Identification of a Thermolabile Component of the Human Neutrophil NADPH Oxidase

A Model for Chronic Granulomatous Disease Caused by Deficiency of the p67-*phox* Cytosolic Component

Richard W. Erickson,* Stephen E. Malawista,‡ Michael C. Garrett,§ Gretchen Van Blaricom,‡ Thomas L. Leto,§ and John T. Curnutte*

**Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037; ‡Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06510; and §Laboratory of Host Defenses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892*

Abstract

Mild heating of human neutrophils inactivates the respiratory burst oxidase, producing a defect in superoxide production and bacterial killing comparable to that seen in patients afflicted with chronic granulomatous disease (CGD). We have now investigated the mechanism and specificity of this inactivation by examining the effect of mild heating on the known oxidase components: the membrane-bound subunits of the cytochrome b_{558} (gp91-*phox* and p22-*phox*) and the two cytosolic oxidase factors (p47-*phox* and p67-*phox*). Heating (46°C for 7.5 min) caused intact neutrophils to lose > 85% of their capacity to produce superoxide, a defect which was localized to the cytosolic, but not the membrane, fraction. Complementation studies with CGD cytosols deficient in either p47-*phox* or p67-*phox* suggested that the defective component of heat-inactivated cytosol was p67-*phox*. This was confirmed by experiments showing that recombinant p67-*phox*, but not p47-*phox*, exhibited lability at 46°C and completely reconstituted oxidase activity of heat-treated cytosol. These studies indicate that mild heating of either intact neutrophils or normal neutrophil cytosol results in a selective inactivation of p67-*phox*, providing a model oxidase system for the extremely rare p67-*phox*–deficient form of CGD. (*J. Clin. Invest.* 1992. 89:1587–1595.) Key words: cytokineplast • heating • phagocyte • p47-*phox* • superoxide

Introduction

Controlled heating of human neutrophils on surfaces produces anucleate, granule-poor cytoplasmic fragments termed cytokineplasts (CKP),[1] which retain the chemotactic and phagocytic function of the parent cells, but lack the capability of undergoing a respiratory burst (1–6). Subsequent studies (7) have shown that the loss of respiratory burst activity in cytoplasts is directly linked to the length of time neutrophils in suspension are preexposed to elevated temperatures (45°C). In the neutrophil, the NADPH oxidase is responsible for the oxidative burst, directing the transport of electrons from NADPH to molecular oxygen according to the following reaction:

$$NADPH + 2O_2 \rightarrow NADP^+ + 2O_2^- + H^+$$

This respiratory oxidase is a multicomponent enzyme complex that is associated with the plasma membrane after activation of the cell by a variety of stimuli (8–10). The absence of a respiratory burst in CKP preparations functionally resembles neutrophils of patients suffering from the inherited disorder chronic granulomatous disease (CGD), in which afflicted individuals are incapable of initiating or sustaining the neutrophil respiratory burst (11). The study of this disease has helped identify at least four oxidase components, two of which are membrane-bound and two of which are cytosolic in the resting neutrophil. In X-linked inheritance, the most common form of CGD (55% of CGD cases [12]), genetic alterations in gene locus Xp21.1 of the X-chromosome result in the complete lack of the 91-kD trans-membrane glycoprotein subunit (gp91-*phox* [for phagocyte oxidase]) of the heterodimeric cytochrome b_{558} (13, 14). This mode of inheritance is also marked by a concomitant loss of the cytochrome 22-kD subunit, p22-*phox* (15–17), although the gene encoding this protein has been localized to chromosome 16 (gene locus 16q24 [18]). Similarly, mutations involving the p22-*phox* gene (18), involving ~ 5% of CGD patients (12), also lead to the absence of both cytochrome subunits (17). In certain rare variant forms of CGD, mutations in the gene for either gp91-*phox* or p22-*phox* lead to a cytochrome complex, which is quantitatively and spectrally normal but is nonfunctional (19–22). Of the remaining 40% of CGD cases in which cytochrome b_{558} is functionally normal, a complete absence of one of the two known cytosolic components of the oxidase is seen: p47-*phox* (35% of CGD cases), encoded on gene locus 7q11.23, and p67-*phox* (5% of CGD cases), encoded on gene locus 1q25 (12, 23–31). Given the apparent similarity of respiratory burst oxidase dysfunction in CKP and CGD neutrophils, we explored the possibility that heated neutrophils might suffer from an acquired defect in one or more oxidase components.

In this report we show that mild heating inactivates the neutrophil respiratory burst oxidase through a mechanism involving the specific thermolability of the p67-*phox* cytosolic component. The rarity of p67-*phox*–deficient CGD patients has made it difficult to study the functional role of p67-*phox*

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Address reprint requests to Dr. Curnutte, Department of Molecular and Experimental Medicine, SBR 12, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037.

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1. *Abbreviations used in this paper:* CGD, chronic granulomatous disease; CKP, cytokineplast; GTPγS, guanosine 5'-*O*-(3-thiotriphosphate); *phox*, phagocyte oxidase (used to designate protein components of the phagocyte respiratory burst oxidase); PIPES, 1,4-piperazinediethanesulfonic acid.

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and to prepare p67-deficient cytosol for use in cell-free complementation studies to identify the molecular defect in CGD patients. We now describe a method which both enables the production of a model p67-*phox*–deficient cytosol system and demonstrates the mechanism by which the respiratory burst oxidase is disabled by mild heat treatment.

Methods

All reagents used were of the best grade commercially available, and were obtained from sources previously described (32).

Preparation of neutrophils. Blood was obtained in most cases by venipuncture from normal donors and CGD patients with their informed consent. Neutrophils were prepared using acid-citrate-dextrose as the anticoagulant, dextran to sediment erythrocytes, and Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden) density-gradient centrifugation to separate mononuclear cells from neutrophils as previously described (33). For experiments requiring purified cytosolic and membrane fractions, neutrophils were obtained by leukapheresis of donors and patients (after acquiring their informed consent) and purified as reported before (34). Normal donors were administered dexamethasone (4 mg orally 12 and 2 h before procedure) to increase neutrophil yields. Neutrophils were maintained at 4°C at all stages of purification after dextran sedimentation.

Intact neutrophil functional studies. Purified neutrophils were heated at 46°C for 7.5 min and assayed for O_2^- production as detailed below, while control neutrophils were kept on ice. Degranulation was assessed by quantitating the extracellular release of vitamin B_{12}–binding protein from phorbol 12-myristate 13-acetate (PMA)-treated neutrophils using a previously described method for measuring the binding of [^{57}Co]vitamin B_{12} to this protein (35, 36). Neutrophil bactericidal activity was measured in vitro using *Staphylococcus aureus* strain 502A as outlined earlier (36, 37).

Neutrophil fractionation. Unstimulated neutrophils were disrupted by nitrogen cavitation and purified membrane and cytosol fractions were prepared as previously described (32–34, 38) and stored at −70°C. Membrane fractions were brought to 1.25×10^9 cell equivalents/ml (~ 5 mg protein/ml) in 5 mM 1,4-piperazinediethanesulfonic acid (PIPES), 50 mM KCl, 1.75 mM $MgCl_2$, 1.5 mM NaCl, 0.5 mM ATP, 0.63 mM EGTA, 0.34 M sucrose, pH 7.3; cytosolic material was prepared at 9×10^7 cell equivalents/ml (~ 2 mg protein/ml) in 10 mM PIPES, 100 mM KCl, 3.5 mM $MgCl_2$, 3 mM NaCl, pH 7.3 (also termed "relax buffer") supplemented with 1 mM ATP and 1.25 mM EGTA. Sodium deoxycholate–solubilized membranes were prepared according to a published method (32) for use in the cell-free assay of superoxide production.

Heat treatment of neutrophils and cell fractions. Purified neutrophils were suspended at 10^8 cells/ml in 25°C phosphate-buffered saline (PBS) containing 138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.3. One volume of this suspension was then added to 3 vol of PBS preequilibrated to 46°C in a siliconized glass vessel equipped with a magnetic stirrer. Aliquots were removed at various times and stored on ice for 15 min before assay for superoxide production.

Purified neutrophil cytosol was removed from storage at −70°C and allowed to come to room temperature before heating. Cytosols were transferred to siliconized glass tubes and heated in a 46°C water bath with constant agitation for up to 20 min. Aliquots were removed at timed intervals, transferred to ice for 5 min, and then allowed to equilibrate at room temperature before analysis in the cell-free oxidase activation system.

Purified membrane vesicles were allowed to thaw at room temperature, and then transferred to a siliconized glass tube for heat treatment. Single-volume aliquots were removed at various times, and diluted with 5 vol relax buffer at 4°C. Samples were then incubated on ice for 10 min before analyzing oxidase activity in the presence of normal cytosol.

Whole-cell superoxide production. The rate of generation of superoxide by intact neutrophils was determined at 37°C in a ThermoMax kinetic microplate reader equipped with a 550±1 nm filter (Molecular Devices Corp., Menlo Park, CA) as previously described (39). In this assay (total volume 0.25 ml), cytochrome *c* reduction in a pair of reactions (one of which contained 15 μg of superoxide dismutase [SOD]) was continuously measured at 550 nm after stimulation of the neutrophil suspension by PMA at a final concentration of 200 ng/ml. Maximum reaction velocities were determined using SoftMax kinetic analysis software (version 2.01, Molecular Devices Corp.). The SOD-inhibitable rate of superoxide production was calculated by subtracting the velocity of the SOD-containing reaction from that measured in its companion reaction without SOD. Absorbance changes were converted to nanomoles O_2^- as described previously for this 550-nm filter (39).

Cell-free assay of oxidase activation. The kinetics of activation of NADPH oxidase were studied in a cell-free system using membranes and cytosol from unstimulated neutrophils as previously described (40). In this assay system, the oxidase is activated at 25°C by the addition of SDS in the presence of NADPH (0.16 mM) and cytochrome *c* (0.1 mM). Reaction mixtures of 0.15 ml total volume were incubated in 96-well Linbro microtitration plates (Flow Laboratories, Inc., McLean, VA) and contained cytosol (0–2×10^6 cell equivalents) and either membrane vesicles (4×10^5 cell equivalents) or deoxycholate-solubilized membranes (1.25×10^6 cell equivalents). The optimal concentration of SDS for activation of the oxidase was 40 μM for experiments using deoxycholate-solubilized membranes and 90 μM for assays using membrane vesicles. Again, measurements of SOD-inhibitable cytochrome *c* reduction were obtained on a Thermomax plate reader, with subsequent determination of maximal velocities by SoftMax software and conversion to nanomoles O_2^- as described above.

Complementation of CGD patient and heat-treated cytosol fractions was accomplished by adding equal volumes of test cytosols (2×10^6 cell equivalents) to the complete cell-free assay using deoxycholate-solubilized normal membranes. This volume of normal cytosol (2×10^6 cell equivalents) was sufficient to generate NADPH oxidase activity > 40 nmol O_2^-/min per 10^7 cell equivalents membranes. All other parameters of the cell-free assay system remained as described above.

The determinations of CGD patient and heat-treated cytosol rate order characteristics were made in the cell-free oxidase system, as previously reported (34, 41). Either relax buffer or an excess (3.6×10^6 cell equivalents) of test cytosol was added to reaction mixtures (0.15 ml) containing normal cytosol in varying amounts (3.6×10^5 to 1.6×10^6 cell equivalents). These cytosol mixtures were assayed for superoxide production using deoxycholate-solubilized normal membranes (1.25×10^6 cell equivalents per reaction). For kinetic studies of heated or p67-*phox*–deficient cytosol reconstituted with recombinant oxidase factors (see below), a similar cell-free assay was employed. Again, varying amounts (3.6×10^5 to 1.6×10^6 cell equivalents) of either heated or p67-*phox*–deficient CGD cytosol were added to 0.15 ml reaction mixtures containing recombinant p47- or p67-*phox* present at a constant stoichiometry of 1.7 μg of recombinant protein per 10^6 cell equivalents cytosol. Assays were carried out using deoxycholate-solubilized normal membranes (1.25×10^6 cell equivalents per reaction) as described above.

Preparation, heat treatment, and cell-free assay of preactivated NADPH oxidase. Purified neutrophils were suspended at 5×10^7 cells/ml in PBS (supplemented with 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, and 7.5 mM glucose) and stimulated with 200 ng/ml PMA for 10 min at 37°C. These activated neutrophils were washed twice with PBS and resuspended at 10^8 cells/ml in 0.34 M sucrose buffered with 10 mM Hepes, pH 7.3. This suspension was sonicated for three consecutive bursts of 10 s each, using a Sonifier cell disruptor model W185 (Heat Systems-Ultrasonics, Inc., Plainview, NY) at a constant power of 50 W. A low-speed centrifugation (500 *g*, 10 min, 4°C) was performed to sediment remaining intact cells and debris, and the resulting supernatant was removed to wet ice for immediate use. This preactivated oxidase was subjected to heat treatment as follows: 1.0 ml of neutrophil sonicate

was added to a 5.0-ml polypropylene test tube prewarmed at either 37 or 46°C. Heated incubations were carried out with constant agitation of the suspension, removing 0.07-ml aliquots directly to iced microcentrifuge tubes at 1 min intervals. NADPH oxidase activity was measured as described above for the cell-free activation assay with the following modifications: no SDS was used, the total assay volume was increased to 0.75 ml, and reactions were run in semi-microcuvettes utilizing a Uvikon model 810 dual-beam spectrophotometer (Kontron Electronics, Inc., Redwood City, CA). Reactions containing 3×10^6 cell equivalents activated oxidase (0.03 ml) were initiated by the addition of 0.16 mM NADPH. The SOD-inhibitable reduction of cytochrome *c* was measured continuously at 550 nm with the SOD-containing reaction in the reference beam. Maximum reaction velocities were determined by visual estimation. In certain experiments, recombinant p47- and p67-*phox* (5 μg of either [or both]) were added to reactions containing preactivated oxidase (3×10^6 cell equivalents) that was heat-inactivated (46°C, 7.5 min) to determine whether oxidase activity could be restored. The complete reaction mixture was then allowed to incubate at 25°C for 3 min before initiation of superoxide production by the addition of NADPH.

Recombinant protein studies. Both recombinant p47-*phox* and p67-*phox* were derived from baculovirus constructs (BV/p47 and BV/p67) as described elsewhere (42). Each of these eukaryotic expression vectors contains the complete coding sequences of one of these two cytosolic oxidase components, enabling production of complete native proteins lacking any additional fusion protein sequences. The recombinant proteins were purified from baculovirus-infected insect cell (Sf9) lysates 3–4 d postinfection, using ion-exchange chromatography. These proteins exhibited the same molecular weights as the native neutrophil proteins and were estimated to be > 90% pure by SDS-PAGE. The pure proteins were diluted to 0.2 mg/ml with relax buffer and heated for various time periods at 46°C, as described above. These solutions contained 0.1 mM dithiothreitol to inhibit oxidative damage of proteins. The cell-free assay system used to test the activity of these recombinant factors was essentially the same as that described above, with modifications (42). Briefly, these reactions (0.1 ml final volume) contained variable amounts of pure recombinant p47-*phox* or p67-*phox*, 10^5 cell equivalents of neutrophil cytosol, and 5×10^5 cell equivalents of deoxycholate-solubilized membranes. The oxidase reactions were activated by addition of 40 μM arachidonic acid instead of SDS. In experiments involving the restoration of heat-treated cytosol with the recombinant factors, the cytosol was heated for 7.5 min at 46°C before use in a reaction containing 10^6 cell equivalents of the heat-treated cytosol, 5×10^5 cell equivalents of deoxycholate-solubilized membranes, and variable amounts (0–1,000 ng) of the recombinant cytosolic factors.

SDS-PAGE and Western blotting. Heat-treated neutrophil-derived cytosol, heated normal cytosol, and recombinant cytosolic proteins were subjected to discontinuous SDS-PAGE prior to electrophoretic transfer to Zeta-Probe (BioRad Laboratories, Richmond, CA) or nitrocellulose blotting membranes, according to a general method (43, 44). An anti–peptide antibody raised in a rabbit against amino acid residues 447–460 (D E P K E S E K A D A N N Q) of the p67-*phox* component of the neutrophil NADPH oxidase was used as the primary antibody for the cytosol immunoblots. The recombinant proteins were probed with goat antibodies raised against these purified factors as described elsewhere (42). In both cases, the bound primary antibodies were detected with appropriate species-specific secondary antibody-alkaline phosphatase conjugates (BioRad Laboratories) using 5-bromo-4-chloro-3-indolyl phosphate and *p*-nitroblue tetrazolium as substrates.

Data analysis and curve fitting. All variances shown are standard deviations, unless otherwise noted. The exponential relationship between cytosol concentration and maximal rate velocity in the cell-free assay system was determined by two methods: (*a*) nonlinear regression analysis power curve fitting using Enzfitter (Elsevier-Biosoft; Cambridge, UK) data analysis software; and (*b*) least-squares linear regressions of log-log transform plots of maximum reaction velocity vs. normal cytosol concentration (34). Enzfitter power curve analyses were based on the equation: $V = K(C^P)$, where V = maximal rate velocity, K = constant, C = cytosol concentration, and P = rate order.

Results

The controlled heating of intact neutrophils at temperatures ranging from 39 to 47°C has been shown to decrease the capability of these cells to produce superoxide (7, 45). For the experiments described in this report, 46°C was chosen as the optimal temperature for inactivating the respiratory burst on the basis of extensive empirical trials. Neutrophils preincubated at 46°C for varying lengths of time were subsequently assayed for their ability to produce superoxide in response to stimulation by PMA at 37°C (Fig. 1). Superoxide production remained largely unaffected after 2 min at 46°C, and then abruptly declined over the next 3 min. By 8 min an almost complete abolishment of superoxide production was observed. Control experiments showed that preincubation at 46°C for up to 10 min did not induce neutrophils to produce superoxide in the absence of PMA, nor did it affect their viability as measured by trypan blue dye exclusion (> 95% viable cells). This pattern of heat-induced inactivation of respiratory burst activity is comparable to that previously described (7).

To obtain a broader understanding of the effect of mild heating on neutrophils, we also studied two other facets of neutrophil function: bacterial killing and degranulation. Table I summarizes these results and compares the functional capabilities of heated neutrophils to those obtained from CGD patients with either the standard or variant form of the disease. A strong resemblence is seen between these heated neutrophils and their CGD counterparts, particularly in the reduced capacity of both heated and CGD neutrophils to kill *S. aureus*.

One of the many possible mechanisms to explain the 46°C inactivation of the respiratory burst was that the NADPH oxidase itself, or one of its subunits, was damaged by the heating. To test this possibility, purified membrane and cytosol fractions from 46°C-treated neutrophils disrupted by nitrogen cavi-

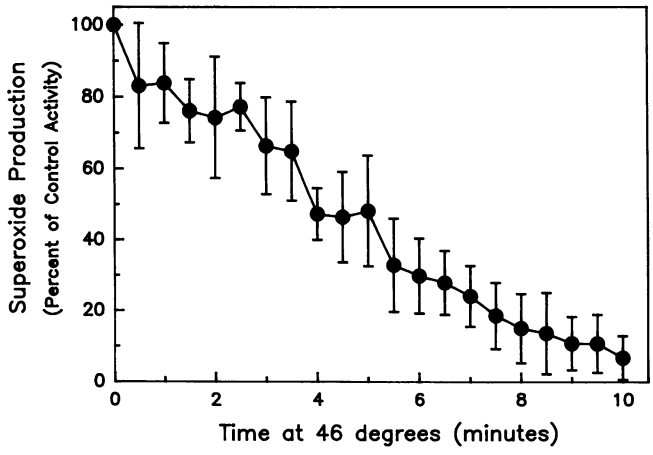

Figure 1. Inhibition of intact neutrophil superoxide production by preincubation at 46°C. Neutrophils were heated at 2.5×10^7 cells/ml in PBS and aliquots removed at 30-s intervals. Superoxide production after stimulation with 200 ng/ml PMA was assayed as described in Methods using a kinetic microplate reader and computer software that calculated the maximal rates of absorbance change at 550 nm. The results shown are the means±SD of three experiments, each performed with neutrophils from a different donor.

Table I. Selected Functional Properties of Heated Neutrophils

Functional assay	Neutrophils		
	Heated	CGD	Variant CGD
	percent control activity		
Superoxide production	5.8±4.2 (n = 4)	0.4±0.8 (n = 12)	5.8 (n = 2)
Bacterial killing:			
1 h	44.6±15.6 (n = 3)	50.9±35.9 (n = 12)	48.8 (n = 2)
2 h	60.9±34.5 (n = 3)	68.5±32.1 (n = 12)	45.7 (n = 2)
Degranulation	45.3±21.0 (n = 3)	Not determined	57.6 (n = 2)

Purified neutrophils were incubated at 4°C (control) or 46°C for 7.5 min. Functional assays were performed as outlined in Methods, and the results are presented as percentage of (unheated) control activity. 14 CGD patients (none of whom were taking interferon-γ at the time) were also studied. 12 of these patients had a complete absence of respiratory burst activity (due to mutations in gp91-*phox*, p22-*phox*, p47-*phox*, and p67-*phox*) while 2 had a variant form of X-linked CGD in which there was a residual level (~5% of normal) of O_2^- production owing to a 95% deficiency of cytochrome *b*. The control level of O_2^- production was 149.2±33.8 nmol/min per 10^7 cells (n = 14). Normal bacterial killing (expressed as the percent colony-forming units killed by incubation with control neutrophils) was 46.7±18.8% (n = 7) after 1 h of incubation, and 66.4±19.0% (n = 7) after 2 h of incubation. Control neutrophils released 42.0±22.7% (n = 7) of their total vitamin B_{12}-binding protein into the extracellular medium (degranulation) after 30 min of exposure to PMA.

tation were analyzed in a cell-free oxidase activation system. As shown in Table II, membranes from both unheated (control) and heat-treated (46°) neutrophils showed normal activity when activated with SDS in the presence of control cytosol. In contrast, cytosolic fractions from heated neutrophils showed a dramatic reduction in activity when compared to that observed with untreated cytosol. In control experiments, membrane fractions from heat-treated neutrophils showed normal activity in the cell-free system when tested at concentrations ranging above and below that shown in Table II (data not shown). In parallel experiments performed with CKP, there was a similar loss of cytosol oxidase activity when assayed in the cell-free oxidase activation system (data not shown).

Given the relatively selective inactivation of the cytosolic fraction, we next investigated whether the effect could be duplicated by 46°C treatment of cytosol and membrane fractions obtained from unheated neutrophils. As shown in Fig. 2, the heated membrane fraction showed only mildly compromised oxidase activity when assayed with control cytosol, while the heat-treated cytosolic fraction demonstrated a time-dependent loss of oxidase activity when assayed using control membrane vesicles. Based upon these data, we found that we could reproducibly generate cytosols inhibited by > 95% by heating at 46°C for 20 min. Addition of 10 μM guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) failed to restore any activity in these heated cytosol preparations, an indication that loss of oxidase activity in 46°C-treated cytosol is not due to thermal damage of this essential nucleotide cofactor (data not shown). A functional defect was thus shown to be inducible in (previously) normal cytosol, indicating the inactivation by heating of a cytosolic oxidase component (or components).

Determination of the heat-labile cytosolic oxidase component was accomplished by cytosol complementation studies in the cell-free assay system (Table III). Cytosols prepared from CGD patients with identical modes of inheritance are incapable of associating with each other in the presence of normal membranes to form activatable oxidase, whereas cytosol fractions prepared from patients with different cytosolic oxidase component deficiencies are capable of complementation in the cell-free oxidase activation system (26, 40). Normal cytosol that was heat-treated at 46°C for 20 min was assayed for complementation with four different cytosol preparations from CGD patients lacking either p47-*phox* (patients CGD_1 and CGD_2) or p67-*phox* (patients CGD_3 and CGD_4). No oxidase activity was detectable in reactions combining heated cytosol with p67-*phox*–deficient cytosol, suggesting that the p67-*phox* component of heated cytosol is functionally inactive. In contrast, heated cytosol was capable of complementing p47-*phox*–deficient cytosols, demonstrating a retention of p47-*phox* function in the heat-treated cytosol.

Kinetic studies were undertaken to determine further functional similarities between heated normal and p67-*phox*–deficient CGD cytosols. We have previously reported (34, 38, 41) that the order of the oxidase-activating reaction with respect to normal cytosol concentration is 2.52±0.09 SE (n = 7), a finding which implies that at least three kinetically independent soluble components (or component complexes) are required for the formation of active oxidase. In these same studies, when oxidase was activated at varying concentrations of normal cytosol in the presence of an excess of p47-*phox* (or p67-*phox*)–deficient cytosol, the yield of active oxidase no longer varied as the 2.5 power of normal cytosol concentration. Instead, oxidase activity increased in direct proportion to the concentration of normal cytosol, indicating that the defective cytosols were contributing all but one of the required kinetic components. The results of similar experiments performed with cytosol from 46°C-heated neutrophils or heat-treated cytosol are shown in Fig. 3, where log-log plots of normal (unheated) cytosol concentration vs. maximum reaction velocity are shown. The slope of each line defines the order of the oxidase-activating reaction with respect to normal cytosol concentration. In the absence of heat-inactivated cytosols, normal control cytosol showed the expected slope of ~ 2.5. The presence of excess heat-treated cytosol altered the dependence of oxidase yield on

Table II. Cell-free Activation of NADPH Oxidase Using Membranes and Cytosol Derived from Control and 46°C-treated Intact Neutrophils

Source of fraction		
Cytosol	Membrane	Superoxide produced
		nmol O_2^-/min per 10^7 cell equivalents membranes
Control	Control	38.5
Control	46°C	28.7
46°C	Control	4.5*
46°C	46°C	2.9

Experiments were carried out as described in Methods. Neutrophils were incubated at 46°C or 4°C (control) for 7.5 min before disruption and preparation of cytosol and membrane fractions. Each reaction (0.15 ml) contained 4×10^5 cell equivalents of membrane vesicles (from either control or 46°C-treated neutrophils) and 2×10^6 cell equivalents of cytosol (either control or 46°C-treated). Superoxide production was activated by the addition of 90 μM SDS. The results shown are representative of three experiments. * The average inhibition of cytosol activity was 94.0±5.6%.

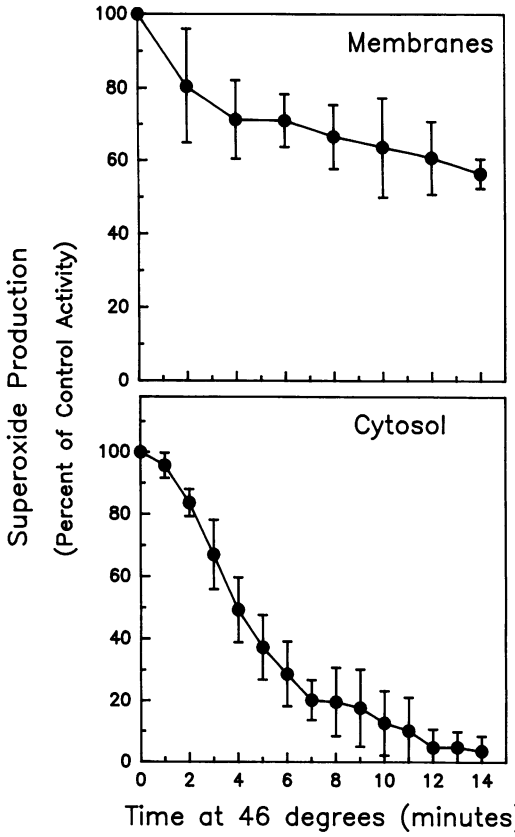

Figure 2. Effect of 46°C heating on oxidase function in membranes and cytosol. Cytosolic and membrane fractions from untreated normal neutrophils were obtained by nitrogen cavitation as described in Methods. These fractions were subjected to 46°C heat treatment, and aliquots were removed at the times indicated for analysis of superoxide production in the cell-free oxidase activation system. In the upper panel, heat-treated membranes (3.2×10^5 cell equivalents) were assayed for oxidase activity with normal untreated cytosol (1.8×10^6 cell equivalents). In the lower panel, heated cytosol (2.5×10^6 cell equivalents) was assayed for activity using normal untreated deoxycholate-solubilized membranes (6.3×10^5 cell equivalents). Control cytosolic and membrane fractions were kept at room temperature for the duration of the time course prior to determination of oxidase activity. Control activity was defined as that observed when cytosol (*lower panel*) or membranes (*upper panel*) incubated at 25°C for 14 min were substituted for the 46°C-treated fraction in the cell-free assay. Control activities were 63.3±12.3 and 73.8±18.2 nmol O_2^-/min per 10^7 cell equivalents membranes for the upper and lower panels, respectively. The results shown are the means±SD of three experiments, each performed with cytosol and membranes from a different donor.

untreated cytosol concentration from 2.5 order to approximately first order. These findings indicate that heated cytosols appear to suffer from a defect in one kinetic component, much like cytosols obtained from CGD patients lacking either p47-*phox* or p67-*phox*. Coupled with the data presented in Tables II and III, these results strongly suggest that heat treatment of neutrophils selectively inactivates the p67-*phox* cytosolic oxidase component.

The specific thermolability of p67-*phox* was directly demonstrated by the 46°C heat treatment of the purified recombinant cytosolic oxidase factors rp47-*phox* and rp67-*phox* (Fig. 4). The effects of heat treatment on the two recombinant factors were assessed under conditions in which the reconstituted oxidase activity was the most sensitive to the addition of these exogenous factors. Previous work has shown that the two recombinant factors (when combined with plasma membranes) do not support oxidase activation without the addition of whole cytosol, indicating the requirement of other soluble component(s) (42). The oxidase activity of reactions (0.1 ml) containing 5×10^5 cell equivalents of membranes and a suboptimum amount of whole cytosol (10^5 cell equivalents) was augmented by > 20-fold by the addition of 1 μg of each of the recombinant cytosolic factors (under these conditions, > 95% of cytochrome *c* reduction was inhibitable by superoxide dismutase). Pretreatment of these recombinant cytosolic factors at 46°C revealed that the p67-*phox* component was markedly sensitive to heat. While rp47-*phox* showed only a slight decrease in its ability to support oxidase activity after preincubation at 46°C for as long as 10 min, heat treatment of rp67-*phox* resulted in a progressive loss of its activity with time (Fig. 4). By 6 min, > 90% of rp67-*phox* activity was lost. These inactivation kinetics closely resemble those observed with cytosol treated in the same manner (Fig. 2), suggesting that there is a direct thermal effect on p67-*phox* that does not involve the participation of other factors in whole cytosol. Western blotting of heated cytosol or the pure recombinant proteins confirmed that the heat-treated p67-*phox* had retained the same apparent molecular weight as intact p67-*phox* (data not shown). It is therefore unlikely that the heat inactivation process involved proteolysis.

The strongest evidence indicating that p67-*phox* is the only oxidase component substantially affected by this mild heat treatment was obtained from experiments in which the recombinant cytosolic factors were used to reconstitute the activity of heat-treated normal cytosol (Table IV). Normal cytosol was heated at 46°C for 7.5 min, yielding activities that were re-

Table III. Complementation among Various Types of CGD Cytosols Deficient in Oxidase Components and 46°C-treated Cytosol

	Superoxide production					
	Cytosol 2					
Cytosol 1	Normal	46°C	CGD_1	CGD_2	CGD_3	CGD_4
	nmol O_2^-/min per 10^7 cell equivalents membranes					
Buffer	44.1	0.4	0.2	0.8	0.1	0.1
46°C		0.4	13.3	21.1	0.6	0.1
CGD_1			1.4	2.1	18.3	25.0
CGD_2				5.1	39.0	33.2
CGD_3					0.1	1.0
CGD_4						0.4

NADPH oxidase was activated in a cell-free system as described in Table II except that each 0.15-ml reaction contained a mixture of 22 μl (2×10^6 cell equivalents) of cytosol 1 (or relax buffer, where indicated) and 22 μl (2×10^6 cell equivalents) of cytosol 2. The heat-treated cytosol was prepared by heating normal cytosol for 20 min at 46°C. Patients 1 and 2 (CGD_1 and CGD_2) were deficient in p47-*phox*; patients 3 and 4 (CGD_3 and CGD_4) were deficient in p67-*phox*. The results shown are representative of six experiments, each performed using heated cytosol from a different normal donor. A total of seven different p47-*phox*–deficient patients and two different p67-*phox*–deficient patients were studied.

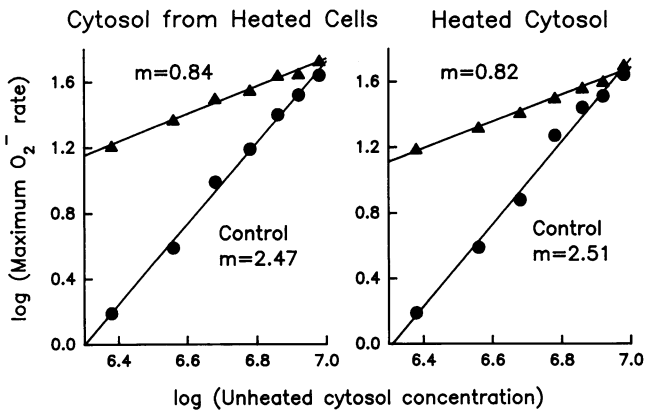

Figure 3. Effect of 46°C-treated cytosols on the order of the oxidase-activating reaction with respect to normal unheated cytosol concentration. The cell-free oxidase assays were carried out as described in Methods, using normal membranes at 1.25×10^6 cell equivalents per 0.15-ml reaction and varying concentrations of normal (unheated) cytosol as shown. The reactions were performed in the absence (●) or presence (▲) of an excess amount of heat-treated cytosol (3.6×10^6 cell equivalents per 0.15-ml reaction). The heat-treated cytosol used in the experiment depicted in the left panel was derived from heated neutrophils, whereas that used in the right panel was prepared by heating normal cytosol at 46°C for 20 min. The slopes of the lines (*m*) reflect the order of the activation reaction with respect to normal (unheated) cytosol concentration. The results in the left panel are representative of two separate experiments; those in the right panel are selected from 10 different experiments. For these 10 determinations, the slopes in the absence and presence of heated cytosol were 2.40±0.08 and 0.98±0.29, respectively.

duced by > 92% when assayed with normal deoxycholate-solubilized membranes in the cell-free system. The addition of increasing amounts of rp47-*phox* failed to restore the oxidase activity of heated cytosol, while almost complete recovery (to activity levels close to those observed with unheated cytosol) was seen with the addition of rp67-*phox*. The quantity of rp67-*phox* needed to attain nearly full restoration of activity (200–400 ng) was close to that required for full reconstitution of p67-*phox*–deficient CGD cytosol (42) and consistent with the level of p67-*phox* estimated to be present in normal neutrophil cytosol (~ 100 ng/10^6 cell equivalents) (42). Recombinant p67-*phox* also reconstituted the kinetic activity of heated cytosol as measured by the rate order constant relating cytosol concentration to oxidase activity. As shown in Fig. 5, heated cytosol supplemented with rp67-*phox* showed a mean rate order of 2.12±0.32, a value similar to those observed with normal cytosol (2.44±0.08) and with p67-*phox*–deficient cytosol supplemented with rp67-*phox* (2.42±0.07). The level of activity in the reconstituted reaction mixtures was less than that seen in experiments 1 and 2 in Table IV where near normal activity was obtained. This apparently is due to variations in the specific activities of different preparations of rp67-*phox*. The reconstitution of both oxidase activity and normal kinetic behavior in heated cytosol by rp67-*phox* provides strong evidence that the defect in oxidase activity produced by mild heating of normal neutrophils is due to the specific thermolability of one of the oxidase components, p67-*phox*.

The susceptibility of p67-*phox* to mild heating led us to investigate whether the loss of function of this component could explain the well-established heat lability of NADPH oxidase in its activated state (46). In these experiments, neutrophils stimulated with PMA and then sonicated were used as a source of activated oxidase. As shown in Fig. 6, this type of oxidase preparation proved to be even more sensitive to heating at 46°C than either intact neutrophils or normal cytosol (Figs. 1 and 2). The level of activity of preactivated oxidase was reduced from 22.0±3.2 to 1.4±0.6 nmol O_2^-/min per 10^7 cell equivalents (n = 3), after heating at 46°C for 7.5 min. Recombinant p47- and p67-*phox* failed to augment the activity of this heated preactivated oxidase: addition of 5,000 ng rp47-*phox* or rp67-*phox* resulted in 1.3±0.3 and 1.2±0.3 nmol O_2^-/min per 10^7 cell equivalents, respectively (n = 3). The addition of 5,000 ng of both recombinant oxidase factors also failed to reconstitute activity (1.21±0.28 nmol O_2^-/min per 10^7 cell equivalents [n = 3]). Unlike the situation observed with cytosol and membranes from unstimulated neutrophils, 46°C heating of preactivated NADPH oxidase produces a defect that cannot be repaired by the addition of rp47- and rp67-*phox*.

Discussion

We have demonstrated that heat treatment of either normal neutrophils or their cytosol selectively inactivates a single NADPH oxidase component, giving rise to a cytosol bearing a close functional resemblance to that derived from a rare class of CGD patients suffering from an inherited loss of p67-*phox*. In this regard, heat inactivation of neutrophil cytosol can provide

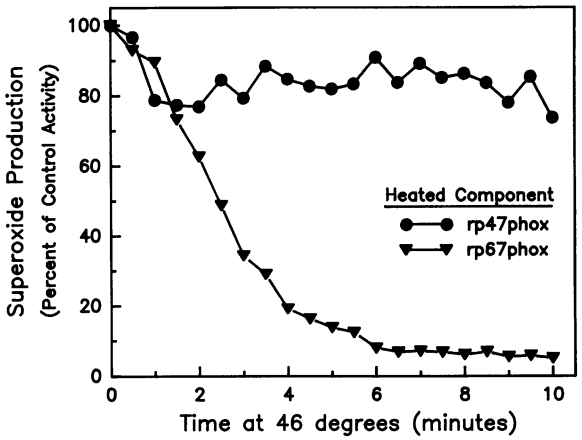

Figure 4. Effect of 46°C heating on recombinant cytosol oxidase factors. Pure recombinant p47-*phox* (●) or p67-*phox* (▼) was heated at 46°C at 30-s intervals for indicated times, as described in Methods. The activities of these proteins were tested in the cell-free oxidase activation system using 1 μg each of rp47-*phox* and rp67-*phox* (either heated or unheated), 10^5 cell equivalents of whole normal cytosol, and 5×10^5 cell equivalents of deoxycholate-solubilized membranes. The data points are averages of duplicate reaction mixtures (assayed simultaneously) obtained in a representative experiment. In the two such experiments performed, the average activities of reactions supplemented with unheated rp47-*phox* or rp67-*phox* were 20.2 and 15.9 nmol O_2^-/min per 10^7 cell equivalent membranes, respectively. The average activity of reactions that lacked recombinant factors was 0.8 nmol O_2^-/min per 10^7 cell equivalents membranes. The kinetics of the rp67-*phox* inactivation were nearly identical in the two experiments: at 2 min, 54.6% and 62.6% of control activity; at 4 min, 22.5% and 19.1%; at 6 min 7.3% and 7.9%; and at 8 min, 8.3% and 6.1%.

Table IV. Reconstitution of 46°C-treated Cytosol Activity by Recombinant p67-phox

Reaction mixture constituents	Quantity	Superoxide production		
		Expt. 1	Expt. 2	Expt. 3
	ng	*percent control activity*		
Heated cytosol		5.1	7.3	0.7
Heated cytosol	200	10.4	9.2	0.7
plus rp47-*phox*	400	15.8	6.9	0.6
	1000	21.9	8.2	0.4
Heated cytosol	200	78.1	96.6	42.9
plus rp67-*phox*	400	87.9	116.9	44.1
	1000	95.6	121.5	70.3

NADPH oxidase was activated in the cell-free system as described in Methods using reaction mixtures (0.1 ml in total volume) which contained deoxycholate-solubilized membranes (5×10^5 cell equivalents), normal cytosol heated at 46°C for 7.5 min (10^6 cell equivalents), and recombinant cytosol factors (rp47-*phox* or rp67-*phox*) in the amounts indicated in the table. Results from three separate experiments are shown, each performed with a different preparation of cytosol. Superoxide production is expressed as the percentage of control activity, which is defined as that measured in the presence of 10^6 cell equivalents of unheated cytosol. * The mean control activity for the three experiments was 29.2±4.3 nmol O_2^-/min/10^7 cell equivalents membranes.

a model system for the oxidase defect in this type of CGD in a manner analogous to the 56°C inactivation of the complement system in serum.

The behavior of heated neutrophils in cell function studies is consistent with the selective inactivation of an oxidase component. The loss of respiratory burst function in heated cells parallels the observed defect in the killing of *S. aureus*, a correlation to be expected given the central role of the respiratory burst in the microbicidal activity of phagocytes. Interestingly, heated neutrophils also demonstrated a reduced capacity to degranulate. Although this effect may be independent of the loss of the neutrophil's respiratory burst activity, the finding of a similar ~ 50% reduction in degranulation in two patients with variant X-linked CGD suggests there may be a linkage of some neutrophil functions to the respiratory burst oxidase (or its components). Although the heat treatment of neutrophils (or neutrophil cytosol) represents a useful model for the oxidase defect in the p67-*phox*–deficient form of CGD, it should not be construed as a strict paradigm for this disease.

The finding that p67-*phox* function is lost by mild heating in vitro raises the question of whether this critical oxidase component might be similarly affected during periods of high fever in vivo. Even though the present studies do not directly address this issue, we have observed that normal cytosol can be heated at 41°C for up to 30 min with no demonstrable decrease in activity in the cell-free oxidase activation system. Based on this finding it does not appear that the thermolability of p67-*phox* seen in our preparations at 46°C has physiological relevance. What effect extended incubations of several hours and longer under fever conditions might have upon neutrophil oxidase function remains to be investigated.

Elucidation of the mechanism by which p67-*phox* is disabled by heat treatment and how this alteration then interferes with oxidase activation will require further studies. Proteolytic degradation of p67-*phox* does not appear to be responsible in light of the finding that heat-inactivated p67-*phox* (in both recombinant and native forms) is still recognizable by immunoblotting and has an unaltered apparent molecular weight. In this respect, heated cytosol differs markedly from p67-*phox*–deficient CGD cytosol, which demonstrates a complete lack of p67-*phox* by immunoblotting. Because heating the pure recombinant p67-*phox* by itself results in defective oxidase function, a heat-induced conformational change in p67-*phox* may be involved.

The baculovirus-derived recombinant NADPH oxidase components, rp47-*phox* and rp67-*phox*, have provided a powerful means to investigate directly defects in the respiratory burst NADPH oxidase. We have shown that the inactivation of NADPH oxidase by controlled heating does not appear to be due to the action of heat shock proteins, as had been previously suggested (45). Instead, loss of oxidase activity can be attributed to the thermolability of p67-*phox* alone, since recombinant p67-*phox* demonstrated the same sensitivity to heat that was observed with whole neutrophil cytosol. These experiments have provided a direct and independent means of testing the kinetically derived model proposing thermal damage to a single soluble oxidase component. In that heating results in the selective inactivation of p67-*phox*, and in that the oxidase activity of heated cytosol can be completely reconstituted by recombinant p67-*phox*, this system should be useful in exploring functional relationships involving genetically engineered struc-

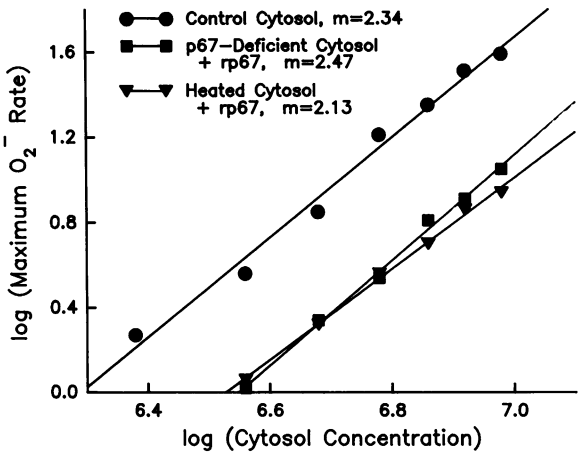

Figure 5. Reconstitution of normal kinetic activity in heated cytosol by recombinant p67-*phox*. Varying amounts of normal cytosol (●) were added to the cell-free oxidase activation assay as outlined in Methods. Similar amounts of heated (▼) or p67-*phox*–deficient (■) cytosol were also assayed, after having been supplemented with recombinant p67-*phox* (1.7 μg/1.0 × 10^6 cell equivalents cytosol). As in Fig. 3, the slopes (*m*) of the linear regressions computed for these data sets represent the order of the oxidase activation reaction with respect to cytosol concentration. The data presented are representative of triplicate experiments, the average rate orders for which are as follows: normal cytosol, 2.44±0.08; heated cytosol supplemented with rp67-*phox*, 2.12±0.32; and p67-*phox*–deficient cytosol supplemented with rp67-*phox*, 2.42±0.07. Neither the heated nor the p67-*phox*–deficient cytosols demonstrated oxidase activity when supplemented with relax buffer or rp47-*phox* instead of rp67-*phox*.

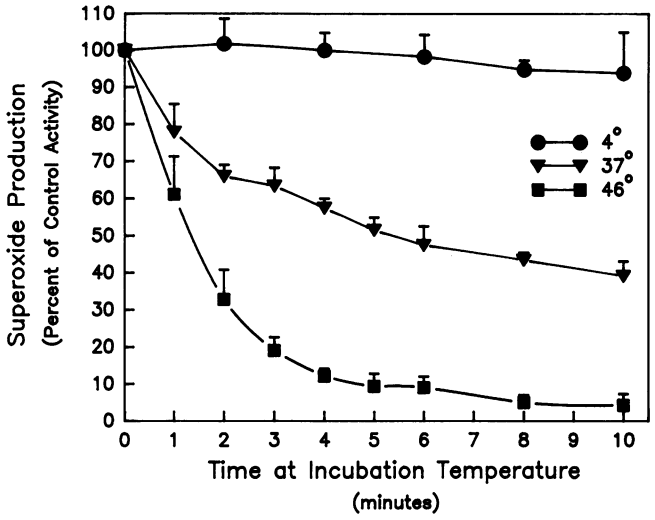

Figure 6. Effect of heating on activated oxidase obtained from stimulated neutrophils. Preactivated NADPH oxidase was prepared as noted under Methods, and subjected to heat treatment at 37 (▼) and 46°C (■). As a control, a portion of the oxidase preparation was kept on ice (●). Aliquots were removed to wet ice after the indicated times of heating and promptly assayed for activity. Reactions were initiated with the addition of 0.16 mM NADPH, and the superoxide dismutase-inhibitable reduction of cytochrome *c* was measured using a dual-beam spectrophotometer. The mean control activity for the three experiments performed was 22.0±3.2 nmol O_2^-/min per 10^7 cell equivalents.

tural variants of p67-*phox*. Furthermore, the finding that rp67-*phox* restores normal kinetic behavior to both heated cytosol and p67-*phox*–deficient CGD cytosol indicates that this type of reconstitution system may be useful for studying the intricate kinetic details of the mechanism by which the NADPH oxidase becomes activated.

NADPH oxidase in its activated state shows a striking sensitivity to mild heating, both at 37°C (Fig. 6 and reference 46) and (especially) at 46°C. The mechanism responsible for this inactivation, however, is not clear. The failure of rp67-*phox* to restore activity does not rule out the possibility that p67-*phox* thermolability is still a causal or contributory factor. For example, rp67-*phox* may not be able to exchange with heat-damaged p67-*phox* in the activated oxidase. Alternatively, the quaternary structure of this form of the oxidase may be disrupted at 46°C and contribute to the loss of enzyme activity in addition to any specific effects on p67-*phox*. Additional experiments aimed at deducing the mechanism by which the preactivated oxidase is damaged by 46°C heating could provide insights into the functional roles of the various oxidase components in the active enzyme. In a broader sense, the heat inactivation of both normal cytosol and preactivated oxidase provides a potentially valuable model system for studying the role that p67-*phox* plays both in activating NADPH oxidase in normal phagocytes and in causing the respiratory burst defect in CGD patients lacking this protein.

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