Monochloramine Inhibits Phorbal Ester-inducible Neutrophil Respiratory Burst Activation and T Cell Interleukin-2 Receptor Expression by Inhibiting Inducible Protein Kinase C Activity*

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Monochloramine derivatives are long lived physiological oxidants produced by neutrophils during the respiratory burst. The effects of chemically prepared monochloramine (NH₂Cl) on protein kinase C (PKC) and PKC-mediated cellular responses were studied in elicited rat peritoneal neutrophils and human Jurkat T cells. Neutrophils pretreated with NH₂Cl (30–50 μM) showed a marked decrease in the respiratory burst activity induced by phorbol 12-myristate 13-acetate (PMA), which is a potent PKC activator. These cells, however, were viable and showed a complete respiratory burst upon arachidonic acid stimulation, which induces the respiratory burst by a PKC-independent mechanism. The NH₂Cl-treated neutrophils showed a decrease in both PKC activity and PMA-induced phosphorylation of a 47-kDa protein, which corresponds to the cytosolic factor of NADPH oxidase, p47phox. Jurkat T cells pretreated with NH₂Cl (20–70 μM) showed a decrease in the expression of the interleukin-2 receptor α chain following PMA stimulation. This was also accompanied by a decrease in both PKC activity and nuclear transcription factor-κB activation, also without loss of cell viability. These results show that NH₂Cl inhibits PKC-mediated cellular responses through inhibition of the inducible PKC activity.

Neutrophils play an important role in the defense against bacterial infections as well as other inflammatory responses. Many different stimuli activate neutrophils, which show a series of responses such as the respiratory burst, cell shape change, aggregation, degranulation, and phospholipid turnover (1). The respiratory burst is a rapid increase in non-mitochondrial oxygen consumption, in which large amounts of superoxide anion (O₂⁻) are produced by the electron transport complex of the respiratory chain (2, 3). Hydrogen peroxide is formed by the dismutation of O₂⁻, and HOCl is formed from H₂O₂ and Cl⁻ by myeloperoxi-dase (4, 5). Chloramines are another type of oxidant produced in significant quantities by activated neutrophils (6). They are formed by the non-enzymatic reaction (I) of HOCl with many endogenous amines such as taurine, ammonia, lysine, and the amino termini of polypeptides (6, 7). Taurine is present in neutrophils at 10–20 mM (8).

R-NH₂ + HOCl → R-NHCl + H₂O

(Reaction I)

Chloramines are less reactive than HOCl, and membrane-impermeable chloramines are relatively long lived oxidants (9). Chloramines react preferentially with sulphydryls and thioethers (10), and some chloramines such as monochloramine (NH₂Cl) are membrane-permeable (11). Neutrophils in concentrations found in blood (2–3 × 10⁶/ml) may easily produce 100 μM chloramine in a short term culture (12). Moreover, chloramines show distinct biological effects such as inhibition of DNA repair (12), inhibition of the generation of macrophage inflammatory mediators (13), and detachment of cultured myocytes (14). Recently we showed that membrane-permeable chloramines derived from the respiratory burst are the primary cause of accelerated turnover of glutathione in activated neutrophils (15). Considering these characteristics, chloramines may have signaling functions in inflammation (9).

One of the key enzymes of the NADPH oxidase activation is protein kinase C (PKC) (16). PKC was originally reported as a ubiquitous, Ca²⁺- and phospholipid-dependent serine/threonine kinase, which is activated transiently (17). To date, 12 isoforms of PKC have been reported, and they are classified into three groups, conventional, novel, and atypical, based on their primary structure and cofactor requirement (18, 19). Neutrophil activation by physiological stimuli such as formyl peptide or complement fragment C₅a begins with interaction with their specific surface receptors (20). This interaction leads to the activation of phospholipase C, which generates two messengers, inositol 1,4,5-triphosphate and 1,2-diacylglycerol. These messengers together lead to the activation of PKC. Among the PKC isoforms, PKCβ is suggested to be responsible for the neutrophil respiratory burst activation (21, 22). Activated PKC phosphorylates one of the cytosolic components of NADPH oxidase, p47phox (23, 24). The phosphorylated p47phox translocates and interacts with membrane component of NADPH oxidase (25, 26), which results in the assembly of the active enzyme.

The tumor promoter phorbol 12-myristate 13-acetate (PMA) induces a PKC-dependent respiratory burst (1, 26). PMA has a structural similarity with the endogenous PKC agonist diacylglycerol and causes a direct and more prolonged activation of

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1 The abbreviations used are: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; IL-2, interleukin-2; IL-2Rα, interleukin-2 receptor α chain; NF-κB, nuclear factor κB; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.
PKC (17). PMA effectively activates neutrophil NADPH oxidase, and the cells produce maximal amounts of superoxide (26). However, this PKC-mediated activation pathway is not the only way of NADPH oxidase activation. Some stimuli such as arachidonic acid (27) or sodium dodecyl sulfate (SDS; 28) also induce a respiratory burst without requiring PKC (29). Arachidonic acid directly interacts with p47phox, changes its conformation, and enables it to assemble active NADPH oxidase (30). Moreover, arachidonic acid-induced respiratory burst in a cell-free reconstituted system is independent of PKC cofactors (Ca2+ and ATP) and is not inhibited by PKC inhibitor (29). Therefore, arachidonic acid is a PKC-independent inducer of respiratory burst.

PKC also has a pivotal role in the activation of T cells. T cells are stimulated by the interaction of a specific antigen with T cell receptor-CD3 complex. This interaction activates the intracellular kinase cascade (31), one of which is mediated by PKC. Activated kinase cascade activates transcription factors (31), which initiate transcription and expression of a variety of molecules including interleukin-2 (IL-2) and the high affinity IL-2 receptor α chain (IL-2Rα). Because IL-2 is a major T cell growth factor, the coordinate production of IL-2 and IL-2 receptor is crucial for T cell proliferation and the immune response (32). T cell stimulation with PMA alone is enough for IL-2R expression (33), and PKCα is responsible for this effect (34). Expression of IL-2Rα is transcriptionally regulated (35) and involves nuclear transcription factor (NF)-κB activation (36). NF-κB activation comprises the phosphorylation of its inhibitory protein IκB (37). The activated NF-κB translocates from the cytosol to the nucleus (38), where it binds to the κB consensus sequence of IL-2Rα gene promoter (36). This binding is essential for NF-κB-mediated gene expression (39, 40).

In this study we show that PMA-induced PKC activity is inhibited by NH2Cl in neutrophils and Jurkat T cells. This inhibition of PKC activity results in distinct biological effects, notably inhibition of the respiratory burst in neutrophils and inhibition of IL-2Rα expression in Jurkat T cells. These results suggest that chloramines may have a physiological function in regions where the respiratory burst is activated.

**EXPERIMENTAL PROCEDURES**

**Materials**

PMA, arachidonic acid, and PKCβ were obtained from Sigma. Dulbecco’s phosphate-buffered saline (Ca2+- and Mg2+-free, pH 7.4; PBS) and the PKC assay system were from Life Technologies, Inc. NF-κB consensus oligonucleotide was from Promega (Madison, WI). T4 polynucleotide kinase and protein molecular weight markers were from Boehringer Mannheim. Phycocyanin-labeled anti-human CD25 (IL-2Rα) antibody was from Immunotech (Westbrook, ME). Calcein A was from Calbiochem. [γ-32P]ATP and [32P]orthophosphoric acid were from NEN Life Science Products. Rabbit polyclonal antibody to human p47phox (raised against glutathione S-transferase-p47phox fusion protein) was a generous gift from Dr. Bernard M. Bahior. All other reagents were of analytic grade or better.

**Cell Preparation**

**Neutrophils**—Rat elicited peritoneal neutrophils were prepared from Sprague-Dawley male rats (250–350 g, from Bantin and Kingman, Fremont, CA) as described previously (15, 41). The collected cells contained about 80% neutrophils based on microscopic examination, and the cell viability was more than 95%, which was assessed by the trypan blue exclusion test.

**Jurkat T Cells**—Jurkat T cell is a cell line of human acute T cell leukemia and was obtained from American Type Culture Collection (clone E6-1, Bethesda, MD). The cell culture medium was RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin-streptomycin, 110 mg/liter sodium pyruvate, and 2 mM l-glutamine (from University of California, San Francisco, cell culture facility), and cells were grown in humidified air containing 5% CO2 at 37 °C.

**Chloramine Inhibition of Protein Kinase C**

Taurine chloramine and monochloramine (NH2Cl) were prepared as described previously (15, 42). The chloramine concentration was determined by 2-nitro-5-thiobenzoate assay (42).

**Pretreatment of the Cells with Chloramine**

Neutrophils were suspended in PBS (5 × 106 cells/ml, and NH2Cl or taurine chloramine was added to get the final concentration of 10–70 μM. After the incubation for 10 min at 37 °C, neutrophils were separated from the PBS by centrifugation and used as described below. Jurkat T cells were suspended in the fresh cell culture medium (1 × 106 cells/ml, and NH2Cl was added to 20–70 μM. In contrast to PBS, this medium alone consumed added NH2Cl. After the incubation for 30 min at 37 °C, Jurkat T cells were used without washing because the medium contained no more detectable chloramine.

**Measurement of the Respiratory Burst**

Neutrophils were resuspended in the assay mixture (1.25 × 106 cells/ml, 1.1 mM p-hydroxyphenylacetaate, 50 μg/ml superoxide dismutase, 50 μg/ml horseradish peroxidase, in PBS), and they were stimulated with either PMA (300 nM, final concentration) or arachidonic acid (100 μM), which was previously dissolved in ethanol at 300 μM and 100 mM, respectively. Preliminary study showed that these concentrations were optimal. The respiratory burst was monitored continuously at 37 °C by H2O2 formation as described previously (15, 43).

**Measurements of PKC Activity**

PKC activity was measured just after the chloramine exposure using a PKC assay system from Life Technologies, Inc. Partial purification with an affinity exchange column was omitted because a preliminary study using crude and partially purified samples showed similar results. Acetylated synthetic peptide from myelin basic protein 4–14 (Ac-Gln-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu) was used for the substrate, which is specific for conventional PKCs (44). Specificity for PKC was established by running the sample and PKC-specific inhibitor peptide PKC19–36 (45) for each sample. PKC activity was expressed as pmol of 32P incorporated/min/106 cells.

**Phosphorylation of 47-kDa Protein**

Phosphorylation of 47-kDa protein in neutrophils was studied as described previously (46), except for the following. Before NH2Cl treatment, neutrophils (1 × 107/ml) were incubated with [γ-32P]orthophosphoric acid (50 μCi/ml) at 30 °C for 30 min in 138 mM NaCl, 2.7 mM CaCl2, and 7.5 mM n-glucose, adjusted to pH 7.5. After the incubation, neutrophils (5 × 106/ml) were added to 30–70 μM NH2Cl in PBS and incubated for 10 min at 37 °C. Then the cells were stimulated with 300 nM PMA for 90 s. The reaction was stopped by the addition of trichloroacetic acid (10% w/v), centrifuged, and the total precipitated protein was dissolved in SDS-PAGE sample buffer. The pH was adjusted to about 7 by NaOH. Precipitated proteins were analyzed by SDS-PAGE using 7.5% gel, stained with Coomassie Brilliant Blue R-250, and dried, and autoradiography was performed.

Phosphorylation of the 47-kDa protein was also studied in the cell-free system using exogenous PKCβ. Cytosolic fractions were prepared from NH2Cl (50 μM)-treated and control neutrophils as described previously (47). Then p47phox was partially purified by passing through a DE52 ion exchange column as described previously (48). Immunoblot analysis showed that virtually all immunoreactive p47phox was recovered from the pass-through fraction. The reaction mixture contained 0.5 mg/ml partially purified protein, 5 units/ml PKCβ, 0.3% (v/v) Triton X-100 mixed micelles containing 10 μM PMA, and 0.28 mg/ml phosphatidylycerine (prepared as described in Ref. 49); 20 mM MgCl2, 1 mM CaCl2, 20 mM ATP (containing 100 μCi/ml [γ-32P]ATP), and 20 mM Tris-HCl (pH 7.5), incubated at 30 °C for 7 min. The reaction was stopped by the addition of SDS-PAGE sample buffer containing 1 μM staurosporine and 1 mM ATP and analyzed by SDS-PAGE and autoradiography.

Immunoblot analysis of p47phox was performed following SDS-PAGE and Western blotting using anti-p47phox antibody. The optical density of the autoradiograms and immunoblots was measured by a Shimadzu CS-9301 PC densitometer and expressed as percent of positive control.

**Measurements of IL-2Rα Expression**

After 30 min of NH2Cl incubation, Jurkat T cells were stimulated with PMA (100 nM) dissolved in dimethyl sulfoxide. Control cells were treated with the same volume of dimethyl sulfoxide (0.1% v/v) without PMA. The cells were maintained in culture condition for 24 h after PMA
Results are the mean ± S.D. for four determinations. *Significantly different from the 0 μM samples (p < 0.05).
creased the PMA-induced IL-2Rα expression (Fig. 5). The NH2Cl pretreatment did not alter cell viability even 24 h after PMA stimulation (data not shown). Results are the mean ± S.D. for four determinations.

PKC Activity in NH2Cl-treated Jurkat T Cells—To correlate the IL-2Rα expression with the inhibition of PKC activation, we measured the inducible PKC activity in NH2Cl-treated Jurkat T cells. Fig. 6 demonstrates that 20–70 μM NH2Cl inhibited inducible PKC activity in Jurkat T cells. Because the cell viability did not change (Table I), this inhibition of PKC activation was not the result of cell death.

NF-κB Activation—The PMA-induced IL-2Rα expression is a multistep response involving kinase cascade activation, NF-κB activation, mRNA synthesis, and protein synthesis (35, 38). We studied NF-κB activation to see if the inhibited PKC activity fails to activate the step after PKC activation. NF-κB activation precedes IL-2Rα expression in Jurkat T cells (36). Consistent with previous reports (40, 55), PMA induced marked activation of NF-κB in Jurkat T cells. Pretreatment of Jurkat T cells with NH2Cl inhibited PMA-induced NF-κB activation (Fig. 7). The inhibition of IL-2Rα expression, NF-κB activation, and PKC activation by NH2Cl all occurred at similar (20–70 μM) NH2Cl concentrations (Figs. 5–7).

Discussion

When neutrophils were pretreated with 50 μM NH2Cl, the PKC-dependent stimulant PMA could not induce respiratory burst, yet the PKC-independent stimulant arachidonic acid induced a complete respiratory burst. This finding strongly suggests that the target of NH2Cl was PKC and not NADPH.
Neutrophils pretreated with 50 mM NH2Cl had no inhibitory effect on either phosphorylation or respiratory burst activity. We further studied if NH2Cl directly damaged p47^phox so that it could not be phosphorylated by PKC. In a cell-free system, exogenously added PKCβ phosphorylated equally well the 47-kDa protein from the control and NH2Cl-treated neutrophils. Both samples contained comparable amounts of immunoreactive p47^phox. This result suggests that even after NH2Cl (50 μM) treatment, p47^phox can be phosphorylated when catalyzed by intact PKCβ.

One explanation for the discrepancy between the 47-kDa protein phosphorylation and the remaining PKC activity is that neutrophils contain phosphoprotein phosphatases that reverse the action of PKC (58, 59). During the PMA-stimulated respiratory burst, p47^phox undergoes a continual cycle of phosphorylation and dephosphorylation (60). Under optimally stimulated conditions the phosphorylation reaction by PKC predominates, which maintains the p47^phox in a phosphorylated state (60) and keeps the NADPH oxidase in the active state. Therefore, if PKC activity decreases, the dephosphorylation reaction by phosphatase may predominate, and p47^phox cannot be maintained in a sufficiently phosphorylated state. Another possible explanation is that NH2Cl may alter the substrate specificity of PKC. In this case, the PKC-catalyzed phosphorylation of p47^phox may be impaired more severely than that of myelin basic protein peptide. This possibility should be studied further. In any case, it is likely that the remaining PKC activity was insufficient to keep the p47^phox phosphorylated enough to allow assembly of an active NADPH oxidase. This would result in the observed substantial inhibition of the respiratory burst activity.

Monochloramine-induced suppression of inducible PKC activity was not restricted to neutrophils. At 20 μM, NH2Cl inhibited the PMA-induced IL-2Ra expression, PKC activation, and the NF-κB activation in Jurkat T cells. Although there are many steps that could result in the inhibition of IL-2Ra expression, we measured NF-κB activation because NF-κB is activated by the phosphorylation of its inhibitory protein IκB (37), and NF-κB activation precedes IL-2Ra gene expression in Jurkat T cells (38). The inhibition of PKC activation and NF-κB activation occurred at similar concentrations of NH2Cl, which suggests that the inhibited PKC activity failed to activate NF-κB.

We studied further if the decrease in NF-κB activation was the result of direct damage of NF-κB protein caused by NH2Cl. Calyculin A is a potent inhibitor of phosphoprotein phosphatase types 1 and 2A, and it activates NF-κB through IκB phosphorylation and degradation in Jurkat T cells (61). Monochlormamine treatment (50 μM) did not affect the calyculin A-induced NF-κB activation. This result showed that NF-κB in NH2Cl-treated Jurkat T cells was still inducible and suggests that NF-κB protein was not a direct target of NH2Cl. Because the NF-κB activation is reported to regulate IL-2Ra gene expression in Jurkat T cells (38), inhibition of PKC activation by NH2Cl seems to be the primary cause of the inhibition of IL-2Ra expression.

Among the PKC isoforms, at least conventional types of PKCs (α, βI, βII, and γ) are likely to be the target of NH2Cl. In
the neutrophils, PKCβ appears to be responsible for NADPH oxidase activation because PKCβ migrates to the membrane fraction upon stimulation (21) and is able to phosphorylate p47phox (22). Although T cells contain both PKCa and PKCβ as well as other PKCs (31), PKCa has been suggested to be responsible for IL-2R expression (34). We used a synthetic myelin basic protein peptide as a substrate for the measurement of PKC activity. This peptide is a good substrate for conventional PKCs but not for novel or atypical PKCs (44). However, it is yet to be resolved whether chloramines also affect novel and/or atypical PKCs.

During the respiratory burst, a variety of chloramine derivatives are detected in the extracellular medium (7). These chloramines are mostly hydrophilic, low molecular weight, mono-N-chloramine derivatives (7) and not likely to be membrane-permeable. However, membrane-permeable chloramines are also likely to be produced because normal human plasma contains 20–40 μM NH$_4$Cl (62). When 2–3 × 10$^6$ml of neutrophils, which is comparable to the concentration in blood, are activated with PMA, the chloramine concentration reaches as high as 100 μM in a short term culture (12). As the neutrophil concentration can be much higher in inflammatory sites, our chloramine concentration can be much higher in inflammatory sites, our neutrophils, PKCα activated with PMA, the chloramine concentration reaches as high as 20–70 μM in a short term culture (12). 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