Pioglitazone restores phagocyte mitochondrial oxidants and bactericidal capacity in chronic granulomatous disease

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Background: Deficient production of reactive oxygen species (ROS) by the phagocyte nicotinamide adenine dinucleotide (NADPH) oxidase in patients with chronic granulomatous disease (CGD) results in susceptibility to certain pathogens secondary to impaired oxidative killing and mobilization of other phagocyte defenses. Peroxisome proliferator–activated receptor (PPAR) γ agonists, including pioglitazone, approved for type 2 diabetes therapy alter cellular metabolism and can heighten ROS production. It was hypothesized that pioglitazone treatment of gp91$^{phox-/-}$ mice, a murine model of human CGD, would enhance phagocyte oxidant production and killing of *Staphylococcus aureus*, a significant pathogen in patients with this disorder.

Objectives: We sought to determine whether pioglitazone treatment of gp91$^{phox-/-}$ mice enhanced phagocyte oxidant production and host defense.

Methods: Wild-type and gp91$^{phox-/-}$ mice were treated with the PPARγ agonist pioglitazone, and phagocyte ROS and killing of *S aureus* were investigated.

Results: As demonstrated by 3 different ROS-sensing probes, short-term treatment of gp91$^{phox-/-}$ mice with pioglitazone enhanced stimulated ROS production in neutrophils and monocytes from blood and neutrophils and inflammatory macrophages recruited to tissues. Mitochondria were identified as the source of ROS. Findings were replicated in human monocytes from patients with CGD after *ex vivo* pioglitazone treatment. Importantly, although mitochondrial (mt)ROS were deficient in gp91$^{phox-/-}$ phagocytes, their restoration with treatment significantly enabled killing of *S aureus* both *ex vivo* and *in vivo*.

Conclusions: Together, the data support the hypothesis that signaling from the NADPH oxidase under normal circumstances governs phagocyte mtROS production and that such signaling is lacking in the absence of a functioning phagocyte oxidase. PPARγ agonism appears to bypass the need for the NADPH oxidase for enhanced mtROS production and partially restores host defense in CGD. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: *Chronic granulomatous disease, phagocytes, mitochondria, oxidants, thioglitazones*

Chronic granulomatous disease (CGD) is a rare disease attributed to mutations in the genes encoding components of the phagocyte nicotinamide adenine dinucleotide (NADPH) oxidase. The inability of phagocytes[1-3] to mount an oxidative burst predisposes to infections with certain bacterial and fungal pathogens, such as *Staphylococcus aureus* and *Aspergillus* species, which are most evident in the lung, skin, lymph nodes, and liver,[4] and even low levels of residual reactive oxygen species (ROS) production by phagocytes are associated with improved survival.[5] In addition to direct oxidative killing of pathogens, phagocyte ROS orchestrate other antimicrobial defenses, such as activation of antimicrobial proteins within the phagolysosome[6] and recruitment of antimicrobial autophagocytic machinery to phagolysosomes. These are deficient in phagocytes from patients with CGD.[7-9]

Pioglitazone and other thioglitazones are so-called nutrient-restricting drugs approved for type 2 diabetes. As peroxisome proliferator–activated receptor (PPAR) γ agonists, they slowly mimic the actions of insulin with increased peripheral glucose disposal. Additionally, their nutrient restriction signaling is associated with altered macrophage metabolism, such as activation of adenosine monophosphate-activated protein kinase (AMPK),[10] and various anti-inflammatory activities.[11,12] Intriguingly, PPARγ agonism has also been associated with enhanced host defense against *Candida albicans*,[13-15] *Staphylococcus aureus*,[16] *Klebsiella pneumoniae*,[17] and *Streptococcus pneumoniae*.[18] Two mechanisms have been proposed: enhanced pathogen containment by means of phagocytosis and encapsulation and enhanced ROS production by phagocytes (or both), although the source was not identified. With regard to ROS production, PPARγ ligands/agonists are reported to decrease expression of NADPH oxidase subunits of 22 and 47 kDa in some cells[19,20] but have also been shown to increase mitochondrial reactive oxygen species (mtROS) output in others.[21,22] Recent data implicating nutrient restriction or "starvation signaling" in the enhancement of mtROS production and entrainment of mtROS

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Abbreviations used
BADGE: Bisphenol A diglycidyl ether
CFU: Colony-forming units
CGD: Chronic granulomatous disease
DHR: Dihydrorhodamine
DPI: Diphenylene iodonium
mtROS: Mitochondrial reactive oxygen species
NADPH: Nicotinamide adenine dinucleotide
PMA: Phorbol 12-myristate 13-acetate
PPAR: Peroxisome proliferator–activated receptor
ROS: Reactive oxygen species
SOD: Superoxide dismutase
WT: Wild-type

for killing of intracellular and extracellular bacteria[23] suggest a possible mechanism through which PPARγ agonists might contribute to host defense.

Given these observations, we questioned whether pioglitazone treatment of gp91$^{phox-/-}$ mice would enhance oxidant production by phagocytes from patients with CGD and, if so, bolster host defense. We found that pioglitazone treatment induced mtROS production in stimulated neutrophils, monocytes, and inflammatory macrophages of mice with CGD, as well as normal mice, and importantly, the restored phagocytes demonstrated significantly enhanced killing of *S aureus* both *in vitro* and *in vivo*. Crosstalk between the NADPH oxidase and mtROS leading to optimal antimicrobial responses by normal phagocytes was also shown. Consequently, in the absence of a functional NADPH oxidase, this signaling is lost but can be restored at the level of mtROS production by pioglitazone treatment.

METHODS

Animals

Male C57BL/6 and gp91$^{phox-/-}$ mice were purchased from the Jackson Laboratory (Bar Harbor, Me) or bred in house. They received care in accordance with the institutional animal care and use committee and were given pioglitazone (10 mg/kg/d); bisphenol A diglycidyl ether (BADGE), a PPARγ antagonist; or vehicle (carboxymethyl cellulose) by oral gavage for 5 days unless otherwise indicated. All agents were well tolerated. Mice were killed with CO_2 inhalation.

Reagents

Pioglitazone, BADGE, MitoTEMPO, phorbol 12-myristate 13-acetate (PMA), diphenylene iodonium (DPI), catalase, and superoxide dismutase (SOD) were from Sigma (St Louis, Mo). Conjugated antibodies to CD115, F4/80, Ly6G, and CD11b were from eBioscience (San Diego, Calif). Antibodies to Nox1, Nox4, Duox, gp91^{phox}, p20^{phox}, p22^{phox}, p47^{phox}, and p67^{phox} were from Santa Cruz Biotechnology (Santa Cruz, Calif). Zymosan, dihydrorhodamine (DHR), MitoTracker Green, and MitoSOX Red were from Life Technologies (Grand Island, NY).

Isolation of blood and peritoneal leukocytes

Red blood cells were lysed (Pharmlyse; BD Biosciences, San Jose, Calif) from whole blood from mice (terminal cardiac puncture) before staining with markers and analysis by flow cytometry (CyAn ADP analyzer, BD Biosciences). Peritoneal cells were harvested by lavage from mice injected intraperitoneally with 1 mg/mL zymosan in PBS. Cells were washed, suspended in PBS (3% FBS), blocked with anti-mouse Fc (CD16/32, eBioscience) for 30 minutes, and stained for 1 hour on ice with conjugated antibodies before flow cytometry.

ROS detection

DHR analysis was performed using phagocytes (10^6) incubated for 15 minutes with 5 μmol/L DHR (PBS, 0.05% gelatin, 0.09% glucose, and 1 mmol/L EDTA) and stimulated with PMA (200 ng/mL) for 15 minutes at 37°C. Cells were washed and analyzed by flow cytometry.

Cytochrome c reduction, measuring superoxide release, was performed as previously described.[24]

mtROS were detected with MitoSOX Red. Cells (10^6) were incubated (37°C) with 25 nmol/L MitoTracker Green in the dark (Dulbecco modified Eagle medium and 10% FBS) followed by 4 μmol/L MitoSox Red (15 minutes each), washed with PBS, and analyzed by flow cytometry or Zeiss LSM 700 confocal microscopy.

Bactericidal assays

***In vitro* assay.** Peritoneal phagocytes (2×10^5, 10 hours after zymosan) were cocultured with 2×10^6 colony-forming units (CFU) of *S aureus* (strain 502A, ATCC #27217) grown overnight in Lauryl Broth and washed twice with saline in 100 μL of RPMI (phenol red free 1% mouse serum) at 37°C for 1 hour. Phagocytes were lysed (1 mL of water [pH 11][25]), bacteria were pelleted at 10,000*g* for 10 minutes (2 times), and washed with 1 mL of saline. Bacterial numbers were determined by using a modified Alamar blue assay.[26] This assay was similarly adapted for killing of *Burkholderia cepacia* (ATCC #15416).

***In vivo* assay.** *S aureus* peritonitis was induced, as described by Pollock et al,[27] with minor modifications. Briefly, mice were injected intraperitoneally with 0.5 mL of 2×10^7 CFU/mL *S aureus* (saline), peritonea underwent lavage at designated times, and cell counts and viable bacteria in lavage fluid and cells (after lysis as above) were determined.

Human blood monocytes

Heparinized blood was obtained from patients with X-linked CGD and healthy control subjects at the National Institutes of Health (Bethesda, Md) after approval of the institutional review board.

The blood was express shipped overnight to Denver, Colorado. PBMCs were isolated using Percoll gradients,[28] plated in X-vivo[10] to adhere monocytes for 2 hours, washed (5 times) to remove nonadherent cells, and cultured at 37°C in a 10% CO_2 atmosphere without and with pioglitazone (10 μmol/L). Cells were then stimulated with PMA and mtROS detected using flow cytometry, as above.

Statistics

Each experiment was performed 3 to 5 times, unless otherwise indicated. Analysis and *P* value calculations were conducted by means of ANOVA (JMP statistical program 4.0.1; SAS Institute, Cary, NC). The Wilcoxon matched-pairs signed-rank test was used for single and multiple comparisons. A *P* value of .05 or less was considered significant. Data are reported as means ± SEMs.

RESULTS

In vivo treatment with pioglitazone enhances blood neutrophil and monocyte ROS production

Wild-type (WT) and gp91$^{phox-/-}$ mice were treated with either pioglitazone or vehicle by oral gavage for 5 days to test whether phagocyte oxidant production would be enhanced/restored. Previously, this treatment resulted in PPARγ activation in inflammatory macrophages and accelerated resolution of sterile peritonitis in mice with CGD.[29] After treatment, blood leukocytes were

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