Original Article

Oral Pretreatment with Ebselen Enhances Heat Shock Protein 72 Expression and Reduces Myocardial Infarct Size

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Reactive oxygen species (ROS) enhance myocardial ischemia-reperfusion (I/R) injury. Ebselen, a seleno-organic glutathione peroxidase (GPx) mimetic, has a protective effect against tissue injury induced by ROS. However, the cardio-protective effect of orally administered ebselen has never been investigated in cardiac I/R injury. We investigated the effects and mechanisms of orally administered ebselen on experimental myocardial infarction. Isolated perfused rabbit hearts underwent 30 min of global ischemia and 60 min of reperfusion, with or without oral administration of ebselen 24 h before I/R, with or without enhanced oxidative stress by H_2O_2 infusion for the first 1 min of reperfusion. The recovery of left ventricular developed pressure (LVDP) was significantly improved, and the myocardial infarct size was significantly reduced by ebselen. The recovery of LVDP and the myocardial infarct size were markedly aggravated by H_2O_2 infusion. These enhancements by H_2O_2 were dose-dependently suppressed by ebselen, along with a reduction in myocardial 8-hydroxydeoxyguanosine levels, a marker for oxidative DNA damage. The myocardial reduced glutathione (GSH) level was preserved by ebselen. Ebselen markedly enhanced myocardial heat shock protein (HSP) 72 expression. The cardioprotective effect of ebselen-induced HSP72 was confirmed by MTT assay in isolated cardiomyocytes using KNK437, a novel HSP inhibitor. In conclusion, an oral administration of ebselen 24 h before I/R provided excellent cardioprotective effects, at least in part through HSP72 induction and GSH preservation. (*Hypertens Res* 2006; 29: 905–913)

***Key Words*: reactive oxygen species, antioxidant, reperfusion injury, 8-hydroxydeoxyguanosine, glutathione**

Introduction

Oxidative stress has been indicated to be the major mechanism of progression of cardiovascular disease, particularly under hypertensive conditions (*1–3*). Myocardial ischemia-reperfusion (I/R) injury is attributed primarily to the production of reactive oxygen species (ROS) (*4*). Percutaneous coronary intervention (PCI) has been widely applied in the treatment of coronary artery disease. For purposes of PCI, it would be desirable to have a clinically available compound that could provide long-lasting suppression of ROS production and attenuation of myocardial I/R injury for use as a pretreatment. More specifically, if such a compound were orally

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available, it would be easier to achieve scheduled PCIs at a day hospital.

Ebselen is a seleno-organic antioxidant with activities that mimic glutathione peroxidase (GPx) as well as direct radical scavenging activities (*5–8*). It has been suggested that ebselen prevents tissue injury caused by ROS (*9–12*), and is one of the promising drugs in cardiac ischemic events. Recently, a cerebroprotective effect of orally administered ebselen was reported in stroke-prone spontaneously hypertensive rats (*12*). However, the cardioprotective effect of ebselen administered orally as a pretreatment has never been verified in myocardial I/R injury. Interestingly, it has been demonstrated that ebselen has the ability to induce the production of cardioprotective heat shock protein (HSP) in cultured cardiomyocytes (*13*). However, this potential cardio-protective mechanism of ebselen has never been investigated *in vivo*.

The primary goal of this study was to clarify whether 1) a single oral application of ebselen provides cardioprotective effects in I/R, and if so, 2) the mechanism involves HSP induction. To accomplish these goals, we used a global ischemic model of isolated rabbit hearts to eliminate some effects of neuro-humoral factors which potentially modify the cardioprotective effects (*14*). Rabbit myocardium is known to lack xanthine oxidase (XO), which is largely responsible for the production of ROS such as H_2O_2 (*15*, *16*). In order to enhance oxidative stress and mimic an *in vivo* condition in humans, we applied H_2O_2 infusion during the first 1 min of reperfusion. We compared the effects of oral ebselen with or without enhanced oxidative stress by H_2O_2 infusion, and examined changes in glutathione (GSH) as well as expression of HSP.

Methods

Isolated Rabbit Heart Preparation

This study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and to the guidelines of the Committee on Laboratory Animals of the Asahikawa Medical College.

Japanese White male rabbits weighing 3.0–3.5 kg were anesthetized with intravenous sodium pentobarbital (40 mg/kg), and were mechanically ventilated with 100% oxygen *via* a positive-pressure respirator (Model SB-460-6; Shimano Co., Tokyo, Japan). The thorax was opened and the heart was quickly removed. Hearts were retrogradely perfused using a Langendorff preparation (*14*) with Krebs-Henseleit solution, gassed with 95% O_2 and 5% CO_2 and paced at 200 beats/min. Left ventricular (LV) pressure, LV systolic pressure (LVSP), LV end diastolic pressure (LVEDP) and LV developed pressure (LVDP: the difference of LVSP and LVEDP) were continuously monitored using an intraventricular latex balloon connected to a transducer (AP-601G; Nihon Kohden Co., Tokyo, Japan).

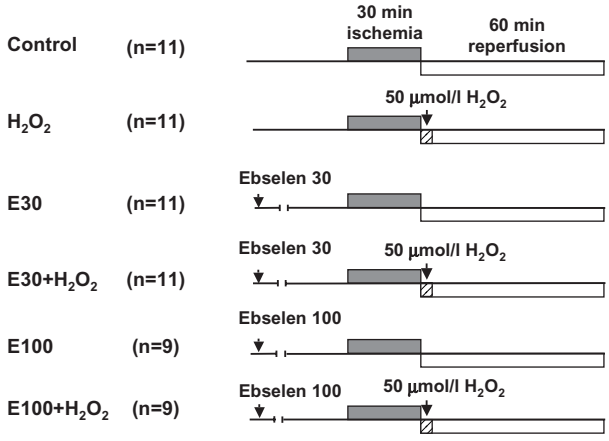

Fig. 1. *Experimental protocol. The control group (*n = *11) underwent 30 min of global ischemia (closed bar) and 60 min of reperfusion (open bar); the H_2O_2 group (*n = *11) underwent ischemia-reperfusion (I/R) with 50 μmol/l hydrogen peroxide infusion for the first 1 min of reperfusion (hatched bar). The ebselen groups received pretreatment with ebselen, 30 mg/kg (E30,* n = *11) or 100 mg/kg (E100,* n = *9) orally 24 h before the I/R protocol and H_2O_2 protocol (E30+H_2O_2,* n = *11; E100+H_2O_2,* n = *9).*

Experimental Protocol

The experimental protocol is summarized in Fig. 1. Rabbits were assigned to 6 groups: the control group (n=11) underwent 30 min global ischemia and 60 min reperfusion (30–60 min I/R); the H_2O_2 group (n=11) underwent 30–60 min I/R with H_2O_2 infusion (50 μmol/l, 15 ml/min) just after reperfusion for 1 min with a syringe pump (syringe infusion pump 22; Harvard Apparatus Inc., Millis, USA) in order to enhance oxidative stress during the initial phase of the reperfusion period. The dose of H_2O_2 was determined by preliminary experiments in which infusions of 10 to 100 μmol/l H_2O_2 were tested (*14*). We found that 50 μmol/l H_2O_2 infusion causes a modifiable enhancement of LV dysfunction, *i.e.*, approximately 40% reduction in LVDP after 60 min of reperfusion. In the ebselen groups, ebselen (gifted by Daiichi Pharma. Co., Tokyo, Japan) 30 mg/kg (E30, n=11) or 100 mg/kg (E100, n=9) was orally administered using a gastric tube 24 h before the 30–60 min I/R procedures. In the ebselen plus H_2O_2 groups, ebselen 30 mg/kg (E30+H_2O_2, n=11) or 100 mg/kg (E100+H_2O_2, n=9) was administered orally using a gastric tube 24 h before the 30–60 min I/R procedure with H_2O_2 infusion (50 μmol/l) for the first 1 min of reperfusion. Five hearts in each group were used for the myocardial infarct size measurement, and the rest were used for further assays. All assays except immunoblotting were performed using samples taken after the 30–60 I/R and H_2O_2 protocols, respectively. Sham group hearts (n=4) underwent neither treatment with the 30–60 min I/R nor treatment with

Table 1. Hemodynamic Parameters

	n	Baseline			Reperfusion 60 min			
		LVSP (mmHg)	LVEDP (mmHg)	LVDP (mmHg)	LVSP (mmHg)	LVEDP (mmHg)	LVDP (mmHg)	%DP (%)
Control	11	120±6	5±1	115±6	89±8*	6±2	82±7*	70±3
H_2O_2	11	118±4	6±1	112±4	91±5	42±7$^{\#}$	49±6*$^{,\#}$	44±6$^{\#}$
E30	11	107±1	5±1	102±4	93±5	3±1	90±3	89±3
E30+H_2O_2	11	100±4	4±1	99±10	70±5*	3±1	69±5*	66±6
E100	9	115±4	5±2	110±4	98±7	4±2	94±7	86±6
E100+H_2O_2	9	112±3	5±2	107±3	98±3	6±3	92±3	85±3

LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; LVDP, left ventricular developed pressure; %DP, % of baseline left ventricular developed pressure; E, ebselen. Values are mean±SEM. *$p<0.01$ *vs*. baseline, $^{\#}p<0.05$ *vs*. Control.

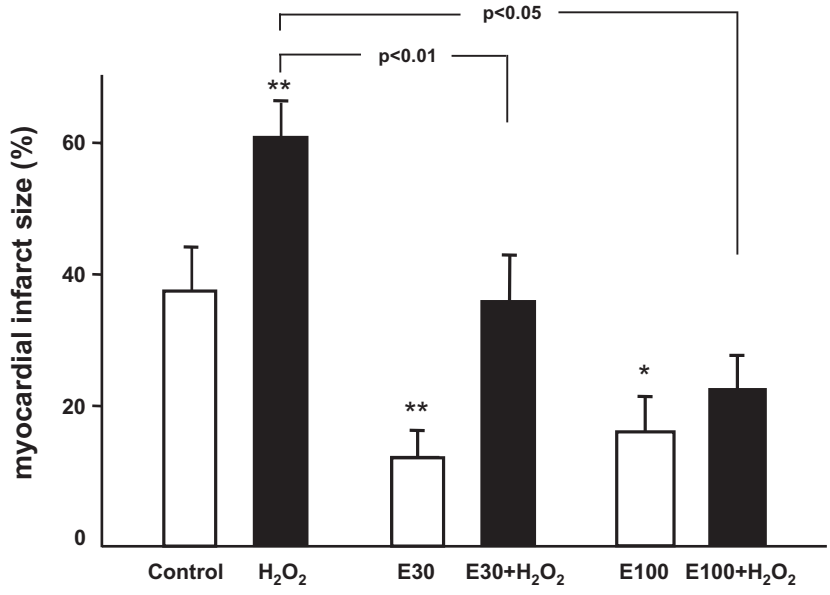

Fig. 2. *The myocardial infarct size was significantly increased in the H_2O_2 groups. Ebselen resulted in a significantly smaller myocardial infarct size compared to that in the Control group. The enhancement of infarct size with H_2O_2 was significantly attenuated in the E30 group, and it was totally abolished in the E100 group. The values shown are the mean±SEM;* n = *5 in each group.* *p < *0.05* vs. *the Control group;* **p < *0.01* vs. *the Control group.*

the 30–60 min I/R +H_2O_2 protocol.

Measurement of Myocardial Infarct Size

Excised hearts were frozen for 2 h at −20°C. The LV was sliced transversely into six to seven sections, approximately 3 mm in thickness. The slices were incubated for 15 min at 37°C in freshly made and pre-warmed solution with 1% triphenyl tetrazolium chloride (TTC) in phosphate buffer (pH 7.4) solution (*17*). Viable tissue was stained brick red and the infarct tissue was left unstained, allowing the differentiation of infracted myocardium (*14*, *18*). Photos were taken and the infarct myocardial area was traced and measured by planimetry with NIH image software (http://rsb.info.nih.gov/nih-image). The myocardial infarct size was calculated by multiplying by the slice thickness, and was expressed as a percentage of LV volume.

Isolation and Hydrolysis of Myocardial DNA and 8-Hydroxydeoxyguanosine Measurement

Measurement of 8-hydroxydeoxyguanosine (8-OHdG) was performed as described previously with minor modification (*19*). Briefly, myocardial samples (300 mg), excised and rapidly frozen in liquid nitrogen, were homogenized and digested. The pelleted DNA was extracted, and the concentration and purity of DNA were determined by UV absorbance. Acetate buffer (pH 4.8) containing 20 μg DNA and nuclease

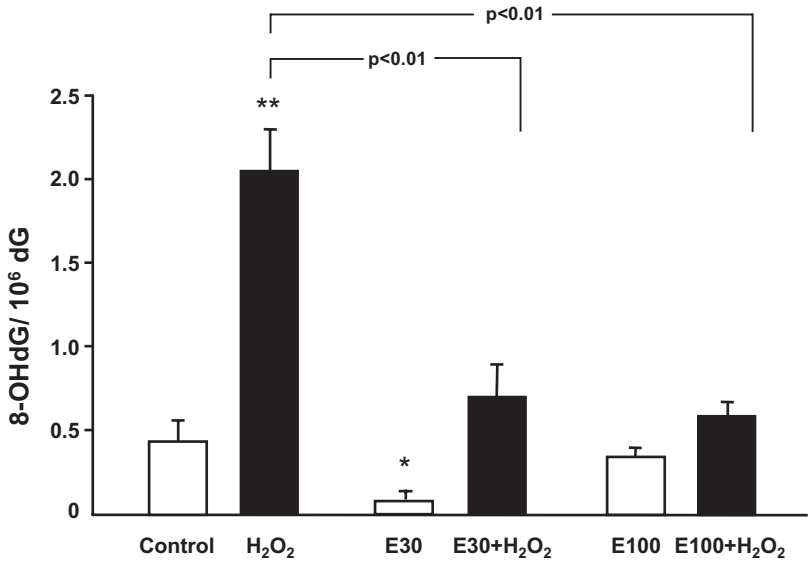

Fig. 3. *Myocardial 8-OHdG levels were measured in samples taken at 60 min after reperfusion. A significant increase in myocardial oxidative DNA damage of 8-OHdG levels in H_2O_2 was seen. These levels were significantly decreased in the combination with ebselen, compared to those in the Control group. The values shown are the mean±SEM;* n = *6 in each group.* *p < *0.05* vs. *the Control group;* **p < *0.01* vs. *the Control group.*

P1 was incubated, then 1 mol/l Tris-HCl (pH 7.4) and 0.65 units of alkaline phosphatase were added under an argon atmosphere. The mixture was filtered and applied to a high performance liquid chromatograph equipped with a Pegasil ODS column (4.6 × 150 mm; Senshu Scientific Co., Tokyo, Japan). Detection of 8-OHdG was performed by electrochemical detection using ECD-100 (Eicom Co., Kyoto, Japan). Oxidative damage of DNA is expressed as the molar ratio of 8-OHdG to 10^6 deoxyguanosine (dG). The amount of dG in each sample was measured with a UV detector at 260 nm (UVD-484MS; Waters Co., Milford, USA).

Measurement of GSH Content

The colorimetric method using a commercially available GSH-400 (Oxis Research, Portland, USA) kit was used for the determination of GSH content. Myocardial samples (200 mg), taken after the 30–60 I/R or H_2O_2 protocol and separated into epicardial and endocardial parts (*14*), were homogenized in 4 volumes of 5% metaphosphoric acid and centrifuged for 10 min at 3,500 rpm, and the supernatants were subjected to assay according to the manufacturer's protocol. The assay was performed in two steps, with the first step leading to the formation of substitution products (thioethers) with the sample mercaptans (GSH) and the second step transforming thioethers to thiones whose absorbance at 400 nm was measured.

Atomic Absorption Spectrometer Analysis

Myocardial samples were taken separately from the epicardial and endocardial parts (*14*). Myocardial homogenates in 50 mmol/l Tris-HCl (pH 7.5), 5 mmol/l EDTA, and 1 mmol/l DTT buffer were centrifuged at 10,000 × *g* for 10 min at 4°C, and supernatant samples of 50 µl were taken for selenium measurement after serial dilution by the conventional graphite-furnace atomic absorption spectrometer method according to the manufacturer's protocol (Zeeman-1400; Hitachi, Ltd., Tokyo, Japan).

Immunoblot Analysis for HSPs

Immunoblot analysis was performed as described previously (*20*). Briefly, myocardial tissue samples (150 mg) from ebselen-treated and sham hearts were taken before I/R and homogenized in 1 ml of 62.5 mmol/l Tris-HCl (pH 6.8) sample buffer containing 2% w/v SDS, 10% glycerol, and 50 mmol/l DTT. Equal amounts of protein (20 µg) were subjected to Tris-glycine-SDS-polyacrylamide gel electrophoresis (8%). After separation by electrophoresis, samples were transferred to nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK) and immunoblotting with anti–HSP 70 monoclonal antibody (Stressgen, San Diego, USA) was performed.

MTT Assay in Cardiomyocytes

Cardiomyocytes were isolated from 1–2 day-old Sprague-Dawley rat ventricles by an enzymatic method and cultured in 96-well plates with 1×10^5 cells (*21*). Twenty-four hours after isolation, the serum-containing medium was changed to Dul-

Table 2. Myocardial Antioxidant Parameters

	GSH content (μmol/l)		Selenium content (μg/dl)	
	Endocardium	Epicardium	Endocardium	Epicardium
Control	24.6±2.1	22.5±5.0	11.8±1.7	11.5±1.4
H_2O_2	7.5±0.7	10.6±1.5	6.7±1.4*	5.9±0.4*
E30	66.5±4.0*	65.5±8.4*	11.0±1.3	10.2±2.1
E30+H_2O_2	30.0±4.3$^{\#}$	33.9±5.8$^{\#}$	10.7±1.4§	10.7±2.1§
E100	61.0±4.5*	62.3±3.6*	13.5±1.7	12.4±1.0
E100+H_2O_2	52.2±5.9$^{\#}$	51.6±4.0$^{\#}$	11.8±1.3§	11.8±0.7§

GSH, glutathione; E, ebselen. Values are expressed as mean±SEM, $n=5$ each, *$p<0.01$ *vs*. Control, $^{\#}p<0.01$ *vs*. H_2O_2, $^{\S}p<0.05$ *vs*. H_2O_2.

becco's modified Eagle's medium/F-12 with a serum substitute (insulin, ascorbic acid). After 24 h of stimulation by ebselen with or without pretreatment with *N*-formyl-3,4-methylenedioxy-benzylidene-γ-butyrolactam (KNK 437; gifted by Kaneka Co., Osaka, Japan), a novel HSP inhibitor (*22*), for 6 h, 1 mmol/l H_2O_2 was added to each well and the plates were incubated for 30 min. Next, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT) 10 μl was added to each well and the plates were incubated for 4 h at 37°C. Finally, isopropranol 100 μl was added to dissolve the formazan and leave a homogenous blue solution suitable for absorbance measurement. Absorbance was read by spectrophotometry at 570/630 nm, and was directly proportional to the number of viable cells.

Statistical Analysis

All results are expressed as the mean±SEM. Differences in all parameters among the experimental groups were analyzed using analysis of variances (ANOVA), followed by post-hoc analysis Fischer test for multiple observations. Differences were considered significant when the *p* values were less than 0.05.

Results

Hemodynamic Parameters

The hemodynamic parameters of LVSP, LVEDP, LVDP and heart rate (HR) were monitored throughout the experiments. The recovery of LVDP at 60 min after reperfusion was significantly suppressed in the Control I/R group, and was markedly exacerbated in the H_2O_2 group in association with a significant elevation of LVEDP ($p<0.01$) (Table 1). The suppressed recovery of LVDP in the H_2O_2 group was partially improved in the E30+H_2O_2 group, and LVDP was almost totally recovered in the E100+H_2O_2 group (Table 1).

Myocardial Infarct Size

Ebselen significantly reduced the volume of myocardial infarct size by 68.3% ($p<0.01$) in the E30 group and by 58.5% ($p<0.05$) in the E100 group. There was a significant increase in myocardial infarct size in the H_2O_2 group of 59.5% ($p<0.01$). This H_2O_2-induced increase in myocardial infarct size was abolished by ebselen in a dose-dependent manner; in the E30+H_2O_2 group by 42.1% ($p<0.01$) and in the E100+H_2O_2 group by 63.6% ($p<0.05$) (Fig. 2).

Myocardial 8-OHdG Levels

Myocardial 8-OHdG levels were significantly increased in Control I/R compared to sham hearts ($0.15\pm0.05\times10^6$ dG, $n=4$) ($p<0.01$). The 8-OHdG levels in the E30 group were significantly decreased to 16.7% of the Control I/R levels, while only a slight and insignificant decrease in the 8-OHdG level was measured in the E100 group (to 64.7% of the Control I/R level). The levels of 8-OHdG increased in the H_2O_2 group by 3.63-fold compared to those in the Control I/R group ($p<0.01$). This increase in 8-OHdG levels in the H_2O_2 group was abolished in a dose-dependent manner, with the level being reduced by 65.7% of the H_2O_2 group in the E30+H_2O_2 group, and reduced by 71.1% of the H_2O_2 group in the E100+H_2O_2 group (Fig. 3).

Myocardial GSH and Selenium Contents

Myocardial GSH levels were significantly decreased in Control I/R compared to sham hearts (41.4±5.0 μmol/l in the endocardium, 41.3±6.6 μmol/l in the epicardium of sham hearts; $p<0.01$; $n=4$). Myocardial GSH levels were more markedly decreased in the H_2O_2 group than in the Control I/R group ($p<0.01$). In contrast, GSH levels were significantly increased in the ebselen-pretreated groups compared to the Control I/R group ($p<0.01$). In the E30+H_2O_2 and E100+H_2O_2 groups, there was a dose-dependent preservation of GSH content compared to the H_2O_2 group (Table 2). GSH contents in the Control I/R group were significantly lower than those in the E30 and E100 groups. Differences between the sham and ebselen-treated hearts prior to I/R were not significant.

Myocardial selenium levels did not change significantly in

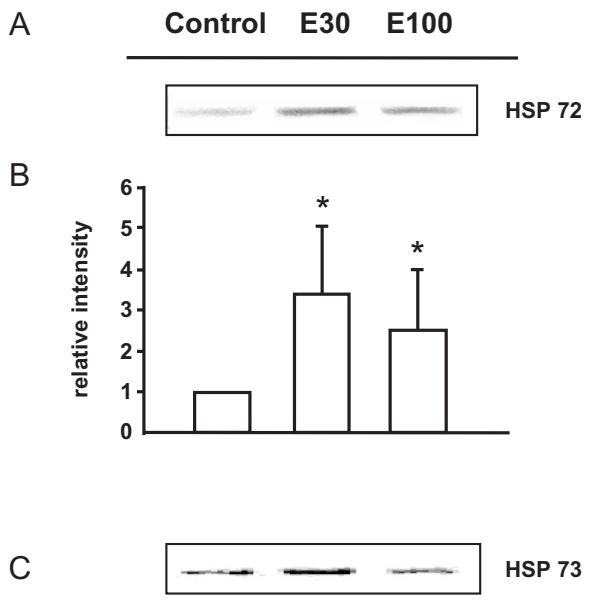

Fig. 4. *Expression of HSPs in rabbit hearts treated with ebselen. All myocardial tissue samples were obtained before the I/R protocol. The Control group consisted of sham rabbit hearts. With respect to the expression of HSPs, HSP72 was significantly upregulated by ebselen (A), particularly in the E30 group, but the level of HSP73 was not changed (C). B summarizes the results of the densitometric quantitative analysis of the autoradiograms (fold-increase* vs. *the Control group), representative of 4 experiments each. Values are expressed as the ratio of relative intensity compared to that of the Control group. Data are the mean±SEM.* *$p < 0.05$ vs. *the Control group.*

Control I/R compared to sham hearts (11.5±1.4 μg/dl in endocardium, 11.8±1.7 μg/dl in epicardium of sham hearts, $n=4$). However, myocardial selenium levels in the H_2O_2 group decreased significanty compared to those of the Control I/R group ($p<0.01$). Myocardial selenium levels were significantly decreased in the H_2O_2 group compared to the Control I/R group ($p<0.01$). The reduced selenium levels in the H_2O_2 group were restored in the E30+H_2O_2 and E100+H_2O_2 groups. There were no statistically significant differences observed between treatment with E30 and that with E100 either in the groups with or without H_2O_2 (Table 2).

HSP72 Expression

Ebselen pretreatment, particularly with E30, caused a marked induction of HSP72 production compared to that in sham rats, when measured in the hearts taken 24 h after ebselen pretreatment before I/R. Myocardial expression of HSP73 was not significantly different among the groups (Fig. 4).

MTT Assay with KNK437

To further confirm the cardioprotective effects of HSP72 induced by ebselen, we performed experiments on neonate rat cultured cardiomyocytes with KNK437, a novel HSP inhibitor (*22*). We observed a dose-dependent cytoprotective effect of ebselen against 1 mmol/l H_2O_2 cytotoxicity as assessed by colorimetric MTT assay for cell survival and proliferation (Fig. 5). Pretreatment with KNK437 6 h before ebselen administration abolished the cytoprotective effects of 30 μmol/l ebselen in a dose-dependent manner (Fig. 5).

Discussion

Ebselen showed excellent cardioprotective effects when administered orally only once 24 h before I/R. Ebselen also attenuated myocardial I/R injury induced by the additional oxidative stress of H_2O_2 infusion at reperfusion. The mechanism of the cardioprotective effects of ebselen was considered, at least in part, to involve HSP72 induction and GSH preservation.

The increase in HSP72 expression induced by oral ebselen pretreatment was associated with subsequent attenuation of myocardial infarct size. In cultured cardiomyocytes, it has been reported that ebselen induces stress proteins, including HSP70 and HO-1, and demonstrates cell-protective effects *in vitro* (*13*). We have reported that the induction of HSP72 confers cell-protective effects in vascular remodeling (*23*). A number of studies have shown that prior induction of HSPs provides protective effects in cardiac I/R (*24–28*). Myocardial injury has been reported to be reduced by a gene transfer of HSP72 in an *in vivo* I/R model (*29*). We here showed for the first time that oral pretreatment with ebselen enhanced HSP72 expression *in vivo* in isolated rat hearts followed by subsequent reduction of myocardial infarct size after I/R. To further confirm the cardioprotective effects of HSP72 induced by ebselen, we performed experiments on neonatal rat cultured cardiomyocytes using KNK437, a novel HSP inhibitor (*22*). This *in vitro* study confirmed that HSP72 played a crucial role in the cardioprotective mechanism of ebselen. Although the difference was not significant, the expression of HSP72 tended to be weaker in the E100 than in the E30 group. Similarly, the cardioprotective effects of ebselen were relatively smaller in the E100 than in the E30 group in terms of myocardial infarct size and myocardial 8-OHdG level. Several studies have demonstrated that a high dose of ebselen has an unfavorable and opposite effect compared to a low dose of ebselen (*7*). In general, high concentrations of antioxidants are not always beneficial, and are actually sometimes harmful, particularly under conditions that are not highly oxidative. In contrast, under highly oxidative conditions, such as the protocol of I/R with H_2O_2 infusion in the present study, the beneficial effects of ebselen appear to be simply dose-dependent.

Intracellular GSH scavenges ROS, which is an important

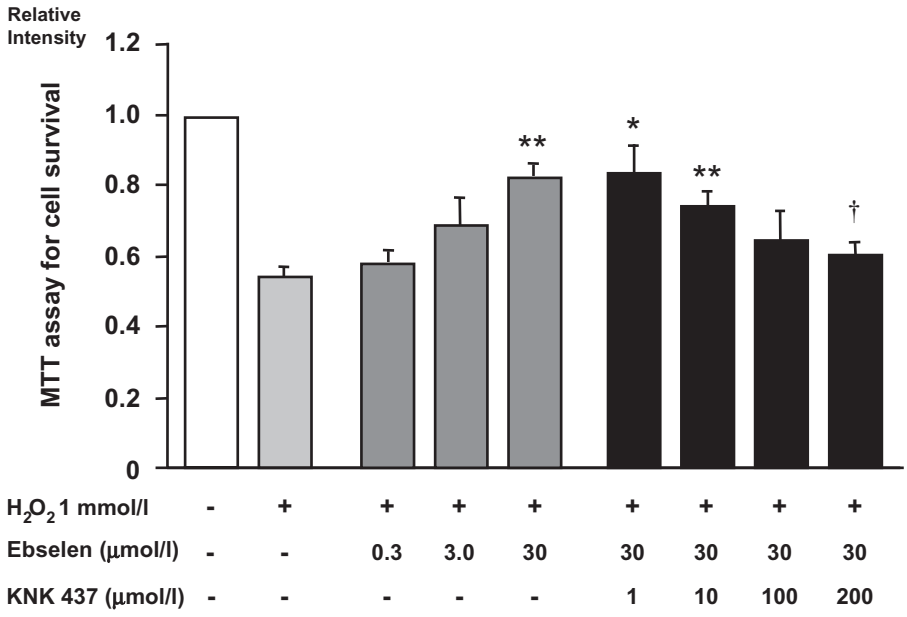

Fig. 5. *Dose-dependent myocardial protective effects of ebselen against 1 mmol/l H_2O_2 cytotoxicity in MTT assay. The pretreatment with KNK437, a novel HSP inhibitor, abolished the cytoprotective effects of 30 μmol/l ebselen in a dose-dependent manner.* *$p < 0.05$ vs. *the Control group;* **$p < 0.01$ vs. *the Control group;* †$p < 0.05$ vs. *the ebselen 30 μmol/l group.*

cell-protective mechanism under the conditions with enhanced oxidative stress (*30–34*). In the present study, the effects of ebselen on the tissue contents of GSH were expressed in the endocardial as well as the epicardial side, transmurally (Table 2). A significant increase in myocardial GSH contents may explain one of the major mechanisms of cardioprotection by ebselen. Activity of glutathione reductase (GR) has been found to be increased by ebselen (*35*), which is a potential mechanism of GSH preservation by ebselen. More importantly, it has been reported that GSH synthesis is impaired (*30*, *31*, *33*, *34*), and the activity of γ-glutamylcysteine synthetase (GGCS), a rate-limiting enzyme in GSH biosynthesis, is decreased in I/R (*32*). The gene encoding GGCS contains an AP-1 site (*36*, *37*), a key region for activating transcription, which is known to be activated by extracellular-signal–regulated kinase (ERK) (*38*). We observed that ebselen activated ERK1/2 and enhanced GGCS protein expression in neonatal cultured rat cardiomyocytes in another series of *in vitro* experiments (data not shown). The improved GSH synthesis could be considered as one of the potential mechanisms of GSH preservation by ebselen in our model of I/R.

Ebselen is a synthetic selenium compound which exhibits a GPx-like activity in the presence of GSH, and decomposes H_2O_2 in a catalytic manner (*39*). Myocardial selenium levels were reduced in H_2O_2-treated groups. The bioavailability of selenium of ebselen has been regarded as low (*7*). Thus, although it may not be the major mechanism of the cardioprotective effects of ebselen, the myocardial content of selenium was preserved in ebselen-treated groups in the present study.

The recovery of LV-developed pressure was facilitated in the ebselen-treated groups, suggesting that ebselen attenuates post-ischemic cardiac dysfunction. It is known that ebselen directly scavenges free radicals (*6*), the major mediators of post-ischemic cardiac dysfunction. Another causative mechanism of post-ischemic cardiac dysfunction is calcium overload (*40–42*). It has been reported that ebselen decreases inositol 1,4,5-triphosphate–induced calcium release from the intracellular calcium pool (*43*, *44*). Thus, ebselen could potentially preserve the cardiac function against calcium overload in I/R. The attenuation of post-ischemic cardiac dysfunction observed in the ebselen-treated groups in the present study may thus have been attributable to suppression of calcium overload as well as to reduction of ROS-induced myocardial damage by ebselen.

Pharmacokinetic studies have demonstrated that ebselen is eliminated within 24 h by glucuronidation, methylation or both, and subsequently *via* bile or renal excretion (*7*). However, there have been no reports on the time course of the tissue distribution of orally administered ebselen. We speculate that orally administered ebselen may have both genomic and non-genomic effects. The former would include the expression of genes such as HSP72, and the latter would include a direct scavenging effect as a seleno-organic compound.

Although the cardioprotective effects of ebselen-induced HSP72 were confirmed by *in vitro* experiments in the present study, the association of HSP72 expression with myocardial infarct size was demonstrated indirectly in an *in vivo* experiment. Further investigations will be needed to confirm this point and the mechanistic associations of HSP72 with regula-

tors of its chaperone activities (*45*).

In conclusion, ebselen administered 24 h before I/R provided excellent cardioprotection with improvement of cardiac functional recovery and reduction of myocardial infarct size. The cardioprotective effect of ebselen is attributable, at least in part, to GSH preservation and induction of HSP72.

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