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Additive pro-oxidative effects of methylmercury and ebselen in liver from suckling rat pups

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Abstract

Oxidative stress has been pointed as an important molecular mechanism for liver injury in methylmercury (MeHg) poisoning. Ebselen, a seleno-organic compound that possesses anti-oxidant properties, is a useful therapeutic agent used in clinical situations involving oxidative stress. Here, we examined the possible in vivo protective effect of ebselen against the pro-oxidative effects of MeHg in liver from suckling rat pups. The effects of MeHg exposure (subcutaneous injections of methylmercury chloride: 2 mg/kg) on the hepatic levels of thiobarbituric acid reactive substances (TBARS) and non-ptotein thiols (NPSH), and on liver glutathione peroxidase (GSHPx) activity, as well as the possible antagonist effect of ebselen (10 mg/kg; subcutaneously) against MeHg effects, were evaluated during the post-natal period. In addition, the possible in vitro interaction between ebselen, glutathione (GSH) and MeHg was investigated by light/UV spectroscopy, with particular attention to the formation of complexes involving ebselen selenol intermediate and MeHg. After in vivo exposure, MeHg and ebselen alone increased hepatic TBARS levels. Moreover, simultaneous treatment with both compounds caused a higher increase in hepatic TBARS levels when compared to the treatments with individual compounds. Liver NPSH decreased after treatments with MeHg and ebselen alone. A significant negative correlation between hepatic TBARS and NPSH was observed. MeHg alone decreased liver GSHPx activity and ebselen, which did not affect this variable per se, reverted this inhibitory effect of MeHg. Light/UV spectroscopy showed that ebselen and GSH form a chemical intermediate that regenerates ebselen after MeHg addition. The presented results show that ebselen abolished the MeHg-induced inhibition on liver GSHPx activity, but did not prevent the oxidative effects of MeHg on liver lipids and NPSH. MeHg affects the in vitro interaction between ebselen and GSH and this phenomenon seems to be responsible for its inhibitory effect toward thiol-peroxidase activity. Additionally, ebselen presents pro-oxidative effects on rat liver, pointing to thiol depletion as a molecular mechanism related to ebselen-induced hepatotoxicity.

Keywords: Methylmercury; Ebselen; Liver; Rat; Lipid peroxidation; Thiol groups; Glutathione peroxidase

1. Introduction

Methylmercury (MeHg) has been shown by many investigators to have toxic effects on animals, as well as on humans, and is believed to cause toxicity by

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multiple mechanisms (for review, see Clarkson, 1997). One of the mechanisms proposed to explain its toxicity is oxidative stress due, at least in part, to its high affinity for endogenous thiols, resulting in the depletion of glutathione (GSH), an important intracellular antioxidant (Yonaha et al., 1983; Sarafian and Verity, 1991).

Of particular importance, is the fact that liver seems to be an important target-organ for MeHg toxicity (Diaz et al., 2001; Bragadin et al., 2002). In fact, MeHg decreases GSH content and glutathione peroxidase (GSHPx) activity in hepatic tissue, leading to increased lipid peroxidation and cell death (Ashour et al., 1993; Lin et al., 1996). In addition, MeHg induces the opening of the permeability transition pore in rat liver mitochondria and this phenomenon represents an important molecular mechanism responsible for apoptosis (Bragadin et al., 2002).

There is a myriad of studies on the neurotoxic effects of MeHg in animals during the early brain post-natal period (Nakai and Satoh, 2002; Farina et al., 2003a; Dare et al., 2003; Goulet et al., 2003). The main toxicological significance of such studies is related to the fact that the exposure of pregnant women to MeHg can indirectly harm their children (Harada, 1995; Weihe et al., 2002) and that the toxic effects of MeHg exposure are higher in developing than in mature organisms (Kostial, 1983; Sakamoto et al., 1993). However, the hepatotoxic effects of MeHg during the early post-natal period have been poorly reported.

Ebselen, 2-phenyl-1,2-benzisoselenazol-3(2*H*)-one, is a seleno-organic compound that mimics the GSH-dependent, hydroperoxide reducing activity of GSHPx (Müller et al., 1984; Klotz and Sies, 2003). Ebselen protects rat liver against ischemia-reperfusion, and alcohol-induced injuries (Ozaki et al., 1997; Kono et al., 2001). However, studies on the effects of ebselen against MeHg-induced toxicity are reported only for brain tissue (Farina et al., 2003a,b).

Taking into account the fact that MeHg, an important environmental contaminant, causes liver damage due to its oxidative properties and ebselen is an anti-oxidant therapeutic agent that protects liver from oxidative stress, the aim of the present study was to evaluate the effects of individual and simultaneous exposures to MeHg and/or ebselen on TBARS, non-ptotein thiols (NPSH) and GSHPx activity in the liver of suckling rat pups. The possible in vitro interaction between ebselen, GSH and MeHg was also investigated with particular attention to the formation of complexes involving ebselen selenol intermediate and MeHg in attempt to explain some of the in vivo biochemical findings after exposure to ebselen and MeHg.

2. Materials and methods

2.1. Chemicals

Methylmercury(II) chloride was obtained from Sigma (St Louis, MO, USA). Ebselen (2-Phenyl-1,2-benzisoselenazol-3[2H]-one) was synthesized based on Engman and Hallberg (1989). All other chemicals were of analytical reagent grade and purchased from Merck (Darmstadt, Germany).

2.2. Animals

Wistar rats obtained from our own breeding colony were maintained at approximately 25 °C, on a 12:12 h light/dark cycle, with free access to food and water. The breeding regimen consisted of grouping three virgin females (90–120 days) with one male for 20 days. Pregnant rats were selected and housed individually in opaque plastic cages. All experiments were conducted in accordance with the guiding principles in the use of animals in toxicology, adopted by the Society of Toxicology in July 1989.

2.3. Treatments

Forty pups, came from five different litter (eight animals per dam), were divided in four experimental groups of 10 animals each; control (group A), MeHg (group B), ebselen (group C), and MeHg plus ebselen (group D). Subgroups of two animals per litter received one of the four differently used treatments (A–D) in order to avoid the possible effects of the different gestations. From post-natal day (PND)-3, pups were treated daily, for 21 days with subcutaneous injections of MeHg and/or ebselen. Ebselen was dissolved in dimethyl sulfoxide (DMSO) to allow for subcutaneous administrations, 1 ml/kg and its dose (10 mg/kg) was based on Farina et al. (2003a). MeHg was dissolved in a $NaHCO_3$ solution (25 mM) to allow for subcutaneous administrations, 1 ml/kg and its

dose (2 mg/kg) was based on Miyamoto et al. (2001). Control rats (group A) received a daily injection of a $NaHCO_3$ solution, 25 mM (1 ml/kg) plus a daily injection of DMSO (1 ml/kg). Group B (MeHg treatment) received a daily injection of MeHg (2 mg/kg) plus a daily injection of DMSO (1 ml/kg). Group C (ebselen treatment) received a daily injection of ebselen (10 mg/kg) plus a daily injection of a $NaHCO_3$ solution, 25 mM (1 m;/kg). Rats treated with MeHg plus ebselen (group D) received a daily injection of MeHg (2 mg/kg) plus a daily injection of ebselen (10 mg/kg). The MeHg and ebselen injections were administered simultaneously, but at different sites to avoid a possible direct chemical interaction.

2.4. Tissue preparation

After the treatment period (21 days), pups were killed by decapitation. Livers were quickly removed, placed on ice and homogenized in 10 volumes of 150 mM NaCl and stored at −70 °C for measurement of TBARS, NPSH, and GSHPx activity.

2.5. Determination of TBARS levels

TBARS were determined in tissue homogenates by the method of Ohkawa et al. (1979), in which malondialdehide (MDA), an end-product of fatty acid peroxidation, reacts with thiobarbituric acid (TBA) to form a colored complex. MDA values were determined with the absorbance coefficient of the MDA–TBA complex at 532 nm = 1.56×10^5 cm/mmol.

2.6. Determination of NPSH levels

To determine NPSH, 500 μl of 10% trichloroacetic acid were added to 500 μl of liver homogenate. After centrifugation (4000 × *g* at 4 °C for 10 min), the protein pellet was discarded and free sulfhydryl groups (–SH) were determined in the clear supernatant (which was previously neutralized with 0.1 M NaOH) by the method of Ellman (1959).

2.7. GSHPx assay

Liver homogenates were centrifuged for 10 min at 15 800 × *g* in an Eppendorf Model 5417 R centrifuge at 4 °C and the supernatant fraction was used in GSHPx assay. GSHPx activity was measured by the method of Pagalia and Valentine (1967). Liver homogenate supernatant (200–400 μg protein) was added to the assay mixture (total volume = 1 ml) and the reaction started by the addition of 0.1 ml of 4 mM H_2O_2 to give a final concentration of 0.4 mM. Conversion of NADPH to $NADP^+$ was monitored continuously at 340 nm for 10 min. GSHPx activity was expressed as nmol of NADPH oxidized to $NADP^+$ per minute per mg protein of liver homogenate supernatant, using an extinction coefficient 6.22×10^6 for NADPH.

2.8. Protein measurement

The protein content of liver homogenate and supernatant was determined by the method of Lowry et al. (1951) using bovine albumin as standard.

2.9. Spectroscopy studies

In order to demonstrate the chemical interaction between ebselen and GSH, ebselen (50 μM) was incubated with GSH (1 mM) in 50 mM phosphate buffer (pH 7.4). The reaction was performed at room temperature (25 °C) in a quartz cuvette and monitored by spectrophotometry (250–400 nm) using a Beckman DU-640 spectrophotometer. In additional experiments, MeHg (1 mM) was added before or after the reaction of ebselen with GSH. The reaction mixture had 1 ml. In parallel, sulfhydryl groups were measured in all tested conditions by the method of Ellman (1959).

2.10. Statistical analysis

Differences between the groups were analyzed by one-way ANOVA, followed by Duncan's multiple range test when ever considered appropriate. In addition, two-tailed Pearson's correlation was performed to identify a possible correlation between TBARS and NPSH.

3. Results

At the end of the treatment, body weight was significantly lower in MeHg-exposed pups when compared to the control group by one-way ANOVA, followed by the Duncan multiple range test, at $P < 0.05$, and

Table 1
Effect of methylmercury (MeHg) and ebselen exposure on body weight and liver-wet weight of 24 day-suckling rat pups

Groups	Control	MeHg	Ebselen	MeHg + Ebselen
Body weight (g)	39.9 ± 1.3 a	30.1 ± 2.9 b	36.0 ± 1.9 a,b	34.1 ± 2.3 a,b
Liver weight (g)	4.3 ± 0.1 a	4.2 ± 0.2 a	4.5 ± 0.1 a	4.6 ± 0.2 a
Ratio liver/body weight	0.107 ± 0.01 a	0.139 ± 0.02 b	0.125 ± 0.01 a,b	0.134 ± 0.13 a,b

From the PND-3, rats were daily injected with MeHg (2 mg/kg body weight; subcutaneously) and/or with ebselen (10 mg/kg body weight; subcutaneously) for 21 days. Data are expressed as mean ± S.E. from 10 animals per group. Values not sharing the same letter are statistically different; $P < 0.05$, by one-way ANOVA, followed by the Duncan multiple range test when F was significant.

ebselen, which did not affect this variable per se, abolished the reduction in body caused by MeHg. Liver-wet weight was not different between groups; however, the ratio of liver weight/body weight was higher in animals treated with MeHg alone when compared to the control group (Table 1).

The hepatic levels of TBARS and NPSH are depicted in Fig. 1A and B, respectively. Both individual MeHg and ebselen treatments increased liver TBARS levels, and an additive increase was observed in this variable when both compounds were administered simultaneously (Fig. 1A). Individual MeHg and ebselen treatments decreased liver NPSH levels and a similar decrease was observed when both compounds were administered simultaneously (Fig. 1B). A negative correlation between NPSH and TBARS was significant at the 0.01 level (two-tailed Pearson's correlation) when all groups were analyzed together (correlation coefficient $= -0.580$; $P < 0.01$). However, when the groups were analyzed individually, the correlation was significant only for pups treated with ebselen alone (correlation coefficient $= -0.672$; $P < 0.05$).

Liver GSHPx activity is represented in Fig. 2. MeHg alone decreased liver GSHPx activity and ebselen, which did not affect this variable per se, abolished the inhibitory effect of MeHg.

In order to find the possible molecular mechanism related to the protective role of ebselen against MeHg-induced inhibition of GSHPx, in vitro experiments of light/UV spectroscopy concerning the chemical interaction between ebselen, GSH and MeHg were carried out. The unique spectral characteristics of ebselen (50 μM, Fig. 3, spectrogram A) was changed after the reaction with the excess of GSH (1 mM), probably due to the formation of ebselen selenol intermediate (Fig. 3, spectrogram B). Interestingly, after the addition of MeHg (1 mM), ebselen was almost totally regenerated (Fig. 3, spectrogram C). The presence of MeHg (1 mM) or the product of the reaction of MeHg with GSH (both at 1 mM)

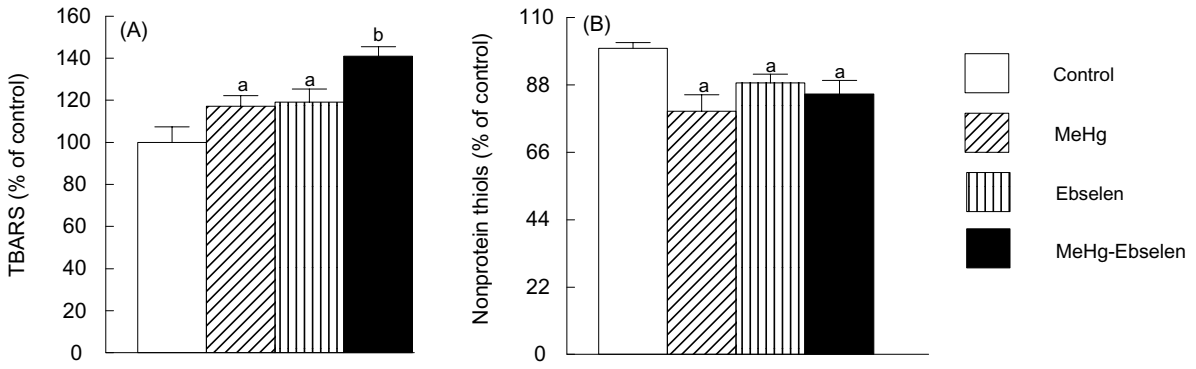

Fig. 1. Effect of methylmercury (MeHg) and ebselen exposure on TBARS (A) and NPSH (B) of 24-day suckling rat pups. For treatment and data details, see Table 1. The absolute control values for TBARS and NPSH were 2.34 nmol MDA/mg protein and 4.77 μmol/g tissue, respectively. Values not sharing the same letter are statistically different; $P < 0.05$, by one-way ANOVA, followed by the Duncan multiple range test when F was significant.

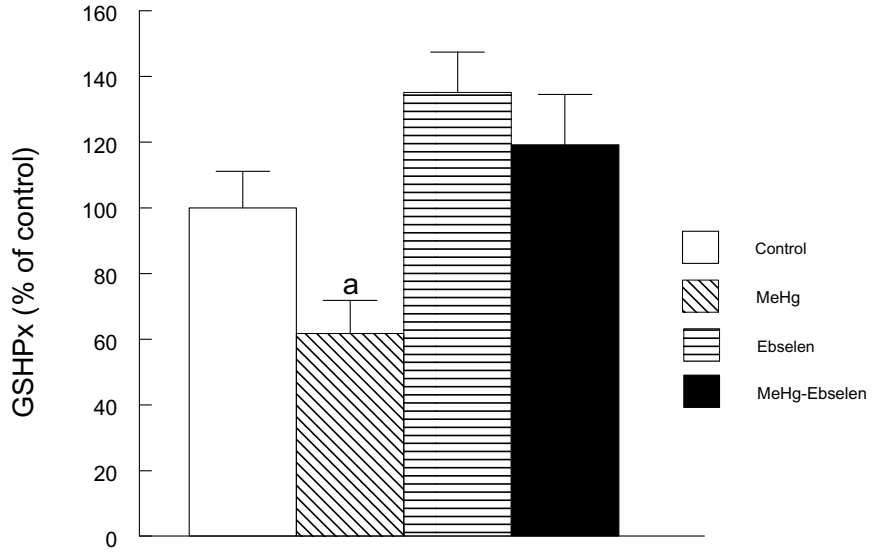

Fig. 2. Effect of methylmercury (MeHg) and ebselen exposure on liver GSHPx activity of 24-day suckling rat pups. For treatment and data details, see Table 1. The absolute control value for GSHPx activity was 9.34 nmol of NADPH oxidation/mg protein/min. Values not sharing the same letter are statistically different; $P = 0.01$, by one-way ANOVA, followed by the Duncan multiple range test when F was significant.

did not change the spectral characteristics of ebselen (Fig. 3, spectrograms D and E, respectively). The same phenomenon was also observed when MeHg (1 mM), GSH (1 mM) and ebselen (50 μM) were added simultaneously (Fig. 3, spectrogram F). MeHg (1 mM), GSH (1 mM) and MeHg + GSH (both at 1 mM) did not presented apparent absorbance in the studied wavelengths (data not shown).

The amount of sulfhydryl groups in the above-mentioned conditions was also determined in an attempt to better investigate the interaction between MeHg, GSH and ebselen. Sulfhydryl groups of GSH (1 mM) were totally oxidized in the presence of MeHg (1 mM) and when MeHg (1 mM) and ebselen (50 μM) were added, independently on the order of addition of the compounds. Ebselen alone (50 μM) oxidized around 90% of added GSH (1 mM) after 1 min at 25 °C (data not shown).

4. Discussion

The hazardous effects of MeHg on liver are well known and seem to be related to thiol depletion that, in turn, lead to increased oxidative stress (Ashour et al., 1993; Lin et al., 1996). Here, we showed that the post-natal exposure to MeHg increases TBARS and decreases NPSH levels on liver of pup rats, agreeing with the previous data obtained on adult animals. In addition to NPSH depletion, MeHg exposure also caused a decrease on GSHPx activity. Since this enzyme detoxifies H_2O_2 and converts lipid hydroperoxides to nontoxic alcohol, it is reasonable to suppose that the increase on liver TBARS levels is related, at least in part, to the observed decrease on GSHPx activity.

Previous works from our laboratory have demonstrated that ebselen presents protective effects against MeHg-induced neurotoxicity under in vivo conditions (Farina et al., 2003a,b). At a dose of 10 mg/kg, ebselen protected against MeHg-induced alterations on glutamate homeostasis on rat pups brain (Farina et al., 2003a). Here, using the same dose used in the previous study, ebselen did not offer any protection against MeHg-induced increase on TBARS levels and decrease on NPSH in hepatic tissue. Contrarily, ebselen alone increased liver TBARS and decreased NPSH. Moreover, simultaneous treatment with both compounds caused a higher increase in hepatic TBARS levels when compared to the treatments with individual compounds. This data suggest that ebselen, at a neuroprotective dose (10 mg/Kg; Farina et al., 2003a)

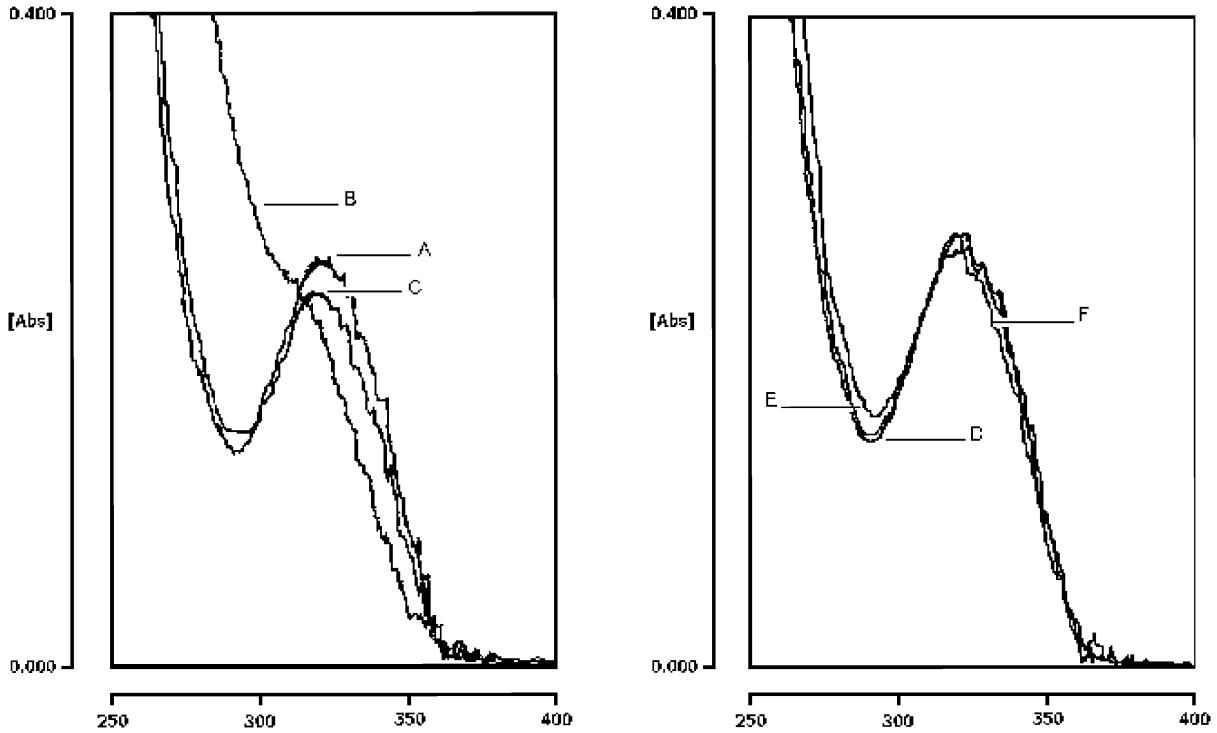

Fig. 3. The comparative spectra of ebselen and the products of its interaction with GSH and/or MeHg. (A) Ebselen (50 μM); (B) Ebselen (50 μM) + GSH (1 mM) + 1 min at 25 °C; (C) Ebselen (50 μM) + GSH (1 mM) + 1 min at 25 °C + MeHg (1 mM) + 1 min at 25 °C; (D) Ebselen (50 μM) + MeHg (1 mM) + 1 min at 25 °C; (E) GSH (1 mM) + MeHg (1 mM) + 1 min at 25 °C + ebselen (50 μM) + 1 min at 25 °C; and (F) Ebselen (50 μM) + GSH (1 mM) + MeHg (1 mM) + 1 min at 25 °C. Absorbance (Abs) was monitored by spectrophotometry (250–400 nm). For details, see materials and methods.

was able to cause oxidative injury on liver of rat pups.

Recent studies have demonstrated the potential toxic effects of ebselen in rats and mice under in vivo conditions (Meotti et al., 2003). Moreover, ebselen induces apoptosis in human hepatoma cell line, $HepG_2$, and this effect seems to be related to its ability to deplete thiols (Yang et al., 2000a,b). In fact, an earlier study has demonstrated that, in addition to its peroxidase-like activity, ebselen can indirectly deplete thiols through the direct oxidation of GSH by its metabolite selenoxide [2-(methylseleninyl) benzanilide] (Akerboom et al., 1995). Since selenoxides are potent thiol oxidants (Farina et al., 2001), our in vivo results, together with previous studies (Akerboom et al., 1995; Yang et al., 2000a,b), reinforce that the pro-oxidative effects of ebselen contributes to its hepatotoxicity.

It is interesting to state that ebselen alone caused an evident, even non-significant, increase in the hepatic thiol peroxidase activity. In addition, ebselen abolished the inhibitory effect of MeHg on liver GSHPx activity, but could offer no protection against the oxidative effects of MeHg. This phenomenon points to a complex process of MeHg-induced liver damage, where oxidative mechanism(s) other than thiol depletion and peroxide generation is (are) involved.

Although evidence shows that MeHg inhibits GSHPx activity (Ashour et al., 1993; Lin et al., 1996), the molecular mechanism related to this inhibitory effect is not known. Taking into account that selenols(ates) (–SeH/–Se^-) are even more highly reactive toward Hg than thiols(ates) (–SH/–S^-) (Sugiura et al., 1978; Clarkson, 1997) and that the selenol group of GSHPx plays a crucial role in its catalytic activity (Ursini and Bindoli, 1987), one could presume

that MeHg inactivates this enzyme due to the direct chemical interaction with selenolate at the active center of the enzyme. Since ebselen selenol is the major ebselen intermediate with peroxidase-like activity, it is possible that MeHg also interacts with its selenolate radical. Here, our in vitro studies confirmed the fact that MeHg interacts with the chemical intermediate formed after the reaction between GSH and ebselen.

A previous study of Cotgreave et al. (1992) showed the formation of ebselen selenol intermediate after the reaction of ebselen with excess of GSH. Using the same experimental conditions (50 μM ebselen + 1 mM GSH in 50 mM phosphate buffer of pH 7.4), we observed that the unique spectral characteristics of ebselen drastically changed after GSH addition, suggesting the formation of the selenol intermediate. The presence of this phenomenon of selenol formation is reinforced by the fact that 50 nmol of ebselen oxidized around 100 nmol of GSH. In agreement with our data, evidence shows that ebselen reacts with equimolar concentrations of GSH to form a sulfur–selenium bond with concomitant opening of the isoselenazol ring and excess GSH then reduces the ebselen selenodisulfide to its selenol(ate) derivative with formation of oxidized glutathione (Jacob et al., 1998).

Interestingly, after the formation of ebselen selenol intermediate, the addition of MeHg regenerates the pattern compound, suggesting the direct chemical interaction of MeHg with selenol(ate) with subsequent closing of the isoselenazol ring. Since MeHg seems to interact with the selenol(ate) group(radical) of ebselen, it is reasonable to presume that it also interacts with the selenol group of GSHPx, decreasing its catalytic activity. Moreover, the evident increase on the GSHPx activity observed in animals of group D (MeHg + ebselen) when compared to animals of group B (MeHg) can be related to two different mechanisms: (1) the peroxidase-like activity of ebselen itself and (2) the direct chemical interaction between ebselen selenol and MeHg, decreasing the free MeHg able to interact with GSHPx.

An elegant study of Kono et al. (2001) showed that ebselen (50 mg/kg twice daily, intragastrically) offers protection against alcohol-induced liver injury in adult rats. Moreover, this study did not report hepatotoxic effects of ebselen. It is important to state that although the ebselen dosage (10 mg/kg, subcutaneously) was 10-fold lower in our study, the pups treated with ebselen alone presented evident signs of liver injury (increased TBARS levels and decreased NPSH levels). Taking into account the fact that ebselen is a lipophilic drug and, as consequence, is well absorbed after intragastric administration, it is reasonable to suppose that the opposing data came from both studies that are not related to high differences on ebselen bioavailability due to different used routes of administration. So, the results of the present investigation indicate that young rats are sensitive to the potential hepatotoxic effects of ebselen, which are at variance with the relative insensitivity of adult rats to short-term treatment with ebselen (Kono et al., 2001). Most importantly, our data demonstrate for the first time the fact that liver of weaning rats are vulnerable to a promising therapeutic anti-oxidant and indicate that further detailed studies are necessary to establish the safety of ebselen in different phases of development and organisms.

5. Conclusion

This paper reinforces the oxidative effects of MeHg on rat liver and could add to our understanding on the molecular mechanisms of ebselen-induced toxicity. The comprehension of such mechanisms is important because: (1) there is a scarcity of toxicity studies of ebselen under in vivo conditions, and (2) ebselen has been reported to show therapeutic efficacy in clinical trials with humans (Lee et al., 1999; Parnham and Sies, 2000; Martinez-Vila and Sieira, 2001). Hence, the possible use of this compound as a pharmacological agent in the near future motivates toxicological studies.

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