The C2-like β-Barrel Domain Mediates the Ca²⁺-dependent Resistance of 5-Lipoxygenase Activity Against Inhibition by Glutathione Peroxidase-1*

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Recently, we reported that in crude enzyme preparations, a monocyte-derived soluble protein (M-DSP) renders 5-lipoxygenase (5-LO) activity Ca²⁺-dependent. Here we provide evidence that this M-DSP is glutathione peroxidase (GPx)-1. Thus, the inhibitory effect of the M-DSP on 5-LO could be overcome by the GPx-1 inhibitor mercaptosuccinate and by the broad spectrum GPx inhibitor iodoacetate, as well as by addition of 13(S)-hydroperoxy-9Z,11E-octadecadienoic acid (13(S)-HPODE). Also, the chromatographic characteristics and the estimated molecular mass (80–100 kDa) of the M-DSP fit to GPx-1 (87 kDa), and GPx-1, isolated from bovine erythrocytes, mimicked the effects of the M-DSP. Intriguingly, only a trace amount of thiol (10 μM GSH) was required for reduction of 5-LO activity by GPx-1 or the M-DSP. Moreover, the requirement of Ca²⁺ allowing 5-LO product synthesis in various leukocytes correlated with the respective GPx-1 activities. Mutation of the Ca²⁺ binding sites within the C2-like domain of 5-LO resulted in strong reduction of 5-LO activity by M-DSP and GPx-1, also in the presence of Ca²⁺. In summary, our data suggest that interaction of Ca²⁺ at the C2-like domain of 5-LO protects the enzyme against the effect of GPx-1. Apparently, in the presence of Ca²⁺, a low lipid hydroperoxide level is sufficient for 5-LO activation.

5-Lipoxygenase (5-LO)[1] catalyzes the initial steps in the biosynthesis of leukotrienes (LTs) and 5(S)-hydro(pero)xyeicosatetraenoic acid (5(S)-H(P)ETE) from arachidonic acid (AA) (for review, see Ref. 1). Due to the pivotal biological functions of

5-LO metabolites (2, 3), the activity of 5-LO is tightly regulated. In intact cells, Ca²⁺ and phosphorylation seem to be primary signals that activate 5-LO. Moreover, the membrane-bound 5-LO-activating protein (FLAP) (4) and the redox state (5, 6) have a strong impact on cellular 5-LO product formation.

In cell-free systems Ca²⁺, ATP, phospholipids (membranes), lipid hydroperoxides (LOOH), and leukocyte-derived proteins have been shown to enhance 5-LO catalysis (reviewed in Ref. 1). However, the degree of stimulation by each of these components depends on the assay conditions, *i.e.* the source of 5-LO (isolated, in crude homogenates or cellular fractions), presence of other cofactors, the concentration of AA, etc. LOOH are of importance for the initial conversion of the active site iron from the ferrous (resting) to the ferric (active) state (7, 8). Accordingly, glutathione peroxidases (GPx) that reduce LOOH inhibit 5-LO product synthesis *in vitro* and in intact cells (5, 9–15), and conditions that are associated with an increased peroxide-tone promote 5-LO product formation (6, 16, 17). Two Ca²⁺ ions bind to the N-terminal C2-like domain of 5-LO with a K_d of 6 μM (18, 19). Half-maximal activation of purified 5-LO was determined at 1–2 μM Ca²⁺, whereas 4–10 μM Ca²⁺ causes maximal activation of the enzyme (20, 21). In intact cells lower concentrations of Ca²⁺ (200–300 nM) seem to be sufficient for 5-LO activation (22, 23). It was shown that Ca²⁺ increases the hydrophobicity of 5-LO (18) and causes 5-LO binding to phosphatidylcholine (PC) vesicles or to cellular membranes (20, 24–26), and that the C2-like domain is important also for membrane association (27, 28). Nevertheless, the mechanisms of how Ca²⁺ stimulates 5-LO activity may involve additional factors.

Several reports state that in cell-free systems 5-LO is catalytically active without Ca²⁺ (see Ref. 29 and references therein), and in intact cells, 5-LO phosphorylation by members of the MAP kinase family stimulate 5-LO product synthesis in the absence of Ca²⁺ (30–32). Mg²⁺ at concentrations that occur in intact cells, can substitute for Ca²⁺ regarding binding and activation of 5-LO *in vitro* (19, 33). We observed considerable 5-LO product synthesis in homogenates of human PMNL and rat basophilic leukemia (RBL)-1 cells in the absence of Ca²⁺, whereas Ca²⁺ was required for 5-LO activity in homogenates of monocytic MM6 cells under the same assay conditions (29, 34). Interestingly, we found that an 80–100 kDa soluble protein from MM6 cells renders 5-LO activity Ca²⁺-dependent. In this study we provide evidence that this M-DSP is GPx-1 and we suggest that Ca²⁺, via interaction with the C2 domain of 5-LO, renders the enzyme resistant against GPx-1, possibly by increasing the affinity toward activating LOOH.

[1] The abbreviations used are: 5-LO, 5-lipoxygenase; AA, arachidonic acid; ATP-PT, ATP affinity column pass-through fraction; BAPTA/AM, [1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetrakis-(acetoxymethyl) ester]; cPLA₂, cytosolic phospholipase A₂; GPx, glutathione peroxidase; 13(S)-HPODE, 13(S)-hydroperoxy-9Z,11E-octadecadienoic acid; LOOH, lipid hydroperoxide; LT, leukotriene; MM6, Mono Mac 6; PC, phosphatidylcholine; PBS, phosphate-buffered saline; PMNL, polymorphonuclear leukocytes; RBL-1, rat basophilic leukemia-1; wt, wild type; HPLC, high performance liquid chromatography.

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EXPERIMENTAL PROCEDURES

Materials—RPMI 1640 medium was from GIBCO and fetal calf serum was obtained from Roche Molecular Biochemicals (Mannheim, Germany). Insulin was a gift from Aventis (Frankfurt, Germany). Human transforming growth factor beta 1 (TGFβ1) was purified from outdated platelets as described (35). Calcitriol was kindly provided by Schering AG (Berlin, Germany). AA, ATP-agarose (A2767), bovine GPx-1, iodoacetate, mercaptosuccinate, GSH reductase, cumene hydroperoxide, ionophore A23187, and ionomycin were from Sigma (Deisenhofen, Germany), HPLC solvents were from Merck (Darmstadt, Germany). Fura-2/AM and 2′, 7′-dichlorofluorescein diacetate (DCF-DA) and [1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl) ester] (BAPTA/AM) were from Calbiochem (Bad Soden, Germany). 13(*S*)-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (13(*S*)-HPODE) was from Cayman, NADPH from Serva.

Cells—RBL-1 cells were maintained in RPMI 1640 medium supplemented with 10 mM HEPES pH 7.4, 10% fetal calf serum, 100 μg/ml streptomycin, 100 units/ml penicillin, 1 mM sodium pyruvate, 1× nonessential amino acids, and 10 μg/ml bovine insulin at a density of 2 × 10⁵ cells/ml. Cells were harvested for experiments 3 days after splitting. MM6 cells were cultured and differentiated with TGFβ and calcitriol as described (36).

Human PMNL were promptly isolated from fresh leukocyte concentrates obtained from healthy donors at St Markus Hospital (Frankfurt, Germany) as described (31). Cells were finally resuspended in PBS plus 1 mg/ml glucose (PG buffer) or PBS plus 1 mg/ml glucose and 1 mM CaCl₂ (PGC buffer) as indicated.

Preparation of 100,000 × g Supernatants and Partial Purification of 5-LO—Freshly isolated PMNL (5 × 10⁸), MM6 cells (2 × 10⁸), or RBL-1 cells (2 × 10⁸), were resuspended in 10 ml of PBS containing 1 mM EDTA. After cooling down on ice for 10 min, cells were homogenized by sonication (3 × 10 s). After centrifugation at 100,000 × g for 70 min at 4 °C, the 100,000 × g supernatant (S100) was applied to an ATP-agarose column, and the column was eluted as described previously (36). Purified 5-LO and ATP affinity column pass-through fractions (ATP-PT) were immediately used for 5-LO activity assays.

Site-directed Mutagenesis, Expression, and Purification of Recombinant 5-LO Proteins—Site-directed mutagenesis of the pT3–5LO plasmid, encoding wild type 5-LO (wt-5-LO), using the Quickchange™ kit from Stratagene yielded the mutated 5-LO plasmid pT3–5LO-N43A-D44A-E46A (referred to as loop2 mut-5LO) (19). Wild type and mutant DNAs were transformed into *Escherichia coli* MV1190, proteins were expressed and purified as described (37) and immediately assayed for activity.

Determination of 5-LO Product Formation; in Vitro Activity Assays—For assays of intact cells in the presence of Ca²⁺, 7.5 × 10⁶ freshly isolated PMNL, 3 × 10⁶ MM6 or 2 × 10⁶ RBL-1 cells were finally resuspended in 1 ml of PGC buffer. The reaction was started by addition of ionophore A23187 and exogenous AA. After 10 min at 37 °C, the reaction was stopped with 1 ml of methanol and 30 μl of 1 N HCl, and 200 ng of prostaglandin B₁ and 500 μl of PBS were added. Formed 5-LO metabolites were extracted and analyzed by HPLC as described (38). 5-LO product formation is expressed as nanograms of 5-LO products per 10⁶ cells, which includes LTB₄ and its all-trans isomers, 5(*S*),12(*S*)-dihydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid (5(*S*),12(*S*)-DiHETE), 5(*S*)-hydroxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HETE), and 5(*S*)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HPETE). 5-HETE and 5-HPETE coelute as one major peak, integration of this peak represents both eicosanoids. Cysteinyl LTs (LTC₄, D₄, and E₄) were not detected and oxidation products of LTB₄ were not determined.

When broken cell preparations or purified 5-LO were assayed, S100 or partially purified 5-LO enzyme (in the presence or absence of ATP-PTs) were diluted in ice-cold PBS containing 1 mM EDTA (final volume, 1 ml) and 1 mM ATP as well as other agents (as indicated) were added. The samples were preincubated for 30 s at 37 °C, and the incubation was started by the addition of AA together with or without 2 mM CaCl₂ and other compounds as indicated. After 10 min at 37 °C, the incubation was stopped with 1 ml methanol and the formed 5-LO products were extracted and analyzed by HPLC as described for intact cells.

SDS-PAGE and Western Blotting of GPx-1—Aliquots (18 μl) of the fractions from the gel-permeation chromatography were mixed with 2 μl of 10× SDS-PAGE sample loading buffer (100 mM Tris, pH 6.8, 10 mM EDTA, 25% (w/v) SDS, 25 mM dithiothreitol) and 4 μl of glycerol/ 0.1% bromphenol blue (1:1, v/v), heated at 95 °C for 5 min and then analyzed by SDS-PAGE on a 12% gel. After electroblot to polyvinylidene difluoride membrane (Amersham Biosciences), blocking with 5% nonfat dry milk for 1 h at room temperature, membranes were washed and

incubated with polyclonal anti-human GPx-1/catalase antibody (Dunn Labortechnik, Asbach, Germany, see Ref. 12) overnight at 4 °C. The membranes were washed and incubated with 1:1,000 dilution of alkaline phosphatase-conjugated IgGs (Sigma) for 2 h at RT. After washing, proteins were visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma) in detection buffer (100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂).

Determination of Cellular Peroxide Formation—Measurement of peroxides was conducted using the peroxide-sensitive fluorescence dye 2′,7′-dichlorofluorescein diacetate (DCF-DA). Freshly isolated PMNL (5 × 10⁶ in 1 ml of PGC buffer), differentiated MM6 cells or RBL-1 cells (3 × 10⁶ cells in 1 ml PGC buffer) were preincubated with DCF-DA (1 μg/ml) for 2 min at 37 °C in a thermally controlled (37 °C) fluorimeter cuvette with continuous stirring in a spectrofluorometer (Aminco-Bowman series 2). The fluorescence emission at 530 nm was measured after excitation at 480 nm. The mean fluorescence data measured 5 min after stimulus addition are expressed as fold increase over unstimulated cells.

Determination of Glutathione Peroxidase Activity—GPx activity in S100 of PMNL, MM6, and RBL-1 cells or fractions of the gel-permeation chromatography was measured according to the indirect GSH reductase-coupled method described by Wendel (39). One unit GPx activity was defined as the conversion of 0.5 μmol of NADPH to NADP⁺ per min at 37 °C and 1 mM GSH. GPx activity is expressed as milliunits/10⁶ cells.

Measurement of Intracellular Ca²⁺ Levels—Cells (1 × 10⁷ in 1 ml of PGC buffer) were incubated with 2 μM Fura-2/AM for 30 min at 37 °C, washed, resuspended in 1 ml of PGC buffer and transferred into a thermally controlled (37 °C) fluorimeter cuvette in a spectrofluorometer (Aminco-Bowman series 2) with continuous stirring. The fluorescence emission at 510 nm was measured after excitation at 340 and 380 nm, respectively. Intracellular Ca²⁺ levels were calculated according to the method of Grynkiewicz *et al.* (40). F_{max} (maximal fluorescence) was obtained by lysing the cells with 1% Triton-X 100 and F_{min} by chelating Ca²⁺ with 10 mM EDTA.

RESULTS

Identification of Glutathione Peroxidase-1 as a M-DSP That Renders 5-LO Activity Ca²⁺-dependent—We attempted to identify the recently described M-DSP, which suppresses the activity of 5-LO in the absence of Ca²⁺ (29). Based on the apparent molecular mass (80–100 kDa) determined by gel-permeation chromatography, it appeared possible that the M-DSP is GPx-1, which was recently identified as a regulator of cellular 5-LO activity in monocytic cells (12). As shown in Fig. 1*A*, GPx-1 protein and GPx activity indeed co-eluted with the 5-LO inhibitory activity of the M-DSP during gel-permeation chromatography.

Next, we investigated if agents that suppress or counteract GPx-1 activity could overcome the effect of the M-DSP. Control experiments confirmed that partially purified 5-LO from MM6 cells alone is catalytically active, regardless of Ca²⁺. When aliquots of the MM6 ATP-PT were added back to 5-LO, 5-LO activity was enhanced about 4-fold in the presence of Ca²⁺. However, without Ca²⁺, 5-LO activity was completely suppressed (Fig. 1*B*). The specific GPx-1 inhibitor mercaptosuccinate (30 μM) (41) as well as the broad spectrum GPx inhibitor iodoacetate (2 mM, not shown) (42), were capable of reconstituting 5-LO activity. Also, 13(*S*)-HPODE (3 μM), that counteracts GPx-1 activity (16) was able to prevent the 5-LO inhibitory action of the MM6-ATP-PT, whereas H₂O₂ (1–100 μM) and 13-*oxo*-octadecadienoic acid (3 up to 30 μM) had no effect. Similarly, 5-LO suppression induced by aliquots of the 80–100 kDa fractions of the gel-permeation chromatography was inhibited by mercaptosuccinate, iodoacetate or 13(*S*)-HPODE (not shown). By contrast, these agents did not alter 5-LO activity in the presence of Ca²⁺ (Fig. 1*B*). Notably, in all these experiments, no exogenous thiols such as GSH or dithiothreitol were added to the incubation mixtures, but it should be noted that the MM6-ATP-PT by itself contains traces of thiols, originally derived from the intracellular environment.

In S100 of MM6 cells, 5-LO is catalytically active only when

A

B

FIG. 1. **GPx co-elutes with the M-DSP that inhibits 5-LO in the absence of Ca^{2+}; effects of mercaptosuccinate, 13(S)-HPODE, and hydrogen peroxide.** *A*, MM6 ATP-PT (5 ml, corresponding to 1×10^8 cells) was applied to a Sephacryl-S200 column (1.6 × 54 cm) equilibrated in PBS/1 mM EDTA, the flow rate was 0.5 ml/min. Fractions (5 ml) were collected and checked for inhibition of partially purified 5-LO in the absence of Ca^{2+}, for GPx activity, and for GPx-1 protein. *B*, partially purified 5-LO, corresponding to 2×10^6 cells, was diluted in ice-cold PBS containing 1 mM EDTA and 1 mM ATP. The MM6-ATP-PT, corresponding to 2×10^6 cells, and mercaptosuccinate (*merc.suc.*, 30 μM) were added as indicated; final volume was 1 ml. After 5 min on ice, samples were prewarmed for 30 s at 37 °C and CaCl$_2$ (2 mM), 13(S)-HPODE (3 μM), or hydrogen peroxide (3 μM) were added as indicated together with 40 μM AA. After another 10 min at 37 °C, 5-LO product formation was determined by HPLC as described. The control represents 5-LO activity in the absence of the MM6 ATP-PT. Results are given as mean ± S.E., $n = 3$.
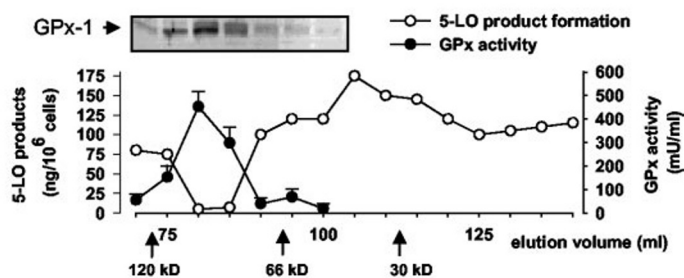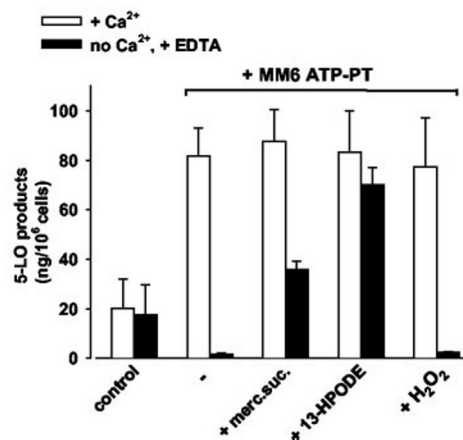
Ca^{2+} is present, but not when Ca^{2+} is omitted (29). Also under such experimental settings mercaptosuccinate, iodoacetate, or 13(S)-HPODE were able to overcome 5-LO suppression in the absence of Ca^{2+} (not shown). In contrast to MM6 cells, Ca^{2+} is not needed for 5-LO activity in S100 from PMNL (34), and these cells exert only low GPx activity (Table I). However, when aliquots of the MM6-ATP-PT were added to S100 of PMNL in the absence of Ca^{2+} (but not in its presence), 5-LO activity was strongly suppressed (Fig. 2*A*). At a fixed concentration of GPx-1, variation of the amount of 5-LO enzyme in the incubation mixture did not alter the degree of 5-LO inhibition. Thus, the amounts of 5-LO products formed in 1 ml incubations containing S100 of PMNL derived from 2.5, 5, 10 or 15 × 10^6 cells was 215 ± 52, 462 ± 147, 745 ± 170, and 988 ± 282 ng/ml, respectively. Inclusion of MM6-ATP-PT corresponding to 2×10^6 cells suppressed the 5-LO activities in all of these incubations by 93–97%. Again, mercaptosuccinate, iodoacetate or 13(S)-HPODE, but not H$_2$O$_2$, counteracted the 5-LO inhibitory effects of the MM6-ATP-PT (Fig. 2*A*). Interestingly, 5-LO activity in PMNL-S100 was considerably suppressed when 5 mM GSH, the main co-substrate of GPx-1, was included, leading to strong catalysis of GPx (Fig. 2*B*). Replenishment of Ca^{2+} restored 5-LO product synthesis under these conditions.

*Bovine GPx-1 Mimics the 5-LO Inhibitory Effect of M-DSP—*Next, we investigated if GPx-1 could mimic the inhibitory effects of the MM6-ATP-PT toward 5-LO. GPx-1, isolated from bovine erythrocytes, was added to S100 of PMNL and 5-LO

TABLE I
GPx-1 activity, 5-LO product synthesis and peroxide formation in PMNL, RBL-1 and MM6 cells

RBL-1 cells were grown in the absence (RBL-1) or presence (RBL-1 + Se) of exogenously added Se^{4+} (100 ng/ml). GPx activity was determined using S100 of PMNL (corresponding to 5-15 × 10^6), MM6 (0.5-2 × 10^6), RBL-1 cells (0.5-2 × 10^6) as described. Results are given as mean + SE, n = 3. Peroxide formation: PMNL (5×10^6), MM6 cells (3×10^6), and RBL-1 cells (3×10^6) were resuspended in 1 ml PGC buffer, DCF-DA (1 μg/ml) was added and after 2 min at 37°C, cells were stimulated with 60 μM AA. Data determined 5 min after addition of AA are expressed as -fold increase of the mean fluorescence +/− S.E. over unstimulated cells, $n = 5$. 5-LO product synthesis: PMNL (7.5×10^6), MM6 cells (2×10^6), and RBL-1 cells (2×10^6) were diluted to 1 ml in PG. Cells were either stimulated with 60 μM AA in presence of 1 mM EDTA, or alternatively with ionophore plus 60 μM AA in presence of 1 mM CaCl$_2$. 5-LO product formation was determined 10 min after addition of the stimuli at 37°C as described. Results are given as mean + S.E., $n = 5$.

Cell type	GPx activity	Peroxide formation	5-LO activity AA	5-LO activity ionophore + AA
	mU/10^6 cells	*fold increase*	*ng/10^6 cells*	*ng/10^6 cells*
PMNL	2.8 ± 1.1	3.9 ± 1.1	57.1 ± 18.9	80.8 ± 2.1
MM6	48.4 ± 10.2	1.7 ± 0.2	2.4 ± 0.4	285 ± 14
RBL-1	12.5 ± 2.1	5.4 ± 1.2	208.3 ± 33.5	398 ± 69
RBL-1 + Se	80.1 ± 15.2	2.9 ± 0.9	33.2 ± 3.1	126 ± 3.5

activity, without supplementation of thiols, was determined. Addition of isolated GPx-1 to PMNL-S100 inhibited 5-LO activity in a dose-dependent manner with an EC$_{50}$ of ~70 mU/ml
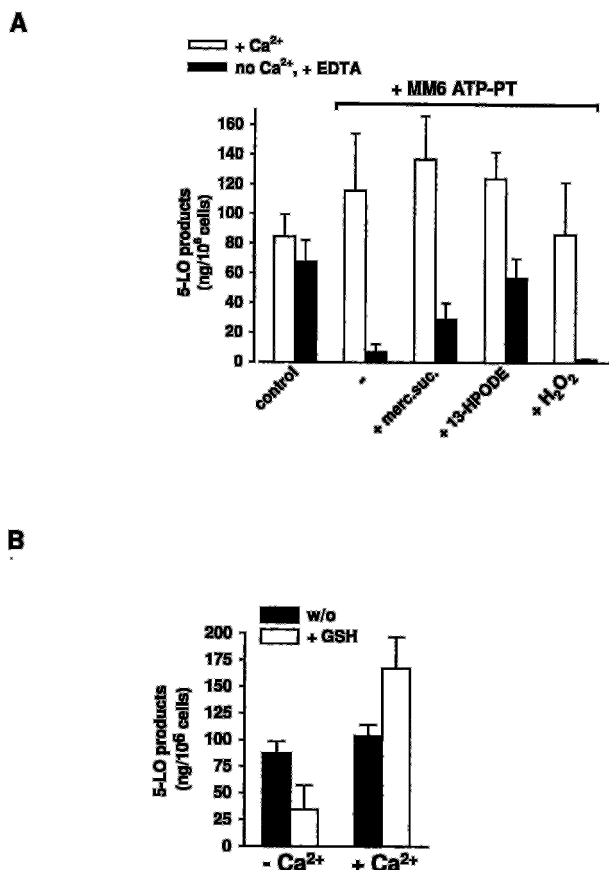
A

B

FIG. 2. **Suppression of 5-LO in S100 of PMNL in the absence of Ca²⁺; reversal by mercaptosuccinate, 13(S)-HPODE, and hydrogen peroxide.** *A*, S100 of PMNL, corresponding to 10^7 cells, was diluted in ice-cold PBS containing 1 mM EDTA and 1 mM ATP. MM6 ATP-PT, corresponding to 2×10^6 MM6 cells and mercaptosuccinate (*merc.suc.*, 30 μM) were added to the samples as indicated; final volume was 1 ml. After 5 min on ice, samples were prewarmed for 30 s at 37 °C and CaCl$_2$ (2 mM), 13(S)-HPODE (3 μM), and hydrogen peroxide (3 μM) were added as indicated together with 40 μM AA. After another 10 min at 37 °C, 5-LO product formation was determined as described. The control represents 5-LO activity in the absence of MM6 ATP-PT. *B*, S100 of PMNL was diluted in ice-cold PBS containing 1 mM EDTA and 1 mM ATP, and 5 mM GSH were added as indicated. After 5 min on ice, samples were prewarmed for 30 s at 37 °C, and CaCl$_2$ (2 mM) was added as indicated together with 40 μM AA. After another 10 min at 37 °C, 5-LO product formation was determined as described. Results are given as mean ± S.E., $n = 4$.

GPx-1 in the absence but not in the presence of Ca²⁺ (Fig. 3*A*). As found for MM6-ATP-PT, the degree of 5-LO inhibition at a fixed amount of GPx-1 (200 mU) was about the same for S100 of 2.5, 5, 10 or 15 × 10^6 PMNL as source of 5-LO (not shown). As shown in Fig. 3*B*, the strong inhibition of 5-LO in PMNL-S100 by 300 mU bovine GPx-1 was almost completely reversed by mercaptosuccinate or 13(S)-HPODE (but not by H$_2$O$_2$), resembling the counteracting effects observed with the MM6-ATP-PT. Again, the agents had no such up-regulatory effects on 5-LO activity when Ca²⁺ was present.

In contrast to the PMNL-S100, for purified 5-LO enzyme (from PMNL, RBL-1, or MM6 cells), addition of isolated bovine GPx-1 failed to suppress 5-LO activity in the absence of Ca²⁺ (Fig. 4), implying that an additional component, present in the PMNL-S100, appears to be operative. Thus, the PMNL-S100 was subjected to ATP affinity chromatography, and the pass through fraction, was separated into a high (>10 kDa) and a low molecular mass (<10 kDa) fraction by gel-permeation chromatography using a PD-10 column (Amersham Biosciences). When the low molecular mass fraction was included in the
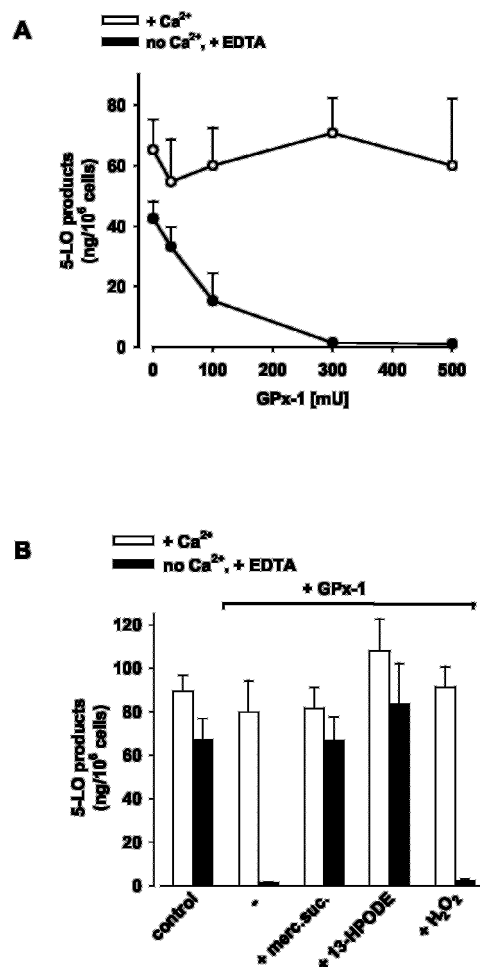
A

B

FIG. 3. **GPx-1 inhibits 5-LO in S100 of PMNL in the absence of Ca²⁺; reversal by mercaptosuccinate and 13(S)-HPODE.** *A*, 5-LO inhibition by GPx-1. The S100 of PMNL, corresponding to 10^7 cells, was diluted in 1 ml of ice-cold PBS containing 1 mM EDTA and 1 mM ATP. Then, GPx-1, isolated from bovine erythrocytes, was added at the indicated amounts. After 5 min on ice, samples were prewarmed for 30 s at 37 °C, and CaCl$_2$ (2 mM) was added as indicated together with 40 μM AA. After another 10 min at 37 °C, 5-LO product formation was determined. *B*, effects of mercaptosuccinate (*merc.suc.*), 13(S)-HPODE, and hydrogen peroxide. The S100 of PMNL, corresponding to 10^7 cells, were diluted in 1 ml of ice-cold PBS containing 1 mM EDTA and 1 mM ATP. Then, GPx-1 (300 mU, isolated from bovine erythrocytes) and mercaptosuccinate (30 μM) were added as indicated. After 5 min on ice, samples were prewarmed for 30 s at 37 °C and CaCl$_2$ (2 mM), 13(S)-HPODE (3 μM), and hydrogen peroxide (3 μM) were added as indicated together with 40 μM AA. After another 10 min at 37 °C, 5-LO product formation was determined as described. Results are given as mean ± S.E., $n = 5$.

incubation mixture (containing purified 5-LO and isolated GPx-1), 5-LO was suppressed by GPx-1 in the absence of Ca²⁺ (but not in its presence) (Fig. 4). The high molecular mass fraction rather increased 5-LO activity regardless of Ca²⁺. Notably, addition of 10 μM GSH (0.307 kDa), could replace the low molecular mass fraction, rendering GPx-1 a potent 5-LO inhibitory enzyme (not shown).

5-LO Product Formation in Intact MM6 Cells: Effects of Ca²⁺ and Inhibition of GPx-1—In order to investigate if Ca²⁺ renders 5-LO activity resistant against inhibition by GPx-1 also in the intact cell, MM6 cells were stimulated for 5-LO product formation under conditions which differentially increase intracellular Ca²⁺. AA (40 μM) was added exogenously as substrate to circumvent phospholipase activity. As shown in Fig. 5*A*, for cells stimulated with 5 μM ionophore A23187, causing rapid and substantial release of intracellular Ca²⁺ ($\approx$400–500 nM Ca²⁺ within 5 s, not shown), 5-LO product synthesis was high
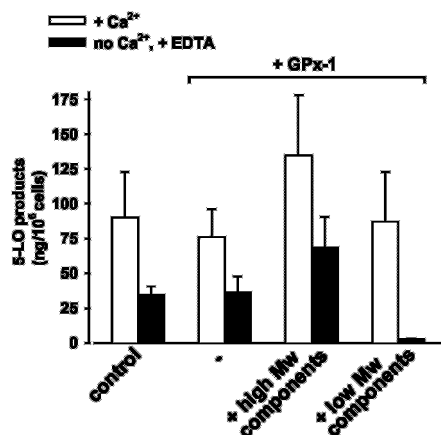
FIG. 4. **Cellular low molecular mass components cooperate with GPx-1 to inhibit 5-LO.** The S100 of PMNL was subjected to ATP-affinity chromatography to obtain partially purified 5-LO and the PMNL ATP-PT, respectively. The PMNL ATP-PT was further separated into a high (>10 kDa) and a low molecular mass (<10 kDa) fraction using a PD-10 column (Amersham Biosciences). Partially purified 5-LO, corresponding to 2×10^6 cells, was diluted in ice-cold PBS containing 1 mM EDTA and 1 mM ATP. Then, GPx-1 (500 mU, isolated from bovine erythrocytes) and aliquots of high and low molecular mass fractions, corresponding to 10^7 cells, were added; final volume was 1 ml. After 5 min on ice, samples were prewarmed for 30 s at 37 °C and $CaCl_2$ (2 mM) was added as indicated together with 40 μM AA. After another 10 min at 37 °C, 5-LO product formation was determined. The control represents 5-LO activity in the absence of GPx-1 and cellular components. Results are given as mean ± S.E., $n = 3$.

(286 ± 14 ng/10^6 cells), and there were no significant upregulatory effects of iodoacetate or 13(S)-HPODE. Mercaptosuccinate is not cell-permeable and thus not suitable for cellular investigations. For cells stimulated with 1 μM thapsigargin, that slowly and moderately elevates Ca^{2+} ($\approx$200 nM Ca^{2+} within 60 s), 5-LO product synthesis was much lower (26.4 ± 5.5 ng/10^6 cells) but inhibition and counteraction of GPx-1 significantly up-regulated 5-LO product synthesis 3- and 2-fold, respectively (Fig. 5B). Moreover, when MM6 cells were incubated with AA alone (which does not elevate the basal Ca^{2+} levels of ~50 nM Ca^{2+}, Ref. 34), 5-LO product synthesis was only 14.4 ± 1.3 ng/10^6 cells, but there was an almost 5-fold increase when GPx activity was blocked by iodoacetate (Fig. 5C). Finally, removal of Ca^{2+} by chelation with BAPTA/AM and EDTA (<10 nM Ca^{2+}) caused lowest 5-LO product synthesis (5.4 ± 1.1 ng/10^6 cells), but inhibition of GPx-1 by iodoacetate or co-addition of 13(S)-HPODE gave 6.5-fold and 3.5-fold enhancements of 5-LO product synthesis, respectively (Fig. 5D). As seen for cell-free systems, hydrogen peroxide was not able to elevate 5-LO product formation in any experiment.

GPx-1 Activity, Peroxide Formation, and 5-LO Product Synthesis in Various Cell Types—The activities of GPx-1 and cellular 5-LO as well as the capacity to elevate the cellular peroxide tone (in response to 60 μM AA) were determined in intact isolated PMNL, MM6 and RBL-1 cells. Cellular 5-LO product synthesis was induced either by stimulation with 60 μM AA after chelation of extracellular Ca^{2+} by EDTA in order to detect Ca^{2+}-independent 5-LO activity, or alternatively with ionophore and 10 μM AA in the presence of Ca^{2+} (compare Ref. 30). As shown in Table I, PMNL and RBL-1 cells exert only low GPx-1 activity and are clearly capable of elevating the cellular redox tone by producing peroxides in response to AA. Interestingly, when the culture medium of RBL-1 cells has been supplemented with 100 ng/ml Se^{4+}, a determinant for GPx-1 expression (43), GPx-1 activity was increased about 6.7-fold, and at the same time the peroxide level in AA-stimulated cells was considerably reduced. Compared with PMNL and RBL-1 cells, the GPx-1 activity was about 17- and 3.8-fold higher in MM6

cells, respectively, and stimulation with AA caused an only marginal increase of the cellular peroxide level. Intriguingly, cells possessing high GPx-1 activity and thus low capacity to elevate the cellular peroxide levels (MM6 or RBL-1 with Se^{4+} supplementation), gave low AA-induced 5-LO product synthesis, whereas 5-LO product formation was high in cells (PMNL and RBL-1 cells) exhibiting low GPx-1 activity and enhanced peroxide levels. Notably, 5-LO product synthesis was substantial in all cell types, when Ca^{2+} levels were elevated by stimulation with ionophore.

Effects of Ebselen on 5-LO Product Synthesis in PMNL—Ebselen, a cell-permeable organo-selenium compound that mimics GPx activity in the presence of thiols (44), was described as an inhibitor of 5-LO (45, 46). PMNL were preincubated with ebselen and 5-LO product synthesis was induced either by stimulation with ionophore in the presence of 10 μM AA and 1 mM Ca^{2+}, or with 60 μM AA in the presence of 1 mM EDTA and 30 μM BAPTA/AM (in order to remove Ca^{2+}). Under conditions where Ca^{2+} is elevated in the cell by ionophore stimulation, the IC_{50} value of ebselen was 12.6 μM (Fig. 6). However, removal of Ca^{2+} leads to a considerable shift of the IC_{50} value to 1.2 μM ebselen.

Interaction of Ca^{2+} with the C2 Domain Protects 5-LO Against Inhibition by GPx-1—It appeared possible that Ca^{2+} could suppress the activity of GPx-1, thereby rendering 5-LO activity resistant against GPx-1. However, we found that Ca^{2+} does not significantly suppress GPx-1 activity in MM6-ATP-PT or the activity of GPx-1 isolated from bovine erythrocytes (not shown), implying that Ca^{2+} rather protects 5-LO against GPx-1, instead of abolishing GPx-1 activity. In order to investigate if a functional C2 domain is important for protection of 5-LO against GPx-1 activity by Ca^{2+}, a mutated 5-LO (N43A, D44A, and E46A, loop2 mut-5LO, Ref. 19), which requires about 10–100 fold higher Ca^{2+} concentrations for binding and stimulation of 5-LO reactions, was tested. wt 5-LO and loop2 mut-5LO were purified and incubated in the absence and in the presence of 10 μM Ca^{2+} with or without soluble fractions of undifferentiated MM6 cells (which are devoid of 5-LO (36) but possess high GPx-1 activity, Ref. 12) and 5-LO activity (at 20 μM AA) was determined. In the absence of Ca^{2+}, the capacity for 5-LO product synthesis was substantial for wt- but also for loop2 mut-5LO, although the specific activity of the wt-5-LO (112.3 μg 5-H(P)ETE/mg of protein) was about 3-fold greater than that of loop2 mut-5LO (36.5 μg 5-H(P)ETE/mg of protein). In agreement with previous studies (19), in the presence of 10 μM Ca^{2+}, the activity of loop2 mut-5LO was unchanged, whereas the activity of wt-5-LO was increased about 2- to 3-fold. To ensure comparable capacities of 5-LO product synthesis in incubations containing either wt- or loop2 mut-5LO, the amounts of wt-5-LO and loop2 mut-5LO were adjusted to obtain comparable 5-HPETE formation. Addition of MM6 soluble fractions in the absence of Ca^{2+} suppressed the activity of both enzymes. Of interest, in the presence of 10 μM Ca^{2+}, MM6 soluble fractions (or GPx-1 plus 10 μM GSH, not shown) suppressed the activity of loop2–5LO, whereas the activity of wt-5-LO (that binds Ca^{2+}) was rather increased (Fig. 7A). Thus, Ca^{2+} binding at the C2 domain protects 5-LO activity against the M-DSP or GPx-1. Importantly, the GPx inhibitors iodoacetate or mercaptosuccinate and also 13(S)-HPODE were able to restore the activity of the loop2 mut-5LO, suppressed by the MM6 soluble fraction in the presence of 10 μM Ca^{2+} (Fig. 7B).

DISCUSSION

We have recently published that in the absence of Ca^{2+}, 5-LO in broken cell preparations of PMNL and RBL-1 cells has considerable catalytic activity, whereas in broken cell preparations of the monocytic cell line MM6, a soluble protein confers
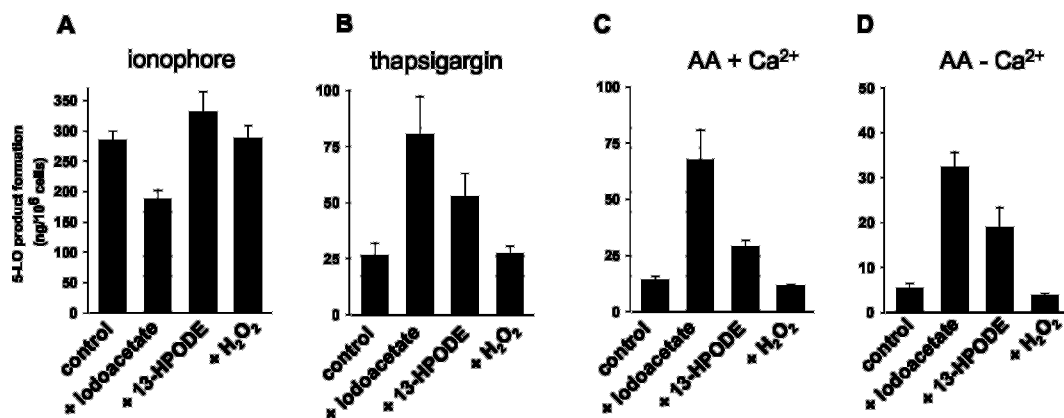
FIG. 5. **Effects of iodoacetate and hydroperoxides on 5-LO product synthesis in intact MM6 cells.** MM6 cells (2×10^6) were resuspended in 1 ml of PG buffer. Iodoacetate (2 mM), 13(S)-HPODE (3 μM), and hydrogen peroxide (3 μM) were added as indicated and cells were incubated at 37 °C. After 5 min, cells were stimulated with the following agents in the presence of 40 μM AA: *A*, 1 mM CaCl$_2$ and 5 μM ionophore A23187; *B*, 1 mM CaCl$_2$ and 1 μM thapsigargin; *C*, 1 mM CaCl$_2$; *D*, 1 mM EDTA and BAPTA/AM. After 10 min at 37 °C, respectively, 5-LO product formation was determined. Results are given as mean ± S.E., $n = 4$.
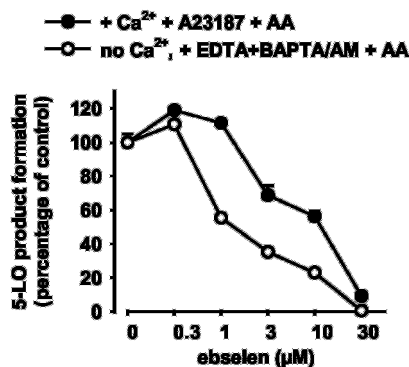

FIG. 6. **Effects of ebselen on 5-LO product synthesis in PMNL.** Freshly isolated PMNL (5×10^6) were resuspended 1 ml of PBS containing 1 mM Ca^{2+} or 1 mM EDTA plus 30 μM BAPTA/AM and ebselen at the indicated concentrations was added. After 30 min at 37 °C, cells preincubated in the presence of Ca^{2+} were stimulated with 2.5 μM ionophore A23187 plus 10 μM AA, cells preincubated in the presence of EDTA and BAPTA/AM were stimulated with 60 μM AA alone. After another 10 min at 37 °C, 5-LO product formation was determined as described. Results are given as mean ± S.E., $n = 5$.

5-LO activity Ca^{2+}-dependent (29, 34). Although we were unable to completely purify this protein in an active state, we provide strong evidence that this M-DSP is GPx-1. First, the apparent size of the M-DSP (80–100 kDa) fits well with the molecular mass (87 kDa) of GPx-1, and the M-DSP shares chromatographic properties with GPx-1 (Fig. 1*A* and Refs. 12 and 29). Second, mercaptosuccinate, a specific inhibitor of GPx-1 (41) as well as the broad spectrum GPx inhibitor iodoacetate (42) reversed the M-DSP-induced 5-LO inhibition in the absence of Ca^{2+}, without significantly affecting 5-LO catalysis in the presence of Ca^{2+} (Figs. 1–3). Similar effects were obtained with 13(S)-HPODE, which counteracts GPx activities. Third, GPx-1 activity in MM6, RBL-1 cells and PMNL is negatively correlated to the cell's capacity to form 5-LO products at low intracellular Ca^{2+}-levels (Table I). Finally, GPx-1, isolated from bovine erythrocytes mimicked the inhibitory effects of the M-DSP with respect to reduction of 5-LO activity, and this GPx-1-mediated 5-LO suppression could be overcome by mercaptosuccinate, iodoacetate, or 13(S)-HPODE.

Members of the GPx family are well recognized endogenous inhibitors of LOs (5, 9, 10, 12, 15, 47), which act by reducing the level of LOOH. A certain level of LOOH is required for the conversion of the active site iron from the ferrous to the ferric state thereby initializing the LO reaction (6–8). Thus, 15-HPETE, 12-HPETE, 5-HPETE, and 13-HPODE, but not H$_2$O$_2$, could stimulate 5-LO *in vitro* (8). In accordance, 13(S)-HPODE but not H$_2$O$_2$ counteracted M-DSP- or GPx-1-induced 5-LO suppression. Although GPx-4 has been reported to inhibit 5-LO activity in differentiated HL-60 and BL41-E95-A cells (11), RBL-2H3 cells (14) and A431 cells (13), it is unlikely that this peroxidase is the M-DSP, since GPx-4 is a monomeric 19 kDa protein (48) that is not sensitive to mercaptosuccinate (5) and requires millimolar concentrations of thiols for efficient inhibition of 5-LO activity (11, 12). Interestingly, Coffey *et al.* (49) reported about a cytosolic protein of mononuclear phagocytes that reduced 5-LO activity in broken cell preparations and prolonged the lag phase of soybean LO, which was reversed by addition of 13(S)-HPODE. Recently we showed that in MM6 cells, GPx-1 but not GPx-4 is involved in the regulation of cellular 5-LO activity (12). For the classical, high turnover rate reduction of peroxides, GPx-1 utilizes GSH and to a lesser extent dithiothreitol in the millimolar range (50, 51). Surprisingly, in this as well as in our previous study (12), reduction of 5-LO activity by GPx-1 occurred also in the absence of millimolar concentrations of GSH or dithiothreitol, and was supported also by 0.5 mM β-mercaptoethanol (12) (which functions as a less efficient cofactor for classical peroxidase activity). Also, protection of 5-LO against inactivation during storage by GPx-1 was supported best by β-mercaptoethanol (46). The reduction of 5-LO activity by M-DSP did not depend on millimolar concentrations of thiols. Thus, sole addition of the 80–100 kDa fraction from gel-permeation chromatography, which should not contain endogenous thiols of low molecular mass, suppressed 5-LO activity in the absence of Ca^{2+}. Presumably, after cell lysis, GPx-1 exists in the reduced selenol form, the species that is responsible for rapid reduction of hydroperoxides. Such hydroperoxide reduction leads to an oxidation of the active- site selenol to selenenic acid, which can oxidize GSH to recover the active selenol form of GPx-1 (52). Possibly, this selenol species could become oxidized during the extended time-consuming purification procedures, explaining why the activity of the M-DSP was lost. Also, GPx-1, purified from erythrocytes, may exist in the selenenic form, explaining why GSH (at least in the micromolar range), supernatants containing traces of thiols, or cellular low molecular mass components were necessary for suppression of 5-LO in the absence of Ca^{2+}. However, for PMNL, which possess only low GPx levels (Table I), 5-LO in S100 was only suppressed when GSH in the millimolar range was present, but also under these experimental settings, Ca^{2+} could protect 5-LO against GPx activity (Fig. 2*B*).
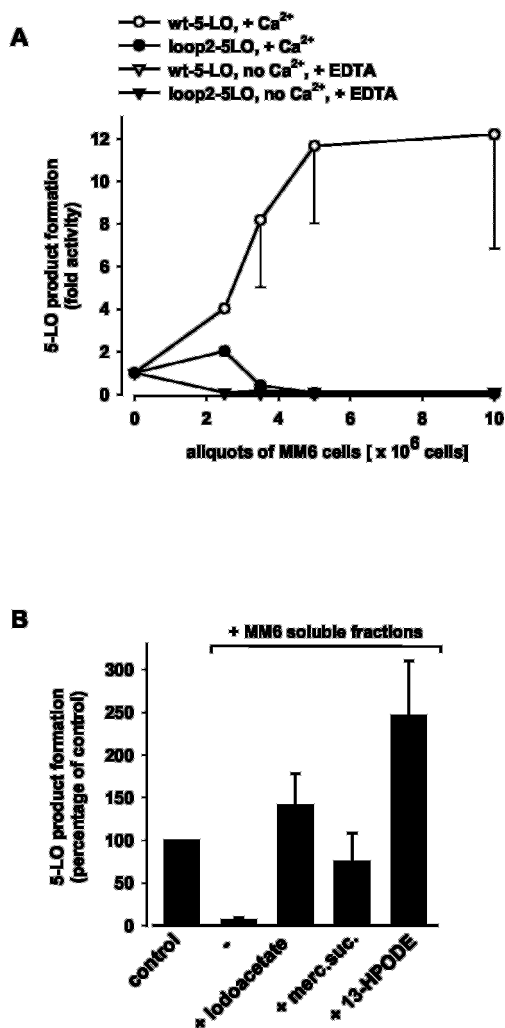
A

○— wt-5-LO, + Ca²⁺
●— loop2-5LO, + Ca²⁺
▽— wt-5-LO, no Ca²⁺, + EDTA
▼— loop2-5LO, no Ca²⁺, + EDTA

B

+ MM6 soluble fractions

FIG. 7. **A functional** Ca^{2+} **binding site within the C2 domain protects 5-LO against inhibition by M-DSP.** *A*, effect of Ca^{2+} on 5-LO activity in the presence of M-DSP. wt-5-LO and loop2–5LO, lacking high affinity for Ca^{2+}, were expressed in *E. coli*. 5-LO proteins were diluted in ice-cold PBS containing 10 μM EDTA and 1 mM ATP. Aliquots of soluble fractions of undifferentiated MM6 cells (devoid of 5-LO) were added as indicated to a final volume of 1 ml. After 5 min, samples were prewarmed for 30 s and $CaCl_2$ (20 μM) was added as indicated together with 20 μM AA. After another 10 min at 37 °C, 5-LO product synthesis was determined as described. *B*, effects of GPx inhibitors and 13(*S*)-HPODE on suppression of loop2 mut-5LO by M-DSP. Loop2 mut-5LO was diluted in ice-cold PBS containing 10 μM EDTA and 1 mM ATP. Aliquots of soluble fractions of MM6 cells (corresponding to 5×10^6 cells), iodoacetate (2 mM), and mercaptosuccinate (30 μM) were added as indicated; final volume was 1 ml. After 5 min, samples were prewarmed for 30 s and $CaCl_2$ (20 μM) and 13(*S*)-HPODE (3 μM) were added together with 20 μM AA. After another 10 min at 37 °C, 5-LO product synthesis was determined. Results are given as mean $\pm$ S.E., $n = 3$, as fold increase over 5-LO product formation of control induced in the absence of M-DSP.

The finding that Ca^{2+} is required to allow 5-LO activity in the presence of GPx-1 *in vitro*, may also be relevant for 5-LO catalysis in intact cells. In fact, the capacities of MM6 cells to form 5-LO products correlate with the intracellular Ca^{2+} levels (Fig. 5, Table I, and Ref. 29). Thus, when the Ca^{2+} supply was substantial, 5-LO product synthesis was prominent, and elimination of GPx activity by iodoacetate or 13(*S*)-HPODE caused no further enhancement. However, when cells were depleted of extracellular Ca^{2+} by chelation, counteraction of GPx-1 potently enhanced the low 5-LO product synthesis. Similarly, only Se^{4+}-supplemented RBL-1 cells (possessing high GPx-1 activity) required elevation of intracellular Ca^{2+} for prominent

5-LO product synthesis, whereas the Ca^{2+} levels played a minor role in cells exerting low GPx-1 activity (Table I). Finally, ebselen, which mimics GPx-1 activity in the presence of thiols (44), suppressed 5-LO activity much more efficiently in PMNL when Ca^{2+} levels were reduced, as compared with conditions where Ca^{2+} was elevated (Fig. 6).

Enzymatic active 5-LO forms the LOOH 5-HPETE and it appeared possible that the effectiveness of GPx-1 to suppress 5-LO activity depends on the ratio of GPx-1 capacity to 5-LO capacity. However, increasing the amounts of 5-LO in the incubation mixtures could not impair the degree of 5-LO inhibition, indicating that the effectiveness of GPx-1 to inhibit 5-LO was seemingly independent of the amounts of 5-LO products (5-HPETE) formed during catalysis. Also, in the incubations of wild-type and loop2 mut-5LO the absolute amounts of 5-LO products formed were about the same, but the enzyme activities were differentially suppressed by GPx-1. Presumably, instead the affinity of 5-LO for LOOH prior to the initiation of the 5-LO reaction determines enzyme activation.

The underlying molecular mechanism of how Ca^{2+} renders 5-LO resistant against GPx-1 activity is not clear. Although conceivable, our data show that the 5-LO protective effect of Ca^{2+} is not related to a direct suppression of GPx-1 activity by Ca^{2+}. Another possibility could be that Ca^{2+} directly facilitates the conversion of the active site iron from the ferrous to the active ferric state at suboptimal LOOH concentrations, for example by alteration of the iron coordination, in particular by the flexible ligands His-367, Asn-554, or a putative water molecule (7). However, no significant effects of Ca^{2+} regarding the redox state of the active site iron were observed when 5-LO was investigated by electron paramagnetic resonance spectroscopy (7), and based on a proposed model of 5-LO (19), the Ca^{2+} binding sites are rather distant from the active site iron.

Ca^{2+} binds to the N-terminal C2 domain of the 5-LO enzyme (19) and such Ca^{2+} binding increases the hydrophobicity of the protein (19) allowing an association with phospholipids or cellular membranes (27, 28). Mutation of Asn-43, Asp-44, and Glu-46 to alanine within the C2 domain of 5-LO (loop2 mut-5LO) caused decreased Ca^{2+} binding and a requirement for higher Ca^{2+} concentrations to stimulate enzyme activity (19). Interestingly, in contrast to wt-5-LO, Ca^{2+} was not able to protect the loop2 mut-5LO against the effect of M-DSP or GPx-1 (Fig. 7*A*). It should be noted that wild type and loop2 mut-5LO exert no significant variations in the uninhibited (no GPx) enzyme activities, neither in the absence nor in the presence of Ca^{2+}. Again, mercaptosuccinate and 13(*S*)-HPODE, but not Ca^{2+}, were capable of counteracting the suppressed activity of mutated loop2 mut-5LO (Fig. 7*B*). Thus, the Ca^{2+} binding sites within the C2 domain seem to mediate the protective effects of Ca^{2+} against GPx-1 and the M-DSP. It was shown that Ca^{2+} lowers the K_m value of 5-LO for enzymatic conversion of AA (33, 53) and preliminary results from our group indicate that Ca^{2+} strongly increases the binding of AA to 5-LO.[2] Along these lines it is conceivable, that in analogy to AA, the Ca^{2+}-mediated increase in hydrophobicity of 5-LO could augment also the affinity toward LOOH, thus allowing 5-LO activation at lower LOOH levels. Experimental data (7, 37, 53, 54) suggest that 5-LO (as other LOs, Ref. 55) may have two fatty acid binding sites, one catalytic and one regulatory, where the latter may be the primary site for LOOH binding. Interestingly, nonredox-type 5-LO inhibitors, which presumably act at such a regulatory fatty acid (LOOH) binding site, require low LOOH levels or GPx activity, respectively, for efficient 5-LO inhibition (37). Investigation of the LOOH binding site of 5-LO

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in relation to Ca^{2+} is in progress in our laboratory.

Taken together our data suggest that Ca^{2+} binding at the C2 domain facilitates 5-LO activation at low LOOH levels, which are controlled by GPx-1. In such a scenario, Ca^{2+} may lead to an increased affinity of 5-LO for LOOH at a putative regulatory fatty acid binding site. 5-LO can be phosphorylated at serine residues by MAPKAPK-2 and ERKs (30, 32), and stimulation of these kinases activated 5-LO in intact cells in the absence of Ca^{2+} (30, 31, 34). Possibly, also phosphorylation could reduce the requirement of LOOH, it is tempting to speculate that Ca^{2+} and phosphorylation may act together to activate 5-LO, by reducing the LOOH requirement for conversion of the active site iron from the ferrous to the ferric form. Alternatively, as discussed before (30), phosphorylation might lead to a small pool of active cellular 5-LO, which could form LOOH, and thus activate the bulk of 5-LO in the cell. In this context it is of interest that phosphorylation-induced 5-LO activity was rather resistant against non-redox-type 5-LO inhibitors (56), which require low LOOH level for efficient 5-LO inhibition (37).

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