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# Selenium Supplementation Can Protect Cultured Rat Cardiomyocytes from Hypoxia/Reoxygenation Damage

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The possibility of enhancing glutathione peroxidase (GPx) activity and cytosolic total antioxidant activity (TAA) in normoxia and hypoxia/reoxygenation (H/R) by the supplementation of different concentrations of sodium selenite (SS) or selenomethionine (SM) was investigated in cultured rat cardiomyocytes. To assess the entity of oxidative stress due to H/R, levels of conjugated dienes containing lipids were determined. In normoxia, GPx activity and TAA increased in parallel with the increase in SS and SM supplementation. H/R did not influence GPx activity but lowered TAA; both SS and SM supplementations were effective in increasing GPx activity, the most effective concentration being 1 $\mu$M. At this SS and SM concentration, TAA returned to a normoxic value. Conjugated diene production, increased by H/R, was reduced by SS and SM supplementation, the 1 $\mu$M concentration appearing to be the most effective one. According to these data Se supplementation represents another possibility to counteract oxidative damage in the myocardium.

KEYWORDS: Selenium; hypoxia/reoxygenation; cultured cardiomyocytes; glutathione peroxidase; oxidative stress

## INTRODUCTION

The recognition that a deficiency of the micronutrient selenium (Se) is harmful to the heart and causes a fatal dilated congestive cardiomyopathy in animals (white muscle disease) and in man (Keshan disease) emphasized the essentiality of this trace element in the diet of mammals and followed a long period in which it was known only for its toxicity.

Selenium is an essential component of glutathione peroxidase (GPx), which plays a critical role in protecting aerobic organisms from oxygen radical initiated injury. A low Se status results in reduced selenium-dependent GPx activity, and Se deficiency has been known to cause extensive oxidative damage in many tissues, including the myocardium (1), in which superoxide dismutase (SOD) and GPx appear to be the most active antioxidant enzymes (2). Therefore, any significant modification of Se status would lead to changes in the activity of the enzyme GPx and have important consequences on the susceptibility of the tissue to oxidative stress (3).

For the general population, the primary pathway of Se intake is food, followed by water. Inorganic selenate and selenite predominate in water, whereas the organic Se compounds selenomethionine (SM) and selenocysteine are the major Se species in cereals and vegetables (4). Although selenium is not an essential element for plants, this mineral is taken up and incorporated by plants into organic compounds; factors that affect the level of Se in plant-derived foods include type of food, soil type, soil conditions, and time of harvest (4).

Many investigators have suggested that diet may play a part in the development of ischemic heart diseases, and many experimental and clinical studies have underscored a role of dietary supply of metal salts (5). With regard to the role of Se, many studies have concentrated on Se deficiency (6), but little is known about the effect of Se supplementation, particularly with respect to cardiac cells and heart diseases.

Ischemic heart disease is one of the major causes of death in most industrialized countries. The pathogenetic mechanisms of myocardial ischemic damage are still not completely understood, but the role of oxygen-derived free radicals in myocardial ischemia/reperfusion has been established. The reactive oxygen species (ROS) may contribute to the genesis of pathophysiological alterations consequent to ischemia/reperfusion in the heart. In this concept, excessive formation of free radicals may overwhelm antioxidant heart defense capacity and damage myocardial cells. It has been reported that the activity of antioxidant enzymes is lower in rat heart than in other tissues such as the liver (7), whereas the susceptibility to oxidative stress is higher in heart than in other tissues (8). Moreover, conditions such as ischemia/reperfusion may weaken the cardiac antioxidant system. Using cultured neonatal cardiomyocytes we recently demonstrated that reoxygenation-induced radical production is related to the duration and severity of the preceding period of hypoxia and that it can be attenuated by antioxidant supplementation (9). In this light, any intervention able to increase

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the activity of antioxidant enzymes, that is, Se supplementation, may be useful for the protection of cardiac cells during ischemia/reperfusion injury.

In this study, we have supplemented cultured neonatal rat cardiomyocytes with scalar concentrations of Se in the form of both sodium selenite (SS) and SM. Some cells have been made hypoxic for 4 h and then reoxygenated for 10 min, and we have evaluated the effect of SS and SM supplementation in both normoxic and hypoxic/reoxygenated conditions by measuring in cell cytosol the activity of GPx and the total antioxidant activity (TAA). To assess the rank of oxidative stress in both unsupplemented and Se-supplemented cardiomyocytes, the entity of formation of oxidized lipids as conjugated dienes was determined.

## MATERIALS AND METHODS

**Materials.** Horse serum (HS), fetal calf serum (FCS), Ham F10 culture medium, SS, SM, 2,2′-azinobis(3-ethylbenzo-6-thiazoline-6-sulfonic acid) (ABTS), glutathione, Trolox, and other biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). SS and SM were dissolved in bidistilled, filtered water at the concentration of 1 mM and kept at 0 °C until use. All other chemicals and solvents were of the highest analytical grade.

**Methods.** Primary heart cell cultures were obtained by isolation of cardiomyocytes from the ventricles of 2−4-day-old Wistar rats, as previously reported (*10*). Cells were seeded in Petri dishes in nutrient mixture Ham F10 supplemented with 10% v/v FCS and 10% v/v HS, and the medium was changed every 48 h. At the first changing of the medium, and subsequently at each medium change, some cells received SS or SM at different concentrations (1 nM−10 μM). Medium pH was checked prior to the addition to cells and eventually adjusted to 7.4. When cells reached complete confluence, the medium was changed to a medium without serum but containing different concentrations of SS or SM, and some culture plates were transferred to a specially designed, airtight, thermostated chamber. The experiments lasted in hypoxia for 4 h and were followed by 10 min of reoxygenation. The hypoxic procedure reduced oxygen from 20 to 5% after 3 min and to <1% after 10 min. The $O_2$ content of the atmosphere inside the chamber was <1% for the duration of the experiment, as measured by an on-line meter (Griffin and George, Fife, U.K.) (*9*). Reoxygenation increased oxygen to 20% within 5 min.

In both normoxic condition and after hypoxia/reoxygenation, GPx activity and TAA were measured in the cytosol of cardiomyocytes. Briefly, cells were washed five times in ice-cold buffer, scraped off in the same, homogenized using an IKA-Werke cell homogenizer at the lowest speed (5/s), and centrifuged at 800*g* for 10 min. The resulting pellet was discarded, and measurements were performed on the supernatant. Protein concentration was assessed according to the method of Bradford (*11*) with bovine serum albumin as a standard.

GPx activity was assayed spectophotometrically according to the method described by Flohe et al. (*12*), which is based on the reduction of oxidized glutathione coupled to the oxidation of NADPH. The disappearance of NADPH was followed at 340 nm. One unit of GPx activity was defined as the amount of enzyme that catalyzes the reduction of 1 μmol of NADPH/min. GPx activity in the cells was expressed as milliunits (mU) per milligram of protein.

TAA was measured using the method of Re et al. (*13*), based on the ability of the antioxidant molecules in the sample to reduce the radical cation of ABTS, determined by the decolorization of ABTS•+ and measured as quenching of absorbance at 740 nm. Values obtained for each sample were compared to the concentration−response curve of a standard Trolox solution and expressed as micromoles of Trolox equivalents (TE).

The appearance of conjugated diene-containing lipids was evaluated as an index of lipid peroxidation using the method of Burton et al. (*14*). Briefly, cells scraped from the culture plates were extracted in chloroform/methanol/water (2:1:1 v/v). The chloroform layers from two extractions were combined and then dried under nitrogen. Samples were
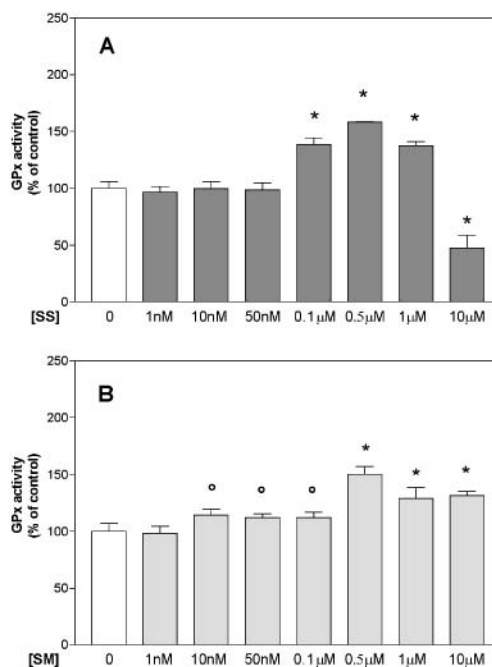


**Figure 1.** GPx activity in normoxic cardiomyocytes supplemented with SS or SM. Cardiomyocytes were grown in medium supplemented with scalar concentrations of SS (A) or SM (B). GPx activity was measured as reported under Methods and is expressed as percent of control unsupplemented cell activity (126.12 ± 9.32 mU/mg of protein = 100%). Data are means ± SD of five different cell cultures. Statistical differences were evaluated using Student′s *t* test to compare supplemented vs unsupplemented cells (°, *p* < 0.01; *, *p* < 0.001) and by two-way ANOVA to compare the effects of SS vs SM (*p* < 0.001).

resuspended in a known volume of acetonitrile, and absorbance was determined at 235 nm.

All data are means ± SD of five different cultures. Statistical differences were evaluated using Student's *t* test.

## RESULTS

Cytosolic GPx activity in normoxic cells supplemented with scalar concentrations of SS or SM is expressed as percent of control unsupplemented cell activity and is reported in parts A and B, respectively, of **Figure 1**.

GPx activity in control cells was 126.12 ± 9.32 mU/mg of protein. SS supplementation in the range of 0.1−1 μM significantly improved GPx activity, whereas the 10 μM concentration caused a significant decrease, below the control value. SM supplementation increased enzyme activity even at lower concentrations (10−50 nM), although the 0.5 μM concentration appeared to be the most effective one.

Cytosolic TAA, expressed as micromoles of TE per milliliter, of normoxic cardiomyocytes, unsupplemented or supplemented with SS or SM, is reported in parts A and B, respectively, of **Figure 2**.

TAA appeared to be higher in 0.1−1 μM SS supplemented cells than in controls and significantly lower in 10 μM SS supplemented ones, according to the observed modifications in GPx activity. SM supplementation increased TAA even at lower concentrations, in agreement with modifications observed in GPx activity.

In normoxic condition the conjugated diene level was not affected by SS or SM supplementation ($A_{235nm}$ = 0.053 ± 0.008).

Hypoxia/reoxygenation did not influence cytosolic GPx activity, which appeared to be similar in normoxic and hypoxic/
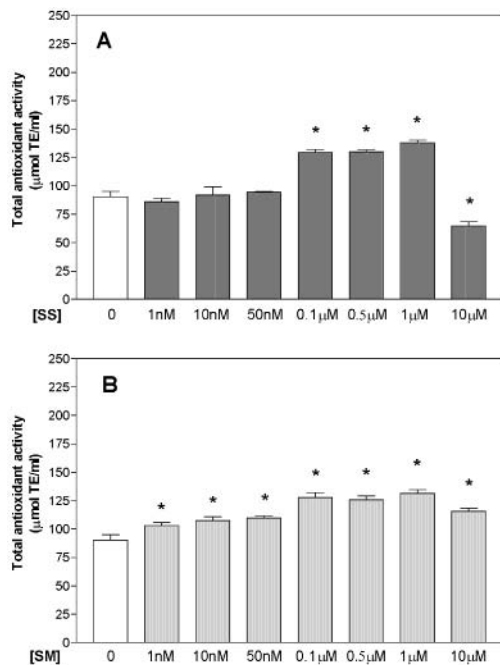
**Figure 2.** Cytosolic TAA in normoxic cardiomyocytes supplemented with SS or SM. Cardiomyocytes were grown in medium supplemented with scalar concentrations of SS (A) or SM (B). TAA was measured as reported under Methods and is expressed as $\mu$mol of Trolox equiv/mL. Data are means $\pm$ SD of five different cell cultures. Statistical differences were evaluated using Student's *t* test to compare supplemented vs unsupplemented cells (*, $p < 0.001$) and by two-way ANOVA to compare the effects of SS vs SM ($p < 0.001$).



**Figure 3.** GPx activity in hypoxic/reoxygenated cardiomyocytes supplemented with SS or SM. Cardiomyocytes were subjected to 4 h of hypoxia followed by 10 min of reoxygenation in the absence or presence of scalar concentration of SS (A) or SM (B). GPx activity was measured as reported under Methods and is expressed as percent of normoxic unsupplemented cell activity (126.12 $\pm$ 9.32 mU/mg of protein = 100%). Data are means $\pm$ SD of five different cell cultures. Statistical differences were evaluated using Student's *t* test to compare supplemented vs unsupplemented cells (°, $p < 0.01$; *, $p < 0.001$) and by two-way ANOVA to compare the effects of SS vs SM ($p < 0.001$).

reoxygenated unsupplemented cardiomyocytes. SS supplementation (**Figure 3A**) in the range of 0.5−1 $\mu$M increased GPx activity, whereas the highest SS concentration (10 $\mu$M) always caused a disruption of cell monolayer during the hypoxic period. Also, SM supplementation at the 0.5−1 $\mu$M concentrations caused an increase in enzyme activity, but to a higher extent than the corresponding SS concentration (**Figure 3B**). Furthermore, the highest SM concentration (10 $\mu$M) did not show any disrupting effect.
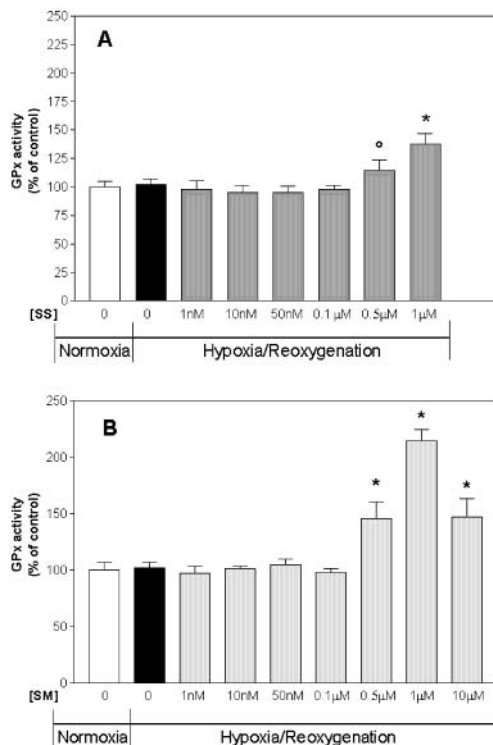
Hypoxia/reoxygenation significantly reduced cardiomyocyte TAA, which was completely restored by the supplementation of either 1 $\mu$M SS or 1 $\mu$M SM (**Figure 4**, parts A and B, respectively).

Conjugated diene production significantly increased in hypoxic/reoxygenated cells. Supplementation with both SS and SM caused a significant decrease in conjugated dienes (**Figure 5**, parts A and B, respectively); in both cases the 1 $\mu$M concentration appeared to be the most effective in reducing lipid peroxidation.

## DISCUSSION

The purpose of the present investigation was to establish whether and to what extent supplementation with Se, in both inorganic and organic forms, enhances the activity of Se-dependent GPx in cultured rat cardiomyocytes. We have verified this hypothesis in both normoxic cardiomyocytes and cardiomyocytes subjected to H/R.
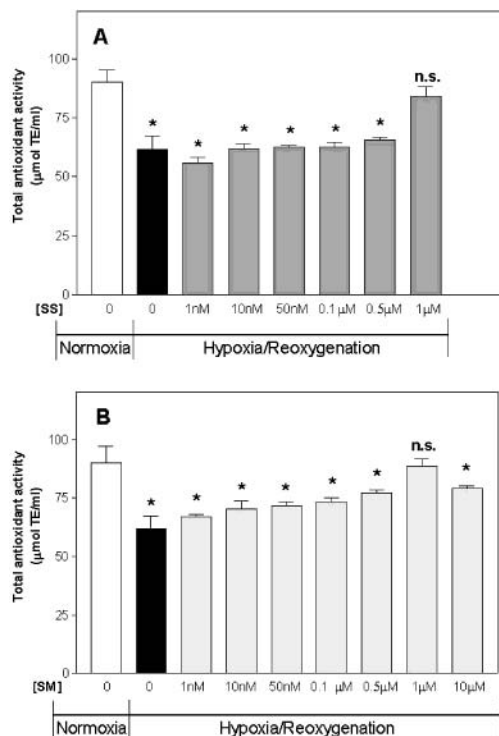
In normoxia, as Se supplementation increases, GPx activity increases. The main difference between SS and SM supplementation is a significant decrease in GPx activity at the highest SS concentration, whereas activity plateaus at the highest SM concentrations. TAA well correlates with the observed modi-

fications in GPx activity, confirming not only that, in normal conditions, Se supplementation can modulate GPx activity but also that the increase in enzyme activity greatly influences the entity of the global defenses against oxidative stress.

According to other authors (*3*, *15*), in our study H/R did not cause a decrease in GPx activity with respect to normoxic cells, but Se supplementation, particularly in the form of SM, was effective in increasing GPx activity also in hypoxic/reoxygenated cardiomyocytes; the most effective concentration was 1 $\mu$M, whereas it was 0.5 $\mu$M in normoxia. Although we did not evaluate GPx expression, many studies indicate that supplementing Se could increase the GPx gene expression level (*16*, *17*), and a Se concentration dependence on the expression of GPx was demonstrated in cultured cells (*16*). It can be hypothesized that, under conditions of oxidative stress, the Se concentration needed for increasing the GPx mRNA level is higher than in normal conditions. A further increase in Se supplementation not only failed to cause a further increase in GPx activity but decreased it in the case of SM and had a disruptive effect on cell monolayer in the case of SS. Similar results were obtained by Gross et al. (*18*), who observed in a porcine kidney epithelial cell line a linear increase in GPx activity as selenite concentration in the medium reached 20 nM; however, when it approached 50 nM, the activity began to decrease. In hypoxia, the effect of 10 $\mu$M SS was a disruption of the cell monolayer, probably due to a synergistic effect of hypoxia and SS supplementation, in agreement with the well-documented higher toxicity of selenite compared with that of

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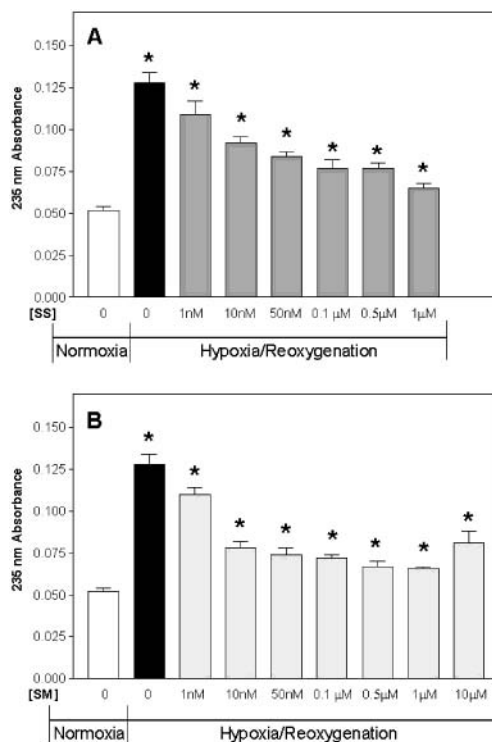*J. Agric. Food Chem.,* Vol. 51, No. 6, 2003  **1739**



**Figure 4.** Cytosolic TAA in hypoxic/reoxygenated cardiomyocytes supplemented with SS or SM. Cardiomyocytes were subjected to 4 h of hypoxia followed by 10 min of reoxygenation in the absence or presence of scalar concentration of SS (A) or SM (B). TAA was measured as reported under Methods and is expressed as $\mu$mol of Trolox equiv/mL. Data are means $\pm$ SD of five different cell cultures. Statistical differences were evaluated using Student's *t* test to compare supplemented vs unsupplemented cells (*, $p < 0.001$) and by two-way ANOVA to compare the effects of SS vs SM ($p < 0.001$).



**Figure 5.** Conjugated diene levels in hypoxic/reoxygenated cardiomyocytes supplemented with SS or SM. Cardiomyocytes were subjected to 4 h of hypoxia followed by 10 min of reoxygenation in the absence or presence of scalar concentration of SS (A) or SM (B). Conjugated diene production was measured as 235 nm absorbance as reported under Methods. Data are means $\pm$ SD of five different cell cultures. Statistical differences were evaluated using Student's *t* test to compare supplemented vs unsupplemented cells (°, $p < 0.01$; *, $p < 0.001$) and by two-way ANOVA to compare the effects of SS vs SM ($p < 0.001$).

SM (*4*). At 1 $\mu$M concentration, SM increased GPx activity more than SS supplementation; this could be due to an effect of methionine per se on GPx activity. Seneviratne et al. (*19*) demonstrated that dietary supplementation with methionine in rats caused a significant increase in myocardial GPx activity, concomitant to an increase in mRNA levels for GPx. Therefore, Se and methionine, administered together as SM, could have a synergistic effect on enzyme activity.

TAA was greatly affected by H/R, and it reverted to normoxic value only when SS or SM was supplemented at 1 $\mu$M concentration, that is, the most effective concentration in increasing GPx activity. Although the exact mechanisms and interactions among various antioxidants are not fully understood, it is possible that one antioxidant may equilibrate with another to establish a cellular redox potential, and thus all endogenous antioxidants may act in concert to protect against oxidative insult (*2*). In our study, due to Se supplementation, GPx activity appears to be one of the main contributors of the TAA: in fact, GPx activity was linearly correlated to TAA in both SS-supplemented ($R^2 = 0.931$, $p < 0.01$) and SM-supplemented ($R^2 = 0.893$, $p < 0.01$) cells.

H/R greatly increased conjugated diene levels, as previously demonstrated (*9*). The overall result of SS and SM supplementation was a reduction of lipid peroxidation. Although this reduction was particularly evident at the 1 $\mu$M SS and SM concentrations, it was present even at SS and SM concentrations that did not cause an increase in GPx activity or in TAA. Conjugated diene level is an index of fatty acid peroxidation, and it is known that lipophilic antioxidants such as vitamin E

protect the acylic groups of phospholipids during oxidative stress. In this work we have not measured vitamin E level, but we can hypothesize a positive effect of Se supplementation also on tocopherol activity, because GPx contributes to the regeneration of tocopheryl radicals (*20*). This vitamin E saving could be activated at low SS and SM concentrations and be responsible for the observed decrease in conjugated diene level.

In conclusion, supplementation with Se, the cofactor of one of the main enzymatic systems involved in cellular antioxidant defense mechanisms, GPx, can modulate some of the sequelae of H/R, such as the increase of lipid peroxidation and the decrease in TAA. It must be noted that our results were obtained after hypoxia followed by a short reoxygenation period. Although the production of free radicals has been shown to persist long into the reoxygenation period, the consensus is in favor of a critical early burst (*21*); the suppression of this early burst by various interventions is accompanied by an attenuation of arrythmias, whereas suppression of free radical production after this burst (i.e., after the first 10 min of reoxygenation) is not (*21*).

In the human being, the severity of ischemia/reperfusion injury and the subsequent level of oxidative stress and the interaction of antioxidants with free radicals as well as among various antioxidants seem to determine the effectiveness of antioxidants for cardioprotection. An increasing number of investigators have demonstrated the importance of GPx in protecting the myocardium from ischemia/reperfusion (*22, 23*). Although results obtained in cultured cells cannot be acritically transposed to humans, our results must be taken in account,

particularly in the light of the very low Se level in patients with myocardial infarction detected in clinical studies (*24*). Therefore, in the case of general Se deficiency in soil, water, and foods, resulting in low Se levels in the population, recommendations for an adequate Se dietary intake, using foods with a suitable Se content or Se-enriched foods, could represent an additional, promising dietary strategy against ischemia/reperfusion injury.

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