Reactive oxygen species–independent activation of the IL-1β inflammasome in cells from patients with chronic granulomatous disease

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Humans with chronic granulomatous diseases (CGDs) due to mutations in p47-phox have defective NADPH activity and thus cannot generate NADPH-dependent reactive oxygen species (ROS). The role of ROS in inflammation is controversial; some in vitro studies suggest that ROS are crucial for secretion of IL-1β via inflammasome activation, whereas mice defective for ROS and patients with CGD have a proinflammatory phenotype. In this study, we evaluated activation of the IL-1β inflammasome in cells from CGD patients. In contrast to previous studies using the small molecule diphenylene iodonium (DPI) as a ROS inhibitor, we found no decrease in either caspase-1 activation or secretion of IL-1β and IL-18 in primary CGD monocytes. Moreover, activation of CGD monocytes by uric acid crystals induced a 4-fold higher level of IL-1β secretion compared with that seen in monocytes from unaffected subjects, and this increase was not due to increased synthesis of the IL-1β precursor. In addition, Western blot analysis of CGD cells revealed that caspase-1 activation was not decreased, but rather was increased compared with control cells. Examination of the effects exerted by the inhibition of ROS activity by DPI revealed that the decrease in IL-1β secretion by DPI was actually due to inhibition of IL-1β gene expression. Thus, inconsistent with the proinflammatory role of ROS, the present findings support the concept that ROS likely dampen inflammasome activation. The absence of ROS in CGD monocytes may explain the presence of an inflammatory phenotype characterized by granulomas and inflammatory bowel disease occurring in CGD patients.

antioxidants | colitis | gout | inflammation | uric acid

There is much recent interest in the processing and release of bioactive IL-1β, especially since the discovery that blockade of IL-1 receptors with the IL-1 receptor antagonist (IL-1Ra) is a very effective treatment for autoinflammatory disorders, such as familial Mediterranean fever (1), familial cold autoinflammatory syndrome (2), Muckle-Wells syndrome (3), neonatal-onset multisystem inflammatory disease (4), hyperimmunoglobulin D syndrome (HIDS) (5), and adult-onset Still's disease (6). Blood monocytes from patients with some of these disorders, especially cryopyrinopathies and HIDS, readily release more IL-1β than do monocytes from unaffected controls (7, 8).

Activation of caspase-1 by the protein complex known as the inflammasome leads to the conversion of proinflammatory IL-1β (pro-IL-1β) to IL-1β (9). Several protein platforms/inflammasomes have been described for the activation of caspase-1, each of which includes members of the NOD-like receptor (NLR) family of proteins (10). The most intensely studied of these are the inflammasomes formed by the NLR family members NLRP3 and NLRP1. Several conditions are thought to be required for the activation of the inflammasome, including the interaction of "danger-signaling" molecules with NLRP components, the induction of K^+ efflux through the P2×7 receptor, and the generation of reactive oxygen species (ROS) (9, 11).

The role of ROS in inflammation is controversial, however. On the one hand, ROS have been suggested to induce NF-κB activation (12, 13), and several in vitro studies have proposed that activation of the inflammasome is strictly dependent on ROS generation (11, 14). On the other hand, other studies have reported anti-inflammatory effects of the NADPH system and ROS (15), and a recent study in mice defective for the generation of ROS strongly suggested anti-inflammatory effects of oxygen species (16). In line with this, patients with chronic granulomatous diseases (CGDs) with defects in the NADPH system and, consequently, defective ROS generation (17) display an inflammatory phenotype characterized by granulomas and Crohn-like colitis (18).

To evaluate the role of ROS in inflammasome activation, we investigated the activation of caspase-1 and the production of IL-1β in the presence of NADPH inhibitors. We also assessed inflammasome activation in cells isolated from CGD patients.

Results

DPI Inhibits Transcription of Proinflammatory Cytokines. We found that LPS induced production of IL-1β and TNF-α from primary human peripheral blood mononuclear cells (PBMCs), as reported previously (19). ROS inhibition by diphenylene iodonium (DPI) decreased production of both IL-1β and TNF-α induced by LPS (Fig. 1*A*). Because TNF-α release is independent of caspase-1 activation, this argues for effects of DPI independent of the inflammasome. Indeed, the effect of DPI was exerted at a transcriptional level, because mRNA for both IL-1β and TNF-α was decreased by DPI (Fig. 1*B*), whereas active caspase-1 was still present (Fig. 1*C*).

Toll-Like Receptor–Induced Production of IL-1β Is Normal in CGD Patients. The Toll-like receptor (TLR) ligands Pam3Cys (TLR2 ligand) and LPS (TLR4 ligand) induced a strong IL-1β response in PBMCs of both healthy volunteers and CGD patients (Fig. 2*A*). Although ROS have been specifically implicated in the activation of the inflammasome (11), when PBMCs from CGD patients who lacked ROS were primed with LPS and subsequently stimulated with the inflammasome activator ATP for 15 min, no difference in the release of IL-1β was seen between cells of healthy individuals and those of CGD patients (Fig. 2*B*). As expected, intracellular pro-IL-1β intracellular concentrations did not differ between healthy volunteers and CGD patients (Fig. 2*B*). IL-18 also is an important proinflammatory cytokine of the IL-1β family that is processed by caspase-1. No differences in IL-18 production were observed between healthy volunteers and

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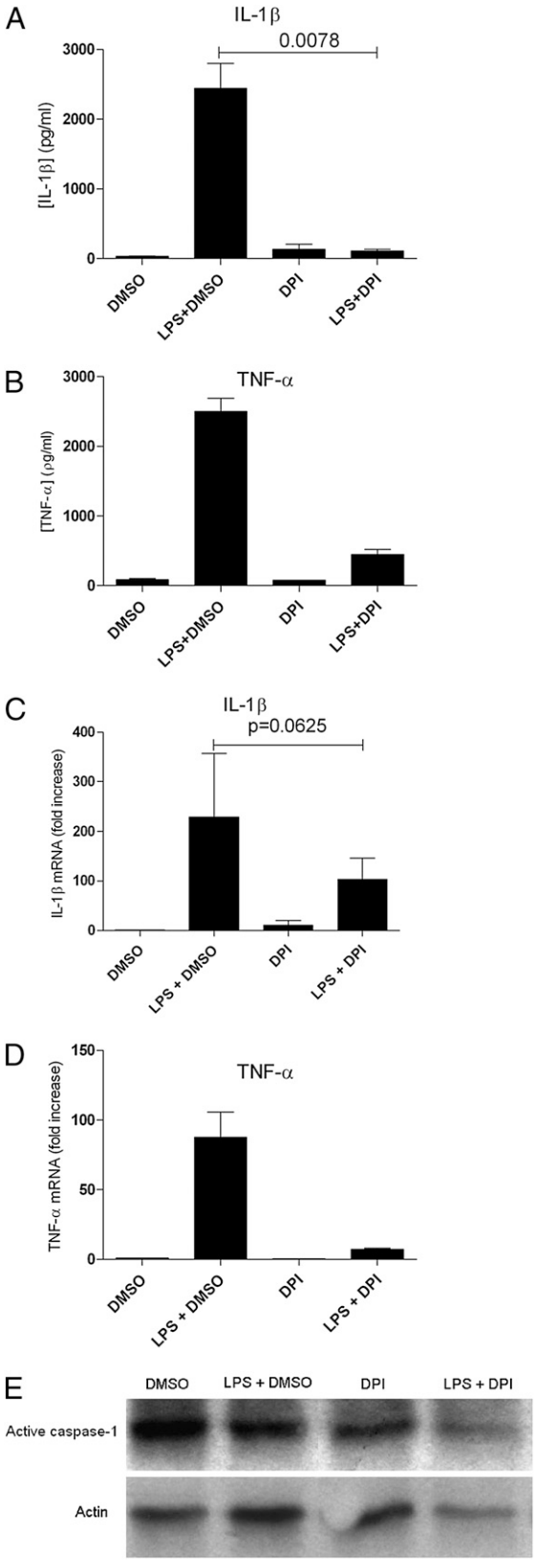

Fig. 1. ROS inhibition decreases production and transcription of IL-1β and TNF-α. (*A*) Monocytes isolated from eight healthy controls were stimulated with LPS in the absence or presence of the ROS inhibitor DPI. In the presence of DPI, IL-1β production was completely inhibited (n = 8). (*B*) TNF-α production also was decreased when LPS-stimulated PBMCs of healthy controls were cultured in the presence of DPI (n = 2). (*C*) PBMCs of healthy controls were stimulated for 4 h with LPS in the absence or presence of DPI. mRNA was isolated from the cell lysates using TRIzol. DPI decreased mRNA expression of IL-1β (n = 6). (*D*) DPI also decreased mRNA expression of TNF-α (n = 2). (*E*) Active p10 caspase-1 was still expressed in cells cultured in the presence of DPI. Data are representative for four healthy volunteers.

CGD patients (5.8 pg/mL vs 4.9 pg/mL), although the very low amounts of IL-18 released by primary monocytes must be noted.

Inflammasome Activation Is Increased in CGD Patients. Despite defective NADPH-dependent ROS generation, more prominent activation of caspase-1 was apparent in monocytes isolated from CGD patients compared with those from healthy volunteers (Fig. 3*A*). The NLRP3 inflammasome has been reported to be activated by uric acid crystals (11, 20), and priming with a TLR stimulus such as LPS is required to accomplish this activation (21). However, PBMCs from CGD patients were able to produce IL-1β when exposed to uric acid alone in the absence of LPS priming, a phenomenon not observed in normal volunteers (Fig. 3*B*), demonstrating increased activation of the inflammasome in cells from CGD patients. In addition, DPI decreased cytokine production in both cells from healthy controls and cells isolated from CGD patients (Fig. 4).

Discussion

Our findings indicate that ROS are not essential for inflammasome activation. This conclusion is based on several lines of evidence. First, treatment of human primary monocytes with the commonly used ROS inhibitor DPI did not inhibit generation of active caspase-1. Second, cells from CGD patients expressed more constitutively active caspase-1 activation. Third, ROS-deficient cells from CGD patients produced more IL-1β after stimulation with urate crystals, a classical inflammasome stimulus.

These findings differ from the results of other recently published studies suggesting that ROS induce inflammasome activation and IL-1β production (11, 14). Those studies were based on the use of ROS inhibitors such as DPI in cell lines or mouse macrophages, however (11, 14, 22). Thus, we investigated the effects of DPI on caspase-1 activation and IL-1β production in human PBMCs. We found that DPI can indeed inhibit IL-1β production in human PBMCs, as reported previously in THP-1 cells and mouse macrophages (11, 14). This inhibition was accompanied not only by lower IL-1β release, but also by decreased TNF-α production, suggesting that DPI can exert its effects independently of the inflammasome. These effects of DPI were exerted at the level of transcription, with repression of mRNA for both TNF-α and IL-1β, whereas the active caspase-1 p35 fragment was normally present. Our data demonstrating that the anti-inflammatory effects of DPI on IL-1β production are exerted at the transcriptional level are in line with the large body of literature reporting that ROS induce NF-κB activation (12, 23, 24). In addition, the clear inhibition of cytokine stimulation by DPI in cells of CGD patients, a system in which ROS production is defective, clearly demonstrates that the effects of DPI on cytokine production are largely independent of NADPH oxydase generation of ROS. Possible additional mechanisms modulated by DPI include inhibition of nitric oxide synthetase and mitochondrial complex 1-dependent (25, 26). Thus, results based on DPI as the sole method of inhibition of ROS production should be interpreted with caution.

In addition to these arguments, it should be kept in mind that the concept that ROS induces inflammasome activation is at odds with the known proinflammatory phenotype of patients with CGD, characterized by the occurrence of sterile granulomas, colitis, and inflammatory skin and urogenital reactions, in which IL-1β is thought to play an important pathogenic role (15). In contrast, our findings of the inhibitory effects of ROS on

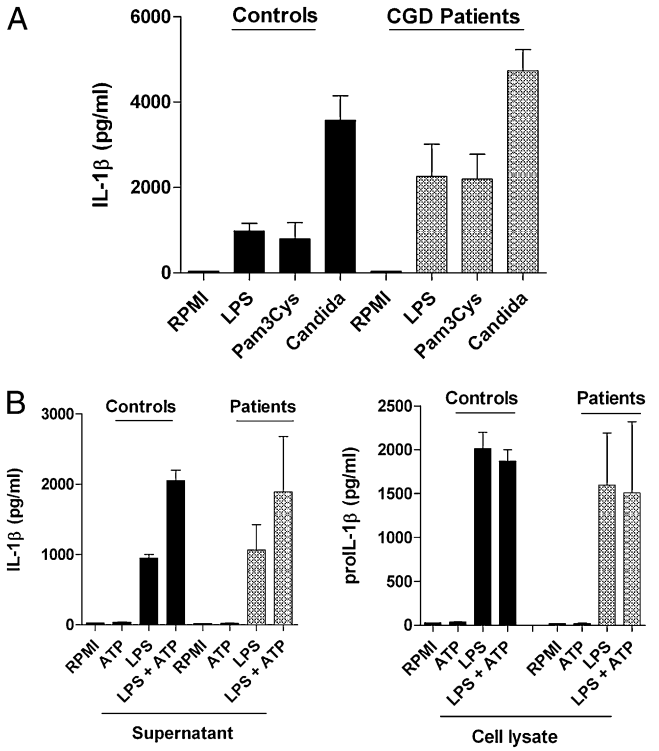

Fig. 2. Inflammasome activation and IL-1β production in CGD patients. (*A*) Monocytes isolated from CGD patients and healthy controls produced IL-1β on stimulation with LPS, Pam3cys, and *Candida*. (*B*) IL-1β stimulation by LPS and ATP was similar in CGD and control individuals. Data are presented as mean ± SEM of five healthy controls and of three CGD patients.

caspase-1 activation agree with a proinflammatory state in CGD, because we found that the release of mature IL-1β was normal and after certain stimuli even increased in cells of CGD patients. Our data do not stand alone; they are in line with several previous studies that have demonstrated consistent up-regulation of various proinflammatory cytokines in cells isolated from CGD patients (27–30).

Unexpectedly, whereas we found that primary monocytes from healthy volunteers did not produce IL-1β in response to uric acid alone, monocytes from CGD patients secreted substantial amounts of IL-1β. In addition, we observed more constitutively activated caspase-1 in monocytes from CGD patients compared with healthy volunteers. In line with our data, there is additional evidence that increased ROS production inhibits caspase-1 activation. Superoxide dismutase 1 (SOD-1) degrades ROS, and thus SOD-1 deficiency results in increased endogenous ROS production. It was recently reported that the increased superoxide production in SOD-1–deficient macrophages specifically inhibits caspase-1 activation by oxidation and glutathionylation (31). SOD-1–deficient mice produced less IL-1β in vivo and were less susceptible to LPS-induced shock (31). Another recent study demonstrated that silencing SOD-1 in human monocytes results in a reduction of IL-1β secretion on stimulation with zymosan (32). Taken together, these data are in agreement with our findings and strongly suggest that ROS inhibit inflammasome activation and, subsequently, IL-1β production.

In conclusion, our findings fo the present study settle the controversy regarding the role of ROS in inflammasome activation in human cells by providing evidence that oxygen radicals have an inhibitory effect on caspase-1 activation and IL-1β release. These data explain the proinflammatory clinical phenotype seen in patients with CGD.

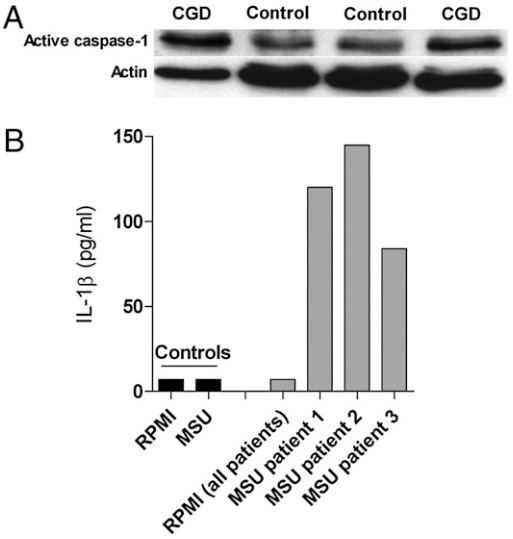

Fig. 3. ROS inhibit inflammasome activation. (*A*) Active p10 caspase-1 was expressed more strongly in unstimulated monocytes from CGD patients than in those from healthy controls. (*B*) NALP3 inflammasome stimulus uric acid crystals stimulated IL-1β release in monocytes isolated from CGD patients, but not in those from healthy controls. Data are presented as mean ± SEM of five healthy controls and of three CGD patients.

Materials and Methods

Patients and Controls. Eight healthy volunteers with no known infectious or inflammatory disorders donated blood as a control group for the assessment of cytokine production capacity. In addition, PBMCs were isolated from three patients with CGD harboring homozygous mutations in the NCF1 gene (p47-phox), in which defective ROS production has been demonstrated. After informed consent was obtained, blood was collected by venipuncture from both patients and volunteers into 10-mL EDTA tubes [BD, Plymouth, UK (art. no. 367525)].

In Vitro Cytokine Production. Separation and stimulation of PBMCs was performed as described previously (33). In brief, the PBMC fraction was obtained by density centrifugation of diluted blood (1 part blood to 1 part pyrogen-free saline) over Ficoll-Paque (Pharmacia Biotech). PBMCs were washed twice in saline and suspended in culture medium supplemented with gentamicin 1%, L-glutamine 1%, and pyruvate 1%. The cells were counted in a Bürker counting chamber, and their number was adjusted to 5×10^6 cells/mL. Then 5×10^5 PBMCs in a volume of 100 μL per well were incubated at 37 °C in round-bottomed 96-well plates (Greiner). After 24 h of incubation with the various stimuli as described below, supernatants were collected and stored at −80 °C until being assayed for IL-1β and TNF-α production. DPI in a concentration of 10 μM was used as an ROS inhibitor (34). Two methodologies were used to assess inflammasome stimulation. One assay used stimulation for 3 h with LPS, followed by ATP-induced IL-1β release for 15 min (35), and a second assay used specific stimulation with LPS-free monosodium urate, also known as uric acid crystals, a putative NLRP3 ligand (20).

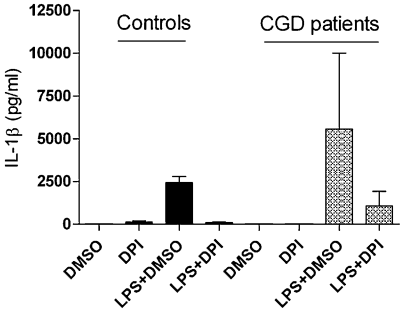

Fig. 4. ROS inhibition decreased IL-1β production in both healthy controls and CGD patients. In both healthy controls and CGD patients, DPI decreased IL-1β production in LPS-stimulated cells. Data are presented as mean ± SEM.

Cytokine Assays. IL-1β and TNF-α concentrations were measured with commercial ELISA kits (R&D Systems). Pro-IL-1β concentrations in the cell lysates were measured by specific ELISA (R&D Systems). The concentration of IL-18 was measured with a BioPlex kit (Bio-Rad).

RT-PCR. Two million freshly isolated PBMCs were incubated with the various stimuli. After 4 h of incubation at 37 °C, total RNA was extracted in 800 μL of TRIzol reagent (Invitrogen). Isolated RNA was reverse-transcribed into cDNA using oligo(dT) primers and M-MLV reverse transcriptase. PCR was performed using an Applied Biosystems 7300 real-time PCR system. The primer sequences for human IL-1β were as follows: sense, 5′- GCC-CTA-AAC-AGA-TGA-AGT-GCT-C-3′; antisense, 5′- GAA-CCA-GCA-TCT-TCC-TCA-G-3′. B2M was used as a reference gene, for which the primers were as follows: 5-ATG-AGT-ATG-CCT-GCC-GTG-TG-3 (forward) and 5-CCA-AAT-GCG-GCA-TCT-TCA-AAC-3 (reverse). PCR conditions were 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min.

Immunoblotting for Caspase-1. For immunoblotting, 10×10^6 cells were lysed in 100 mL of lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 1% Triton X-100, 40 mM a-glycerophosphate, 50 mM sodium fluoride, 200 mM sodium vanadate, 10 mg/mL leupeptin, 10 mg/mL aprotinin, 1 mM pepstatin A, and 1 mM phenylmethylsulfonyl fluoride]. The homogenate was frozen, then thawed and centrifuged at 4 °C for 10 min at "15,000 × *g*", and the supernatant was taken for Western blot analysis. Equal amounts of protein were subjected to SDS/PAGE using 10% and 15% polyacrylamide gels at a constant voltage of 100 V. After SDS/PAGE, proteins were transferred to nitrocellulose membrane (0.2 mm). The membrane was blocked with 5% (wt/vol) milk powder in PBS for 1 h at room temperature, followed by incubation overnight at 4 °C with a caspase-1 p10 antibody (SC-515; Santa Cruz Biotechnology) in 5% BSA/TBS/Tween 20. After overnight incubation, the blots were washed three times with TBS/Tween 20 and then incubated with HRP-conjugated goat anti-rabbit antibody at a dilution of 1:10 000 in 5% (wt/vol) milk powder in PBS for 1 h at room temperature. After being washed three times with TBS/Tween 20, the blots where developed with ECL (GE Healthcare) according to the manufacturer's instructions.

Statistical Analyses. Differences between groups were analyzed using the Mann-Whitney *U* test for unpaired data and the Wilcoxon signed-rank test for paired data. Differences were considered statistically significant at $P \leq 0.05$. Data represent the cumulative results of all experiments performed and are presented as mean ± SEM.

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