

# Novel Functions of Mitochondrial Phospholipid Hydroperoxide Glutathione Peroxidase (PHGPx) as an Anti-Apoptotic Factor

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Phospholipid hydroperoxide glutathione peroxidase (PHGPx) which is a selenoprotein and a key enzyme in the protection of biomembranes exposed to oxidative stress. Involvement of mitochondrial PHGPx in apoptosis was studied using RBL2H3 cells that overexpressed mitochondrial PHGPx. The elevation of hydroperoxides in mitochondria, release of cytochrome c, the activation of caspase and the fragmentation of DNA associated with apoptosis sequentially occurred in control cells upon exposure of cells to hypoglycemia with 2-deoxyglucose (2DG). Overexpression of mitochondrial PHGPx prevented the rise of hydroperoxide in the earliest event of apoptosis and then blocked apoptosis. Mitochondrial PHGPx overexpressing cells were also resistant to apoptosis induced by etoposide, staurosporine and UV irradiation, which mediate apoptosis through the mitochondrial pathway. These results indicate that mitochondrial PHGPx might play a critical role as an anti-apoptotic factor in the mitochondrial-death pathways. PHGPx was exclusively expressed in the mitochondria of human spermatozoa. We found dramatic decrease in the expression of mitochondrial PHGPx in the spermatozoa of 10% of infertile males. Mitochondrial PHGPx-deficient spermatozoa lost the mitochondrial membrane potential and showed an abnormal mitochondrial morphology. Failure of the expression of mitochondrial PHGPx might cause a dysfunction of the mitochondria in spermatozoa and a defect in spermatogenesis in which apoptosis occurs.

**Key words** — glutathione peroxidase, apoptosis, mitochondria, spermatozoa, infertile

Mitochondria are a major physiological source of reactive oxygen species (ROS), which can be generated during mitochondrial respiration. It has recently been shown that ROS modulate the physiological state of cells and influence cell death.<sup>1)</sup> A relationship between ROS and apoptosis has been suggested by many experimental findings. Pro-oxidant agents, such as hydrogen peroxide, diamide, etoposide and semiquinones<sup>2,3)</sup> induce apoptosis. Other apoptotic stimuli such as treatment with tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) and ceramide also elevate intracellular levels of ROS.<sup>4,5)</sup> Antioxidants, such as *N*-acetylcysteine (NAC) suppress apoptosis by acting as scavengers of ROS, and their actions provide additional evidence that ROS act as signaling molecules to initiate apoptosis. Mitochondrial antioxidant enzymes strictly regulate the production of ROS in mitochondria. Two types of glutathione peroxidase (GPx), namely, cytosolic GPx (cGPx) and

phospholipid hydroperoxide glutathione peroxidase (PHGPx), are located in mitochondria. PHGPx is the only known intracellular antioxidant enzyme that can directly reduce peroxidized phospholipid and cholesterol in membrane.<sup>6)</sup> Therefore, PHGPx that can reduce lipid hydroperoxide rather than cGPx is thought to contribute to the enzymatic defenses against oxidative damage to mitochondrial membrane.<sup>7)</sup> However, the role of PHGPx in mitochondria has not been fully characterized.

We conducted a series of experiments to clarify the role of PHGPx in mammalian cells using established non-mitochondrial PHGPx overexpressing cells and mitochondrial PHGPx overexpressing cells.<sup>8–10)</sup> Overexpression of non-mitochondrial PHGPx suppresses the activity of 5-lipoxygenase by reducing lipid hydroperoxides in nucleus,<sup>8)</sup> while overexpression of mitochondrial PHGPx also protects the cell from necrotic death caused by chemical hypoxia.<sup>9)</sup>

The present report reviews our recent research on the novel functions of mitochondrial PHGPx at

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two points. We first demonstrated that hydroperoxide produced in mitochondria play a crucial role in the initiation of the apoptosis<sup>10</sup> and mitochondrial PHGPx suppressed the apoptotic cell death. A second study demonstrated that expression of mitochondrial PHGPx is extremely low in spermatozoa of infertile males and, as an anti-apoptotic factor, it could participate in the spermatogenesis which apoptosis might be involved at various stages of spermatogenesis.

### Induction of Apoptosis by Hypoglycemia

Cell death of RBL2H3 cells was induced by chemical anoxia with 2DG.<sup>11</sup> Numerous cells were induced by apoptosis after incubation with 2DG for 8 h and accounted for approximately 80% of the total within 12 h. Overexpression of mitochondrial PHGPx in RBL2H3 markedly prevented the apoptosis and more than 90% remained viable at 12 h. Overexpression of non-mitochondrial PHGPx, on the other hand, failed to prevent apoptosis.

Release of cytochrome *c* and the activation of caspase-3, a common effector of apoptosis, were detectable in control cells at 4 h and 6 h after the start of exposure to 2DG, respectively, while cytochrome *c* release and caspase activation were completely inhibited by the overexpression of mitochondrial PHGPx.

To rule out the possibility that resistance to apoptosis of mitochondrial PHGPx-overexpressing cells was due to factors other than the overexpression of mitochondrial PHGPx, we investigated 2DG-induced apoptosis of mitochondrial PHGPx-overexpressing cells after treatment of cells with diethylmalate (DEM). DEM depletes the intracellular glutathione and inhibits the activity of glutathione peroxidase. Resistance of mitochondrial PHGPx-overexpressing cells to apoptosis was abolished upon inhibition of PHGPx activity by DEM. Thus, overexpression of PHGPx in mitochondria appeared to contribute to protection from 2DG-induced apoptosis.

### Production of Superoxide and Hydroperoxide in Response to 2DG

The release of cytochrome *c* and the activation of caspase-3 were inhibited in mitochondrial PHGPx-overexpressing cells. Thus, the interruption of the apoptotic-signaling pathway in RBL2H3 cells seems to occur prior to the release of cytochrome *c*. Marked increases in the levels of intracellular superoxide and hydroperoxide were observed prior to

cytochrome *c* liberation after the exposure of cells to 2DG. Superoxide was detectable in control and mitochondrial PHGPx-overexpressing cells within 1 h after the exposure to 2DG and its level increased at a steady rate for up to 4 h. However, there were no differences in rates of production of superoxide among control and mitochondrial PHGPx-overexpressing cells. Hydroperoxide also accumulated in whole cells and also in mitochondria after exposure to 2DG. It accumulated more slowly than superoxide in whole cells and its production was detected at 2h after the addition of 2DG. Overexpression of mitochondrial PHGPx significantly suppressed the production of hydroperoxide. In isolated mitochondria, the elevation of hydroperoxide level was observed in control cells, however, no rise in hydroperoxide was found in mitochondria overexpressed PHGPx.

Considerable evidence has accumulated to suggest that ROS in mitochondria might act as mediators of apoptosis.<sup>12</sup> The ROS from mitochondria might be responsible for a close association between the dysfunction of mitochondria and cell death.<sup>13</sup> This possibility is supported by the observation that TNF $\alpha$ -, Fe<sup>2+</sup>-, amyloid  $\beta$ -peptide or alkaline-mediated apoptosis are blocked by Mn-SOD, which scavenges superoxide that has leaked from the mitochondrial respiratory chain.<sup>14</sup> 2DG-induced apoptosis was blocked when accumulation of hydroperoxide in mitochondria was prevented by overexpression of mitochondrial PHGPx. This suggests that production of hydroperoxide in mitochondria might be an important early trigger of apoptosis.

### Effects of Various Apoptotic Agonists on the Apoptosis of Mitochondrial PHGPx-Overexpressing Cells

Studies of Apaf-1 and caspase-9 knockout mouse revealed that two independent apoptotic pathways operate upstream of the critical activation of caspase-3.<sup>15,16</sup> In the first pathway involved in Fas-induced-apoptosis, the activation of caspase-3 by caspase-8 is independent of any proapoptotic factors in mitochondria. In the second pathway (mitochondrial-death pathway) involved in various forms of apoptotic agents such as etoposide, staurosporine and UV irradiation, caspase-3 is activated by the mitochondrial apoptotic factors such as cytochrome *c*, caspase-9 and Apaf-1. Overexpression of mitochondrial PHGPx prevented apoptosis *via* the mitochondrial-death pathway upon treatment with etoposide, UV irradiation, staurosporine or glucose deprivation.

By contrast, the Fas-mediated apoptotic pathway was intact in mitochondrial PHGPx-overexpressing cells as well as control cells. Mitochondrial PHGPx is irrelevant to Fas-induced apoptosis. Thus, mitochondrial PHGPx selectively prevented apoptosis *via* the mitochondrial-death pathway, in which production of hydroperoxide is apparently one of the key apoptotic signals. Recent reports also showed the generation of hydroperoxide by the treatment with etoposide or staurosporine.<sup>17,18)</sup>

Release of cytochrome *c* from mitochondria is generally accepted as a crucial step in the activation of apoptosis in various model systems,<sup>19)</sup> However, it is poorly understood. Yang and Cortopassi demonstrated that canonical inducers of a mitochondrial permeability transition (MPT), such as *t*-butyl hydroperoxide (100  $\mu$ M), induce the swelling-dependent release of cytochrome *c* from isolated mitochondria.<sup>20)</sup> ROS might induce the dissociation of cytochrome *c* from inner membranes of mitochondria that contain significant amounts of highly unsaturated fatty acid at the initial step.<sup>21)</sup> At the following step, oxidation of mitochondrial membrane lipids might trigger the opening of MPT pores, including the adenine nucleotide translocator (ANT), with release of cytochrome *c* from mitochondria.<sup>22)</sup> The present study demonstrates that the generation of ROS is an early and critical event in apoptosis.

### Failure of the Expression of PHGPx in the Spermatozoa of Infertile Males

Human spermatozoa in ejaculated semen contain large amounts of PHGPx. Immunostaining revealed that PHGPx was mainly distributed in mitochondria of the mid-piece of human spermatozoa, confirming previous studies of rat epididymal spermatozoa by Godeas *et al.*<sup>23)</sup> The abundance of PHGPx in spermatozoa suggests that this unique antioxidant enzyme might be involved in the function and development of spermatozoa for the following reasons. The first is that PHGPx is one of the major selenoproteins in spermatides and selenium deficiency results in impairment of both the production and motility of spermatozoa. The second point is that extensive peroxidation of lipid in spermatozoa might be connected with the dysfunction of spermatozoa of infertile males.<sup>24)</sup>

We found a dramatic decrease in the expression of PHGPx in spermatozoa from 7 of 73 infertile men by immunoblotting analysis. The amounts of PHGPx were from 20% to 10% of those in the spermatozoa of fertile volunteers. No defect in the expression of

PHGPx in spermatozoa was found in 31 normal fertile volunteers, nor were there decreases in expression of other mitochondrial proteins, such as Mn-SOD and voltage-dependent anion channel (VDAC).

When infertile men with PHGPx-defective spermatozoa were classified according to the criteria of the World Health Organization, all of seven subjects with PHGPx-defective spermatozoa belonged to the group with oligo-asthenozoospermia in which both the number and the motility of spermatozoa are significantly below normal. These observations suggest that insufficient expression of PHGPx in spermatozoa might lead to serious impairment of fertilization.

The membrane potential in mitochondria of PHGPx-deficient spermatozoa is decreased as compared to that of normal spermatozoa. The ultrastructure of mitochondria was assessed by transmission electron microscopy and morphology of the mitochondria in the mid-piece of PHGPx-defective spermatozoa was extremely variable and abnormal compared to that of normal spermatozoa. Mitochondrial dysfunction could be a direct cause of the impairment of the spermatozoa functions. Especially, insufficient expression of mitochondrial PHGPx in spermatozoa might lead to serious impairment of fertilization.

Another major change associated with reduced expression of PHGPx was a marked decrease in the number of spermatozoa in the ejaculate of infertile males. Spermatogenesis is a complex process and little information is available about its regulation at the molecular level. Recent studies with knockout mice suggest that apoptosis might be closely linked to this regulation. Mutation of genes for other proteins in the Bcl-2 family such as *Bax*,<sup>25)</sup> *bcl-2*<sup>26)</sup> and *bcl-xL*<sup>27)</sup> leads to the accumulation of pre-meiotic germ cells and disruption of the germ cell differentiation. When PHGPx activity and the expression of mRNA for PHGPx in the rat testis was diminished by the administration of ethane dimethanesulfonate (EDS),<sup>28)</sup> EDS accelerated the apoptosis of germ cells during the formation of rat pachytene spermatocytes and spermatides.<sup>29)</sup> These results suggest the involvement of mitochondria PHGPx as an anti-apoptotic factor in spermatogenesis, and they also suggest that failed induction of the expression of mitochondrial PHGPx as an anti-apoptotic factor in late spermatocytes might have a major effect on spermatogenesis.

## CONCLUSION

We propose that mitochondrial PHGPx as one kind of selenoprotein participate in the regulation of apoptosis through mitochondria death pathway. Our results also indicate that lipid hydroperoxide produced in mitochondria might be the earliest signaling molecules to initiate apoptosis. The failure of the expression of mitochondrial PHGPx would cause diseases in which apoptosis is involved. Our observations suggest a new role for mitochondrial PHGPx in male fertility, and the clinical significance of PHGPx-expression in spermatozoa for infertile males was also demonstrated.

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