Rapid Inhibition of Leptin Signaling by Glucocorticoids

in Vitro and in Vivo*

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Elevated secretion of glucocorticoids (GCs) or hypersensitivity to GCs has a permissive effect on the development of obesity and leads to abnormalities of body fat distribution. Recent studies demonstrated that GCs act as antagonists of leptin in rodents. However, little is known about the interaction between GCs and leptin signaling. In the present study, we investigated the effects of GCs on leptin action in vitro and in vivo. GCs rapidly inhibited the leptin-induced STAT3 phosphorylation in a dose- and time-dependent manner, as assayed by Western blotting using anti-phosphospecific-STAT3 in human hepatoma cell lines (Huh7) transiently expressing long form leptin receptor. GCs also inhibited the leptin-induced JAK2 tyrosine phosphorylation but unaltered the specific binding of [125I]-leptin to the cells. Parallel experiments, however, demonstrated that the inhibitory effects of GCs were not observed in either IL-6- or LIF-induced STAT3 phosphorylation. Furthermore, we examined the feeding behavior and hypothalamic leptin signaling following intracerebroventricular (icv) infusion of GCs prior to icv leptin infusion in Sprague-Dawley rats. The food intake after 24 h of icv leptin injection increased 3-fold in GCs-treated animals. In addition, central infusion of GCs resulted in a marked reduction of hypothalamic STAT3 phosphorylation in response to icv infusion of leptin. To clarify the molecular mechanism by which GCs rapidly reduce leptin-induced JAK/STAT signaling, we examined the intracellular signal transduction pathway potentially mediated by GCs. PD98059, a specific MEK inhibitor, blocked the inhibitory effects of GCs on leptin-induced JAK/STAT activation in Huh7 cells. These results suggest GCs antagonize leptin action by a rapid inhibition of the leptin-induced JAK/STAT pathway partly via MAPK cascade.

Leptin, a hormone secreted by adipocytes (1), acts via a long form of the leptin receptor (OBRb) expressed in specific nuclei of the hypothalamus (2) and regulates food intake and energy expenditure (3–5). Based on sequence homology, OBR belongs to the class I cytokine receptor family, which includes receptors for interleukin-6 (IL-6),1 leukocyte inhibitory factor (LIF), and granulocyte-colony-stimulating factor (G-CSF) (6, 7). OBRb has a long cytoplasmic domain containing several motifs for signal transduction through the JAK/STAT pathway (8). Leptin-OBRb complex formation leads to the induction of tyrosine phosphorylation through its association with JAK2 (8, 9). The activated JAK2 phosphorylates a distal tyrosine (Tyr-1138) (10) on the receptor, similar to STATs proteins (8–10). Although leptin can activate STAT1, STAT3, STAT5, and STAT6 (11, 12), it only activates STAT3 in the rodent hypothalamus (13). Rodents with defective leptin receptors such as db/db mice and Zucker (fa/fa) rats result in morbid obesity and extreme leptin resistance (14, 15). Although mutations of the human leptin receptor gene have been identified (16), obesity in humans is rarely due to defective receptors (17). Most obese humans have elevated serum leptin concentrations that do not induce the expected responses, suggesting reduced sensitivity to leptin (18). Several studies (19–21) reported the mechanism for leptin resistance, including reduced leptin transport across the blood-brain barrier and impaired signaling downstream of the leptin receptor. Orexigenic molecules were reported, and some of them antagonized leptin action in vivo (3, 22, 23).

Hypercortisolism resulting from exogenous administration or endogenous overproduction of glucocorticoids (GCs) is known to cause central obesity associated with hyperinsulinemia and also hyperleptinemia (24). GCs-mediated regulation of cytokine production and action has been extensively investigated. GCs inhibit the transcription of most proinflammatory cytokines such as IL-1, IL-2, IL-6, IL-8, interferon-γ, and tumor necrosis factor-α (25). In addition to the inhibition of a cytokine expression, GCs can often negatively regulate its subsequent signaling pathway (26–28). In contrast, GCs stimulate the expression of the ob gene and leptin secretion in rodents or humans (29, 30). Recent studies demonstrated the ability of GCs to modulate leptin action in vivo. Adrenalectomy enhances the effects of intracerebroventricular (icv) administration of leptin on food intake and body weight in normal rats (31). In the same study, the supplementation of adrenalectomized rats with dexamethasone (DEX) inhibited the enhanced leptin action. It was also demonstrated that central DEX injection induces obesity associated with hyperleptinemia in normal rats (32). These findings suggest that GCs act as counter-regulatory hormones of leptin. However, the molecular basis for the cross-talk between the OBRb and GCs pathway has remained unclear. In the present study, we report the acute effects of DEX on the leptin-induced JAK/STAT pathway in vitro and in vivo. We also

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Abbreviations used are: IL-6, interleukin-6; GCs, glucocorticoids; LIF, leukocyte inhibitory factor; G-CSF, granulocyte-colony-stimulating factor; icv, intracerebroventricular; DEX, dexamethasone; JAK, Janus kinase; STAT, signal transducers and activators of transcription; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; GH, growth hormone.
Western Blotting—In the in vitro study, 40 μl of the cell lysates was mixed with SDS sample buffer, and in the in vivo study, it was a 200-μg protein of the supernatant. The mixture was boiled for 5 min before the samples were applied to SDS-PAGE. After electrophoresis, proteins were transferred to a nitrocellulose membrane and blocked by incubation for 1 h at room temperature with 5% non-fat dry milk in Tris-buffered saline, 0.1% Tween 20 (TBST). The membranes were incubated with anti-phospho-STAT1, STAT3, STAT5, STAT6, or JAK2 antibody (1:1,000) in 5% milk in TBST overnight at 4 °C. After washing three times at room temperature, the membranes were reacted with horseradish peroxidase-conjugated anti-rabbit immunoglobulin (1:2,000) in 1% milk in TBST for 60 min at room temperature, and again washed six times with TBST. The targeted proteins were detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce) following the instructions of the manufacturer. The membranes were stripped in Restore Western Blot Stripping Buffer (Pierce) according to the manufacturer’s instructions. The membranes were then reprobed with anti-STAT1, STAT3 (1:1,000), STAT5, STAT6 (1:200), or JAK2 (1:1,000) antibodies.

Results

Effects of DEX on STAT3 Phosphorylation in Vitro—It is well known that administration of GCs to rodents and humans results in obesity associated with hyperleptinemia in addition to hyperinsulinemia. In this study, we examined the effects of DEX on leptin-induced JAK/STAT signaling pathway using human hepatoma cell lines (Huh7). Due to a lack of endogenous leptin receptor, we transiently transfected OBRb cDNA to Huh7 cells (33). Leptin has been shown to activate STAT1, STAT3, STAT5, and STAT6 in other cell lines (8, 12). We tested which STAT proteins might be involved in leptin signaling in Huh7 cells. Huh7 cells transiently expressing long form leptin receptor (OBRb) were stimulated with 1 nM of leptin at room temperature for 15 min. The cell lysates were subsequently immunoblotted with antibodies for phospho-STAT1, STAT3, STAT5, or STAT6. The bands could only be detected with the antibody for phospho-STAT3. When these membranes were stripped and reprobed with each respective anti-STAT antibody, STAT1, STAT3, STAT5, and STAT6 proteins were all detected in the Huh7 cells (Fig. 1A). We thus tested whether DEX affects the leptin-induced STAT3 phosphorylation in Huh7 cells. The Huh7 cells transiently expressing OBRb were pretreated with various concentrations of DEX sequentially stimulated with leptin. The cell lysates were immunoblotted with anti-phospho-STAT3 antibodies.

To investigate the dose response of inhibitory effects of DEX on leptin-induced STAT3 phosphorylation, the cells expressing OBRb were pretreated with 0–10 nM DEX for 1 h at 37 °C before 1 nM leptin stimulation for 15 min at room temperature. A 1-h DEX pretreatment inhibited the leptin-induced STAT3 phosphorylation in a dose-dependent manner. A significant inhibitory effect of DEX was detected from 0.1 nM DEX pretreatment (Fig. 1B). The leptin-induced STAT3 phosphorylation was completely inhibited by 10 nM DEX pretreatment. Furthermore, to determine the time course of DEX inhibitory effects on leptin-induced STAT3 phosphorylation, the cells were pretreated in 10 nM DEX for various times. The inhibition of leptin-induced STAT3 phosphorylation was detected after a 5-min pretreatment with 10 nM DEX and inhibited completely after 1 h (Fig. 1C). All membranes were stripped and reprobed with anti-STAT3 antibodies. The total amount of STAT3 protein was not affected by DEX.

OBRb is a member of the class I cytokine receptor superfamily, which includes receptors for IL-6, LIF, prolactin, erythropoietin, and growth hormone (GH). We performed parallel experiments with Huh7 cells expressing endogenous IL-6 and LIF receptor. DEX showed no effect on IL-6- or LIF-induced
STAT3 phosphorylation in either a dose- or time-dependent manner (Fig. 1, D and E).

**Effects of DEX on JAK2 Phosphorylation in Vitro**—JAK2 is presumed to be STAT3 tyrosine kinases involved in signaling by the cytokine subfamily to which OBRb belongs. We therefore examined whether DEX affects leptin-induced JAK2 tyrosine phosphorylation by Western blot analysis with anti-phospho-JAK2 antibody. Because Huh7 cells did not have enough endogenous JAK2 protein to be detected by using Western blotting, both JAK2 and OBRb were transiently co-transfected into the cells. When Huh7 cells co-transfected with JAK2 and OBRb were stimulated with 1 nM of leptin for 5 min, JAK2 tyrosine phosphorylation was increased to a greater extent compared with that in cells co-transfected with empty vector and OBRb. The degree of JAK2 tyrosine phosphorylation in response to leptin appears to be correlated with the relative amount of JAK2 protein. However, leptin-induced STAT3 phosphorylation in cells co-transfected with JAK2 and OBRb was almost equal to that in cells co-transfected with empty vector and OBRb (Fig. 2A). In parallel experiments, we examined IL-6- or LIF-induced JAK2 tyrosine phosphorylation in Huh7 cells transfected with empty vector or JAK2 cDNA. We could not detect the tyrosine phosphorylation of endogenous JAK2 in Huh7 cells transfected with empty vector after stimulation by IL-6 or LIF. Each ligand markedly stimulated the tyrosine phosphorylation of JAK2 in cells transfected with JAK2 (Fig. 2B). Similar to the results obtained for STAT3 phosphorylation mediated via leptin, the amount of JAK2 protein did not affect STAT3 tyrosine phosphorylation in response to either ligand. Next, we examined the effects of DEX on leptin-induced JAK2 phosphorylation using Huh7 cells co-transfected with JAK2 and OBRb. The cells expressing both JAK2 and OBRb were incubated with or without 10 nM DEX for 1 h and stimulated with 1 nM leptin for 5 min. DEX completely inhibited the
leptin-induced JAK2 tyrosine phosphorylation in cells co-expressing JAK2 and OBRb, as assayed by Western blotting using anti-phosphospecific JAK2 (Fig. 2C). The membrane was stripped and reprobed with anti-JAK2 antibodies. The total amount of JAK2 protein was not affected by DEX. We did not determine the tyrosine kinase activity of JAK2 in this study. In general, the catalytic activity of JAK2 was stimulated by phosphorylated tyrosine at position 1007 (34). We used anti-phosphospecific JAK2 antibody that recognizes this critical site for JAK2 kinase activity. Therefore, phosphorylation of JAK2 should be correlated with the activity of JAK2 in the present study. We next examined the effects of DEX on leptin-induced STAT3 phosphorylation in Huh7 cells co-transfected with JAK2 and OBRb. Similar to previous results (Fig. 1, B and C), DEX attenuated the phosphorylation of STAT3 in cells co-expressing JAK2 and OBRb (Fig. 2C).

Effects of Dexamethasone on 125I-Leptin Binding—To test whether DEX decreases the cell surface expression of leptin receptor in Huh7 cells transiently expressing OBRb, we studied the effects of DEX on 125I-leptin binding to the cells. Cells were incubated with 0–100 nM of DEX for 1 h and then subjected to 125I-leptin binding assay. DEX pretreatment had no significant effects on the specific binding of 125I-leptin to the cells expressing OBRb (Fig. 3).

The Effects of icv Infusion of DEX on Leptin Action in Vivo—To test whether DEX antagonizes the inhibitory effect of leptin in vivo, we measured the cumulative food intake 24 h after the infusion in Sprague-Dawley rats. As expected, a single icv infusion of leptin significantly suppressed food intake compared with the vehicle-infused control rats. Cumulative food intake 24 h after combined icv infusion of leptin and DEX with 1 nM of leptin for 5 min. Cell lysates were separated by 8% SDS-PAGE and analyzed by immunoblotting with an anti-phosphospecific JAK2 antibody and then with an anti-JAK2 antibody. PBS, phosphate-buffered saline. B, Huh7 cells were co-transfected with empty vector (0.5 μg) or JAK2 (0.5 μg). Cells were stimulated with 1 nM of IL-6 or LIF for 5 min. Cell lysates were subjected to Western blotting using anti-phosphospecific JAK2 antibody and then with an anti-JAK2 antibody. C, Huh7 cells expressing both OBRb (1.5 μg) and JAK2 (0.5 μg) were incubated with 10 nM of DEX for 60 min at 37 °C and subsequently stimulated with 1 nM of leptin for 5 or 15 min. Cell lysates were subjected to Western blotting using anti-phosphospecific JAK2 antibody, then with an anti-JAK2 antibody or anti-phosphospecific STAT3 antibody, and then with an anti-STAT3 antibody. These data are representative of at least three independent experiments.
DEX has been shown to modulate the activity of several protein kinases, including protein kinase C (35) and mitogen-activated protein kinase (MAPK) (36). To determine whether the inhibitory effects of DEX on leptin-induced STAT3 phosphorylation are mediated through these kinases, we investigated the inhibitory effects of DEX by using the specific inhibitors of protein kinase C and MAPK. Rottlerin, an inhibitor of protein kinase C, and SB202190, a p38 MAPK inhibitor, had no effect on the DEX inhibition of leptin-induced STAT3 activation (Fig. 5A). In contrast, when 1 μM of PD98059, a MEK inhibitor, was added to the cells prior to treatment with DEX, the inhibitory effect of DEX on leptin-induced STAT3 phosphorylation was blocked. PD98059 by itself had no effect on the STAT3 phosphorylation (Fig. 5B). We also determined whether PD98059 affects the inhibition of signaling events upstream of STAT3 phosphorylation. We thus investigated the effect of PD98059 on leptin-induced JAK2 phosphorylation that is inhibited by DEX. Pretreatment of PD98059 prevented DEX-mediated inhibition of JAK2 phosphorylation. PD98059 itself did not alter the total amount of JAK2 protein or tyrosine phosphorylation of JAK2 (Fig. 5C).

**DISCUSSION**

The current data demonstrate that DEX inhibits the leptin-induced STAT3 signaling pathway in a cultured cell line and also in the rat hypothalamus. GCs cause an increase in the level of ob gene expression and leptin secretion in isolated adipose tissue from rodents and humans (29, 35). On the other hand, GCs decrease gene expression for most cytokines including IL-6 and LIF (25). Furthermore, several studies suggested that GCs have a major ability to modify the actions of leptin in vivo (31, 32). However, the molecular mechanisms responsible for this antagonism remain unclear. In the present study, a rapid inhibitory effect of DEX on the leptin-induced STAT3 phosphorylation was observed from 0.1 nM of DEX pretreatment in cultured cells expressing OBRb. This concentration in blood level can be considered within the physiologically relevant range (37). Little is known about the pharmacokinetics of DEX in the central nervous system of humans. One study reported the concentration of DEX in plasma and central nervous system after intravenous administration of DEX to rabbits (38). Comparing these two concentrations, central nervous system/plasma ratio is around 10% after 6 h from DEX administration. This inhibitory effect of DEX was not observed in other cytokine receptors such as IL-6 and LIF receptor. We are unable to explain the difference between leptin and the IL-6 or LIF system with regard to the effects of DEX. Although OBR is a member of the class I cytokine receptor family, it has been demonstrated that OBR transduces the signal via its homodimerization without gp130, the signal transducing component for IL-6 and LIF receptor (39). Furthermore, the structural analysis revealed that the extracellular domain of OBR contains two repeating cytokine receptor domain/fibronectin type 3 domains, in contrast to other members of the class I family (40).

Hypersecretion of corticosterone has been observed in genetically obese rodents (41) and obese humans (42). Continuous central administration of GCs to normal rats resulted in obesity associated with hyperleptinemia, in keeping with the increase in hypothalamic neuropeptide Y levels (32). A central bolus injection of low dose leptin showed no significant effects in normal rats, but significantly decreased food intake and body weight in adrenalectomized rats (31). In the present study, an icv bolus administration of GCs (DEX) to normal rats attenuated the inhibitory effects of exogenous leptin on food intake. In agreement with those in vitro findings, GCs markedly inhibit leptin-induced STAT3 phosphorylation in the rat hypothalamus without affecting the total amount of STAT3 protein (Fig. 4A).

Fig. 4. Effects of pretreatment with icv DEX (5.0 μg) on the response to exogenous leptin. A, cumulative food intake was measured during the 24-h period after infusion of either vehicle, leptin (7.5 μg), or DEX (5.0 μg) and leptin in male Sprague-Dawley rats. Values are means ± S.E. of four to six rats per group. p value < 0.05 was considered to be statistically significant. B, immunoblot of hypothalamic STAT3 tyrosine phosphorylation in male Sprague-Dawley rats. Rats pretreated with or without DEX for 24 h were killed 30 min after infusion of either vehicle or leptin. Tissue lysates were separated by 8% SDS-PAGE and analyzed by immunoblotting with an anti-phosphospecific STAT3 antibody and then with an anti-STAT3 antibody.

increased 3-fold compared with icv leptin infusion alone (Fig. 4A). Next we examined the effect of DEX on the leptin signaling in the hypothalamus. Western blotting was carried out using anti-phospho-STAT3 antibody as a primary antibody in the hypothalamus at 30 min after icv infusion of leptin. Pretreatment of icv DEX infusion for 12 h prior to infusion of leptin decreased the leptin-induced STAT3 phosphorylation in the hypothalamus without affecting the total amount of STAT3 protein (Fig. 4B).

**The Mechanism of the DEX Effects on JAK/STAT Signaling Pathway**—The mechanism by which DEX inhibited leptin-induced JAK/STAT signaling was further examined. DEX rapidly inhibited the leptin-induced STAT3 phosphorylation, suggesting that these effects did not require de novo protein synthesis.
hypothalamus. These results suggest the short term effects of GCs on food intake may be partly mediated via an inhibition of central leptin signaling.

The findings in the present study demonstrated that the inhibitory effect of GCs occurs at JAK2 upstream of STAT3 in Huh7 cells. DEX has been reported to cause a rapid decrease of the cell surface number in GH receptors, and subsequently to inhibit the tyrosine phosphorylation of JAK2 in fibroblasts (43). GH receptor is a member of the cytokine receptor superfamily that includes receptors for IL-6, LIF, G-CSF, prolactin, and leptin. We have shown previously that in contrast to GH receptor, no significant effects on leptin binding were observed in DEX-pretreated Chinese hamster ovary cells stably expressing OBRa or OBRb (44). Consistent with previous findings, the present observations showed DEX had no effects on the cell surface number of OBRb in Huh7 cells transiently expressing OBRb. It was reported previously that GCs inhibit IL-2-induced JAK/STAT signaling that suppresses the expression of IL-2 receptor and JAK3 in primary human T cells (27). A more recent study demonstrated that inhibition of STAT1 expression as a mechanism by which GCs inhibit INF-α-induced STAT3 activation in Chinese hamster ovary cells (46). Recently, we reported ethanol inhibits leptin-induced STAT3 phosphorylation partly via p38 MAPK, activated by inflammatory cytokines and environmental stresses in Huh7 cells (47). A more recent study (48) demonstrated that the targeted disruption of c-Jun amino-terminal kinase 1 (JNK1), also called stress-activated protein kinase, shows reduced adiposity and increased insulin sensitivity associated with enhanced insulin receptor signaling in both genetically and diet-induced obese mice. These findings suggest that MAPKs may play an important role in the modification of insulin and leptin action.

In conclusion, the present results provide evidence for a direct cross-talk between the leptin and GCs signaling pathways in vitro and in vivo. In Huh7 cells, GCs rapidly inhibit the leptin-induced JAK/STAT pathway partly mediated via MAPK cascades. In vivo GCs attenuate hypothalamic leptin signal transduction pathway and reduce the sensitivity in response to central administration of leptin.

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Fig. 5. Effects of the MEK inhibitor, PD98059, on the DEX inhibition of leptin-induced JAK2 and STAT3 tyrosine phosphorylation. A and B, Huh7 cells expressing OBRb were incubated with Rottlerin (10 μM), SB202190 (1 μM), or PD98059 (1 μM) for 45 min prior to treatment with 10 nM of DEX. Cells were stimulated with 1 nM of leptin for 15 min. Cell extracts were separated by 8% SDS-PAGE and analyzed by immunoblotting with an anti-phosphospecific STAT3 antibody and then with an anti-STAT3 antibody. C, Huh7 cells expressing both OBRb (1.5 μg) and JAK2 (0.5 μg) were incubated with 1 μM of PD98059 for 45 min prior to treatment with 10 nM of DEX. Cells were stimulated with 1 nM of leptin for 5 min. Cell extracts were separated by 8% SDS-PAGE and analyzed by immunoblotting with an anti-phosphospecific JAK2 antibody and then with an anti-JAK2 antibody. These data are representative of at least three independent experiments.
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