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8-Oxo-7,8-dihydroguanosine triphosphate(8-oxoGTP) down-regulates respiratory burst of neutrophils by antagonizing GTP toward Rac, a small GTP binding protein

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Abstract
8-Oxo-7,8-dihydroguanosine triphosphate (8-oxoGTP) has been regarded simply as a oxidative mutagenic byproduct. The results obtained in this study imply that it may act as a down-regulator of respiratory burst of neutrophils. Human neutrophils treated with PMA produced superoxides and at the same time, the cytosol of these cells was intensely immunostained by 8-oxo-7,8-dihydroguanosine(8-oxoG) antibody, indicating that 8-oxoG-containing chemical species including 8-oxoGTP are produced. Human neutrophil lysates treated with PMA also produced superoxides, which was stimulated by GTPγS but inhibited by 8-oxoGTPγS. Moreover, 8-oxoGTPγS suppressed the stimulatory action of GTPγS. Likewise, GTPγS stimulated Rac activity in neutrophil lysates but 8-oxoGTPγS and GDP inhibited it. The inhibitory effect of GDP was one tenth that of 8-oxoGTPγS. Here again, 8-oxoGTPγS also suppressed the stimulatory action of GTPγS on Rac activity. These results imply the possibility that 8-oxoGTP is formed during respiratory burst of neutrophils and limits neutrophil production of superoxides by antagonizing GTP toward Rac.

Keywords: *8-Oxo-7,8-dihydroguanine, 8-oxoGTP, NADPH oxidase, Rac, GTP-binding proteins, neutrophils*

Abbreviations: *8-oxoGTP, 8-oxo-7,8-dihydroguanosine triphosphate; 8-oxoG, 8-oxo-7,8-dihydroguanosine; NADPH, nicotinamide adenine dinucleotide phosphate; PMA, phorbol myristate acetate*

Introduction

Reactive oxygen species (ROS) attack guanine bases in DNA and form 8-oxo-7,8-dihydroguanine [1], which acts as a potent mutagen [2–4]. ROS also attack guanine bases of guanosine triphosphaste (GTP) and form 8-oxo-7,8-dihydroguanosine triphosphate (8-oxoGTP). This is evidenced by the presence of 8-oxodGTPases, MutT [5] and MTH1 [6] which hydrolyze 8-oxoGTP (8-oxodGTP) into 8-oxoGMP(8-oxodGMP) and prevent the use of these oxidized nucleotides into DNA synthesis. Thus, they have been also regarded simply as mutagenic oxidative byproducts. In a previous study [7], we tested the interaction between 8-oxoGTP and Rac, a small GTP-binding protein *in vitro*, and found that it inactivates Rac while GTP activates it. This result indicates that the biological natures of 8-oxo-GTP and GTP differ and suggests that 8-oxo-GTP is not a simple by-product but may be a biologically active molecule. But we do not know the meaning or significance of this activity of 8-oxo-GTP.

Phagocytic leukocytes such as neutrophils [8] and macrophages [9] phagocytose invading bacteria.

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During phagocytosis, NADPH oxidase is activated to produce superoxides and other ROS, which are used for killing the engulfed bacteria. This phenomenon is known as respiratory burst and also provoked by chemicals such phorbol myristate acetate (PMA) [10] and fMLP [11]. The activation of NADPH oxidase undergoes several processes, which are formation of GTP–Rac complex by replacement of GDP bound to Rac with GTP [12], phosphorylation of three cytoplasmic subunits of this oxidase, i.e. $p67^{phox}$, $p47^{phox}$ and $p40^{phox}$ [13–15], migration of the GTP–Rac complex [16] and the three phosphorylated subunits [17] to the membrane and then their association with $gp91^{phox}$ of a complex of $gp91^{phox}$/22^{phox} present in membrane. The resulting active form of NADPH oxidase is a complex of the Rac bound to GTP and the five subunits (three cytoplasmic and two membranous). Rac–GTP, as a structural component in the active oxidase complex, plays essential roles in maintaining the active state of this enzyme [18,19]. The predominant form of Rac in human neutrophils is Rac2 [20], whereas Rac1 is found in the macrophages of mice and guinea pigs [21].

The main function of ROS produced in this respiratory burst is to kill invading bacteria. However, ROS can also attack membranous lipids [22], nuclear DNA [23,24] and other important components of neutrophils [25,26] and can be auto-toxic or auto-lethal. Thus, these cells should have a means of regulating the production of ROS. It was reported that 8-oxo-7,8-dihydroguanine is formed in DNA [27] of ROS-producing neutrophils. This observation highly suggests that 8-oxo-7,8-dihydroguanine is also formed in cytosolic GTP, producing 8-oxoGTP during respiratory burst of neutrophils. Therefore, the respiratory burst of phagocytic leukocytes such as neutrophils and macrophages may be one of the cellular situations where a large amount of 8-oxoGTP production is expected.

Here, we have two questions; is 8-oxoGTP really formed in neutrophils during respiratory burst? and what is the role of this oxidatively modified GTP if it is formed in this cellular event? In this study, attempts were made to provide some information for these questions by demonstrating the formation of 8-oxo-7,8-dihydroguanosine(8-oxoG) containing compounds in PMA-treated human neutrophils immunohistochemically and by observing the 8-oxoGTPγS effects on the production of superoxides by NADPH oxidase and Rac activity in PMA-treated human neutrophil lysates. We found that PMA-treated neutrophils showed immunostained cytosols with much higher intensity than non-treated cells and 8-oxoGTPγS inhibited the PMA-induced superoxide production and Rac activation in neutrophil lysates while GTPγS stimulated both. We suggest the possibility that 8-oxoGTP is formed during respiratory burst of neutrophils and 8-oxoGTP formed may contribute to limiting ROS production by antagonizing GTP toward Rac.

Materials and methods

Materials

Human neutrophils were obtained from healthy volunteers (25–35 years old). Guanosine 5′-[γ-thio]-triphosphate (GTPγS; a non-hydrolysable analog of GTP) were obtained from Sigma (St Louis, USA). Anti 8-oxo-7,8-dihydroguanosine(8-oxoG) antibody was from PharMingen (Flanklin Lakes, USA). Cy-3 conjugated secondary antibody was from Jackson ImmunoResearch Laboratories Inc. (West Groves, USA). Rac activation assay kits were from Upstate Biotechnology (Lake Placid, USA). All chemicals used in this study were of analytical grade. 8-Oxo-7,8-dihydroguanosine 5′-[γ-thio] triphosphate(8-oxoGTPγS) was custom-made by Trilink Biotechnologies (San Diego, USA).

Preparation of human neutrophils and neutrophil lysates

Human neutrophils of over 95% purity were prepared as previously described [28] using acid–citrate–dextrose as the anticoagulant, dextran to sediment erythrocytes, and Ficoll-Hypaque density gradient centrifugation to separate mononuclear cells from neutrophils. Neutrophils in pellets were suspended at 10^8 cells/ml in phosphate buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4 at pH 7.4. The above procedures were all carried out at 4°C. The cells prepared were used in intact form or lysate. To prepare lysates, the neutrophil suspensions were sonicated for 4×15 s in ice using a Branson sonicator at 40 W to disrupt cells, and then centrifuged at 10,000*g* to remove nondisrupted cells and particulates, including nuclei. After protein determinations, supernatants were kept in ice and used within 8 h.

Measurement of superoxide production in neutrophils or neutrophil lysates

Superoxide generation by NADPH oxidase were determined by measuring superoxide dismutase (SOD) inhibitable ferricytochrome C reduction as previously described [29] with slight modifications. When effects of GTPγS or 8-oxoGTPγS on superoxide production was observed, neutrohpil lysates were used since these nucleotides can not have access to neutrophil cytoplasm. Briefly, neutrophil lysates (100 μg protein/ml) were mixed with various concentrations (0–100 μM) of GTPγS or 8-oxoGTPγS for 15 min at room temperature (RT) in 0.5 ml of PBS supplemented with 1 mM $MgCl_2$; the latter was added to augment binding between Rac and GTPγS/8-oxoGTPγS. PMA (1 μg/ml) and arachidonic acid (1.5 mM) were then added for 5 min; arachidonic

acid plays as an important role in NADPH oxidase activity in cell free systems [30]. Mixtures were then transferred to photometric cuvettes containing 100 μM ferricytochrome *C* and 250 μM NADPH in 0.5 ml of PBS. Rates of absorbance changes were recorded for about 10 min at 550 nm using a spectrophotometer (Uvicon 933, Kontron instrument, Italy). The same experiment was performed but with 50 μg SOD added in the photometric cuvettes containing 100 μM ferricytochrome *C* and 250 μM NADPH. Differences between the rates of absorbance changes were taken to indicate superoxide production. We also observed superoxide production from intact neutrophils when we compared the time course of superoxide production to that of formation of 8-oxoguanosine-containing compounds. In this case, neutrophils (1×10^5 cells/ml) were suspended in 0.5 ml of PBS containing PMA (1 μg/ml) and then transferred to photometric cuvettes containing 100 μM ferricytochrome *C* and 250 μM NADPH in 0.5 ml of PBS. The procedures thereafter were the same as above.

Assay of Rac activity

Rac activation assays were conducted as described previously [31] using Rac activation kits (Upstate Biotechnology, Lake Placid). First, according to the manufacturer' instruction, neutrophil lysates were prepared by suspending neutrophils (1×10^6/ml) in a magnesium-containing lysis buffer (MLB; 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1% Igepal CA-630, 2% glycerol, 10 μg/μl aprotinin, 10 μg/μl leupeptin, 25 mM NaF, 1 mM Na_3VO_4 and 25 mM Hepes, pH 7.5) for 30 min on ice and then centrifuged at 10,000*g* for 15 min. Lysates were then collected and protein concentrations were determined. Lysates (500 μg) were treated with GDP, GTPγS, 8-oxoGTPγS or GTPγS + 8-oxoGTPγS of various concentrations in 1 ml MLB containing 10 mM EDTA for 15 min at 30°C. To stabilize binding between Rac and guanine nucleotides, $MgCl_2$ was then added to 60 mM and mixtures were cooled on ice. Activated Rac was affinity-precipitated by adding 5 μg of GST-PAK-PBD (a fusion protein of glutathione-S-transferase and p21-activated protein kinase 1 p21-binding domain) conjugated to glutathione–agarose beads for 1 h at 4°C. After washing with MLB three times, beads were resuspended in 20 μl of 2 × SDS sample buffer, boiled for 5 min, and electrophoresed in 10% SDS-PAGE. Gels were transferred to nitrocellulose membranes and Rac2 on membranes was detected by Western blotting using a mouse polyclonal anti-Rac2 antibody in the Rac activation kits.

Evaluation of 8-oxo-7,8-dihydroguanosine (8-oxoG)-containing chemical species levels

We cannot measure cellular level of 8-oxoGTP since an assay specific to 8-oxoGTP has not been established. Thus, levels of 8-oxoGTP in neutrophils before and after stimulation with PMA were indirectly evaluated as 8-oxoG-containing chemical species immunocytochemistry using monoclonal anti 8-oxoG antibody [32]. Briefly, neutrophils prepared as described above (1×10^5 cells/ml) were incubated in 0.5 ml PBS with/without PMA (1 μg/ml). At every five min, aliquots (50 μl) were taken and washed with ice cold PBS twice. Neutrophils on slides were then fixed for 15 min with PBS containing 4% paraformaldehyde and 0.03 M sucrose, permeabilized with PBS containing 0.1% Triton X-100 for 20 min, blocked with BSA(1 mg/ml) in PBS for 1 h at 4°C, treated with anti 8-oxoG antibody (1:100–200) for 1 h and then treated with Cy-3 conjugated secondary antibody (1:100) for 1 h. After each step, cells were washed with PBS 2–3 times. Immunofluorescence images were photographed using a Bio-Rad confocal system (Lsm 510, Carl Zeiss, Germany) and fluorescence intensities were quantified using a BAS 2500 imaging analyzer (Fuji, Japan). Some samples were stained with Cy-3 without anti 8-oxo G antibody to validate the immune specificity of this staining. Unless otherwise mentioned, experiments were done at RT.

Results

Increased levels of anti 8-oxoG monoclonal antibody detectable chemical species in the cytosol of PMA-stimulated neutrophils

First, we tried to provide evidence for 8-oxoGTP formation during respiratory burst of neutrophils. Unfortunately, however, we are unable to monitor the concentration of 8-oxoGTP in cells as no specific assay has been established. As an alternative approach to explore this issue, an indirect method was tried, involving the visualization of 8-oxoG-containing chemical species immunocytochemically using anti 8-oxoG monoclonal antibody. This method was applied to neutrophils treated with PMA (Figure 1A). Using this method, we found that we could stain even resting cells not treated with PMA (0 min in Figure 1A). At 5 min after PMA stimulation, the cells were stained very heavily, thereafter the staining was being light. The staining was observed exclusively in cytoplasm. When anti 8-oxoG antibody was omitted from the experimental procedure, cells were not stained indicating that the staining observed was due to the presence of 8-oxoG-containing compounds. Staining intensities are quantified in Figure 1B. Figure 1C shows superoxide production with time after treating neutrophils with PMA. Superoxide production was also at a maximum at 5 min and decreased thereafter. This result is quite similar to the time course of intensity changes of the immunohistochemical staining shown in Figure 1B.

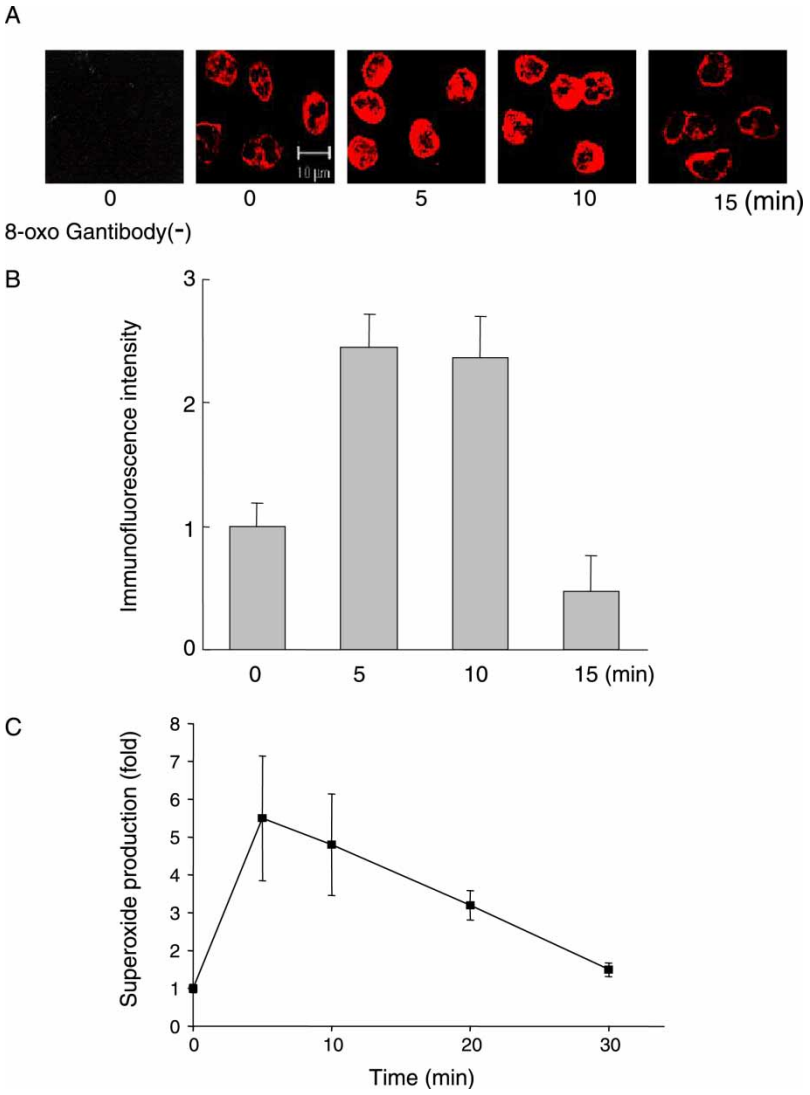

Figure 1. Effect of PMA on immunostaining by anti 8-oxoguanosine(8-oxoG) antibody in human neutrophils. After stimulation with PMA, the levels of 8-oxoG-containing chemical species in neutrophils were determined by immunostaining using anti 8-oxoG antibody as described in Materials and Methods. Immunofluorescence was photographed under a confocal microscope (panel A) and fluorescence intensities were quantified using a BAS imaging analyzer (panel B). During immunostaining, superoxide production was also monitored in PMA-treated neutrophils (panel C). The ordinate indicates the fold of zero time production of superoxides. Details are described in Materials and Methods. Data are means ± SD ($n = 3$).

However, we needs an explanation for how the cytoplasm of the neutrophils was stained by PMA stimulation in a topological point of view that superoxide anions are released outside of cells when stimulated by PMA while the superoxide anions are released into phagosomes when stimulated by phagocytosis. This may be due to the intracellular entry of H_2O_2 that is formed outside of cells from dismutation of the extracellular superoxide anions. H_2O_2 can permeate into cells since it has do charge [43]. We observed such staining in mouse microglial cells (BV2) under the similar condition where BV2 was stimulated by lipopolysacharides [44] and when these cells were treated with H_2O_2 [data not shown].

Effects of GTPγS and 8-oxoGTPγS on NADPH oxidase activity

Results are shown in Figure 2. First, in order to observe the effect of PMA on NADPH oxidase, we treated neutrophil lysates with PMA alone. Under this condition, superoxide production (NADPH oxidase activity) was 10.75 ± 1.12 nmol/min/mg protein. We then studied the effects of GTP and 8-oxoGTP on this basal production (basal activity) of superoxides using the non-hydrolysable analogs, GTPγS and 8-oxoGTPγS in place of GTP and 8-oxoGTP. To observe the effect of GTPγS, neutrophil lysates were treated with PMA in the presence of various concentrations of GTPγS. As shown in Figure 2,

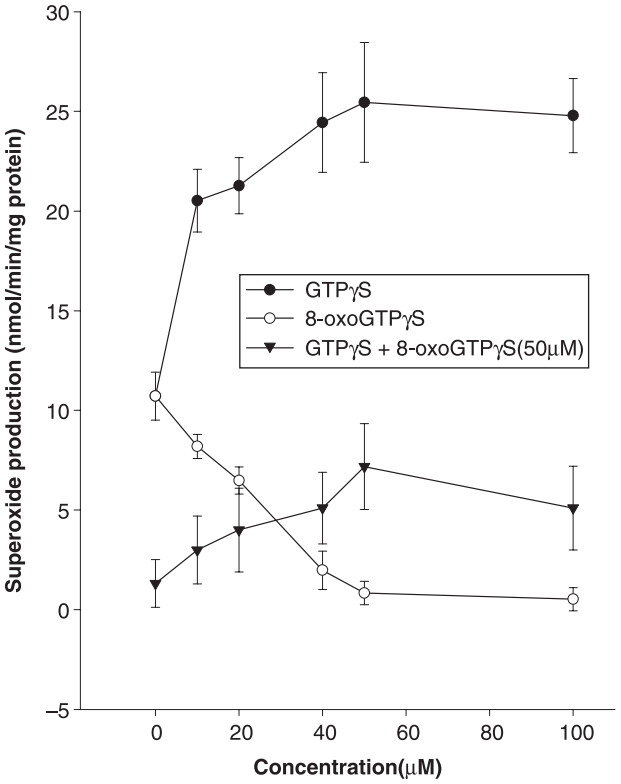

Figure 2. Effects of GTPγS and 8-oxoGTPγS on the superoxide production in human neutrophil lysates. The preparation of human neutrophil lysates and rate of superoxide generation were described in Materials and Methods. Aliquots of the neutrophil lysates (50 μg protein) were mixed with various concentrations of GTPγS, 8-oxoGTPγS, or GTPγS + 8-oxoGTPγS for 15 min in 0.5 ml of PBS supplemented with 1 mM $MgCl_2$ and then treated with PMA (1 μg/ml) and arachidonic acid(1.5 mM) for 5 min. Whole mixtures were then transferred to photometric cuvettes containing ferricytochrome *C* and NADPH in 0.5 ml of PBS. Absorbance changes were recorded for about 10 min at 550 nm. The same experiments were duplicated with SOD in photometric cuvettes. Differences between rates in the two experiments were regarded as rates of superoxide production. The details are described in Materials and Methods. The data shown are means ± SD ($n = 3$).

the addition of 10 μM GTPγS to the PMA-treated lysates markedly increased the basal superoxide production to 20.52 ± 1.57 nmol/min/mg protein. At GTPγS concentrations over 10 μM, superoxide production continued to increase and at 50 μM, maximum production (25.45 ± 7.07 nmol/min/mg protein) was observed. In contrast to GTPγS, 8-oxoGTPγS was shown to inhibit the basal production in a dose-dependent manner, and the basal superoxide production was eventually abolished at 8-oxoGTPγS concentrations over 50 μM. The inhibitory effect of 8-oxoGTPγS was also clearly demonstrated by the finding that the stimulatory effect of GTPγS on superoxide production, i.e. the superoxide production versus GTPγS concentration curve, was effectively suppressed in the presence of 8-oxoGTPγS.

Effects of GTPγS and 8-oxoGTPγS on Rac activity

We studied the effects of GTPγS and 8-oxoGTPγS on the Rac activity in human neutrophil lysates. Human neutrophil lysates showed Rac (Rac2) activity without adding PMA, GTP or GTPγS (Figure 3A). GTPγS and 8-oxoGTPγS were also tested on this endogenous activity of Rac2 in neutrophil lysates. As shown in Figure 3A, GTPγS increased the endogenous activity of Rac (the band indicated by control) dose-dependently, as indicated by an increase in the band density. In contrast to GTPγS, 8-oxoGTPγS reduced this basal activity in a dose-dependent manner. Figure 3A also shows inhibition of endogenous Rac activity by GDP, a physiologic Rac inhibitor but in the mM range, whereas 8-oxoGTPγS inhibited

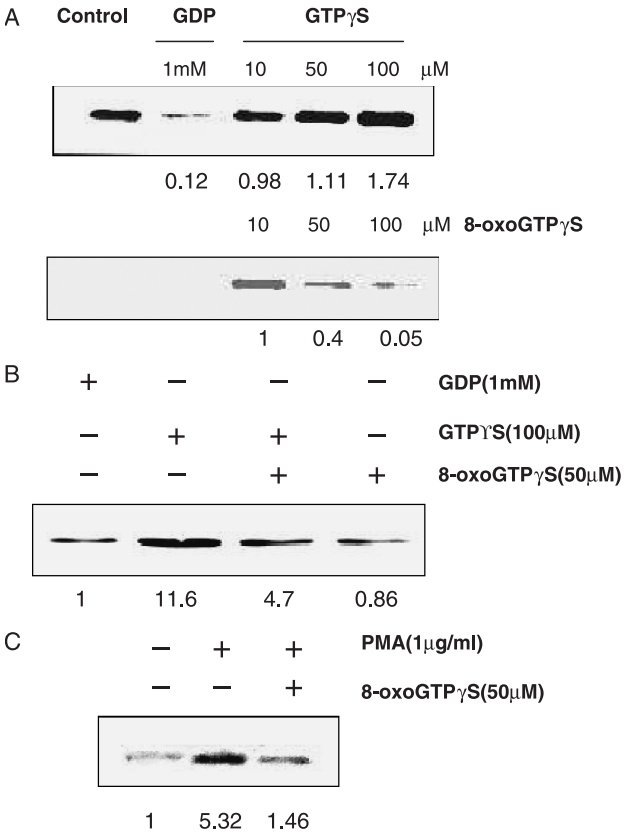

Figure 3. Effects of GTPγS and 8-oxoGTPγS on Rac activity in human neutrophil lysates. Rac activation assays were carried out as described by the manufacturer using an assay kit. Neutrophils were lysed in a magnesium-containing lysis buffer (MLB) on ice for 30 min. Supernatants collected as lysates (500 μg) were treated with GDP, GTPγS, 8-oxoGTPγS (panel A), or GTPγS + 8-oxoGTPγS (panel B) of various concentration in 1 ml MLB. The activated Rac2 was affinity-precipitated by adding GST-PAK-PBD (a fusion protein of glutathione-S-transferase and p21-activated protein kinase 1 p21-binding domain to glutathione conjugated-agarose beads. Beads were then resuspended in a sample buffer, boiled for 5 min, and electrophoresed in 10% SDS-PAGE. Gels were transferred to nitrocellulose membranes and Rac was detected by Western blotting using a mouse monoclonal anti-Rac2 antibody. Details are described in Materials and Methods. Numbers shown below the bands indicate band intensities measured by a BAS imaging analyzer. Data are representatives of 2–3 experiments.

in the μM range. In order to examine the inhibitory effect of 8-oxoGTPγS on Rac more clearly, its effect was tested in the presence of GTPγS (Figure 3B). We observed that 8-oxoGTPγS (100 μM) reduced the Rac band density generated by GTPγS (100 μM) almost completely to the level of the endogenous activity observed in the presence of GDP (1 mM). We observed that 8-oxoGTPγS was able to inhibit the PMA-induced activation of Rac (Figure 3C). This result is consistent with the observation that PMA-induced production of superoxides was inhibited by 8-oxoGTPγS (Figure 2).

Discussion

In this study, we observed that PMA treatment stimulated human neutrophils to produce superoxides (Figure 1C) and at the same time, induced to increase the intensity of immunostaining by 8-oxoG antibody in the cytosol of PMA-treated cells (Figure 1A,B), indicating that 8-oxoG-containing chemical species including 8-oxoGTP are formed by ROS generated during respiratory burst. The temporal changes of immunostaining density and superoxide production were quite similar (Figure 1). In human neutrophil lysates (Figure 2), it was observed that GTPγS stimulated PMA-induced production of superoxides but 8-oxoGTPγS inhibited it. Moreover, 8-oxoGTPγS suppressed the stimulatory action of GTPγS. Likewise, GTPγS stimulated Rac activity in neutrophil lysates but 8-oxoGTPγS and GDP inhibited it (Figure 3A). The inhibitory effect of GDP was one tenth that of 8-oxoGTPγS (Figure 3A). Here again, 8-oxoGTPγS also suppressed the stimulatory action of GTPγS on Rac activity (Figure 3B). This kinetic data shows that V_{max} was reduced by 8-oxoGTPγS from 25.45 ± 7.07 to 7.18 ± 3.01 nmol/min/mg protein, suggesting 8-oxoGTPγS is not a competitive inhibitor. However, the experimental condition does not allow further kinetic analysis toward Km of GTPγS and Ki of 8-oxoGTPγS since neutrophil lysates seem to be contaminated with a considerable amount of endogenous GTP. As shown in Figure 2, superoxides were produced by PMA with no addition of GTP or GTPγS and this basal production could be inhibited completely in the presence of 8-oxoGTPγS over 50 μM. Therefore, Rac activation assay using purified Rac protein is more suitable for estimation of Km and Ki of GTPγS and 8-oxoGTPγS, respectively.

All aerobic cells contain 8-oxodGTPase [5,6]. This enzyme hydrolyzes 8-oxodGTP (and 8-oxoGTP) into 8-oxodGMP(8-oxoGMP) and PPi, and thus, prevents their incorporation into DNA or RNA. The presence of 8-oxoGTPase indicates that 8-oxoguanine is formed also in cellular GTP (and/or dGTP) under oxidative stress although the cellular levels of 8-oxoGTP cannot not be monitored as no specific assay is available. In support of this, Figure 1 shows the formation of 8-oxoguanosine-containing species in PMA-treated neutrophils immunocytochemically using anti 8-oxoG monoclonal antibody. Of course, the signals shown in Figure 1A are likely to reflect mainly oxidized RNA since immunocytochemical studies for oxidative DNA damage using anti 8-oxoG monoclonal antibody show that the intensity of this cytoplasmic signal is decraesed when cell or tissue specimen are treated with RNase [45]. However, the two facts support that 8-oxoGTP is one of the chemical species giving rise to this signal; (1) guanine bases in RNA are highly susceptibile to ROS attack as indicated by the observation that the relative amounts of oxidized transcripts in Alzheimer's disease brains was reported to reach 30–70% for mRNA species [46] and (2) the intracellular concentration of ribonucleotides is very high, which was reported to reach up to 10 mM although it varies with condition and cell types [47]. In addition, the inhibitory effect of 8-oxoGTPγS on Rac activity was 10 times that of GDP, a natural Rac inhibitor (Figure 3A,B). Thus, these results imply the possibility that 8-oxoGTP is formed during respiratory burst of neutrophils and possibly it, if accumulated, may play a role as a negative regulator of ROS production by antagonizing GTP toward Rac.

Regulation and termination of NADPH oxidase activity has not been clearly understood [for review, see Ref. 33]. One known regulating mechanism is conversion of GTP/GDP-bound states of Rac [for review, see Ref. 34]. The GTP-bound form of Rac has a high GTPase activity and can stimulate NADPH oxidase, whereas the GDP-bound form is inactive. GEF (guanine-nucleotide exchange factor) [35]activates Rac (GTPase) by stimulating the exchange of GDP for GTP, thus, allowing Rac to activate NADPH oxidase. GAP (GTPase activating protein) [36] and GDI (GDP dissociation inhibitor) [37] play negative roles by enhancing the formation of GDP on Rac and keeping it bound to Rac, respectively. In addition to guanine nucleotide-binding states of Rac, protein kinase A and phosphatases are likely to play a negative role by preventing the phosphorylation of the cytoplasmic subunits of the oxidase as indicated by the findings that cAMP/cAMP analogs [38] and okadaic acid [11] are shown to diminish the activity of NADPH oxidase and to inhibit phosphorylation of $p47^{phox}$. However, it is not clearly illucidated how the actions and interactions of these individual proteins are controlled in a concerted manner for regulation of NADPH oxidase activity.

In addition to these proteins, 4-hydroxynonenal [39] is known to inhibit NADPH oxidase. Inhibition by this aldehyde has been suggested to be of physiological significance because it is a major product of lipid peroxidation, and therefore, is present in tissues under oxidative attack. The inhibition by 4-hydroxynonenal was reversed by dithiothreitol, suggesting that the

blockade of –SH groups on proteins is responsible for the action of this aldehyde. Figure 3 shows the accumulation of anti 8-oxoG antibody detectable compounds in PMA-treated neutrophil cytosols, which suggests that the formation of 8-oxoGTP during respiratory burst exceeds the removal capacity of 8-oxoGTPase. Figure 2 shows that 8-oxoGTP inhibits the action of GTP, a key molecule in activation of NADPH oxidase. These data suggest that 8-oxoGTP also appears to be an inhibitor of physiological relevance. In the present study, we have focused on 8-oxoGTP rather than 8-oxodGTP for two reasons. One is that Rac is controlled by GTP and not by dGTP and thus, 8-oxoGTP may interfere with the action of GTP more efficiently than 8-oxodGTP, and the other reason is that compared to 8-oxodGTP, the pool size of cellular ribonucleotides is a hundred times larger than that of deoxyribonucleotides [40] thus, a significant amount of 8-oxoGTP is produced in the cytoplasm under oxidative stress.

However, our hypothesis is still cautious. The fluorescent signal increased only two-fold above the basal level upon stimulation of NADPH oxidase. Under this condition, the level of oxidized NTPs is probably very low. Therefore, it seems unlikely that such a small increase can inhibit Rac when seeing the results in Figure 3. To prove this, several questions should be solved. The first involves the monitoring of the cellular level of 8-oxoGTP and the demonstration of 8-oxoGTP in an amount sufficient to influence Rac activity. However, unfortunately, no 8-oxoGTP assay is available. The second involves identifying the site of action of 8-oxoGTP. Rac itself is the most possible candidate since GTP is the substrate of GTPase of Rac. However, the possibilities exist that 8-oxoGTP may interact with GEF, GDI or GAP. The third involves the termination of the action of 8-oxoGTP. Hydrolysis by 8-oxoGTPase could be one mechanism, but hydrolysis by GTPase of Rac remain to be determined.

In this study, 8-oxoGTP was tested only on NADPH oxidase. In addition to ROS production, Rac is also associated with other neutrophils functions, namely phagocytosis [8], chemotactic movement [41] and degranulation/secretion [42]. It would be interesting to determine whether these functions are also affected by 8-oxoGTP. However, it is difficult to prove these actions of 8-oxoGTP since intact cells must be used. We are currently examining some methods, such as electroporation and transfection to introduce 8-oxoGTP molecules into intact neutrophils.

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