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Transforming growth factor-β1 induces the non-classical secretion of peroxiredoxin-I in A549 cells ☆

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Abstract

Recent studies found that peroxiredoxin-I (Prx-I) is secreted from A549 cells although it does not contain a signal peptide and is known to be a cytosolic protein. Transforming growth factor-β1 (TGF-β1) treatment dramatically enhanced Prx-I secretion from A549 cells, and this effect was not inhibited by brefeldin A. Further investigation revealed that A549 cells constitutively secrete TGF-β1. Furin, a TGF-β1-converting enzyme, was also highly activated in A549 cells. Ectopic expression of α_1-antitrypsin Portland (α_1-PDX), a potent furin inhibitor, blocked both TGF-β1 activation and Prx-I secretion. Our findings collectively suggest that non-classical secretion of Prx-I is induced by TGF-β1, which is constitutively activated by furin in A549 cells.

Keywords: Peroxiredoxin-I; ER/Golgi-independent secretory pathway; Non-classical secretion; A549 cells; TGF-β1; Furin

Many proteins are constitutively or inducibly secreted from cells. Typical secretory proteins are directed to the endoplasmic reticulum (ER) by N-terminal signal peptides [1]. From the ER, these proteins are processed for vesicle transport in the Golgi apparatus and then transported to the plasma membrane, where the vesicles fuse to the plasma membrane, releasing the proteins into the extracellular space; this process is known as the classical or ER/Golgi-dependent secretory pathway [2,3]. However, many proteins are also secreted in the absence of a signal peptide; this is called the non-classical or ER/Golgi-independent secretory pathway [4]. These proteins are insensitive to brefeldin A, an inhibitor of the classical secretory pathway [5,6]. However, the molecular mechanism and the molecules involved in this pathway have not been clearly elucidated.

Peroxiredoxin (Prx) is a new type of antioxidant protein capable of reducing hydrogen peroxide, peroxinitrite, and organic hydroperoxide by using thioredoxin (Trx) as an electron donor [7–9]. In addition to acting as antioxidant enzymes, Prxs regulate H_2O_2-mediated signaling cascades and are associated with diverse cellular functions, such as cell proliferation, differentiation, immune responses, growth control, apoptotic processes, and numerous unidentified functions [10–13]. The six mammalian Prx isoforms (Prx-I to VI) can be classified into three groups based on the number and position of the Cys residues that participate in catalysis (2-Cys, atypical 2-Cys, and 1-Cys) [8,9]. Among them, only Prx-IV contains the N-terminal signal sequence that identifies it as a classical secretory protein [14]. We previously showed that Prx-I was overexpressed in A549 lung adenocarcinoma cells and human lung cancer tissues [15]. A subsequent report identified Prx-I in a significant number of serum samples from lung cancer patients and in A549-conditioned media [16], even though Prx-I lacks a signal peptide and is known to exist in the cytosol. Here, we investigated the non-classical secretion

☆ *Abbreviations:* α1-PDX, α1-antitrypsin Portland; ER, endoplasmic reticulum; FGF, fibroblast growth factor; IL-1β, interleukin-1β; Prx, peroxiredoxin; PAGE, polyacrylamide gel electrophoresis; TGF-β1, transforming growth factor-β1; Trx, thioredoxin.

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of Prx-I. In A549 cancer cells, we found that addition of transforming growth factor-β1 (TGF-β1)-induced secretion of Prx-I, and that TGF-β1 is constitutively activated by the elevated activity of furin, a TGF-β1-converting enzyme [17,18].

Materials and methods

Cell culture and preparation of conditioned media. Human cells including BEAS 2B, A549, MCF7, Hep3B, HeLa, and SH-SY5Y (obtained from the American Type Culture Collection) were cultured in RPMI 1640 (GibcoBRL, Grand Island, NY) supplemented with 10% fetal bovine serum at 37 °C under a humidified atmosphere of 95% air and 5% CO_2 (v/v). Cultured cells were harvested at 90–95% confluence and washed twice in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4). For preparation of conditioned media, cells grown in 60 mm dishes were washed three times with serum-free RPMI 1640 and then covered with 3 ml of serum-free media with or without TGF-β1 (Calbiochem, Darmstadt, Germany), IL-6 (Promega, Madison, WI) or brefeldin A (Epicentre Biotechnologies, Madison, WI). The conditioned media were collected and centrifuged at 15,000 rpm for 15 min for removal of non-adherent cells and debris. The supernatants were concentrated to a final volume of 100 μl using a 10 kDa cutoff Centricon (Millipore, Billerica, MA).

Immunoblotting and antibodies. Protein samples were incubated with SDS sample buffer at 95 °C for 5 min and then subjected to SDS–PAGE. The resolved proteins were transferred to a nitrocellulose membrane, which was incubated for 2 h with 5% (w/v) non-fat dried milk in TBST solution (25 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% (w/v) Tween 20). The membrane was washed with TBST, incubated with polyclonal primary antibody for 1 h, and then incubated with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Immunoreactive proteins were visualized using ECL reagents (Amersham Bioscience, Uppsala, Sweden). Primary antibodies used in this study were anti-Prx-I and anti-Prx-IV antibodies from LabFrontier (Seoul, Korea), anti-tubulin, anti-TGF-β1, and anti-PARP antibodies from Santa Cruze Biotechnology (Santa Cruze, CA), and anti-furin antibody from Alexis (Carlsbad, CA).

Real-time reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from BEAS 2B and A549 cells using a guanidinium thiocyanate–phenol chloroform solution (RNA STAT-60t; TELTEST, Friendswood, TX). Complementary DNA was made from 1 μg of total RNA in a reaction volume of 20 μl using Iscript (Bio-Rad, Hercules, CA). Real-time RT-PCR analyses were performed on a CHROMO4 instrument (MJ Research, Boston, MA). Each 50 μl reaction volume contained 1 μl of cDNA template, 1× QuantiTect SYBR green PCR Master Mix (Qiagen, Hilden, Germany), and 0.4 pM of each TGF-β1-specific primer (forward, 5′-CCGGGTTATGCTGGTTGTA-3′ and reverse, 5′-ACATTGACTTCCGCAAGGAC-3′). The PCR conditions consisted of 30 cycles of 94 °C for 10 s, 57 °C for 30 s, and 72 °C for 30 s. The results were analyzed using the Opticon Monitor software (MJ Research, Boston, MA), with data normalized with regard to the expression levels of actin (the internal standard).

Furin activity assay. A549 or BEAS 2B cells were sonicated in buffer containing 50 mM Tris, pH 7.0, and 2 mM $CaCl_2$. Each protein extract (15 μg) was incubated with 100 μM pERTKR-MCA (Peptide International, Osaka, Japan) for 30 min. Furin activity was measured at 380 nm for excitation and at 460 nm for emission using a fluorometer (SpectraMax; Molecular Devices, Sunnyvale, CA).

Adenovirus-mediated overexpression of flag-α1-PDX. At 50% confluence, A549 cells were infected with adenovirus-flag-α1-PDX (Affinity BioReagents, Golden, CO) and cultured for 8 h with agitation every 30 min. Cells were cultured in fresh media for 18 h and then in serum-free media for 8 h. These media were collected as conditioned media. Cells were harvested and directly lysed with SDS-sample buffer, and ectopic expression of flag-α1-PDX was confirmed by immunoblotting with anti-flag antibody (Sigma, St. Louis, MO).

Results

Non-classical secretion of Prx-I from A549 adenocarcinoma cells is enhanced by TGF-β1

Since we previously found that Prx-I is secreted from A549 lung cancer cells but not from non-cancerous BEAS 2B lung cells [16], we examined Prx-I secretion in other cancer cells such as breast cancer cells (MCF7), hepatocarcinoma cells (Hep3B), cervical carcinoma cells (HeLa), and neuroblastoma cells (SH-SY5Y). Cells were cultured in serum-free medium for 12 h and the conditioned media were analyzed by Western blotting. Prx-I was observed only in the A549-conditioned media but not in the other conditioned media (Fig. 1A). Tubulin was not detected in the conditioned media (Fig. 1A), which indicates the absence of cell lysis resulting from physical damage. The lack of tubulin was confirmed in all conditioned media

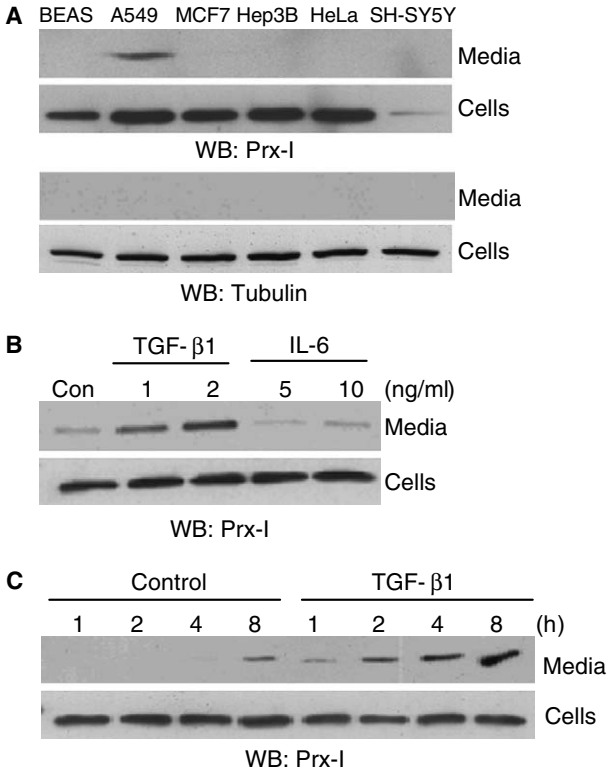

Fig. 1. TGF-β1 enhances the secretion of Prx-I from A549 cells. (A) Prx-I was secreted from A549 lung cancer cells but not from non-cancerous BEAS 2B lung cells or other cancer cells such as MCF7, Hep3B, HeLa, and SH-SY5Y cells. Tubulin was not detected in the conditioned media. (B) Prx-I secretion from A549 cells was increased by treatment with TGF-β1 but not IL-6. A549-conditioned media were prepared by culturing cells for 8 h in serum-free media in the absence and presence of TGF-β1 (1 and 2 ng/ml) or IL-6 (5 and 10 ng/ml). Proteins in the conditioned media and cells were separated by 12% SDS–PAGE, and Western blot analysis was performed with an anti-Prx-I antibody. (C) A549-conditioned media prepared in the absence (control) and presence of 2 ng/ml TGF-β1 were collected at 1, 2, 4, and 8 h, and analyzed by Western blotting with an anti-Prx-I antibody. This set of immunoblots is representative of three independent experiments.

used in this study. Interestingly, Western blotting revealed that the expression levels of Prx-I were similar in A549, MCF7, Hep3B, and HeLa cells (Fig. 1A), indicating that overexpression of Prx-I does not account for its secretion from A549 cells. Because cytokines such as TGF-β1 and IL-6 are known to be elevated in lung cancer [19–22], we examined whether these cytokines could induce secretion of Prx-I from A549 lung cancer cells. A549 cells were cultured in the presence of TGF-β1 (1 and 2 ng/ml) or IL-6 (5 and 10 ng/ml), and Western blotting was used to detect Prx-I levels in the conditioned media. Our results revealed that TGF-β1 dose dependently enhanced Prx-I secretion, whereas IL-6 had no such effect (Fig. 1B). Cells treated with 2 ng/ml TGF-β1 showed detectable secretion of Prx-I after 1 h versus 8 h in control cultures (Fig. 1C). The time-dependent secretion of Prx-I was clearly observed from A549 cells after TGF-β1 treatment (Fig. 1C). Quantitative analysis from three independent experiments determined that $0.3 \pm 0.1\%$ of total cellular Prx-I was secreted into media in 8 h culture in the presence of 2 ng/ml TGF-β1. Prx-I secretion was not observed in other cancer cells such as MCF7, Hep3B, and HeLa cells even after TGF-β1 treatment (data not shown).

Western blotting also revealed that Prx-IV, which is the only peroxiredoxin isoform containing a signal peptide, was secreted from A549 cells, but this secretion was not enhanced by treatment with 2 ng/ml TGF-β1 (Fig. 2A). To examine whether the increased secretion of Prx-I could be an indirect result of cell death, we detected the 85 kDa fragment generated by proteolytic processing of PARP during cell death. The fragment could be detected in control cells cultured for 24 h in serum-free media but not in cells cultured in the presence of TGF-β1 (Fig. 2B), indicating that TGF-β1 actually reduced cell death while increasing Prx-I secretion.

When A549 cells were cultured in the presence of 1 or 2 μg/ml brefeldin A, a specific inhibitor of the classical ER/Golgi pathway, TGF-β1-induced Prx-I secretion was unchanged, whereas TGF-β1-induced MMP-9 secretion, which is known to be mediated by the classical secretory pathway, was completely blocked (Fig. 2C).

TGF-β1 is constitutively expressed and activated in A549 cells

Since TGF-β1-induced Prx-I secretion, we examined mRNA level of TGF-β1 in BEAS 2B and A549 cells. Quantitative real-time RT-PCR revealed that the mRNA levels of TGF-β1 in A549 cells were approximately 2.5-fold those found in BEAS 2B cells (Fig. 3A). In addition, most of the proTGF-β1 was processed into active TGF-β1 in A549 cells, whereas processed TGF-β1 was hardly detected in BEAS 2B cells (Fig. 3B). Secreted TGF-β1 was also detected in conditioned media from A549 cells but not from BEAS 2B cells (Fig. 3B). TGF-β1 seems to be constitutively activated and secreted in A549 cells, which may induce non-classical secretion of Prx-I.

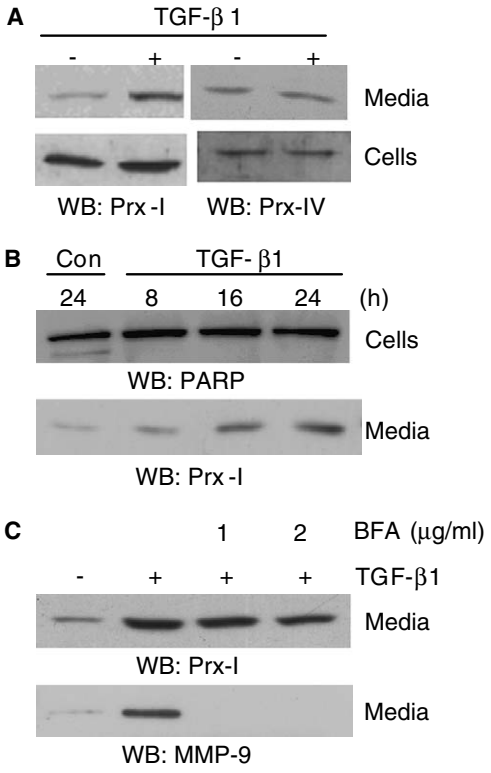

Fig. 2. Non-classical secretion of TGF-β1 in A549 cells. (A) Prx-IV was also secreted from A549 cells, but was not altered by TGF-β1 treatment. (B) PARP fragments (indicative of cell death) were detected in lysates from control A549 cells cultured with serum-free media for 24 h (Con) but not from TGF-β1-treated cells. (C) Prx-I secretion was enhanced by TGF-β1 (lanes 1 and 2), and this effect was not inhibited by 1 or 2 μg/ml brefeldin A (BFA, lanes 3 and 4). TGF-β1-induced MMP-9 secretion was completely inhibited by brefeldin A (lower panel). These immunoblots are representative of three independent experiments.

Activated furin mediates the activation of TGF-β1 in A549 cells

Since the majority of TGF-β1 was activated in A549 cells, we examined the possible involvement of furin, a known TGF-β-converting enzyme [17,18]. Furin is expressed as a 110 kDa precursor that is activated by auto-proteolytic processing. Western blotting revealed that the 97 kDa activated furin predominated in A549 cells, whereas the 110 kDa furin precursor was primarily seen in BEAS 2B cells (Fig. 4A). Measurement of furin activity using the fluorogenic substrate, pERTKR-MCA, revealed that furin activity was ~3-fold higher in A549 cells than in BEAS 2B cells (Fig. 4B). Furthermore, when flag-tagged α1-PDX, a potent furin inhibitor [23], was ectopically expressed in A549 cells, both TGF-β1 activation and Prx-I secretion were effectively blocked (Fig. 4C).

Discussion

In the early 1990s, it was first reported that IL-1 and galectin could be exported from cells even though these proteins lacked signal peptides [24,25]. Since then,

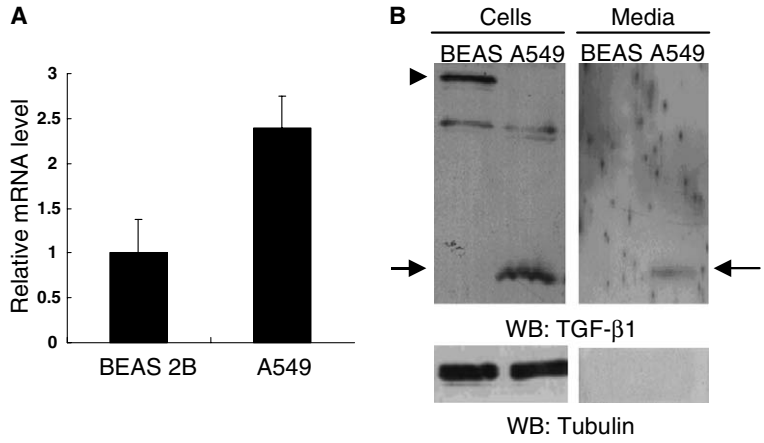

Fig. 3. TGF-β1 is up-regulated and constitutively activated in A549 cells. (A) Real-time RT-PCR showed that the mRNA levels of TGF-β1 were 2.5-fold higher in A549 cells than in BEAS 2B cells. Data were obtained from three independent experiments and the histogram shows the mean ± SD. (B) Majority of pro-TGF-β1 was processed into active TGF-β1 (arrow) in A549 cells, but remained in its precursor form in BEAS 2B cells (arrowhead). Secreted TGF-β1 could be detected in A549-conditioned media. These immunoblots are representatives of three independent experiments.

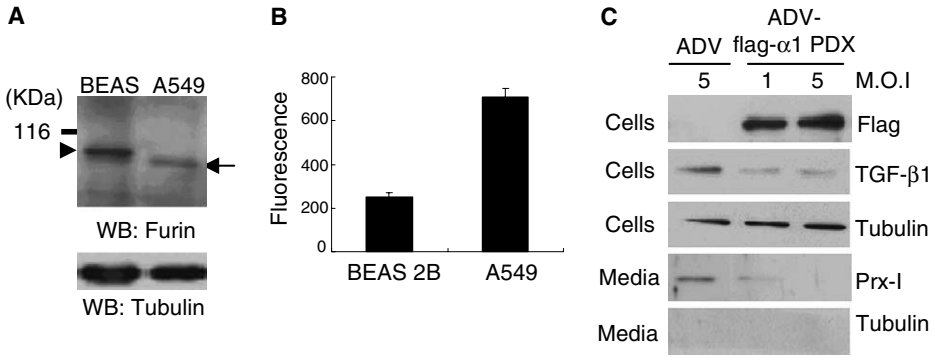

Fig. 4. Furin activates TGF-β1 in A549 cells. (A) Western blotting with an anti-furin antibody revealed higher levels of processed mature furin (arrow) in A549 cells, but high levels of pro-furin (arrowhead) in BEAS 2B cells. (B) Furin activity was ~2.5-fold higher in A549 cells than in BEAS 2B cells, as measured by cleavage of a fluorogenic substrate (pERTKR-MCA). Data were obtained from three independent experiments and the histogram shows the mean ± SD. (C) A549 cells were infected with adenovirus encoding flag-tagged α1-PDX (1 or 5 M.O.I.), and cells and conditioned media were prepared and analyzed by Western blotting with anti-flag, anti-TGF-β1, anti-tubulin, or anti-Prx-I antibody. The levels of activated TGF-β1 in cells and secreted Prx-I in media were dramatically reduced in cells expressing α1-PDX. These immunoblots are representatives of three independent experiments.

non-classical ER/Golgi-independent secretion has been reported for an increasing number of proteins, including the pro-angiogenic mediators FGF-1 and FGF-2 [26,27], macrophage migration inhibitory factor (MIF) [28], nuclear proteins such as HMGB1 [29], viral proteins [30,31], and various parasitic surface proteins potentially involved in the regulation of host cell infection [32]. Here, we demonstrate that Prx-I should be included in this list, as it is secreted from A549 lung cancer cells through the non-classical pathway. Although we observed Prx-I secretion only from A549 cells, a previous proteomic study detected Prx-I secretion from mouse astrocytes [33], indicating that Prx-I may be secreted from other cells.

Non-classical secretion is regulated through various mechanisms [4]. For example, secretion of IL-1β is induced upon activation of monocytes [26], while secretion of MIF can be induced by a variety of stimuli, including inflammatory stimuli such as lipopolysaccharides and tumor necrosis factor [28]. In addition, secretion of galectin-1 is regulated during differentiation [34], and the secretion of FGF-1 is triggered under stress conditions such as heat shock [35]. Since Prx-I secretion was observed in A549 lung cancer cells, and cytokines such as TGF-β1 and IL-6 are elevated in lung cancer patients [19–22], we examined whether these cytokines could induce Prx-I secretion. We found that addition of TGF-β1 to the culture media dramatically induced Prx-I secretion from A549 lung cancer cells but not from the other tested cancer cells (MCF7, Hep3B, and HeLa). This TGF-β1-induced Prx-I secretion was not inhibited by brefeldin A, which inhibits the classical pathway by blocking protein transport from the ER to the Golgi [5,6]. TGF-β1 mRNA expression and activation levels were constitutively higher in A549 cells than in BEAS 2B cells. The increased secretion of Prx-I in TGF-β1-treated cells was not associated either with cell lysis or with cell death because not only tubulin was not detected in the conditioned media but also a PARP cleavage assay indicated that TGF-β1 actually increased cell viability. Collectively,

these findings indicate that constitutive secretion of activated TGF-β1 induces non-classical secretion of Prx-I from A549 cells.

TGF-β superfamily is composed of many multifunctional growth factors including TGF-βs, activins, inhibins, bone morphogenetic proteins, and others. Among the highly similar isoforms of TGF-βs (TGF-β1, -β2, and -β3), TGF-β1 is frequently up-regulated in human cancers, including lung cancer [19,20,36,37]. TGF-β1 has potent effects on cell growth and differentiation. In addition, TGF-β1 strongly induces extracellular matrix synthesis, angiogenesis, and immune response. Secreted Prx-I may associate with the functions of TGF-β1. Recently, it was reported that secreted molecules from *Fasciola hepatica*, one of which is Prx-I, induce alternative activation of macrophage and cytokine release [38].

Proteolytic processing of the TGF-β1 precursor (pro-TGF-β1) is an essential step in the formation of biologically active TGF-β1, and furin is known to act as a TGF-β1-converting enzyme [17,18]. We found that the majority of furin existed in the active form in A549 cells but not in BEAS 2B cells. Furthermore, ectopic expression of α_1-PDX, a potent furin inhibitor [23], blocked both activation of TGF-β1 and secretion of Prx-I. These results strongly suggest that activated furin is responsible for activation of TGF-β1, which induces Prx-I secretion in A549 cells. However, TGF-β1-induced Prx-I secretion via activated furin seems to be specific to A549 lung cancer cells. What remains to be explained is why the specific TGF-β1-induced, furin-mediated, Prx-I secretion is observed only A549 cells despite the fact that activated furin is present in many other cancer cells [39].

Thioredoxin is another antioxidant enzyme known to be secreted by the non-classical pathway [40]. The Prxs, which are also called thioredoxin peroxidases, are closely associated with thioredoxin-dependent reactions. Other antioxidant enzymes, including superoxide dismutase, glutathione peroxidase, and reduced glutathione, are known to exist in the extracellular space [41]. It is interesting to note that Prx-I secretion is specific to A549 lung cancer cells and that circulating serum levels of Prx-I were found in a significant number of lung cancer patients [16]. Thus, it is possible that Prx-I acts along with the other antioxidants to enhance the extracellular defense system of cancer cells against oxidative attack.

In sum, our findings show for the first time that Prx-I is secreted from A549 lung cancer cells by non-classical ER/Golgi-independent pathway and its secretion is induced by TGF-β1. These results suggest that overexpression and non-classical secretion of Prx-I in lung cancer cells may result in high antioxidant capacities and consequent high resistances to cytotoxic drugs and radiation.

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