RESEARCH ARTICLE

Dietary selenium intake influences Cx43 dephosphorylation, TNF-α expression and cardiac remodeling after reperfused infarction

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Scope: Post-infarct left ventricular dysfunction and cardiac remodeling are the primary causes of chronic heart failure in industrialized countries. In the present study, we examined the influence of dietary selenium intake on cardiac remodeling after reperfused myocardial infarction and explored one of the possible mechanisms.

Methods and results: Rats were fed a diet containing either 0.05 mg/kg (Low-Se, group of rats receiving the low-selenium diet) or 1.50 mg/kg (group of rats receiving the high-selenium diet) selenium. At the end of the 5th week of the diet, rats were subjected to transient (1 h) coronary ligation followed by 8 days of reperfusion. Infarct size and cardiac passive compliance were increased in the Low-Se group compared with group of rats receiving the high-selenium diet. Similarly, indices of cardiac remodeling (thinning index and expansion index) were more altered in Low-Se hearts. These adverse effects of the Low-Se diet on cardiac remodeling were accompanied by an increase in cardiac TNF-α content, a decreased activity of antioxidant seleno-enzymes and an increase in connexin-43 dephosphorylation.

Conclusion: Dietary selenium intake influences post-infarct cardiac remodeling even when provided within the range of physiological values. Our data suggest that the cardioprotective effect of selenium might be mediated by a reduced oxidative stress, a lower connexin-43 dephosphorylation, and a decreased TNF-α expression.

Keywords:
Cardiac remodeling / Connexin-43 / Reperfused myocardial infarction / Selenium / TNF-α

1 Introduction

Heart failure (HF) is a leading cause of morbidity and mortality in developed countries. Because of increased life-span of the population, its prevalence is growing, particularly among the elderly. HF may be due to any structural or functional disorder that affects the cardiac muscle, and consequently undermines the ability of the ventricles to fill up and pump blood adequately [1]. Common causes of HF include myocardial infarction (MI) and other forms of ischemic heart disease that remain the most frequent cause of HF, hypertension, dilated cardiomyopathy, obesity, and valvular heart disease. HF has its origin rooted in an adverse structural, biochemical, and molecular remodeling of the myocardium that affects cardiomyocytes, extracellular matrix, as well as coronary vasculature [2]. It has been recently suggested that a dyshomeostasis of macrominerals, such as calcium (Ca^{2+}) and magnesium (Mg^{2+}), micro-minerals, such as zinc (Zn^{2+}) and selenium (Se^{2+}), might contribute to such pathological remodeling, irrespective of the etiological origins of HF [2]. For the most part, these

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Abbreviations: CSA, cross-sectional area; **Cx43,** connexin-43; **DI,** dilation index; **GSH-Px,** glutathione peroxidase; **HF,** heart failure; **High-Se,** group of rats receiving the high-selenium diet; **Low-Se,** group of rats receiving the low-selenium diet; **LV,** left ventricle; **MI,** myocardial infarction; **ROS,** reactive oxygen species; **TBS-T,** Tris-buffered saline Tween-20; **TI,** thinning index; **TrxR,** thio-redoxin reductase

nutrients are from dietary origin and insufficient dietary intake or increased urinary and fecal wasting may rapidly lead to alterations in their homeostasis. The resulting nutrient imbalance is at the origin of a common pathophysiological response, based on the development of an oxidative stress with a production of reactive oxygen species (ROS) and nitrogen species that overwhelm endogenous antioxidant defenses [3]. This oxidative stress contributes to contractile failure, structural damage in cardiac cells, apoptosis, interstitial fibrosis, and the development of a proinflammatory phenotype [2, 3]. Thus, it is widely admitted that oxidative stress may play a major role in the pathophysiological events that are responsible for ventricular remodeling and progression toward HF [2, 3]. Besides, oxidative stress is also involved in various cardiovascular diseases, including atherosclerosis, hypertension, and in the aging process. Therefore, any strategy aimed at limiting oxidative stress might constitute a logical therapeutic approach to prevent adverse remodeling of the ventricular myocardium. During the last decade, several experimental studies have underlined the role of antioxidant enzymes in the cellular protection against oxidative stress. Among these enzymes, the two seleno-proteins glutathione peroxidase (GSH-Px) and thioredoxin reductase (TrxR) have received much attention [4]. Thus, in the Keshan province of The People Republic of China, where the soil is very poor in selenium, the consumption of vegetables locally grown is associated with the appearance of a dilated cardiomyopathy in children, known as Keshan's disease, which is often reversible after selenium supplementation [5]. Although there has been yet no conclusive evidence from epidemiological studies to support the role of selenium and selenoenzymes in patients with HF, several experimental studies on various experimental models of myocardial ischemia and infarction have suggested that a high-selenium diet can significantly reduce post-ischemia reperfusion injury [6–10]. In this study, we investigated the effect of two different levels of dietary selenium intake within the range of physiological values in an animal model of reperfused MI to elucidate whether selenium intake may play a role in post-infarction cardiac remodeling and the evolution toward HF. Moreover, we explored the possible mechanism by which high-selenium supply could exert its cardioprotective effects.

2 Materials and methods

2.1 Animals and diets

In rats, only very low selenium diets containing less than 0.04 mg Se/kg have been shown to lead to the development of selenium deficiencies, and administration of high-selenium diets up to 2.50 mg Se/kg did not exhibit any toxic effect [10]. On this basis, in the present study, male Wistar rats (188 ± 6 g body weight at the beginning of the experiment, Charles River, France) were randomly assigned to one of the two following experimental groups: a low-selenium group (Low-Se), receiving a standard diet containing 0.05 mg of selenium/kg of food for 5 wk, and a high-selenium group (High-Se), receiving the same diet containing 1.50 mg of selenium/kg of food for 5 wk. Selenium content in both diets was adjusted by sodium selenite (Sigma, Lyon, France) in addition to a low selenium-containing basic-diet (TD 92163, Harlan Teklad, France). Rats were housed under the conditions of constant temperature, humidity, and standard light/dark cycle (12/12 h). They had free access to deionized water and food. They received human care in compliance with the guidelines formulated by the European Community for use of experimental animals (L358–86/609/EEC). Diets were replaced every day, and body weights were determined weekly for the 5-wk duration of the experiment. All protocols involving living animals were performed under the license from the French authorities (license number A38018).

2.2 Reperfused MI

Rats were anaesthetized intraperitoneally with a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg). They were incubated and mechanically ventilated (tidal volume: 1 mL/100 g body weight; ventilation rate: 65 strokes/min) with a mixture of Isoflurane (0.5%; AErrane®, Lessins, Belgium) and oxygen (20%) in room air (79.5%). Experimental myocardial ischemia was performed as described previously [11]. A left thoracotomy was performed at the fourth intercostal space and the left coronary artery was ligated 1–2 mm from its origin. After 1 h of occlusion, the ligation was removed and the left coronary artery was reperfused. The chest cavity was compressed to remove any air before being hermetically sealed. Rats were then maintained under the same diets as prior to surgery for 8 days of reperfusion. The same procedure was followed for sham-operated control animals (Sham), but the coronary ligation was not tied.

2.3 *Ex-vivo* determination of left ventricular pressure–volume curves

Eight days after the surgical induction of infarction, rats were anaesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg) and a PE-30 catheter was inserted into the left ventricle (LV) *via* the right carotid artery for the determination of *in vivo* LV end-diastolic pressure. Passive pressure–volume characteristics of the LV were evaluated postmortem as described previously [12]. A saturated solution of potassium chloride was injected into the vena cava until the heart stopped. The heart was then excised and a cannula, connected to a pressure transducer (Statham P23XL), was inserted 5 mm into the LV through the aorta. The right and left atrioventricular junctions, the pulmonary artery, and

vena cava were ligated and physiological saline was infused in the LV at a constant flow rate of 0.68 mL/min, whereas intraventricular pressure was continuously monitored from 0 to 30 mmHg. The operating LV end-diastolic volume was determined from the LV pressure–volume curve and defined as the volume corresponding to a filling pressure equal to *in vivo* end-diastolic pressure.

2.4 Assessment of infarct size

Hearts were frozen in liquid nitrogen and cut at −20°C with a cryostat (Microm HM505E, Microm International GmbH, Walldorf, Germany). Three successive 20-μm thick transverse sections were obtained at 5.5 mm from the basis of the ventricles of each frozen heart. Frozen sections were stained using nitro blue tetrazolium (0.04% in 0.05 mol/L sodium succinate buffer, pH 7.6). Necrotic and non-necrotic tissues were distinguished by the absence or presence of staining respectively, and infarct size was calculated using ImageJ software [12].

2.5 Assessment of cardiac geometry

For each ventricular section, the cross-sectional area (CSA) of the LV cavity and the CSA of the entire LV (LV CSA) were measured using planimetry. For each heart, the ratio of LV cavity CSA to LV CSA corresponds to an index of LV cavity dilation index (DI). The thickness of the infarcted wall and septum was measured on cross-sections. The thinning index (TI) was defined as the ratio of the thickness of the infarcted wall to the thickness of the septum. The infarct expansion index was estimated from the ratio of DI to TI [11].

2.6 Plasma and cardiac samples

Eight days after the surgical induction of infarction, rats were anaesthetized with sodium pentobarbital (60 mg/kg), blood was collected on heparinized tubes, and immediately centrifuged at $2000 \times g$ for 15 min at room temperature. Hearts were removed, quickly frozen at liquid nitrogen temperature, and homogenized in a phosphate buffer containing protease and phosphatase inhibitors. Plasma samples and myocardial extracts were stored at −80°C until assay.

2.7 Plasma selenium content and cardiac seleno-enzyme activities

Plasma selenium content was evaluated by gas chromatography coupled to mass spectrometry as described previously [7].

The activities of the seleno-enzymes GSH-Px and TrxR were determined in supernatants from frozen cardiac homogenates centrifuged at $2000 \times g$ for 10 min (4°C). GSH-Px activity was assessed by the modified method of Flohe and Günzler [13] and TrxR activity was assessed on the supernatants by the modified method of Arner *et al.* [14]. Enzyme activities were expressed in international units *per* milligram protein (IU/mg prot.).

2.8 Assessment of total and dephosphorylated Connexin-43

Total and dephosphorylated connexin-43 (Cx43) were detected in myocardial samples by Western blot analysis according to the method previously described by Jeyaraman *et al.* [15]. Frozen myocardial samples (50 mg) were homogenized in a phosphate buffer containing protease and phosphatase inhibitors. After the determination of the total protein content in each sample (BCA-200 kit, Pierce), 20 μg of proteins were loaded onto 10% polyacrylamide gels and subjected to electrophoresis according to standard protocol. Proteins were thereafter electroblotted onto PVDF membranes for 1 h. Membranes were stained with Ponceau S red (Sigma-Aldrich, l'Isle d'Abeau Chesnes, France) as a confirmation of equal loading between lanes. The blots were then blocked by immersion in Tris-buffered saline Tween-20 (TBS-T) consisting of 20 mmol/L Tris-HCl, pH 7.4, 137 mmol/L NaCl, and 0.05% Tween-20, and containing 10% nonfat milk, for 1 h at room temperature. Subsequently, they were washed four times for 7 min with TBS-T containing 1% nonfat milk, and incubated with an anti-total Cx43 (35800, Zymed Laboratories, USA) or an anti-dephosphorylated Cx43 (CX1B1, Zymed Laboratories) mouse monoclonal antibody diluted to a concentration of 1/750 or 1/1500, respectively, in TBS-T containing 1% nonfat milk for 1 h, at room temperature. The blots were then washed four times for 7 min with TBS-T containing 0.5% nonfat milk, and incubated with a peroxidase-conjugated rat anti-mouse polyclonal antibody (Interchim, France) diluted to a concentration of 1/2000 in TBS-T containing 0.5% nonfat milk for 1 h, at room temperature. After four times of 7-min washes with TBS-T, the signal was visualized using chemiluminescence (West Pico ECL kit, Pierce, France). Semi-quantitative analysis of the signal was performed by exposure of the blots to films. The films were scanned and band intensities (Arbitrary Units) were measured with NIH AutoExtractor 1.51.

2.9 Estimation of myocardial TNF-α

TNF-α was assessed using ELISA kits (Rat-TNF-α DY510, R&D Systems, UK), and expressed as pg/mg protein.

2.10 Statistical analysis

Values are expressed as means ± SEM. One-way analysis of variance (ANOVA) was performed to determine the significant differences between groups. The significance of the difference between the means of the groups was tested with Fisher's a posteriori protected least significant difference test (PLSD). Western blot intensities were compared using the nonparametric test of Mann–Whitney. The p-value of 0.05 was considered as the threshold of statistical significance.

3 Results

3.1 Effect of selenium intake on selenium status and seleno-protein content

Plasma selenium content and cardiac GSH-Px activity were almost twice higher in the group of rats fed the high-selenium diet compared with rats fed the low-selenium diet (Table 1). Reperfused MI had no significant effect on plasma selenium and cardiac GSH-Px activity in both groups of rats. TrxR activity was also higher in rats fed the high-selenium diet compared with rats fed the low-selenium diet (Table 1). Moreover, reperfused MI by itself increased TrxR activity. This phenomenon, which was statistically significant in the group of rats fed the low-selenium diet (0.68 ± 0.07 versus 0.90 ± 0.04, $p < 0.05$), was considerably reduced in the group of High-Se rats.

3.2 Pressure–volume curves

LV pressure–volume curves measured ex vivo in potassium-arrested hearts reflect both the geometry and the mechanical properties of cardiac walls (Fig. 1A). After 8 days of reperfusion, a significant rightward shift of the curves for both groups of rats (high- and low-selenium diets) was found compared with the corresponding sham-operated animals, indicating an increase in LV compliance. This shift was more pronounced in the group of rats fed the low-selenium diet ($p < 0.05$). In addition, reperfused MI induced an increase in operating LV end-diastolic volume ($p < 0.05$) in rats fed a low-selenium diet, whereas this variable remained unaffected in rats fed the high-selenium diet (Fig. 1B).

Table 1. Effect of selenium intake on plasma selenium content and cardiac seleno-enzyme activities

	Plasma Se (microM)	GSH-Px activity (IU/mg prot.)	TrxR activity (IU/mg prot.)
Low-Se Sham	3.55 ± 0.31	264 ± 46	0.68 ± 0.07
Low-Se myocardial ischemia	3.63 ± 0.22	348 ± 35	0.90 ± 0.04†
High-Se Sham	6.23 ± 0.27*	617 ± 87*	1.77 ± 0.27*
High-Se myocardial ischemia	6.18 ± 0.29*	601 ± 66*	2.02 ± 0.32*

Mean ± SEM (n = 7–9/group). Groups are defined in the text. Enzyme activities were assessed on cardiac extracts. *$p < 0.05$ versus the corresponding Low-Se group. †$p < 0.05$ versus Low-Se Sham.

3.3 Cardiac necrosis and remodeling

As shown in Fig. 2A, infarct size was significantly ($p < 0.05$) higher in Low-Se (55.8 ± 4.14%) than in High-Se rats (35.3 ± 3.19%).

Reperfused infarction 8 days after surgery led to a significant decrease in the thickness of the LV-free wall in both Low-Se (1.51 ± 0.09 mm versus 0.46 ± 0.07 mm, $p < 0.01$) and High-Se (1.63 ± 0.06 mm versus 0.92 ± 0.10 mm, $p < 0.01$) rats. The high-selenium diet significantly limited the thinning of the LV-free wall, induced by reperfused infarction (Fig. 2B and C). DI, which reflects the adverse increase in ventricular volume after MI, was similar in sham and myocardial ischemia rats from both experimental groups (Low-Se: Sham = 0.83 ± 0.02; IM = 0.88 ± 0.02; High-Se: Sham = 0.84 ± 0.02; IM = 0.89 ± 0.02). Expansion index, which reflects the thinning of the infarcted wall and the dilation of the cavities, was significantly increased after myocardial ischemia in both Low-Se and High-Se rats (Fig. 2D). Nevertheless, this phenomenon was exacerbated by low-selenium diet ($p < 0.05$).

3.4 Cardiac TNF-α and Cx43

Cardiac TNF-α content was significantly increased 8 days after reperfused myocardial ischemia in rats fed a low-selenium diet ($p < 0.05$), whereas this cytokine was not modified in rats fed a high-selenium diet (Fig. 3).

Ischemia/reperfusion did not affect total Cx43 cardiac content measured 8 days after reperfused myocardia ischemia (Fig. 4A and B). Reperfused myocardial ischemia induced a marked dephosphorylation of Cx43 in the Low-Se group as attested by Western blot analysis (Fig. 4A and C). On the contrary, no measurable difference of dephosphorylated Cx43 was detected in hearts from the High-Se group (Fig. 4C).

Therefore, it can be concluded that the High-Se diet prevented Cx43 dephosphorylation after reperfused myocardial ischemia.

4 Discussion

The major finding in this study is that dietary selenium intake influences the final size of necrosis and the remodeling process of the heart evaluated 8 days after reperfused infarction in the rat.

The underlying mechanisms may be multiple. One possibility is that selenium could protect the heart by contributing to maintain an efficient antioxidant status. Another likely reason may be the prevention of Cx43

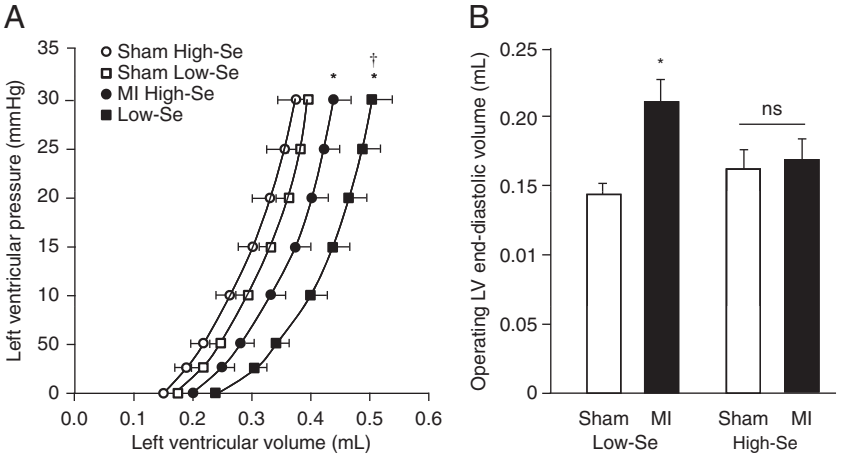

Figure 1. Effect of dietary selenium intake on passive cardiac compliance 8 days after reperfused myocardial ischemia. LV pressure–volume curves obtained ex vivo after potassium arrest during saline infusion over a pressure range of 0–30 mmHg (A). Operating LV end-diastolic volume (B). $n = 7$–11 *per* group. $^{*}p < 0.05$ versus the corresponding Sham group; $^{\dagger}p < 0.05$ *versus* myocardial ischemia High-Se.

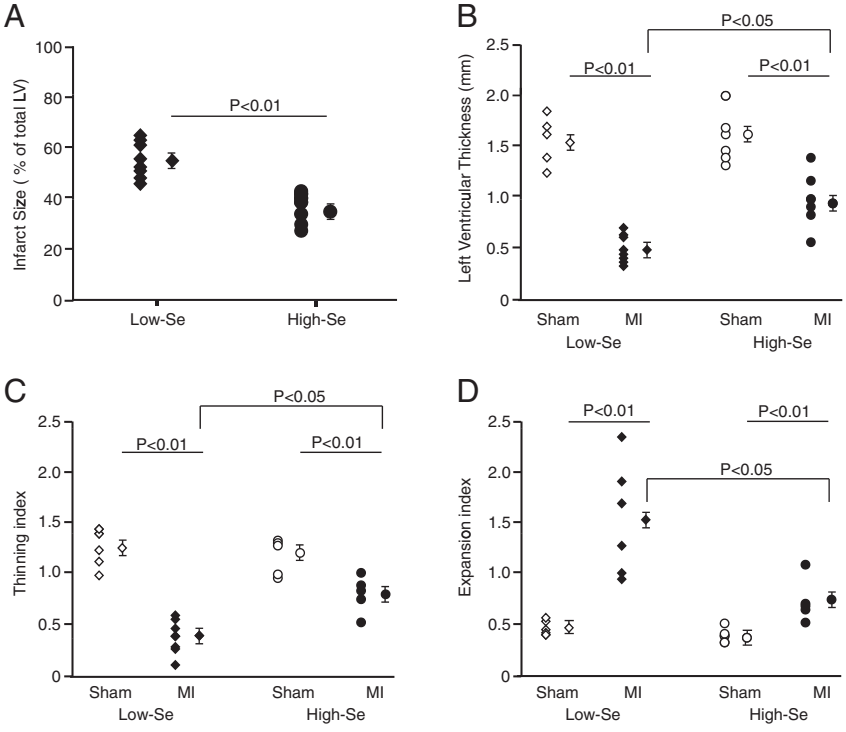

Figure 2. Effect of dietary selenium intake on cardiac morphometric variables 8 days after reperfused myocardial ischemia. Infarct size expressed as a % of total LV (A). Minimal thickness of infarcted LV (B). TI defined as the ratio of the thickness of the infarcted wall to the thickness of the septum wall (C). Expansion index estimated from the ratio of DI to TI (C). $n = 5$–8 *per* group.

dephosphorylation that could limit the spread of necrosis and reduce inflammation as supported by the reduced level of TNF-α in the group of High-Se rats.

4.1 Oxidative stress and cardiac pathology

It is now widely admitted that excessive production of ROS, such as superoxide anions ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), plays a major role in the pathogenesis of cardiovascular diseases, such as ischemic heart disease and HF. In myocardial ischemia, a burst of ROS production from endothelial cells and cardiomyocytes can exacerbate local inflammatory response and influence nearby neutrophils, leading to a chain reaction of ROS generation [16, 17]. When production of ROS overwhelms the capacity of endogenous antioxidant defences, oxidative stress develops, causing harmful effects to the myocardium, including contractile failure and ultrastructural alterations [18]. Several authors have postulated that oxidative stress would be one of the major actors of the pathophysiological mechanism of cardiac remodeling and progression toward HF [3]. Many experimental studies have reported that antioxidant therapy may be a useful approach in the field of cardioprotection and for reversing post-infarction remodeling [18]. In the 90 s, several compounds belonging to the class of synthetic nonenzymatic catalytic antioxidants have been shown to protect myocardial tissue against ischemia/reperfusion injury [19, 20] by reducing infarct size and

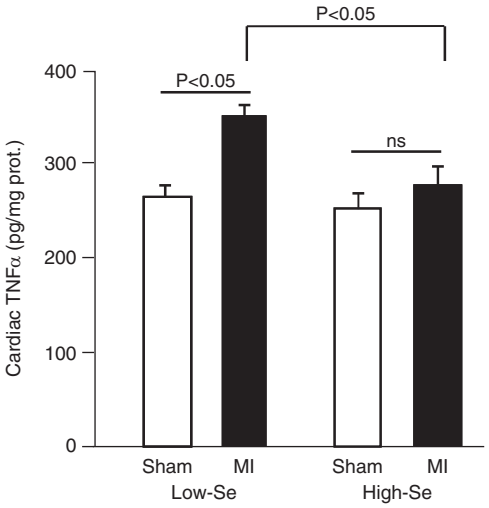

Figure 3. Effect of dietary selenium intake on cardiac TNF-α content measured 8 days after reperfused myocardial ischemia. $n = 8–12$ *per* group.

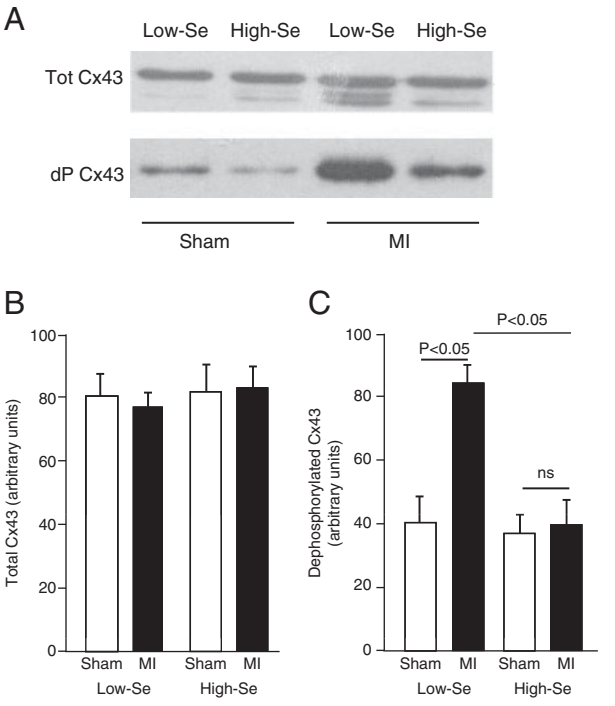

Figure 4. Effect of dietary selenium intake on cardiac Cx43 dephosphorylation, 8 days after reperfused myocardial ischemia. Western blot analysis of total Cx43 (Tot Cx43) and dephosphoylated Cx43 (dP Cx43) (A). Quantification of total Cx43 (B) and dephosphorylated Cx43 (C). $n = 8–12$ *per* group.

reperfusion arrhythmias. However, the beneficial effect of antioxidant therapy has been shown to be strongly influenced by the timing of administration and the dose of the antioxidant agent used [21]. Moreover, direct positive evidences of the efficiency of antioxidant treatments in reversing cardiac remodeling in patients are still lacking.

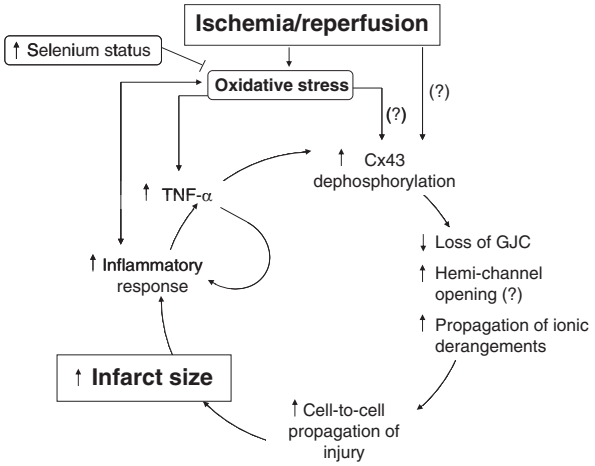

Figure 5. Proposed role for gap-junction channels (GJC) and Cx43 in infarct size limitation by high-dietary selenium intake.

4.2 GSH-Px, TrxR, and selenium

In recent years, selenium research has attracted tremendous interest because of its important role in antioxidant seleno-enzymes, such as GSH-Px and TrxR systems, for protection against oxidative stress [22]. In cardiac cells, mainly two isoforms of seleno-dependent GSH-Px (GSH-Px 1 and GSH-Px 4) are expressed. They catalyze the reduction of hydrogen peroxide and organic hydroperoxides and therefore prevent the formation of more toxic ROS. The thioredoxin system regulates the activity of various enzymes including those that function to counteract oxidative stress within the cell. TrxR expression can be induced by oxygen and oxidative stress in many cell types [23], nevertheless our results indicate that exogenous selenium supply can be rate limiting for the synthesis of TrxR after reperfused MI.

Our study shows that low-selenium intake results in a major decline in cardiac GSH-Px and TrxR activities, probably contributing to increased infarct size and adverse cardiac remodeling after reperfused ischemia. Consistently with these findings, a recent study has demonstrated that high-dietary selenium is clearly associated with lower cardiac oxidative stress and increased GSH-Px and TrxR expression, as well as reduced HF severity and mortality in ageing spontaneously hypertensive rats [24].

4.3 Cx43 and post-infarct remodeling

After myocardial ischemia, fibroblasts are responsible for orchestrating the complex remodeling process that conditions the mechanical quality of the scar.

Recent studies have reported the importance of heterocellular gap junction intercellular communication between cardiac myocytes and surrounding fibroblasts after MI [25, 26]. Connexin-43, the major ventricular gap-junction

protein, has been shown to be a major determinant of both myocardial infarct size [27] and cardiac remodeling [25] after experimental MI in mice. A study on Cx43-deficient mice has shown that reduced expression of Cx43 is associated with improved ventricular remodeling after MI [25]. Nevertheless, it is now well established that gap-junction functions are not only regulated through connexin expression, and various mechanisms including calcium concentration, pH, and transjunctional potential are involved [26]. In addition, changes in connexin phosphorylation also affect gap junction intercellular communication [25] and PKC-dependent phosphorylation of Cx43 on Ser368 or Ser262 inhibits gap-junctional communication [28, 29].

Previous studies from our group have established that dietary selenium intake prevents the dephosphorylation of Ser368-Cx43 induced by short-term regional ischemia, in isolated perfused rat hearts [9]. In addition, both selenium intake [8] and Cx43 expression [27] have been shown to be the determinants of myocardial infarct size *in vivo*. The results of the present study suggest, for the first time to our knowledge, that the cardioprotective effect of high-selenium intake on infarct size and post-infarct remodeling might be mediated, at least in part, by the prevention of Cx43 dephosphorylation.

The mechanism by which Cx43 inhibition could avoid adverse cardiac remodeling still remains hypothetical (Fig. 5). Nevertheless, it has been suggested that gap junctions could be implicated in cell death due to the ''bystander effect'' in which gap junctions spread a death signal between dying cells and those adjoining them [28]. Increasing Cx43 phosphorylation, or preventing its dephosphorylation, could therefore limit gap junction intercellular communication by controlling Cx43 trafficking and degradation or by closing the channels [28]. This might result in reducing the spread of cardiac injury during ischemia and, in turn, limit infarct size. In addition, several studies have shown that Cx43 downregulation or inhibition in the site of skin wounding decreases inflammation and improves tissue repair [30]. In our study, the prevention of Cx43 dephosphorylation by high-selenium status might have prevented adverse remodeling through its anti-inflammatory action, as attested by the reduction of TNF-α cardiac content.

In conclusion, the present study shows that high pre-ischemic selenium intake reduces myocardial infarct size *in vivo* in rats and reduces adverse remodeling. In addition, our data suggest that this beneficial effect might be related to (i) the preservation of cellular redox status, (ii) the prevention of Cx43 dephosphorylation, and (iii) the reduction of proinflammatory cytokine overexpression. Further studies are now required to verify the hypothesis that increased anti-oxidant status associated to high-selenium intake could prevent Cx43 dephosphorylation after MI, limiting both infarct size and proinflammatory cytokine overexpression, and therefore decreasing adverse cardiac remodeling.

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The authors have declared no conflict of interest.

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