Acute Suppressive Effect of Hydrocortisone on p47^{phox} Subunit of Nicotinamide Adenine Dinucleotide Phosphate Oxidase

Paresh Dandona, Ahmad Aljada, Husam Ghanim, Priya Mohanty, Wael Hamouda, and Waddah Al-Haddad

We have recently demonstrated that reactive oxygen species (ROS) generation by polymorphonuclear leukocytes (PMNL) and mononuclear cells (MNC) is inhibited following the intravenous administration of hydrocortisone. This is associated with a parallel decrease in intranuclear NFκB, known to modulate inflammatory responses including ROS generation. We have also shown that the plasma levels of interleukin-10 (IL-10), an anti-inflammatory and immunosuppressive cytokine produced by TH2 cells, are also increased after hydrocortisone administration. In this study, we have investigated the effect of hydrocortisone on p47^{phox} subunit, a key component of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, in MNC and the pharmacodynamics of this effect with ROS generation and plasma IL-10 levels. p47^{phox} subunit protein levels in MNC showed a progressive decrease after hydrocortisone administration. It reached a nadir at 4 hours and increased thereafter to a baseline level at 24 hours. ROS generation also decreased, reached a nadir between 2 and 4 hours, and returned to a baseline level at 24 hours. IL-10 concentrations increased, peaked at 4 hours, and reverted to the baseline levels at 24 hours. In conclusion, p47^{phox} subunit suppression may contribute to the inhibition of ROS generation in MNC after hydrocortisone administration. This suppression occurs in parallel with the suppression of NFκB and an increase in IL-10 plasma levels. Therefore, it would appear that the decrease in intranuclear NFκB and an increase in IL-10 may cause the inhibitory modulation on p47^{phox} subunit and ROS generation by MNC following hydrocortisone and other glucocorticoids.

GLUCOCORTICOIDS in pharmacologic concentrations have been shown to inhibit reactive oxygen species (ROS) generation by leukocytes, in vitro.[1-3] We have recently demonstrated that following the intravenous administration of hydrocortisone at a commonly employed dose (100 mg) in clinical medicine, there is rapid and marked inhibition of ROS generation by polymorphonuclear leukocytes (PMNL)[4] and mononuclear cells (MNC).[5] Associated with this decrease is the induction of protein IκB,[6] which may account for at least some of the anti-inflammatory and immunosuppressive effects of glucocorticoids through binding to NFκB, which is a transcription factor responsible for the transcription of proinflammatory cytokines and ROS generation.[7,8] In addition, total cellular NFκB content also decreases after hydrocortisone. We have recently described an acute stimulatory effect of hydrocortisone on interleukin-10 (IL-10), an immunosuppressive cytokine secreted by TH2 cells; IL-10 suppresses TH1 cells and the proinflammatory cytokines secreted by them.[9-11]

Since the assay system used in our ROS generation studies largely detects the superoxide ($O_2^{\cdot}-$) radical,[12] which is produced by the conversion of molecular O_2 by the enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase,[13] we have now hypothesized that hydrocortisone inhibits the one integral protein of this enzyme, p47^{phox} subunit. NADPH oxidase consists of 4 other subunits, which assemble to form this enzyme.[14-18] Equimolar amounts of p47^{phox} and p67^{phox} in the cytosol combine to form a highly basic 250-kd protein, containing 2 SH3 domains.[19,20] During activation, the p47^{phox} and p67^{phox} complex migrates from the cytosol to the membrane where this complex combines with cytochrome b_{558}, which, in turn, consists of gp91^{phox} and p22^{phox}.[21] Rac 2, the fifth component of the enzyme, migrates to the membrane in association with p47^{phox} subunit, which, without it, is unable to reach the membrane.[21,22] Thus, p47^{phox} subunit is cardinal to the functional integrity of NADPH oxidase. The activation of NADPH oxidase requires the combination of p47^{phox} and p67^{phox} complex to cytochrome b_{558}, followed by phosphorylation of the p47 ^{phox} moiety within the complex of the enzyme.[13,21]

This study describes the acute suppressive effect of hydrocortisone on p47^{phox} in MNC and compares the pharmacodynamics of the effects of hydrocortisone with those on ROS generation by MNC and the stimulatory effect on plasma IL-10 concentrations.

MATERIALS AND METHODS

Subjects

Four normal subjects (age range, 33 to 54 years; mean, 43 ± 9 years) volunteered for the study. None of the subjects had a previous history of significant chronic illness, including endocrine disease. None had previously taken corticosteroids. None had taken aspirin or other nonsteroidal anti-inflammatory drugs for 1 week. All subjects presented at our clinical research center between 8 and 9 AM. An indwelling canula was placed in the antecubital vein and a blood sample was drawn. A dose of 100 mg hydrocortisone was then injected into the vein through the canula. Sequential blood samples were then drawn at 1, 2, 4, 8, and 24 hours.

Preparation of MNC

Blood samples were collected with EDTA as an anticoagulant. A 3.5-mL quantity of the anticoagulated blood sample was carefully layered over 3.5 mL of PMN medium (Robbins Scientific, Sunnyvale, CA) in a 15-mL centrifuge tube. Samples were centrifuged at 450 × *g*, in a swing-out rotor for 30 minutes at 22°C. At the end of the centrifugation, 2 bands separate out at the top of the red blood cell pellet. The top band consists of MNC while the bottom consists of PMNL. The MNC band was harvested with a Pasteur pipette and the

From the Division of Endocrinology, Diabetes and Metabolism, State University of New York at Buffalo; and Kaleida Health, Buffalo, NY.

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Address reprint requests to Paresh Dandona, MD, Director, Diabetes-Endocrinology Center of Western New York, 3 Gates Circle, Buffalo, NY 14209.

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cells were repeatedly washed with Hank's balanced salt solution (HBSS). This method yields greater than 95% pure MNC suspension and was tested repeatedly to validate the method. Thereafter, random checks were made to ensure the purity of the preparations.

Immunoblotting

Total cell lysates were prepared by adding 1 mL of boiling lysis buffer (1% sodium dodecyl sulfate [SDS], 1 mmol/L sodium orthovanadate, 10 mmol/L Tris, pH 7.4) to the MNC pellets. Cell homogenates were boiled for an additional 5 minutes and centrifuged at 14,000 $\times$ *g* for 5 minutes. Total protein concentrations were determined using bicinchoninic acid (BCA) protein assay (Pierce, Rockland, IL). Twenty micrograms of total cell lysate was electrophoresed on 10% SDS polyacrylamide gels (SDS-PAGE). The proteins were transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked for 1 hour in 5% nonfat dry milk in 0.1% Tween–Tris-buffered saline buffer (TTBS) and then incubated for 1 hour with a monoclonal antibody against p47^{phox} protein (Transduction Labs, Lexington, KY). The membrane was washed 4 times for 15 minutes each with TTBS and then incubated with peroxidase-conjugated goat anti-mouse immunoglobulin for 1 hour. Finally, the membrane was washed and developed using super signal chemiluminescence reagent (Pierce).

Electrophoretic Mobility Shift Assay

DNA-binding protein extracts were prepared from MNC as previously described.[6] Total protein concentrations were determined using BCA protein assay. NFκB gel retardation assay was performed using NFκB binding protein detection kit (Life Technologies, Long Island, NY). Briefly, the double-stranded oligonucleotide containing a tandem repeat of the consensus sequence for the NFκB binding site was radiolabeled with γ-P^{32} by T4 kinase. Then, 5 μg of the nuclear extract were mixed with the incubation buffer and the mixture was preincubated at 4°C for 15 minutes. Labeled oligonucleotide (60,000 cpm) was added and the mixture was incubated at room temperature for 20 minutes. Samples were then applied to wells of 6% nondenaturing polyacrylamide gel. The gel was dried under vacuum and exposed to x-ray film. Densitometry was performed using Bio-Rad molecular analyst software (Hercules, CA).

Measurement of ROS Generation

Five hundred microliters of MNC (2×10^5 cells) were delivered into a Chronolog Lumi-Aggregometer plastic flat-bottom cuvette (Chronolog, Havertown, PA) to which a spin bar was added. Fifteen microliters of 10 mmol/L luminol was then added followed by 1.0 μL of 10 mmol/L formylmethionylleucinylphenylalanine (fMLP). Chemiluminescence was recorded for 15 minutes (a protracted record after 15 minutes did not alter the relative amounts of chemiluminescence produced by various blood samples). Our method, developed independently, is similar to that published by Tosi and Hamedani.[12] In this assay system, the release of superoxide radical as measured by chemiluminescence has been shown to be linearly correlated with that measured by the ferricytochrome C method. We further established that, in our assay system, there is a dose-dependent inhibition of chemiluminescence by superoxide dismutase and diphenylene iodonium (DPI, data not shown), a specific inhibitor of NADPH oxidase, the enzyme responsible for the production of superoxide radicals. The specific inhibitory effect of DPI on NADPH oxidase has been established by Hancock et al.[23] Our assay system is exquisitely sensitive to DPI-induced inhibition at nanomolar concentrations.

Effect of IL-10 on ROS Generation, In Vitro

IL-10 was incubated at 50 and 500 pg/mL with MNC for 30 minutes. MNC were then challenged with fMLP as described above and ROS generation was measured.

IL-10 Assay

Plasma IL-10 concentration was measured by an enzyme-linked immunosorbent assay (ELISA) with a kit obtained from R&D systems (Minneapolis, MN). The sensitivity of this assay is 1.5 pg/mL, the intra- and interassay variations are 4.3% and 7.5%, respectively.

Statistical Analysis

Chemiluminescence due to ROS generation is highly variable from one subject to another. Therefore, for sequential comparisons, all values were normalized to 100% for baseline time point and the following values were expressed as percent of basal. Statistical analysis was carried out by analysis of variance (ANOVA) for repeated measures using SigmaStat software (Jandel Scientific, San Rafael, CA). Plasma IL-10 concentrations were also compared by ANOVA for repeated measures; the concentrations are expressed as mean $\pm$ SE.

RESULTS

Immunoblots for MNC homogenates showed a decrease in p47^{phox} at 1 hour, which progressively decreased further to a nadir at 4 hours. Thereafter, it increased at 8 hours and returned to baseline levels at 24 hours (Fig 1). ROS generation by MNC

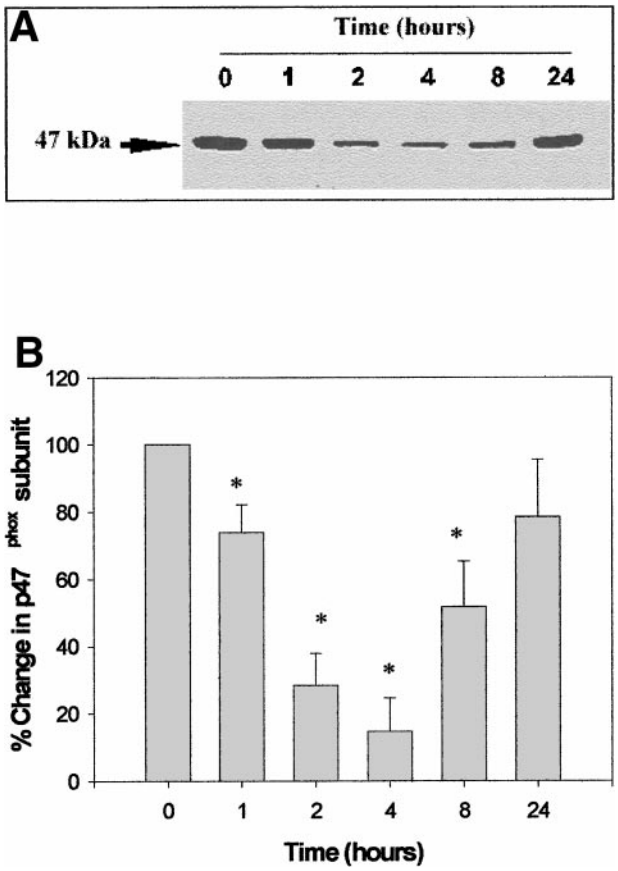

Fig 1. (A) Western blot shows the relative expression of p47^{phox} subunit in MNC (mean $\pm$ SE). Note that p47^{phox} expression is inhibited at 1 hour after 100 mg injection of hydrocortisone. Maximum inhibition is observed at 2-4 hours. p47^{phox} levels returned to basal levels 24 hours postinjection (*$P < .05$). This blot is a representative of 4 different experiments with 4 different subjects. (B) Relative expression of p47^{phox} subunit levels as measured by densitometry.

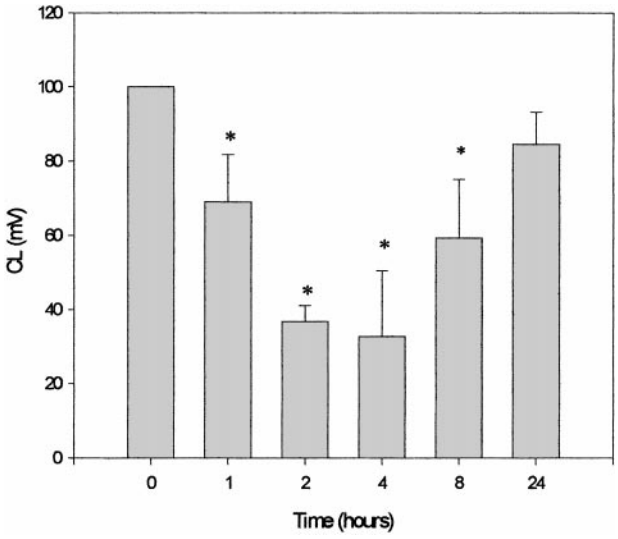

Fig 2. Inhibition of ROS generation by MNC following hydrocortisone injection (mean ± SE). The nadir of ROS generation was between 2-4 hours (n = 4). Note that ROS generation by MNC at 24 hours was similar to that at baseline (*$P < .05$).

also decreased at 1 hour, reached a nadir between 2 and 4 hours, recovering partially at 8 hours, and returned to baseline level at 24 hours (Fig 2). The inhibition was highly significant ($P < .001$). IL-10 concentrations increased significantly at 1 hour, peaked at 4 hours, and reverted to baseline levels at 8 hours (Fig 3). The increase was highly significant ($P < .001$). Intranuclear NFκB decreased after hydrocortisone injection with a peak effect at 4 hours, a partial recovery at 8 hours, and a return to baseline at 24 hours (Fig 4; $P < .01$). Preincubation of MNC with IL-10 at 50 and 500 pg/mL for 30 minutes did not change the magnitude of ROS generation.

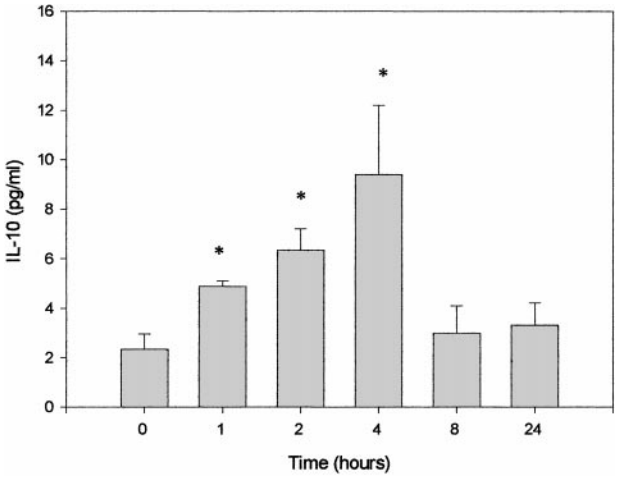

Fig 3. Sequential mean ± SE of IL-10 concentrations before and after 100 mg hydrocortisone injection (n = 4). Note that the increase was observed at 1 hour, peaked at 4 hours, and reverted to baseline at 8 hours. The increase was highly significant (*$P < .05$).

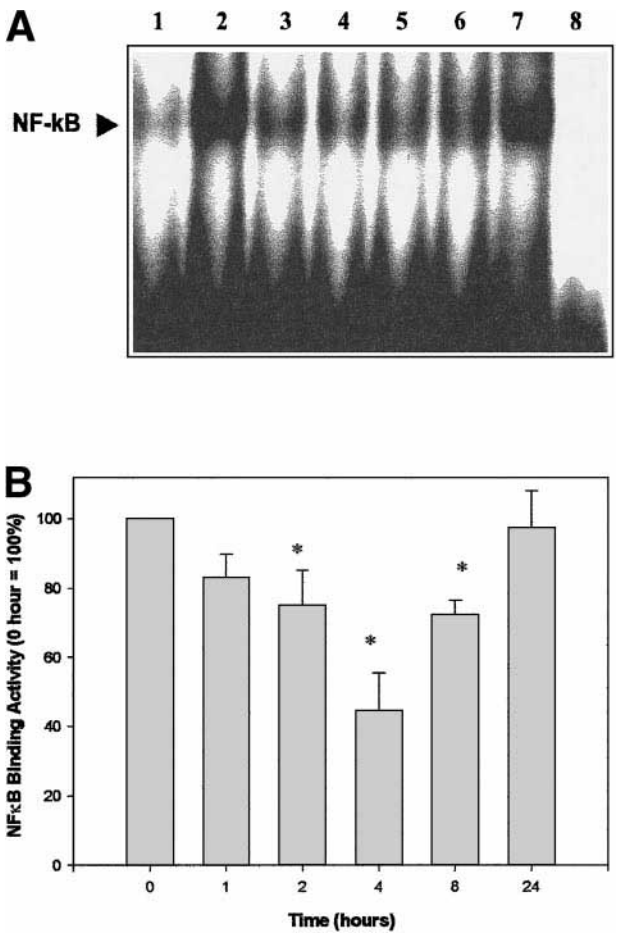

Fig 4. (A) Gel shift assay shows the relative NFκB binding to the double-stranded oligonucleotide containing NFκB DNA binding site. Lanes: (2) 0 hours, (3) 1 hour, (4) 2 hours, (5) 4 hours, (6) 8 hours, and (7) 24 hours. Lane (1) shows the inhibition of NFκB binding using 2 μL of competitor oligonucleotide in the baseline sample nuclear extract. Lane (8) is radiolabeled NFκB double-stranded oligonucleotide binding site without any nuclear extract. (B) Relative NFκB binding to double-stranded oligonucleotide containing NFκB DNA binding site as measured by densitometry. All values were normalized to 100% for baseline time point and the following values were expressed as percent of basal. The results are presented as mean ± SE (*$P < .05$).

DISCUSSION

Our data demonstrate clearly for the first time that hydrocortisone causes an acute reduction in $p47^{phox}$ subunit, a key constituent protein of NADPH oxidase, within 1 hour after hydrocortisone injection. These data also show for the first time that $p47^{phox}$ subunit can be used as a molecular marker of NADPH oxidase–generated ROS, in vivo, following acute pharmacologic intervention. The peak effect of hydrocortisone was observed at 4 hours with partial recovery at 8 hours and a return to baseline at 24 hours after hydrocortisone injection. The magnitude of reduction in $p47^{phox}$ subunit and the pharmacodynamics of this effect parallel those of ROS generation by MNC, whose peak effect was observed between 2 and 4 hours. It is probable, therefore, that the reduction in $p47^{phox}$ subunit contributes to a reduction in NADPH oxidase units,

which is reflected in a rapid fall in superoxide radical generation following hydrocortisone injection. Whether other component units of NADPH oxidase, like p67^{phox}, cytochrome b_{558}, and Rac2 protein, also diminish following hydrocortisone, requires further investigation. It is of interest that α-tocopherol (vitamin E) also causes a decrease in p47^{phox} expression after incubation of MNC with α-tocopherol, in vitro.[24] The congenital absence of p47^{phox} subunit results in chronic granulomatous disease.[25] This is a disease characterized by inability of the body to deal with infections.[25] Similar to the effect of hydrocortisone on ROS, the effect on plasma IL-10 concentrations also started at 1 hour, peaked at 4 hours, began a decline at 8 hours, and returned to baseline at 24 hours. This parallels the effect of hydrocortisone on p47^{phox} subunit and, therefore, it would appear that the inhibitory modulation on p47^{phox} subunit and the stimulatory effect on IL-10 may have similar underlying mechanisms. IL-10 is an immunosuppressive cytokine secreted by TH2 lymphocytes; it inhibits TH1 cells whose secretory products, IL-2 and interferon gamma, normally stimulate monocyte-macrophages to secrete tumor necrosis factor alpha and ROS.[10,11,26] We have recently demonstrated that plasma IL-10 concentrations increase after the administration of hydrocortisone[9] or dexamethasone.[27] It was possible that this increase in IL-10 may have impacted MNC to cause a suppression of p47^{phox} subunit and ROS generation, since IL-10 inhibits formyl peptide receptor expression in leukocytes.[28] However, we eliminated this possibility by testing the ability of IL-10 to inhibit ROS generation by MNC by demonstrating that IL-10 at even 500 pg/mL was not able to change ROS generation by MNC.

The rapid fall in p47^{phox} subunit following hydrocortisone administration suggests that p47^{phox} subunit is a rapidly turning over protein and that hydrocortisone probably induces ubiquitination of p47^{phox} subunit. Our study does not address the issue of the translocation of p47^{phox} subunit from the cytosol to the membrane as affected by hydrocortisone. While our observations show that hydrocortisone causes a diminution in total p47^{phox} subunit expression by MNC, it is possible that hydrocortisone also inhibits the translocation of p47^{phox} subunit from the cytosol to the membrane and thus causes a reduction in active NADPH oxidase units. This issue requires further investigation. The pharmacodynamics of the effect of hydrocortisone on p47^{phox} subunit mimic those of hydrocortisone on IκB and NFκB.[6] It is therefore possible that glucocorticoid-induced IκB biosynthesis and a concomitant decrease in total cellular NFκB result in a diminished intranuclear NFκB, which may negatively modulate the transcription of p47^{phox} subunit gene. Since ROS may be one of the major mediators of injury in inflammation, the suppression of p47^{phox} subunit by glucocorticoids may be an important mechanism underlying the anti-inflammatory and tissue protective effect of these drugs. Glucocorticoids have previously been shown to induce IκB and to reduce intranuclear NFκB in certain cell lines, in vitro.[7] Glucocorticoid receptor bound to glucocorticoids also form dimers with NFκB, thus inhibiting NFκB-mediated transcription of proinflammatory factors.[29]

In conclusion, hydrocortisone causes a predictable suppression of p47^{phox} subunit, a key component protein of NADPH oxidase. This may explain the inhibition of ROS generation by hydrocortisone. These events parallel the increase in plasma IL-10 concentrations and the reduction of NFκB by hydrocortisone. The reduction in p47^{phox} subunit would contribute to the inhibitory effect of hydrocortisone (and possibly other glucocorticoids) on NADPH oxidase and ROS generation.

REFERENCES

1. Levine PH, Hardin JC, Scoon KL, et al: Effect of corticosteroids on the production of superoxide and hydrogen peroxide and the appearance of chemiluminescence by phagocytosing polymorphonuclear leukocytes. Inflammation 5:19-27, 1981

2. Niwa Y, Somiya K, Miyachi Y, et al: Luminol-independent chemiluminescence by phagocytes is markedly enhanced by dexamethasone, not by other glucocorticosteroids. Inflammation 11:163-174, 1987

3. Umeki S, Soejima R: Hydrocortisone inhibits the respiratory burst oxidase from human neutrophils in whole-cell and cell-free systems. Biochim Biophys Acta 1052:211-215, 1990

4. Dandona P, Suri M, Hamouda W, et al: Hydrocortisone induced inhibition of reactive oxygen species (ROS) by polymorphonuclear (PMN) leucocytes. Crit Care Med 27: 2442-2444, 1999

5. Dandona P, Thusu K, Hafeez R, et al: Effect of hydrocortisone on oxygen free radical generation by mononuclear cells. Metabolism 47: 788-791, 1998

6. Aljada A, Ghanim H, Assian E, et al: Increased IκB expression and diminished nuclear NFκB in human mononuclear cells following hydrocortisone injection. J Clin Endocrinol Metab 84:3386-3389, 1999

7. Scheinman RI, Cogswell PC, Lofquist AK, et al: Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids. Science 270:283-286, 1995

8. Schulze-Osthoff K, Los M, Baeuerle PA: Redox signalling by transcription factors NF-kappa B and AP-1 in lymphocytes. Biochem Pharmacol 50:735-741, 1995

9. Dandona P, Aljada A, Garg R, et al: Increase in plasma interleukin-10 following hydrocortisone injection. J Clin Endocrinol Metab 84:1141-1144, 1999

10. Moore K W, O'Garra A, de Waal Malefyt R, et al: Interleukin-10. Annu Rev Immunol 11:165-190, 1993

11. Mosmann TR: Properties and functions of interleukin-10. Adv Immunol 56:1-26, 1994

12. Tosi MF, Hamedani A: A rapid, specific assay for superoxide release from phagocytes in small volumes of whole blood. Am J Clin Pathol 97:566-573, 1992

13. Babior BM: The respiratory burst oxidase. Adv Enzymol Relat Areas Mol Biol 65:49-95, 1992

14. Maly FE, Schuerer-Maly CC, Quilliam L, et al: Restitution of superoxide generation in autosomal cytochrome-negative chronic granulomatous disease (A22(0) CGD)-derived B lymphocyte cell lines by transfection with p22phox cDNA. J Exp Med 78):2047-2053, 1993

15. Segal AW, Jones OT, Webster D, et al: Absence of a newly described cytochrome b from neutrophils of patients with chronic granulomatous disease. Lancet 2:446-449, 1978

16. Chanock SJ, Faust R, Barrett D, et al: O_2^- production by B lymphocytes lacking the respiratory burst oxidase subunit p47phox after transfection with an expression vector containing a p47phox cDNA. Proc Natl Acad Sci USA 89:10174-10177, 1992

17. Zhen L, King AA, Xiao Y, et al: Gene targeting of X chromosome-linked chronic granulomatous disease locus in a human myeloid leukemia cell line and rescue by expression of recombinant gp91phox. Proc Natl Acad Sci USA 90:9832-9836, 1993

18. Sekhsaria S, Gallin JI, Linton GF, et al: Peripheral blood pro-

genitors as a target for genetic correction of p47phox-deficient chronic granulomatous disease. Proc Natl Acad Sci USA 90:7446-7450, 1993

19. Park JW, Ma M, Ruedi JM, et al: The cytosolic components of the respiratory burst oxidase exist as a M(r) approximately 240,000 complex that acquires a membrane-binding site during activation of the oxidase in a cell-free system. J Biol Chem 267:17327-17332, 1992

20. Park JW, Benna JE, Scott KE, et al: Isolation of a complex of respiratory burst oxidase components from resting neutrophil cytosol. Biochemistry 33:2907-2911, 1994

21. el Benna J, Ruedi JM, Babior BM: Cytosolic guanine nucleotide-binding protein Rac2 operates in vivo as a component of the neutrophil respiratory burst oxidase. Transfer of Rac2 and the cytosolic oxidase components p47phox and p67phox to the submembranous actin cytoskeleton during oxidase activation. J Biol Chem 269:6729-6734, 1994

22. Heyworth PG, Curnutte JT, Nauseef WM, et al: Neutrophil nicotinamide adenine dinucleotide phosphate oxidase assembly. Translocation of p47-phox and p67-phox requires interaction between p47-phox and cytochrome b558. J Clin Invest 87:352-356, 1991

23. Hancock JT, White JI, Jones OT, et al: The use of diphenylene iodonium and its analogues to investigate the role of the NADPH oxidase in the tumoricidal activity of macrophages in vitro. Free Radic Biol Med 11:25-29, 1991

24. Cachia O, Benna JE, Pedruzzi E, et al: Alpha-tocopherol inhibits the respiratory burst in human monocytes. Attenuation of p47(phox) membrane translocation and phosphorylation. J Biol Chem 273:32801-32805, 1998

25. Smith RM, Curnutte JT: Molecular basis of chronic granulomatous disease. Blood 77:673-686, 1991

26. Howard M, O'Garra A, Ishida H, et al: Biological properties of interleukin 10. J Clin Immunol 12:239-247, 1992

27. Dandona P, Mohanty P, Hamouda W, et al: Effect of dexamethasone on ROS generation by leucocytes: A pharmacodynamic study. Clin Pharm Ther 66:58-65, 1999

28. Thivierge M, Parent JL, Stankova J, et al: Modulation of formyl peptide receptor expression by IL-10 in human monocytes and neutrophils. J Immunol 162:3590-3595, 1999

29. McKay L, Cidlowski J: Molecular control of immune/inflammatory responses: Interactions between nuclear factor-κB and steroid receptor-signaling pathways. Endocr Rev 20:435-459, 1999