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Original Article

Activities of Cytosolic Glutathione Peroxidase and Phospholipid Hydroperoxide Glutathione Peroxidase in Rat Salivary Glands

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Distribution of cytosolic glutathione peroxidase (cGPx) activity and phospholipid hydroperoxide glutathione peroxidase (PHGPx) was observed in rat salivary glands. Among three major glands, high cGPx activity was observed in submandibular and sublingual glands; activity in the parotid gland was about 30% that of other glands. In intracellular fractions, cGPx activity was mainly distributed in the cytosolic fraction. On the other hand, PHGPx activity was in the following order: submandibular, parotid and sublingual glands. PHGPx activity in the sublingual gland was relatively lower than the other glands in contrast to cGPx activity. PHGPx activity was widely distributed in cytosolic, mitochondrial and microsomal fractions. Activities in parotid mitochondrial and sublingual cytosolic fractions were relatively lower than the other fractions, although those in the submandibular gland were almost the same among the three fractions. In addition, PHGPx activities in both cytosolic and mitochondrial fractions showed less preference toward the phospholipid head-group, which is effective for the reduction of various phospholipid hydroperoxides formed in cells. The gland-specific distribution of cGPx and PHGPx may be caused by differences in oxygen consumption depending on saliva-secretory ability and distribution of PHGPx isozymes. In addition, it is suggested that cGPx and PHGPx in salivary glands share a role in the reduction of hydroperoxide as an antioxidant; cGPx reduces hydrophilic hydroperoxide in cytosol but PHGPx reduces phospholipid hydroperoxide in various compartments of cells.

Key words: Hydroperoxide, Cytosolic glutathione peroxidase, Phospholipid hydroperoxide glutathione peroxidase, Salivary gland

INTRODUCTION

Oxygen consumption in the salivary gland is increased when saliva secretion is induced by adrenergic and cholinergic secretagogues[1,2]. During aerobic metabolism in cells, reactive oxygen species are constantly generated as by-products. They cause cell damage by reacting easily with cellular components such as lipids, proteins and DNA; in particular, unsaturated lipid is one of the major targets of reactive oxygen species[3]. In salivary glands, indeed, considerable amounts of lipid hydroperoxide have been detected, even in the resting (unstimulated) state[4~7]. In particular, it is possible that phospholipid hydroperoxide formed in the membrane bilayer affects the saliva secretion system because membrane fusion occurred at the final step of exocytosis in saliva secretion.

To prevent oxidative damage caused by reactive oxygen species and hydroperoxides, antioxidant enzymes such as catalase, superoxide dismutases and several glutathione peroxidases (GPxs) exist in cells. In particular, lipid hydroperoxides are thought to be reduced by GPxs such as cytosolic or classic glutathione peroxidase[8,9] (cGPx), phospholipid hydroperoxide glutathione peroxidase[10] (PHGPx) and glutathione *S*-transferase isozymes[11].

Previously, we investigated hydroperoxide-reducing activities by GPxs in the submandibular gland, taking advantage of their substrate specificities, and suggested that cGPx and PHGPx have a shared role in reducing intracellular hydroperoxide, while glutathione *S*-transferase is less important to reduce hydroperoxides[12]; however, few data are available on the comparison of GPx activities in the salivary glands.

In this study, therefore, we investigated the distribution of cGPx and PHGPx activities in three major salivary glands with regard to their shared role in reducing intracellular hydroperoxides.

MATERIALS AND METHODS

1. Materials

Phosphatidylcholine (from soybean, type III-S) and 13-lipoxygenase (EC 1.13.11.12, from soybean, type IV) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Phosphatidylcholine (dilinoleoyl) and phosphatidylethanolamine (dilinoleoyl) were from Doosan Serdary Research Laboratories (Toronto, Canada) and Avanti Polar Lipids (Alabaster, AL, USA), respectively. Glutathione (reduced form) and NADPH were from Kohjin Co. (Tokyo, Japan). Glutathione reductase (from yeast) was from Oriental Yeast Co. (Tokyo, Japan). Triton X-100 (peroxide-free grade) was from Roche Molecular Biochemicals (Mannheim, Germany). *tert*-Butyl hydroperoxide (*t*-BuOOH) and 5,5'-dithiobis(2-nitrobenzoic acid) were from Aldrich Chemical Co. (Milwaukee, WI, USA) and Dojindo Laboratories (Mashiki, Kumamoto, Japan), respectively. Other chemicals were of the highest grade available from commercial

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sources.

2. Preparation of cell homogenates

Male Wistar rats, 9-10 weeks old, obtained from a local breeder, were maintained with free access to water and Oriental MF solid chow (Oriental Yeast Co.), under a 12-h light/dark cycle. All procedures for handling the rats in this study were approved by Institutional Animal Care and Use Committee of Asahi University (#05-003). After overnight fasting, all animals were killed by exsanguination under light anesthesia with diethyl ether. Immediately, salivary glands were dissected out and washed with ice-cold saline. The tissues were freed from blood vessels and connective tissues at 0-4°C.

Homogenates were prepared at 0-4°C, as described previously[12]; tissues from 3 to 4 animals were used for one preparation. Mitochondrial and microsomal fractions were obtained from the homogenates by differential centrifugation as 20,000×*g*-20 min pellets of 1,000×*g*-10 min supernatant and 105,000×*g*-60 min pellets of 20,000×*g*-20 min supernatant, respectively. The 105,000×*g* supernatant was used as the cytosol fraction. The pellets were suspended in 0.1 M Tris-HCl buffer (pH 7.4) with a glass-pestle homogenizer. These samples were stored at −85°C until assayed.

3. Preparation of phospholipid hydroperoxides

Phosphatidylcholine hydroperoxide (PCOOH) and phosphatidylethanolamine hydroperoxide (PEOOH) were enzymatically prepared from the corresponding phospholipids using 13-lipoxygenase[13], as described previously[12]. Hydroperoxidation of phospholipid was checked with a PeroxiDetect Kit (Sigma Chemical Co.) based on the formation of Fe^{3+}-xylenol colored adducts using *t*-BuOOH as the standard. Almost 100% and more than 62% of linoleoyl moieties were hydroperoxidized in these methods using phosphatidylcholine and phosphatidylethanolamine, respectively. As traces of deoxycholate remaining in the final preparations affect the activities of PHGPx[14], the same batch of substrate was used for comparative experiments.

4. Assays

The activities of GPxs were measured using a spectrophotometric assay[13]; oxidation of glutathione was recorded at 340 nm in the presence of glutathione reductase and NADPH, as described previously[12]. The standard assay mixture contained 30 μM *t*-BuOOH or phospholipid hydroperoxide, 3 mM glutathione (reduced form), 0.2 mM NADPH, 1.5 units of glutathione reductase, 5 mM EDTA, 10 mM sodium azide, 0.1% Triton X-100 (peroxide-free) and an appropriate amount of enzyme source in 1.0 ml of 0.1 M Tris-HCl buffer (pH 7.4); Triton X-100 did not affect activities at this concentration[12]. The reaction was initiated by addition of the hydroperoxide substrate and carried out at 37°C in duplicate. Phospholipid hydroperoxide was added as 15 μl of methanolic solution. Non-specific NADPH oxidation measured without hydroperoxide substrate was subtracted to calculate hydroperoxide-reducing activities.

Protein concentration was determined by the method of Lowry *et al.*[15] using bovine serum albumin (fraction V, Pierce Chemical Co., Rockford, IL, USA) as the standard. Glutathione was measured according to the method of Tietze[16] using 5,5'-dithiobis(2-nitrobenzoic acid)[17]. Phospholipid concentration was estimated by the method of Bartlett[18], as modified by Marinetti[19].

Statistical analysis was performed using Student's *t*-test.

RESULTS AND DISCUSSION

As GPxs require reduced-form glutathione as a cofactor for the reduction of hydroperoxide, glutathione content in the submandibular gland was measured, comparing with that in the liver. As shown in Table 1, the submandibular gland contained about 0.5 mg/g tissue (3 μg/mg protein) glutathione and most existed as the reduced form. This is thought to be sufficient for the action of GPxs in the submandibular gland, although the content was 1/4-fold that in the liver, in which glutathione metabolism is quite active.

Figure 1 shows cGPx and PHGPx activities in the homogenates of three major salivary glands. The functional distribution of enzymes was determined by measuring catalytic activity in this study because the amount of PHGPx protein detected by an immunoassay does not correspond to activity distribution[13]. High cGPx activity, reducing *t*-BuOOH, was observed in submandibular and sublingual glands. cGPx activity in parotid gland homogenate was 26.9% of that in the submandibular gland and 32.6% of that in the sublingual gland. On the other hand, PHGPx activity, reducing PCOOH, was in the following order: submandibular, parotid and sublingual glands. Among the three glands, PHGPx activity in the sublingual gland was relatively lower than in other glands (53.4% of parotid and 38.1% of submandibular glands), in contrast to cGPx activity.

The gland-specific distribution of cGPx and PHGPx activities among the three glands may be caused by differences in oxygen consumption, depending on saliva-secretory ability. Among the

Table 1 Glutathione content in submandibular gland and liver

Tissue	Glutathione	Content	
		(mg/g tissue)	(μg/mg protein)
Submandibular gland	Reduced form	0.508	2.96
	Oxidized form	0.0057 ± 0.0016	0.033 ± 0.0095
	Total	0.513 ± 0.0269	2.99 ± 0.157
Liver	Reduced form	2.26	11.3
	Oxidized form	0.0329 ± 0.0017	0.165 ± 0.0086
	Total	2.29 ± 0.159	11.5 ± 0.80

Content of reduced-form glutathione was calculated by subtracting oxidized-form glutathione from the total glutathione content. Oxidized forms are shown as reduced-form-equivalent values. Data are expressed as the mean ± S.E. of three different preparations, each assayed in duplicate.

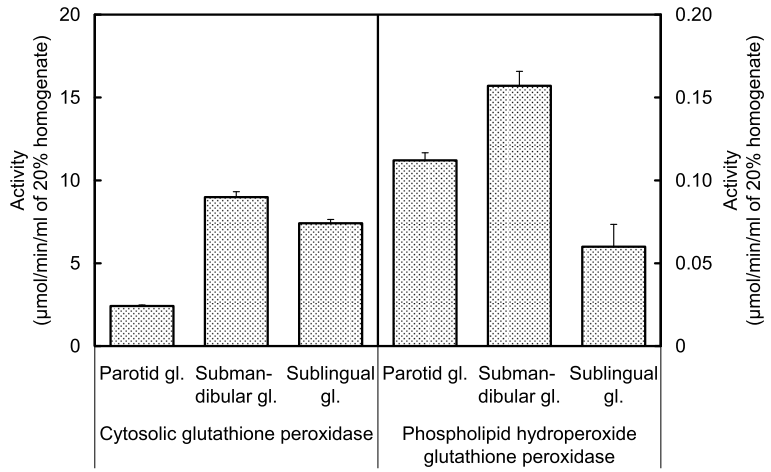

Fig. 1 Activities of cytosolic glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase in salivary gland homogenates. Activities were assayed using the following substrates: *tert*-butyl hydroperoxide (*t*-BuOOH) for cytosolic glutathione peroxidase and phosphatidylcholine hydroperoxide (PCOOH) prepared from soybean phosphatidylcholine for phospholipid hydroperoxide glutathione peroxidase. Data are expressed as the mean ± S.E. of three different preparations, each assayed in duplicate.

three glands, for example, oxygen consumption was highest in the submandibular gland both in the basal and secretagogue-stimulating states[20]. Because reactive oxygen species are constantly generated as by-products during aerobic metabolism, the submandibular gland may contain high cGPx and PHGPx activities to protect against cell damage caused by high oxygen consumption.

Figures 2 and 3 show the intracellular distribution of cGPx and PHGPx activities in the salivary glands. The highest cGPx activity was observed in the cytosolic fraction in each gland (Fig.2). Although cGPx is commonly referred to as 'cytosolic', it is known to be distributed not only in cytosol but also in mitochondria and other organelles[21, 22], and the activities in mitochondrial and microsomal fractions of salivary glands were quite lower than in the cytosolic fraction. High cGPx activity in the cytosolic fraction is effective to reduce hydrophilic hydroperoxides. On the other hand, it is difficult to reduce hydrophobic hydroperoxides such as phospholipid hydroperoxide in membranes by the action of cGPx. Although van Kuijk *et al.*[23] suggested that fatty acid hydroperoxide released from phospholipid hydroperoxide by phospholipase A_2 are reduced by cGPx, phospholipase A_2 activities in parotid[24, 25] and submandibular glands (data not shown) are much lower than PHGPx activities by which phospholipid hydroperoxides are reduced directly[26]; therefore, it is thought that cGPx primarily reduces hydrophilic hydroperoxides formed in the cytosol of salivary gland cells.

On the other hand, PHGPx activity was widely distributed in cytosolic, mitochondrial and microsomal fractions (Fig.3). In the submandibular gland, especially, PCOOH-reducing activities were almost the same among the three fractions. The activities

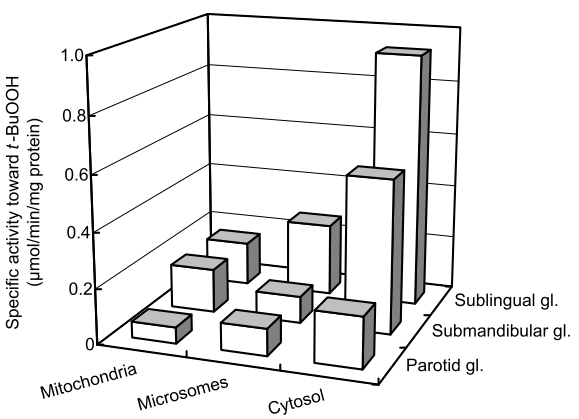

Fig. 2 Distribution of cytosolic glutathione peroxidase activity in salivary glands. Activities were assayed using *tert*-butyl hydroperoxide (*t*-BuOOH) as substrates. Data are expressed as the mean of three different preparations, each assayed in duplicate.

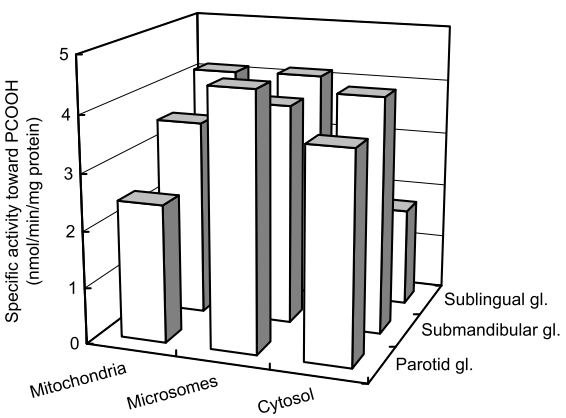

Fig. 3 Distribution of phospholipid hydroperoxide glutathione peroxidase activity in salivary glands. Activities were assayed using phosphatidylcholine hydroperoxide (PCOOH) prepared from soybean phosphatidylcholine as substrates. Data are expressed as the mean of three different preparations, each assayed in duplicate.

of the mitochondrial fraction in the parotid gland and cytosolic fraction in the sublingual gland were relatively lower than in the other fractions. These differences may be constrained by the presence of isozymes[27]. Although it is reported that a substantial amount of PHGPx is present in membranes as an inactive form[13], PHGPx in membrane fractions of salivary glands is thought to be very active.

Figure 4 shows cytosolic PHGPx activity toward PCOOH and PEOOH. In this experiment, dilinoleoyl types of phosphatidylcholine and phosphatidylethanolamine were used as original forms to avoid the effects of fatty acyl composition in phospho lipids. Almost the same activities between PCOOH and PEOOH were observed in cytosolic PHGPx of the three glands. The ratios of activities between PCOOH and PEOOH were as follows: 0.938 (parotid), 0.840 (submandibular) and 1.19 (sublingual). Similar results were obtained for mitochondrial PHGPx activity (Fig.5). The activity ratios between PCOOH and PEOOH were as follows: 1.11 (parotid), 0.842 (submandibular) and 0.702 (sublingual). Significant difference was observed only in the mitochondrial fraction of the sublingual gland. Although some activities did not fully correspond with Fig. 3, this may have been caused by batch differences of the phospho lipid hydroperoxide substrate, as trace deoxycholate remaining in the substrate affects PHGPx[14].

Less preference of PHGPx toward the phospholipid headgroup is effective to reduce various phospholipid hydroperoxides formed in cells. As phosphatidylcholine and phosphatidylethanolamine occupies more than 70% of membrane phos pholipids[28], PHGPx is able to reduce most phospholipid hydro peroxides formed in phospholipid membranes. In addition, hydroperoxides derived from minor phospholipids, such as phosphatidic acid and phosphatidylglycerol, were also reduced by PHGPx in our preliminary experiments (data not shown). It is known that PHGPx also reduces hydrophilic or small-molecule hydroperoxides such as H_2O_2 and *t*-BuOOH; however, PHGPx

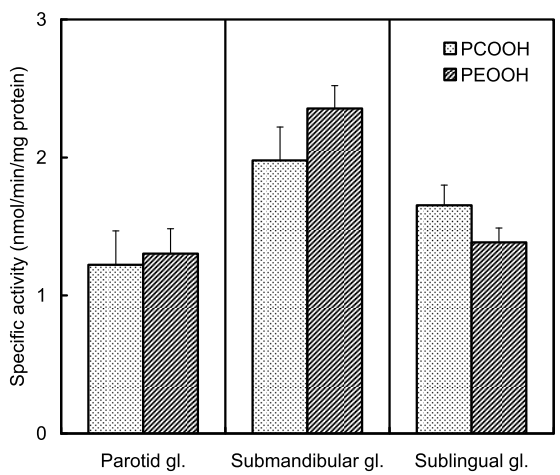

Fig. 4 Specific activities of phospholipid hydroperoxide glu tathione peroxidase toward phosphatidylcholine hydro peroxide (PCOOH) and phosphatidylethanolamine hydroperoxide (PEOOH) in cytosolic fractions of salivary glands. Phospholipid hydroperoxides were prepared from dilinoleoyl-phospholipids. Data are expressed as the mean ± S.E. of three different preparations, each assayed in duplicate. No significant difference ($p<0.05$) was observed between glands.

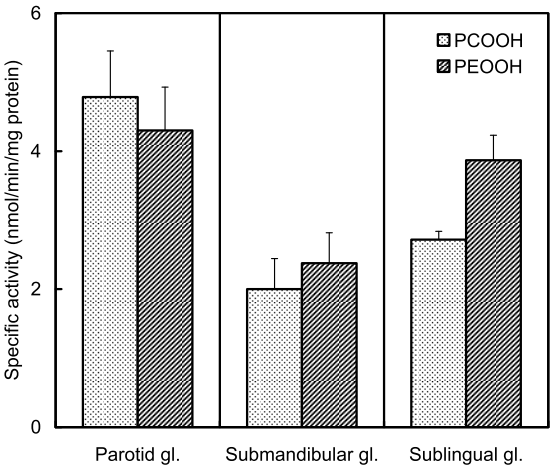

Fig. 5 Specific activities of phospholipid hydroperoxide glu tathione peroxidase toward phosphatidylcholine hydro peroxide (PCOOH) and phosphatidylethanolamine hydroperoxide (PEOOH) in mitochondrial fractions of salivary glands. Phospholipid hydroperoxides were prepared from dilinoleoyl-phospholipids. Data are expressed as the mean ± S.E. of three different preparations, each assayed in duplicate. Significant difference ($p<0.05$) was observed in the sublingual gland but not in parotid and submandibular glands.

activity was much lower than cGPx activity, especially in the cytosolic fraction. Furthermore, apparent second order rate constants of PHGPx for these compounds are much higher than that of cGPx[26]; therefore, it is though that PHGPx primarily reduces phospholipid hydroperoxides in membrane fractions. Furthermore, Zhang *et al.*[29] suggest that a house keeping role was more likely for PHGPx than other GPxs because PHGPx activity is well conserved in different age groups compared with cGPx and glutathione *S*-transferase. On the other hand, functions of PHGPx other than as an antioxidant have been revealed in various tissues[30~32]; however, the functions of cytosolic PHGPx in salivary glands are unclear at present.

In this paper, we demonstrated that PHGPx activity was widely distributed in cytosolic and membrane fractions, whereas cGPx activity was mainly distributed in the cytosolic fraction, suggesting that cGPx and PHGPx share a role in the reduction of hydroperoxide in salivary glands[12]. Further studies are necessary to clarify the functional significance of differences in the distribution profiles of GPxs and the physiological functions of enzymes other than as antioxidants in salivary glands.

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ラット唾液腺の細胞質型グルタチオンペルオキシダーゼ活性およびリン脂質ヒドロペルオキシドグルタチオンペルオキシダーゼ活性

八代耕児　北村圭司　神谷真子
亀山泰永　藤田　厚

ラット唾液腺における細胞質型グルタチオンペルオキシダーゼ（cGPx）とリン脂質ヒドロペルオキシドグルタチオンペルオキシダーゼ（PHGPx）の分布を検討した。三大唾液腺のうち，顎下腺と舌下腺では高い cGPx 活性が認められたが，耳下腺では他の唾液腺の約30%の活性であった。cGPx は主として細胞質画分に分布した。一方，PHGPx 活性は顎下腺，耳下腺，舌下腺の順に高い活性を示した。舌下腺の PHGPx 活性は cGPx 活性とは異なり，他の唾液腺よりかなり低かった。PHGPx 活性は細胞質画分，ミトコンドリア画分，ミクロソーム画分に広く分布した。顎下腺ではこれらの画分でほぼ同程度の活性を示したが，耳下腺細胞質画分と舌下腺ミトコンドリア画分の活性は他の画分より低かった。細胞質画分とミトコンドリア画分の PHGPx 活性はリン脂質種に対する特異性が低く，細胞内で生じる種々のリン脂質ヒドロペルオキシドの還元処理に好都合である。cGPx と PHGPx の腺特異的な分布は，唾液分泌能に依存する酸素消費量の違いや PHGPx アイソザイムの分布差から生じるものと考えられる。さらに，唾液腺の cGPx と PHGPx は抗酸化酵素として役割を分担しながらヒドロペルオキシドの消去を担っており，cGPx は細胞質で生じる水溶性ヒドロペルオキシドを，PHGPx は細胞内の種々の場で生じるリン脂質ヒドロペルオキシドを還元処理していることが示唆された。

キーワード：ヒドロペルオキシド，細胞質型グルタチオンペルオキシダーゼ，リン脂質ヒドロペルオキシドグルタチオンペルオキシダーゼ，唾液腺

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