Regulation of the thyroid NADPH-dependent H₂O₂ generator by Ca²⁺: studies with phenylarsine oxide in thyroid plasma membrane

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Pig thyroid plasma membranes contain a Ca²⁺-dependent NADPH:O₂ oxidoreductase, the thyroid NADPH-dependent H₂O₂ generator. This provided the H₂O₂ for the peroxidase-catalysed synthesis of thyroid hormones. The effect of the trivalent arsenical, phenylarsine oxide (PAO), on the NADPH oxidase was studied. PAO caused two directly related dose-dependent effects with similar half-effect concentrations of PAO (3 nmol of PAO/mg of protein): (i) partial inactivation of H₂O₂ formation by the Ca²⁺-stimulated enzyme, and (ii) desensitization of the enzyme activity to Ca²⁺. PAO had no effect on membranes that had been Ca²⁺-desensitized by α -chymotrypsin treatment. The NADPH oxidase in membranes treated with excess PAO had the same $V_{\max}$ with and without Ca²⁺. This value was half the $V_{\max}$ of the native enzyme. However, the K_m for NADPH determined with Ca²⁺ (18 μ M, identical with that of the native

enzyme) was approx. one-third of the K_m measured without Ca²⁺, showing the direct action of Ca²⁺ on the PAO–enzyme complex. PAO had the same effects, partial inactivation and Ca²⁺ desensitization, on the NADPH:ferricyanide oxidoreductase activity of the NADPH oxidase, suggesting that PAO acts on the flavodehydrogenase entity of the enzyme. Both partial inactivation and Ca²⁺ desensitization were completely and specifically reversed by 2,3-dimercaptopropanol, partly reversed by dithiothreitol and not reversed by 2-mercaptoethanol, indicating that PAO binds to vicinal thiol groups. These results suggest that thiol groups are involved in the control of thyroid NADPH oxidase by Ca²⁺; PAO bound to vicinal thiols might alter the structure of the enzyme so that electron transfer occurs without Ca²⁺ but more slowly.

INTRODUCTION

Thyroid hormones are synthesized at the apical surface of the follicular cells during or after fusion of exocytotic vesicles with the apical membrane. The final two reactions, iodination of tyrosyl residues and coupling to form the hormone, are catalysed by thyroid peroxidase in the presence of H₂O₂. Production of H₂O₂ is a limiting step of hormone synthesis in the presence of physiological concentrations of iodide [1]. An NADPH:O₂ oxidoreductase, called NADPH oxidase by analogy with the respiratory-burst NADPH oxidase of phagocytes, is the H₂O₂ generator system that is associated with thyroperoxidase in pig thyroid cells [2]. The NADPH oxidase in these cells is induced by thyrotropin via the cAMP cascade, and the rate at which the oxidase appears in thyrotropin-treated cells agrees with the functional characteristics of these cells as indicated by their iodination capacity. This enzyme is an integral membrane flavoprotein [3,4].

The Ca²⁺ ionophore A23187 was used on pig thyroid cells to show that Ca²⁺ triggers the thyroid NADPH oxidase [5], and signalling pathways mediated by Ca²⁺ have been implicated in the regulation of H₂O₂ production in thyroid cells from several species [6–8]. We have shown that micromolar concentrations of Ca²⁺ reversibly regulate H₂O₂ production by thyroid NADPH oxidase with the use of pig particles [9] and purified thyroid plasma membranes [10,11] and that this effect occurs by a direct action of Ca²⁺ on the membrane enzyme.

However, limited proteolysis of the pig thyroid particulate fraction by α -chymotrypsin, or heat treatment with Zn²⁺ in the presence of proteinase inhibitors, gives an NADPH oxidase that is fully active in the absence of Ca²⁺; it is thus a Ca²⁺-desensitized

enzyme [12], and the activation by limited proteolysis is irreversible. The activation by Zn²⁺ was not reversed by Zn²⁺ chelators such as 1,10-phenanthroline and EGTA, but dithiothreitol (DTT) partly reversed it. This last result suggests that thiol groups are implicated in the activation by Ca²⁺.

Tervalent arsenoxides react with two thiol groups that are either adjacent in the protein sequence or close together in the folded protein (i.e. vicinal dithiols) and form stable dithioarsine rings. Vicinal cysteinyl residues interact with arsenoxides in several systems, including lipoamide in the pyruvate dehydrogenase complex [13], Cys³¹–Cys¹⁸⁴ in lecithin–cholesterol acyl transferase [14], the redox-active pair of Cys residues in the active site of thioredoxin [15,16] and the adjacent Cys residues in the reduced nicotinic acetylcholine receptor [17]. Vicinal thiol groups are believed to be implicated in the interaction of arsenoxides with a variety of enzyme systems, such as phosphotyrosyl protein phosphatases [18], the hexose transport system [19], the enzymes involved in the activation of neutrophil [20,21] or macrophage [22] NADPH oxidase, and in the association of NADPH oxidase subunits in the neutrophil membrane [23], because the complexes with arsenoxides can be specifically reversed by vicinal dithiol competitors but not by monothiols.

The present study examines the effects of phenylarsine oxide (PAO) on the NADPH oxidase of thyroid plasma membranes. The Ca²⁺/NADPH-dependent formation of H₂O₂ catalysed by the thyroid membrane fraction is sensitive to PAO, which allowed electron transfer from NADPH to O₂ to occur without Ca²⁺, but at a lower rate than with the native enzyme. The PAO effects are specifically and fully reversed by British anti-Lewisite (2,3-dimercaptopropanol; DMP). Our results suggest that vicinal thiols of the flavodehydrogenase entity of the NADPH oxidase

are implicated in the activation of the H_2O_2 generator system by Ca^{2+} .

MATERIALS AND METHODS

Materials

NADPH and freeze-dried horseradish peroxidase (grade 1) were purchased from Boehringer (Mannheim, Germany); scopoletin was from Serva (Heidelberg, Germany); CHAPS, FAD, $\text{K}_3\text{Fe}(\text{CN})_6$, cytochalasin B, DMP, 2-mercaptoethanol, antipain, aprotinin, leupeptin, pepstatin, benzamidine, trypsin inhibitor and tosyl-lysylchloromethane-treated α -chymotrypsin were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.); PAO was from Aldrich-Chemie (Steinheim, Germany).

Preparation of pig thyroid plasma membranes

The preparation of plasma membranes from pig thyroid glands was adapted from Nakamura et al. [10] as previously described [11]. An NADPH:cytochrome *c* oxidoreductase activity, not related to H_2O_2 production, was removed from freshly prepared plasma membranes by extraction with CHAPS [24]. The plasma membranes (5–10 mg/ml) were washed once with 10 mM CHAPS in 50 mM sodium phosphate buffer, pH 7.2, containing 2 mM MgCl_2 , 0.25 M sucrose (buffer A) and a cocktail of protease inhibitors (4 $\mu\text{g}/\text{ml}$ leupeptin, 4 $\mu\text{g}/\text{ml}$ antipain, 0.1 mg/ml benzamidine, 50 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ pepstatin and 1 $\mu\text{g}/\text{ml}$ trypsin inhibitor). The membrane fraction was collected by centrifugation at 10000 *g* for 15 min at 4 °C and resuspended in buffer A in the presence of protease inhibitors. Membranes were quick-frozen in liquid nitrogen and stored at –80 °C. The specific activity of the membranes was approx. 600 nmol of $\text{H}_2\text{O}_2/\text{h}$ per mg of protein.

Treatment of the membrane fraction with α -chymotrypsin

Membrane fraction was pelleted by centrifugation at 8000 *g* for 15 min at 4 °C, resuspended (10 mg/ml protein) in 20 mM Tris/HCl, pH 8, containing 0.1 mM MgCl_2 , 10 μM FAD, 2 mM CaCl_2 , 1 mM CHAPS and 1 mM tosyl-lysylchloromethane-treated α -chymotrypsin (2 *N*-benzoyl-L-tyrosine ethyl ester units/ml) and incubated for 10 min at 30 °C. Proteolysis was stopped by adding 1.5 mM PMSF. The treated membrane fraction was pelleted by centrifugation at 8000 *g* for 15 min at 4 °C and resuspended (5–6 mg/ml protein) in buffer A plus 0.5 mM CaCl_2 , 0.4 mM EGTA and 1 mM NaN_3 . Aliquots were quick-frozen in liquid nitrogen and stored at –80 °C.

Treatment of the membrane fraction with PAO

The membrane fraction (1 ml, 30–50 $\mu\text{g}/\text{ml}$ protein) was routinely preincubated for 10 min at 0 °C in 2 ml Eppendorf tubes in 50 mM sodium phosphate, pH 7.25, containing 0.5 mM CaCl_2 , 0.4 mM EGTA, 1 mM NaN_3 and 10 μM FAD (buffer B), and 10 μl of freshly prepared PAO solution (at different concentrations in DMSO). DMP, which reacts with free PAO, could not be used to stop the preincubation of the membranes with PAO because it reversed the effects of PAO on membranes (see the Results section). Preincubation could be stopped only by centrifugation and washing. The preincubation time used allowed complete reaction with PAO, whatever the PAO concentration tested. The membrane fraction was pelleted by centrifugation at 8000 *g* at 4 °C for 10 min in a Hettich 30 RF centrifuge. The membrane pellet was washed once with 2 ml of 50 mM sodium

phosphate buffer, pH 7.25, containing 0.5 mM CaCl_2 , 0.4 mM EGTA, 1 mM NaN_3 , 10 μM FAD and 1 mM CHAPS. The final pellet was resuspended in 50 μl of 200 mM sodium phosphate buffer, pH 7.25, containing 0.5 mM CaCl_2 , 0.4 mM EGTA, 1 mM NaN_3 and 10 mM CHAPS, and residual activity was measured immediately. The final pellet was resuspended as before but without Ca^{2+} and with 7.5 mM EGTA for studying the effect of the absence of Ca^{2+} . Control membranes were treated under the same conditions, except that PAO was omitted.

Measurement of H_2O_2 production

H_2O_2 formation was measured routinely by incubating samples of membrane fraction at 30 °C in 1 ml of 200 mM sodium phosphate, pH 7.25, containing 1 mM NaN_3 , 0.4 mM EGTA, 0.5 mM CaCl_2 and 0.5 mM CHAPS. The reaction was started by adding 0.1 mM NADPH; 100 μl samples were collected at various times up to 20 min and mixed with 10 μl of 2.4 M HCl to stop the reaction by destroying the remaining NADPH. The amount of H_2O_2 in each sample was measured by the decrease in scopoletin fluorescence in the presence of horseradish peroxidase in a Perkin–Elmer MPF43A spectrofluorimeter, as previously described [9]. Initial rates of H_2O_2 formation were determined on eight samples from each test and expressed as nanomoles of $\text{H}_2\text{O}_2/\text{h}$ per ml of membrane.

H_2O_2 formation catalysed by membranes with or without Ca^{2+} was assayed: the incubating medium contained either 8 mM CaCl_2 and 7.5 mM EGTA, or 7.5 mM EGTA alone.

Measurement of NADPH:ferricyanide oxidoreductase activity

Membrane fraction was incubated at 30 °C in 1 ml of 200 mM sodium phosphate buffer, pH 7.25, containing 1 mM NaN_3 , 0.1 mM ferricyanide and 7.5 mM EGTA, with or without 8 mM CaCl_2 . The reaction was started by adding 0.1 mM NADPH; aliquots (100 μl) were collected at various times up to 20 min and mixed with 10 μl of 2.5 mM DTT and 100 μl of 2% (w/v) SDS to stop the reaction. The initial rates of ferricyanide-dependent NADPH oxidation were determined from eight aliquots of each assay by following the decrease in NADPH fluorescence at pH 8.0 in a Perkin–Elmer MPF43A spectrofluorimeter. The excitation and emission wavelengths were 340 and 453 nm.

NADPH:ferricyanide oxidoreductase activity was calculated from the change in fluorescence determined from the linear portion (up to 15–20 min) of the reaction curve. The change in fluorescence of a blank containing all reactants except the membrane fraction, corresponding to the rate of chemical NADPH oxidation in the presence of ferricyanide, was systematically subtracted. It represented approx. 7% of the initial fluorescence. The activity is expressed as nmol of NADPH oxidized/h per ml of membrane fraction.

Determination of protein content

Protein concentration was determined by the method of Bradford [25].

RESULTS

Effects of PAO on H_2O_2 formation: partial inhibition and Ca^{2+} desensitization

The initial rate of H_2O_2 formation by the PAO-treated membranes was measured in the presence of Ca^{2+} (Figure 1). Parallel experiments were performed with different amounts of protein membrane fraction (35–435 $\mu\text{g}/\text{ml}$) and different concentrations

Figure 1 Effect of increasing concentrations of PAO on H_2O_2 formation by thyroid membrane NADPH oxidase in the presence of Ca^{2+}

The membrane fraction was preincubated for 10 min at 4 °C with PAO in buffer B. Parallel experiments were performed with 35 (★), 87 (●), 143 (■) and 435 (▲) μg of protein per ml. The membranes were centrifuged and washed as described in the Materials and methods section; residual H_2O_2 -forming activity was measured with Ca^{2+} plus 100 μM NADPH; inhibition is plotted as a function of the concentration of PAO, and (inset) as a function of the amount per mg of membrane protein.

Figure 2 Ca^{2+} desensitization of NADPH-dependent H_2O_2 formation by PAO

(A) H_2O_2 formation as a function of PAO concentration: membrane fraction (●, ○; 36 $\mu\text{g}/\text{ml}$ protein) or membrane fraction treated with α -chymotrypsin (▲, △) was incubated for 10 min at 4 °C with different concentrations of PAO in buffer B. The membrane fraction was centrifuged and washed. Residual H_2O_2 -forming activity was measured with (●, ▲) or without (○, △) Ca^{2+} plus 100 μM NADPH. (B) Ca^{2+} desensitization as a function of PAO concentration. Ca^{2+} desensitization (%) = $100 \times (\text{activity measured in the absence of } \text{Ca}^{2+}) / (\text{activity measured in the presence of } \text{Ca}^{2+})$.

of PAO. When the activity was plotted against PAO concentration for different fixed amounts of membranes, the activities decreased in parallel with the PAO concentration, to reach a plateau at about half the activity of the membranes treated without PAO (control membrane). The half-maximum inactivatory effect increased with the protein membrane concentration. A smooth dose-response curve was obtained when the PAO concentration was expressed in nmol/mg of membrane protein (Figure 1, inset); half-inactivation was obtained with 3 nmol of PAO/mg of membrane protein.

The initial rate of H_2O_2 formation catalysed by PAO-treated membranes was measured in the absence of Ca^{2+} (Figure 2A). Activity increased with the PAO concentration until a plateau was reached in excess PAO (30 nmol/mg of protein). This plateau activity was approx. 80 % of the inhibited activities observed for the same PAO-treated membranes in the presence of Ca^{2+} . This indicates a desensitization to Ca^{2+} of the PAO-treated NADPH

Figure 3 Effect of Ca^{2+} on the kinetic constants of PAO-treated NADPH-dependent H_2O_2 generator (Lineweaver-Burk plot)

Membrane fraction was treated with PAO (220 nmol/mg of protein) as described in the Materials and methods section. The NADPH-dependent H_2O_2 -generating activity was measured in the absence (○) or in the presence (●) of Ca^{2+} . The H_2O_2 -generating activity of the control membrane fraction was measured in the presence (▲) of Ca^{2+} . The NADPH concentration was determined by measuring the absorbance at 340 nm.

oxidase. This desensitization was quantified using the initial rate of H_2O_2 formation in the absence of Ca^{2+} expressed as a percentage of the initial rate of H_2O_2 formation measured in the presence of Ca^{2+} . This value was plotted against the PAO concentration (Figure 2B). The half-effect was obtained at 10 nmol of PAO/mg of protein. (i.e. a value slightly higher than the inhibitory value). However, PAO had no effect on α -chymotrypsin-treated membranes (Figure 2A).

As illustrated in Figure 3, excess NADPH substrate inhibited H_2O_2 formation, therefore more than 100 μM NADPH could not be used to measure the enzyme activity. The H_2O_2 formation catalysed by membranes treated with excess PAO (220 nmol/mg) was measured at different NADPH concentrations with or without Ca^{2+} . The same V_{max} was obtained, corresponding to 100 % Ca^{2+} desensitization for an infinite concentration of NADPH. However, the K_m was 18 μM in the presence of Ca^{2+} and 50 μM in its absence (Figure 3). The PAO-treated membranes had a K_m decreased by half in the presence of Ca^{2+} , which was similar to that of the control membrane.

Reversibility of PAO effects by thiol reagents

To determine whether vicinal thiol groups were involved in the PAO effect on NADPH oxidase, attempts were made to reverse the effects with monothiol or dithiol. Membranes pretreated with 2 μM PAO were incubated with increasing concentrations of dithiols (DMP and DTT) or monothiol (2-mercaptoethanol) (Figure 4A). The membranes were extensively washed and H_2O_2 formation was measured in the presence of Ca^{2+} . The inhibition of H_2O_2 formation was completely reversed by 200 μM DMP, whereas 40 % of the inhibition was reversed by 1 mM DTT; 1 mM 2-mercaptoethanol was without effect (Figure 4A). Neither DMP, DTT nor 2-mercaptoethanol affected the rate of H_2O_2 formation with the thiol concentrations used in the absence of PAO.

Control membranes and PAO-treated membranes (40 nmol of PAO/mg of protein) were incubated in parallel experiments

Figure 4 Effects of DMP (●), DTT (○) and 2-mercaptoethanol (▲) on the PAO-treated membrane fraction

(A) Membrane fraction (50 μg/ml protein) was incubated with 2 μM PAO (40 nmol of PAO/mg of protein) for 10 min at 4 °C; DMP, DTT or 2-mercaptoethanol was added at the indicated concentration and incubation was continued for 10 min at 4 °C. Membrane was collected by centrifugation, washed once with buffer B plus 1 mM CHAPS, with the addition of the appropriate thiol reactant, and washed again with buffer B plus 1 mM CHAPS. The H₂O₂-forming activity of the PAO-treated membrane fraction was measured in the presence of Ca²⁺ and expressed as a percentage of that of the control membrane fraction incubated under the same conditions. (B) Reversal of desensitization to Ca²⁺ of NADPH oxidase activity by DMP. The Ca²⁺ desensitization is the initial rate of H₂O₂ formation measured in the presence of EGTA, expressed as a percentage of the initial rate measured in the presence of Ca²⁺.

Table 1 Effect of PAO on the NADPH:ferricyanide reductase activity of the membranes

Membrane fractions were incubated with PAO (220 nmol/mg of protein) as described in the Materials and methods section. Residual NADPH-oxidizing activities were measured in the presence of 70 μM NADPH with or without Ca²⁺. Results are means ± S.E.M. for three experiments.

	NADPH oxidation in the presence of K ₃ Fe(CN) ₆			
	+ Ca ²⁺		- Ca ²⁺	
	(nmol/h per ml)	(%)	(nmol/h per ml)	(%)
Membranes (control)	4217 ± 137	100	448 ± 67	10.5
PAO-treated membranes	2507 ± 118	59	1966 ± 166	46.5

with or without 1 mM DMP. The H₂O₂ formation was measured with or without Ca²⁺. The results indicated that PAO-treated membranes recovered their Ca²⁺ sensitivity when post-incubated with DMP; hence they again needed Ca²⁺ for H₂O₂ formation (Figure 4B).

Effect of PAO on NADPH:ferricyanide oxidoreductase activity

Thyroid NADPH oxidase transfers electrons from NADPH to an artificial acceptor, ferricyanide [11,24]. This catalysis needed micromolar Ca²⁺ concentrations. The ability of PAO-treated membranes to reduce ferricyanide was tested. PAO had a dose-dependent inhibitory effect when this activity was measured in the presence of Ca²⁺. As with H₂O₂ formation, inhibition was not complete and the residual NADPH:ferricyanide oxidoreductase activity was 50–60 % of that of the control membrane fraction. The half-inhibitory effect was obtained with 3–4 nmol of PAO/mg of protein (results not shown). The capacity of the PAO-treated membrane to reduce ferricyanide increased with PAO concentration in the absence of Ca²⁺. The ferricyanide reductase activity of membranes treated with an excess of PAO

(220 nmol/mg of protein) was partly inhibited and Ca²⁺-desensitized (Table 1).

DISCUSSION

Thyroid NADPH oxidase is a transmembrane enzyme that catalyses the transfer of two electrons from cytoplasmic NADPH to O₂ on the colloid side of the apical thyrocyte membrane, where H₂O₂ is produced. Unlike neutrophil cytochrome *b*-558, this transfer occurs without release of O₂⁻, and the molecular nature of the thyroid membrane-associated electron transport chain remains unknown. The thyroid H₂O₂ generator has a flavodehydrogenase component or domain with FAD as prosthetic group [4]. This entity could also transfer electrons from NADPH to an artificial scavenger such as ferricyanide without any H₂O₂ formation [11]. NADPH-dependent H₂O₂ formation, as well as NADPH:ferricyanide oxidoreductase activity, requires micromolar concentrations of Ca²⁺. This Ca²⁺-dependent activation process is completely reversible. The H₂O₂ generator could be Ca²⁺-desensitized, i.e. fully active in the absence of Ca²⁺, by limited proteolysis or Zn²⁺ treatment at 40 °C [12], and our previous results indicate that activation of the H₂O₂ generator by Ca²⁺ proceeds via an autoinhibitory domain or an inhibitory component.

The present study shows that the membrane H₂O₂ generator is sensitive to micromolar concentrations of PAO, with partial inhibition of H₂O₂ formation when the activity is measured with Ca²⁺, and activation of H₂O₂ formation when the activity is measured without Ca²⁺.

The residual activity obtained with an excess of PAO in the presence of Ca²⁺ is 40–50 % of the H₂O₂ formation obtained with the membrane control. A similar half-inhibitory value (3 nmol of PAO/mg of protein) was obtained when the concentration of PAO was expressed on the basis of membrane protein concentration, showing that hydrophobic PAO is dissolved in the lipophilic compartment (i.e. the lipidic phase of the membranes). An identical phenomenon was observed for the inhibitory effect of diphenylene iodonium, a lipophilic flavoprotein reactant on either neutrophil [26] or thyroid NADPH oxidase [4].

The production of H₂O₂ by PAO-treated membranes became Ca²⁺-independent. The half-effect of this Ca²⁺ desensitization was obtained for 10 nmol of PAO/mg of protein; this value was 3-fold higher than that needed to obtain the half-effect of the inhibition. Treating membranes with excess PAO gave a Ca²⁺-desensitized NADPH oxidase that catalysed H₂O₂ formation at half the rate.

The kinetic constants of the PAO-treated enzyme with NADPH as substrate were compared in the presence and absence of Ca²⁺. The turnover for H₂O₂ formation was unchanged but the *K_m* for NADPH was 18 μM with Ca²⁺ and 50 μM without it. A higher concentration of NADPH was needed to saturate the system in the absence of Ca²⁺ than in its presence. These results indicate that Ca²⁺ acts on NADPH binding. On the PAO–enzyme complex, NADPH is more accessible to its binding site in the presence of Ca²⁺ than in its absence. These results seem to be similar to those obtained for a particulate system in which thiol groups were suggested to be involved in Ca²⁺ desensitization for H₂O₂ formation by Zn²⁺ treatment [12]. Both Zn²⁺ and PAO treatments could give an enzyme insensitive to Ca²⁺ for its enzymic activity but with different *K_m* values for NADPH in the absence of Ca²⁺ (50 μM) and in its presence (18 μM).

The difference between the PAO concentrations giving half-inhibition and half-desensitization could be because H₂O₂ formation was measured at the same NADPH concentration (100 μM), which represents five times the *K_m* for measurements

with Ca^{2+} and twice the K_m without Ca^{2+} . In these conditions the activity measured without Ca^{2+} is underestimated by 20% (Michaelis equation) of the activity measured with Ca^{2+} . When this is allowed for, the values for the half-effect are similar. Therefore the Ca^{2+} desensitization induced by PAO and the inhibition of H_2O_2 formation seem to be directly related.

The NADPH:ferricyanide oxidoreductase activity of the PAO-treated membranes is also partly inhibited when measured in the presence of Ca^{2+} , and activated in the absence of Ca^{2+} compared with control membranes under the same Ca^{2+} incubation conditions. The half-effects of these phenomena were obtained at PAO concentrations similar to those for NADPH-dependent H_2O_2 formation, showing that PAO effects occur before the O_2 reduction step.

The way in which PAO binds to proteins is still unclear. Monothiol and dithiol can bind to *p*-amino-PAO with different affinities, depending on the nature of the ring formed [27–30]. PAO reacts with vicinal thiol groups to form a stable five-ring structure. The reaction was reversed by the vicinal dithiol competitor DMP, whereas monothiol or dithiol that did not present the correct space between thiol groups were ineffective. The inhibition of thyroid NADPH oxidase activity by PAO was completely reversed by the 1,2-dithiol DMP in a 100-fold molar excess over PAO. A 500-fold molar thiol excess of 2-mercaptoethanol was without effect. In contrast with another system [31–33], in which a small excess (2–50) of the 1,4-dithiol DTT completely reversed the PAO inhibition, a large excess of DTT (250–500-fold) only partly reversed the inhibition of NADPH oxidase by PAO, suggesting that there is a precise space between the vicinal thiols of NADPH oxidase that react with PAO. The large excess of DMP needed to reverse the PAO inhibition completely, compared with the concentrations needed to reverse arsenoxide inhibition in other systems [23,29], might be due to the nature of the hydrophobic target site of PAO, which could be difficult to reach by DMP. The complete reversibility of the inhibition by DMP shows that PAO does not disrupt the integrity of the plasma membrane or cause any irreversible damage to electron transport under these conditions. The reversal of inhibition by DMP was also accompanied by the recovery of Ca^{2+} sensitivity of the membrane NADPH oxidase. The selective reversal by DMP suggests that vicinal thiols are involved in the partial inhibition and in Ca^{2+} desensitization of the NADPH oxidase, and provides further evidence that these two effects are directly related.

PAO did not inhibit H_2O_2 formation by proteolysed NADPH oxidase, which was fully active in the absence of Ca^{2+} . Proteolysis alters the entity or the domain liable to activation by Ca^{2+} , and could eliminate groups implicated in PAO binding or destroy the conformation needed for binding. As the system is completely activated, it is also possible that PAO is still bound but causes no inhibition. Binding studies with radiolabelled PAO and purified NADPH oxidase are needed to determine exactly what happens.

The importance of thiol groups for thyroid NADPH oxidase was investigated previously by blocking these groups with specific reagents [34]. H_2O_2 formation catalysed by the PAO-treated enzyme and by the proteolysed NADPH oxidase, on which PAO has no action, are both completely inhibited by 0.1 mM *p*-hydroxymercuribenzoate (results not shown). Thus the thiol groups needed for enzyme activity are different from those implicated in PAO binding and regulation by Ca^{2+} .

In conclusion, PAO acts on the flavodehydrogenase entity of the thyroid NADPH oxidase to give an enzyme able to transfer electrons from NADPH to O_2 without Ca^{2+} . Electron transfer occurs half as fast in this PAO-modified enzyme as in the native NADPH oxidase, and Ca^{2+} makes the binding of NADPH to the

Figure 5 Diagram showing the hypothetical PAO target site on the thyroid plasma membrane NADPH oxidase

(a) In the absence of Ca^{2+} , the autoinhibitory protein component or domain (Inh) prevents NADPH from reaching its binding site and/or the electron transfer from NADPH to the NADPH flavodehydrogenase entity (Fp). (b) Submicromolar Ca^{2+} concentrations allow electrons to flow from NADPH through the components of the H_2O_2 generator to molecular O_2 and so to generate H_2O_2 . (c) Binding of PAO to vicinal thiols alters the structure of the enzyme so that electron transfer occurs without Ca^{2+} , but more slowly (dashed line). (d) Ca^{2+} facilitates the binding of NADPH to the PAO–NADPH oxidase. The turnover of the PAO–NADPH oxidase with and without Ca^{2+} is unchanged.

active site easier. Figure 5 illustrates this action of PAO. The results suggest that vicinal thiols are essential to the activation of NADPH oxidase by the direct action of Ca^{2+} . However, the presence of these vicinal thiols must be confirmed by structural analysis. Cys-to-Ala scanning mutagenesis was recently used to identify the putative vicinal thiols that mediate arsenoxide binding in arginyl-tRNA protein transferase, and these results suggest that the binding of PAO to the transferase is not mediated by vicinal thiols [35]. This study on the effects of PAO on H_2O_2 production provides further evidence that thyroid NADPH oxidase differs from cytochrome *b*-558, the only well-characterized NADPH oxidase known so far. PAO does not inhibit O_2^- production by preactivated NADPH oxidase in isolated membranes or intact neutrophils [23], but acts on thyroid membrane by interacting with the Ca^{2+} -regulating domain that modulates the NADPH-dependent H_2O_2 production.

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