Inhibition of Neutrophil Superoxide Production by Uremic Concentrations of Guanidino Compounds

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Abstract. In uremia, diminished reactive oxygen intermediate production is an important consequence of impaired neutrophil function. The effects of guanidino compounds, which are known uremic toxins, on neutrophil reactive oxygen intermediate production in vitro were studied. Neutrophils from healthy volunteers were exposed for 3 h to individual guanidino compounds or mixed guanidino compounds (GCmix), at concentrations observed in uremic plasma. After removal of the guanidino compounds, the neutrophils were activated by adhesion, N-formylmethionylleucylphenylalanine, phorbol myristate acetate, or opsonized zymosan, and superoxide production was measured by monitoring lucigenin chemiluminescence. The direct effects of guanidino compounds on superoxide production in activated neutrophils were also measured. The energy status (ATP and creatine phosphate), antioxidant status (total glutathione), and glycolytic flux (lactate production) were measured. GCmix pretreatment decreased superoxide production in activated neutrophils (activated by N-formylmethionylleucylphenylalanine or zymosan) by 50% (P < 0.01), decreased ATP concentrations by 60% (P < 0.05), and inhibited glycolytic flux (lactate production) by 45% (P < 0.01) but did not alter glutathione concentrations. Simultaneous GCmix exposure and activation did not inhibit NADPH oxidase activity in cell lysates but inhibited superoxide formation in zymosan-activated intact neutrophils; this inhibition was reversed after removal of the guanidino compounds. Guanidosuccinic acid, guanidinopropionic acid, and guanidinobutyric acid, when tested individually, were each as potent as GCmix. The inhibition of neutrophil superoxide generation by guanidino compounds results from decreased energy status. Micromolar concentrations of guanidino compounds significantly inhibit neutrophil metabolism, with serious implications for the functions of neutrophils in host defenses.

Infection remains a major cause of death for patients with uremia (1–3), and the mortality rate attributable to infection for dialysis patients is 5 to 35% (2). The biologic features of neutrophils have been widely studied in uremia, and defects of phagocytosis, generation of reactive oxygen intermediates, chemotaxis, ATP generation, hexose monophosphate shunt activity, and antioxidant enzyme activity (4–6), as well as accelerated apoptosis (7), have all been described. Studies of leukocyte microbicidal activity in uremic patients have yielded conflicting results, because of technical and patient population variations and because the identity and mode of action of the uremic compounds responsible for leukocyte dysfunction are still unclear (reviewed in reference 3). Vanholder et al. (8) tested many uremic compounds and concluded that p-cresol was responsible for decreasing pentose phosphate pathway activity and reactive oxygen intermediate production in neutrophils, although those authors did not directly examine superoxide production.

Uremic metabolites containing a guanidino group are of major interest because of their ability to interact with various reactive species and act as metabolic toxins (9). Although guanidino compounds are removed during dialysis, concentrations remain significantly higher in patients undergoing hemodialysis or peritoneal dialysis, compared with healthy subjects (9). These compounds are cytotoxic and can inhibit natural killer cell activity (10). Guanidinopropionic acid (GPA) decreases creatine kinase activity in cardiac muscle, and a role for this compound in uremic cardiomyopathy was recently suggested (11,12).

In view of the renewed interest in guanidino compounds, we investigated neutrophil free radical production, antioxidant status, and energy status after exposure to a variety of these compounds, at concentrations that are pathophysiologically relevant in uremia. We demonstrated that the energy status of uremic leukocytes is compromised by these compounds, with subsequent impairment of their ability to generate superoxide and peroxynitrite.

Materials and Methods

Reagents
ATP luminescence kits were purchased from Bio Orbit (Turku, Finland), and NADPH was obtained from Boehringer Mannheim (Lewes, United Kingdom). Lymphoprep cell isolation medium was obtained from Nycomed Ltd. (Birmingham, United Kingdom).
Hanks’ balanced salt solution (HBSS) and Hepes were purchased from Life Technologies (Paisley, United Kingdom). Histopaque, lucigenin, all guanidino compounds, 5,5-dithio-bis(2-nitrobenzoate) (DTNB), N-formylmethionylleucylphenylalanine (fMLP), phorbol myristate acetate (PMA), zymosan, creatine phosphokinase, glutathione reductase, and all other substrates were obtained from Sigma Chemical Co. (Poole, United Kingdom). All other chemicals were of analytical grade.

**Neutrophil Preparation**

Blood samples were withdrawn from the antecubital veins of healthy volunteers, into syringes containing 100 U/ml heparin, and were placed in sterile tubes. Neutrophils were prepared by the Histopaque-Lymphoprep double-layer method. After the removal of platelet-rich plasma by centrifugation at 220 × g for 20 min, the blood was made up to the original volume with Ca²⁺/Mg²⁺-free HBSS, carefully placed on a Histopaque (density, 1.119 g/ml)/Lymphoprep (density, 1.077 g/ml) double gradient, and then centrifuged at 450 × g for 16 min. The neutrophil layer was aspirated and washed twice with Ca²⁺/Mg²⁺-free HBSS. This method avoids hypotonic erythrocyte lysis, thus preventing artifactual activation of neutrophils and expression of adhesion molecules (13).

**Chemiluminescence Measurements**

Chemiluminescence was monitored using a luminometer incorporating a gallium arsenide photomultiplier tube (cooled to −20°C). The neutrophil samples (2 ml in 35-mm sealed dishes) were maintained in a light-tight chamber at 37°C.

Light emission was characterized using selective enhancers of chemiluminescence (20 μM lucigenin for superoxide and 20 μM luminol for hydrogen peroxide and peroxynitrite). Luminol chemiluminescence resulting from hypochlorous acid was 95% quenched by the use of 5% fetal calf serum throughout.

**ATP and Creatine Phosphate Measurements**

The ATP content of neutrophils was measured using the luciferin-luciferase firefly luminescence method (14). A perchloric acid extract (100 μl) was added to a cuvette containing 40 μl of ATP-monitoring reagent and 200 μl of 0.1 M Tris-acetate, pH 7.8.

Increases in baseline chemiluminescence were measured with an LKB Wallac 1250 luminometer, using the addition of 5 μl of a 5 μM ATP solution for internal standardization. The ATP content of each sample was calculated on the basis of the chemiluminescence of the corresponding internal standard.

After ATP measurements, 5 μl of a 5 U/ml creatine phosphokinase solution was added, and the increases in chemiluminescence (ATP formation) during a 15-min period were measured. The concentrations of creatine phosphate were calculated on the basis of the chemiluminescence of creatine phosphate internal standards.

**Total Glutathione and Lactate Measurements**

Neutrophil glutathione concentrations were measured as total intracellular glutathione (reduced glutathione plus oxidized glutathione) levels, using a method based on the cyclic reduction of oxidized glutathione by NADPH and glutathione reductase and oxidation by DTNB (15). The glutathione levels were determined on the basis of the rates of reduction of DTNB to 5-thio-2-nitrobenzoate in the presence of NADPH. A 100-μl sample was added to 900 μl of 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM ethylenediaminetetraacetate, 30 μM DTNB, 200 μM NADPH, and 2.5 U/ml glutathione reductase. The rates of DTNB reduction were monitored with a LKB Ultraspec spectrophotometer at 412 nm. Total glutathione concentrations were calculated using a 0.1 to 2 μM standard calibration curve and were expressed as oxidized glutathione equivalents. Lactate production during a 3-h period (glycolytic flux) was measured spectrophotometrically in perchloric acid cell plus medium extracts, by monitoring (at 340 nm) NAD⁺ reduction with lactate dehydrogenase in a glycine-hydrazine buffer, pH 9.5.

**NADPH Oxidase Assays**

The direct effects of guanidino compounds on NADPH oxidase activity were examined. Resting or fMLP-activated neutrophils, in HBSS with 10 mM Hepes, pH 7.2, were lysed by sonication and freeze-thawing. Extracts were used immediately for measurements of lucigenin chemiluminescence in the presence of 100 μM NADPH; in parallel experiments, the disappearance of NADPH was measured at 340 nm in a Cary 1E spectrophotometer. Assays were performed in the presence of guanidino compounds at concentrations 1 and 3 times those observed for uremic patients.

**Experimental Protocols**

**Pretreatment of Neutrophils with Guanidino Compounds.** Neutrophil suspensions (1 × 10⁶/ml) were incubated in Ca²⁺/Mg²⁺-free HBSS containing 5% fetal calf serum, in the presence or absence of guanidino compounds, for 3 h at 37°C. This time was chosen on the basis of preliminary time course experiments in which a 3-h period yielded the most consistent effects, without alteration of ATP and superoxide levels, in control cells. Concentrations of guanidino compounds were adjusted to those reported for the plasma of uremic patients (9) and were as follows: guanidinosuccinic acid (GSA), 20 μM; methylguanidine (MG), 10 μM; GPA, 5 μM; guanidinobutyric acid (GBA), 5 μM; guanidinoacetic acid (GAA), 5 μM. A mixture of these compounds (GC mix) at these concentrations was also studied.

After incubation, neutrophils were centrifuged at 220 × g for 10 min and then resuspended in HBSS containing 1 mM Hepes, 4 mM bicarbonate, and 5% fetal calf serum, pH 7.2. The cell density for each treatment group was confirmed, after incubation, by measuring neutrophil DNA contents using the fluorometric method of Downs and Wilfinger (16).

Neutrophil suspensions were immediately used for chemiluminescence measurements. Cells were transferred into 35-mm culture-grade dishes with lucigenin or luminol (20 μM). The chemiluminescence resulting from adherence to the dish was measured for 30 min, by which time the chemiluminescence had returned to baseline levels. Neutrophil activation was then induced using opsonized zymosan A (40 μg/ml), PMA (20 nM), or fMLP (20 ng/ml). Measurements were obtained at time 0 (the time of either the addition of neutrophils to the dish or the addition of agonists) and at 5-min intervals thereafter for up to 60 min, by which time the chemiluminescence had decreased to baseline levels. A representative time course is presented in Figure 1; in the other figures, results are expressed as the sum of all readings obtained during a 1-h period.

**Direct Effects of Guanidino Compounds on Neutrophils.** Untreated neutrophils were transferred to dishes containing guanidino compounds at the concentrations described, and lucigenin chemiluminescence was monitored during adherence and then zymosan activation. In a separate experiment, the medium was removed 30 min after zymosan activation, the cells were washed once with warmed medium, and then the medium was replaced with fresh, guanidino compound-free medium, with lucigenin and zymosan, while a temperature of 37°C was maintained. The production of chemiluminescence was then monitored again.
Effects of Guanidino Compounds on Metabolic Intermediates.

ATP, creatine phosphate, lactate, and glutathione levels were all measured as indices of the energy status and the antioxidant status of the cells. Neutrophils (10^6 cells) in HBSS were allowed to adhere to 9-mm sulfonated glass coverslips, which were then incubated, with or without guanidino compounds, in 24-well tissue culture plates for 3 h. Each coverslip was then removed, lightly rinsed with HBSS, and immediately immersed in 100 \( \mu l \) of 1 M ice-cold perchloric acid. After being shaken for 1 min (for extraction of cellular metabolites), samples were adjusted to pH 6 to 7.4 with potassium hydroxide and triethanolamine. These extracts were centrifuged at 4000 \( \times \) g for 1 min and then used immediately for ATP and creatine phosphate measurements. Glutathione levels were measured in aliquots of the same extracts, which had been immediately frozen and were thawed just before use. Lactate levels were measured in extracts of cells plus medium obtained in parallel experiments.

Results

Chemiluminescence Measurements of Superoxide and Peroxynitrite

A time course of lucigenin and luminol chemiluminescence in fMLP-activated neutrophils is presented in Figure 1. After 60 min, the chemiluminescence returned to baseline levels. Lucigenin chemiluminescence was abrogated by superoxide dismutase, and luminol chemiluminescence was 30% inhibited by 200 \( \mu M \) \( L - (1\text{-iminoethyl})\text{ornithine} \), a nitric oxide (NO) synthase inhibitor.

Effects of Guanidino Compounds on Superoxide Generation

Adherent Neutrophils. The production of superoxide (lucigenin chemiluminescence) after neutrophil adhesion was inhibited by preincubation of the cells for 3 h with GSA, GPA, or GCmix but was unaffected by preincubation with GBA, MG, or GAA (Figure 2). The numbers of cells adhering to the dishes, as assessed by DNA measurements, were unchanged with guanidino compound treatment.

Activated Neutrophils. Superoxide generation in neutrophils activated with zymosan or fMLP and treated with GCmix was significantly inhibited, compared with controls. GSA, GPA, and GBA alone each produced inhibition that matched that produced by GCmix, whereas MG and GAA were without effect (Figure 2). Similar results were obtained using cells activated with latex beads (data not shown). However, when cells were activated with PMA, no inhibition with GCmix was noted. Parallel inhibition of chemiluminescence was observed when luminol was used instead of lucigenin (Figure 1), indicating that the generation of peroxynitrite from the reaction of NO with \( O_2^- \) was not altered by the presence of guanidino compounds. The addition of guanidino compounds at concen-
trations observed in normal plasma (20% of uremic values) produced no effect on superoxide production, in either adherent or activated neutrophils (data not shown).

**Direct Effects of Guanidino Compounds on Neutrophil Superoxide Production**

The addition of GC mix at the time of neutrophil adhesion produced no significant effect on lucigenin chemiluminescence (83 ± 15% of control values). However, the guanidino compounds exhibited marked effects on neutrophil lucigenin chemiluminescence after zymosan activation (Figure 3, inset). GC mix produced 40% inhibition of superoxide production and a 30-min delay in the start of chemiluminescence emission. When GC mix was quickly rinsed from the cells and replaced with fresh medium (Figure 3, arrow), there was an initial decrease caused by temperature fluctuations during the manipulation; subsequent neutrophil superoxide production exceeded that of untreated neutrophils. However, if neutrophils were reexposed to GC mix after rinsing, superoxide production remained inhibited. NADPH oxidase activities measured in resting and PMA-activated neutrophil lysates exhibited no changes after the addition of guanidino compounds, even when the concentrations of the compounds were increased threefold greater than the standard GC mix (data not shown).

**Neutrophil Metabolic Intermediates**

**Energy Status (ATP, Creatine Phosphate, and Lactate).** The steady-state (3-h) intracellular ATP concentration in resting neutrophils was 2.10 ± 0.70 nmol/10⁶ cells. This concentration was significantly decreased by GSA, GPA, GBA, and GC mix (P < 0.01) (Figure 4A). The other guanidino compounds tested did not alter the neutrophil ATP concentrations. The changes produced by GC mix, GSA, and GPA persisted for up to 6 h. Creatine phosphate concentrations were below the level of detection (<0.1 nmol/10⁶ cells) in both control and treated neutrophils. The lactate production in 3 h (in the cells and medium), which is a measure of glycolytic flux, was decreased by 40 to 50% by GSA, GPA, GBA, and GC mix (P < 0.01) (Figure 4C).

**Antioxidant Status.** The intracellular glutathione concentration in resting neutrophils was 1.14 ± 0.42 nmol/10⁶ cells, and this was not significantly changed by treatment with individual guanidino compounds or GC mix (Figure 4B).

**Discussion**

The formation of reactive oxygen intermediates by neutrophils is needed to maintain defenses against bacterial infection. This process involves the initial formation of superoxide by neutrophil NADPH oxidase and the subsequent generation of the bactericidal agents hypochlorous acid and peroxynitrite. Our data demonstrated that pretreatment of neutrophils with guanidino compounds inhibited the production of superoxide through metabolic effects (decreases in ATP levels and glycolytic flux) and that the presence of guanidino compounds during neutrophil activation decreased superoxide production during phagocytosis. These direct effects were not attributable to decreases in the numbers of adherent cells, inhibition of NADPH oxidase activity, or quenching of lucigenin chemiluminescence.

Lucigenin and luminol chemiluminescence measurements

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**Figure 3.** Direct effects of guanidino compounds on superoxide production by zymosan-activated neutrophils. Neutrophils without (◇) and with (■, ▲) GC mix were activated with zymosan. After 30 min of activation (arrow), the medium was replaced with HBSS (◇, ■) or HBSS containing GC mix (▲). (Inset) Uninterrupted profile of chemiluminescence (CL) in neutrophils activated with zymosan in the presence or absence of GC mix. Each value is the average of 100 readings obtained at that time point, and results are representative of four separate experiments. In all cases, the SD was <2%.
were used in combination, to evaluate the relative contributions of superoxide and NO during neutrophil activation. Peroxynitrite, the product of NO and superoxide, produces luminol chemiluminescence with a high quantum yield, whereas lucigenin produces light only from superoxide. Differential changes in these two chemiluminescence signals indicate peroxynitrite formation, because light from lucigenin decreases during the rapid consumption of superoxide by NO, whereas that of luminol increases. It has been suggested that some guanidino compounds can affect NO synthase activity (17,18); however, the pronounced inhibition of both lucigenin and luminol signals indicated that no changes in NO production occurred during the treatment of neutrophils with guanidino compounds. Therefore, either intracellular arginine concentrations were saturating for NO synthase or these guanidino compounds do not act as substrates for this enzyme, because the compounds tested here were without effect on NO synthase activity.

ATP in neutrophils is generated by glycolysis, in which lactate is formed from glucose; during neutrophil activation or phagocytosis, the ATP concentrations decrease rapidly (19). We observed that guanidino compounds had significant negative effects on glycolytic flux, producing inhibition of >40%. This compromises the maintenance of the energy status, especially during cellular activation. There was a strong correlation between decreased ATP levels and superoxide production in neutrophils \( r = 0.906, P < 0.005 \), and guanidino compounds that had no effect on ATP concentrations also had no effect on neutrophil superoxide production. The relationship between energy status and superoxide production has been described (19), and we propose that this phenomenon explains the abnormal changes we observed.

When ATP consumption is high, creatine kinase can transfer the high-energy phosphate of creatine phosphate to ADP, to maintain ATP concentrations. Inhibition of creatine kinase thus prevents the mobilization of high-energy phosphate stores. Inhibition of creatine kinase activity by GPA has been observed in cardiac muscle (11,12) and brain (20), and the possibility that guanidino compounds could competitively inhibit the creatine kinase reaction cannot be discounted. The creatine kinase reaction is rate-limited, in the direction of ATP synthesis, by the size of the guanidino compound pool. However, as we have confirmed in our study, creatine phosphate pools are very small in neutrophils (19), reflecting the absence of mitochondrial-derived ATP. It seems, therefore, that disruption of creatine phosphate metabolism is not responsible for the depletion of energy stores in neutrophils exposed to guanidino compounds.

The addition of the guanidino compounds tested did not seem to impose any oxidative stress on neutrophils, because they were able to maintain normal thiol status. Glutathione concentrations were maintained despite the decreases in the levels of ATP, which is an essential cofactor for glutathione biosynthesis. In contrast, previous studies showed an association between the biosynthesis of guanidino compounds and oxidative stress, as indicated by the generation of free radicals (21).

Guanidino compounds affected both adherence and phagocytic activity (zymosan or latex beads) and G protein-linked receptor-mediated events (fMLP activation). However, the effectiveness of PMA, which acts via a cascade involving protein kinase C and results in assembly of the multicomponent enzyme NADPH oxidase, was unchanged by guanidino compounds. This finding indicates that the site of guanidino compound action is upstream of protein kinase C activation.

Adenosine inhibition of neutrophil superoxide similarly exhibits differences with PMA and fMLP activation (22), and it is possible that the accumulation of adenosine (a product

Figure 4. Effects of guanidino compounds on resting neutrophil ATP (A), glutathione (B), and lactate (C) levels. Neutrophils adherent to 9-mm coverslips were treated with guanidino compounds for 3 h. Neutralized perchloric acid extracts of cells (or cells plus medium for lactate measurements) were analyzed. Each value is the mean ± SEM of six separate experiments. *\( P < 0.05 \), **\( P < 0.01 \), versus control.
of ATP breakdown) during the inhibition of glycolysis by guanidino compounds is responsible for the effects reported. In addition, G protein coupling to fMLP receptor activation is strongly dependent on high-energy phosphate transfer from ATP to GDP, which in guanidino compound-treated cells is impaired by the lowered energy status. In contrast, the enhanced neutrophil oxidative bursts with fMLP stimulation reported in cases of chronic renal insufficiency (23) are probably related to neutrophil priming factors present during chronic renal insufficiency and are not associated with alterations in the energy status.

In summary, GSA, GPA, and GBA, at micromolar concentrations typical of uremic plasma, have profound effects on neutrophil energy metabolism. We think this explains their inhibitory effects on phagocytic and fMLP-induced superoxide production by neutrophils and may thus account for the impaired defenses against bacterial infection in cases of uremia.

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