0.2 mmol/L [γ-³²P]ATP. MLCK was diluted into this reaction, which was then incubated at room temperature for 30 min. Forty microliters from this reaction was spotted on Whatman chromatography paper, followed by three washes with 75 mmol/L phosphoric acid, a 5-min 95% ethanol wash, and air-drying. Scintillation counting was performed to measure incorporated ³²P.

Data Analysis

Statistical analyses were performed using the SPSS software (ver. 13 for Windows; SPSS Inc., Chicago, IL, USA). Significance was determined by a one-way ANOVA using a significance level of P<0.05. The data were assessed using the Tukey–Kramer method for multiple comparisons. All values are expressed as the means±SD.

Results

Se Levels in Blood and Uterine Tissues

The effects of dietary Se on the Se levels in blood and uterine smooth muscle are shown in Fig. 1. Compared with the NG, the Se levels in blood and uterine smooth muscle tissues were increased significantly in the HG and reduced significantly in the LG at each sampling time. In line with the duration of treatment, the Se levels in blood and uterine smooth muscle tissues steadily increased in the HG and the NG. Compared with the Se level in blood, the uterine smooth muscle Se level behaved slightly differently. The Se concentration in both blood and uterine smooth muscle tissues reached a plateau after 40 days in the NG. In the HG, the Se concentration in blood also reached a plateau after 40 days, but the level of Se in uterine smooth muscle tissues continued to increase. The Se concentrations in blood and uterine smooth muscle tissues significantly decreased in the LG over time, but this dissipation was slower in blood.

The Concentration of Ca²⁺ in Uterine Tissues

The Ca²⁺ concentration of uterine smooth muscle tissues was detected by atomic absorption spectroscopy. There was an obvious linear correlation between Se and Ca²⁺ concentration. This result is shown in Fig. 2. With increasing doses of dietary Se, the effect was obvious. Compared with the NG, the Ca²⁺ concentration of uterine smooth muscle tissues was increased significantly in the HG and reduced significantly in the LG at 20, 40, or 60 days. The uterine smooth muscle Ca²⁺ concentration reached a plateau after 40 days in the NG, while in the HG, the Ca²⁺ concentration continued to rise until the end of the experiment. There was a sustained decrease of Se levels in the LG.

Effect of Se on the Expression of CaM

CaM has a key role as a Ca²⁺ signal-regulatory protein. The mRNA and protein expression levels of CaM were measured by quantitative RT-PCR and Western blot analysis in the present study. Compared with the NG, there was a significant reduction (P<0.05) in CaM mRNA in the LG. The CaM mRNA level was increased in the HG, but the difference from the NG was not statistically significant (P>0.1). During the experiment, the CaM mRNA level continued to decrease in the LG, slightly increased in the HG, and did not change in the NG. The results are shown in Fig. 3b.
The protein level of CaM was significantly increased in the HG and significantly decreased in the LG compared to the NG (Fig. 3a). Over the course of the experiment, the expression of CaM protein continually increased in the HG and continually decreased in the LG.

The Influence on the Expression and Activity of MLCK

MLCK is a key signaling molecule for smooth muscle contraction. MLCK mRNA was measured by quantitative RT-PCR (Fig. 4b); compared with the NG, there was a significant reduction \((P<0.05)\) in MLCK mRNA in the LG. MLCK mRNA was increased in the HG, but it was not significantly different \((P>0.1)\) from the NG. During the course of the experiment, the MLCK mRNA level continued to decrease in the LG, slightly increased in the HG, and did not change in the NG.

MLCK protein was measured by Western blot (Fig. 4a). Compared with the NG, the expression of CaM protein was increased significantly in the HG and suppressed in the LG. The level of MLCK protein continued to increase until the end of experiment in the HG and continued to decrease in the LG. The activity of the MLCK protein was also assayed (Fig. 4c). Compared with the NG, MLCK had a higher catalytic activity in the HG \((P<0.01)\), but the catalytic activity was significantly reduced in the LG \((P<0.01)\).

Discussion

Selenium is an essential nutrient for humans and animals [22]. Many diseases are caused by Se deficiency [23]. Many forms of cardiac and skeletal muscle disorders that are defined as nutritional muscular dystrophy are caused by Se deficiency [24, 25]. However, data on the relationship between Se and smooth muscle function, especially for the uterine smooth muscle, are lacking.

The Se concentrations in blood and uterine smooth muscle are most commonly used as the biomarkers of Se status in various parts of the body [26]. In the present study, the Se concentrations in blood and uterine smooth muscle tissues were significantly increased in the HG and significantly reduced in the LG at 20, 40, and 60 days \((P<0.05)\), and the magnitude of these changes steadily increased over the course of the experiment. It is significant that the Se concentrations in the blood and uterine smooth muscle tissues were affected by the Se intake. This result was in agreement with previous studies on other tissues, including smooth muscle tissues [27]. The Se level in blood reached a plateau after 40 days in the HG. In the LG, the rate of Se dissipation in blood was lower than in uterine smooth muscle. Thus, we inferred that the blood serves as a Se reservoir and has a buffering action on the dietary intake of Se before it can reach systemic tissues. The dose of dietary selenium intake significantly influenced the Se concentration in uterine tissues.

Ca\textsuperscript{2+} ions were detected by atomic absorption spectrophotometry. The increased Ca\textsuperscript{2+} concentration correlated with the increased content of dietary Se. Over the course of the experiment, the Ca\textsuperscript{2+} concentration of uterine smooth muscle tissues continued to increase in the HG and continued to
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Fig. 3 The expression of CaM in uterus tissues. a Western blotting was performed to detect CaM protein levels in the low level group (LG), normal level group (NG), and high level group (HG). b qPCR was performed to detect the mRNAs of CaM; β-actin was used as a control. Values are presented as the means±SEM (n=10). *P<0.01, significantly different from the NG. **P<0.01, significantly different from the 20-day sampling point in the same group.

decrease in the LG. This result is in agreement with a previous study of the effects of Se on the intracellular calcium concentration [28]. It was suggested that Se was involved in calcium channel mobilization [29]. CaM has a key role as a Ca²⁺ signal-regulatory protein [30]. In the present study, CaM mRNA did not increase significantly.

Fig. 4 Effect of Se on the expression and activity of MLCK. a Western blotting was performed to detect MLCK protein levels in the low level group (LG), normal level group (NG), and high level group (HG). b qPCR was performed to detect the mRNAs of MLCK; β-actin was used as a control. c The activity level of isolated MLCK in uterus smooth muscle tissues. Values are presented as the means±SEM (n=10). *P<0.01, significantly different from the NG. **P<0.01, significantly different from the 20-day sampling point in the same group.
with enhanced Se intake, but CaM protein expression did increase. Over the course of the experiment, the expression of CaM mRNA and protein continued to increase. These results indicated that CaM protein expression was influenced by Se intake, and the increased Ca²⁺ concentration was the direct result of the increased levels of CaM. Se initially played an indirect role in increasing the Ca²⁺ concentration.

Smooth muscle contraction, unlike skeletal and cardiac muscle contraction, is a slow process. Enormous variability exists between different smooth muscle cells, making it difficult to draw general conclusions concerning their signaling mechanisms [31]. MLCK is a key protein in the smooth muscle contraction process [32, 33]. Contraction is achieved through the phosphorylation of the P chain of myosin by MLCK and can be activated by Ca²⁺/CaM [34]. In the present study, the expression of MLCK mRNA and protein in uterine smooth muscle tissues increased significantly with increased Se intake. The activity of MLCK was also significantly increased. Our results showed that the expression of MLCK was promoted by increasing the Se concentration in uterine smooth muscle tissues, and the activity of MLCK was promoted by increasing Ca²⁺/CaM levels, which were induced by increased dietary Se. Because there is no troponin in smooth muscle, the smooth muscle contraction process is achieved through the phosphorylation of the P chain of myosin by MLCK [35]. In the present study, the expression and activity of MLCK were promoted by increasing the Se content in uterine smooth muscle tissues. This result indicates that Se could promote uterine smooth muscle contraction.

In conclusion, the increase of dietary Se, the Se concentrations in blood and uterine smooth muscle tissues were obviously increased. Meanwhile, the Ca²⁺ and CaM levels were also increased. These results confirmed that the Ca²⁺ and CaM levels were regulated by Se in uterine smooth muscle. We also found that Se promoted the expression and activity of MLCK in uterine smooth muscle tissues. Therefore, it was concluded that Se plays a major role in regulating the contraction process of the uterine smooth muscle.

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