Glucocorticoids Sensitize the Innate Immune System through Regulation of the NLRP3 Inflammasome

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Background: The ability of glucocorticoids to regulate the expression and function of the NOD-like receptors is unknown. Results: Glucocorticoids enhance the expression and function of NLRP3, promoting the secretion of IL-1β in response to ATP. Conclusion: Glucocorticoids sensitize the innate immune system to specific stimuli. Significance: This work demonstrates a novel, proinflammatory role for glucocorticoids, enhancing the activation of the innate immune system in response to danger signals.

Glucocorticoids have long been recognized as powerful anti-inflammatory compounds that are one of the most widely prescribed classes of drugs in the world. However, their role in the regulation of innate immunity is not well understood. We sought to examine the effects of glucocorticoids on the NOD-like receptors (NLRs), a central component of the inflammasome and innate immunity. Surprisingly, we show that glucocorticoids induce both NLRP3 messenger RNA and protein, which is a critical component of the inflammasome. The glucocorticoid-dependent induction of NLRP3 sensitizes the cells to extracellular ATP and significantly enhances the ATP-mediated release of proinflammatory molecules, including mature IL-1β, TNF-α, and IL-6. This effect was specific for glucocorticoids and dependent on the glucocorticoid receptor. These studies demonstrate a novel role for glucocorticoids in sensitizing the initial inflammatory response by the innate immune system.

The actions of endogenous and synthetic glucocorticoids are mediated through the glucocorticoid receptor (GR), a member of the nuclear receptor superfamily of ligand-dependent transcription factors (5). Consistent with the physiological actions of glucocorticoids, the GR is ubiquitously expressed and is essential for life (6). In the absence of ligand, the GR is primarily cytoplasmic as part of large multiprotein complex (7, 8). Binding of glucocorticoids induces a conformational change in GR, exposing nuclear localization sequences and subsequent import into the nucleus (9). Once in the nucleus, GR can positively or negatively regulate gene transcription through one of three mechanisms: 1) directly binding to DNA at glucocorticoid response elements; 2) physical association with other transcription factors (tethering); or 3) a combination of DNA binding and tethering (10). The anti-inflammatory role of glucocorticoids has been attributed largely to the ability of GR to tether to and “trans-repress” the activities of the proinflammatory molecules AP-1 and NF-κB (11). In addition, it is becoming apparent that glucocorticoids also can induce the expression of a number of other anti-inflammatory molecules (12). Globally, this is reflected in the inhibition of a number of physiological responses following an inflammatory insult, including inhibition of vascular dilation and permeability (13), a decrease in leukocyte migration (14), and induction of apoptosis (14, 15). However, not all actions of glucocorticoids are immunosuppressive, and emerging data suggests that glucocorticoids may also have proinflammatory actions. For example, basal levels of glucocorticoids are required for the production of epinephrine by the adrenal glands and for proper signaling by norepinephrine both of which mediate stress-induced increases in immune activity (16, 17). Additionally, glucocorticoids have been shown to have a priming effect and can augment the immune response to LPS (18). Finally, the TNF-α-mediated increase of Toll-like receptor 2 is enhanced by glucocorticoids (19).

The innate immune system is regarded as the first line of defense that is able to discriminate between self and non-self.

* This work was supported, in whole or in part, by a National Institutes of Health grant 1ZIAES090057-15 (intramural research program of the NIEHS).
‡ The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.
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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 44, pp. 38703–38713, November 4, 2011
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JOURNAL OF BIOLOGICAL CHEMISTRY 38703
Glucocorticoids Enhance NLRP3 Expression and Function

To accomplish this, the innate immune system employs a family of receptors known as pattern recognition receptors (e.g., Toll-like receptors). These receptors are able to detect and activate the innate immune system to a wide variety of invariant microbial motifs known as pathogen-associated molecular patterns or endogenous molecules as the result of injury known as danger-associated molecular patterns (20). Recently, a new class of intracellular pattern recognition receptors has also been described, known as the NOD-like receptors (NLRs). This family consists of 22 members in humans and is broken up into three subfamilies; the NOD family (NOD1–5 and class II trans-activator); the NLRP family (NLRP1–14); and the IPAF family (NLRC4 (ICE protease activating factor) and neuronal apoptosis inhibitory protein) (21). Of the 22 members, NLRP1, NLRP3, and NLRC4 are able to assemble and form a complex known as the inflammasome (21). In response to a wide variety of bacterial and viral pathogens, inflammasomes regulate the activation of caspase-1 and the subsequent maturation and release of proinflammatory cytokines such as IL-1β and IL-18 (22). The current understanding of inflammasome activation in macrophages suggests that two signals are required: 1) a priming signal, usually through TLR activation (e.g., LPS), which enhances the transcription of IL-1β and 2) the detection of an inflammasome “agonist” (23, 24).

NLRP3 is one of the most well-characterized NLRs and is activated by a broad range of molecules, including bacterial and viral RNA, bacterial products (e.g., LPS and muramyl dipeptide), and particulate matter (e.g., silica and aluminum) (25). It is currently unknown how the NLRP3 inflammasome is able to respond to such a wide variety of agonists; however, it is widely accepted that low intracellular K+ or the generation of reactive oxygen species (ROS) are triggers for activation (26, 27). Of significant interest is the fact that the NLRP3 inflammasome is also activated by a number of endogenous molecules released following cellular damage or elevated in disease states, including extracellular ATP (23), hyaluronan (28), amyloid β (29), glucose (30), and uric acid (31). Activation of the NLRP3 inflammasome by these danger-associated molecular patterns indicates that there is a potential link between the innate immune system and the physiological response to danger and stress. Although there has been much work regarding molecules that activate the NLRP3 inflammasome, there is very little known regarding the transcriptional regulation of NLRP3. The promoter region of NLRP3 contains binding sites for AP-1, Sp1, Ets, and Myb (32), and expression is enhanced following activation of NF-κB (33, 34). Therefore, we sought to determine the actions of glucocorticoids on regulating both the expression and function of NLRP3. We show that NLRP3 is a glucocorticoid-responsive gene in cultured and primary macrophages. This regulation was specific to differentiated macrophages and was associated with increased levels of NLRP3 intracellularly and enhanced maturation and release of IL-1β in response to extracellular ATP. In addition, glucocorticoids also enhanced the secretion of pro-IL-6 and TNF-α and anti-inflammatory cytokines (IL-10) in LPS-primed macrophages stimulated with ATP. Together, these data demonstrate that glucocorticoids can enhance the initial inflammatory response, providing evidence for a proinflammatory role for glucocorticoids specifically in response to a danger signal.

EXPERIMENTAL PROCEDURES

Reagents—Dexamethasone (Dex), RU486, estradiol, aldosterone, and testosterone were all purchased from Steraloids, Inc. RPMI was purchased from In Vitrogen. Heat-inactivated fetal calf serum was purchased from Atlanta Biologicals. Charcoal-stripped heat-inactivated FBS and the human inflammatory cytokine bead array kit were purchased from Thermo Fisher Scientific. Ultra pure Escherichia coli (0111:B4 strain) was purchased from InvivoGen. Penicillin/streptomycin, HEPES (pH 7.0), and ATP were purchased from Sigma Aldrich. Anti-caspase-1, anti-IL-1β, and anti-GR pS211 antibodies were from Cell Signaling Technology. Anti-NLRP3 was purchased from Axxora, LLC. The anti-GR antibody was produced in our laboratory (35). Phorbol 12-myristate 13-acetate was purchased from EMB Biosciences. Ac-YVAD-CMK was purchased from Enzo Life Sciences. TaqMan® RT-PCR primer probes were purchased from Applied Biosystems.

Cell Culture—THP-1 cells were maintained in RPMI medium supplemented with 10% heat-inactivated FBS, 50 µM β-mercaptoethanol, 25 mM HEPES (pH 7.0), and 100 units/ml penicillin/streptomycin (complete RPMI). THP-1 cells were differentiated into macrophages as described previously (26). Briefly, 2 × 10⁶ cells per well of a 6-well dish (Costar) were incubated with 0.5 µM phorbol 12-myristate 13-acetate for 3 h at 37 °C in serum-free RPMI supplemented with 25 mM HEPES (pH 7.0). Adherent cells were then washed with PBS and cultured overnight in complete RPMI. The following morning, adherent cells were washed twice with PBS, and 2 ml of RPMI supplemented with 10% charcoal-stripped FBS was added in the absence or presence of 1 µg/ml of UltraPure LPS for 1 h prior to the addition of the indicated agonists. Where indicated, cells were treated with 1 µM RU486 (5 min) or 10 µM YYAD (0.5 h) prior to the addition of Dex (100 nM) or ATP (500 µM). For siRNA experiments, THP-1 cells were resuspended at 2 × 10⁶ cells/ml in 4D-Nucleofector™ X (Lonzza) and mixed with 600 pmol of either non-targeting control or NLRP3-specific ON-TARGETplus SMARTpool siRNAs (Dharmacon) and electroporated per the manufacturer’s instructions.

Isolation of Bone Marrow Macrophages—All of the experiments were performed in accordance with the NIHES, National Institutes of Health Institutional Care and Use Committee. Wild type C57BL/6 mice were obtained from The Jackson Laboratory. Marrow was flushed from femoral, and tibia bones using bone marrow media (DMEM supplemented with 2 mM l-glutamine, 10% L929-conditioned media, 10% FBS, and 100 units/ml penicillin/streptomycin). Cells were then centrifuged (2200 rpm, 5 min, 4 °C) and resuspended in 1 ml of ammonium-chloride-potassium lysis buffer for 1 min on ice. The volume was then adjusted to 10 ml with sterile PBS. Cells were centrifuged as above, counted, and plated at 1.5 × 10⁶ cells/well of a 12-well plate. Cells were cultured at 37 °C and 7% CO₂ in 1 ml of bone marrow medium/well and fed on Day 5 with 0.5 ml bone marrow medium. On the morning of day 6, cells were washed with PBS, and 1 ml of stripped DMEM (2 mM l-glutamine and 10% charcoal-stripped FBS) was added 1 h prior to the addition.
of dexamethasone. Cells were stimulated for 6 h with dexamethasone, and duplicate samples were processed for real-time PCR and Western blotting.

Isolation of Primary Human Monocytes—Protocol and procedures were approved by the NIEHS Institutional Review Board. Healthy volunteers were recruited to the NIEHS Clinical Research Unit and underwent phlebotomy. Whole blood (50 ml) was collected, and monocytes were isolated using Ficoll (Sigma Aldrich). Monocytes (2 × 10⁶/well of a six-well plate) were allowed to attach for 2 h at 37 °C in complete RPMI medium. Adherent cells were washed with PBS, and 2 ml of complete RPMI supplemented with GM-CSF (40 μg/ml) was added. Cells were allowed to differentiate in this medium for 7 days. On day 8, cells were washed with PBS and placed in stripped RPMI (RPMI supplemented with 10% charcoal-stripped FBS) for 1 h prior to the addition of Dex (100 nM, 6 h).

Real-time PCR Analysis—Following stimulation, cellular RNA was harvested from cells using the Qiagen RNeasy mini-kit. mRNA was amplified using the TaqMan™ universal one-step RT-PCR procedure on the 7900HT sequence detection system with specific primer probe sets from Applied Biosystems (Foster City, CA). Each primer probe set was analyzed in duplicate from at least three separate sets of RNA. Total mRNA levels were normalized to cyclophillin B (PPIB) and are presented relative mRNA levels (mean ± S.E.).

Western Blot Analysis—Following the indicated treatments, cells were washed twice with PBS and directly lysed in 2X SDS-PAGE sample buffer supplemented with β-mercaptoethanol. Samples were sonicated and boiled for 5 min, and then an equal volume was separated by 4–20% ReadyGel Tris-Gly gels (Bio-Rad). Proteins were then transferred to nitrocellulose and incubated with primary antibodies to NLRP3, GR, caspase-1, IL-1β (all 1:1000), or β-actin (1:20,000) overnight at 4 °C. Blots were washed extensively and incubated with a mixture of goat anti-rabbit Alexa Fluorophore 680-conjugated (Molecular Probes) and goat anti-mouse IRDye 800 conjugated secondary (Rockland Immunochemicals) antibodies at 1 h at room temperature and developed using the Odyssey LiCor imaging system. To monitor the release of mature IL-1β (the p17 isoform), an equal volume of cell culture supernatant was mixed with 5X SDS buffer, boiled for 5 min, and then separated on 4–20% ReadyGel Tris-Gly gels (Bio-Rad). Protein was then transferred to nitrocellulose and incubated with anti-IL-1β (1:500) overnight at 4 °C. Blots were developed using the Amersham Biosciences ECL Plus chemiluminescent detection system (GE Healthcare).

Flow Cytometry—The release of pro- and anti-inflammatory cytokines into cell culture supernatant was analyzed using the BD™ Cytometric Bead Array human inflammatory cytokines kit (BD Biosciences) per the manufacturer’s instructions. Briefly, 50 μl of cell culture supernatants were incubated with the capture beads for 1.5 h at room temperature. Following calibration, capture beads were washed extensively, and data were collected on the LSR II (BD Biosciences) and analyzed using the BD CBA Software available through BD Biosciences. Absolute measurements of each cytokine were determined from a standard curve provided by the manufacturer.

**RESULTS**

Glucocorticoids Induce Rapid and Sustained Increase in NLRP3 mRNA and Protein—Based on the above results, we used THP-ΜΦ as a model system and determined the effects of glucocorticoids on the expression of NLRP3 over 24 h. Dexamethasone led to a rapid induction of NLRP3 mRNA (within 1 h) that was sustained for up to 8 h (Fig. 2A, left panel). This expression also led to a 2.5-fold increase in NLRP3 protein within 4 h that was sustained for up to 8 h (Fig. 2A, right panel). Consistent with the induction of mRNA (Figs. 1A and 2B), the presence of LPS did not affect the ability of Dex to induce the expression of NLRP3 protein (Fig. 2B, right panel). The expression of NLRP3 was also induced to a similar extent as Dex by the naturally occurring glucocorticoid, cortisol (Fig. 2C). We hypothesized that the rapid induction of NLRP3 by glucocorticoids was a primary effect of GR-dependent transcription rather than a secondary effect. To evaluate this question, we pretreated cells with cyclohexamide to inhibit protein synthesis prior to glucocorticoid treatment, effectively eliminating secondary effects of glucocorticoid signaling. Neither the induction of IL12 (Fig. 2D) nor NLRP3 (Fig. 2E) was affected by cyclohexamide pretreatment, suggesting that ligand-bound GR directly enhances the regulation of the NLRP3 gene. These data indicate that NLRP3 is rapidly and directly regulated by glucocorticoids.
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A. GILZ

- Relative mRNA
- Veh vs. Dex

B. NLRP3

- Relative mRNA
- Veh vs. Dex

C. NR3C1

- Relative mRNA
- THP vs. THP-MΦ

D. Human MΦ

- GR
- Actin

E. Human MΦ

- Relative NLRP3 mRNA
- Veh vs. Dex

F. Murine MΦ

- Relative NLRP3 mRNA
- Veh vs. Dex
Glucocorticoids Specifically Regulate NLRP3 but Not Other Inflammasome Components—Based on the rapid and direct regulation of NLRP3 expression by glucocorticoids, we hypothesized that glucocorticoids potentially prime the innate immune system to respond to cellular damage and stress. Therefore, we examined the effect of glucocorticoids on the release of IL-1β in response to extracellular ATP (23). ATP has the potential to activate the NLRP3 inflammasome by one or a combination of mechanisms: 1) potassium efflux through P2X receptors (23); 2) generation of ROS (41); and 3) large pore formation allowing agonists to enter the cell (42). We initially determined whether the presence of ATP would affect the glucocorticoid-dependent increase in NLRP3. Fig. 3A shows that ATP alone had no effect on the levels of NLRP3 mRNA and, when administered concomitantly with Dex, did not affect the glucocorticoid-dependent increase in NLRP3 mRNA or protein (Fig. 3A). In fact, the combined treatment led to a slight increase in expression over Dex alone (Fig. 3A). We then examined two other components of the NLRP3 inflammasome, IL-1β and caspase-1. Glucocorticoids have been shown to reduce the transcription of IL-1β in other cells (43, 44); however, in LPS-primed THP-MΦ, Dex had no effect on the levels of IL-1β mRNA or protein (Fig. 3B). In contrast, ATP significantly enhanced the level of IL-1β mRNA in THP-MΦ, which was reflected by an increase in the 32-kDa proform of IL-1β (IL-1β (p32)) intracellularly (Fig. 3B). When applied concomitantly, Dex was able to blunt the ATP-dependent increase in IL-1β mRNA (Fig. 3B). The decrease in the levels of IL-1β (p32) may be reflective of enhanced turnover. Finally, Dex treatment resulted in a slight decrease in caspase-1 mRNA, but there was no change in caspase-1 at the protein level by any of the treatments tested (Fig. 3C).

Glucocorticoids Enhance Release of IL-1β and Sensitize NLRP3 Inflammasome to Extracellular ATP—We evaluated the activity of the NLRP3 inflammasome in response to extracellular ATP, we assayed for the release of the mature form of IL-1β (IL-1β (p17)) into cell culture supernatants of LPS-primed THP-MΦ. Treatment of THP-MΦ for 6 h with ATP (500 µM) enhanced the release of the mature form of IL-1β (p17) (Fig. 4A). Dex alone or in combination with ATP significantly enhanced the release of IL-1β (p17) (Fig. 4A), consistent with previous results demonstrating that the level of NLRP3 expression determines its activity (33). Pretreatment with the GR antagonist, RU486, inhibited the GR-dependent increase in NLRP3 and release of IL-1β, providing further evidence for a direct effect of GR in regulating NLRP3 expression and function (Fig. 4A).

To distinguish whether the enhanced secretion of IL-1β was specific to glucocorticoids or was affected by other steroids, LPS-primed THP-MΦ were treated with a variety of steroids in presence of extracellular ATP. Only concomitant treatment with glucocorticoids (Dex or cortisol) led to an increase in the release of IL-1β (Fig. 4B, upper panel), which was inhibited by the GR antagonist RU486. Furthermore, Dex led to a dose-dependent increase in IL-1β release (Fig. 4B, lower panel). We examined whether glucocorticoids could also sensitize the cells to ATP, THP-MΦ were either pretreated with glucocorticoids or stimulated with lower concentrations of ATP. Consistent with a role in priming macrophages to respond to ATP, glucocorticoid pretreatment significantly enhanced the release of IL-1β to shorter exposures of ATP (500 µM) (Fig. 4C, upper panel) and sensitized the cells to release IL-1β in response to lower concentrations of ATP (Fig. 4C, lower panel). In the absence of Dex, there were no detectable levels of IL-1β released following a 6 h stimulation with either 50 or 100 µM ATP (Fig. 4C, lower panel); however, Dex treatment led to a significant increase in IL-1β secretion with both 50 and 100 µM ATP (Fig. 4C). To confirm that the enhanced secretion of IL-1β is dependent on NLRP3, THP-1 cells were transfected with non-targeting control or NLRP3-specific siRNAs. Despite only being able to achieve an ~50% reduction in the level of NLRP3 (Fig. 4D), the ATP-dependent and glucocorticoid-enhanced secretion of IL-1β was reduced significantly as compared with non-targeting control (Fig. 4D). Finally, pretreatment with the specific caspase-1 inhibitor Ac-YVAD completely inhibited the ATP-dependent and glucocorticoid-enhanced release of IL-1β (Fig. 4E), consistent with assembly and activation of the NLRP3 inflammasome. Moreover, Ac-YVAD did not inhibit the glucocorticoid-dependent increase in NLRP3 (Fig. 4E). Thus, the glucocorticoid-dependent increase of NLRP3 is dependent on GR and enhances the maturation and release of IL-1β (p17). Additionally, glucocorticoids sensitize macrophages to respond to a wide range of ATP concentrations and durations, enhancing IL-1β release to potentially promote the initiation of the inflammatory response.

Glucocorticoids Enhance Secretion of Proinflammatory Cytokines in Response to ATP Stimulation—The studies described above led us to ask whether glucocorticoids would enhance the release of other proinflammatory cytokines in response to ATP. Acute stress has been shown to prime the immune system and enhance the release of TNF-α, IL-1β, and IL-6 (45, 46). Additionally, it recently has been shown that glucocorticoids enhance the release of IL-6 from endothelial cells in response to ATP (47). Therefore, supernatants from LPS-primed THP-1 and THP-MΦ were assayed for the secreted forms of the following pro- and anti-inflammatory cytokines by flow cytometry: IL-8, IL-1β, IL-6, IL-10, TNF-α, and IL-12 p70. In the undifferentiated state, cytokine release from THP-1 was mini-

FIGURE 1. Glucocorticoids enhance the expression of NLRP3 in human macrophages. THP-1 (2 × 10^6) cells were either left untreated (THP) or differentiated into macrophages by phorbol 12-myristate 13-acetate (PMA) (THP-MΦ) and treated as described under "Experimental Procedures." The level of GILZ mRNA (A), NLRP3 mRNA (B), or GR mRNA (C) was normalized to cyclophilin B (PPIB), and data are presented as relative mRNA (mean ± S.E.; n = 4). THP or THP-MΦ were harvested and blotted for total GR or actin (D). Shown are representative blots from four independent experiments. Human monocytes (2 × 10^6) were differentiated to macrophages and treated as described under "Experimental Procedures." The level of NLRP3 mRNA (E, left panel) was normalized to cyclophilin B (PPIB), and data are presented as relative mRNA (mean ± S.E.; n = 3). Duplicate samples were processed for Western blot (E, right panel). Shown are representative blots for NLRP3 and actin. Bone marrow monocytes (1.5 × 10^6) were differentiated to macrophages and treated as described under "Experimental Procedures." The level of NLRP3 mRNA (F, left panel) was normalized to cyclophilin B (PPIB), and data are presented as relative mRNA (mean ± S.E.; n = 4). Duplicate samples were processed for Western blot (F, right panel). Shown are representative blots for NLRP3 and actin. Veh, vehicle.
mal and not affected by ATP or Dex (supplemental Fig. S1). In contrast, the secretion of IL-1β from THP-MΦ was evident following a 6-h treatment with either ATP or Dex alone (Fig. 5, A and B). Consistent with our previous results (Fig. 4), this was enhanced significantly when ATP and Dex were administered concomitantly (Fig. 5, A and B). Interestingly, ATP and Dex

FIGURE 2. Glucocorticoids rapidly and directly increase the levels of NLRP3 mRNA and protein. Following differentiation, THP-MΦ were treated with Dex (100 nM) in the absence of LPS priming (A). Samples were processed for RT-PCR analysis of NLRP3 (mean ± S.E., n = 3) or Western blotting of NLRP3 and GR (right panel). Shown is a representative Western blot from one of three independent experiments. Following differentiation, THP-MΦ were treated with Dex (100 nM) in the presence of 1 µg/ml of LPS (B). Following differentiation, THP-MΦ were treated with Dex (100 nM) or cortisol (Cort; 500 nM) over 24 h (C). Samples were processed for Western blotting of NLRP3. Shown is a representative Western blot from one of three independent experiments. THP-MΦ were incubated with 10 µg/ml cyclohexamide (CHX) for 1 h prior to the addition of Dex (100 nM) for 1 and 6 h. Cellular RNA was isolated and total mRNA for GILZ (D) and NLRP3 (E) was determined as described previously (mean ± S.E., n = 3).

A.

B.

C.

D.

E.
alone resulted in the accumulation of TNF-α in cell culture supernatants. Similar to IL-1β, this was also significantly enhanced by the concomitant administration of ATP and Dex (Fig. 5, A and B). We also observed an increase in the secretion of IL-6 by concomitant treatment of ATP and Dex (47); however, the absolute level of IL-6 secreted was much lower than IL-1β or TNF-α (Fig. 5). ATP was also able to induce the secretion of IL-10, whereas Dex by itself did not have an effect on IL-10 secretion (Fig. 5, A and B). However, when administered concomitantly, Dex significantly enhanced the ATP-mediated secretion of IL-10. These data suggest that glucocorticoids are enhancing signaling events in response to ATP stimulation. ATP induced the secretion of IL-8, but this was not changed in the presence of Dex. Finally, there was no detectable change in the amount of IL-12 detected. Together, these results show that glucocorticoids enhance the secretion of specific pro-inflammatory cytokines from macrophages in response to extracellular ATP.

DISCUSSION

Glucocorticoids are central components in the ability of an organism to maintain homeostasis in response to stress. The ability of glucocorticoids to resolve inflammation, which is itself a stressor, has been at the heart of their use therapeutically. However, as inflammation is a defense mechanism by which a pathogen is eliminated, it would seem counterproductive for glucocorticoids to generally suppress the initial response to insult or injury. Here, we show that glucocorticoids positively regulate the expression of NLRP3 in macrophages, which, in turn, leads to enhanced maturation and release of IL-1β in response to extracellular ATP. Furthermore, glucocorticoids also enhance the ATP-dependent secretion of the cytokines TNF-α and IL-6, providing evidence...
Glucocorticoids sensitize the NLRP3 inflammasome to extracellular ATP and enhance inflammasome function. A, THP-MΦ were pretreated for 1 h with 1 μg/ml LPS in medium supplemented with 10% charcoal-stripped FBS. Cells were then stimulated with ATP (500 μM), Dex (100 nM), or ATP and Dex for 6 h. Where indicated, Ru486 was added to the cells 5 min before Dex stimulation. The maturation and release of IL-1β (p17) was determined in cell culture supernatants by Western blot. Shown are representative blots from four independent experiments.

B, upper panel: THP-MΦ were treated with Dex (100 nM), cortisol (Cort; 500 nM), aldosterone (Aldo; 1 μM), estradiol (Est; 10 nM), testosterone (Test; 10 nM), or ethanol (Veh) for 6 h in the presence of ATP (500 μM), and maturation and release of IL-1β was determined. Shown are representative blots from three independent experiments. Lower panel: LPS-primed THP-MΦ were left untreated (−) or treated with 0, 1, 10, 33.3, and 100 nM Dex in the presence of ATP (500 μM) for 6 h, and the maturation and release of IL-1β (p17) was determined. Shown are representative blots from three independent experiments.

C, upper panel: LPS-primed THP-MΦ were pretreated with Veh (H2O) or Dex (100 nM) for 0, 3, 4, 5, and 5.5 h prior to the addition of ATP (500 μM). IL-1β release was determined as described above. Shown are representative blots from four independent experiments. Lower panel: LPS-primed THP-MΦ were concomitantly treated with Veh (H2O) or Dex and either 100 or 50 μM ATP for 6 h. Shown are representative blots from four independent experiments.

D, 96 h following electroporation of either non-targeting control (NTC) or NLRP3-specific siRNA, LPS-primed THP-MΦ were left untreated (−) or treated with Veh (H2O) or Dex (100 nM) for 6 h in the presence of ATP (500 μM) for 6 h. Shown are representative blots from four independent experiments.

E, LPS-primed THP-MΦ were pretreated for 30 min with either Veh (dimethyl sulfoxide) or YVAD (10 μM) prior to the addition of ATP, Dex, or ATP + Dex. Shown are representative blots from three independent experiments.
FIGURE 5. Glucocorticoids enhance the release of the proinflammatory cytokines, TNF-α, and IL-6 from THP-Mφ. A. THP-Mφ were left untreated (−) or primed with LPS for 1 h prior to stimulation with ATP, Dex, or ATP + Dex for 6 h. Cell culture supernatants were harvested, and cytokine levels were determined by flow cytometry using a cytometric bead array as described under “Experimental Procedures.” Shown are representative flow cytometric plots from five individual experiments. B, cytokine levels were quantitated as described under “Experimental Procedures.” Data are expressed as mean ± S.E. (n = 5).
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that glucocorticoids enhance the proinflammatory response in response to a danger signal.

The inflammasome is a large, multiprotein complex that is responsible for the activation of caspase-1 and subsequent cleavage of pro-IL-1β and pro-IL-18 into their mature, biologically active forms. Of the 22 NLRs that have been identified to date, three have been shown to truly activate caspase-1: NLRP1, NLRP3, and NLRC4 (25). Although NLRP1 and NLRC4 have only been shown to respond to bacterial toxins, NLRP3 has a much broader spectrum of agonists that it can respond against (25). This includes products released by cellular damage or stress and, thus, could provide a link between the initiation of inflammation and the hypothalamic-pituitary-adrenal axis. The specific role of GR in macrophages has been studied using a tissue-specific, conditional deletion of GR. In line with the anti-inflammatory properties of glucocorticoids, mice lacking GR in myeloid cells were more susceptible to LPS-induced death (48) and inflammation associated with contact allergy (49). Interestingly, we show that NLRP3 expression is induced by glucocorticoids in both cultured and primary human and mouse macrophages but not in monocytes (Fig. 1). Furthermore, this effect was rapid and direct (Fig. 2). The difference in responsiveness between THP-1 and THP-MΦ is not due to a lack of GR expression (Fig. 1, C and D) or ability to induce gene transcription, as GILZ mRNA was increased to a similar, if not greater, extent as compared with macrophages (Fig. 1A). These data are consistent with observations demonstrating that glucocorticoids directly affect innate immunity by enhancing the expression of TLR2 (19). Together, our findings raise the possibility that the stage of myeloid differentiation and the cellular environment dictates the actions of GR from a pro- or anti-inflammatory perspective.

An increase in cellular NLRP3 levels is necessary but not sufficient for processing and release of mature IL-1β (33). Consistent with this view, we show that the glucocorticoid-dependent increase in NLRP3 levels enhances IL-1β secretion in response to extracellular ATP (Figs. 4 and 5). The release of submicromolar concentrations of ATP into the extracellular space is commonly associated with autocrine and paracrine signaling, regulating a variety of cellular functions (50, 51). On the other hand, activation of the inflammasome in vitro has typically been associated with millimolar concentrations of extracellular ATP (52) and is thought to induce pore formation and large changes in the intracellular ionic milieu (42). The finding that glucocorticoids not only enhance the expression and activity of NLRP3 but also sensitize cells to lower concentrations of ATP (Fig. 4C) has considerable implications for processes that result in micromolar levels of extracellular ATP in vivo, such as inflammation (53), damaged and dying tissue (54), or chemotherapy (55). In addition to changes in NLRP3, induction of P2Y2 by glucocorticoids could be contributing to this sensitization and providing another pathway for ATP and GR to cross-talk (47).

Systemic administration of glucocorticoids has generally been associated with suppression of the immune system, particularly in response to LPS (3). However, in certain instances, glucocorticoids can enhance the secretion of IL-1β, TNF-α, and IL-6 in vivo or in cultured cells if administered prior to the LPS challenge (45, 46). Here, we have shown that glucocorticoids can enhance the secretion of these cytokines, which is largely dependent on the presence of extracellular ATP (Fig. 5). These data support our hypothesis that glucocorticoids are enhancing the immune response following detection of damaged or dying cells. However, we cannot rule out that other potential signaling pathways downstream of GR activation also are involved. For instance, glucocorticoids can generate ROS (56) and could contribute to NLRP3 activation in two ways: 1) GR-dependent induction of NLRP3 and and 2) enhancing ROS signaling downstream of ATP. Consistent with this is the observation that Dex alone was able to promote IL-1β maturation from THP-MΦ (Fig. 5). Additionally, glucocorticoids can induce the expression of TXNIP (57), which is able to associate with and activate NLRP3 following ROS generation in response to glucose (30).

The ability of glucocorticoids to sensitize the NLRP3 inflammasome to ATP could also play a role in enhancing IL-1β release during sterile inflammation. For example, NLRP3 is activated by elevated levels of glucose (30), cholesterol crystals (58), and amyloid β (29) while also participating in insulin resistance (59) and type 2 diabetes (60). It is well known that, in the periphery, elevated levels of glucocorticoids, either from exogenous or endogenous sources, are associated with elevated glucose levels, insulin resistance, diabetes, and atherosclerosis (61). Furthermore, in the central nervous system, glucocorticoids have been shown to be proinflammatory, increasing the levels of IL-1β and TNF-α in microglial cells of the hippocampus (18). It is interesting to hypothesize that the data presented here provide a link between glucocorticoids, NLRP3 activation, and progression of these metabolic and CNS diseases.

In conclusion, we have shown that glucocorticoids rapidly induce the expression of NLRP3 mRNA specifically in cultured and human and mouse macrophages. This is associated with enhanced IL-1β secretion in response to extracellular ATP and is specific for glucocorticoids as only Dex and cortisol enhanced expression and dose-dependently enhanced function. The enhanced expression of NLRP3 primes the inflammasome to respond to lower concentrations of ATP. In addition, glucocorticoids also enhance the secretion of the proinflammatory cytokines TNF-α and IL-6 in response to extracellular ATP, providing evidence for a model by which glucocorticoids enhance the inflammatory response to specific danger signals.

Acknowledgment—We thank Dr. Robert Oakley for critically evaluating this manuscript.

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