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Susceptibility to Hypoxia/Reoxygenation of Aged Rat Cardiomyocytes and Its Modulation by Selenium Supplementation

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Since in the aged heart an increased basal production of reactive oxygen species (ROS) has been demonstrated, and the resistance to ROS attack could be ameliorated by antioxidant supplementation, we verified the protective effect of selenium, as sodium selenite (SS) or seleno methionine (SM), in cultured rat cardiomyocytes aged in vitro. In normoxia, glutathione peroxidase (GPx) activity and total antioxidant activity were higher in old than in young cardiomyocytes, suggesting the existence of a compensatory increase of antioxidant defenses. When aged cells were submitted to hypoxia/reoxygenation, GPx activity was not modified; while total antioxidant activity decreased, conjugated diene level increased. Selenium supplementation, particularly as SM, was able to increase GPx, and consequently total antioxidant activity, and to decrease conjugated diene production. The observed ability of selenium supplementation to protect aged cardiomyocytes from hypoxia/reoxygenation damage underlines the importance of an optimal selenium dietary intake, particularly in the elderly.

KEYWORDS: Selenium; aging; hypoxia/reoxygenation; cardiomyocytes; glutathione peroxidase

INTRODUCTION

Aging is a progressive and universal process originating endogenously that manifests itself during postmaturational life. Many pathologies, including cardiovascular diseases, become more frequent with increasing age (*1*), the aging process itself becoming part of the pathophysiological mechanism (*2*).

Numerous cellular and molecular modifications accompany the senescence of the cardiovascular system and contribute to its decreased resistance against stress-induced injury (*2*). In humans, age-related changes in cardiovascular function and structure are remarkable for changes in pump function and increased vascular afterloading (*3*). There is also evidence for a reduction in the number of cardiac myocytes with advancing age (*3*). Subcellular changes with aging include certain regulatory factors of excitation−contraction−relaxation coupling (i.e., calcium handling) (*4*), modulation by adrenergic receptor stimulation (*4, 5*), and changes in the generation and sensitivity to the damaging effects of reactive oxygen species (ROS).

In the aged heart, the increased basal production of ROS could be paralleled by increased ROS-sensitivity (*3*) and reduced antioxidant capacity (*2*). Overall, these changes could lead to a diminished capacity of the heart to adapt to physiological (i.e., heavy exercise) or pathological stress (i.e., ischemia and reperfusion) causing a further increase in ROS production.

Recently, the possibility of the existence of a compensatory antioxidant defense system to counteract oxidative stress-associated vascular aging has been postulated (*6*), and an increase in the activities of glutathione peroxidase (GPx), glutathione reductase, and glutathione transferase has been reported in the heart of aged rats after ischemia/reperfusion (*7*).

In light of the nutritional recommendations on antioxidant intake for the prevention of ROS-related diseases, in a previous study (*8*) we demonstrated the protective effect of a selenium supplementation in both inorganic and organic form on hypoxia/reoxygenation damage in young cultured cardiomyocytes. Since a compensatory increase in antioxidant defenses during aging has been suggested (*6*), the first aim of this work was to evaluate GPx activity, total antioxidant activity, and conjugated diene level as an index of lipid peroxidation in cultured cardiomyocytes aged in vitro, in normoxic condition, and after hypoxia/reoxygenation. Primary myocyte cultures derived from newborn rat heart provide a convenient cellular model system for studies on age-related effects on heart cells. The minimal cell division in these cultures enables studies on the same cells for an extended time without the complications arising in cell lines from continuous cell division and from changes related to the number of passages (*9*).

The second aim of this work was to verify a possible positive action of selenium also in aged cells, by supplementing both normoxic and hypoxic/reoxygenated cardiomyocytes with scalar concentrations of selenium in both inorganic (sodium selenite, SS) and organic form (seleno methionine, SM). Since nutritional essentiality of dietary selenium for mammals has been estab-

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lished, but the exact requirement of selenium needed to obtain maximal GPx activity is still controversial, we have also verified the amount of SS or SM able to increase GPx activity in aged cardiomyocytes submitted to hypoxia/reoxygenation.

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). Sodium selenite and SM were dissolved in bidistilled, filtered water at the concentration of 1 mM and kept at 0 °C until use. All the 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 100-mm i.d. Petri dishes at the density of 2×10^6 cells/mL in nutrient mixture Ham F10 supplemented with 10% v/v FCS and 10% v/v HS, and the medium was changed every 48 h. Cardiomyocytes were randomly divided in control and supplemented groups; supplemented cells received SS or SM at different concentrations (1 nM−10 μM) for the first time at the first medium change and subsequently at each medium change. Medium pH was always checked and eventually adjusted at 7.4. All cells reached complete confluence at day 5 and were grown for 7 days ("young" cells) or for 14 days ("old" cells) in the above-mentioned conditions; then, some of them were turned to hypoxia in a specially designed, airtight, thermostated chamber. Cells were maintained in hypoxia for 4 h, and then were turned again to normoxia by 10 min reoxygenation. The hypoxic procedure reduced oxygen from 20 to 5% after 3 min and to less than 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 online meter (Griffin and George, Fife, U.K.) (*11*). Reoxygenation increased oxygen to 20% within 5 min. During hypoxia/reoxygenation, cells were maintained in Ham F10 medium without serum but containing the previous concentrations of SS or SM. Serum deprivation did not affect cell viability, as previously demonstrated (*12*).

In both normoxic condition and after hypoxia/reoxygenation, cytosolic GPx activity and total antioxidant activity were measured. Briefly, cells were scraped off in ice-cold buffer, homogenated, 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 Bradford (*13*) with bovine serum albumin as a standard.

Glutathione peroxidase activity was assayed spectophotometrically according to the method described by Flohe et al. (*14*), which is based on the reduction of oxidized glutathione coupled to the oxidation of NADPH. The disappearance of NADPH is followed at 340 nm. One unit of GPx activity was defined as the amount of enzyme that catalyses the reduction of 1 μmol NADPH $\times$ min^{-1}. GPx activity in the cells was expressed as milliunits (mU) $\times$ mg protein^{-1}.

Total antioxidant activity was measured using the method of Re et al. (*15*), on the basis of the ability of the antioxidant molecules in the sample to reduce the radical cation of ABTS, determined by the decolorization of ABTS$^{\circ+}$ and measured as quenching of absorbance at 740 nm. Values obtained for each sample were compared to the concentration−response curve of standard trolox solutions and were expressed as μmol trolox equivalent (TE).

The appearance of conjugated diene-containing lipids was evaluated as an index of lipid peroxidation using the method of Burton et al. (*16*). 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 resuspended in a known volume of acetonitrile and the absorbance was determined at 235 nm.

All data are means ± SD of five different cultures. Statistical differences were evaluated using the Student's t test, the one-way analysis of variance, the two-way ANOVA, and linear correlation analysis (Graph Pad Prism, version 4.0, Graph Pd software Inc.)

Table 1. Glutathione Peroxidase Activity, Total Antioxidant Activity, and Conjugated Diene Levels in Young and Old Cardiomyocytes in Normoxia and Hypoxia/Reoxygenation[a]

	glutathione peroxidase activity (mU/mg protein)	total antioxidant activity (μmol TE/mL)	conjugated dienes (235-nm absorbance)
young cells, normoxic	127.39 ± 10.04	90.17 ± 4.89	0.052 ± 0.002
old cells, normoxic	189.75 ± 5.66	101.81 ± 5.88	0.048 ± 0.004
young cells, hypoxia/reoxygenation	126.51 ± 7.32	61.72 ± 5.72	0.128 ± 0.006
old cells, hypoxia/rexygenation	195.78 ± 5.04	85.23 ± 8.99	0.102 ± 0.003

[a] Glutathione peroxidase activity, total antioxidant activity, and conjugated diene level were measured as reported in Methods and expressed as indicated. Data are means ± SD of at least five different cultures. Statistical analysis was by the two-way ANOVA and revealed for GPx a significant effect of age ($p < 0.001$), a not significant effect of pO_2, and a not significant interaction between the two parameters; for total antioxidant activity a significant effect of age ($p < 0.001$), a significant effect of pO_2 ($p < 0.001$), and a not significant interaction between the two parameters; for conjugate diene level a significant effect of age ($p < 0.001$), a significant effect of pO_2 ($p < 0.001$), and a significant interaction between the two parameters ($p < 0.001$).

RESULTS

In **Table 1**, GPx activity, total antioxidant activity, and conjugated diene formation in normoxic and hypoxic/reoxygenated young and old cardiomyocytes are reported.

Glutathione peroxidase activity was significantly higher in old cells than in young ones, and hypoxia/reoxygenation did not influence the enzyme activity either in young or old cardiomyocytes with respect to their normoxic counterparts. As a result, in normoxia total antioxidant activity was higher in old than in young cells; hypoxia/reoxygenation greatly reduced total antioxidant activity in both young and old cells. Hypoxia/reoxygenation caused an increase in lipid peroxidation independently of cell age, although more evident in young than in old cells.

The activity of GPx in old hypoxic/reoxygenated cells, unsupplemented or supplemented with scalar concentrations of SS or SM, is reported in **Figure 1**.

Supplementation with SS at the concentrations 1−50 nM did not influence GPx activity, while higher concentrations caused a significant increase compared to unsupplemented cells. The highest SS concentration (10 μM) always caused a disruption of cell monolayer. Cell viability was measured by the Trypan blue exclusion and cell damage was revealed only in 10 μM SS supplemented cells (data not shown).

Compared to unsupplemented cells, SM supplemented cardiomyocytes revealed a significant increase in GPx activity, more evident in the range 0.1−1 μM SM. At equal concentration, SM influenced GPx activity more than SS, and the highest SM concentration (10 μM) did not show any disrupting effect on cell monolayer, although being less effective than lower SM concentrations.

In hypoxic/reoxygenated cells, no significant differences in total antioxidant activity were detected in 1−50 nM SS supplemented cells compared to unsupplemented ones, while total antioxidant activity increased at the highest SS concentrations (**Figure 2A**). SM supplementation was able to increase total antioxidant activity at all the concentrations used with the 1 μM being the most effective (**Figure 2B**). At equal concentration, SM supplementation influenced total antioxidant activity more greatly than SS supplementation.

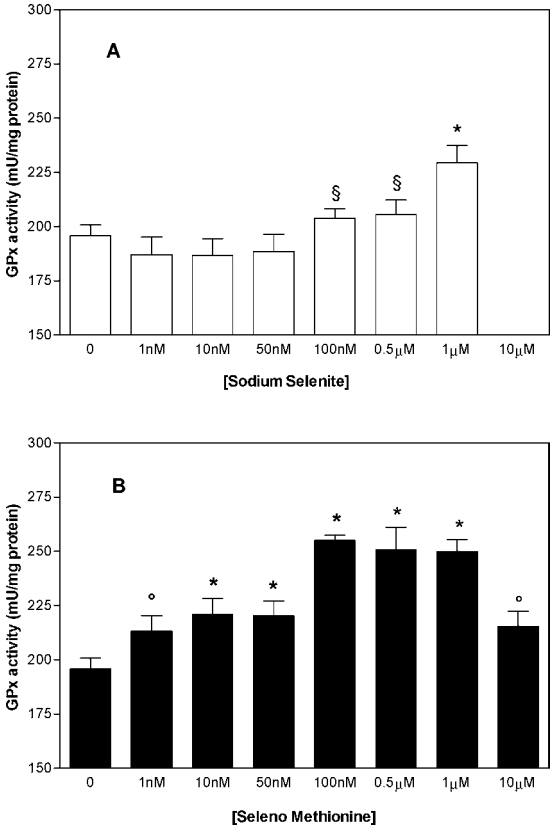

Figure 1. Glutathione peroxidase activity in hypoxic/reoxygenated old cardiomyocytes supplemented with sodium selenite or seleno methionine. Old cardiomyocytes were subjected to 4 h of hypoxia followed by 10 min of reoxygenation in the absence or presence of scalar concentrations of sodium selenite (**A**) or seleno methionine (**B**). Glutathione peroxidase activity was measured as reported under Methods. Data are means ± SD of five different cell cultures. Statistical differences were evaluated using the Student's t test to compare supplemented vs unsupplemented cells: §, $p < 0.05$; °, $p < 0.01$; *, $p < 0.001$ by the one-way analysis of variance to compare among different sodium selenite ($p < 0.001$) and seleno methionine concentrations ($p < 0.001$) and by two-way ANOVA to compare the effect of sodium selenite vs seleno methionine ($p < 0.001$).

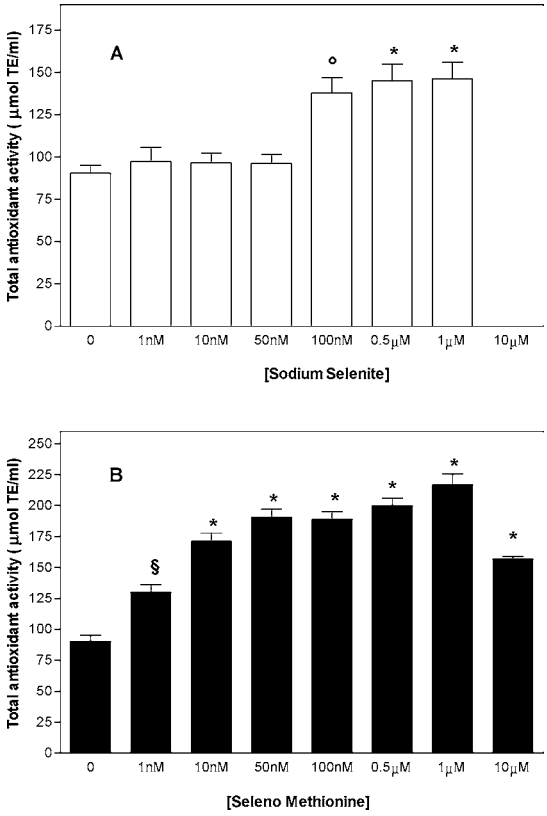

Figure 2. Total antioxidant activity in hypoxic/reoxygenated old cardiomyocytes supplemented with sodium selenite or seleno methionine. Old cardiomyocytes were subjected to 4 h of hypoxia followed by 10 min of reoxygenation in the absence or presence of scalar concentrations of sodium selenite (**A**) or seleno methionine (**B**). Total antioxidant activity was measured as reported under Methods and is expresodium seleniteed as μmol of trolox equiv/mL. Data are means ± SD of five different cell cultures. Statistical differences were evaluated using the Student's t test to compare supplemented vs unsupplemented cells: §, $p < 0.05$; °, $p < 0.01$; *, $p < 0.001$ by the one-way analysis of variance to compare among different sodium selenite ($p < 0.001$) and seleno methionine concentrations ($p < 0.001$) and by two-way ANOVA to compare the effect of sodium selenite vs seleno methionine ($p < 0.001$).

In hypoxic/reoxygenated cells, supplementation with SS caused a significant decrease in conjugated dienes only at the highest concentrations (0.1−1 μM) (**Figure 3A**), and the 1 μM concentration appeared the most effective in reducing lipid peroxidation. SM supplementation reduced conjugated diene production even at 50 nM concentration, and the 0.5 μM and 1 μM were the most effective in reducing lipid peroxidation (**Figure 3B**). Although in hypoxia/reoxygenation Se supplementation was not able to reduce conjugated dienes to levels comparable to normoxia, the Se organic form appeared the most effective.

DISCUSSION

Although it has been reported that senescent heart may undergo some ultrastructural and biochemical changes that favor a greater ROS production, therefore becoming more susceptible to ischemia/reperfusion injury (*17, 18*), it has also been reported that aging enhances antioxidant defense capacity in the myocardium, modulating the susceptibility of senescent heart to imposed oxidative stress (*7, 19*). Our data in cultured rat cardiomyocytes confirm this last observation; in fact, in normoxic condition, GPX activity increased in old cells probably in response to an enhancement in ROS production; as a consequence, total antioxidant activity increased, causing an invariance in conjugated diene levels. As previously observed in young cardiomyocytes (*8*), and also in old cells, hypoxia/reoxygenation did not modify GPX activity, while total antioxidant activity significantly decreased, and conjugated diene level significantly increased, but to a lesser extent. This increase in conjugated diene level may therefore be accounted to an increased ROS production during aging and not to a reduction of antioxidant defenses. Similar results were obtained by Leichtweis et al. (*7*) in ischemic/reperfused rat hearts, in which the higher malondialdheyde levels after ischemia/reperfusion observed in old animals were not coupled to lower GPx activity. It has been reported that, during aging, normoxic ROS production in the heart is increased (*20, 21*); the increased antioxidant defenses herein reported in normoxic old cardiomyocytes counteract ROS overproduction, preventing oxidative damage. When exogenous stimuli such as hypoxia/reoxygenation cause a further increase in ROS production, antioxidant defenses may be not sufficient to counterbalance it and to prevent lipid peroxidation. A plethora of studies (for a review, see ref *22*) have indicated that ischemia followed by reperfusion generates ROS, and chemiluminescence and line scan imaging in liver

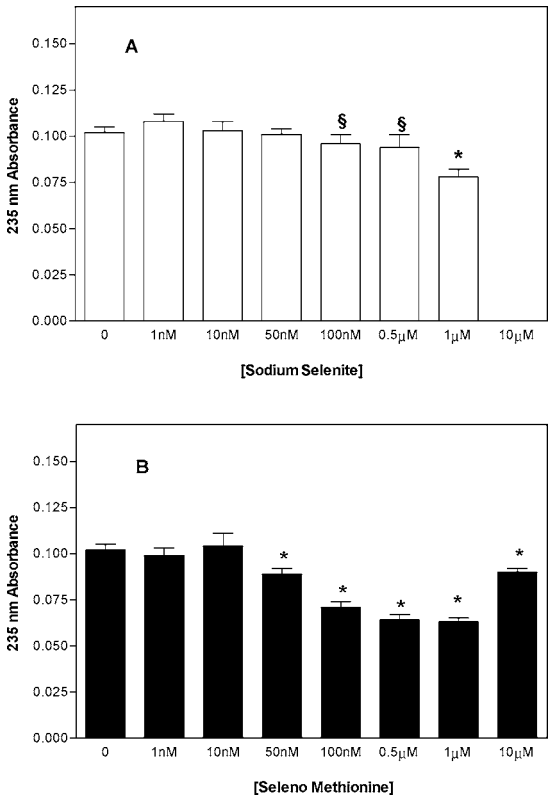

Figure 3. Conjugated diene levels in hypoxic/reoxygenated old cardiomyocytes supplemented with sodium selenite or seleno methionine. Old cardiomyocytes were subjected to 4 h of hypoxia followed by 10 min of reoxygenation in the absence or presence of scalar concentrations of sodium selenite (**A**) or seleno methionine (**B**). Conjugated diene production was measured as 235-nm absorbance as reported under Methods. Data are means ± SD of five different cell cultures. Statistical differences were evaluated using the Student's t test to compare supplemented vs unsupplemented cells: §, $p < 0.05$; *, $p < 0.001$ by the one-way analysis of variance to compare among different sodium selenite ($p < 0.001$) and seleno methionine concentrations ($p < 0.001$) and by two-way ANOVA to compare the effect of sodium selenite vs seleno methionine ($p < 0.001$).

have demonstrated that this ROS production is further increased by aging (*21*).

In light of the highest ROS production in aged cells, any intervention causing an increase in antioxidant defenses could be useful to reduce hypoxia/reoxygenation damage. A recent research has demonstrated that the effects of aging are exacerbated by poor nutritional habits (*3*); a healthy diet or a dietary supplementation with antioxidants could therefore ameliorate the resistance to ROS attack in aged cells.

In a previous work (*8*), we demonstrated that supplementation with selenium in both inorganic and organic forms is able to enhance GPx activity in cultured myocytes, thus increasing total antioxidant activity and reducing lipid peroxidation. In this work, we supplemented with the same concentrations of SS and SM cultured cardiomyocytes aged in vitro. In old cells, SM supplementation appeared much more efficient than SS in increasing GPx activity, being active even at the lowest concentration used (1 nM). The highest increase in GPx activity was detected at the 100 nM SM supplementation, while in young cells it was at the 1 μM one (*8*). Although we did not evaluate GPx expression, many studies indicate that supplementing Se could increase the GPx gene expression level (*23, 24*), and a Se concentration dependence on the expression of GPx was demonstrated in cultured cells (*23*). Seleno methionine supplementation increased GPx activity more than SS supplementation; this could be due to an effect of methionine per se on GPx activity. It has been demonstrated (*25*) that dietary supplementation with methionine in rats caused an 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. In both SS and SM supplemented old cardiomyocytes, a linear correlation was found between total antioxidant activity and GPx activity (SS supplemented cells: $r = 0.832$, $p < 0.05$; SM supplemented cells: $r = 0.859$, $p < 0.01$). Although the exact mechanisms and interactions among the different antioxidants are not fully understood, in old cardiomyocytes GPx appears to be one of the main contributors to the antioxidant defenses.

Conjugated diene production was significantly inversely correlated to both total antioxidant activity (SS: $r = -0.803$, $p < 0.05$; SM: $r = -0.773$, $p < 0.05$) and GPX activity (SS: $r = -0.971$, $p < 0.001$; SM: $r = -0.907$, $p < 0.01$). These data clearly demonstrate that the possibility to counteract lipid peroxidation strongly relies on total antioxidant activity, which in turn is strongly related to GPx activity. In cultured old cardiomyocytes, the supplementation with appropriate amounts of Se, in both inorganic and organic forms, is able to increase the enzyme activity.

It is estimated that by 2035, nearly one in four individuals will be 65 years of age or older. The definition of the specific characteristics of the cardiovascular aging process and of the targets for preventive or therapeutic interventions relevant to age-associated changes is very important. The old heart operates on the razor's edge, and old cardiomyocytes have a reduced tolerance to acute oxidative stress. ROS production in the heart has been reported to increase with age (*20*); membrane polyunsaturated fatty acids (PUFAs) undergo lipid peroxidation by ROS, and lipid peroxidation, per se, may be viewed as an "amplifier" for the initial ROS and, furthermore, the reactive aldehydes generated in this process may well act as "toxic second messengers" of the complex chain reaction that follow ROS production (*26*). Therefore, the intervention commonly applied in clinical practice directed to increase cardiomyocytes ω-3 PUFA content, major targets of lipid peroxidation, although having positive effects may, on the other site, exacerbate ROS deleterious effect on the aged heart if not counterbalanced with appropriate intervention to increase antioxidant defenses. Our data demonstrate that Se supplementation, particularly in its organic form, is able to increase GPx, and consequently total antioxidant activity, in old cardiomyocytes, which appear less prone to lipid peroxidation. Although clinic studies are needed, our data suggest the importance of an optimal Se dietary intake, particularly in the elderly.

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