Effects of Preconditioning with Ebselen on Glutathione Metabolism and Stress Protein Expression

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ABSTRACT
Selenium induces several proteins, including glutathione and stress proteins. These proteins have been shown to be cardioprotective against oxidative injury. To determine whether ebselen, a seleno-organic compound, can also induce these proteins and exert cardioprotective action, we examined the effects of preconditioning with ebselen on glutathione metabolism and stress protein expression and on myocyte injury induced by oxidative stress. Treatment of cultured cardiac myocytes with ebselen (0.3–30 μM) for 24 hr increased the reduced glutathione content. Glutathione reductase activity, but not glutathione peroxidase activity, was significantly elevated in a dose-dependent manner. Pretreatment with ebselen increased the expression of such stress proteins as heat shock protein 70 and heme oxygenase-1 (heat shock protein 32) in cardiac myocytes, as assessed by Western blotting. Expression of heat shock protein 70 was increased only at a higher dose of ebselen (30 μM), whereas expression of heme oxygenase-1 was markedly increased at a lower dose of ebselen (3 μM). Under these conditions, the myocyte injury induced by hydrogen peroxide or simulated ischemia/reperfusion, assessed by the release of lactate dehydrogenase into the culture medium, was reduced by ebselen pretreatment in a dose-dependent manner. Results indicated that cardiac myocytes pharmacologically preconditioned with ebselen for 24 hr exhibited resistance to oxidative injury, possibly via the up-regulation of glutathione metabolism and the expression of stress proteins.

Murry et al. (1986) first described “ischemic preconditioning,” in which sublethal repeated ischemia protects the heart against subsequent sustained ischemia and reperfusion injury. Recent reports have described “late preconditioning,” in which repeated brief episodes of ischemia exert cardioprotective effects not only immediately after, but also 24 hr after, induction of sublethal ischemia in a biphasic manner (Kuzuya et al., 1993; Marber et al., 1993; Sun et al., 1995). Late preconditioning can also be induced by treatment with heat stress (Currie et al., 1993; Hutter et al., 1994) and cytokines (Brown et al., 1990; Maulik et al., 1993). Late preconditioning has been shown to be related to the expression of stress proteins such as HSPs (Marber et al., 1993; Hutter et al., 1994) and antioxidant enzymes (Currie and Tanguay, 1991; Hoshida et al., 1993; Das et al., 1993). In coronary bypass surgery and unstable angina, the cardioprotective effect of late preconditioning would be more useful than ischemic preconditioning, because it could be induced by administration of pharmacological agents.

The present study focused on a unique agent, ebselen, and on how the late preconditioning effect protects cells against oxidative stress. Ebselen exhibits a potent antioxidant action and mimics the activity of GPX (Muller et al., 1984; Maiorino et al., 1992; Sies, 1993; Hoshida et al., 1994a). Our present objectives were 1) to examine the late effects of ebselen on glutathione metabolism and stress protein expression and 2) to evaluate the cardioprotective effect of ebselen pretreatment 24 hr before the induction of cell injury by oxidative stress in vitro by using rat cultured neonatal cardiac myocytes.

Materials and Methods
Isolation of myocardial cells. Cardiac myocytes were isolated from the rat neonatal heart as previously reported (Yamashita et al., 1994). In brief, hearts of newborn Wistar-Kyoto rats were immersed in PBS (pH 7.4, containing NaCl 137 mM, Na2HPO4 10.6 mM, KH2PO4 2.1 mM and K2HPO4 1.1 mM) and 2.5 mM CaCl2. Minced ventricles were placed in PBS containing 0.1% collagenase, and the dissociated cells were plated for 1 hr at 37°C, after which the unattached myocytes were collected. The myocytes were suspended in DMEM containing 25 mM glucose and 10% FBS and plated on

ABBRVIATIONS: DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; GPX, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; GR, glutathione reductase; HO, heme oxygenase; HSP, heat shock protein; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline.
culture dishes at a density of $3.1 \times 10^5$ cells/cm$^2$. Nonmyocyte contamination of primary cultures 48 hr after isolation consisted of approximately 10% of the total cell population.

**Cell sampling.** After 24 hr of culture in DMEM with FBS, the medium was replaced with serum-free DMEM, 10 mg/ml insulin and 10 mg/ml transferrin with or without ebselen (0, 0.3, 3.0 and 30 μM) dissolved in DMSO. Final concentration of DMSO was less than 0.001%. Incubation conditions were 37°C, with perfusion by a normoxic gas mixture (95% room air, 5% CO$_2$; pO$_2$ 143 mm Hg). After 24 hr in serum-free medium, cell samples for the measurement of glutathione content, glutathione-related enzyme activity, and the expression of stress proteins were collected as described below. To measure myocyte injury by H$_2$O$_2$, cultured myocytes pretreated with ebselen 24 hr were exposed to H$_2$O$_2$ (100 and 300 μM) for 1 hr, and LDH activity in the culture medium was measured by the standard method. To investigate further the cardioprotection to ischemic injury, myocytes pretreated with ebselen for 24 hr were transferred to an ischemic medium adapted from Esumi et al. (1991), 137 mM NaCl, 3.8 mM KCl, 0.49 mM MgCl$_2$, 0.9 mM CaCl$_2$, 2 mM HEPES (pH 7.4) that contained 150 mM NaCl, 0.05% (w/v) 4-chloro-1-naphtol (Stressgen, Vitoria, Canada), and in- ducible HSP70 (1991), 137 mM NaCl, 3.8 mM KCl, 0.49 mM MgCl$_2$, 0.9 mM CaCl$_2$, 2 mM HEPES) supplemented with 10 mM 2-deoxyglucose, 0.75 mM sodium dithionate, 12 mM KCl and 20 mM lactate, pH 6.5, and incubated for 24 hr at 37°C. Three hours after the medium was replaced with serum-free DMEM, LDH activity in the cultured medium was assayed. This ischemic medium is designed to simulate the extracellular milieu of myocardial ischemia. The extent of cell injury was expressed as a percentage of LDH activity of cultured medium over that of cardiac myocytes that were exposed to Tween-20 (0.2%) for 1 hr.

**Measurement of the glutathione redox state.** The content of GSH and of GSSG in cultured myocytes was determined as previously described (Hoshida et al., 1994a; 1994b). After the addition of 10 volumes of 2.5% 5-sulfosalicylic acid, cultured myocytes were centrifuged for 3 min and the supernatants were stored at −80°C. This enzymatic recycling assay, which uses 5,5′-dithiobis (2-nitrobenzoic acid) and GR to determine total GSH, is sensitive and specific. For the determination of GSSG, the supernatants were pretreated with 2-vinylpyridine before the addition of 5,5′-dithiobis(2-nitrobenzoic acid). The activities of GPX and GR were measured as previously described (Hoshida et al., 1993; 1994a). One unit of GPX or GR activity was defined as the amount of enzyme that catalyzed the reduction of 1 nM of NADPH per min.

**Western blot analysis.** Protein levels of stress proteins were evaluated by Western blotting as previously described (Hoshida et al., 1996c). Protein samples (20 μg/well) were resolved by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12.5% (HO-1) or 8.5% (HSP70) discontinuous gels. The separated polypeptides were electrophotorecursively transferred to a nitrocellulose membrane. After treatment with primary and secondary antibodies, the electroblot was incubated for 30 min at 37°C in 20 mM PBS (pH 7.4) that contained 150 mM NaCl, 0.05% (w/v) 4-chloro-1-naphthol and 0.015% (v/v) H$_2$O$_2$. HO-1 was detected with a polyclonal rabbit anti-rat HO-1 IgG antibody (Stressgen, Victoria, Canada), and inducible HSP70 was visualized with a monoclonal mouse anti-human HSP70 IgG antibody (C92) (Stressgen). The relative levels of Western blots were determined using densitometry. The anti-HSP70 antibody (C92) is specific to inducible HSP70 and does not cross-react with constitutive protein (HSC70).

**Materials.** The cell culture apparatus was purchased from Becton Dickinson (Mountain View, CA). The chemicals and reagents for cell culture were purchased from Gibco Laboratories (Gaithersburg, MD), Sigma Immunochimicals (St. Louis, MO) and Wako (Osaka, Japan).

**Statistical analysis.** Data are expressed as mean ± S.E.M. The measurement of glutathione concentration and glutathione-related enzyme activity was repeated using five batches of cell culture (n = 5). Myocyte injury was assessed using six batches of cell culture (n = 6). There was no significant difference between batches. Analysis of variance (ANOVA) with Scheffe’s test was used for statistical analysis. A level of P < .05 was accepted as statistically significant.

**Results**

**Ebselen treatment and glutathione metabolism.** Treatment with ebselen for 24 hr significantly increased the content of both GSH and GSSG in cardiac myocytes in a dose-dependent manner (fig. 1). The content of GSH in cardiac myocytes treated with 30 μM ebselen showed an approximate 3-fold increase compared with that in control cells. GSH content in cardiac myocytes treated with 30 μM ebselen was significantly increased even after 6 hr of incubation (data not shown). There was no significant difference in GSH content between cells treated with 0.3 μM ebselen and the control cells. The GSH/GSSG ratio in cardiac myocytes did not differ significantly among those treated with the different doses of ebselen.

To investigate further the effect of 24-hr ebselen treatment on glutathione metabolism, we examined glutathione-related enzyme activity. GPX activity in cells treated with 0.3 μM ebselen was slightly higher than that in control cells or cells treated with 3.0 or 30 μM ebselen, but the difference did not reach a significant level. In contrast, GR activity in cardiac myocytes treated with ebselen for 24 hr significantly exceeded that in control cells in a dose-dependent fashion (fig. 2). Treatment with 0.3 μM ebselen caused no change in GR activity.

**Ebselen-induced expression of stress proteins.** Expression of HSP70 in cardiac myocytes treated with 30 μM, but not 0.3–3.0 μM, ebselen for 24 hr increased significantly compared with that in control cells (fig. 3 A and B). However, ebselen induced HO-1 expression in a dose-dependent manner. A low level of HO-1 or HSP70 was detected in untreated cells (fig. 3 A and B).

**Ebselen pretreatment and cardiac myocyte injury.** The injury to cardiac myocytes that was induced by H$_2$O$_2$, as assessed by the release of LDH in the culture medium, was reduced in a dose-dependent manner by pretreatment with ebselen 24 hr before the addition of H$_2$O$_2$ (fig. 4). After exposure to simulated ischemic medium followed by control medium, the cytoprotective effect of cardiac myocytes pretreated with ebselen was also observed in a dose-dependent manner (fig. 5). Pretreatment with ebselen at 30 μM significantly protected the cardiac myocytes against the oxidative

[Image] Fig. 1. Content of GSH and GSSG in cultured cardiac myocytes treated with ebselen 24 hr earlier. Data are expressed as mean ± S.E.M. of five batches of cultured myocytes. *P < .05 vs. without ebselen, †P < .05 vs. ebselen 0, 0.3, 3.0 μM.
injury induced by H$_2$O$_2$ or simulated ischemic medium. Pretreatment with ebselen did not result in significant LDH release, which indicates that it did not produce significant cell damage (data not shown).

**Discussion**

**Ebselen-induced protein expression.** Selenium has been shown to increase intracellular GSH (Dalvi and Rob-
neonatal cardiac myocytes (Hoshida et al., 1996c). Reduction in intracellular GSH content can also enhance HO-1 synthesis (Kwok and Sutherland, 1989). However, the addition of GSH to cardiac myocytes did not prevent the increase in HO-1 expression induced by 30 μM ebselen in the present study (data not shown). Because HSP70 expression in cardiac myocytes treated with ebselen increased significantly only at 30 μM, the kinds of stress proteins expressed by ebselen pretreatment would differ with the dose of this agent used.

Ebselen-induced cytoprotection. We previously reported the acute effect of ebselen on myocardial ischemia-reperfusion injury in a canine model (Hoshida et al., 1994a). In the present study, pretreatment with ebselen effectively protected against H₂O₂- or simulated ischemic medium-induced injury of rat cardiac myocytes. Late preconditioning is believed to result from the induction of cardioprotective proteins such as stress proteins. Treatment of cardiac myocytes with ebselen significantly increased GSH content and GR activity, but not GPX activity. These alterations in glutathione metabolism by ebselen pretreatment may be related to the drug’s cardioprotective effect.

Induction of stress proteins, including HSP70 and HO-1, may be a possible additive mechanism for the cardioprotection provided by ebselen. HSP70 has been shown to be cardioprotective in the late preconditioning phenomenon induced by sublethal ischemia and heat stress (Marber et al., 1993; Hutter et al., 1994). HO-1 has been shown to be induced by oxidative stress (Keyse and Tyrrell, 1989) and is an HSP (Ewing and Maines, 1991). HO-1 degrades heme to biliverdin, which is converted to an antioxidant, bilirubin (Stevens and Small, 1976; Stocker et al., 1987). The potential antioxidant defense provided by the induction of HO-1 may add to the powerful defense provided by enhanced endogenous glutathione. HO-1 has been shown to be expressed in ischemic/reperfused myocardium (Maulik et al., 1996).

Significance of pharmacological preconditioning. Some kinds of preconditioning, such as hypercholesterolemia and atherosclerosis, which are common in humans, can exacerbate the severity of myocardial injury induced by ischemia in animal models (“pathological preconditioning”) (Hoshida et al., 1996a; 1996b). In contrast, brief episodes of repeated ischemia produce cardioprotection that is acquired soon after sublethal ischemia as well as in the later phase (24 hr) after it (Kuzuya et al., 1993; Marber et al., 1993; Sun et al., 1995). Beside ischemia, treatment with heat stress or cytokines has been shown to be effective in reducing myocardial injury resulting from prolonged myocardial ischemia and reperfusion 24 to 48 hr after the treatment (“late preconditioning”) (Brown et al., 1990; Maulik et al., 1993). The late preconditioning may be more effective in suppressing the propagation of myocardial necrosis in patients with unstable angina or coronary artery bypass surgery as compared with classical ischemic preconditioning. Treatment with agents such as ebselen may hold promise for the pharmacological preconditioning to render the myocardial tissue more resistant to ischemia and reperfusion.

Treatment of cardiac myocytes with ebselen for 24 hr reduced the cell injury induced by oxidative stress. Ebselen affected glutathione metabolism to increase intracellular GSH content and GR activity in a dose-dependent manner. The expression of stress proteins such as HSP70 and HO-1 was also increased. Ebselen shows promise as an agent to increase tolerance to ischemia and reperfusion injury. Further studies are needed to clarify the precise mechanisms by which ebselen protects cardiac myocytes against oxidative stress-induced damage in late preconditioning.

References


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