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A thin layer chromatographic method for determining the enzymatic activity of peroxidases catalyzing the two-electron reduction of lipid hydroperoxides

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Abstract

Thiol-dependent peroxidases catalyzing the reductive detoxification of lipid hydroperoxides (LOOHs) are crucial antioxidant components of mammalian cells. There is a growing interest in manipulating expression of such enzymes to better understand their biological roles. A new approach for determining their cellular activity is described, whereby LOOH reduction kinetics are tracked by high performance thin layer chromatography with peroxide-sensitive tetramethyl-*p*-phenylenediamine detection (HPTLC-TPD). The approach was tested on a tumor cell transfectant clone (7G4) over-expressing selenoperoxidase GPx4. Timed incubation of Triton-solubilized 7G4 cells with GSH and peroxidized phosphatidylcholine (PCOOH), followed by lipid extraction, HPTLC-TPD and densitometry revealed an exponential decay of PCOOH at a rate ~80-times greater than that for GPx4-deficient controls (VC). A TPD-detectable cholesterol hydroperoxide (7α-OOH) was also reduced much faster by 7G4 than VC extracts. Spraying with H_2SO_4 after TPD revealed both 7α-OOH loss and resolved diol product (7α-OH) accumulation, the kinetics of which were identical. The approach described is relatively convenient, highly specific, and much more sensitive than conventional assays for cellular LOOH reducing enzymes.
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Keywords: Peroxidase activity; Thin-layer chromatography; Phospholipid hydroperoxide; Cholestrol hydroperoxide

1. Introduction

Lipid hydroperoxides (LOOHs) are important intermediates arising during exposure of biomembranes and lipoproteins to oxidizing enzymes (e.g. lipoxygenases) or reactive oxygen species (ROS) such as hydroxyl radical, peroxynitrite, and singlet oxygen [1,2]. Once formed, phospholipid-, glycolipid-, cholesterol-, and cholesteryl ester-derived hydroperoxides are subject to reductive degradation, which either enhances or diminishes their prooxidant potential. Iron-catalyzed one-electron reduction converts LOOHs to free radical species, which can trigger damaging free radical (chain) peroxidation reactions [2,3]. However, two-electron reduction catalyzed by various non-heme peroxidases converts LOOHs to relatively innocuous alcohol (LOH) derivatives [2]. In addition, LOOHs may undergo spontaneous or protein-facilitated translocation to other membrane or lipoprotein sites [4,5], where one- or two-electron turnover could ensue, resulting in dissemination of peroxidative stress on the one hand and attenuation on the other. The degree of partitioning between the one- and two-electron pathways will determine how a system responds to a LOOH challenge. If enzymatic antioxidant defenses are inadequate, chain reactions can proceed, resulting in amplification of peroxidative injury. In mammalian cells, enzymes that can catalyze the direct reductive detoxification of LOOHs include (i) selenium-dependent glutathione peroxidase type-4 (GPx4 or PHGPx) [6,7], (ii) non-selenium α-type glutathione S-transferases [8,9], and (iii) non-selenium glutathione or thioredoxin peroxidases containing, respectively, one or two active site cysteine residues, and collectively referred to as peroxiredoxins [10–12]. The role of these enzymes in preventing LOOH damage or regulating

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LOOH signaling activity is under intensive investigation [2,7,9–12]. Molecular genetic approaches such as gene silencing, supplementation (knock-in), or deletion (knock-out) typically call for measurement of expressed enzyme activity in target cells. Many workers still employ a classic coupled enzymatic assay with glutathione reductase or thioredoxin reductase, using NADPH as the reporting reductant [9,11,12]. However, this type of assay is not very sensitive and absolute specificity is not guaranteed. In this report, we describe a highly sensitive and selective TLC-based approach for determining cellular LOOH peroxidase activity. The method was tested on a GPx4 transfectant clone of a breast tumor line, using either peroxidized phosphatidylcholine (PCOOH) or a cholesterol hydroperoxide (7α-OOH) as substrate.

2. Materials and methods

2.1. General materials

Butylated hydroxytoluene (BHT), desferrioxamine (DFO), cholesterol (Ch), bovine brain phosphatidylcholine (PC), *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TPD), and Chelex-100 were obtained from Sigma Chemical Co. (St. Louis, MO). The photosensitizing dye aluminum phthalocyanine disulfonate ($AlPcS_2$) was supplied by Dr. J. van Lier (University of Sherbrook) as a gift. A stock mixture of protease inhibitors (Complete-Mini^{TM}) was obtained from Roche Diagnostics GmbH (Mannheim, Germany).

2.2. Preparation of PCOOH, 7α-OOH, and 7α-OH

PCOOH was prepared by singlet oxygen-mediated photoperoxidation of PC. A chloroform solution of PC (2 mg/ml) in a 10-ml beaker was irradiated in the presence of 5 μM $AlPcS_2$ at 4 °C, using a 90-W quartz-halogen light source and a fluence rate of 25 mW/cm^2 [13]. The typical exposure time was 30 min (fluence ∼40 J/cm^2). PCOOH was separated from parent lipid by normal-phase HPLC, using a silica column (250 mm × 4.6 mm; 5 μm particles) from Supelco (Bellefonte, PA). The mobile phase consisting of isopropanol/hexane/water (20:15:4, v/v) was delivered isocratically at a flow rate of 1.0 ml/min. Absorbance of the effluent at 206 and 234 nm was monitored, the latter reflecting hydroperoxide-conjugated dienes. PCOOH species emerged in a single broad peak exhibiting maximal A_{234}/A_{260} ratio. The PCOOH fraction was isolated and analyzed iodometrically for total peroxide content.

Cholesterol hydroperoxides were generated by $AlPcS_2$-sensitized photoperoxidation of Ch in liposomal form [13,14]. 3β-Hydroxycholest-5-ene-7α-hydroperoxide (7α-OOH) was separated from other peroxides in two chromatographic steps: (1) reversed-phase HPLC using a C_{18} column (250 mm × 4.6 mm; 5 μm particles) from Beckman (San Ramon, CA), methanol/isopropanol/acetonitrile/water (70:12:11:7, v/v) as the mobile phase, and UV (212 nm) detection; (2) normal-phase HPLC using a Beckman silica column (250 mm × 10 mm; 5 μm particles), hexane/isopropanol (95:5, v/v) as the mobile phase, and UV (212 nm) detection. Cholest-5-ene-3β,7α-diol (7α-OH) was generated by triphenylphosphine reduction of 7α-OOH and isolated by reversed-phase HPLC [15]. Product identities were confirmed by proton NMR and mass spectrometry [15].

2.3. Iodometric analysis

Total peroxide content of stock PCOOH or 7α-OOH solutions was determined by iodometric assay. Each sample was dried under argon and dissolved in 0.3 ml of deaerated acetic acid/chloroform (3:2, v/v). To this was added 20 μl of 0.45 M KI in deaerated water. After a 10 min reaction period, the solution was mixed with 0.9 ml of 20 mM cadmium acetate to minimize autoxidation of remaining iodide. Following centrifugation, the 334 nm absorbance of triiodide that had been stoichiometrically generated from peroxide was measured. LOOH quantitation was based on an extinction coefficient of 22,500 M^{-1} cm^{-1} for triiodide [16].

2.4. Cell culture

COH-BR1 cells, a human breast tumor epithelial line, were obtained from Dr. J. Doroshow (City of Hope Cancer Center). Wild type cells that had been transfected with a construct encoded for only geneticin resistance or geneticin resistance plus the mitochondrial form of human GPx4 were grown in DME/F12 medium supplemented with 60 nM sodium selenite and geneticin (0.5 mg/ml) [17]. A *gpx*4-transformed clone (7G4) and a vector control clone (VC) were used in this study. Using 7α-OOH as a substrate and reversed-phase high-performance liquid chromatography with mercury cathode electrochemical detection [HPLC-EC(Hg)] for analysis, we showed previously that the GPx4 activity of 7G4 cells was at least 80-times greater than that of VC counterparts and that the bulk of this activity was localized in mitochondria [17,18]. HPTLC-based activity determinations were carried out on exponentially growing cells.

2.5. GPx4 assay reaction conditions

Cells were lysed in Chelex-treated PBS buffer [25 mM sodium phosphate/125 mM NaCl (pH 7.4)] containing 1.0 mM EDTA, 0.1 mM DFO, 25 μM BHT, 0.3% (w/v) Triton X-100, and protease inhibitors (Complete-Mini^{TM}, 1 tablet/10 ml of buffer). Assays were carried out using either PCOOH or 7α-OOH as peroxide substrate. Reaction mixtures (0.5 ml) contained 5.0 mM GSH, cell lysate (100 μg of protein with PCOOH; 750 μg of protein with 7α-OOH), and 40 μM PCOOH or 100 μM 7α-OOH (added last) in pH 7.4 Chelexed PBS containing 50 μM DFO and 1.0 mM EDTA (PBS/DFO/EDTA). At various times during incubation at 37 °C, 0.1 ml samples were removed and extracted

with 0.4 ml of ice-cold chloroform/methanol (2:1, v/v), as described [13,14]. After centrifugation, 0.45 ml of the organic phase was recovered, dried under nitrogen, and stored at −20 °C until analyzed for remaining PCOOH or in the case of 7α-OOH, remaining peroxide and generated 7α-OH.

2.6. HPTLC procedures

Sample extracts from PCOOH-containing assay mixtures were analyzed for the peroxide by means of high-performance thin layer chromatography with TPD spray detection (HPTLC-TPD). This approach is based on peroxide-induced oxidation of TPD to a radical cation chromophore (Wurster's blue), as catalyzed by iron and/or other redox metal ions on the HPTLC plate [19]. Silica-gel 60 HPTLC plates (10 cm × 10 cm; 0.2 mm layer thickness) obtained from EM Science (Gibbston, NJ) were dried at 110 °C for 1 h before use. Dried samples were dissolved in hexane/ethanol (4:1, v/v) and applied to a plate in a hairline nitrogen stream, using a Linomat IV programmable applicator (Camag Scientific, Wilmington, NC). Chromatography was carried out using chloroform/methanol/acetic acid/water (100:75:7:4, v/v) as the mobile phase. Immediately thereafter, the plate was dried under a stream of argon and sprayed with a fine mist of freshly prepared 1% (w/v) TPD in methanol/water/acetic acid (50:50:1, v/v). After flushing again with argon, the plate was clamped to a clear glass cover in order to minimize air exposure. When analyte signal intensity appeared maximal relative to background (typically ∼15 min), densitometric scanning was carried out using an Alpha Innotech MultiImager (San Leandro, CA). A dose–response curve obtained with a PCOOH standard was used for quantifying assay peroxide, measurements being restricted to the linear dynamic range [19]. PCOOH molecular species are not resolved from one another or from their reduction products, PCOHs [19]; however, only the peroxides are detected by this approach.

For assays performed with 7α-OOH, sample extracts were applied to silica-gel 60 HPTLC plates and chromatographed using benzene/ethyl acetate (1:1, v/v) as the mobile phase. In this case, the peroxide substrate is well separated from alcohol product, 7α-OH (R_f ∼ 0.42 and 0.25, respectively), allowing either or both to be determined in the assay. Three different detection methods were tested initially, using mixtures of 7α-OOH and 7α-OH standards: (i) spraying with 18N H_2SO_4 from a glass atomizer, followed by heating at 110 °C for 15 min; (ii) spraying with TPD as described for PCOOH; or (iii) spraying with TPD and then with 18N H_2SO_4, followed by heating. These are referred to as HPTLC-H_2SO_4, HPTLC-TPD, and HPTLC-TPD/H_2SO_4, respectively. The first of these proved suitable for only 7α-OH, the second for only 7α-OOH, while the last permitted simultaneous measurement of both analytes at relatively high sensitivity (see Section 3). Analytes were quantified via densitometric scanning (see above), using loads within the linear dynamic range established with the standards.

3. Results

3.1. HPTLC-TPD-based determination of cellular GPx4 activity using PCOOH as a substrate

The suitability of the HPTLC-TPD approach [19] for determining cellular LOOH peroxidase activity was tested initially on GPx4, using photochemically generated PCOOH as the peroxide substrate. GPx4 can catalyze the GSH-dependent two-electron reduction of various phospholipid-, cholesterol-, and cholesteryl ester-derived hydroperoxides in solubilized as well as membrane- or lipoprotein-bound form [6,7,20]. This activity distinguishes it from GPx1, a more abundant intracellular selenoperoxidase which can act only on more polar species such as H_2O_2 and fatty acid hydroperoxides [2,7]. A clone (7G4) of breast tumor COH-BR1 cells that had been stably transfected with a construct encoded for mitochondrial GPx4 was tested for GSH-dependent PCOOH reducing activity in comparison with a vector control (VC). As shown by the HPTLC-TPD profile in Fig. 1A, the 7G4 cell extract produced a much more rapid disappearance of

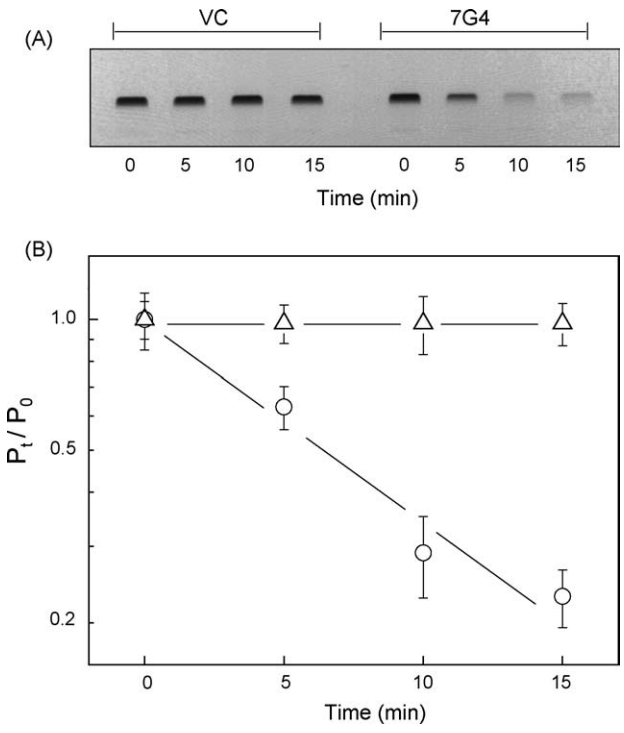

Fig. 1. Cellular GPx4-catalyzed reduction of PCOOH, as assessed by HPTLC-TPD: comparison of 7G4 and VC activities. Each assay mixture (0.5 ml) contained 100 μg of 7G4 or VC cell protein, 5 mM GSH, 60 μM PCOOH, and 0.05% Triton X-100 in PBS/DFO/EDTA at 37 °C. At various reaction times, samples were extracted and recovered lipid fractions were subjected to HPTLC-TPD analysis. (A) PCOOH band intensity after 0, 5, 10, and 15 min of incubation. Sample load: ∼5 nmol of initial PCOOH (0-min); 20 μg of cell protein per lane (equivalent to $\sim 1.3 \times 10^5$ cells). (B) First-order plot depicting the time course of PCOOH loss for 7G4 cells (△) and VC cells (◯). Densitometrically determined PCOOH at time-zero and time-t is denoted by P_0 and P_t, respectively. Data points are means from duplicate experiments.

the PCOOH band over a 15 min reaction period than the VC extract. No significant peroxide loss was observed when either GSH or cellular material was omitted from the assay mixtures (results not shown), ruling out any non-enzymatic contributions. Although not resolved from PCOOH, the alcohol product (PCOH) is essentially unreactive with TPD [19], and therefore did not interfere with the assay. Sample application with direct spraying, i.e. without chromatographic separation, was ruled out as a simplified option because cellular material that otherwise remained at the origin gave a background color which reduced sensitivity. Plots of densitometrically determined PCOOH as a function of incubation time are shown in Fig. 1B. The analyzed amounts were within the linear dynamic range previously established for this peroxide [19]. With 7G4 extracts, PCOOH level decayed exponentially over at least a 15 min reaction period, the apparent first-order rate constant being $(44.3 \pm 6.0) \times 10^{-3}$ min^{-1}. By contrast, PCOOH loss was barely detectable with VC extracts, the rate constant based on measurements extending to 1 h (not shown) being $(0.5 \pm 0.1) \times 10^{-3}$ min^{-1}, i.e. only 1.1% that of 7G4 cells. Specific enzymatic activities (nmol min^{-1} mg^{-1}), based on initial rates (calculated from the rate constants and starting PCOOH levels) and total cellular protein, were as follows: 20.3 ± 2.7 for 7G4 and 0.25 ± 0.05 for VC. (The kinetic parameters are mean ± S.D. of values from three separate experiments.) Activity of wild type COH-BR1 cells (not shown) was found to be no different from that of the VC clone. We tentatively attribute this low basal activity to GPx4, cognizant that certain glutathione S-transferases and peroxiredoxins can also catalyze phospholipid hydroperoxide reduction [9–12]. However, GPx1, which was previously shown by coupled assay to be expressed at the same high level in 7G4 and VC cells [17], could not have interfered in the HPTLC-TPD assay (Fig. 1) because it cannot act on phospholipid hydroperoxides [2]. The PCOOH-based HPTLC-TPD assay represented in Fig. 1 is estimated to be at least 50-times more sensitive than a coupled enzymatic assay employing NADPH and glutathione reductase [7]. This would be an obvious advantage for measuring cellular peroxidase activity, e.g. screening transfectant clones for expressed levels of activity.

3.2. Simultaneous determination of 7α-OOH and 7α-OH using HPTLC with H_2SO_4, TPD, or TPD/H_2SO_4 detection

A GPx4 assay based on reduction of a cholesterol hydroperoxide isomer, 7α-OOH, was also developed. The advantage in this case is that both substrate (7α-OOH) and product (7α-OH) could be tracked during an assay incubation, since they are well resolved by HPTLC [14,21,22]. Responsiveness was examined first, using mixtures of 7α-OOH and 7α-OH standards varying in mol ratio from 100:1 to 1:100, and detecting the separated species with H_2SO_4/heat alone, TPD alone, or TPD followed by H_2SO_4/heat. As shown in Fig. 2A, 7α-OOH appeared as a brownish-purple band upon

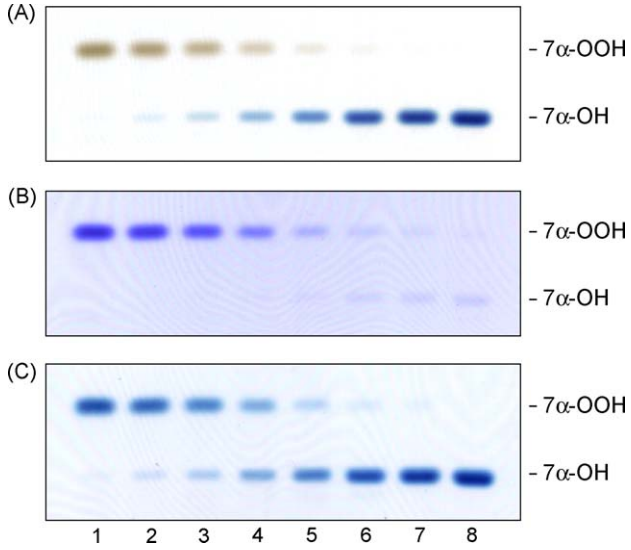

Fig. 2. HPTLC profiles of various 7α-OOH and 7α-OH standard mixtures. The numbered lanes represent the following amounts (nmol) of 7α-OOH and 7α-OH, respectively: (1) 10.0, 0.1; (2) 7.5, 0.25; (3) 5.0, 0.5; (4) 2.5, 1.0; (5) 1.0, 2.5; (6) 0.5, 5.0; (7) 0.25, 7.5; (8) 0.1, 10.0. Analytes were detected by spraying with (A) H_2SO_4 followed by warming; (B) TPD alone; (C) TPD followed by H_2SO_4 and warming. (B) and (C) represent the same TLC plate. R_f values are as follows: 7α-OOH, 0.42; 7α-OH, 0.26.

H_2SO_4/heat treatment, whereas less mobile 7α-OH appeared as a blue band of much greater intensity at the same analyte load. Spraying a duplicate HPTLC plate with TPD revealed a distinct blue band for 7α-OOH (Fig. 2B), as observed previously [14,21]. By contrast, 7α-OH gave a barely detectable (non-specific) response, its densitometrically determined intensity being <0.1% that of 7α-OOH at the same load, and therefore, negligible (compare lanes 1 and 8). When the same plate was subsequently sprayed with H_2SO_4 and heated, both 7α-OOH and 7α-OH appeared as sharp blue bands, which intensified progressively with increasing load (Fig. 2C). Especially noteworthy in the latter case is the color change and intensification of the 7α-OOH band compared with its appearance after H_2SO_4/heat treatment alone (Fig. 2A). This change is attributed to the quantitative conversion of 7α-OOH to 7α-OH on the plate when it was first sprayed with TPD. The likely mechanism is an iron-catalyzed two-electron reduction of 7α-OOH by the chromogen [19]. Subsequent exposure to H_2SO_4/heat gave the same strong blue color as observed when 7α-OH was analyzed directly (compare Fig. 2C and A). Dose–response plots were constructed following densitometric scanning of the various bands shown in Fig. 2. Best-fit equations for these plots over various analyte ranges, along with correlation coefficients and detection limits, are shown in Table 1. Responsiveness increased approximately linearly with applied amount of analyte up to ~5 nmol, above which there was progressive flattening, which was more pronounced with 7α-OH than with 7α-OOH. Consequently, for optimal sensitivity in measuring GPx4 activity with 7α-OOH, no more than 5 nmol of initial peroxide was applied to a TLC plate.

Table 1
Parameters for HPTLC analysis of standard 7α-OOH and 7α-OH mixtures[a]

	H_2SO_4[b]		TPD[c]	TPD/H_2SO_4[d]	
	7α-OOH	7α-OH	7α-OOH	7α-OOH	7α-OH
Best-fit equation[e]	$y = 5.3x - 0.16x^2$	$y = 26.4x - 2.64x^2$	$y = 1.3 + 11.6x - 0.37x^2$	$y = 0.3 + 11.9x - 0.45x^2$	$y = 26.1x - 2.56x^2$
Range (nmol)	1–10	0.25–10	0.25–10	0.25–10	0.25–10
Correlation coefficient	0.98	0.99	0.99	0.99	0.98
Detection limit (nmol)	1.0	0.2	0.2	0.2	0.2

[a] Data are from the chromatograms represented in Fig. 2.
[b] Detection by spraying with H_2SO_4 and warming.
[c] Detection by spraying with TPD.
[d] Detection by spraying with TPD followed by H_2SO_4 and warming.
[e] Equation representing the best-fit of plotted dose–response data.

3.3. HPTLC-TPD/H_2SO_4-based determination of cellular GPx4 activity using 7α-OOH as a substrate

In a manner analogous to that described using PCOOH as substrate (Fig. 2), we compared the 7α-OOH reducing activity of 7G4 versus VC cells. The incubation conditions were similar to those used for the PCOOH system, but cell protein and starting peroxide levels were set higher to compensate for the fact GPx4 is less active on cholesterol hydroperoxides than phospholipid counterparts [23]. For optimal substrate and product signal strength (cf. Fig. 2), we used TPD/H_2SO_4 treatment for detection rather than H_2SO_4 alone. As shown by the HPTLC-TPD/H_2SO_4 profile in Fig. 3A, incubation of 7α-OOH in the presence of GSH and a 7G4 cell extract resulted in a relatively rapid disappearance of the peroxide band with corresponding accumulation of 7α-OH product. A VC extract at the same protein level reduced 7α-OOH much more slowly, in agreement with the PCOOH findings (Fig. 2). Fig. 3B shows plots of the densitometrically determined analyte levels as a function of incubation time. For both cell clones, there was no significant difference in the apparent first-order kinetics of 7α-OOH decay and 7α-OH buildup over a 1 h period ($p > 0.50$ by regression analysis). However, the reaction rate constant for 7G4 was ~44-times greater than that for VC [$(39.2 \pm 0.4) \times 10^{-3}$ min^{-1} versus $(0.9 \pm 0.2) \times 10^{-3}$ min^{-1}]. Calculated specific enzymatic activities [nmol min^{-1} (mg cell protein)$^{-1}$] were as follows: 6.83 ± 0.81 for 7G4 and 0.15 ± 0.03 for VC (mean ± S.D. of values from three separate experiments). A more complex HPLC-EC(Hg)-based assay carried out previously [17] revealed approximately the same activity differences between these clones. However, in that case, only 7α-OOH loss could be monitored. The observed agreement between the kinetics for 7α-OOH loss and 7α-OH accumulation (Fig. 3B) confirms that the substrate/product stoichiometry was as expected for a GPx4-catalyzed reaction [6]. Knowing this, one could opt to measure enzymatic activity by tracking only peroxide decay (TPD alone) or only alcohol buildup (H_2SO_4/heat alone). However, the assurance afforded by combined treatment detection, which requires very little additional effort, argues strongly in favor of it for determining enzymes that can catalyze cholesterol hydroperoxide reduction.

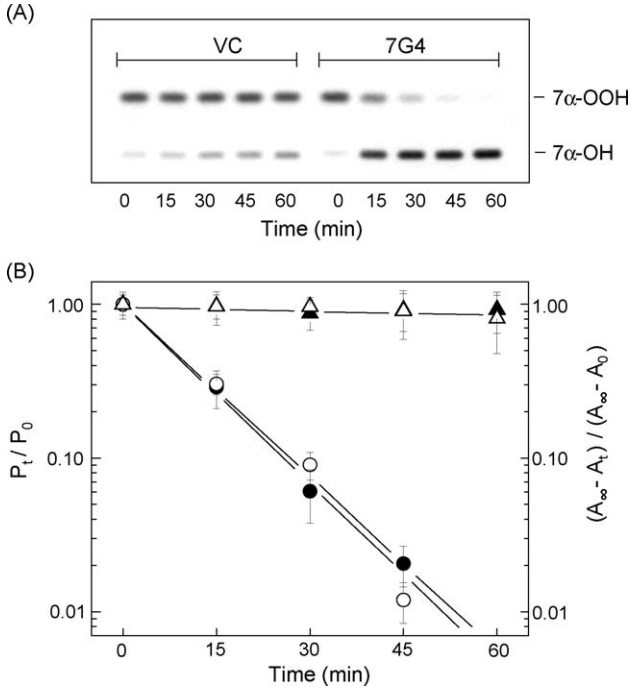

Fig. 3. Cellular GPx4-catalyzed reduction of 7α-OOH, as assessed by HPTLC-TPD/H_2SO_4: comparison of 7G4 and VC activities. Each assay mixture (0.5 ml) contained 750 μg of 7G4 or VC protein, 5 mM GSH, 100 μM 7α-OOH, and 0.05% Triton X-100 in PBS/DFO/EDTA at 37 °C. At the indicated times, samples were extracted and lipid fractions analyzed by HPTLC-TPD/H_2SO_4. (A) Chromatogram showing 7α-OOH and 7α-OH band intensities as a function of incubation time. Sample load: ~5 nmol of initial 7α-OOH (0-min); 150 μg of cell protein (equivalent to ~1.0×10^6 cells). (B) First-order plot showing the time course of 7α-OOH disappearance (◯, △) and 7α-OH appearance (●, ▲) for 7G4 cells (◯,●) and VC cells (△, ▲). P_0 and P_t denote 7α-OOH level at time-zero and -t, respectively. A_0, A_t, and A_∞ denote 7α-OH level at time-zero, -t, and -infinity, respectively. Mean ± deviation of values from duplicate experiments are plotted.

4. Discussion

It has long been known that endogenous or exogenous LOOHs can kill cells via direct oxidative damage to vital targets [2]. However, there is growing evidence that LOOHs,

like H_2O_2, can also act in more subtle fashion as stress signaling molecules [2,24,25]. In this capacity, they may either mediate a growth response or apoptotic death response, depending on the level of oxidative pressure [2]. Mammalian cells typically express a variety of antioxidant enzymes, some of which act in preventative fashion (e.g. by detoxifying LOOH-generating ROS) and others in reparative fashion (e.g. by reducing LOOHs to redox-inactive LOHs which, in the case of phospholipids, are subsequently hydrolyzed and reacylated) [2]. The second category includes thiol-dependent non-heme enzymes with peroxidase or peroxidase-like activity, prominent examples being GPx4, the α-class glutathione S-transferases (αGSTs), and the one- or two-cysteine peroxiredoxins (Prxs) [6–12]. NADPH is a crucial component in the sustained action of these enzymes. It serves as a substrate for GSSG reductase, which regenerates GSH for GPx4, αGST, or one-cysteine Prx activity [26], and also for thioredoxin reductase, which regenerates reduced thioredoxin for two-cysteine Prx activity [27]. LOOH-reducing activity of an over- or under-expressed peroxidase in a cell extract is commonly assessed by coupling this enzyme with the appropriate reductase and measuring the rate of NADPH oxidation (A_{340} decay) [7–12]. Though relatively simple and straightforward, this approach has at least two disadvantages: (i) the sensitivity is quite low, meaning that a relatively large amount of cellular material is needed for the assay (e.g. at least 1 mg of 7G4 protein/ml, which corresponds to $\sim 7 \times 10^6$ cells); (ii) absolute specificity for peroxidatic activity cannot be assumed; e.g. one needs to check for possible interference by NADPH dehydrogenases (diaphorases).

The HPTLC approach that we describe, though more complex than the coupled enzymatic assay, is highly specific for LOOH-based peroxidatic activity because NADPH is not required and reductant (e.g. GSH)-dependent loss of LOOH is tracked directly. An added advantage is that one can monitor either peroxide loss, alcohol product buildup, or both simultaneously, providing that their relative mobilities are sufficiently different. This was clearly demonstrated for a GPx4-overexpressing cell clone (7G4), using the cholesterol hydroperoxide 7α-OOH, which is well resolved from 7α-OH by HPTLC. In terms of specificity, GPx4 is the only enzyme known to catalyze cholesterol hydroperoxide reduction [7,28], so use of 7α-OOH provides another advantage if GPx4 is the enzyme of interest. The best analytical approach developed for the 7α-OOH-based assay is HPTLC-TPD/H_2SO_4, which affords maximal color yield for both the peroxide substrate and diol product. We estimate that for a given reaction mixture, this approach is up to 50-times more sensitive than the conventional coupled enzymatic assay. One could improve the sensitivity of the HPTLC-based assay by using radiolabeled 7α-OOH and tracking the reaction by radioimaging. In keeping with this, we showed previously, using photoperoxidized [^{14}C]Ch-containing liposomes and purified testicular GPx4, that the kinetics of total cholesterol hydroperoxide loss (7α/β-OOH plus three other isomers) matched those of total diol accumulation [28]. However, preparation of [^{14}C]7α-OOH that is both radioactively- and peroxide-pure requires considerable effort [13], making this alternative rather impractical compared with TPD/H_2SO_4 detection. It is important to note that we previously developed a GPx4 assay in which 7α-OOH was determined by HPLC-EC(Hg) analysis [13,17]. Though estimated to be at least 100-times more sensitive than HPTLC-TPD/H_2SO_4, HPLC-EC(Hg) has clear limitations in terms of equipment availability and complexity of operation. Moreover, only the peroxide substrate can be monitored by this approach.

We showed that an assay based on PCOOH as the peroxide substrate is also amenable to HPTLC analysis. Since TPD-inert PCOH was not separated from PCOOH under the chromatographic conditions used, only the TPD-reactive peroxide could be monitored. It is unlikely that phospholipase A_2 (PLA_2)-catalyzed hydrolysis of *sn*-2 fatty acyl hydroperoxide groups, followed by reduction, could have contributed to the observed PCOOH loss because PLA_2 requires Ca^{2+} and this would have been EDTA-bound in the assay system. It is of interest that simultaneous determination of PCOOH and PCOH in a GPx4 assay system has been reported [29], but in that case the analytes were separated by Ultracarb-5 ODS HPLC and detected by measuring conjugated diene absorbance at 232 nm. In principle, it should be possible to adapt the HPLC conditions described [29] to HPTLC, thus allowing a substrate/product relationship to be clearly established, as demonstrated for 7α-OOH (Fig. 3). Determination of the separated analytes could be accomplished by spraying with a lipophilic fluorophore such as 1,6-diphenylhexatriene [30], followed by fluorescence scanning.

In summary, we have developed a new chromatographic method for assessing the activity of cellular non-heme peroxidases capable of reducing and detoxifying phospholipid- or cholesterol-derived hydroperoxides. Using this approach in its simplest form, i.e. as HPTLC-TPD, one can directly measure the kinetics of LOOH loss for a given amount of cell extract and thus obtain a specific enzymatic activity. HPTLC-TPD is more sensitive and informative than the widely used coupled enzymatic assay, and is particularly well suited for determining the activities of upregulated or overexpressed antioxidant peroxidases such as GPx4, Prxs, and αGSTs.

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