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Identification of Alkylation-Sensitive Target Chaperone Proteins and Their Reactivity with Natural Products Containing Michael Acceptor

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Molecular chaperones have a crucial role in the folding of nascent polypeptides in endoplasmic reticulum. Some of them are known to be sensitive to the modification by electrophilic metabolites of organic pro-toxicants. In order to identify chaperone proteins sensitive to alkyators, ER extract was subjected to alkylation by 4-acetamido-4'-maleimidyl-stilbene-2,2'-disulfonate (AMS), and subsequent SDS-PAGE analyses. Protein spots, with molecular mass of 160, 100, 57 and 36 kDa, were found to be sensitive to AMS alkylation, and one abundant chaperon protein was identified to be protein disulfide isomerase (PDI) in comparison with the purified PDI. To see the reactivity of PDI with cysteine alkylators, the reduced form (PDI_{red}) of PDI was incubated with various alkylators containing Michael acceptor structure for 30 min at 38°C at pH 6.3, and the remaining activity was determined by the insulin reduction assay. Iodoacetamide or *N*-ethylmaleimide at 0.1 mM remarkably inactivated PDI_{red} with *N*-ethylmaleimide being more potent than iodoacetamide. A partial inactivation of PDI_{oxid} was expressed by iodoacetamide, but not *N*-ethylmaleimide (NEM) at pH 6.3. Of Michael acceptor compounds tested, 1,4-benzoquinone (IC_{50}, 15 μM) was the most potent, followed by 4-hydroxy-2-nonenal and 1,4-naphthoquinone. In contrast, 1,2-naphthoquinone, devoid of a remarkable inactivation action, was effective to cause the oxidative conversion of PDI_{red} to PDI_{oxid}. Thus, the action of Michael acceptor compounds differed greatly depending on their structure. Based on these, it is proposed that PDI, one of chaperone proteins in ER, could be susceptible to endogenous or xenobiotic Michael acceptor compounds *in vivo* system.

Key words: PDI, Chaperone, Michael acceptor, Inactivation, Oxidative conversion, Quinone, 4-Hydroxy-2-nonenal

INTRODUCTION

Cells respond to a variety of environmental stresses including physical or chemical impacts by the induction of heat shock or stress proteins, which bind to denatured or aggregated cellular proteins, thereby facilitating their refolding in the cytoplasm and luminal compartment of the endoplasmic reticulum (Calvert *et al.*, 2003). Many stress proteins are constitutively expressed, and can function as molecular chaperones. Meanwhile, molecular chaperones bind to nascent proteins, promote proper protein folding, and prevent the aggregation of nonnative and misfolded proteins in ER (Freedman *et al.*, 1994; Noiva, 1994; Gilbert, 1997; Wiest *et al.*, 1997). Such a role is performed mainly by the chaperones such as GRP78 (MW 72 kDa, BiP), oxygen regulated protein 150 (ORP150; MW 111 kDa), calreticulin (MW 48 kDa), calnexin (MW 65 kDa), and protein disulfide isomerase (MW 57 kDa). In addition, a recently discovered, ubiquitously expressed ER protein, ERP29, may complement this group of ER chaperones (Koen and Hanzlik, 2002). PDI is of particular importance for protein folding in the ER where it catalyzes the formation and isomerization of disulfide bonds (Freedman *et al.*, 1988; Freedman *et al.*, 1994). There are different PDI family members such as ERP72, CaBP1 or ERP60, abundant in mammalian tissues (Kim *et al.*, 2002). A common characteristic of these PDI family proteins is that they contain cysteine residue of low pKa value, highly nucleophilic at physiological pHs. Previously, the enzymes which contain the cysteine residue in the active site were observed to be

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susceptible to Michael acceptor compounds (Bastyns and Engelborghs, 1992; Sok *et al.*, 1993; Ahn and Sok, 1996). In this espect, PDI_{red} was supposed to be modified by Michael acceptor compounds.

α,β-Unsaturated aldehydes, corresponding to endogenous Michael acceptor compounds, are produced, as a result of the β-scission of lipid alkoxy radicals, from the reaction of carbon-centered lipid radicals with molecular oxygen during oxidative stress (Esterbauer *et al.*, 1991; Montine, 2002). Endogenous α,β-unsaturated aldehydes, such as 4-hydroxy-2-nonenal or acrolein, are known to exert an adverse action as electrophilic toxins, capable of covalently modifying proteins as well evidenced by the alkylation of cysteine and histidine residues by α,β-unsaturated aldehydes (Uchida and Stadtman, 1994; Gan and Ansari, 1998). Recently, they are known to activate the antioxidant response element DNA (Tjalkens *et al.*, 1998), responsible for the induction of antioxidant enzymes, in cells subjected to oxidative stress. Nevertheless, there has been no study on the interaction of PDI with α,β-unsaturated aldehydes.

The cytotoxicity of many small, relatively unreactive organic compounds has been associated with their biotransformation to electrophilic metabolites, which then covalently bind to cellular protein nucleophiles. In animal experiment, seven ER proteins including GRP 78, Erp29, and protein disulfide isomerase family proteins were identified as targets for reactive metabolites of bromobenzene (Koen and Hanzlik, 2002). Also, PDI is a target protein for reactive metabolites of acetaminophen (Zhou *et al.*, 1996) and methoxychlor (Zhou *et al.*, 1995). However, most of the studies on the target of metabolites have emphasized their covalent binding to lysine residues of target proteins despite a recent report (Kim *et al.*, 2000) that some chaperone proteins with cysteine residues of low pKa values are sensitive to alkylation at low physiological pHs. Therefore, it has been intriguing to find whether chaperon proteins with low pKa cysteine residue are modified by Michael acceptor compounds at low physiological pHs. For this purpose, 4-acetamido-4-maleimidyl-stilbene-2,2'-disulfonate (AMS) will be beneficial as an alkylator, since AMS has been employed as a selective modifier of cysteine residue (Kobayashi *et al.*, 1997). In this study, we attempted to identify ER chaperone proteins susceptible to Michael acceptor compounds, and define the mode for the interaction between PDI and Michael acceptor compounds.

MATERIALS AND METHODS

Materials

GSH (reduced glutathione), GSSG (oxidized glutathione), glutathione reductase, insulin, iodoacetic acid, iodoacetamide, acrylamide, *N*-ethylmaleimide (NEM), benzoquinone, naphthoquinone, phenylisothiocyanate and dithiothreitol (DTT) were purchased from Sigma Chemical Co. (St. Louis. MO, USA), and *tert*-2-hexenal was from Aldrich Chemical Co (Milwauki, WI, USA). 4-Hydroxy-2-nonenal was from Calbiochem Co. (La Jolla, CA, USA). 4-Acetamido-4-maleimidyl-stilbene-2,2'-disulfonate (AMS) was procured from Molecular Probes (Eugene, OR, USA).

Preparation of liver endoplasmic reticulum (ER) and treatment of ER with AMS

Livers were removed and homogenized in ice-cold 20 mM Tris buffer, pH 8.0, containing 0.15 M NaCl, 250 mM sucrose, and 1 mM DTT. The homogenate, gauze-filtered, was successively centrifuged at 25,000 g (30 min) and 100,000 g (60 min). The final microsomal pellet was resuspended and rehomogenized in 50 mM Tris buffer (pH 7.5) containing 25 mM KCl, 5 mM $MgCl_2$ and 0.5% Triton X-100 to prepare the ER extract. The ER extract was incubated with 10 mM AMS at 38°C for 60 min.

Purification of PDI and preparation of PDI_{red} or PDI_{oxid}

PDI was purified from bovine liver homogenate according to the previous procedure (Rigobello *et al.*, 2000) employing ammonium sulfate fractionation (55-93%), CM Sephadex C-50 chromatography, and DEAE Sephacel chromatography, and finally FPLC. The form of the finally purified PDI was determined to be mainly PDI_{oxid} according to AMS treatment/SDS-PAGE analysis (Kobayashi *et al.*, 1997). PDI_{red} was prepared by incubating PDI (40 nmoles) with 10 mM DTT in 0.5 mL of 50 mM MES (pH 6.3) for 30 min at 38°C, and then passed through Sephadex G-25 (1.5×20 cm), which was eluted with the same buffer. Separately, the purified PDI was incubated with GSSG (2 mM) in 50 mM Tris (pH 7.0) for 30 min at 38°C to accomplish the further oxidation of the purified PDI.

Assay of PDI activity

PDI activity was determined according to the insulin reduction assay (Rigobello *et al.*, 2000); PDI solution was added to 200 mL of 0.1 M phosphate buffer (pH 7.0) containing 2 mM EDTA and insulin (1 mg/mL). The enzyme reaction was started by addition of 2 mL of dithiothreitol (0.05 M). After 30 min incubation, PDI activity was determined as an increase of O.D. at 578 nm due to the reduction of insulin disulfides and the subsequent precipitation of the insulin β-chain. Separately, the PDI activity was determined by GSH-insulin transhydrogenation assay (Rigobello *et al.*, 2000); the enzyme reaction was started

by adding PDI solution to 200 μL of the above reaction mixture containing 0.12 mM NADPH, glutathione reductase (2.8 units/mL), insulin (30 μM), GSH (8 mM), and EDTA (5 mM), and the formation of NADP was monitored at 340 nm.

Determination of PDI form, PDI_{red} or PDI_{oxid}

The redox form of PDI, PDI_{red} or PDI_{oxid} was determined according to the modification by 4-acetamido-4'-maleimidyl-stilbene-2,2'-disulfomate (AMS), and then SDS-PAGE analysis (Kobayashi *et al.*, 1997); PDI (0.6 nmol/mL) was incubated with 10 mM AMS in 50 μL of 50 mM Tris buffer, pH 8.0 containing 1% SDS and 1mM EDTA at 38°C for 60 min, and then an aliquot was subjected to SDS-PAGE (12% acrylamide) to analyze redox forms of PDI.

Inactivation of PDI_{red} or PDI_{oxid} by iodoacetamide or *N*-ethylmaleimide

PDI_{red} (0.6 nmol/mL) was incubated with iodoacetamide or *N*-ethylmaleimide (100 μM) in 50 mM Tris buffer (pH 6.3) at 38°C for 30 min, and then, an aliquot (20 μL) of the mixture was taken and subjected to insulin reduction assay. Separately, PDI_{oxid} was incubated with iodoacetamide or *N*-ethylmaleimide of 100 μM at pH 7.0 for 30 min.

Inactivation of PDI_{red} by alkylators including michael acceptor compounds

PDI_{red} (0.6 nmol/mL) was incubated with each alkylator of various concentrations (10, 30 or 100 μM) in 50 mM MES buffer (pH 6.3) for at 38°C for 30 min, and then, an aliquot (20 μL) of the mixture was taken and subjected to insulin reduction assay. Additionally, the concentration-dependent inactivation of PDI_{red} by benzoquinone was examined by incubating PDI_{red} (0.6 nmol/mL) with benzoquinone (0-100 μM) as described above.

GSSG or quinone-induced oxidative conversion of PDI_{red} to PDI_{oxid}

PDI_{red} (3 nmol/mL) was preincubated with GSSG (0.1 or 1.0 mM) in 50 mM MES buffer (pH 6.3) at 38°C, and 30 min later, an aliquot (20 μL) of the mixture was subjected to the alkylation by NEM (1 mM) in 50 mM Tris buffer (pH 7.0). Finally, the remaining activity was determined, and expressed as a percentage of total activity at an initial period; the remaining activity (%) corresponds to the relative amount of PDI_{oxid} generated from PDI_{red}. Additionally, the concentration-dependent effect of 1,4-benzoquinone on oxidative conversion of PDI_{red} was examined by incubating PDI_{red} (0.6 nmol/mL) with benzoquinone of various concentrations (0-30 μM), and measuring the remaining activity as described above.

RESULTS AND DISCUSSION

Identification of ER proteins sensitive to the modification by AMS

Previously, metabolites of organic compounds had been reported to modify proteins through covalent binding to histidine, cysteine or lysine residues. At physiological pHs, the cysteine residues with a low pKa value could be more sensitive to alkylators than the histidine or lysine residues (Kim *et al.*, 2000). In the present experiment, AMS, a selective modifier of cysteine residues, was employed to detect chaperone target proteins containing cysteine residue sensitive to alkylation. First, the Triton X-100 extract of ER was incubated with 10 mM AMS for 30 min at 38°C, and the mixture was subjected to SDS/PAGE analysis, followed by Coomassie Blue staining. Although the protein pattern of AMS-treated sample appears to be similar to that of the non-treated control, the Rf value of at least four bands (bands A, B, C, and D) varies noticeably between the two preparations (Fig. 1) Thus, the proteins modified by AMS can be distinguished from the non-modified control proteins over a wide range of molecular masses. The M.W. of proteins, which migrated to higher mass, were determined to be 160 kDa, 100 kDa, 57 kDa and 36 kDa; there are two major bands with estimated molecular masses of ~100 kDa and ~57 kDa, and two less-abundant bands

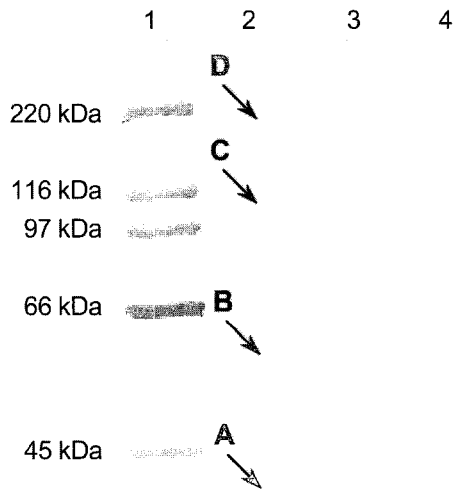

Fig. 1. PAGE analysis of ER proteins subjected to AMS/PAGE. The ER extract was incubated with 10 mM AMS in 50 μL of 50 mM Tris buffer (pH 8.0) at 38°C. After 60 min, an aliquot of the mixture was subjected to SDS-PAGE analysis, and compared with non-treated sample. Lane 1, marker proteins; lane 2, non-treated control; lane 3, after AMS treatment (bands A, B, C or D; 36 kDa, 57 kDa, 100 kDa or 160 kDa); lane 4, purified PDI.

with masses of ~160 kDa and ~36 kDa. Since AMS could modify cysteine or lysine residues at pHs used (pH 8.0), it was assumed that chaperone proteins containing multiple cysteine or lysine residues would be susceptible to AMS modification. To identify chaperone proteins susceptible to AMS, the chaperone proteins, which migrated to higher Rf value in PAGE analysis, were searched among chaperone proteins present in ER (Gething and Sambrook, 1992; Koen and Hanzlik, 2002). Abundant resident chaperone proteins in the ER are calnexin (65 kDa), BiP (90 kDa), calreticulin (48 kDa), GRP94 (92 kDa), GRP78 (72 kDa), and PDI (57 kDa), which had been reported to be modified by Sulfo-NHS biotin (Calvert *et al.*, 2003), specific for primary amine group. However, the bands corresponding to molecular weights of these chaperone proteins except PDI (band C, ~57 kDa) were not affected remarkably by the AMS modification, based on the change of molecular mass. In SDS-PAGE study employing the commercially available or purified PDI, the protein of band B was found to be identical to PDI in the molecular behavior. In a previous study, chaperone proteins modified by bromobenzene metabolite were identified to be PDI family proteins and BiP (Koen and Hanzlik, 2002). Separately, the metabolites of acetaminophen were found to label amino group of two proteins, PDI and calreticulin (Zhou *et al.*, 1996). In addition, radioactive 1,4-benzoquinone was found to label PDI in human bronchial epithelial cells (Lame *et al.*, 2003). Thus, PDI is suggested to be one of chaperone proteins commonly modified by electrophilic alkylaors. Furthermore, PDI might be a primary target for alkylators, since it is one of major ER-resident proteins comprising 3-5% of microsomal protein. PDI is one of a large family of proteins (Primm and Gilbert, 2001), homologous to thioredoxin (Holmgren, 1985), which include ERP72, PDI58, ERP60 or CaBP1, abundant in mammalian tissues (Kim *et al.*, 2002). The common property of PDI isozymes is that they contain two or three thioredoxin motifs (CysGlyHisCys), characterized by the existence of cysteine residue of a low pKa value, which is highly susceptible to electrophilic modifiers at low physiological pHs (Hawkins and Freedman, 1991; Kim *et al.*, 2000). The alkylative modification of PDI family proteins will result in the loss of PDI function; it is a multifunctional chaperone protein having, peptide and protein binding, Ca^{2+}-binding, and ATPase activities in addition to disulfide-isomerase activity (Ferrari and Söling,1999).

Sensitivity of PDI_{oxid} or PDI_{red} to AMS modification

In an attempt to characterize the modification of PDI by AMS, two redox forms of PDI, PDI_{red} and PDI_{oxid}, were exposed to AMS at pH 8.0, and the modified PDI was subjected to PAGE analysis. As shown in Fig. 2, it was found that the band of PDI_{red} migrated to a higher mass after the treatment with AMS, whereas PDI_{oxid} was resistant to the

Fig. 2. Determination of PDI form, PDI_{red} or PDI_{oxid}. PDI (0.6 nmol/mL), PDI_{red} or PDI_{oxid}, was incubated with 10 mM AMS in 50 mL of 50 mM Tris buffer, pH 8.0 containing 1 % SDS and 1 mM EDTA at 38°C for 60 min, and then an aliquot was subjected to SDS-PAGE (12% acrylamide) to analyze redox forms of PDI. Lane 1, PDI_{red}; lane 2, PDI_{red} + AMS; lane 3, PDI_{oxid} + AMS.

modification by AMS. This indicates that the thiol group of cysteine residue in the thioredoxin (CXHC) of active site in PDI_{red} is sensitive to AMS modification. Meanwhile, the resistance of PDI_{oxid} to AMS modification suggests that the thioredoxin box in the active site of PDI_{oxid}, where the cysteine residues in the thioredoxin (CXHC) box exists as a disulfide linkage, is resistant to AMS modification. This indicates that the migration of the AMS-treated PDI_{red} to a higher mass band is related to the modification of cysteine residue, but not the other amino acid residues. These led to the notion that only the reduced form of PDI can be inactivated by selective thiol modifiers.

Selective inactivation of PDI_{red} by thiol modifier at low pHs

To define the relationship between the alkylative modification of PDI and the loss of PDI activity, PDI_{red} was incubated with thiol modifiers, iodoacetamide or NEM, at pH 6.3, and the remaining activity was determined by insulin reduction assay. As shown in Fig. 3, NEM at 0.1 mM inactivated PDI_{red}, but not PDI_{oxid}, suggesting a selective inactivation of PDI_{red} by NEM at pH 6.3. Similarly, iodoace-

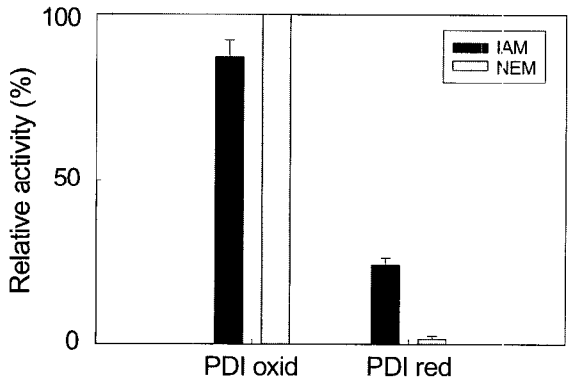

Fig. 3. Inactivation of PDI_{red} or PDI_{oxid} by iodoacetamide or *N*-ethylmaleimide. PDI_{red} (0.6 nmol/mL) was incubated with iodoacetamide or N-ethylmaleimide (100 μM) in 50 mM Tris buffer (pH 7.0) for at 38°C for 30 min, and then, an aliquot (20 μL) of the mixture was taken and subjected to insulin reduction assay. In the same way, PDI_{oxid} was incubated with iodoacetamide or *N*-ethylmaleimide (100 μM) at pH 7.0 for 30 min. Data are expressed as a mean ± SD (bar) value of triplicate sets.

tamide also inactivated PDI_{red}, but it was less potent than NEM. Moreover, iodoacetamide decreased the activity of PDI_{oxid} to a slight extent, suggesting that another amino acid resuidue, probably histidine residue, would be modified by iodoacetamide at pH 6.3. Thus, PDI_{red}, but not PDI_{oxid}, can be inactivated selectively by NEM. Therefore, it was supposed that NEM could be employed as a selective modifier of PDI to determine the ratio of dithiol group to disulfide group in active site of PDI molecule. In this regard, we examined the effect of GSSG, endogenous oxidant, on the oxidation of PDI_{red} using either NEM treatment/insulin reduction assay or iodoacetamide treatment/insulin reduction assay. For this purpose, PDI_{red} was incubated with either NEM (0.1 mM) or iodoacetamide (0.1 mM) in the presence of GSSG (0.1 or 1.0 mM), and 30 min later, the reaction mixture was subjected to insulin reduction assay. As shown in Fig. 4, GSSG at 0.1 mM and 1.0 mM protected PDI_{red} from the inactivation by NEM to an extent of approximately 71% and 85%, respectively, suggesting that the corresponding amount of PDI_{red} was converted to PDI_{oxid} during the preincubation with GSSG. Thus, it is suggested that once the dithiol group in the active site of PDI_{red} is oxidized by GSSG, the oxidized PDI is resistant to alkylation. Meanwhile, the pretreatment with GSSG at 0.1 mM and 1.0 mM protected PDI_{red} from the inactivation by iodoacetamide to an extent of approximately 25% and 52%, respectively. In comparison, the protective action of GSSG was more remarkable with the NEM treatment assay than the iodoacetamide treatment assay. This might comply with the notion that NEM is a selective modifier of cysteine residue in the thioredoxin box, while iodoacetamide could modify histidine residue in addition to cysteine residue.

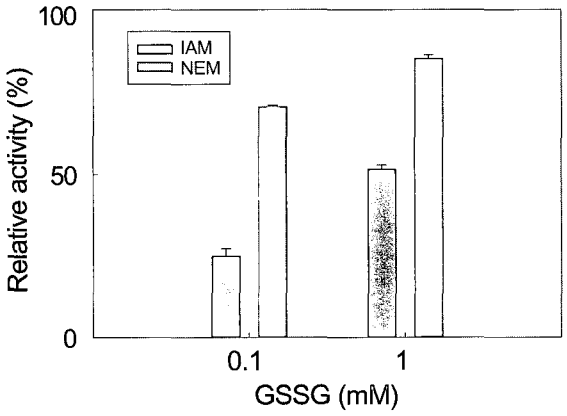

Fig. 4. GSSG -induced oxidative conversion of PDI_{red} to PDI_{oxid}. PDI_{red} (3 nmol/mL) was incubated with iodoacetamide or *N*-ethylmaleimide (0.1 mM) in the presence of GSSG (0.1 or 1.0 mM) in 50 μL of 50 mM MES buffer (pH 6.3) at 38°C, and 30 min later, an aliquot (20 μL) was subjected to the insulin reduction assay. Data are expressed as a mean ± SD (bar) value of triplicate sets.

Inactivation of PDI_{red} by Michael acceptor compounds

Because reactive cysteine residues with low pKa values are primary sites for the electrophilic modifiers at low pHs, the possible inactivation of PDI_{red}, which possesses a low pKa cysteine residue, by various Michael acceptor compounds was examined. For this purpose, PDI_{red} was incubated with various thiol modifiers including Michael acceptor compounds at pH 6.3, and the remaining activity was determined by the insulin reduction assay (Table I). First, the inactivation of PDI_{red} by various alkylators with aromatic moiety was investigated at pH 6.3. Of Michael acceptor compounds used, the most potent was 1,4-benzoquinone, showing 94% inactivation at 30 μM, followed by 1,4-naphthouinone, possessing 42% inactivation at 30 μM, and anthracene-1,4,9,10-tetraone (1,4,9,10-ATO) with a negligible inactivation at 0.1 mM. Thus, the inactivating potency of quinone-type Michael acceptor compounds depended on the size of quinone moiety rather than the redox potential, indicating that the active site of PDI may express a limited accessibility. In related study (Fig. 5), where the concentration-dependent inactivation of PDI_{red} was assessed, 1,4-benzoquinone was found to possess an IC_{50} value of approximately 15 μM. From these, it is suggested that 1,4-benzoquinone might be one of potent Michael acceptor compounds capable of modifying the thioredoxin motif of PDI_{red}. Next, Michael acceptor com-

Table I. Inactivation of PDI_{red} by alkylators including Michael acceptor compounds. PDI_{red} (0.6 nmol/mL) was incubated with each alkylator of various concentrations (10, 30 or 100 μM) in 50 mM MES buffer (pH 6.3) for at 38°C for 30 min, and then, an aliquot (20 μL) of the mixture was taken and subjected to insulin reduction assay.

Compound	Conc. (mM)	Inactivation (%)
1,4-Benzoquinone	0.03	94.0±0.6
Bromobenzene	0.1	< 5
1,4,9,10-ATO	0.1	< 5
1,2-Naphthoquinone	0.03	< 5
	0.1	18.7±2.0
1,4-Naphthoquinone	0.03	9.2±1.3
	0.1	42.1±2.7
2-Furaldehyde	0.03	< 5
Dihydrocoumarin	0.1	< 5
Phenylisothiocyanate	0.1	< 5
4-Hydroxy-2-nonenal	0.01	18.5±9.8
	0.03	44.0±5.1
tert-2-Hexenal	0.1	< 5
Acrylamide	0.1	< 5
AMS	0.01	10.6±1.9
	0.03	45.6±2.7

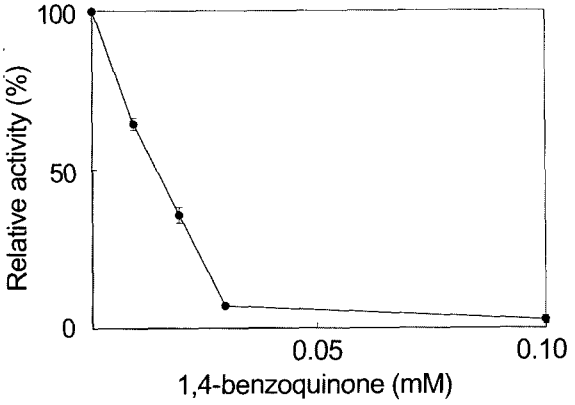

Fig. 5. Concentration-dependent inactivation of PDI_{red} by 1,4-benzoquinone. PDI_{red} (0.6 nmol/mL) was incubated with 1,4-benzoquinone of various concentrations (0-100 μM) in 50 mM MES buffer (pH 6.3) for at 38°C for 30 min, and then, an aliquot (20 μL) was subjected to insulin reduction assay. The remaining activity was expressed as a percentage of control. Data are expressed as a mean ± SD (bar) value of triplicate sets.

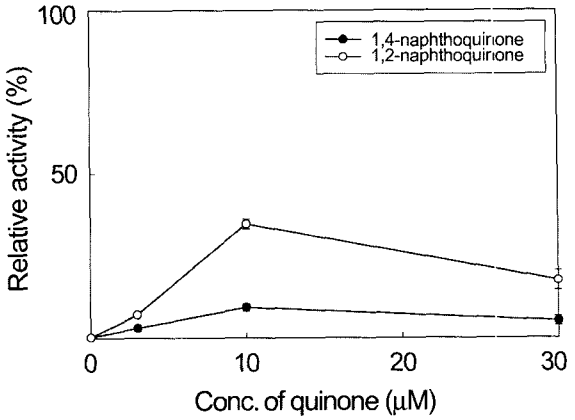

Fig. 6. Quinone-induced oxidative conversion of PDI_{red} to PDI_{oxid}. PDI_{red} (3 nmol/mL) was preincubated with each naphthoquinone (0-30 μM) in 50 mM MES buffer (pH 6.3) at 38°C, and 30 min later, an aliquot (20 μL) of the mixture was taken and subjected to the alkylation by NEM (1 mM) in 50 mM Tris buffer (pH 7.0). Finally, the aliquot of the mixture was subjected to the assay of remaining activity, which depends on the oxidative conversion of PDI_{red} to PDI_{oxid}. The relative activity was expressed as a percentage of PDI_{oxid} among total PDI activity.

pounds bearing aliphatic chain were tested for the inactivation of PDIred. After 30 min exposure, 4-hydroxy-2-nonenal at 10 μM and 30 μM was found to inactivate PDI_{red} by 18.5% and 44%, respectively, whereas *tert*-2-hexenal and acrylamide at 1 mM showed no remarkable inactivation. It is noteworthy that PDI_{red} can be inactivated by 4-hydroxy-2-nonenal at concentrations as low as 10 to 30 μM. *In vivo* system, the a lengthy exposure of PDI_{red} to 4-hydroxy-2-nonenal could lead to a gradual reduction of PDI activity, up to a cytotoxic level. In contrast, less electrophilic Michael acceptors such as phenylisothiocyanate or 2-furaldehyde showed no inactivation. In addition, alkylators such as bromobenzene or dihydrocoumarin had no remarkable inactivation at concentrations used. Taken together, it is suggested that the reactivity of Michael acceptor with PDI_{red} may be governed by the size as well as electrophilicity of Michael acceptor.

Quinones-induced oxidative conversion of PDI_{red} to PDI_{oxid}

In further study, the structural modification of PDI_{red} by naphthoquinines, 1,2- or 1,4-naphthoquinones, was examined more extensively. As shown in Table I, two naphthoquinones had no inactivating action up to 30 μM, in contrast to the strong inactivating potency of 1, 4-benzoquinone. In comparison, the inactivating potency of 1,2-naphthoquinone was smaller than that of 1,4-naphthoquinone. Thus, it seemed that the position of keto group was important for the electrophilicity of naphthoquinone. Next, the possible oxidative conversion of PDI_{red} to PDI_{oxid} in the presence of quinones was investigated, since naphthoquinone analogues had been known to generate oxidants. First, PDI_{red} was exposed to naphthoquinone of various concentrations (1-30 μM) in the buffer of pH 6.3 at 38°C, and 30 min later, the aliquot was subjected to NEM treatment/insulin reduction assay. As demonstrated in Fig. 6, a part of PDI_{red} was converted to PDI_{oxid} during the exposure to two naphthoquinones in a concentration-dependent manner up to 10 μM, but at 30 μM, the formation of PDI_{oxid} decreased. In comparison, 1,2-naphthoquinone was more efficient than 1,4-naphthoquinone in converting PDI_{red} to PDI_{oxid}. Thus, it seems that naphthoquinone, more effective in inactivating PDI_{red}, was less efficient in the oxidative conversion of PDI_{red}. These led to the idea that there may be a reverse relationship between the inactivating action of quinones and their oxidative activity. Consistent with this, 1,4-benzoquinone, highly potent in inactivating PDI_{red}, failed to exert the oxidative conversion. The relatively low concentration of 1,2-naphthoquinone required for the effective oxidative conversion may suggest that endogenous naphthoquinone could be involved in the oxidation of PDI_{red}. In this respect, the role of vitamine K, a naphthoquinone analogue, was tested for the effect on PDI_{red}, but it had no effect on PDI_{red} in respect with the inactivating activity as well as the oxidative action. Overall, it appears that the thioredoxin motif of PDI shows a limited accomodation in the interaction with quinones.

Taken together, it is suggested that PDI_{red} is one of ER chaperone proteins, susceptible to Michael acceptor compounds *in vivo* system. The sensitivity of PDI_{red} to Michael acceptor compounds is based on the interaction between the reactive thiolate group of PDI_{red} and the electrophilic group of Michael acceptor compounds. The high reactivity of some Michael acceptor compounds toward PDI_{red} at pH

6.3 is ascribed to the low pKa value of cysteine residue in the active site of PDI_{red}. This might also apply to other proteins with low pKa cysteine residue, similar to the previous observation with the alkylation of intracellular proteins (Bastyns and Engelborghs, 1992; Kim *et al.*, 2000). Therefore, the cytotoxicity of Michael acceptor compounds such as benzoquinone or 4-hydroxy-2-nonenal in ER may be at least partially related to the inactivation of PDI_{red}, necessary for the oxidative folding of proteins in ER (Freedman et al., 1994). In addition, it is also possible that the modification of PDI by these alkylators could cause the alteration in its chaperone and/or antichaperone activities (Cai *et al.*, 1994). Meanwhile, the oxidative conversion of PDI_{red} was more remarkable with 1,2-naphthoquinone rather than 1,4-naphthoquinone. Thus, a specific structure of quinone compounds may be required for the effective coupling with PDI in the oxidation relay. This might be implicated in the facilitated oxidative folding of nascent polypeptides. In a different view point, quinone compounds might interfere with the redox system involving PDI, which needs further clarification. Taken together, it is proposed that endogenous or xenobiotic Michael acceptor compounds may affect the function of PDI as a oxidoreductase and a chaperone/antichaperone protein as well as a hormone reservoir *in vivo* system (Cai *et al.*, 1994; Puig and Gilbert, 1994). Conversely, PDI, rich in endoplasmic reticulum (Primm and Gilbert, 2001), could lower the toxicity of Michael acceptor compounds by removing them at least according to a stoichiometrical removal. Then, it remains to be revealed how PDI modified by these alkylators would be removed in ER. Finally, it will be of interest to see whether such an action of Michael acceptor compounds may be extended to different PDI family members such as ERP72, CaBP1 or ERP60 abundant in mammalian tissues (Kim *et al.*, 2002).

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