Periodic NADH oxidase activity associated with an endoplasmic reticulum fraction from pig liver. Response to micromolar concentrations of retinol

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Abstract

An endoplasmic reticulum fraction from pig liver enriched in transitional endoplasmic reticulum vesicles capable of forming 50–60 nm buds in the presence of ATP and retinol was assayed for retinol-responsive oxidation of NADH and cleavage of a dithiodipyridine (DTDP) protein disulfide–thiol interchange substrate. Maxima for the two activities alternated giving rise to a 24 min period. The NADH oxidase activity was inhibited by micromolar and submicromolar concentrations of retinol. Retinol at 0.1 mM stimulated the activity. The inhibition was confined to two activity maxima separated in time by about 5 min. In contrast, with the DTDP substrate, the activity was stimulated by retinol and the stimulations were in the part of the oscillatory pattern where retinol inhibition of NADH oxidation was observed. The findings support an earlier proposed mechanism whereby retinol exerted opposing effects on NADH oxidation and protein disulfide reductions. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: NADH oxidase; Transitional endoplasmic reticulum; Protein disulfide–thiol interchange; Retinol

1. Introduction

Much effort has been devoted to understanding how retinoids, and especially retinol, regulate cell proliferation and differentiation [1]. Mechanisms based on regulation of gene expression, while relevant to retinoic acid which combines with the retinoid binding family of nuclear receptors, are less relevant to retinol for which a nuclear receptor is unknown ([2], see, however, [3]). In addition, secretion and carbohydrate processing of secreted proteins are affected by all-trans-retinol [4,5] but less so by retinoic acid.

Work from our laboratory has focused on vesicular traffic between the endoplasmic reticulum and the Golgi apparatus [5]. The numbers of transition vesicles budding from transitional endoplasmic reticulum (TER) in the vicinity of Golgi apparatus were increased in rats fed retinol in excess [6]. Using preparations enriched in elements of the TER of rat liver, retinol at physiological concentrations was shown both to bind to [7] and to enhance the budding of
transition vesicles even in a completely cell-free sys-

The endoplasmic reticulum fractions that transfer
membrane to Golgi apparatus do so via 50–70 nm
vesicles. The vesicle-producing fractions consist of
part rough (with ribosomes) and part smooth (lack-
ing ribosomes) regions defined as TER.

Our attention has focused most recently on a fam-

ily of NOX (for cell surface-associated NADH oxi-
dase) proteins involved in cell enlargement, vesicle
budding and in physical membrane displacements
in general [10]. NOX activity has been associated
primarily with plasma membranes where roles in

cell enlargement [11] and endocytosis [12] are impli-
cated. The activity is also present in elements of the
TER [13,14] and in Golgi apparatus [15,16]. NOX
proteins exhibit both hydroquinone [17] and protein
disulfide–thiol interchange (protein disulfide isomer-
ase) [18,19] activities. The hydroquinone can be re-
placed by NAD(P)H in the oxidative portion of the
activity cycle [19]. The two manifestations of NOX
activity alternate giving rise to 24 min periodic oscil-
lations in each of the two activities [11,17].

The NOX proteins respond to retinol. Oxidation
of NADH is inhibited by micromolar and submicro-
molar concentrations of retinol [20]. With TER, on
the other hand, the protein disulfide–thiol inter-
change activity is stimulated by micromolar and sub-
micromolar concentrations of retinol [13]. Both these
activities are attributable to the same polypeptide
chain as demonstrated with recombinant NOX [11].
Thus, the two activities of the same protein appear to
respond differently to retinol. A mechanism based on
measurement of disulfide oxidation and reduction
estimated with 5,5′-dithiobis-(2-nitrobenzoic acid)
(Ellman’s reagent) was proposed [19] whereby the
opposing oxidative and reductive NOX reactions of
the activity cycle responded differently to retinol. The
oxidative portion appeared to be inhibited (hence in-
hibition of NADH oxidation) [20] whereas the reduc-
tive portion was stimulated (hence stimulation of
protein disulfide–thiol interchange) [13]. The two
manifestations of NOX activity alternate giving rise
to 24 min periodic oscillations in each of the two
activities [11,21–23]. In this report, observations are
extended to a TER fraction prepared from pig liver
in which a more detailed analysis of the response to
retinol has been conducted.

2. Materials and methods

2.1. Materials

Unfrozen pig livers were purchased at the Boiler-

maker Butcher Block (Purdue University). Unless
specified otherwise, chemicals were from Sigma
Chemical Company (St. Louis, MO, USA).

2.2. Cell culture

HeLa S cells were grown as described [24] on min-

imal essential medium (Jolik modified) with gluta-
mine (244 mg/l) and phosphate (1.3 g/l Na2HPO4)
plus 5% donor horse serum but without CaCl2. Gen-
tamicin sulfate (50 mg/l) and sodium bicarbonate
(2 g/l) were added.

2.3. Isolation of TER

Fresh pig liver (4–5 g) was cut and minced into ca.
0.5 cm³ pieces and homogenized using a Polytron
homogenizer (20 ST, Brinkman Instruments) in two
volumes of a homogenization medium containing
37.5 mM Tris–maleate buffer (pH 6.4), 0.05 M su-
crose, 5 mM MgCl₂ and 1% dextran (V220 kDa) for
40 s [25]. The homogenate was filtered through two
layers of miracloth and then centrifuged at 1100
Ug for 15 min. The supernatant was centrifuged at
80 000 Ug for 20 min. To remove contaminating plas-
ma membranes from the pellet, aqueous two-phase
partition was used modified from Sandelius et al.
[26]. The two-phase system contained 6.4% dextran
T-500, 40% polyethylene glycol 3350, 20 mM K3PO4
(pH 6.8) and 1 M sucrose. The two phases were
separated after centrifugation at 15000 ×g for 30 min
to reduce the amount of dextran T-500. The pellet
contained the pig liver microsomes.

For isolation of translational endoplasmic reticu-
lum, the lower phase pellets following removal of
plasma membrane by aqueous two-phase partition
were resuspended and layered onto discontinuous
sucrose gradients consisting of layers of 1.3 M,
1.5 M and 2.0 M sucrose. The gradients were centri-
fuged for 90 min at 90000 ×g. The material from the
1.3 M sucrose/sample interface (TER) was removed
and collected by centrifugation at 80,000 × g for 20 min.

2.4. Preparative cylindrical SDS-PAGE

A Bio-Rad Model 491 prep cell system (12% cylindrical gel) was used. For 5 ml pig TER supernatant after proteinase K digestion, the stacking gel volume was approximately 10 ml. The elution/electrophoresis buffer contained 30.30 g/l Tris base, 144 g/l glycine and 10.0 g/l SDS. The elution rate was 0.75 ml/min at 15 W. The fraction volume was 3.75 ml. The total number of fractions collected was 90. NADH oxidase activities were assayed on every fifth fraction.

2.5. Protein determination

Protein content was determined by the bichinchoninic acid/copper assay (BCA) [27]. BCA reagent was obtained from Pierce Technology Corporation (Ise-lin, NJ, USA).

2.6. Preparation of retinoid solutions

The stock retinoid solutions (0.1 M) were prepared by dissolving 5.8 mg all-trans-retinol or all-trans-retinoic acid in 200 μl absolute ethanol. Appropriate dilutions were made with absolute ethanol. The solutions were prepared and used fresh under yellow light to minimize photo destruction. A control assay with the same amount of ethanol without retinol was carried out in series for each retinol addition. To monitor the purity and concentration of retinol or all-trans-retinoic acid, absorption spectra were determined with a double beam spectrophotometer (UV 160, Shimadzu, Kyoto, Japan) over the range 200–400 nm. Solutions with a single maximum at 325 nm (retinol) or 351 nm (retinoic acid) and an absorbance of approximately 1.0 at 3 μM final concentration in ethanol were used.

2.7. Spectrophotometric assay for NADH oxidase activity

NADH oxidase activity was determined from the disappearance of NADH measured at 340 nm with a reference wavelength of 430 nm at 37°C with constant stirring using an SLM Aminco DW-2000 MV-VIS dual beam spectrophotometer with continuous recording of two 5 min intervals once a steady state was reached. Alternatively, paired Hitachi Model U32O spectrophotometers were used with NADH disappearance measured at 340 nm at 37°C with stirring and with continuous recording over 1 min at intervals of 1.5 min for 90 min to monitor periodic activity. The reaction mixture contained in a final volume of 2.5 ml, 25 mM Tris-Mes buffer (pH 7.2), 1 mM KCN to inhibit any contaminating mitochondrial oxidase activity, 50–100 μg TER protein and 150 μM NADH. Preparations to be assayed were incubated with 100 μM reduced glutathione (prepared fresh daily), followed after 10 min by the addition of 0.03% hydrogen peroxide. After 10 min, rate measurements were initiated. A millimolar extinction coefficient of 6.21 was used to calculate the NADH disappearance.

2.8. Spectrophotometric assay of dithiodipyridine (DTDP) cleavage as a measure of protein disulfide–thiol interchange activity of the NOX protein

The assay in 50 mM Tris-Mes buffer (pH 7.0) containing approximately 0.1 mg of TER protein was preincubated with 0.5 μmol 2,2′-DTDP in 5 μl of DMSO to react with endogenous reductants present with the TER [28]. After 10 min, a further 3.5 μmol DTDP was added in 35 μl DMSO to start the reaction. The final reaction volume was 2.5 ml.

The reaction was monitored from the increase in absorbance at 340 nm using a Hitachi Model U3210 spectrophotometer. The change in absorbance was recorded as a function of time by a chart recorder. The specific activity was calculated using a millimolar absorption coefficient of 6.21 cm−1 recognizing that 2 mol of product were generated for each mol of substrate cleaved.

2.9. Statistical analysis

Effects of detergents and retinoids on the NADH oxidase activity were analyzed by two sample t-test using a GraphPAD InStat software version 1.11a (1990 GraphPAD Software, United States Department of Agriculture).
3. Results

3.1. Morphology of TER fraction and response to retinol

The appearance of the TER fractions utilized in this study (Fig. 1) and the response to retinol (Table 1) identify the fractions as containing elements of the TER. The vesicles consist of part rough (with ribosomes), part smooth (lacking ribosomes) microsomes (Fig. 1A) capable of forming ca. 60 nm buds when treated with ATP plus retinol (arrows, Fig. 1B).
Quantitation of the numbers of buds produced as a result of the incubation (Table 1) demonstrated an approximately 10-fold greater proportion (4.5 per 100 vesicles) of vesicles with buds generated by incubation with ATP plus retinol as compared to incubation with ATP but where the retinol was replaced by an equivalent amount of ethanol (0.45 per 100 vesicles).

3.2. NADH oxidase activity of TER fraction and response to retinol and retinoic acid

When TER fractions were assayed for NADH oxidase activity (Fig. 2), retinol inhibited over the ranges of $10^{-9}$–$10^{-6}$ M, compared to ethanol alone. The activity was stimulated by $10^{-4}$ M retinol.

To ensure that the results with retinol were not the result of oxidation of retinol to form retinoic acid during the assay and to verify the retinol specificity of the response, the effect of retinoic acid was determined (Fig. 3). NADH oxidase activity of pig liver TER did not show the same response to retinoic acid as to retinol. Retinoic acid did not affect NADH oxidase activity of pig liver TER at submicromolar concentrations and inhibited the activity at higher concentrations ($10^{-5}$ and $10^{-4}$ M) as observed previously for the NADH oxidase activity of HeLa plasma membrane vesicles [20].

3.3. Subfractionation of TER

To attempt to resolve the retinol-responsive NADH oxidase into inhibited and unresponsive components, the isolation procedure of del Castillo-Olivares [29] was followed. The TER fractions were
treated with 0.1 M sodium acetate, pH 5 for 1 h and the released material was treated at 70°C for 10 min. The resultant supernatant, ca. 20-fold enriched in NADH oxidase (16 nmol/min/mg protein vs. 0.8 nmol/min/mg protein), was used as the starting material for preparative SDS-PAGE. The solubilized material demonstrated the same pattern of response to retinol as did the starting TER.

NADH oxidase activities of pooled fractions 17–19, fractions 27–29 and fractions 37–38 were inhibited beginning at a retinol concentration of $10^{-9}$ M (Table 2). The maximal inhibitions were observed at $10^{-7}$ M and/or at $10^{-6}$ M. NADH oxidase activity of all the pooled fractions was stimulated at $10^{-4}$ M. While discrete bands with retinol-inhibited NADH oxidase activity were found, there was no resolution into retinol-responsive and retinol-unresponsive fractions with both fractions having NOX activities.

Alternatively, the crude pig liver TER was digested with proteinase K (see Fig. 4) and then analyzed.

Table 1
Response of pig liver TER to all-trans-retinol in the presence or absence of ATP

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vesicles per 100 vesicles with 60 nm buds</th>
<th>Δ Incubation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (not incubated)</td>
<td>2.70 ± 0.40</td>
<td>–</td>
</tr>
<tr>
<td>Ethanol</td>
<td>3.15 ± 0.15</td>
<td>0.45</td>
</tr>
<tr>
<td>Ethanol+ATP</td>
<td>3.10 ± 0.7</td>
<td>0.40</td>
</tr>
<tr>
<td>Retinol $10^{-7}$ M</td>
<td>4.40 ± 0.70*</td>
<td>1.70</td>
</tr>
<tr>
<td>Retinol $10^{-3}$ M+100 μM ATP</td>
<td>7.20 ± 0.80**</td>
<td>4.50</td>
</tr>
<tr>
<td>Retinol $10^{-3}$ M</td>
<td>4.50 ± 0.60*</td>
<td>1.80</td>
</tr>
<tr>
<td>Retinol $10^{-3}$ M+100 μM ATP</td>
<td>7.70 ± 0.60**</td>
<td>5.00</td>
</tr>
</tbody>
</table>

TER fractions were incubated for 15 min at 37°C with $10^{-7}$ or $10^{-3}$ M retinol with or without 100 μM ATP. The ability of the membrane fragments to generate buds in an ATP- and retinol-dependent manner as illustrated in Fig. 1 identifies the fragments as TER.

*Significantly greater than control *(P < 0.005) or **(P < 0.0001).
directly by preparative SDS-PAGE gel electrophoresis. Eighty fractions were collected. Every fifth fraction was assayed for NADH oxidase activity and analyzed by SDS-PAGE. The fractions showing NADH oxidase activity with a retinol-inhibited response were concentrated. Fractions 5–20 were inhibited by retinol at a concentration of $10^{-7}$ M and corresponded to a molecular weight (MW) of 15–29 kDa (not shown).

### Table 2

NADH oxidase activities of fractions from preparative SDS-PAGE (nmol/min/mg protein)*

<table>
<thead>
<tr>
<th>Fraction number</th>
<th>MW</th>
<th>Retinol concentration (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$0$</td>
</tr>
<tr>
<td>17–19</td>
<td>14.5</td>
<td>0.84</td>
</tr>
<tr>
<td>27–29</td>
<td>28.3</td>
<td>5.15</td>
</tr>
<tr>
<td>37–38</td>
<td>36</td>
<td>11.02</td>
</tr>
</tbody>
</table>

*Protein content was based on the starting endoplasmic reticulum fraction.

#### 3.4. Oscillatory pattern of NADH oxidase activity of pig liver TER

The oscillatory pattern of activity was used to identify and analyze the NOX activity from pig liver TER. Oscillations of NADH oxidase activity of crude pig liver TER exhibited maxima (arrows) at intervals of 24 min before and after proteinase K digestion (Fig. 4). The results showed that the oscil-
latory NADH oxidase activity of pig liver TER was resistant to proteinase K. While the pig liver TER may contain multiple protein components with NADH oxidase activity, at least one is proteinase K-resistant and periodic and another is not periodic and proteinase K-susceptible.

With regard to retinol modulation, the oscillation of NADH oxidase activity of crude pig liver TER exhibited maxima at 24 min intervals before retinol addition. After addition of retinol at a final concentration of $10^{-7}$ M, portions of the periodic NADH activity were inhibited (Fig. 5). Within the activity pattern, several oscillations were observed, each appearing at 24 min intervals (Fig. 5). In the presence of retinol, the inhibition involved principally the oscillatory component labeled 1 with less of an effect on the oscillatory components labeled 2 and 3.

When analyzed in greater detail, a total of five oscillatory components were resolved within each 24 min period (Fig. 6). With NADH as substrate for the oxidative activity (Fig. 6A), the oscillatory components labeled 1 and to a lesser extent the os-
cillatory component labeled 2 were most evident. With DTDP as substrate for the protein disulfide–thiol interchange activity (Fig. 6B), the oscillatory components labeled 2, 3 and 4 were most evident. As reported previously for recombinant tNOX protein [11], the mean times of the dominant oscillatory components of the two activities were 12 min out of phase with each other. The measurements of Fig. 6A,B were made simultaneously with two aliquots of the same TER preparation using two spectrophotometers in parallel. With NADH as substrate, the two main oscillations were at 18, 42 and 66 min and at 24, 48 and 72 min. In addition, there were three minor oscillations to complete each 24 min period. With DTDP as substrate, the major oscillations were at 6, 30, 54 and 79 min, at 9, 33, 57 and 81 min, and at 12, 36, 60 and 84 min. These major oscillations with DTDP as substrate corresponded exactly to the minor oscillations of the oxidative activity with NADH as substrate. The minor oscillations with

Fig. 7. Time series (decomposition) analyses of the data of Fig. 6 to verify the reproducibility of the recurring 2+3 pattern of activity oscillations.
the DTDP substrate were at 18, 42 and 66 min and at 24, 48 and 72 min which corresponded to the times of the major oscillations in NADH oxidation. Thus the two major oscillations of NADH oxidation align with the two minor oscillations of DTDP cleavage and the three major oscillations of DTDP cleavage along with the three minor oscillations of NADH oxidation. This repeating 3+2 pattern of activity occurred reproducibly and has been validated by time series decomposition analyses (Fig. 7) as described in Foster et al. [23]. The inhibition by retinol with NADH oxidation was primarily with the first one or two major oscillations of the 2+3 pattern where the inhibition was virtually complete (Fig. 4).

With the DTDP substrate, there was a 25% overall increase in activity in the presence of 10⁻⁷ M retinol. This increase was largely due to an enhanced activity displaced approximately 3 min after the tripartite maximum of activity with DTDP. The stimulated activity in the 3+2 pattern of DTDP breakdown corresponded very closely with the inhibited component of the 2+3 pattern of NADH oxidation.

4. Discussion

The activities of the NOX proteins, proteins whose activities are implicated in physical membrane displacements such as cell enlargement and membrane budding [10], emerge as being retinol-responsive. As reviewed in Section 1, the NOX proteins are unique in that they exhibit two complex activity patterns initially considered to alternate as based on studies with recombinant human NOX [11]. Both activities were attributable to the same polypeptide chain and exhibited periodic maxima and minima. It now appears that each 24 min period of activity in well synchronized preparations can be resolved into five oscillating components. For NADH oxidation, representative of the oxidative electron transport (NOX) activity, two oscillating components of elevated activity alternate with three oscillating components of lower activity. When DTDP cleavage representative of protein disulfide–thiol interchange activity is measured, the oscillating components 1 and 2 which are major components with NADH as substrate are now minor components whereas components 3, 4 and 5...
which were minor components with NADH as substrate are now major. Both activities exhibit the same five oscillating components but when NADH oxidation is at a maximum, DTDP cleavage is at a minimum and vice versa.

The above oscillatory pattern of activity was observed initially with plasma membrane vesicles or recombinant NOX. It now also appears to be characteristic of NOX activities present in internal membranes including both TER as reported here as well as Golgi apparatus [16].

The effect of retinol on NOX activity as well as on vesicle budding is distinct from that of retinoic acid. While NADH oxidase of plasma membranes was inhibited by both retinol and retinoic acid, much higher concentrations of retinoic acid than retinol were required to inhibit [20]. Retinoic acid at micromolar or submicromolar concentrations was without effect on vesicle budding of TER, a response given only by retinol when several retinoids were compared [9]. These findings agree with those of the present study where retinol stimulated both vesicle budding and inhibited NADH oxidase activity at concentrations where retinoic acid was without effect.

A second characteristic of the NOX protein is an ability to catalyze protein disulfide-thiol interchange. This activity is exhibited both by plasma membranes [18] and endoplasmic reticulum vesicles [13] and stimulated by retinol but not by retinoic acid [13].

An explanation for opposing responses (stimulation of one activity and inhibition of the other) by retinol was provided earlier by Morré et al. [14] where the oxidative and reductive parts of the NOX cycle were shown by direct measurement of thiols and disulfides to respond differently to retinol. Conversion of -S-S- to -SH by oxidation of NADH was inhibited by retinol whereas reconversion of -SH to -S-S- was stimulated by retinol. This difference is reflected as well in the present findings where retinol at 0.1 μM inhibited oxidation of NADH and stimulated cleavage of DTDP. The DTDP substrates were developed to serve as a convenient alternative to activation of ribonuclease as an assay for the protein disulfide-thiol interchange activity of the NOX proteins [28].

One consideration that has been problematic with the retinol response of the NOX protein of the TER was that the inhibition was small and incomplete. Both stimulations and inhibitions were in the range of 25–30% of the total activity. One possibility, considered early, was that multiple NOX isoforms were present, only one of which was retinol-responsive. This possibility was not supported by attempts to isolate the NOX activity from TER (e.g. Table 2). Despite the fact that at least two MW regions with NOX activity were observed, oxidation of NADH was inhibited by retinol for both. The alternative explanation was that, for some reason, the NOX activity of each polypeptide chain was only partially inhibited. This explanation is supported by findings of this paper. The activity of the NOX protein pulses through at least five oscillating components per cycle, two where oxidation of NADH is emphasized and three where protein disulfide-thiol interchange is emphasized. Apparently only one of these components is inhibited by retinol when NADH oxidation is measured. The same oscillatory component where NADH oxidation is inhibited by retinol corresponds to the component in the 2+3 pattern of oscillations where DTDP cleavage is stimulated by retinol.

The apparent lack of sensitivity of at least a component of the NOX activity of the pig liver TER is not unexpected in view of the fully documented resistance of a drug-responsive cancer NOX isoform from the HeLa cell surface to a variety of proteases including proteinase K [29,30]. The activity remaining in pig liver TER after proteinase K is clearly oscillatory and retinol-responsive. The component that is susceptible to digestion by proteinase K has not been characterized.

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