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NADH oxidase activity (NOX) and enlargement of HeLa cells oscillate with two different temperature-compensated period lengths of 22 and 24 minutes corresponding to different NOX forms

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Abstract

NOX proteins are cell surface-associated and growth-related hydroquinone (NADH) oxidases with protein disulfide–thiol interchange activity. A defining characteristic of NOX proteins is that the two enzymatic activities alternate to generate a regular period length of about 24 min. HeLa cells exhibit at least two forms of NOX. One is tumor-associated (tNOX) and is inhibited by putative quinone site inhibitors (e.g., capsaicin or the antitumor sulfonylurea, LY181984). Another is constitutive (CNOX) and refractory to inhibition. The periodic alternation of activities and drug sensitivity of the NADH oxidase activity observed with intact HeLa cells was retained in isolated plasma membranes and with the solubilized and partially purified enzyme. At least two activities were present. One had a period length of 24 min and the other had a period length of 22 min. The lengths of both the 22 and the 24 min periods were temperature compensated (approximately the same when measured at 17, 27 or 37°C) whereas the rate of NADH oxidation approximately doubled with each 10°C rise in temperature. The rate of increase in cell area of HeLa cells when measured by video-enhanced light microscopy also exhibited a complex period of oscillations reflective of both 22 and 24 min period lengths. The findings demonstrate the presence of a novel oscillating NOX activity at the surface of cancer cells with a period length of 22 min in addition to the constitutive NOX of non-cancer cells and tissues with a period length of 24 min.

Keywords: Hydroquinone (NADH) oxidase; Tumor associated hydroquinone (NADH) oxidase; Oxidation of NADH; Cell enlargement; Growth; Temperature compensation; Ubiquinone; HeLa cell; Coenzyme Q

1. Introduction

A growth factor- and hormone- or drug-responsive oxidation of both NADH and hydroquinones catalyzed by a family of proteins referred to as NOX proteins has been found associated with plasma membranes of the animal and plant cell surface [1,2]. With rat hepatomas [3] and with cancer cells in culture [4], a constitutively activated component of NADH oxidation was observed which was no longer

Abbreviations: HeLa, human cervical carcinoma cell line; NOX, plasma membrane-located hydroquinone (NADH) oxidase with protein disulfide–thiol interchange activity; CNOX, constitutive NOX; tNOX, tumor-associated NOX; LY181984, *N*-(4-methylphenylsulfonyl)-*N*′-(4-chlorophenyl)urea; LY181985, *N*-(4-methylphenylsulfonyl)-*N*′-(4-phenyl)urea; capsaicin, 8-methyl-*N*-vanillyl-6-noneamide

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dependent upon hormones or growth factors for activation. This constitutively activated component (tNOX) was responsive to certain drugs such as the antitumor sulfonylurea, LY181984 [5], and the capsaicinoid quinone site inhibitor, capsaicin [4,6]. The constitutive [2,7] and the constitutively activated [4] oxidation of NADH were differentially responsive to thiol reagents [8,9].

A second enzymatic activity associated with the NOX proteins was that of protein disulfide–thiol interchange [10]. An unusual feature of both the oxidation of NADH and of the protein disulfide–thiol interchange activity catalyzed by the constitutive CNOX proteins was that the two activities alternated every 12 min giving rise to oscillations with a period of 24 min [11–14]. Reported here is a similar oscillatory activity observed with the NOX proteins from HeLa cells. With both plants and CHO cells, the period length of the oscillations observed with whole cells or tissues, plasma membranes and the purified protein was that the period length was independent of temperature (temperature compensated). A function of NOX proteins as ultradian (less than 24 h) drivers of circadian timekeeping has been postulated [13,15].

The plasma membrane-associated NOX proteins appear to be involved in the enlargement phase of cell growth [11]. Normally, compounds that stimulate the cell surface NADH oxidase stimulate cell enlargement whereas compounds that inhibit the cell surface NADH oxidase inhibit cell enlargement [11].

Because the NOX proteins are localized at the external cell surface [16], it was considered unlikely that NADH was a physiological substrate for the activity [11,12]. Rather, the electron donor appeared to be hydroquinone (reduced coenzyme Q) [12]. The acceptor is most likely oxygen or protein disulfides [17]. The second enzymatic activity of the NOX proteins, to cleave and reform disulfide bonds, led to restoration of activity to scrambled and inactive ribonuclease [10]. This activity also resulted in cleavage of dithiodipyridyl substrates to provide a simple, direct spectrophotometric assay [18]. tNOX has been cloned (GenBank accession No. AF207881). The protein when expressed in *Escherichia coli* continues to show both enzymatic activities which oscillate with a regular period length of 22–23 min. In this report, we demonstrate that the pattern of NOX activities from a cancer cell line, HeLa (human cervical carcinoma), is complex and contrasts with that of CHO cells [19], for example, by exhibiting at least two major activity components, one with a period length of 24 min corresponding to CNOX and one with a period length of 22 min corresponding to tNOX. The period length of NADH oxidation by solubilized NOX preparations from HeLa cells, HeLa cell plasma membranes and intact HeLa cells was independent of temperature (temperature compensated). The latter is a characteristic most often associated with biological timekeeping.

2. Materials and methods

2.1. Materials

Chemicals used in this study were from Sigma (St. Louis, MO) unless indicated otherwise. Bicinchoninic acid (BCA) was from Pierce (Rockford, IL).

2.2. Growth of cells

The cells were grown in Dulbecco's modified Eagle's medium (D-MEM) (Joklik modified) (Gibco BRL, Gaithersburg, MD) with L-glutamine and D-glucose (4500 mg/l) and without calcium chloride, sodium bicarbonate, phenol red and sodium pyruvate, plus 5% donor horse serum. Gentamicin sulfate (50 mg/l), pluronic F69 prill and sodium bicarbonate (2 g/l) were added. Cells were grown at 37°C with 5% CO_2 in 3 l flasks. Cells were collected by centrifugation for 6 min at $1400\times g$. Attached HeLa cells (ATCC CCL2) were from the American Type Culture Collection (ATCC). They were grown in 25 cm flasks in minimal essential medium (MEM) (Gibco BRL), pH 7.4, at 37°C with 10% fetal bovine serum (heat-inactivated), plus 50 mg/l gentamicin sulfate.

2.3. Purification of plasma membranes from cultured cells

Cultured cells were collected by centrifugation for 6 min at $1400\times g$. The cell pellets were resuspended in 0.2 mM EDTA in 1 mM $NaHCO_3$ in an approximate ratio of 1 ml per 10^8 cells and incubated on ice

for 10–30 min to swell the cells. Homogenization was with 7–8 ml aliquots with a Polytron homogenizer (Brinkmann) for 30–40 s at 10 500 rpm, using a ST-10 probe. To estimate breakage, the cells were monitored by light microscopy before and after homogenization. At least 90% cell breakage without breakage of nuclei was achieved routinely.

The homogenates were centrifuged for 10 min at $175\times g$ to remove unbroken cells and nuclei and the supernatant was centrifuged a second time at 1.4×10^6 g·min (e.g. 1 h at $23\,500\times g$) to prepare a plasma membrane-enriched microsome fraction. The supernatant was discarded and the pellets were resuspended in 0.2 M potassium phosphate buffer in a ratio of approx. 1 ml per pellet from 5×10^8 cells. The resuspended membranes were then loaded onto the two-phase system constituted on a weight basis consisting of 6.6% (w/w) Dextran T-500 (Pharmacia) and 6.6% (w/w) polyethylene glycol 3350 (Fisher) in a 5 mM potassium phosphate buffer (pH 7.2) for aqueous two-phase separation as described [10]. The upper phase, enriched in plasma membranes, was diluted 5-fold with 1 mM sodium bicarbonate and the membranes were collected by centrifugation. The purity of the plasma membrane was determined to be $>90\%$ by electron microscope morphometry. The yield was 20 mg plasma membrane protein from 10^{10} cells.

2.4. Spectrophotometric assay of NADH oxidase

NADH oxidase activity was determined as the disappearance of NADH measured at 340 nm in a reaction mixture of 25 mM Tris–MES buffer (pH 7.2), 1 mM KCN and 150 μM NADH at 37°C and continuous stirring [7] using paired Hitachi U3210 spectrophotometers or a SLM Aminco DW-2000 spectrophotometer (Milton Roy, Rochester, NY) in the dual wavelength mode of operation with continuous recording and 430 nm as reference. Assays were initiated by addition of NADH. A millimolar extinction coefficient of 6.22 was used to determine specific activity. Assays were for 1 min and were repeated on the same sample every 1.5 min for the times indicated. Alternatively, absorbance values were recorded every 10 s for 90 min and then averaged over 1.5 min intervals. The rate of NADH oxidation for each 1.5 min then was estimated from the equation:

Rate of nth 1.5 min interval =

$$-[A_{340\cdot(n-1)}-A_{340\cdot(n+1)}]/2$$

Proteins were estimated by the bicinchoninic acid method [20] with bovine serum albumin as standard.

2.5. Growth measurements

Cells were grown for 24 h at 37°C on glass coverslips placed in small culture dishes with medium. Direct measurements of cells attached to the coverslips were with an Olympus Vanox-S Microscope, Model AHBS, using bright field and/or differential interference contrast optics. The microscope was coupled to a Hamamatsu CE400-07 video camera system with a Hamamatsu (700 horizontal lines) camera head for viewing and data recording. As required, Hamamatsu Argus-10 and Argus-20 real time digital contrast and low light enhancement image processing were used. Increases in area were monitored for 90 to 120 min. In order to maintain the proper growth environment for the cells, a chamber was constructed to allow fresh medium to flow under the coverslip. Transparent tape and grease were applied along the edges of the slide to secure the coverslip to avoid movement as medium flowed through the space. The coverslip was placed upside-down over the slide to avoid having to focus through the medium. The cells were regularly supplied with fresh medium. Temperature control was provided by a brass block with a viewing port that was placed over the microscope slide and through which water was circulated. Temperature at the slide surface was monitored by means of a thermocouple.

Using freeze frame technology the tape was stopped at 1 min intervals. The cell perimeter was then digitally traced. Cell areas were calculated using the Hamamatsu Argus-10, equipped with the appropriate software. Each frame was measured three times. The measurements were then averaged and standard deviations were determined using Microsoft Excel. Due to the irregular geometry of the attached cells, no attempt was made to calculate volumes from the primary data.

2.6. Solubilized tNOX

To a pellet of HeLa S cells (whole cells) from 10 l of culture medium, 6 ml of 0.1 M sodium acetate, pH 5.0, were added. The cells were grown and shipped frozen by Cellex Biosciences (Minneapolis, MN). The cells were thawed at room temperature and diluted 1/1 (original pellet volume in pH 5.0, 0.1 M sodium acetate) and incubated at 37°C for 1 h to release the protein. The cells were removed by centrifugation (17 000 rpm, 60 min, Sorvall) and the cell-free supernatants containing the protein of interest (fully active and drug inhibitable) were refrozen and stored in 1 ml aliquots at −70°C.

Approx. sixteen 1 ml aliquots of the above supernatant material were thawed at room temperature and heated to 50°C for 10 min in 1 ml aliquots for uniform heating. The denatured proteins were removed by centrifugation (6000 rpm, 5 min, Eppendorf centrifuge in 1 ml aliquots). Full activity was retained from this step [21]. The preparation is 10–20-fold enriched in tNOX compared to HeLa cell plasma membranes.

The above method was used by del Castillo et al. [21] to release the tNOX protein from HeLa cells. Binding appears to be non-covalent and reversible. The protein apparently lacks a transmembrane spanning domain [22]. The low pH release is neither accelerated by added cathepsin nor inhibited by protease (cathepsin) inhibitors [23]. The released NOX proteins and the plasma membrane-associated NOX have similar enzymatic properties and respond to inhibitors and exhibit similar molecular weights on SDS–PAGE.

2.7. Statistical analysis

Results were analyzed using a fast Fourier transform and decomposition fits. User-defined fast Fourier transforms in SigmaPlot 5.0 (SPSS) were used to determine the period length of the NADH oxidase activity. Decomposition fits were used to predict seasonal patterns based on the period length established by Fourier analysis.

Decomposition was carried out by fitting a trend line to the data, then detrending the data if necessary, by subtracting out the trend component. The data, with or without detrending, were then smoothed by subtracting a centered moving average of length equal to the length of the cycle. The median value of the period was determined by Fourier analysis. Finally, the time series were decomposed into cyclic and error components. The decomposition fits were used to validate the cyclic oscillatory pattern and to demonstrate that minor intervening fluctuations also recurred within each period as part of a reproducible pattern. The model type was additive and the model component setting was selected to be seasonal only [24]. The decomposition fits used MINITAB, a statistical package.

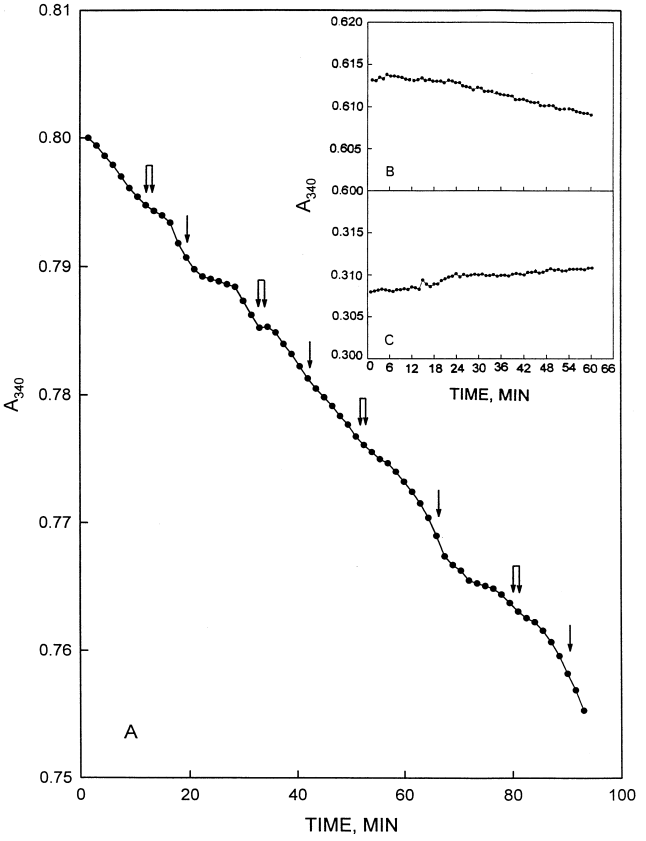

Fig. 1. Time course of NADH oxidation by a preparation solubilized and partially purified from the surface of HeLa cells (0.044 mg total protein). As a measure of NADH oxidation, the decrease in absorbance at 340 nm was measured at 10 s intervals and averaged over 1.5 min. The arrows show midpoints of periods of rapid oxidase activity. At least two sets of oscillations at intervals of approx. 24 min (single and double arrows) are indicated. Control experiments are shown in the inset. (A) Complete reaction mixture. (B) Spectrophotometric assay with NADH plus all reactants but without HeLa extract. (C) With HeLa extract plus all reactants but without NADH.

3. Results

3.1. Measurement of NADH oxidase

The oxidation of NADH, as determined from the decrease in absorbance at 340 nm using the SLM DW-2000 spectrophotometer with a reference cuvette, was approximately linear with time but with a discernible periodic departure from linearity as the rates appeared to slow or accelerate (Fig. 1). At least two sets of fluctuations (single and double arrows) each spaced approx. 24 min apart were observed. A clear periodic variation in reaction rate was seen when the rate of change was displayed (Fig. 2A). Maxima denoted by single arrows were at intervals of approx. 24 min. Two sets of minor oscillations exhibited maxima at intervals of 22 min (double arrows). Fourier analysis (Fig. 2B) of the data of Fig. 2A revealed maxima at frequencies of 0.042 and 0.08 cycles/min corresponding to period lengths of 24 and 12 min respectively. With the reagents alone (no enzyme source) or the enzyme plus all reactants but

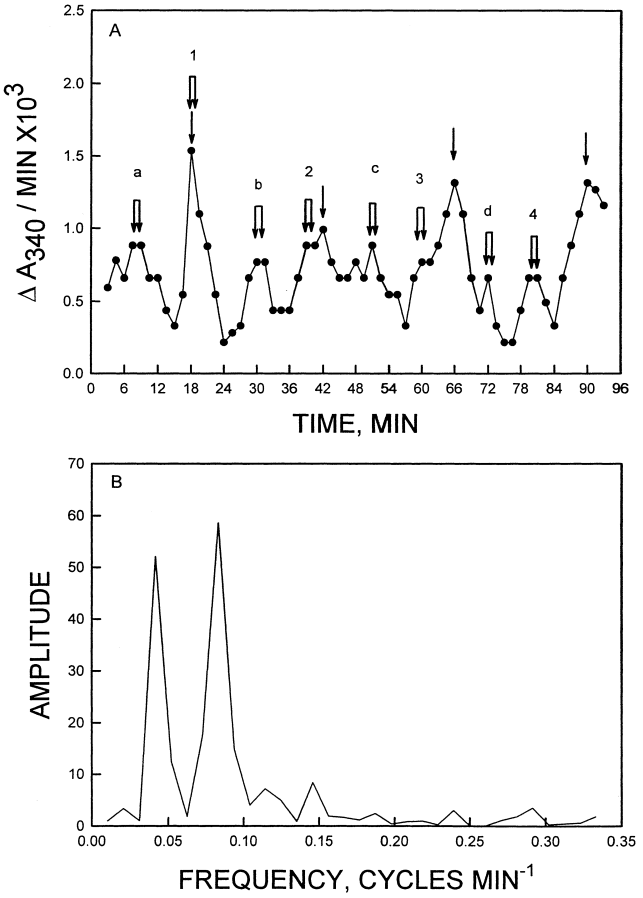

Fig. 2. Rates of NADH oxidation determined by numerical averaging from data of Fig. 1. Absorbance readings were recorded every 10 s as described for Fig. 1 and averaged over 1.5 min intervals. The rate of NADH oxidation for each 1.5 min interval was estimated from the equation: rate of nth 1.5 min interval $= -[A_{340\cdot(n+1)} - A_{340\cdot(n+1)}]/2$. (A) Three sets of oscillations are displayed. For one, maxima (single arrows) are indicated at 18, 42, 66 and 90 min with a period length of about 24 min. Also observed are two sets of oscillations (a–d and 1–4) with period lengths of about 22 min (double arrows). (B) Fourier analysis of the rate data of A yielded frequencies of 0.042 and 0.08 corresponding to period lengths of 24 and 12 min. This basic experiment was repeated more than 20 times with the solubilized and partially purified preparations from the HeLa cell surface. All show patterns of oscillations in NADH oxidase activity similar to that shown here.

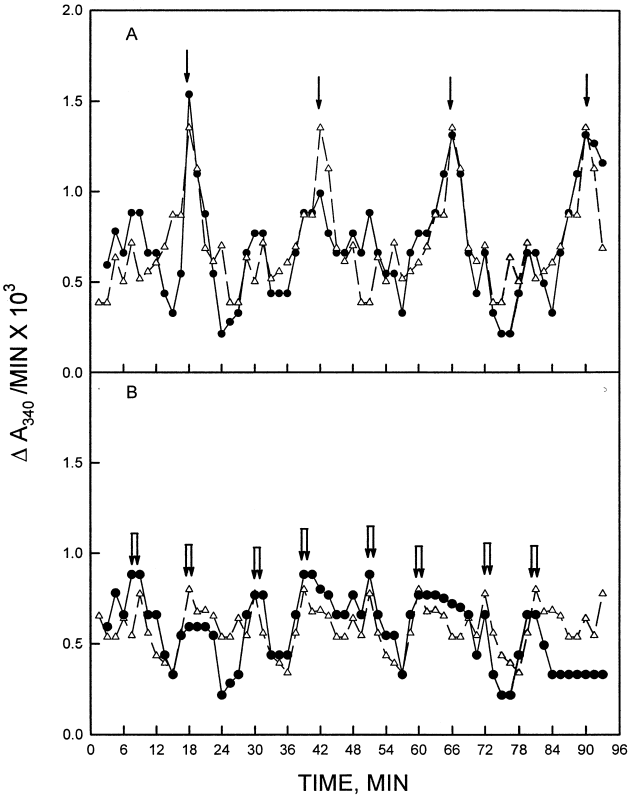

Fig. 3. Decomposition analysis of the rate of NADH oxidation of Fig. 2A. The decomposition fits (open triangles, dotted lines) generated a predictor repeating pattern of the observed oscillations using the period length of 24 min determined from fast Fourier analysis (Fig. 2B). (A) The dominant maxima corresponded to a period length of 24 min. (B) The maxima of the oscillation of period length 24 min were subtracted and the data were reanalyzed to demonstrate that the remaining minor oscillations best fitted a period length of 22 min.

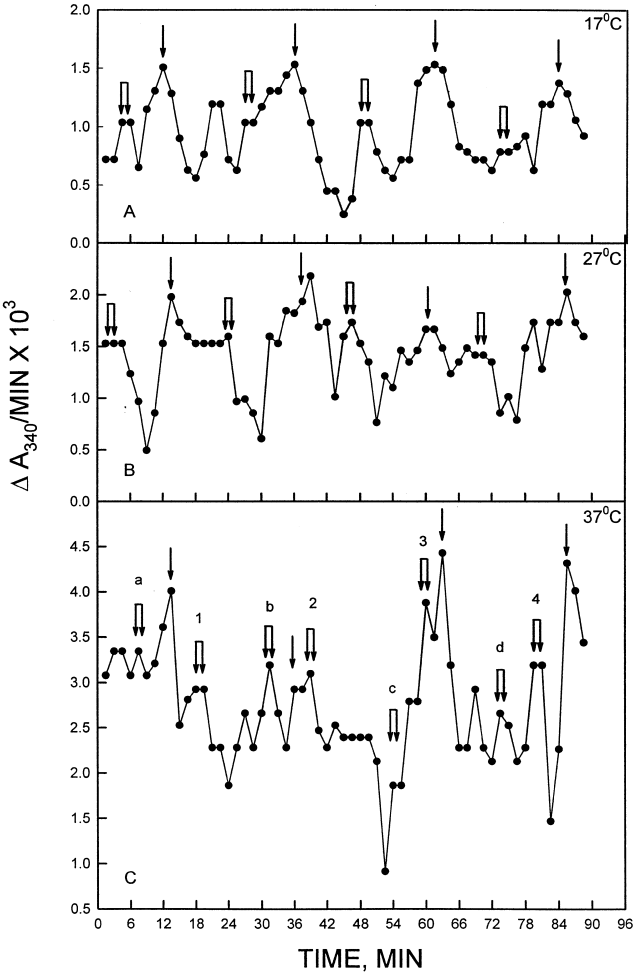

Fig. 4. Rate of oxidation of NADH by a solubilized and partially purified preparation of the HeLa cell surface comparable to that of Figs. 1–3 but assayed at three different temperatures. (A) 17°C. (B) 27°C. (C) 37°C. Arrows indicate rate maxima. A similar period length is seen at all three temperatures despite an increasing amplitude with increasing rise in temperature. Single arrows are spaced at 24 min intervals. Double arrows are spaced at 22 min intervals. In C, two sets of maxima (1–4 and a–d) with a 22 min period length were evident.

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lacking NADH, no periodic fluctuations were observed (Fig. 1B,C).

Verifications of period lengths were provided by decomposition fits of the data of Fig. 2A (Fig. 3). When fitted using the 24 min period length provided by Fourier analysis (Fig. 2B), an exact fit was observed with the major period (single arrows). However, if the contributions of the major peaks were subtracted and the data reanalyzed using a 22 min period length a correspondence with the minor peaks was now revealed (Fig. 3B).

3.2. Effect of temperature (solubilized tNOX)

With the solubilized and partially purified tNOX from HeLa cells, the period length was unchanged with temperature, being the same at 17°C, 27°C and 37°C (Fig. 4), as confirmed by Fourier analysis (Fig. 5). In contrast, the reaction rate doubled with each 10°C rise in temperature (Table 1).

Table 1
Period length and mean amplitude for oxidation of NADH of HeLa as a function of temperature for solubilized proteins, plasma membranes and whole cells

Cells	Temperature (°C)	Period length (min)		Mean amplitude[a]
		Major	Minor	
Solubilized proteins	17	23.8 ± 0.8	22.8 ± 1.5	0.36
	27	24.1 ± 1.2	22.5 ± 0.8	0.83
	37	24.0 ± 0.9	21.3 ± 0.6	1.75
Plasma membranes	17	23.5 ± 1.3	22.7 ± 0.6	0.40
	27	23.8 ± 0.9	22.1 ± 0.8	0.80
	37	24.3 ± 1.3	22.4 ± 1.0	2.20
Cells	17	24.3 ± 0.6	21.8 ± 0.4	0.11
	27	24.0 ± 0.8	21.3 ± 1.0	0.25
	37	24.4 ± 0.8	21.8 ± 0.6	0.53
Mean		24.0 ± 0.3	22.1 ± 0.6	

[a]Amplitudes are averages of all rates minus the mean minimum value determined for the three to four periods analyzed. Units are A_{340}/min × 10^3 for solubilized proteins, nmoles/min/mg protein for plasma membranes and nmoles/min/10^6 cells for whole cells.

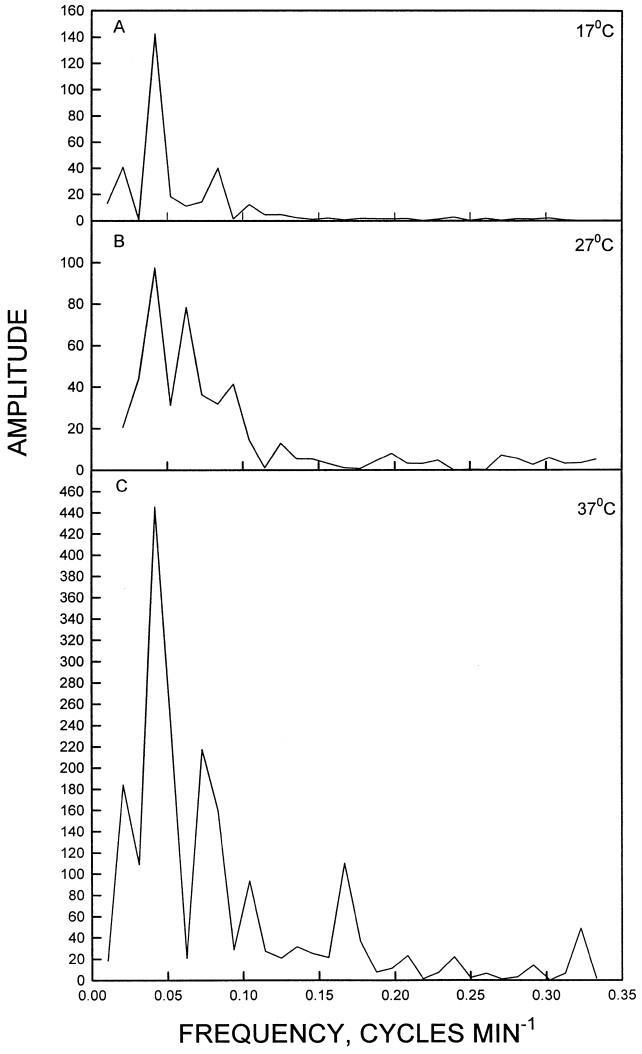

Fig. 5. Fourier analyses of data of Fig. 4. The maximum amplitude coincided with a frequency of 0.0417 cycles/min at all three temperatures to give a period length of 24 min.

3.3. Plasma membranes

Plasma membranes isolated from HeLa cells also exhibited periodic oscillations in the rate of oxidation of NADH (Fig. 6). For temperature studies with the isolated plasma membranes, rates were estimated from data generated with the SLM DW-2000 spectrophotometer in the dual wavelength mode of operation. Sample and reference cells were prepared in an identical manner except for the addition of NADH to the sample cuvette. Single absorbance readings were collected every minute and rates were determined by numerical averaging as for Fig. 2A.

The result with plasma membranes was a very complicated pattern of oscillations. However, one period length of 24 min (single arrow) and two periods of length of 22 min (double arrows) were fitted to the data (Fig. 6). Fourier analysis revealed a 24 min major period as among the principal frequencies at all three temperatures (Fig. 7, arrows). This was most evident for 17° C (Fig. 7A). For 27°C and 37°C an additional frequency of 0.032 cycles/min or a period length of 31 min (Fig. 7B,C) was obtained from the Fourier analyses. This apparently arose from the chance displacement of the 24 min period and one of

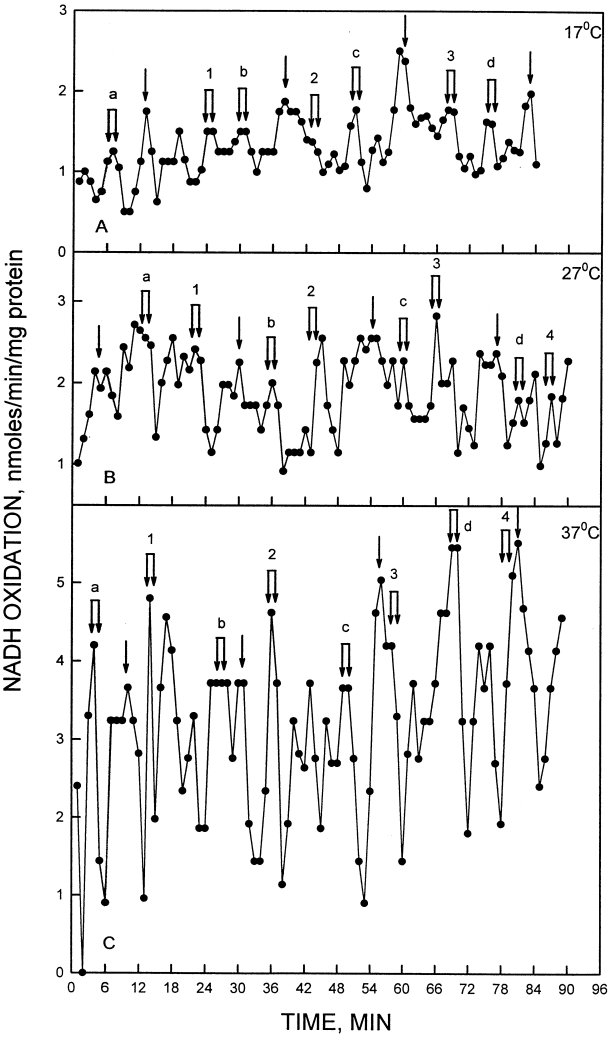

Fig. 6. Rate of oxidation of NADH by plasma membrane preparations from HeLa cells and response to temperature. (A) 17°C. (B) 27°C. (C) 37°C. The data were collected as single absorbance readings every minute and the rates were calculated by numerical averaging. The maxima marked by single arrows generate a period length of 24 min. The maxima marked by double arrows generate period lengths of 22 min.

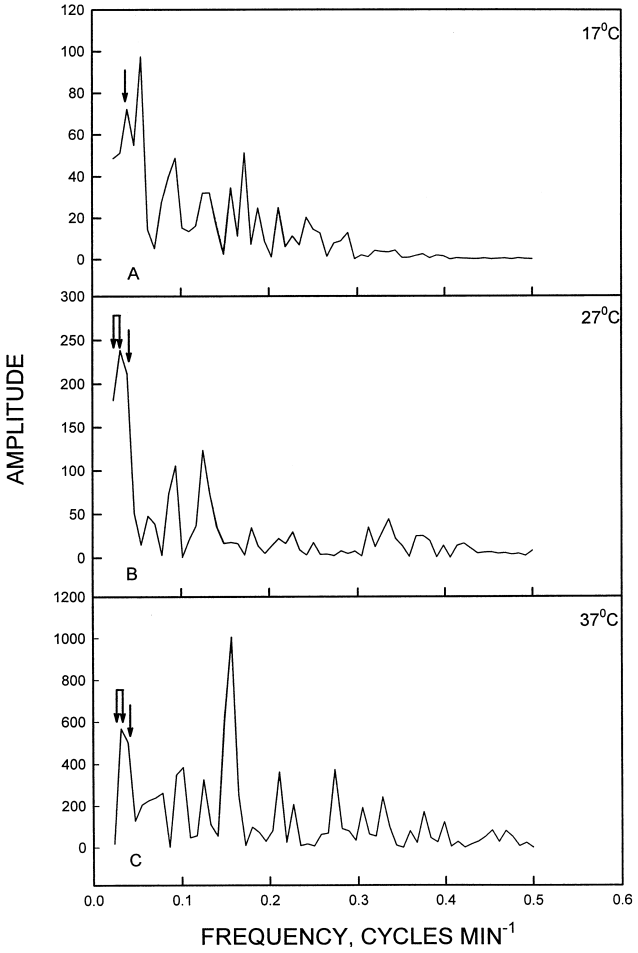

Fig. 7. Fourier analyses of data of Fig. 6. Amplitudes coinciding with a frequency of 0.042 cycles/min (arrows) corresponding to a period length of 24 min were obtained at all three temperatures. A second amplitude of 0.032 cycles/min (period length of 31 min) at 27°C (B) and 37°C (C) shown at the double arrows seems to have arisen as a hybrid between the maxima separated by 24 min and one of the maxima separated by 22 min but displaced from each other by 31 min (see text).

the 22 min periods by about 7 min such that a hybrid period length of 31 min was generated along with the usual 24 min period length.

3.4. Cells

With intact HeLa cells, the results were similar to those with the solubilized extracts (Fig. 1). These experiments were possible because the binding site for the NADH is at the external surface of the plasma membrane [16]. The NADH oxidase activity was periodic with an average period length of about 24 min for the major period (single arrows, Fig. 8) at all three temperatures of 17°C, 27°C and 37°C. In parallel with data for the solubilized preparations and for plasma membranes, also present were two sets of minor oscillations with a period length of about 22 min (double arrows, Fig. 8). Fourier analysis confirmed a major period at all three temperatures of 0.042 cycles/min corresponding to a period length of 24 min (Fig. 9). For each of the three temperatures the period lengths appeared to remain the

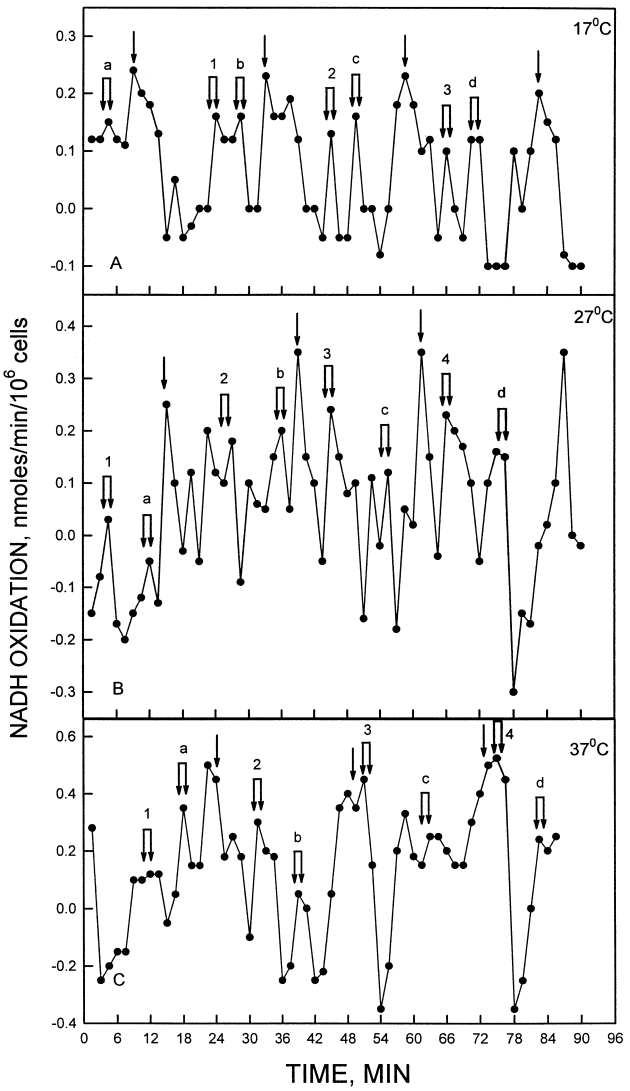

Fig. 8. Oxidation of NADH with intact HeLa cells to show periodicity and temperature compensation of period length. (A) 17°C. (B) 27°C. (C) 37°C. Single arrows indicate the oscillation with maxima separated by 24 min. Double arrows indicate oscillations with maxima at intervals of 22 min.

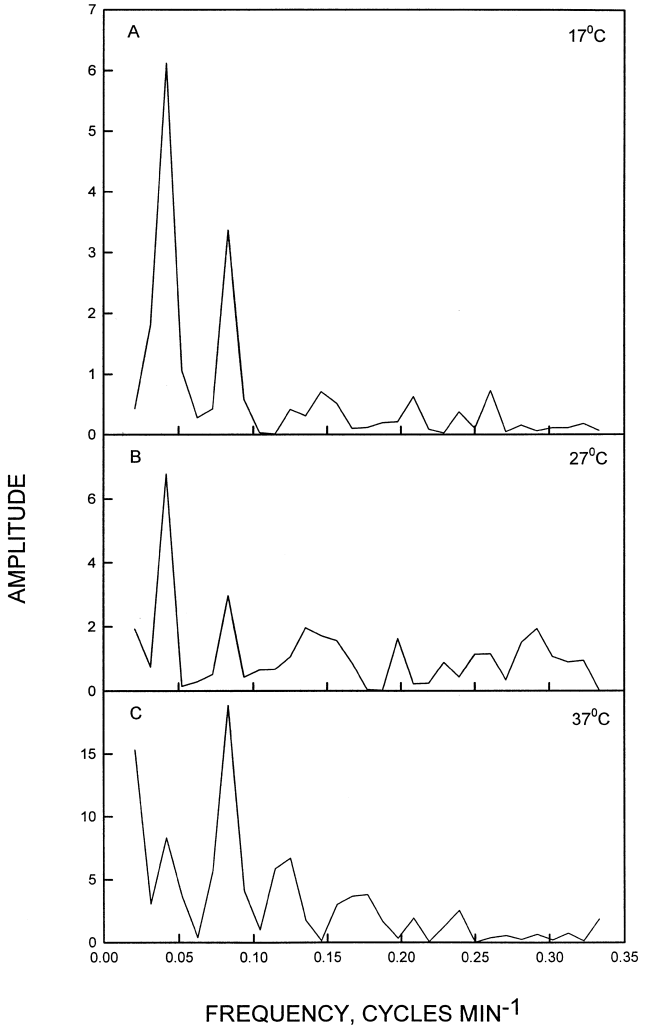

Fig. 9. Fourier analyses of data of Fig. 8. The maximum amplitude corresponded with a frequency of 0.0417 cycles/min at all three temperatures (period length of 24 min).

same despite the 2-fold increase in amplitude for each 10°C rise in temperature (Table 1).

3.5. Statistical analyses

The periodic nature of the oscillations was verified by Fourier analyses (Figs. 2B,5,7 and 9). The maximum amplitude for solubilized tNOX (Figs. 2B and 5), plasma membranes isolated from HeLa cells (Fig. 7), and for HeLa cells (Fig. 9) coincided with a frequency of 0.042 cycles/min or a period length of approx. 24 min.

An important characteristic of the oscillatory behavior of the cell surface NADH oxidase was that the period length was independent of temperature (temperature compensated). These findings, summarized in Table 1, were based on Fourier analyses and decomposition fits of the rate data comparing 17, 27 and 37°C. NADH oxidase rates comparing 17°C (Fig. 4A), 27°C (Fig. 4B) and 37°C (Fig. 4C) for solubilized tNOX showed that while the rate of NADH oxidation approximately doubled for each 10°C rise in temperature ($Q_{10} \approx 2$), the period length

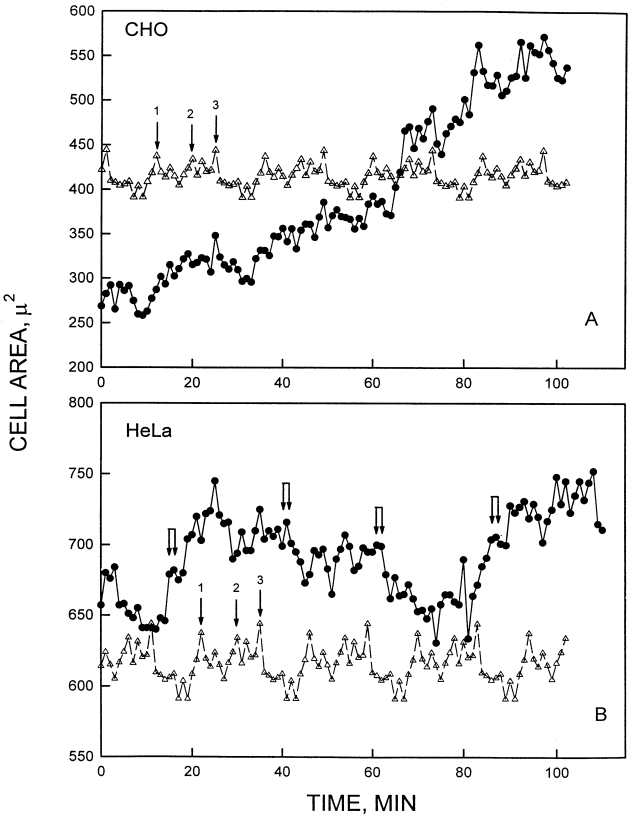

Fig. 10. Periodicity of growth (increase in area) of cells in culture at 37°C determined using video-enhanced microscopy. Measurements were at 1 min intervals over 110 min. Periods of rapid cell enlargement alternated with periods of relaxation where cell areas actually decreased. (A) CHO cells. As observed previously, the enlargement phase of CHO cells consists of three maxima (labeled 1, 2 and 3) separated by about 5 min [19]. Single arrows coincide with the positions of maximum rates of enlargement spaced 24 min apart. Recurrent positions of maxima were determined from the detrended decomposition fits (open triangles, dotted lines). (B) HeLa cells. A more complex pattern of oscillatory components is displayed. Single arrows identify the oscillatory components spaced 24 min apart that correspond to those observed with CHO cells as determined from the detrended decomposition fits (open triangles, dotted lines) with CHO cells. However, in between are a second set of oscillations (double arrows) spaced 22 min apart.

was unchanged. Similarly, NADH oxidation for plasma membranes isolated from the HeLa cells when assayed at 17°C (Fig. 6A), 27°C (Fig. 6B) and 37°C (Fig. 6C) or for intact HeLa cells at 17°C (Fig. 8A), 27°C (Fig. 8B) and 37°C (Fig. 8C) also approximately doubled for each 10°C rise in temperature while the period length remained constant.

Fourier analyses provide information on the length of the 24 min major period whereas decomposition fits show the reproducibility of the pattern of periodicity that includes the two sets of minor oscillations with period lengths of 22 min (Fig. 3). Thus, not only do the major 24 min fluctuations coincide with the predicted values, but the minor oscillations between the major peaks are also reproduced.

3.6. Cell enlargement

Increase in cell area, like NADH oxidation, fluctuates with alternating rapid and slow rates. The data of Fig. 10A for CHO cells which lack tNOX show a single 24 min period of alternating maxima in rates of cell enlargement followed by minima. As observed previously, cell enlargement of CHO cells is not a simple sine function but each maximum, based on decomposition fits, is seen to consist of three separate oscillations, on average, labeled 1, 2 and 3 (Fig. 10A). The three maxima occur about 5 min apart and those in one set of oscillations are separated from comparable maxima in the subsequent set of oscillations by 24 min. A similar set of oscillations marked by single arrows is discernable with HeLa cells (Fig. 10B). However, with HeLa cells the pattern of growth oscillations is more complex reflecting the complexity of the oscillation patterns found with the oxidation of NADH. Single arrows indicate oscillations separated by 24 min whereas the double arrows denote a second set of oscillations with a period length of 22 min (Fig. 10B).

4. Discussion

4.1. NADH oxidase

Our laboratory has described an NADH:protein disulfide reductase (NADH oxidase) associated with the plasma membrane of HeLa cells that is inhibited by both capsaicin [4] and by antitumor sulfonylureas [5,25]. The activity is measured by oxidation of NADH with the electron acceptor being either protein disulfides [7] or molecular oxygen [17]. The protein also appears to function in protein disulfide–thiol interchange [10].

Dai et al. [26] reported a highly significant correlation of NADH oxidase activity of plasma vesicles from HeLa cells with growth of HeLa cells in culture for a variety of synthetic retinoids as well as *all-trans* retinoic acid and *all-trans* retinol. This suggested that inhibition of the plasma membrane NADH oxidase activity may be related to inhibition of growth of HeLa cells in culture.

4.2. Ultradian clock

This study showed that the external NOX activity exhibited a characteristic oscillatory behavior of constant period length and variable amplitude. The protein represents a potential time-measuring driver of ultradian (less than 24 h) or circadian rhythms. The enzymatic activity accelerates and decelerates with a periodicity of about 24 min or 60 times per 24 h day. The actual period may be somewhat less and correspond more closely to the free running day in animals of 23.7 h [27].

The periodicity is retained by the solubilized and purified enzymatic activities and is fully temperature compensated. The latter is a hallmark of the biological clock. The response of the period was remarkably unaffected by temperature even though amplitude increased by a factor of about two for each 10°C rise in temperature between 17 and 37°C (Table 1). A thorough search of the literature revealed that this extraordinary degree of temperature compensation of our protein is the first ever described with the potential to function as a temperature-compensated ultradian clock driver.

Since blanks without NADH or without an enzyme source did not exhibit oscillatory absorbance changes (Fig. 1, inset), the oscillations observed with the complete system are inherent in the cell surface NADH oxidase protein itself and are not a function of machine variation or more complex environments. The oscillatory behavior is similar for

partially purified NOX proteins released from the HeLa cell surface as for the NOX activity of isolated plasma membrane vesicles and of whole cells. Another interesting observation is that the NOX activity of isolated plasma membrane vesicles showed one major and two minor periods. This suggested that at least two, possibly three, different NOX activities were present in each of the three sources of NOX from HeLa cells that did not cross-entrain. Although the differences were only marginally significant, the two minor oscillations may have different period lengths. In those experiments where two minor sets of oscillations were observed, the period length of the oscillations labeled a–d was 22.5 ± 0.6 min whereas the period length of those labeled 1–4 was 21.5 ± 0.6 min.

The ultradian cycle of the NADH oxidase may very well be one component of higher order, including circadian, cycles. Cycle amplitude in HeLa cells seems to vary during the day with the maxima between 10.00 h and 14.00 h. An overall maximum occurred at midday.

The approx. 24 min period length of the constitutive NOX protein is not unique to HeLa cells but has been observed with soybean, a plant source [13], CHO cells [19], sera of cancer patients [28], and with bovine milk fat globule membrane [29] which is a derivative of the plasma membrane of bovine mammary epithelial cells. It may very well be a general characteristic of plasma membranes. Non-cancer sources, however, lack the set of minor oscillations with the approx. 22 min period length which may typify cancer cells.

In living systems, oscillatory states are relatively common. At the level of the whole organism, the dominant periodic processes are the circadian rhythms ubiquitous in all eukaryotic systems [30] and the cell division cycle [31]. The oscillations in activity of the NOX proteins occur more slowly than those of metabolism, but more quickly than circadian oscillations and belong to the category of oscillations broadly defined as ultradian with period lengths of less than 24 h [32].

Temperature compensation and entrainment are two major properties of biological timekeeping [33]. Temperature compensation means that period lengths remain constant with changes in temperature. Entrainment means that the phase of the oscillations could be synchronized or shifted by light or other environmental factors. The plant NOX activity is entrained by light [34]. For the milk fat globule membranes, when preparations from different batches exhibiting oscillatory patterns with maxima displaced by several minutes were mixed, the mixtures initially displayed both sets of maxima and minima. However, if the preparations were incubated together for several hours, a common pattern of oscillations with only a single maximum every 24 min was the result [29].

Except for the NOX proteins, temperature-compensated oscillations in enzymatic activities are largely unknown. Cell division of synchronous cultures of *Acanthamoeba castellanii* is temperature-dependent (Q_{10} of about 2, typical of biochemical reactions) and an integer multiple of the period of the respiratory rhythm [35].

Ultradian oscillations with periods between 5 min and 4 h have been described [36] and suggested to have timekeeping functions. Under some circumstances, an ultradian clock appears to exert control over cell division times [37]. Yet, how a temperature-compensated clock can determine the temperature-dependent duration of the cell cycle [37], or control cell cycle progression [38], remains unclear.

The operation in HeLa cells of at least two oscillatory patterns, one potentially cancer specific and with a period length of less than 24 min, is of interest in the context of altered rhythms observed with patients with cancer. Murine and human data have indicated that tumors and tumor-bearing hosts may exhibit nearly normal or markedly altered circadian rhythms. Amplitude damping, phase shifts, and/or period length (τ) change, including appearance of ultradian rhythms with $\tau < 20$ usually become more prominent at late stages of cancer development [39]. Rhythm alterations seem to depend upon tumor type, growth rate and level of differentiation, both in animal and in human tumors. Ultradian rhythms usually worsen in the course of cancer progression. Thus, NOX oscillations with a period length different from those of non-cancer cells may have important therapeutic implications.

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