Elevated Superoxide Generation in Mononuclear Phagocytes by Treatment With 1α Hydroxyvitamin D₃: Changes in Kinetics and in Oxidase Cytosolic Factor p47¹

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ABSTRACT

The effect of 1α-hydroxyvitamin D₃ (1αOHD₃) treatment on the superoxide production of phagocytic cells in patients undergoing continuous ambulatory peritoneal dialysis (CAPD) was studied. A 3-day treatment of CAPD patients with 3 µg per day of 1αOHD₃ (high-dose treatment) significantly increased NADPH oxidase and killing activities in peritoneal macrophages and peripheral blood monocytes (P<0.001) as compared with low-dose (0.25 µg/day of 1αOHD₃) treatment or nontreatment. The high oxidase activity observed in macrophages and monocytes after the treatment with 1αOHD₃, correlated significantly to the increase in the amount of the cytosolic factor p47 of the NADPH oxidase as detected by western blotting analysis. Superoxide production by the peripheral blood neutrophils of these patients only slightly increased with 1αOHD₃ treatment, and the amount of p47 was not affected by 1αOHD₃ administration. In order to evaluate the significance of the oxidase cytosolic factor in dictating oxidase activity, a reconstitution of NADPH oxidase was conducted by mixing macrophage cytosols and membranes in a cell-free system. The addition of macrophage cytosol from patients on high-dose treatment to macrophage membranes from patients in all of the categories of treatment resulted in significantly higher (P<0.001) superoxide production as opposed to the macrophage cytosol from nontreated patients. These results suggest that 1,25(OH)₂D₃ causes an increase in NADPH oxidase activity in the peritoneal macrophages and monocytes of CAPD patients by inducing synthesis and elevating the amount of the cytosolic factor p47. The increased killing of staphylococci by these cells is probably due to the elevation of superoxide generation, which is the major mechanism for the killing of invasive pathogens.

Key Words: Phagocytes, vitamin D, superoxide production

A n increasing number of patients with end-stage renal failure undergo treatment by continuous ambulatory peritoneal dialysis (CAPD). Despite a marked improvement in CAPD techniques, peritonitis remains a major complication of this method, consequently motivating in-depth research in the field of host defense of the peritoneal cavity (1,2). Mononuclear phagocytes constitute the predominant cell type found in the peritoneal dialysate from noninfected patients undergoing acute or chronic peritoneal dialysis (3,4) and therefore are the first line of cellular defense against microorganism invasion in CAPD patients. These patients usually have a low serum level of the active form of vitamin D (Vit D) because of the failure of renal synthesis and the loss of Vit D metabolites in the peritoneal fluid (5,6). These patients are usually treated with the synthetic analog of 1,25(OH)₂D₃, namely, 1αOHD₃.

The phagocyte respiratory burst, which generates superoxide, is one of the most important mechanisms for killing invasive microbial pathogens. The enzyme responsible for the production of superoxide is an NADPH oxidase. The activated enzyme is a multicomponent electron transport chain, which includes a membrane-bound cytochrome b558 (7–9) containing an NADPH-binding site and both FAD and heme electron transfer moieties. In addition, superoxide-generating activity depends on the presence of three cytosolic factors: 47 kd (p47), 67 kd (p67) and rac 2, a ras-related GTP-binding protein (10–14).

Several studies have reported that peripheral blood monocytes treated with 1,25(OH)₂D₃ for several days demonstrated a significant increase in H₂O₂ secretion relative to control cells (15–18). In our previous in vitro study (19), we demonstrated that 1,25(OH)₂D₃ increases the superoxide generation and killing activity of peritoneal macrophages from normal subjects and CAPD patients. An increase in peritoneal macrophage activity could be important in the prevention and therapy of peritonitis. The aim of this work was to study the effect of 1αOHD₃ treatment on the produc-
tion of superoxide and the killing activity in the peripheral blood neutrophils, peripheral blood monocytes, and peritoneal macrophages of CAPD patients and to determine the mechanism by which this Vit D metabolite affects, in vivo, phagocyte function.

METHODS

Patients

Eight nondiabetic patients undergoing CAPD for 18 months, and peritonitis free for at least 3 months before the study, were investigated. All patients were treated with four peritoneal dialyses exchanges per day (20). The dialysis solution (Dianeal, Travenol, Israel) contained: 132 mEq/L sodium, 3.5 mEq/L calcium, 1.5 mEq/L magnesium, 102 mEq/L chloride, 35 mEq/L lactate, and 1.5 or 4.25% glucose. Three exchanges per day were performed with Dianeal containing a glucose concentration of 1.5%, and one exchange was performed with 4.25% glucose. None of the patients had undergone parathyroidectomy or renal transplant and were treated by a low dose of 0.25 μg of 1αOH,2 per day. 1αOH,2 was discontinued in the research group for 1 wk (nontreatment) and was resumed with a high dose of 3 μg/day for 3 days (high-dose treatment). Plasma calcium concentration was determined daily during high-dose administration and 3 days after treatment was stopped.

Peripheral monocytes and neutrophils from eight control subjects with normal renal function were studied. Superoxide generation and killing were measured in parallel to the CAPD patients. The CAPD patients and the controls were age and sex matched.

Phagocyte Isolation

Peripheral blood neutrophils were separated by Ficoll/Hypaque centrifugation dextran sedimentation and hypotonic lysis of erythrocytes. Peripheral blood monocytes were obtained in >95% purity by Ficoll/Hypaque centrifugation and separation on Percoll gradient as described previously (21). Cell number and viability were determined by trypan blue exclusion. Peritoneal macrophages were separated as previously described (19). The complete effluent from 24 h was centrifuged (150 g, 20 min). Cells were washed twice in RPMI-1640 medium (Bet Haemek, Israel) and resuspended in RPMI medium that contained 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, 10 mg/mL streptomycin, and 12.5 U/mL nystatin (Biological Industries, Bet Haemek, Israel) at 37°C in a humidified atmosphere of 5% CO2 and 12.5 U/mL nystatin (Biological Industries, Bet Haemek, Israel) at 37°C in a humidified incubator (5% CO2 in air). The monocytes were prepared by placing 1 mL (106 cells/mL) onto plastic layers consisted of 95% macrophages as determined by monocyte-specific monoclonal antibodies against mac 1.

Superoxide Anion Measurements

The production of superoxide anion (O2-) by intact cells was measured as the superoxide dismutase-inhibitable reduction of ferricytochrome c (22) by a modification of the microtiter plate technique of Pick and Miltz (23). Cells were suspended (2 x 10⁶ cells/well) in 100 μL of Hanks’ balanced salts solution (HBSS) containing 150 μM ferricytochrome c. Cells were stimulated by the addition of 1 mg/mL OZ or 10⁻⁷ M FMLP, and the reduction of ferricytochrome c was followed by the change of absorbance at 550 nm every 5 min on a Thermomax Microplate Reader (Menlo Park, CA). The maximal rates of superoxide generation were determined and expressed as nanomoles of O2⁻/10⁶ cells per 10 min with the extinction coefficient E550=21 M⁻¹ cm⁻¹.

Bactericidal Activity

The bactericidal activity of phagocytes (macrophages, monocytes, or neutrophils) for Staphylococcus aureus was examined according to a previously described method (19). Briefly, the bacterial suspension (10⁵ microorganisms in 100 μL of broth) was preopsonized with 10% pooled normal serum in HBSS for 60 min at 37°C. The suspensions were centrifuged, and the bacterial pellets were resuspended in 1 mL of HBSS. One hundred microliters of preopsonized bacteria was mixed with an equal volume of phagocytes suspension (1 x 10⁶ cells/mL HBSS) in polypropylene counting tubes (bacteria-to-cell ratio, 10:1). Serial 10-fold dilutions of the mixtures were prepared immediately after the mixing (Time 0) and after incubation, in a shaking bath, for 30, 60, and 90 min at 37°C. Triplicate 20 μL from the appropriate dilutions was plated on nutrient agar, and bacterial colonies were counted after 24 h of incubation at 37°C. Control tests were performed by incubating bacteria without macrophages. The results were expressed as percent killed bacteria (percent decrease in bacterial colonies versus control) after 90 min of incubation (at plateau phase).

Isolation of Membrane and Cytosol Fractions

Membrane and cytosol fractions were prepared as described previously (22). Phagocytes were treated with 5 mM diisopropyl fluorophosphate for 1 h at 18°C, washed, suspended at 10⁶ cells/mL in relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, 1.25 mM EGTA, 1 mM ATP, 10 mM PIPES; pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride and 100 μM leupeptin at 4°C, and sonicated three times for 10 s, resulting in about 95% cell breakage. After centrifugation (5 min; 15,500 g) to remove granules, nuclei, and unbroken cells, the supernatant was centrifuged in a Beckman airfuge (Beckman Instruments, Fullerton, CA) (30 min; 134,000 g) to obtain a cell membrane pellet and a cytosol supernate. Membranes were suspended at 10⁶ cell equivalents/mL in 0.34 M sucrose/half-strength relaxation buffer containing 1 mM dithiothreitol. Solubilized membrane and cytosol were stored at -70°C without loss of activity.

Immunoblot Analysis

The immunoblot detection of cytosolic and membrane NADPH oxidase components was performed as described (22). Samples were solubilized in 2X sample buffer (12% sodium dodecyl sulfate, 8 M urea, 250 mM Tris, 8 mM EDTA, 0.2 mM leupeptin, 2 mM phenylmethylsulfonyl fluoride; pH 6.9). The amount of protein in each sample was quantitated with the Pierce BCA protein assay (Pierce Chemical Co., Rockford, IL) with bovine serum albumin standards. Cytosols (5 x 10⁶ cell equivalents/lane) were analyzed by electrophoresis on 10% polyacrylamide gels. The samples from the different treatments of the same patient were run on the same gel. The resolved proteins were electrophoretically

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transferred to nitrocellulose, which was stained with Fast green to detect protein banding, and then blocked in 5% nonfat dry milk in Tris-buffered saline. Two groups of rabbit sera reactive with either p47 or p67 were raised by immunizing with recombinant p47 or p67 (a gift of T.L. Lobo, NIH, Bethesda, MD). The blots were incubated in Tris-buffered saline-1% gelatin containing goat antiserum to either p47 or p67. Immunoblots were incubated with 1 mg/mL peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulin G (Biomakor, Rehovot, Israel) and developed with 4-chloro-1-naphthol and H2O2.

The relative changes of each oxidase component were quantitated by the use of densitometry in a reflectance mode (Hoefer: Hoefer Scientific Instruments, San Francisco, CA). The lanes were integrated over the width and the thickness of the bands. Cytosol from neutrophil monocytes and macrophages was serially diluted and immunoblotted. The detection of p47 and p67 was linear in the range of 0.25 to 15 mg of cytosol protein for neutrophils, which corresponds to 106 to 1.5 x 105 cell equivalents, and 0.25 to 30 mg of cytosol protein for monocytes and macrophages, which corresponds to 105 to 104 cell equivalents. These measurements are adequate to determine the changes of each individual oxidase component by different treatments but not for comparison between the various components.

Cell-Free Superoxide Generation Assay

This was performed as described (22) in a 96-well flat-bottom microplate. Wells contained solubilized macrophage membranes (5 x 106 cell equivalents) plus macrophage cytosol (106 cell equivalents), in a final volume of 100 l of reaction mixture. The reaction mixture contained: 0.15 mM ferricytochrome c, 4 mM MgCl2, 10 mM flavin adenine dinucleotide, 1 mM EGTA, 200 lM NADPH, and 40 lM arachidonic acid in 75 mM potassium phosphate at pH 7.0. Control wells contained 2.5 lM of superoxide dismutase. The reaction was followed at 22°C for 20 min. Light absorbance at 550 nm was determined every 5 min.

1,25(OH)2D3 Determination

Serum samples obtained from the CAPD patients were submitted to lipid extraction by acetonitrile. The 1,25(OH)2D3 fraction was isolated by a Sep-Pak separation with C18 and silica columns (24). The 1,25(OH)2D3 fraction was submitted to a radioimmunoassay with a sheep antibody against 1,25(OH)2D3 (25). Results are expressed as picograms of 1,25(OH)2D3 per milliliter of serum ± SE.

Analysis of Data

All densitometry observations presented here were confirmed in at least three or four separate experiments performed on different preparations of cells. The differences in means were analyzed by a paired t test. The plots were drawn as least-squares regression lines and tested by analysis of variance.

RESULTS

Table 1 summarizes the serum levels of 1,25(OH)2D3 in CAPD patients during the three phases of the study: (1) low-dose treatment of 1a(OH)D3, (2) nontreatment, and (3) high-dose treatment. Serum levels of 1,25(OH)2D3 in nontreated and low-dose-treated patients were 18.9±2.7 and 19.6±2.1, respectively, and were significantly below the normal values of healthy subjects, 25 to 50 pg/mL. After 3 days of 3 lM/day 1a(OH)D3 therapy, the serum levels of the VD metabolites were normalized (40.3±4.45). No significant changes in serum calcium concentration levels were observed during any of the phases of the study. Serum calcium concentrations before treatment were 9.3, 9.1, and 10.2 mg%, and after 3 days of 3 lM/day 1a(OH)D3 therapy, they were 9.1, 8.8, and 10.7 mg%, respectively.

Figure 1A illustrates the superoxide generation of peritoneal macrophages during the three phases of the experiment. The rates of superoxide production by macrophages obtained from patients receiving low-dose treatment of 1a(OH)D3 were 4.90±0.7 or 4.30±0.4 nmol of O2/106 cells per minute induced by 1 mg/mL OZ or by 10-7 M FMLP, respectively. There was a slight nonsignificant reduction in the rates of superoxide production in the nontreated patients compared with those receiving low-dose treatment with 1a(OH)D3. The correction of serum 1,25(OH)2D3 concentration by high-dose 1a(OH)D3 administration caused a highly significant increase (P<0.001) in the production of superoxide by macrophages (11.2±1.5 and 11.8±1.1 nmol of O2/106 cells per minute, by OZ or by FMLP, respectively). The superoxide production of unstimulated macrophages was not affected by the different treatments with 1a(OH)D3. Figure 1B presents a representative sodium dodecyl sulfate-polyacrylamide gel electrophoresis immunoblot analysis of the cytosolic proteins p47 and p67 of the normal values of healthy subjects, 25 to 50 pg/mL. After 3 days of 3 lM/day 1a(OH)D3 therapy, the serum levels of the VD metabolites were normalized (40.3±4.45). No significant changes in serum calcium concentration levels were observed during any of the phases of the study. Serum calcium concentrations before treatment were 9.3, 9.1, and 10.2 mg%, and after 3 days of 3 lM/day 1a(OH)D3 therapy, they were 9.1, 8.8, and 10.7 mg%, respectively.

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Figure 2 shows the superoxide production and expression of the cytosolic proteins p47 and p67 of the

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**Table 1. 1,25(OH)2D3 levels in the serum of CAPD patients undergoing different doses of 1a(OH)D3 replacement therapy**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1,25(OH)2D3 serum levels (pg/mL)</th>
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<tbody>
<tr>
<td>0.25 µg of 1a(OH)D3/day (N)</td>
<td>19.6 ± 2.1</td>
</tr>
<tr>
<td>Discontinued Vii D Administration (L)</td>
<td>18.9 ± 2.7</td>
</tr>
<tr>
<td>3 µg of 1a(OH)D3/day (H)</td>
<td>40.3 ± 4.45</td>
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*The serum levels of 1,25(OH)2D3 are the mean ± SE of the patients studied. N, 0.25 µg of 1a(OH)D3/day; L, discontinued Vii D administration; H, 3 µg of 1a(OH)D3/day. P<0.001 for the difference between the levels of 1,25(OH)2D3 for patients receiving high-dose treatment and no treatment. P<0.005 for the difference between the levels of 1,25(OH)2D3 for patients receiving high-dose treatment and low-dose treatment. P=0.02 for the difference between the low-dose and nontreated patients. Normal range of 1,25(OH)2D3 serum levels are pg/mL.*
TABLE 2. Relative changes of oxidase components determined by densitometry

<table>
<thead>
<tr>
<th>Oxidase Protein</th>
<th>p47</th>
<th>p67</th>
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<tr>
<td>Macrophages</td>
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<tr>
<td>0.25 μg of 1a(OH)D3/day</td>
<td>696 ± 61</td>
<td>373 ± 31</td>
</tr>
<tr>
<td>Discontinued Vit D Administration</td>
<td>690 ± 45</td>
<td>310 ± 52</td>
</tr>
<tr>
<td>3 μg of 1a(OH)D3/day</td>
<td>1,271 ± 76*</td>
<td>290 ± 38</td>
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<tr>
<td>Monocytes</td>
<td></td>
<td></td>
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<tr>
<td>0.25 μg of 1a(OH)D3/day</td>
<td>580 ± 69</td>
<td>301 ± 25</td>
</tr>
<tr>
<td>Discontinued Vit D Administration</td>
<td>635 ± 21</td>
<td>397 ± 31</td>
</tr>
<tr>
<td>3 μg of 1a(OH)D3/day</td>
<td>989 ± 45*</td>
<td>367 ± 48</td>
</tr>
<tr>
<td>Neutrophils</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25 μg of 1a(OH)D3/day</td>
<td>799 ± 37</td>
<td>352 ± 51</td>
</tr>
<tr>
<td>Discontinued Vit D Administration</td>
<td>781 ± 79</td>
<td>297 ± 30</td>
</tr>
<tr>
<td>3 μg of 1a(OH)D3/day</td>
<td>825 ± 73</td>
<td>335 ± 41</td>
</tr>
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</table>

* The determinations by densitometry of the relative amounts of the cytosolic factors are an average of the patients studied. Units are arbitrary units of density with higher numbers representing a darker band on the immunoblot. The quantitative measurements are adequate to determine the relative changes of each individual oxidase component by the different conditions but not for comparison between the various components, because of the different characteristics of the antibodies used.

** Significant differences between the expression of p47 in high-dose treatment and discontinued or low-dose treatment with 1α(OH)D3, P < 0.001.

*** Significant differences between the expression of p47 in high-dose treatment and discontinued or low-dose treatment with 1α(OH)D3, P < 0.005.

The rate of superoxide production by monocytes of patients on low-dose treatment (6.83 ± 0.7 or 5.35 ± 0.5 nmol of O2/10⁶ cells per minute, stimulated by 1 mg/mL OZ or by 10⁻⁷ M FMLP, respectively) was significantly lower (P<0.005) than that in healthy controls (9.22±0.4 or 7.50±0.7 nmol of O2/10⁶ cells per minute, stimulated by 1 mg/mL OZ or by 10⁻⁷ M FMLP, respectively). In correlation with the increase in the activity, there was an increase in p47 expression in the monocytes of the patients receiving high-dose treatment (Figure 2B). The increases of both activity and p47 expression are lower than those seen in peritoneal macrophages. The levels of the NADPH-oxidase cytosolic factor p67 remained constant during all three phases of the study. Superoxide generation by unstimulated monocytes was similar in the monocytes of the patients undergoing the different treatments and of the controls.

In contrast to the peritoneal macrophages and peripheral blood monocytes, the peripheral blood neutrophils in patients were not significantly affected by 1αOHD₃ administration (Figure 3A). The amount of oxidase cytosolic factors was not affected by the different 1αOHD₃ treatments as detected by immunoblot analysis (Figure 3B).

Figure 4 describes the reconstitution of NADPH oxidase by mixing macrophage cytosols and membranes, in the presence of arachidonate in a cell-free assay, in order to evaluate the significance of the oxidase cytosolic factors in dictating the rate of NADPH oxidase activity. The maximal rates of superoxide production by mixing cytosols and membranes from nontreated patients (2.1±0.5 nmol/10⁶ cells per minute) was significantly lower (P<0.001) than that produced by mixing macrophage cytosols and membranes from patients undergoing high-dose treatment.
Figure 2. (A) The effect of 1αOHD₃ treatment on superoxide production by peripheral blood monocytes of CAPD patients. The results are expressed as percentage of control: the rate of superoxide production in monocytes of patients receiving low-dose treatment. The mean±SE of superoxide production by low dose-treated patients' monocytes is: 6.83±0.7 or 5.35±0.5 nmol of O₂/10⁶ cells per minute, stimulated by 1 mg/ml OZ or by 10⁻⁷ M FMLP, respectively. *Significant differences between the activity in high-dose treatment and discontinued Vit D metabolite treatment, P<0.005. (B) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis immunoblot analysis of the cytosolic proteins p47 and p67 of the NADPH oxidase in peripheral blood monocytes of a representative CAPD patient. N, 0.25 µg of 1α(OH)D₃/day; L, discontinued Vit D administration; H, 3 µg of 1α(OH)D₃/day. Other patients showed similar results.

Figure 3. (A) The effect of 1αOHD₃ treatment on superoxide production by peripheral blood neutrophils of CAPD patients. The results are expressed as percentage of control: the rate of superoxide production in monocytes of patients receiving low-dose treatment. The mean±SE of superoxide production by low dose-treated patients' neutrophils is: 12.45±1.7 or 10.30±0.9 nmol of O₂/10⁶ cells per minute, stimulated by 1 mg/ml OZ or by 10⁻⁷ M FMLP, respectively. **Differences between the activity in high-dose treatment and discontinued Vit D metabolite treatment, P=0.02. (B) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis immunoblot analysis of the cytosolic proteins p47 and p67 of the NADPH oxidase in peripheral blood neutrophils of a representative CAPD patient. N, 0.25 µg of 1α(OH)D₃/day; L, discontinued Vit D administration; H, 3 µg of 1α(OH)D₃/day. Other patients showed similar results.

(3.4±0.6 nmol/10⁶ cell equivalents per minute). When macrophage cytosols from nontreated patients were added to macrophage membranes from patients on high-dose treatment, a low activity was obtained (2.35±0.4 nmol/10⁶ cell equivalents per minute). These latter values were similar to those produced by the addition of macrophage membranes to cytosols from nontreated patients. By contrast, when macrophage cytosols of patients undergoing high-dose treatment with 1αOHD₃ were mixed with macrophage membranes from nontreated patients, a high activity of 4.0±0.4 nmol/10⁶ cell equivalents per minute was detected. These results were similar to those obtained by the addition of membranes and cytosol from pa-
The results are the mean±SE from three experiments. There is a significant difference (P<0.001) in the activities obtained by the addition of Dcyt+Dmem or Dcyt+Cmem from that of Ccyt+Cmem or Ccyt+Dmem.

Patients undergoing high-dose treatment with 1αOHD₃ (3.4±0.6 nmol/10⁹ cell equivalents per minute). Similar results were obtained when the experiments were performed with macrophages from low dose–treated controls instead of macrophages of nontreated patients.

Staphylococcal killing by phagocytic cells was studied in the CAPD patients receiving the different treatments with 1αOHD₃. As shown in Figure 5, the high-dose treatment with 1αOHD₃ caused a significant increase (P<0.001) in staphylococcal killing by peritoneal macrophages (from 43±5 to 68±4%) or peripheral monocytes (from 53±7 to 78±7%). The killing by peripheral monocytes from nontreated CAPD patients or from low-dose 1αOHD₃ treatment was compared with that of healthy controls (70±8%) and was found to be significantly reduced (P<0.005). Staphylococcal killing by peripheral neutrophils from CAPD patients receiving the different 1αOHD₃ treatments was similar to that of peripheral neutrophils from healthy controls (83±9%) and was not significantly affected by the high-dose treatment.

DISCUSSION

This study demonstrates that the correction of serum levels of 1,25(OH)²D₃ of CAPD patients causes a significant increase in superoxide production and the killing capacity of peritoneal macrophages and peripheral blood monocytes. This treatment with 1αOHD₃ did not cause any priming of the cells, avoiding spontaneous release of superoxide, which could cause damage of the peritoneum. Because we have previously demonstrated that 1,25(OH)₂D₃ accumulates in the dialysate of CAPD patients (26), these data may well have important clinical implications. There is increasing evidence showing that at local sites of inflammation the hormonal form of Vit D₃, 1,25(OH)₂D₃, is important to the up-regulation of the immune response. Consequently, in the peritoneal microenvironment of CAPD patients, the immunoregulatory role of this hormone might be exhibited in the macrophages, strengthening the host defense mechanism against the invasion of microorganisms. The sources of 1,25(OH)₂D₃ in the peritoneal cavity stem from the losses of this metabolite from the circulation together with the Vit D–binding protein or, alternatively, by its intraperitoneal synthesis by the macrophages. We (27) and others (28) have recently demonstrated that the synthesis of 1,25(OH)₂D₃ by the peritoneal macrophages depends on the presence of 25–α hydroxylase and that losses of 25(OH)D₃ into the dialysate is a steady source of substrate to this conversion. This endogenous synthesis of 1,25(OH)₂D₃ was found to be enhanced during peritonitis.

The increase in oxidase activity in peripheral monocytes and peritoneal macrophages correlates with the augmented expression of p47 as detected by immunoblot analysis. However, p67 oxidase cytosolic factor was not affected by treatment with 1αOHD₃. The enhanced oxidase activity of the macrophages and monocytes observed in patients treated with high doses of 1αOHD₃ appears to be due to the raised amount of the p47 cytosolic factor, as indicated by the maximal rates of superoxide production in a cell-free system (Figure 4) and by the immunoblot analysis. This conclusion is supported by our previous study (22,29) and by others (30), which showed a correlation between the amount of p47 and the levels of superoxi-
Elevated Superoxide Generation

We have previously shown (29) that the loss of the ability of cultured monocytes to produce superoxide correlated with the loss of p47 expression in immunoblot analysis. The loss of both superoxide production and immunologically detectable p47 in cultured monocytes was prevented by the addition of 1,25(OH)2D3. Moreover, monocytes cultured for 4 days in the presence of 1,25(OH)2D3 showed higher activity and an increase in detectable p47 compared with fresh monocytes. The increased killing capacity of peritoneal macrophages and peripheral blood monocytes of CAPD patients after high-dose treatment of 1αOHD3 is probably due to the increase in NADPH oxidase activity, which is the major mechanism for the killing of invasive pathogens.

The normalization of serum levels of 1,25(OH)2D3 by high-dose treatment of 1αOHD3 significantly increased oxidase activity and the expression of p47 in macrophages and monocytes but only slightly affected oxidase activity and did not affect p47 expression in neutrophils. This difference in p47 expression between neutrophils and monocytes/macrophages may be related to the fact that protein synthesis is needed and it may be present in monocytes/macrophages that have a longer lifespan. Neutrophils have a very limited capacity for de novo protein biosynthesis and are short-lived end-stage cells, and the bulk of neutrophil-derived proteins are believed to be synthesized and packaged during myeloid development. In accordance with our results, it was recently reported (31) that 4-wk replacement therapy with 0.5 μg/day of 1αOHD3 in chronic hemodialysis patients increased superoxide generation in monocytes only and did not affect polymorphonuclear cells. The elevation in p47 expression is not the sole mechanism responsible for the increase in NADPH oxidase activity. 1,25(OH)2D3 was shown to modulate protein kinase C (PKC) in several cell types (32,33). PKC activity is known to be involved in the activation of the NADPH oxidase (34). An impaired killing activity was demonstrated (35) in the phagocytes of patients with end-organ resistance to 1,25(OH)2D3. The authors suggested that 1,25(OH)2D3 affects killing through its effect on the synthesis of proteins that regulate intracellular calcium metabolism. Such effect of 1,25(OH)2D3 in elevating intracellular calcium concentration was shown by us in HL-60 cells (36). This hypothesis may also be relevant to the effect of 1,25(OH)2D3 on oxidase activity as shown in our study, because oxidase activity induced by OZ or FMLP is a Ca2+-dependent process (37). The slight improvement capacity of neutrophils to generate superoxide shown in our study may be a result of the effect of 1,25(OH)2D3 on PKC or on intracellular Ca2+ concentration.

In conclusion, the high correlation between the rates of superoxide production and the levels of p47 suggests that p47 determines the activity of the NADPH oxidase. The 1αOHD3 therapy normalizing the serum level of 1,25(OH)2D3 causes a significant increase in the levels of the oxidase cytosolic factor p47 in mononuclear phagocytes. This study suggests that this induction of p47 is responsible for the increase in NADPH-oxidase activity in peritoneal macrophages and peripheral blood monocytes of CAPD patients treated with 1αOHD3. The increase of superoxide production and killing capacity in this type of cells may have a clinical relevance to the prevention and treatment of peritonitis. Moreover, because peritoneal macrophages are able to synthesize 1,25(OH)2D3, these results suggest an autocrine role for 1,25(OH)2D3 in the peritoneal cavity of CAPD patients. The mechanism by which 1,25(OH)2D3 regulates the expression of p47 merits further study.

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REFERENCES


