Endogenous glucocorticoids released during acute toxic liver injury enhance hepatic IL-10 synthesis and release

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Swain, Mark G., Caroline Appleyard, John Wallace, Howard Wong, and Tai Le. Endogenous glucocorticoids released during acute toxic liver injury enhance hepatic IL-10 synthesis and release. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G199–G205, 1999.—Endogenous glucocorticoids are known to play a role in the regulation of the inflammatory response possibly by modulating pro- and anti-inflammatory cytokine expression. We examined endogenous glucocorticoid secretion, hepatic damage, tumor necrosis factor-α (TNF-α), and interleukin-10 (IL-10) mRNA expression and release in rats treated with carbon tetrachloride (CCl4) after treatment with vehicle or a glucocorticoid receptor antagonist (RU-486). Rats treated with CCl4 demonstrated striking elevations of plasma corticosterone levels. Inhibition of endogenous glucocorticoid activity by pretreatment with the glucocorticoid receptor antagonist RU-486 resulted in augmented CCl4-mediated hepatotoxicity, as reflected by histology and serum transaminase levels, which were independent of alterations in serum TNF-α levels or hepatic mRNA expression. CCl4 treatment resulted in enhanced hepatic IL-10 mRNA expression and elevated serum IL-10 levels, which were markedly attenuated by glucocorticoid receptor blockade. In summary, significant endogenous glucocorticoid release occurs during acute toxic liver injury in the rat and suppresses the inflammatory response independent of effects on TNF-α but possibly by upregulating hepatic IL-10 production.

INFLAMMATORY PROCESSES are capable of stimulating endogenous glucocorticoid secretion by activating the hypothalamic-pituitary-adrenal axis (13). This activation has been postulated to occur via proinflammatory cytokines [e.g., interleukin-1 (IL-1), IL-6, tumor necrosis factor-α (TNF-α)] that act to stimulate all levels of the hypothalamic-pituitary-adrenal axis (13). Glucocorticoids (corticosterone in the rat) released from the adrenal cortex during the inflammatory process are known to play a role in the regulation of the inflammatory cytokine expression. We examined endogenous glucocorticoid secretion, hepatic damage, tumor necrosis factor-α (TNF-α), and interleukin-10 (IL-10) mRNA expression and release in rats treated with carbon tetrachloride (CCl4) after treatment with vehicle or a glucocorticoid receptor antagonist (RU-486). Rats treated with CCl4 demonstrated striking elevations of plasma corticosterone levels. Inhibition of endogenous glucocorticoid activity by pretreatment with the glucocorticoid receptor antagonist RU-486 resulted in augmented CCl4-mediated hepatotoxicity, as reflected by histology and serum transaminase levels, which were independent of alterations in serum TNF-α levels or hepatic mRNA expression. CCl4 treatment resulted in enhanced hepatic IL-10 mRNA expression and elevated serum IL-10 levels, which were markedly attenuated by glucocorticoid receptor blockade. In summary, significant endogenous glucocorticoid release occurs during acute toxic liver injury in the rat and suppresses the inflammatory response independent of effects on TNF-α but possibly by upregulating hepatic IL-10 production.

Carbon tetrachloride (CCl4) is a hepatotoxic compound that is used widely as an inducer of acute and chronic experimental liver disease (35). Single-dose treatment of rats with CCl4 induces acute liver injury characterized by zone 3 liver cell necrosis and steatosis (35). TNF-α has been suggested as playing a role in liver cell damage due to CCl4 (9), as well as playing a central role in recovery from CCl4 hepatotoxicity (1, 3, 4). Furthermore, endogenous glucocorticoid secretion has been shown to be capable of modulating TNF-α synthesis and release in endotoxin-treated rats and mice (23, 36). Alternatively, administration of pharmacological doses of glucocorticoid appears to be able to increase the endogenous release of the anti-inflammatory cytokine IL-10 in humans and mice (19, 30, 32). Moreover, exogenous administration of IL-10 has been shown to protect the liver from toxic damage induced by a number of hepatic insults (17, 18).

Therefore, in this series of experiments we quantified the magnitude of endogenous glucocorticoid secretion in rats treated acutely with CCl4 and examined the effects of the inhibition of endogenous glucocorticoid activity on the extent of CCl4-induced hepatotoxicity as well as on TNF-α and IL-10 synthesis and release.

METHODS

Animal model. Male Sprague-Dawley rats weighing ~200 g were obtained from Charles River (Ponette Claire, QC, Canada). All animals were housed in a light-controlled room maintained at 22°C with a 12:12-h day-night cycle and were given free access to food and water. The rats were handled regularly to avoid handling stress during experiments. All animals were treated humanely under University of Calgary Animal Care Committee guidelines.

The model of acute liver injury used was that due to CCl4 treatment (35). Rats were fasted for 12 h before and for 6 h after CCl4 (Sigma Chemical, St. Louis, MO) treatment. CCl4 (0.3 ml/200 g body wt in corn oil 1:1) or corn oil vehicle were given by gavage, rats were killed 0, 12, and 24 h later, blood samples were obtained by vena caval puncture (under halothane anesthesia) for TNF-α and transaminase level (ALT) determination, and liver samples were removed for histological examination. For plasma corticosterone level determination rats were killed by decapitation within 30 s of removal from their cages, and trunical blood was collected.

To examine the effects of endogenous glucocorticoid activity inhibition on CCl4-induced hepatotoxicity, rats were treated with vehicle (0.2 ml of 0.9% saline) or RU-486, a glucocorticoid receptor antagonist (15) kindly provided by Roussell UCLA (10 mg/kg body wt in 0.9% saline). Vehicle or RU-486 was administered intraperitoneally every 12 h, starting 12 h before CCl4 gavage (28).

Serum transaminase and corticosterone determinations. Plasma alanine aminotransferase (ALT) levels were determined using a commercially available kit (Sigma). Plasma...
corticosterone levels were determined using a sensitive RIA as described previously (29).

Plasma cytokine assays. Plasma TNF-α bioactivity was determined using a highly sensitive assay modified from Mosmann (20). This assay determines TNF-α cytotoxicity against WEHI 164 murine fibrosarcoma cells (CRL 1751, American Type Culture Collection, Rockville, MD) in the presence of actinomycin D and uses 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma) as colormetric end point. Briefly, 5 x 10⁴ cells were incubated in 96-well microtiter plates at 37°C with 5% CO₂ in 50 µl RPMI 1640 medium (GIBCO, Gaithersburg, MD) containing 10% fetal bovine serum, 1% glutamine, and 1% penicillin-streptomycin solution (1:1). Actinomycin D (25 µl; 30 mg/ml) was added to each well followed by samples of filtered rat plasma (50 µl) or purified recombinant human TNF-α as standards (R&D Systems, Minneapolis, MN). The cells were incubated for 24 h at 37°C, and then 25 µl of sterile MTT were added to each well. After incubation for 3 h at 37°C, the reaction was stopped by adding 100 µl lysis buffer (20) and the plates were read at 620 nm using a spectrophotometric plate reader. One unit of TNF-α activity was defined as the concentration at which 50% of the cells were lysed.

Plasma IL-10 levels were determined using a commercially available ELISA specific for rat IL-10 following manufacturer’s instructions (Medicorp, Montreal, QC, Canada).

Steady-state liver TNF-α and IL-10 mRNA levels by semi-quantitative RT-PCR. Total RNA was extracted from liver tissue by the acid guanidinium isothiocyanate method (21). The final RNA concentrations were determined by absorbance using a GeneQuant spectrophotometer (Pharmacia, Piscataway, NJ). The reverse transcription and PCR reactions were performed as described previously by us (27) using the “primer dropping” method of Wong et al. (34). Briefly, cDNA was generated by a reverse transcription reaction by incubating 1 µg of total RNA, 1× PCR buffer (in mM, 10 Tris-HCl, pH 9.0, 50 KCl, and 1.5 MgCl₂), 1 nM each of deoxynucleotide triphosphates (dATP, dGTP, dCTP, and dTTP), 20 units placental ribonuclease inhibitor (RNA guard, Pharmacia), 160 units of Superscript-reverse transcriptase (GIBCO-BRL, Burlington, ON, Canada), and 100 pmol of random hexamer oligodeoxynucleotides (Pharmacia). Reaction mixtures were preincubated 10 min at 21°C before cDNA synthesis. The reverse transcription reactions were carried out for 50 min at 42°C and were heated to 95°C for 5 min to terminate the reaction. Reactions were performed in an AmpliTaq I thermal cycler (Barnstead/Thermolyne, Dubuque, IA).

Multiplex PCR reactions were performed using the primer dropping method as described by Wong et al. (34) in 50 µl reaction volumes containing 2 µl of RT reaction product as template DNA, 1× PCR buffer, 80 µM of each deoxynucleotide, and 20 pmol of each specific 5’ and 3’ primer pair. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was amplified as an internal control for TNF-α and RPL 19 cDNA for IL-10. Two units of Taq DNA polymerase (Pharmacia) were added to each tube during the first denaturation step, and equal aliquots of secondary primer sets (20 pmol GAPDH or RPL 19) were added to the appropriate cycle number by the primer dropping method of Wong et al. (34). Briefly, cDNA was generated by a reverse transcription reaction by incubating 1 µg of total RNA, 1× PCR buffer (in mM, 10 Tris-HCl, pH 9.0, 50 KCl, and 1.5 MgCl₂), 1 nM each of deoxynucleotide triphosphates (dATP, dGTP, dCTP, and dTTP), 20 units placental ribonuclease inhibitor (RNA guard, Pharmacia), 160 units of Superscript-reverse transcriptase (GIBCO-BRL, Burlington, ON, Canada), and 100 pmol of random hexamer oligodeoxynucleotides (Pharmacia). Reaction mixtures were preincubated 10 min at 21°C before cDNA synthesis. The reverse transcription reactions were carried out for 50 min at 42°C and were heated to 95°C for 5 min to terminate the reaction. Reactions were performed in an AmpliTaq I thermal cycler (Barnstead/Thermolyne, Dubuque, IA).

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Aliquots of PCR reaction products (~10 µl) equalized to given equivalent signals from the GAPDH or RPL 19 mRNA were electrophoresed through 2% agarose gels (Ultrapure, Pharmacia) containing 0.2 µg/ml of ethidium bromide. Gels were visualized under ultraviolet light, photographed under Polaroid film, and analyzed by computer densitometric scanning of the images using a Hewlett-Packard Scanjet I & IIc scanner, DeskScan II software, and National Institutes of Health imaging software. The intensities of ethidium bromide fluorescence signals were determined from the areas under the curve for each peak.

The primers used for RT-PCR as well as the number of PCR cycles and PCR product size were as follows: TNF-α sense, 5’- CCACAGCCTTCTCTGCTACT-3’ and antisense, 5’-CCACACTTCTACCTCGGTTCTCT-3’ (32 cycles with 1,063-bp product); GAPDH sense, 5’-CGGAGTCAAGGTTGTTCGTATG-3’ and antisense, 5’-AGCCCTTCTCCATGTGTTGAAGAC-3’ (22 cycles with 306-bp product); IL-10 sense, 5’-GAGAGAAGCTGAGACCCCTC-3’ and antisense, 5’-GCTTGCACGGGTGGTGAAG-3’ (31 cycles with 161-bp product); RPL 19 sense, 5’-AGATGCTCTAGGCTCAGAAG-3’ and antisense, 5’-TCTCCTGTCCTAGACCTGC-3’ (22 cycles with 501-bp product).

Adrenalectomy studies. To further assess the role of endogenous glucocorticoids in CCl₄-induced hepatotoxicity we used rats completely devoid of endogenous glucocorticoids due to adrenalectomy. Adrenalectomized and shad adrenalectomized rats were obtained from the supplier and were studied 3–4 days after their adrenalectomy or sham adrenalectomy. Adrenalectomized rats were maintained with 0.9% saline for drinking water. Adrenalectomized and shad adrenalectomized rats were treated with CCl₄ (0.3 ml/200 g body wt by gavage) and mortality recorded over the ensuing 24 h. Another group of adrenalectomized rats was pretreated with corticosterone suspended in 500 µl of polyethylene glycol 300 (Sigma) at a dose of 10 mg/kg given subcutaneously at the time of CCl₄ gavage and 12 h later. This dose of corticosterone produces stress levels of circulating corticosterone in adrenalectomized rats (20).

Liver histology. Liver tissue was collected in Formalin, sectioned, and stained with hematoxylin and eosin. Liver sections were examined under light microscopy in a blinded fashion.

Statistical analyses. Data are expressed as means ± SE. For comparisons between two means a Student’s t-test was performed. For comparisons among more than two means an ANOVA followed by Student-Newman-Keuls test was performed. For comparison of mortality data a Fishers exact test was performed. Statistical significance was achieved if P ≤ 0.05.

RESULTS

CCl₄-induced corticosterone secretion. Plasma corticosterone levels were similar in oil-gavaged control rats 12 and 24 h after gavage (Fig. 1). However, CCl₄ treatment resulted in striking elevations in plasma corticosterone levels at all time points after gavage compared with control levels at similar time points (Fig. 1).

RU-486 treatment and CCl₄-induced hepatotoxicity. Serum ALT levels were significantly elevated 24 h after CCl₄ treatment in both vehicle- and RU-486-treated rats compared with basal levels (Fig. 2). However, serum ALT levels were strikingly higher in RU-486-
treated rats compared with vehicle-treated rats 24 h after CCl₄ gavage (Fig. 2).

Histological examination of liver specimens revealed a marked reduction in steatosis but enhanced liver cell necrosis in RU-486-treated compared with vehicle-treated CCl₄-gavaged rats, which was most pronounced 24 h after CCl₄ administration (Fig. 3).

Glucocorticoid secretion and survival after CCl₄. Adrenalectomized and sham adrenalectomized rats were gavaged with CCl₄ and subsequently assessed 12 and 24 h later for survival. Throughout the 24-h observation period no CCl₄-treated sham adrenalectomized rats died (n = 7). However, by 24 h after CCl₄ administration five of seven adrenalectomized rats had died (P = 0.02). Administration of corticosterone to adrenalectomized CCl₄-treated rats completely prevented CCl₄-induced mortality in this group (n = 7).

Hepatic TNF-α mRNA expression and plasma TNF-α levels. The bioassay used in this study to detect circulating TNF-α is very sensitive, and serum levels of TNF-α in control rats similar to those detected in our experiments have been previously reported using this assay (10). Plasma TNF-α bioactivity increased significantly and to a similar degree 12 h after CCl₄ gavage in vehicle- and RU-486-treated rats (Fig. 4). Plasma TNF-α bioactivity, however, had returned to levels similar to control rats by 24 h in both vehicle- and RU-486-treated animals (Fig. 4).

TNF-α mRNA expression was not detected in control livers. However, a striking increase in TNF-α mRNA expression was noted after CCl₄ administration with levels peaking 12 h after CCl₄ gavage in both vehicle- and RU-486-treated CCl₄-gavaged rats (Fig. 5), consistent with previous reports (8). However, hepatic TNF-α mRNA expression was similar in vehicle- and RU-486-treated rats 12 and 24 h after CCl₄ administration.
Hepatic IL-10 mRNA expression and plasma IL-10 levels. IL-10 is an anti-inflammatory cytokine that has recently been shown to be capable of protecting the liver from experimental hepatotoxicity (17, 18). Furthermore, recent evidence suggests that glucocorticoids can induce IL-10 synthesis and release (25, 30, 32). IL-10 was undetectable in plasma of control rats, consistent with observations of others (18). However, CCl4 treatment resulted in a striking increase in plasma IL-10 levels by 12 h and this remained unchanged at 24 h post-CCl4 (Fig. 6). Rats pretreated with RU-486 demonstrated a markedly attenuated increase in plasma IL-10 levels in response to CCl4 12 h after CCl4 treatment (Fig. 6). By 24 h the plasma IL-10 levels in RU-486-pretreated CCl4-gavaged rats were similar to levels observed in CCl4-gavaged rats which were not pretreated with RU-486 (Fig. 6).

Low-level IL-10 mRNA expression was detectable in liver tissue from control rats. CCl4 administration resulted in a 6.5-fold increase in hepatic IL-10 expression by 12 h, which was still evident 24 h after CCl4 gavage (Figs. 7 and 8). However, CCl4 administration in RU-486-treated rats resulted in no significant change in hepatic IL-10 mRNA expression compared with untreated controls (Figs. 7 and 8).

Fig. 4. Plasma tumor necrosis factor-α (TNF-α) levels as determined by bioassay in normal rats or in vehicle- or RU-486-treated CCl4-gavaged rats 12 and 24 h after gavage; n = 6 rats/group. *P < 0.05 and **P < 0.01 vs. untreated control rats. Plasma TNF-α bioactivity was similar in control and vehicle- and RU-486-treated rats 24 h after CCl4 gavage.

Fig. 5. Hepatic TNF-α mRNA levels (corrected for glyceraldehyde-3-phosphate dehydrogenase mRNA expression) expressed as arbitrary densitometric units in vehicle- or RU-486-treated CCl4-gavaged rats 12 and 24 h after gavage; n = 6 rats/group. All values are similar. TNF-α mRNA was not detectable in normal liver. Hepatic TNF-α mRNA levels at both 12- and 24-h time points for vehicle- and RU-486-treated groups were not significantly different from each other.

Fig. 6. Plasma interleukin-10 (IL-10) levels as determined by ELISA in vehicle- or RU-486-treated CCl4-gavaged rats 12 and 24 h after gavage; n = 9 rats/group for 12-h groups and 6 rats/group for 24-h groups. Normal rats had no detectable IL-10 in plasma. *P < 0.01 vs. all other groups.
**DISCUSSION**

Acute hepatocellular necrosis induced by CCl4 is a widely used model of acute liver injury (35). We have shown that acute hepatic inflammation induced by CCl4 is a potent activator of endogenous glucocorticoid secretion and that enhanced glucocorticoid release plays a significant modulatory role with respect to the development of hepatic inflammation and overall survival after CCl4 treatment in rats. Furthermore, endogenous glucocorticoid release appears to be necessary for early hepatic release of the anti-inflammatory cytokine IL-10 in response to acute CCl4 hepatotoxicity.

The release of endogenous glucocorticoids from the adrenal gland during the inflammatory response plays a central role in the control of inflammation (reviewed in Ref. 6). However, the role played by endogenous glucocorticoid secretion in modulating hepatic inflammation and the mechanisms underlying this modulation are poorly documented. In the current study we have identified that a striking increase in plasma corticosterone levels accompanies the hepatic inflammatory response due to CCl4 administration. Moreover, the rise in plasma corticosterone levels precedes histological or biochemical indexes of CCl4-induced hepatotoxicity. This hypothesis was confirmed by the demonstration of enhanced CCl4-mediated lethality in adrenalectomized rats (which is completely prevented by corticosterone treatment) and an augmentation of the biochemical and histological progression of CCl4-induced hepatotoxicity in rats where endogenous glucocorticoid receptors had been blocked by RU-486 treatment (RU-486 is a glucocorticoid receptor antagonist; 15). RU-486 is also a progesterone receptor antagonist (18). Given that progesterone can modulate inflammation by repressing NF-kappa B activity (reviewed in Ref. 31), some of the biological effects observed in our studies using RU-486 may possibly be due in part to effects of RU-486 on progesterone-mediated responses.

Recent work has also suggested a possible beneficial role of TNF-\(\alpha\) in the response of the liver to damage. TNF-\(\alpha\) appears to play a critical role in hepatic repair after injury. TNF-\(\alpha\) stimulates hepatic DNA and RNA synthesis and hepatic mitosis (3) and is of central importance in hepatic regeneration after partial hepatectomy in rats (1). Moreover, enhanced hepatic TNF-\(\alpha\) levels have recently been shown to be necessary for the induction of early-immediate genes and in liver repair following CCl4-induced hepatotoxicity (4). The discrepancy between adrenalectomy and RU-486 effects on TNF-\(\alpha\) release has been examined by Pettipher et al. (24) who found that elevated plasma TNF-\(\alpha\) levels in endotoxin-treated mice are due to effects of released adrenal catecholamines, not glucocorticoids.

IL-10 is an anti-inflammatory cytokine, which plays a critical role in the natural defense against endotoxin-induced toxicity (2) and which has recently been shown to be capable of attenuating galactosamine-endotoxin- and concanavalin-A-induced liver injury in mice (17, 18). The mechanisms underlying the hepatoprotective effects of IL-10 are poorly understood. IL-10 may attenuate CCl4-induced hepatotoxicity through its well-documented ability to decrease the production of many proinflammatory cytokines and chemokines (reviewed in Ref. 26). Furthermore, IL-10 can inhibit the recruitment of neutrophils to sites of inflammation by down-regulating the expression of endothelial cell adhesion molecules (14). Because neutrophils are known to contribute to CCl4-mediated hepatotoxicity (35), diminished neutrophil accumulation in the liver would be expected to attenuate CCl4-induced liver damage.
The liver appears to be the major source of circulating IL-10 (33). Moreover, glucocorticoids enhance IL-10 secretion from Th1 lymphocytes, and the administration of glucocorticoids to patients can increase serum IL-10 levels (25, 30, 32). Therefore, endogenous glucocorticoids may modulate inflammatory processes, at least in part, by enhancing IL-10 production. Our findings are consistent with this hypothesis. Endogenous IL-10 mRNA expression and IL-10 release were strikingly attenuated 12 h after CCl4 administration in rats receiving RU-486. By 24 h after CCl4 administration in RU-486-treated rats, plasma IL-10 levels were similar in RU-486 pretreated and nonpretreated groups, despite the observation that hepatic IL-10 mRNA levels remained low 24 h after CCl4 in RU-486-treated animals. Given that IL-10 has known hepatoprotective effects against hepatotoxicants (17, 18) these data suggest that a relative lack of glucocorticoid-driven endogenous IL-10 secretion early in the course of acute CCl4 hepatotoxicity may predispose rats to more profound hepatic damage induced by hepatotoxicants such as CCl4. The finding that at 24 h post-CCl4 treatment plasma IL-10 levels were similar in RU-486 pretreated and nonpretreated rats, despite a persistent decrease in hepatic IL-10 mRNA expression in the RU-486-pretreated rats, suggests that endogenous glucocorticoids have a complex modulatory role with respect to IL-10 production. Specifically, despite low hepatic mRNA levels in RU-486-pretreated rats 24 h after CCl4 gavage, IL-10 secretion appears to have normalized. However, given that serum transaminase levels and histological changes are significantly worse in CCl4 gavaged RU-486-pretreated rats (compared with rats not treated with RU-486) 24 h after CCl4 treatment, the early hepatic IL-10 responses may be of critical importance with respect to hepatotoxicity evident at later time points.

In summary, we have demonstrated that acute liver necrosis due to CCl4 is associated with enhanced endogenous glucocorticoid secretion, which attenuates the early hepatic inflammatory response through a mechanism that appears to be independent of hepatic and serum TNF-α levels and possibly by enhancing local IL-10 production.

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