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New Concepts

Biochemical Basis for the Biological Clock†

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ABSTRACT: NADH oxidases at the external surface of plant and animal cells (ECTO-NOX proteins) exhibit stable and recurring patterns of oscillations with potentially clock-related, entrainable, and temperature-compensated period lengths of 24 min. To determine if ECTO-NOX proteins might represent the ultradian time keepers (pacemakers) of the biological clock, COS cells were transfected with cDNAs encoding tNOX proteins having a period length of 22 min or with C575A or C558A cysteine to alanine replacements having period lengths of 36 or 42 min. Here we demonstrate that such transfectants exhibited 22, 36, or 40 to 42 h circadian patterns in the activity of glyceraldehyde-3-phosphate dehydrogenase, a common clock-regulated protein, in addition to the endogenous 24 h circadian period length. The fact that the expression of a single oscillatory ECTO-NOX protein determines the period length of a circadian biochemical marker (60 X the ECTO-NOX period length) provides compelling evidence that ECTO-NOX proteins are the biochemical ultradian drivers of the cellular biological clock.

Our laboratory has described a family of NAD(P)H oxidase (NOX)[1] proteins that exhibit both an oxidative and a protein disulfide isomerase-like activity (*1*, *2*). These proteins are characterized by the property, unprecedented in the biochemical literature, of having two distinct biochemical activities, hydroquinone (NAD(P)H) oxidation and protein disulfide−thiol interchange, that alternate (*1*−*5*). Present in both plants and animals, they have no flavin, heme, or non-heme iron prosthetic groups and do not require ancillary proteins for activation (*1*).

They are now referred to as ECTO-NOX proteins because of their cell surface location (*6*) and to distinguish them from the *phox*-NOX proteins of host defense (*7*). They achieve protease (including proteinase K) resistance, impart protease resistance to protease-susceptible proteins, bind copper, and form amyloid, all of which are characteristics of prions (*8*). While activities have been most often measured as oxidation of NADH, the physiological substrate for the activity appears to be hydroquinones of the plasma membrane such as reduced coenzyme Q_{10} (*9*).

At least two forms of ECTO-NOX activities have been distinguished on the basis of response to hormones, growth factors, capsaicin (8-methyl-*N*-vanillyl-6-noneamide) (*10*, *11*), and certain other quinone-site inhibitors or potential

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[1] Abbreviations: ECTO-NOX, cell-surface and growth-related NADH oxidase with protein disulfide−thiol interchange activity; tNOX, tumor-associated and drug-responsive ECTO-NOX of cancer cells; CNOX, constitutive and drug-unresponsive ECTO-NOX of noncancer cells; ttNOX, truncated tNOX; Capsaicin, 8-methyl-*N*-vanillyl-6-noneamide; EGTA, ethylene glycol-bis(*β*-aminoethyl ether); HBS, Hepes-buffered saline; HMG-CoA, 2-hydroxy-2-methylglutaryl-coenzyme A.

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quinone-site inhibitors with anticancer activity (*1*, *12*). The constitutive ECTO-NOX, designated CNOX, is hormone-responsive and refractory to the quinone-site inhibitors (*1*). A tumor-associated NOX (tNOX) is unregulated, refractory to hormones and growth factors, and responds to inhibitors (*1*). CNOX proteins are widely distributed and exhibit activity oscillations with a period length of 24 min (*13*). tNOX proteins are cancer-specific and exhibit oscillations with a period length of about 22 min, ca. 2 min shorter than those of CNOX (*2*,*14*).

The period lengths of the activity oscillations of the NOX proteins are independent of temperature (temperature compensated) (*3*−*5*, *14*), and their phases are entrainable (*5*, *14*). These two characteristics, temperature compensation and entrainment (coupling the intrinsic clock to environmental cues), are two defining hallmarks of the biological clock (*15*, *16*). ECTO-NOX synchrony through entrainment is achieved through autosynchrony in solution (*5*), by coupling to red (plants) (*17*) and blue (plants and animals) (*18*) light photoreceptors and in direct response to melatonin (unpublished).

To investigate possible relationships between the regular oscillations of activity of the NOX proteins and the circadian clock, COS-1 cells (SV-40-transformed African monkey kidney cell line) were stably transfected as described (*19*) with a truncated form of tumor-associated NOX (ttNOX) with a period length of 22 min, a C575A replacement of ttNOX with a period length of 36 min, or a C558A replacement of ttNOX with a period length of 42 min (*2*, *19*). As an endogenous biochemical marker to monitor the circadian rhythm, the activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a common clock-regulated housekeeping protein (*20*), was used.

The C558A and C575A amino acid replacements were by site-directed mutagenesis within a fully active and truncated tNOX cDNA (*19*). The truncated recombinant tNOX protein consisted of amino acid residues 220−610 of the full-length tNOX sequence. NdeI and BamHl restriction sites were incorporated to accommodate subcloning into protein expression vector pET-11b for amplification and expression (*21*). The oligonucleotides used for C558A were 5′-GCAAGCATTGAATACATCGCTTCCTACTTGCA-CCGTCTTG-3′ (forward), 5′-CAAGACGGTGCAAGTAG-GAAGCGATGTATTCAATGCTTGC-3′ (reverse). For C575A, the oligonucleotides were 5′-CCAGCGATGTG-GAGGCCCTCATGGGTAGACTCC-3′ (forward), 5′-G-GAGTCTACCCATGAGGGCCTCCACATCGCTGG-3′ (reverse). DNA sequencing was utilized to confirm the correctness of all replacements.

For measurement of the period length of the circadian day with GAPDH activity as the indicator, COS cells were plated a day before the experiment at 4×10^5 cells per 100-mm dish. For each experiment, triple dishes were prepared for each time point. After 2 days, the cells were exposed to ordinary laboratory lighting for 10 min to entrain the NOX period, covered with foil, and placed back into the incubator. Time zero for GAPDH measurements was at 8:00 a.m. the following morning.

Glyceraldehyde-3-phosphate dehydrogenase was assayed as described (*22*). The conditions were 0.1 M Tris-HCl, 0.5 mM EGTA, pH 8.0, 10 mM $Na_2HA_5O_4$, 2 mM NAD^+, and 3 mM glyceraldehyde-3-phosphate. NAD^+ reduction was determined from the increases in absorbance at 340 nm at 37 °C measured over 300 s. A millimolar extinction coefficient of 6.2 nm cm^{-1} was used to calculate NADH disappearance.

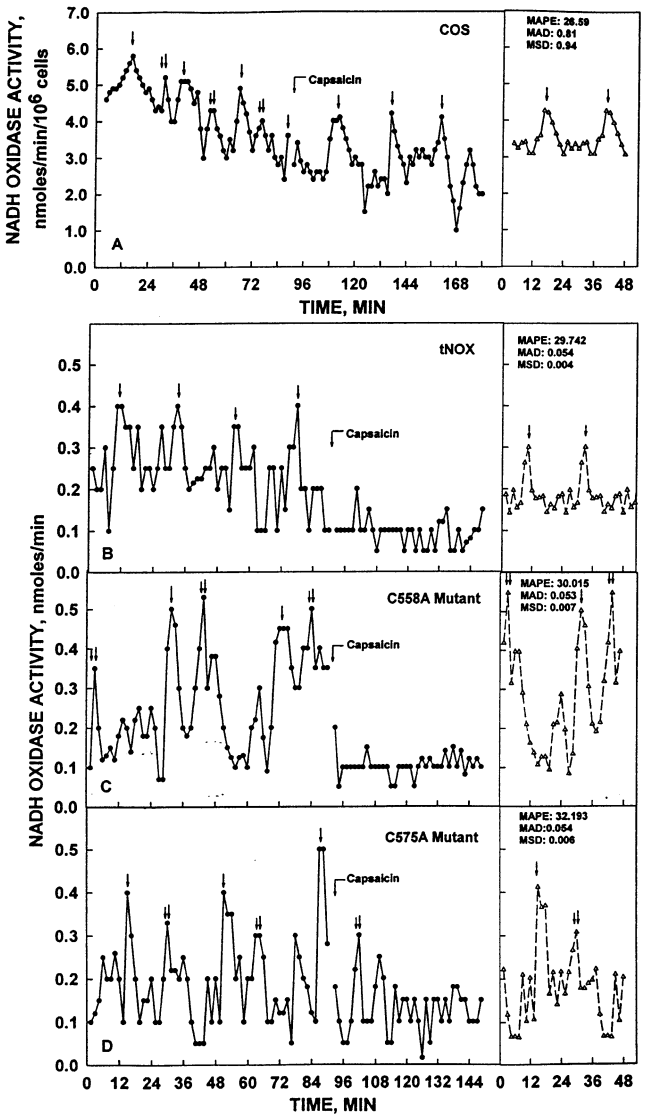

FIGURE 1: Rate of NADH oxidation by the cell surface NADH oxidases (ECTO-NOX proteins) of cultured COS cells. (A) Wild-type COS cells. The activity with a period length of 24 min (single arrows) is resistant to capsaicin, as is characteristic of the constitutive NOX (CNOX). Activity with a period length of 22 min (double arrows) is no longer observed after capsaicin addition, as is characteristic of tNOX (drug inhibition distinguishes tNOX from CNOX). (B) Recombinant tNOX. The period length is 22 min (single arrows). (C) Recombinant C558A replacement tNOX. The maxima are a doublet (single and double arrows) with a period length of 42 min. Both members of the doublet were inhibited by capsaicin. (D) Recombinant C575A replacement tNOX. The maxima are a doublet (single and double arrows) with a period length of 36 min. Both members of the doublet were inhibited by capsaicin. The decomposition fits (open triangles) for two (A and B) or 1 (C and D) full periods show the reproducibility of the patterns of oscillations. The mean standard deviations of panels B−D averaged ±2.4%. The two peak patterns were noted previously as a feature of NOX function.

Proteins were estimated by the bicinchoninic acid method (*23*) with bovine serum as the standard.

Statistical analyses used fast Fourier transform and decomposition fits. To determine the period length, fast Fourier

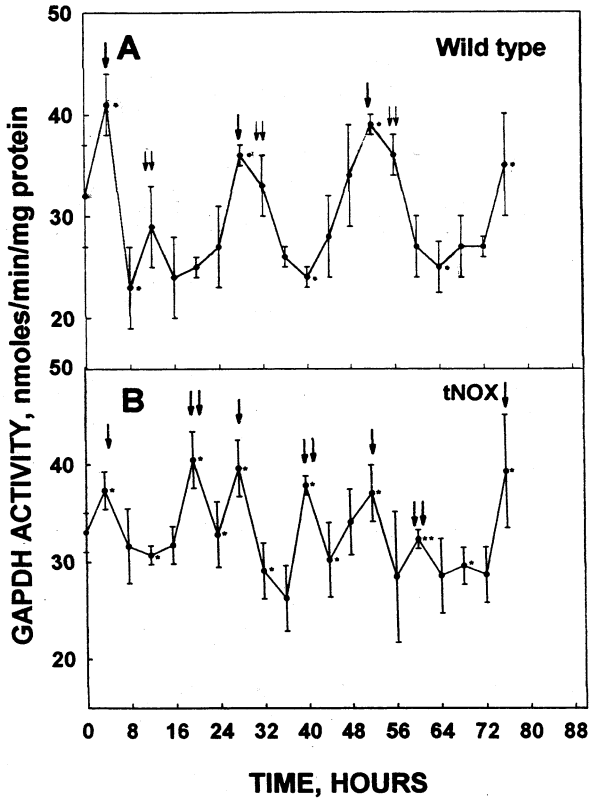

FIGURE 2: Period length of the circadian clock in COS cells as determined from measurements of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity. (A) Glyceraldehyde-3-phosphate dehydrogenase activity of wild-type COS cells oscillated with a circadian period of 24 h (single arrows). A possible minor period of 22 h in length is indicated at the small double arrows. (B) COS cells transfected with tNOX cDNA exhibit two circadian period lengths. One of 24 h (single arrows) corresponds to the CNOX period × 60, and one of 22 h (double arrows) corresponds to the tNOX period of 22 min ×60. Maxima and minima (asterisks) were highly significant for A ($p < 0.0012$) and significant ($p < 0.04$) for B ($p < 0.08$ where marked by the double asterisk).

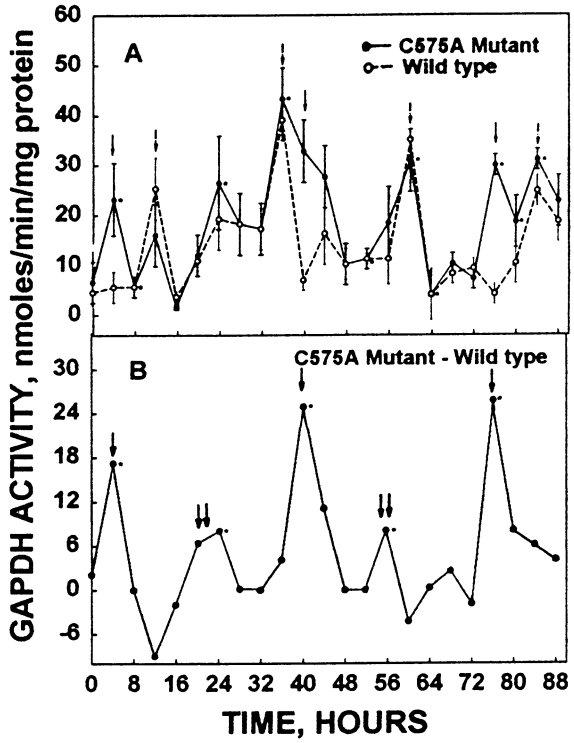

FIGURE 3: Length of the circadian period of COS cells in response to transfection with the C575A replacement having a tNOX activity with a 36 min period length. (A) COS cells transfected with the C575A replacement (solid symbols, lines and arrows) exhibited GAPDH maxima (solid arrows) absent from wild type (open symbols, dashed lines and broken arrows). Maxima and minima (asterisks) were significantly different ($p < 0.005$). (B) GAPDH activities of wild-type COS cells subtracted from those carrying the C575A replacement both showed a major (single arrows) and a minor (double arrows) circadian period length of 36 h (ECTO-NOX period X 60). The maxima (asterisks) were significantly different from basal values ($p < 0.03$).

user-defined transforms were with SigmaPlot 8.0. Decomposition fits used a MINITAB program to predict oscillatory patterns based on the period length established by the Fourier analyses. The decomposition fits served to evaluate the reproducibility of the oscillatory patterns and yielded mean average percentage error (MAPE), a measure of the periodic oscillation, mean average deviation (MAD), a measure of the absolute average deviations from the fitted values, and mean standard deviation (MSD), the measure of standard deviation from the fitted values.

For the cDNA used in the transfection experiments, the patterns of oscillations given by the recombinant proteins generated in *Escherichia coli* are shown in Figure 1B−D. For the recombinant tNOX, the period length was 22 min (Figure 1B). For the C558A replacement, the period length was 42 min (Figure 1C), and for the C575A replacement, the period length was 36 min (Figure 1D).

Wild-type COS cells in culture exhibit an oscillatory pattern of ECTO-NOX activities comparable to those seen with other transformed cell lines (*1, 2, 14*) (Figure 1A). The principal maxima corresponding to CNOX appeared at intervals of 24 min (single arrows) and were resistant to inhibition by capsaicin. Maxima with a period length of about 22 min, no longer evident after capsaicin addition, corresponded to the tNOX protein (double arrows). The presence of two distinct NOX activities having period lengths of 22 min (capsaicin inhibited) and 24 (capsaicin resistant) min, respectively, within a homogeneous population of cancer cells was investigated in detail by Wang et al. (*14*).

When assayed for GAPDH activity at 4 h intervals over 76 (Figures 2 and 4) or 88 (Figures 3 and 5) h, experiments in triplicate reproduced a major 24 h circadian rhythm of alternating maxima and minima with nontransfected (wild type) COS cells (Figure 2A). The GAPDH activity of COS cells transfected with tNOX retained the wild-type pattern (Figure 2B, single arrows) plus a second set of maxima with a period length of 22 h (Figure 2B, double arrows).

The circadian pattern of GAPDH activity for transfectants carrying the C575A replacement with a 36 min period showed a pattern of circadian activity oscillations distinct from that of wild type (Figure 3A). When the wild-type activity for that particular experimental series was subtracted from that of the C575A replacement activity, the differences due to the C575A replacement were seen as a new set of major oscillations spaced at intervals of 36 h (Figure 3B) (ECTO-NOX period X 60).

The circadian pattern of GAPDH activity for COS cells expressing the C558A replacement where the NOX activity oscillated with a period length of 42 min was reproduced in two independent experiments. Both showed a complex pattern of GAPDH activity changes with a major circadian

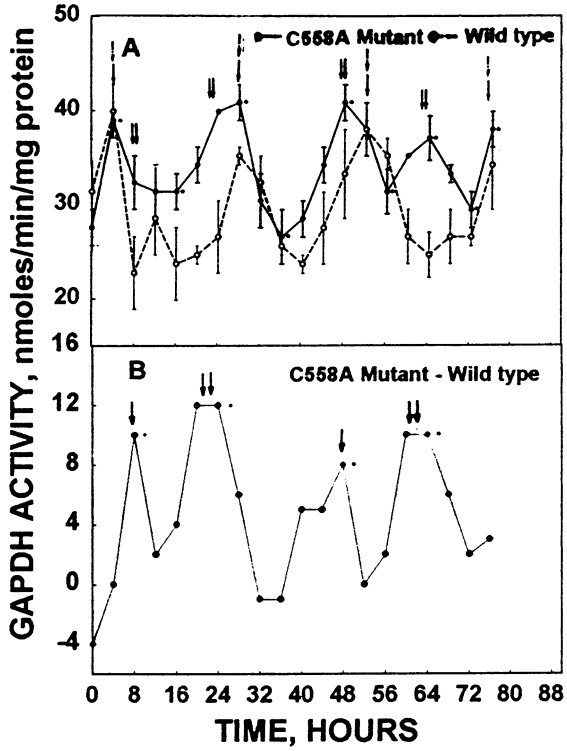

FIGURE 4: Length of the circadian period of COS cells transfected with the C558A replacement having a tNOX activity with a period length of 42 min. (A) COS cells transfected with the C558A replacement (solid symbols, lines and arrows) compared to wild-type (open symbols, dashed lines and broken arrows). Maxima and minima (asterisks) were significantly different ($p < 0.008$). (B) When the GAPDH activities of wild-type COS cells were subtracted from those carrying the C558A mutant, the difference showed double maxima (double arrows in panel A) with a circadian period length of 40−42 h (ECTO-NOX period × 60), where the maxima (asterisks) were significantly different from the basal values ($p < 0.005$).

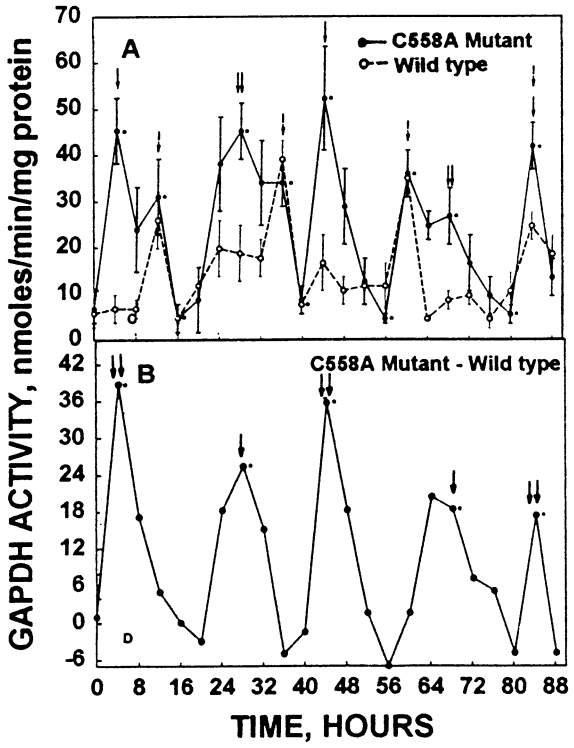

FIGURE 5: Repetition of the experiment of Figure 4 carried out in parallel with the experiment of Figure 3. (A) COS cells transfected with the C558A replacement with tNOX activity with a 42 min period (solid symbols, double arrows) compared to wild type (open symbols, single arrows). The differences comparing maxima and minima (asterisks) were highly significant ($p < 0.004$). (B) GAPDH activities of wild-type COS cells were subtracted from those carrying the C558A mutant; the difference showed double maxima with a circadian period length of 40−42 h (ECTO-NOX period × 60). The differences between activity values at maxima (asterisks) were highly significant ($p < 0.007$) compared to basal values.

period 24 h in length, plus a second circadian period with maxima at intervals of 40−42 h (Figures 4 and 5). Subtraction of the wild-type GAPDH activity from that of the C558A mutant activity clearly revealed the oscillations in GAPDH activity with a period length of 40−42 h (Figures 4B and 5B).

Figures 2−5 were reproduced in duplicate determinations each within three repetitions. Differences between maxima and minima determined by Student's t-test were significant to highly significant. Also significant to highly significant were the differences observed when wild-type specific activities were subtracted from the specific activities of the mutants. Figures 2 and 4 were from the same set of experiments. Figures 3 and 5 were from a different series. Yet the wild-type profiles were very similar. A difficulty in distinguishing between a 40 h period length and a 42 h period length arose because data were collected at 4 h intervals. What might have been oscillatory maxima spaced at intervals of >40 but <44 h might have appeared spaced at 40 h because of the 4 h sampling interval. In addition to the two repetitions with GAPDH activity, HMG-CoA reductase activity, assayed as described (*24*), showed a similar altered pattern of response with the C558A replacement compared to wild type (not shown).

The opportunity to test the hypothesis that the NOX proteins function as ultradian biochemical oscillators that underlie the cellular biological clock was made possible by identification of key regions of the tNOX protein confirmed by site directed mutagenesis. In several of the cysteine replacements, a normal rate of oscillating NADH oxidase activity was retained, but the period length of the oscillations was increased from 22 to 36 min or 42 min. Overexpression of these tNOX proteins in COS cells resulted in a proportionate influence on the length of the circadian period. Current clock models suggest operation of a transcriptional feedback loop with time delays in protein translation and transport back to the nucleus and where activities fluctuate as a function of the light-dark cycle (*25*, *26*). Feedback loops seem essential to the construction of a true 24 h time-keeping system capable of coordination of the many aspects of cell function under circadian control (*27*−*32*). However, they may not be the actual clock drivers. Even in organisms carrying mutations in time-keeping genes that affect molecular rhythmicity, aspects of circadian control remain. Despite considerable conservation among feedback loop genes (*27*−*29*), their operation at all times in all cells of all organisms that exhibit circadian rhythms remains to be established. In contrast, the CNOX proteins appear as universal cell constituents that keep time over widely varying conditions and without interruption.

Consistent with our findings are experiments that show the clock-related transcription factors, NPAS2 and Clock, to be intracellular redox sensors (*25*). DNA binding activities

of NPAS2 and Clock are influenced by the redox status of NAD(H) and/or NADP(H) (*33*). Since NOX proteins function as terminal electron acceptors of plasma membrane electron transport (*1*), their activities provide a significant mechanism for regeneration of cytosolic $NAD(P)^+$ from NAD(P)H (*34*).

The correlation between expression in COS cells of ECTO-NOX activities of different period lengths (NOX period X 60) and circadian GAPDH activity show that expression of a single protein through cDNA transfection determines the circadian period length. Thus, we conclude that the NOX proteins are the ultradian biochemical drivers of the cellular biological clock.

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