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Upregulations of Glucocorticoid-Induced Leucine Zipper by Hypoxia and Glucocorticoid Inhibit Proinflammatory Cytokines under Hypoxic Conditions in Macrophages

Yan Wang,*1 Yuan-Yuan Ma,*1 Xing-Lei Song,* Hao-Yu Cai,* Ji-Cheng Chen,* Liang-Nian Song,† Rui Yang,* and Jian Lu*

Hypoxia and inflammation often develop concurrently in numerous diseases, and the influence of hypoxia on natural evolution of inflammatory responses is widely accepted. Glucocorticoid-induced leucine zipper (GILZ) is thought to be an important mediator of anti-inflammatory and immune-suppressive actions of glucocorticoid (GC). However, whether GILZ is involved in hypoxic response is still unclear. In this study, we investigated the effects of hypoxic exposure and/or the administration of dexamethasone (Dex), a synthetic GC on GILZ expression both in vitro and in vivo, and further explored the relationship between GILZ and proinflammatory cytokines IL-1β, IL-6, and TNF-α under normoxic and hypoxic conditions. We found that hypoxia not only remarkably upregulated the expression of GILZ, but also significantly enhanced Dex-induced expression of GILZ in macrophages and the spleen of rats. ERK activity is found involved in the upregulation of GILZ induced by hypoxia. Inhibiting the expression of GILZ in RAW264.7 cells using specific GILZ small interfering RNA led to a significant increase in mRNA production and protein secretion of IL-1β and IL-6 in hypoxia and abrogated the inhibitory effect of Dex on expression of IL-1β and IL-6 in hypoxia. We also found that adrenal hormones played pivotal roles in upregulation of GILZ expression in vivo. Altogether, data presented in this study suggest that GILZ has an important role not only in adjusting adaptive responses to hypoxia by negatively regulating the activation of macrophages and the expression of proinflammatory cytokines, but also in mediating the anti-inflammatory action of GC under hypoxic conditions. The Journal of Immunology, 2012, 188: 222–229.

Hypoxia and inflammation often present simultaneously in numerous diseases, such as the high mountain sickness, ischemic diseases, chronic inflammatory diseases, and tumor progressions (1–6). Immune cells are exposed to hypoxia during systemic hypoxia and when they migrate to hypoxic pathological sites (7). Oxygen availability is proved to be a critical regulator of functional behaviors of the immune cells. Recent studies demonstrated that hypoxia can activate macrophages, dendritic cells, and monocytes, which are involved in inflammatory response by altering gene expression and cytokine (such as IL-1β, IL-10, and IL-8, etc.) secretion (8–11). Hypoxic stress could affect several independent transcriptional regulators related to adaptive and inflammatory responses, in which hypoxia-inducible transcription factor-1 (HIF-1) and NF-kB are ones that play the central roles (12–14). Although the existence of the relationship between tissue hypoxia and inflammatory response is widely accepted, ways to achieve precise regulations of hypoxia on the immune cells and the exact influence of hypoxia on the inflammatory responses remain elusive.

Glucocorticoids (GCs) secretion in response to atmospheric hypoxia due to a high altitude in humans has been extensively documented. GCs play essential roles in the body’s adaptation to hypoxic environments and in homeostasis maintenance by regulating the expression of genes (15, 16) and have therefore been widely used to treat the high mountain sickness (16, 17) and severe tissue damage due to hypoxia and ischemia (18, 19). The effect of GC is due, at least in part, to its immunosuppressive and anti-inflammatory effects (20, 21). Most of the anti-inflammatory effects of GC are mediated by GC receptor (GR), which can modulate gene expression through either a direct interaction with GC response element in the promoter region of target genes (22) or an interference with other transcription factors, such as NF-kB and AP-1, to inhibit their transcriptional activity (23–25). In recent years, more researchers are interested in the pathophysiologic mechanism behind the interaction between hypoxia and glucocorticoid-mediated cellular responses. It was reported that the expressions of several target genes of HIF-1 were mediated by GC (26); until now, however, only a few target genes of GR are known to be involved in the GC action in tissue and systemic adaptation to hypoxia.

Glucocorticoid-induced leucine zipper (GILZ) is a 137-aa protein that was originally identified as a dexamethasone (Dex)-inducible gene. GILZ is widely distributed in various tissues (27, 28) and highly expressed in tissues of the immunological system (such as the thymus, bone marrow, spleen, and lymph nodes) (27). The first suggestion that GILZ plays an important role in GC immunomodulation came from the discovery that GC
upregulates GILZ, mainly in lymphoid organs, and GILZ expression selectively protects T cells from apoptosis induced by treatment with anti-CD3 mAb (27).

GC-induced upregulation of GILZ inhibits the activation of monocytes/macrophages by LPS and production of inflammatory mediators and proinflammatory cytokines (29). Therefore, GILZ is thought to be a new kind of important mediator of GC action. Recent studies have demonstrated that the anti-inflammatory and immunosuppressive roles of GILZ were achieved by inhibiting NF-κB–mediated gene transcription in macrophages and T cells (13, 30, 31). Some GILZ targets have been identified, including AP-1, Raf-1, and Ras, to be associated with GC effects (32, 33). GILZ was also reported to mediate the anti-inflammatory effect of Dex in human airway epithelial cells (34).

Recent extensive studies have revealed that hypoxia activates macrophages in inflammation (4, 11). Unfortunately, we still do not know how hypoxia regulates the function of immune cells in vivo to prevent them from damaging due to overactivation. In an attempt to elucidate whether GILZ is involved in the hypoxic response and mediates the role of GC in adaptation to hypoxia, we investigated the effect of hypoxia and Dex alone or together on the expression of GILZ in vitro and vivo and further explored the association of GILZ with expressions of proinflammatory cytokines IL-1β, IL-6, and TNF-α in RAW264.7 cells under hypoxia exposure and/or Dex administration.

Materials and Methods

Cell culture

The murine macrophage cell line RAW264.7 was cultured in DMEM (Gibco) supplemented with 10% FBS (PAA Laboratories) at 37˚C under a humidified atmosphere of air containing 5% CO2. Cells were grown to ∼70% confluence and rinsed three times with PBS, then cultured for 12 h in low-glucose DMEM medium containing 5% dextran-coated charcoal-treated FBS to avoid possible interference by serum steroids. Before experiment, the medium was changed to fresh low-glucose DMEM containing 5% steroid-free FBS again, and the cells were incubated in an anaerobic system (Forma Scientific) with different oxygen concentrations (1–3% O2) for different periods of time. Control cells in normoxia were maintained at 37˚C in humidified air containing 5% CO2 for the same time. Oxygen levels were constantly monitored with an oxygen sensor. RAW264.7 cells were incubated with 50–200 µM CoCl2 for 12 h or 200 µM CoCl2 for different times in low-glucose DMEM containing 5% steroid-free FBS. PD98059 inhibitor (100 nM), p38 inhibitor SB203580 (10 µM), ERK inhibitor PD98059 (20 µM), and JNK inhibitor SP600125 (20 µM) were added 1 h before hypoxic stimulation.

Isolation of mouse peritoneal macrophages

BALB/C mice, at 4–8 wk of age, were housed in accordance with guidelines of the Institutional Animal Care Committee of the Second Military Medical University in Shanghai, China. Animals were selected at this age, as they yield a robust number of primary macrophages for subsequent experiments. Macrophages were harvested by peritoneal lavage as described previously (35). Cells were cultured in 10% FBS/DMEM at 37˚C for 3 h. After washing off nonadherent cells, adherent monolayer macrophages were cultured overnight in complete medium. Cells were then exposed to hypoxia as RAW264.7 cells.

Animals and hypoxia exposure

The protocol for the studies was approved by the Institutional Animal Care Committee of the Second Military Medical University in Shanghai, China. The animal care facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Sprague Dawley rats, weighing from 200 and 250 g, were obtained from Shanghai Laboratory Animal Commission. All animals were acclimatized in our animal laboratory for at least 7 d before the experiment, being fed a standard laboratory chow and water.

The randomly selected rats were put in a normobaric hypoxia chamber (40 L; Yangyuan hyperbaric oxygen chamber company, Shanghai, China) and flushed with 5% O2 (a gas mixture of 8% O2 and 92% N2) for different times (36). After hypoxia exposure termination, animals were immediately anesthetized and killed. The spleens and lungs were removed for GILZ detection.

![FIGURE 1. Effect of low oxygen or CoCl2 treatment on the expression of GILZ in RAW264.7 cells. RAW264.7 cells were incubated for 12 h at normal oxygen tension (21% O2) or in different oxygen concentrations (1, 2, or 3% O2). GILZ mRNA was measured by real-time PCR (A). RAW264.7 cells were incubated in hypoxia (1% O2) for the indicated time periods, and GILZ mRNA (B) and protein (C) were measured by real-time PCR and Western blot, respectively. RAW264.7 cells were incubated with different concentrations of CoCl2 for 12 h or 200 µM CoCl2 for different times; GILZ protein was measured by Western blot (D). GAPDH was used as a normalization control for real-time PCR and β-actin as a loading control for Western blot. GILZ protein expression was quantified by densitometric analysis. The results were expressed as fold over normoxia (0 h) controls and representative of three independent experiments. *p < 0.05, **p < 0.01 versus 0 or normoxia.](http://www.jimmunol.org/)

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Adrenalectomy and Dex supplement

The animals were anesthetized by i.p. injection with 3% pentobarbital sodium (1 ml·kg⁻¹ body weight), and adrenal glands were removed by the dorsal approach as described by Fleshner et al. (37). Sham animals underwent the same surgery, except the adrenal glands were left intact (37). Adrenalectomized (ADX) rats were given 0.9% saline ad libitum to compensate for sodium loss after the operation and allowed to recover for 1 wk before Dex supplement and/or hypoxia exposure.

The ADX rats were injected i.m. with 5 mg/kg body weight of Dex (Sigma-Aldrich) dissolved in a 0.9% NaCl solution. Control ADX rats were treated with 0.9% NaCl alone (38). At the same time of Dex supplement, hypoxia exposure was done in the ADX rats of the combined administration group. Rats were anesthetized and killed at 12 h after treatment, and spleens were isolated for GILZ detection.

RNA extraction and real-time PCR analysis

After treatment, total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA), and 2 μg total RNA was reverse transcribed using Reverse Transcription Reagents (Fermentas) in accordance with the manufacturer’s instructions. The mRNA levels of the indicated genes were analyzed in triplicate using SYBR Green PCR Master Mix (Toyobo) on a Mastercycler ep realplex (Eppendorf). The primer sequences used in the PCR reactions were:

- For GILZ in rat: 5’-GCTCAGCCGGGACTGGAACT-3’ (forward) and 5’-AGCG-TGGTGGCCCTAGACAA-3’ (reverse)
- For GILZ in mouse: 5’-CAGCAGCACTCACTGAGAC-3’ (forward) and 5’-ACCACATCCCCTGAGAC-3’ (reverse)
- For IL-1β in mouse: 5’-GACGTGGAAACTGGCAGAAGAG-3’ (forward) and 5’-TTGGTGGTGTTGAGTGTGAG-3’ (reverse)
- For IL-6 in mouse: 5’-TAGTCCTTCCTACCCCAATTTCG-3’ (forward) and 5’-TTGGTGCCTTACCTCTCCTC-3’ (reverse)
- For TNF-α in mouse: 5’-CAATTGT-TACTCAACTGGGACG-3’ (forward) and 5’-GGTTGGCTAGGGTTGCAG-3’ (reverse)
- For GAPDH in mouse: 5’-ATGGTGCTGCAGAAGAGATG-3’ (forward) and 5’-TGGCTGGGGTGTTGAGTGTGAG-3’ (reverse)
- For GAPDH in rat: 5’-ATGGTGCTGCAGAAGAGATG-3’ (forward) and 5’-TGGCTGGGGTGTTGAGTGTGAG-3’ (reverse)

Thermal cycling conditions consisted of an initial denaturing step (95˚C, 2 min) followed by 40 cycles of denaturing (95˚C, 15 s), annealing (60˚C, 15 s), and extending (72˚C, 45 s). The specified mode of reaction was controlled with the melting curve. The mRNA levels were normalized to GAPDH (internal control) using the formula $\Delta\Delta$CT = $\Delta$CT (gene of Dex and/or hypoxia-treated group) − $\Delta$CT (gene of untreated group) and expressed as relative fold of change using the formula: $2^{-\Delta\Delta\text{CT}}$.

Western blot analysis

Whole-cell or tissue extracts were prepared in RIPA lysis buffer, and protein extracts were equally loaded on 12% SDS-polyacrylamide gel and trans-
ferred to nitrocellulose membrane (Millipore). The blots were stained with 0.2% Ponceau S red to ensure equal protein loading. After blocking with 5% nonfat milk in TBST, the membranes were incubated with primary Ab against GILZ (1:500; eBioscience) overnight at 4°C followed by AP-conjugated secondary Ab (1:1000; Chinagen) or HRP-conjugated secondary Ab (1:5000; Rockland Immunochemicals). All blots were reprobed with anti-β-actin Ab (1:10000; Sigma-Aldrich Chemicals) to confirm equal loading among samples. Detection was visualized using the NBT/BCIP staining kit (Beyotime) or ECL assay kit (Pierce) following the manufacturer’s recommended protocol. Subsequently, the relative expression of the protein bands of GILZ (~17 kDa) was quantified by scanning densitometry (ImageJ software; National Institutes of Health). All Western blot densitometry data are expressed relative to levels of the loading control β-actin.

**Small interfering RNA knockdown**

ONTARGET plus SMARTpool small interfering RNA (siRNA) to GILZ (Mouse TSC22D3) was purchased from Dharmacon (Lafayette, CO) along with the ONTARGET plus siCONTROL nontargeting pool to be used as a control. The 200,000 RAW264.7 cells were transfected with 0.1 μM siRNA in a 12-well plate using Dharma FECT4 transfection reagent (Dharmacon) following the manufacturer’s instructions. After the transfection, the cells were allowed to recover for 24 h before Dex treatment and subsequent hypoxia exposure. GILZ knockdown was monitored by Western blot.

**ELISA**

Commercially obtained ELISA kits were used to quantify the levels of immunoreactive mouse IL-1β, IL-6, and TNF-α (R&D Systems, Minneapolis, MN) released from cells into culture media under various experimental conditions. The culture media were collected, centrifuged at 14,000 rpm for 5 min at 4°C, and used in ELISA in 100-μl aliquots. The assays were carried out according to the manufacturer’s instructions in at least three independent experiments. The sensitivity of IL-1β, IL-6, and TNF-α in these assays was 3.8, 5.5, and 7.8 pg/ml, respectively.

**Statistical analysis**

Data are expressed as mean ± SD of at least three determinations. Statistical significance between experimental groups was analyzed by ANOVA, and the significance level was set at p < 0.05.

**Results**

**Hypoxia upregulates the expression of GILZ in RAW264.7 cells**

To determine whether hypoxia regulates GILZ expression, we observed the expression of GILZ mRNA in mouse macrophage RAW264.7 cells exposed in different hypoxic conditions (1, 2, and 3% O2) for 12 h by real-time PCR. As shown in Fig. 1A, GILZ mRNA was significantly upregulated under all oxygen levels for 12 h, and the maximum increase was seen at the 1% O2 level. Then we examined the expression of GILZ in RAW264.7 cells exposed to 1% O2 level for different time intervals (0–24 h) by real-time PCR and Western blot. Increased GILZ mRNA was detected as early as 8 h and peaked after 12 h (2.7-fold of the normoxic controls; p < 0.01) of hypoxic stimulation (Fig. 1B). A similar result was observed in GILZ protein (Fig. 1C).

We also examined the effects of chemical inducers of hypoxia (CoCl2 and dimethylxoylallyl glycine [DMOG]) on GILZ expression at different concentrations for different intervals. The results show that in cells treated with 200 μM CoCl2 for 12 h, GILZ protein was significantly augmented (Fig. 1D), but DMOG did not increase the expression of GILZ mRNA and protein (data not shown).

**Upregulation of GILZ by hypoxia inhibits the expression and secretion of proinflammatory cytokines in RAW264.7 cells**

We next examined the expression of IL-1β mRNA in macrophage RAW264.7 cells exposed to hypoxia (1% O2) for different time durations. As shown in Fig. 2A, hypoxia exposure resulted in a rapid increase of IL-1β mRNA, and its peak level (1.9-fold of that in normoxic controls; p < 0.01) was observed after hypoxia exposure for 4 h.

Then we investigated the relationship between the upregulated GILZ expression by hypoxia and the expression of proinflammatory cytokines in macrophage RAW264.7 cells using specific GILZ siRNA. A significantly decreased GILZ protein expression in normoxia and hypoxia exposures was confirmed by Western blot after GILZ siRNA intervention (Fig. 2B). An inhibiting effect of GILZ siRNA to the expression of GILZ significantly increases IL-1β mRNA (Fig. 2C) as well as IL-1β protein levels in culture medium detected by ELISA assay in normoxia and hypoxia exposures (Fig. 2D). The expression of other two proinflammatory cytokines, IL-6 and TNF-α, was further examined, and we found that hypoxia exposure dramatically

FIGURE 3. Hypoxia exposure results in a potentiation of Dex-induced GILZ expression and the inhibition effect of the IL-1β and IL-6 expression in macrophage RAW264.7 cells. RAW264.7 cells were cultured with 100 nM Dex for various time points (0–48 h) (A) or cultured in the presence or absence of 100 nM Dex under normoxic or hypoxic conditions for 12 h. mRNA (B) and protein (C) expression of GILZ was analyzed by real-time PCR and Western blot. RAW264.7 cells were transfected with GILZ siRNA or control siRNA. After 24 h recovery, cells were incubated in hypoxia at 1% oxygen or normoxia with 100 nM Dex for 12 h. At the end of culture, GILZ knockdown was monitored at the protein level by Western blot (D), and the mRNA expression of IL-1β (E) and IL-6 (F) was measured by real-time PCR. GAPDH was used as a normalization control for real-time PCR and β-actin as a loading control for Western blot. GILZ protein expression was quantified by densitometric analysis. The results were expressed as fold over normoxia or control siRNA in normoxia and representative of three independent experiments. *p < 0.05, **p < 0.01 versus 0, control, or control siRNA in normoxia, p < 0.05, ##p < 0.01 versus hypoxia or control siRNA in hypoxia, $p < 0.05 versus Dex or control siRNA plus Dex in normoxia, $p < 0.05, $$p < 0.01 versus control siRNA plus Dex in hypoxia.
upregulated the expression of IL-6, and inhibitory of GILZ expression by siGILZ significantly increased IL-6 mRNA and protein levels in hypoxia (Fig. 2E, 2F). TNF-α mRNA was also slightly increased in hypoxia, and the maximal level of TNF-α mRNA was ∼1.5-fold of that in the control cells after 12 h of hypoxia exposure (p < 0.05). The inhibition of GILZ expression also resulted in an increase in TNF-α mRNA, but the difference was not statistically significant (Fig. 2G, 2H). These results indicated that upregulation of GILZ expression by hypoxia negatively controls the activation of macrophages and contributes to inhibition of IL-1β and IL-6 productions and secretions.

Hypoxia coinduces the expression of GILZ with Dex and enhances the effect of Dex in RAW264.7 cells

Because GILZ is considered an important mediator of GC action in anti-inflammatory effects and immune suppressions, we examined whether GILZ mediated the adaptive role of GC under hypoxia condition. Results demonstrated that Dex alone markedly induced the GILZ mRNA in a time-dependent manner. Approximately 5-fold induction of GILZ mRNA in RAW264.7 cells was observed following 100 nM Dex treatment for 12 h, and continuously increased mRNA for 48 h (21.5-fold of the control; p < 0.01) were detected (Fig. 3A). The treatment of RAW264.7 cells with hypoxia (1% O2) or 100 nM Dex increased GILZ mRNA to 2.6- and 4.3-fold of those in control cells, respectively (p < 0.01). The cotreatment of cells with hypoxia and Dex resulted in a 5.9-fold increase in GILZ mRNA (p < 0.01) (Fig. 3B). Similar results were also observed in GILZ protein levels (Fig. 3C).

We further examined whether the anti-inflammatory effect of Dex was mediated by its induction of GILZ in normoxia and hypoxia exposures. As shown in Fig. 3D, the upregulation of GILZ proteins induced by Dex in normoxia and hypoxia exposures were obviously inhibited by GILZ siRNA. Further experiments demonstrated that Dex significantly inhibited the expressions of IL-1β and IL-6 at the mRNA level, and the inhibitory effects were amplified in hypoxia exposure. Blocking the expression of GILZ by GILZ siRNA significantly attenuated the inhibitory effect of Dex on the expression of IL-1β and IL-6 under hypoxic conditions (Fig. 3E, 3F). These results indicate that GILZ may be an important mediator of the anti-inflammatory action of Dex, and hypoxia can enhance the effect of Dex by coinduction of GILZ expression in macrophages.

ERK activity is involved in the induction of GILZ by hypoxia in RAW264.7 cells

Because hypoxia has been reported to be able to activate kinases of the MAPK family, such as ERK, JNK, p38MAPK, and the PI3K/Akt pathway in RAW264.7 cells and several other cells (39–41), we investigated the effect of inhibitors of these pathways on the expression of GILZ in hypoxia to determine whether these signaling pathways are involved in induction of GILZ by hypoxia. The inhibitor concentrations were decided according to previous studies in RAW264.7 cells (42–44). In these experiments, RAW264.7 cells were incubated with wortmannin (PI3K inhibitor), SB203580 (p38 inhibitor), SP600125 (JNK inhibitor), or PD98059 (ERK inhibitor) 1 h before hypoxic stimulation, and then the expression of GILZ was detected by Western blot. As shown in Fig. 4, administration of cells with inhibitor of PI3K or p38 had no effect on GILZ expression in hypoxia. JNK inhibitor slightly decreased the expression of GILZ, whereas ERK inhibitor markedly inhibited the expression of GILZ in hypoxia, indicating that ERK activity is involved in the induction of GILZ by hypoxia in RAW264.7 cells.

Hypoxia induced the expression of GILZ alone and with Dex in mouse peritoneal macrophages

We further isolated mouse peritoneal macrophages and examined the effect of hypoxia on GILZ expression and found significantly increased GILZ mRNA when primary macrophages were exposed to hypoxic conditions with different concentrations of oxygen (1, 2, and 3% O2) for 12 h (Fig. 5A). GILZ protein production of cells in hypoxia (2% O2) increased to ∼3-fold of that in normoxic controls (p < 0.01) (Fig. 5B). Further experiments showed that, as was the case with RAW264.7 cells, hypoxia and Dex, which each alone could upregulate the expression of GILZ, could also coinduce GILZ mRNA expression and protein production in primary macrophages (Fig. 5C, 5D).

Hypoxia upregulates the expression of GILZ in spleen and lung of rats

GILZ is highly expressed in the immune system, such as the spleen. To determine the in vivo changes to further evaluate the upregulation of GILZ by hypoxia, we examined the effect of hypoxia on GILZ expression in the spleen and lung, which are rich in immune cells, especially macrophages (34, 45). The rats were exposed to hypoxia (8% O2) for different time durations, and the expression of GILZ in the two organs was detected. As shown in Fig. 6A, GILZ mRNA was significantly upregulated in the spleen of rats exposed to hypoxic conditions, and maximal increase of GILZ mRNA (2.6-fold of normoxic control; p < 0.05) was reached at 8 h after hypoxic exposure. Also increased was the GILZ protein level, with the maximal induction appearing at 12 h (3.3-fold of the control; p < 0.01) (Fig. 6B). The GILZ mRNA in lung was found upregulated following hypoxia exposure for 12 h as well.
Hypoxia-induced GILZ expression is dependent on adrenal hormones in the spleen of rats

It has been reported that hypoxia exposure results in high levels of endogenous GCs (mainly corticosterone in rats) in vivo. In this study, ADX rats were used in which endogenous adrenal hormones were eliminated to examine the effect of hypoxia on the expression of GILZ with and without supplementing with Dex, a synthesized GC, which has a high affinity to the GR but not to the mineralocorticoid receptor. As shown in Fig. 7A, GILZ mRNA in the spleen of the ADX rats was decreased to a 44% ($p$, 0.01) level of that in the sham group in normoxia and did not significantly increase following hypoxia exposure. Administration of ADX rats with 100 nM Dex resulted in a significant elevation in GILZ mRNA in normoxia (11.9-fold of that in the ADX group; $p$, 0.01), and the treatment of ADX rats with both Dex and hypoxia led to an even higher expression of GILZ mRNA (15.7-fold of that in the ADX group; $p$, 0.01). A similar result was observed at the protein level (Fig. 7B). These results indicate that adrenal hormones play pivotal roles in upregulation of GILZ expression in vivo under both physiological and hypoxic conditions. Moreover, Dex-induced expression of GILZ can be amplified by hypoxic exposure in vivo.

**Discussion**

Recent studies have demonstrated that macrophages and other immune cells can be activated by hypoxia through altering gene expressions and then undergo marked phenotypic changes to be involved in inflammation response, which is proven to be related to hypoxic tissue damage in hypoxic areas (4, 8, 11). Indeed, in the current study, we demonstrated that hypoxia could upregulate the expression of proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α. Meanwhile, we also noticed that the level of IL-1β mRNA was rapidly increased by hypoxia and gradually decreased with extended hypoxia exposure. Moreover, the level of IL-1β induced (Fig. 6C), indicating that upregulation of GILZ expression by hypoxia does occur in vivo.

**Hypoxia-induced GILZ expression is dependent on adrenal hormones in the spleen of rats**

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FIGURE 7. Additive effect of hypoxia and Dex to upregulate the expression of GILZ in rat spleen. Adult male Sprague-Dawley rats underwent ADX or sham operation; sham group (n = 9) and ADX group (n = 6) stayed in normoxic environment; rats in ADX and hypoxia group (n = 6) stayed in normal-pressure hypoxia chamber filled with 8% O2 and 92% N2 for 12 h, i.m. injected with Dex at a dose of 5 mg/kg alone (n = 6), or together with hypoxia (n = 6) at random. The levels of mRNA and protein of GILZ in rat spleen after different treatments were assessed by real-time PCR (A) and Western blot (B), respectively. GILZ protein expression was quantified by densitometric analysis. The results were expressed as fold over sham group. Each bar represents mean ± SD. **p < 0.01 versus sham group, #p < 0.01 versus ADX group, ##p < 0.01 versus ADX and Dex group.

by hypoxia is not so high. Therefore, we presumed that there might be a protective mechanism of preventing immune cells from overactivation and overexpression of proinflammatory cytokines, alleviating hypoxic injury of cells and tissues in an environment with a lower oxygen level.

It has been proven that GILZ is a mediator of GC action and plays an anti-inflammatory role at least by inhibiting NF-κB–mediated activation of transcription in macrophages and T lymphocytes (13, 30, 31). In this study, we found that hypoxia significantly upregulated the expression of GILZ at both the mRNA and protein levels in macrophage RAW264.7 cells and mouse peritoneal macrophages, and the same effect on GILZ expression was found in the spleen and lung tissues of rats as well. Moreover, hypoxia and Dex could coinduce the expression of GILZ both in vitro and in vivo. Further study demonstrated that inhibiting the expression of GILZ by siRNA markedly increased the expression of IL-1β and IL-6 at the mRNA and protein levels in hypoxia. We also found that Dex could inhibit the expression of IL-1β and IL-6 in RAW264.7 cells under hypoxic condition, and inhibiting the GILZ expression by siRNA could abrogate the effect of Dex. Data presented in this study indicate that GILZ may function as an integrator of hypoxia and GC signaling pathways. It plays an important role not only in the adaptive response to hypoxia by negatively controlling the activation of macrophages and reducing the production of proinflammatory cytokines, but also in mediating the anti-inflammatory effect of GC under a hypoxic environment.

It is well known that HIF-1α plays a central role by regulating the expression of its target genes in hypoxia (15). However, we were not able to find classical HIF-responsive elements in the promoter region of the GILZ gene with the help of several software programs such as Transcription Element Search Software, TFSSEARCH, and TRED for predicting transcription factor binding sites in DNA sequences. Furthermore, DMOG, a cell-permeable prolyl-4-hydroxylase inhibitor that can stabilize HIF-1α, did not increase the expression of GILZ mRNA and protein (data not shown), suggesting that upregulation of GILZ might not be mediated by HIF-1α directly but via other signal transduction pathways or transcription factors. Because hypoxia can activate multiple signaling pathways and has been reported to activate ERK, JNK, and p38, which belong to the MAPK family and PI3K pathway in RAW264.7 cells and some other cells (13, 39–41), we investigated the effect of inhibitors of these pathways on GILZ expression in hypoxia and found that only the ERK inhibitor could markedly inhibit the expression of GILZ in hypoxia, indicating that ERK activity is involved in the induction of GILZ by hypoxia in RAW264.7 cells.

In the present experiments, we found that hypoxia could even upregulate the expression of GILZ in macrophages cultured in the medium containing 5% steroid hormone-free calf bovine serum. However, an upregulation of GILZ by hypoxia in the spleen was observed only in intact rats rather than ADX ones, indicating that upregulation of GILZ by hypoxia is independent of adrenal hormones in vitro; however, in vivo adrenal hormones play pivotal roles in the upregulation of GILZ expression. Yachi and colleagues (46) also reported that upregulation of GILZ in the medial prefrontal cortex and hippocampus of the brain induced by water immersion restraint stress was dependent on activation of the hypothalamic–pituitary–adrenal axis. The reason for the discrepancy between the data obtained in vitro and in vivo is still to be uncovered. It has been reported that positive and negative regulators of GILZ were present. Positive regulators, such as GC, mineralocorticoid (aldosterone) (27, 47), as well as TGF-β, IL-10, IL-4, and IL-13 (31, 48, 49), stimulate the expression of GILZ, whereas negative regulators, such as some proinflammatory cytokines, have an inhibitory effect (34). One possible explanation is that increased inhibitory hormone or factor(s) by hypoxia in vivo antagonize the GILZ-upregulating effect of hypoxia in the absence of adrenal hormones in ADX rats. Although we did not find a significant change of GILZ expression in ADX animals exposed to hypoxia, we observed a marked increase in GILZ mRNA in ADX animals exposed to both hypoxia and Dex compared to Dex alone. How hypoxia enhances the Dex-induced GILZ expression in vivo is still unclear. GILZ is a target gene of GR, and its promoter region contains several GC response elements. A previous study has reported that hypoxia could upregulate the expression of GR and enhance its transcriptional activity in human renal proximal tubular epithelial cells (50). Therefore, further studies are expected to clarify whether enhanced GR activity is involved in the increased expression of GILZ by Dex in hypoxia.

In conclusion, in the current study, we found that hypoxia not only upregulated the expression of GILZ, but also amplified the Dex-induced expression of GILZ in vivo and in vitro. Upregulation of GILZ expression by hypoxia, in which ERK activity is involved, significantly inhibited the expression of proinflammatory cytokines in macrophages. These findings implicated that GILZ might have a protective role by preventing immune cells from overactivation and overproduction of proinflammatory cytokines in hypoxic microenvironments in some diseased areas; moreover, it may be involved in systemic adaptation to hypoxia as an important mediator of GC action.
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Disclosures
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References