Hydroxyurea Induces the Gene Expression and Synthesis of Proinflammatory Cytokines In Vivo

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ABSTRACT

The anticancer agent hydroxyurea (HU) was previously found to cause dose-dependent adrenal activation in the rat. The increased secretion of corticosterone (CORT) that results appeared to protect animals against HU toxicity, which was dramatically enhanced in adrenalectomized (ADX) rats. Similarities with the endocrine and toxicological profiles of proinflammatory cytokines such as interleukin (IL)-1 and tumor necrosis factor (TNF) led us to suggest that these effects of HU might be mediated by an increased synthesis of these cytokines. The goal of this study was therefore to demonstrate that HU induces the gene expression and synthesis of proinflammatory cytokines in vivo. Intact and ADX rats were treated with HU, mRNA was extracted from spleen cells 2 and 24 hr after treatment and message levels for IL-1α, IL-2, IL-4, IL-6, TNFα and interferon-γ were evaluated using the reverse transcriptase-polymerase chain reaction technique. In some experiments, circulating levels of CORT and TNF were also measured. We found that transcripts of the proinflammatory cytokines, TNF, IL-6 and (though less clearly) IL-1α, were expressed in the majority of intact rats treated with HU but were absent or less evident in most controls. In contrast, gene expression of IL-2, IL-4 and interferon-γ was not influenced by drug treatment. Adrenalectomy markedly enhanced the effects of HU. Twenty-four hours after administration of the drug, the expression of TNF and IL-6 mRNAs was still higher in ADX rats compared with intact animals. Parallel measurements of plasma CORT levels revealed that gene expression of IL-1α and, to a lesser extent, TNF was inversely related to levels of circulating CORT. Adrenalectomy per se caused a significant increase in plasma TNF levels compared with intact controls. Hydroxyurea elicited significant increases in circulating TNF in both ADX and intact rats. These findings lend support to our working hypothesis and provide an explanation for both the rise in glucocorticoid secretion induced by HU in intact rats and the increase in lethality observed in animals with disruptions of the hypothalamo-pituitary-adrenal axis.

HU is an inhibitor of ribonucleotide phosphate reductase (Nocentini, 1996) that has long been used in the treatment of neoplastic diseases, such as chronic myelogenous leukemia (Chabner et al., 1996). Renewed interest in HU has been kindled by the finding that the drug inhibits HIV-type 1 replication in vitro (Lori et al., 1994), and clinical studies with the drug are currently under way in HIV-infected patients.

We became interested in HU while studying the influence of anticancer drugs on the adrenocortical function in the rat. Unlike other anticancer agents such as cisplatin, asparagi-

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ABBREVIATIONS: HU, hydroxyurea; CORT, corticosterone; CRH, corticotropin-releasing hormone; HPA axis, hypothalamo-pituitary-adrenal axis; HYX rats, hypophysectomized rats; ADX rats, adrenalectomized rats; TNF, tumor necrosis factor; IL-1, interleukin-1; IL-2, interleukin-2; IL-4, interleukin-4; IL-6, interleukin-6; IL-12, interleukin-12; INF-γ, interferon-γ; HIV, human immunodeficiency virus; RT-PCR, reverse transcriptase-polymerase chain reaction; EBSS, Earle’s balanced salt solution.
acute mortality is unlikely to be explained by the established mechanisms of HU toxicity in intact animals, including bone marrow depression. The lack of major histopathological and hematological changes in HYX rats treated with HU at 300 mg/kg/day for 4 days (Argentino-Storino et al., 1992; Navarra et al., 1996) suggests the occurrence of additional effects in animals with disruption of the HPA axis, mediated by deregulation of functional parameters. It is consistent with this hypothesis that HU causes cardiovascular collapse in HYX rats, which presumably contributes to the early death of the treated animals (Preziosi, unpublished observation).

It is known that the toxicity of xenobiotics may involve the secondary production of certain cytokines, including TNF, IL-1, IL-6, IL-12, INF-γ and leukemia inhibitory factor (Ryffel, 1995). Interestingly, the exogenous administration of IL-1 and TNF, two major proinflammatory cytokines, may mimic the effects of HU in eliciting acute stimulation of the HPA axis through an increase in CRH secretion (Bernardini et al., 1990; Busbridge and Grossman, 1992). Moreover, the toxic effects of these cytokines are more severe in ADX animals and include the worsening of an endotoxic-like shock (Bertini et al., 1988; Dinarello, 1991) that resembles the clinical conditions of ablated rats treated with HU. These similarities led us to hypothesize that 1) proinflammatory cytokines could mediate the activation of the HPA axis induced by HU in intact rats and 2) drug-induced synthesis of proinflammatory cytokines might account for the increased HU toxicity observed in rats with disruption of the HPA axis (Preziosi et al., 1992). Although a stimulatory effect of HU on the in vitro production of IL-1 has been described (Matsushima et al., 1986), the effect of the drug on cytokine synthesis in vivo has never been investigated.

In this study, intact and ADX rats were treated with 800 mg/kg HU, a dose that elicits maximum increase in plasma CORT levels in intact rats and the highest lethality in ADX and HYX animals (Navarra et al., 1990). Total mRNA was extracted from spleen cells 2 and 24 hr after treatment, and message levels for a variety of cytokines, including IL-1α, IL-2, IL-4, IL-6, TNF-α and INF-γ, were evaluated using the RT-PCR technique. Changes in the actual cytokine synthesis were confirmed by assessment of circulating protein. In some experiments, plasma CORT levels were also measured with a specific radioimmunoassay.

**Materials and Methods**

**Experimental procedures.** Male Wistar rats weighing 200 to 250 g were acclimatized for a period of 7 days in a room maintained at a temperature of 23°C ± 1.5°C with a relative humidity of 65% ± 2%. The animals were exposed to 12 hr of light (06.00–18.00) followed by 12 hr of dark and had free access to food pellets and water. Lumbar adrenalectomy, as described by Pomeau-Delille (1953), was performed on some of the rats. These animals were given normal saline instead of water and were used 1 week after surgery. On the day of the experiment, intact and ADX rats were treated p.o. with 800 mg/kg HU (Sigma Biochemical Co., St. Louis, MO) or corresponding volumes of vehicle and were decapitated 2 or 24 hr later. In some experiments, trunk blood was collected for the measurements of circulating levels of TNFα and CORT. Spleens were rapidly dissected and kept in ice-cold EBSS (Sera-Lab Ltd, Crawley Down, Sussex, UK) containing penicillin G sodium 15 µg/ml and streptomycin sulfate 25 µg/ml (both from Sigma). Spleens were processed for mRNA extraction on the day of the experiment. RNA preparation and detection of cytokine transcripts by RT-PCR. These procedures have been previously described in detail (Campanile et al., 1993; Campanile et al., 1994). Briefly, 5 × 10⁶ spleen cells were subjected to RNA extraction by the guanidium thiocyanate-phenol-chloroform procedure. Purified total RNA was incubated with 0.5 µg of oligo(dt) (Pharmacia, Uppsala, Sweden) for 3 min at 65°C and chilled on ice for 5 min. Each sample was then incubated for 2 hr at 42°C after addition of 20 U of RNase inhibitors (Boehringer-Mannheim Italia Spa, Milan, Italy), 1.5 mM deoxynucleoside triphosphates, 7.5 µM of avian myeloblastosis virus reverse transcriptase (Boehringer-Mannheim) and reverse transcriptase buffer [50 mM Tris-HCl (pH 8.3), 8 mM MgCl₂, 30 mM KCl and 10 mM DTT, final concentrations] in a final volume of 20 µl. The cDNA was diluted to a total volume of 75 µl with Tris-EDTA buffer (10 mM Tris-HCL, 1 mM EDTA, pH 8.0) and frozen at −20°C until use.

Amplification of synthesized DNA was carried out using cytokine-specific primers synthesized according to rat cytokine gene sequences present in a data bank (GenBank database release 88.0). IL-1α (5′-ATG CCC AAA GTT CCT GAC TTG TTT-3′ and 5′-CCT CAG CAA CAC AGG CTT GTC T-3′), IL-2 (5′-ATG TAC AGG ATC AGC CTG GCA G-3′ and 5′-TCA TTG AGA TGA TGG AAG A-3′), IL-4 (5′-ATG GGT CTC AAC CCC CAC CCT GT-3′ and 5′-GAC TAA CTC AGC CTC GAC GAA GTA G-3′), IL-5 (5′-ATG AAT CTC TCC GCC GCA GAG A-3′ and 5′-CAT CAC GTG TGT TGC CGA GTA GAT CTC T-3′), TNFα (5′-ATG AGC AAG CAA ACC ATG ATC CGA-3′ and 5′-CC AAA GTA GAC CTG CCC GGA CTC-3′), INF-γ (5′-TGA AGC GTA GGC TAT CTC TGG-3′ and 5′-CGA CTT CTC CGC TTC AG-3′) and β-actin-specific 5′ and 3′ antisense primers were used. Briefly, 1 to 5 µl of cDNA was added to a reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 3.0 mM MgCl₂, 0.01% gelatin, 0.2 deoxynucleoside triphosphates, 1 µM each primer and 0.5 U of AmpliTaq polymerase (Perkin-Elmer Corp., Hayward, CA). Each 20-µl sample was overlaid with 25 µl of mineral oil (Sigma) and incubated in a DNA Thermal Cycler 480 (Perkin-Elmer Corp.) for a total of 30 cycles: 1 min at 94°C, 1 min at 67°C or 60°C (β-actin), and 1 min at 72°C. The amplified DNA size, as compared with a positive control, was 625 bp for IL-1α, 501 bp for IL-2, 398 bp for IL-4, 638 bp for IL-6, 460 bp for INF-γ, 692 bp for TNFα and 540 bp for β-actin. The positive controls, with the expected size, consisted of the amplification products from mRNA of ConA-stimulated rat spleen cells. The β-actin primers were used as a control for both reverse transcription and the PCR reaction itself and also for comparing the amount of products from samples obtained with the same primer. The RT-PCR fragments were analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. RT-PCR-assisted mRNA amplification was repeated at least twice for at least two separately prepared cDNA samples for each experiment. Data are representative of at least two different experiments. Under the conditions employed, control samples from naive rats showed, as a rule, no background cytokine mRNA levels, so that the magnitude of the response to treatment could be demonstrated easily.

**TNF-α assay.** TNF bioactivity in sera was measured as cytotoxic activity to WEHI 164 clone 13 murine fibrosarcoma cells (Espévak and Nissen-Meyer, 1986) obtained through the courtesy of P. van der Bruggen and Ludwig Institute for Cancer Research, Brussels, Belgium. The assay was performed as described, in the presence of L-ClO₂ to optimize sensitivity to TNF-mediated toxicity (Beyaert et al., 1989) and using a tetrazolium-based colorimetric assay to estimate mortality of WEHI cells (Campanile et al., 1993). TNF titers were expressed as picograms per milliliter, calculated by reference to a standard curve constructed with known amounts of rTNFα (Genzyme, Cambridge, MA). Data are means ± S.E. of triplicate determinations.

**Radioimmunoassay for plasma CORT.** This technique has been described in detail elsewhere (Navarra et al., 1990).
Statistics. Data from TNFα assays were expressed as means ± S.E. and were analyzed by analysis of variance and subsequent modified Student’s t test for multiple comparisons among group means. Differences were taken as significant if P < .05.

Results

Experiments in intact rats. In this series of experiments, the animals were treated with HU (n = 8) or vehicle (controls, n = 8) and sacrificed 2 hr after treatment, when the drug-induced increase in circulating CORT reaches a peak (Preziosi, unpublished observation). Message levels of IL-1α, IL-2, IL-4, IL-6, TNFα and INF-γ were assessed. The following pattern of expression emerged: 1) Transcripts of TNF and IL-6 were expressed in the majority of rats treated with HU but were absent or less evident in most controls. A similar pattern, though less clear, could be observed for IL-1α. 2) mRNA encoding IL-2 and INF-γ was undetectable in both control and HU-treated rats. 3) By contrast, IL-4 mRNA was expressed by most animals in both groups (fig. 1).

Taken collectively, these results show that treatment with HU is specifically associated with an increased gene expression of those cytokines that have been more frequently reported to elicit acute stimulation of the HPA axis (Sapolsky et al., 1987; Bernardini et al., 1990; Navarra et al., 1991).

Experiments in ADX rats. These experiments were carried out as above, and message levels of IL-1α, IL-2 and TNF were evaluated. We found that 1) IL-2 mRNA was not expressed in either control (n = 5) or HU-treated rats (n = 5), 2) IL-1α transcript was detectable in HU-treated animals to a much greater extent than in controls and 3) although control rats expressed TNF mRNA, such expression was considerably higher in ADX rats (fig. 2).

In these experiments, adrenalectomy appeared to enhance the gene expression of proinflammatory cytokines, TNF in particular, induced by HU treatment. It is conceivable that adrenalectomy per se could induce proinflammatory cytokines gene expression as a consequence of various mechanisms. Infectious disease states fostered by adrenalectomy might induce the expression of proinflammatory cytokines. Besides, endogenous glucocorticoids down-regulate TNF and IL-1 gene expression. Therefore, it was impossible to determine whether HU-treated ADX rats express proinflammatory cytokines to a larger extent than likewise-treated intact rats. To clarify this point, we used both intact and ADX rats in the same experiments to compare the effects of HU on the two groups.

Experiments in intact and ADX rats. Both intact and ADX rats were treated with HU and sacrificed 2 and 24 hr after treatment to establish the time course of cytokine gene expression. Message levels of IL-1α, IL-4, IL-6 and TNF were assessed. Trunk blood was also collected for parallel plasma CORT assays. Two hours after treatment, weaker expressions of TNF, IL-1α and IL-6 mRNAs were observed in HU-treated intact rats compared with those found in the ADX group. In particular, and at some variance with the results in figure 1, intact rats did not express significant IL-1α mRNA at 2 hr after HU treatment. Conversely, plasma CORT levels were almost undetectable in ADX rats, whereas they were elevated in intact animals (fig. 3).

Twenty-four hours after administration of HU, no major difference in IL-1α was evident between ADX and intact animals, whereas the expression of TNF mRNA was still high in ADX rats. Similarly, IL-6 message levels remained higher in the ADX group compared with intact rats (fig. 3). Circulating CORT in intact rats had returned to values in the range of those usually found in untreated rats under basal conditions (Navarra et al., 1990), which confirms that gene expression of IL-1α and, to a lesser extent, that of TNF are inversely related to CORT levels.

Whereas the pattern of expression of mRNA for IL-1α, IL-6 and TNF appeared to be related to both HU treatment and plasma CORT levels, that of IL-4 seemed to be directly related only to the latter parameter. High levels of IL-4 mRNA were detected 2 hr after HU treatment. These levels were reduced, but still detectable, 24 hr after drug administration, in parallel with decreased plasma CORT levels. Virtually no IL-4 message was expressed in ADX rats (fig. 3).

TNF-α assay. From the foregoing experiments, it emerged that HU administration is specifically associated with increased expression of mRNAs encoding the proinflammatory cytokines, TNF, IL-6 and IL-1α, in intact and particularly...
ADX rats. It was therefore of interest to ascertain whether synthesis and release of cytokine protein paralleled variations in cytokine gene expression. Circulating levels of TNF seemed to be a suitable marker of HU-induced increase in cytokine production, because 1) IL-1α is commonly considered a membrane-associated form of IL-1 (Dinarello, 1991), and its circulating levels might not represent a reliable index of protein synthesis; and 2) levels of IL-6 mRNA more elevated at 24 hr than at 2 hr after HU treatment (figure 3) suggested that gene expression and synthesis of this cytokine might reflect the overlapping effects of HU and other proinflammatory cytokines, i.e., IL-1 and TNF (Dinarello, 1991; Chrousos, 1995), which are first induced by HU.

Intact and ADX rats were treated with HU or vehicle, and trunk blood was collected 2 hr after treatment. Figure 4 shows that both HU and adrenalectomy significantly increased plasma TNF bioactivity ($P < 0.01$ and $P < 0.05$, respectively). Treatment of ADX rats with HU elicited a further increase in TNF levels compared with ADX rats that received vehicle.

**Discussion**

The results of this study provide the first experimental evidence that HU modulates gene expression and synthesis of proinflammatory cytokines *in vivo*. This effect is markedly enhanced by adrenalectomy. A complex interplay appears to exist between the effects of HU on cytokine gene expression and its ability to induce adrenocortical activation. In fact, those cytokines whose gene expression is up-regulated by HU have been previously reported to stimulate adrenocortical secretion in the rat (Sapolsky et al., 1987; Bernardini et al., 1990; Navarra et al., 1991). The acute effects of proinflammatory cytokines on the HPA axis (e.g., the activation observed within 1 hr after the i.v. injection of IL-1, TNF or IL-6) are believed to occur via direct action at the hypothalamic level, where they provoke an increase in CRH secretion (Busbridge and Grossman, 1992). There is general agreement that circulating cytokines, whose levels increase in various disease states, are able to elicit the adrenocortical response, although the mechanisms through which they signal across the blood-brain barrier are still controversial. Besides, CNS-borne cytokines (Koenig, 1991) can also play a role in releasing CRH. The present evidence, together with our previous findings in stalk-sectioned rats (Navarra et al., 1990), is consistent with the notion that HU increases the levels of circulating cytokines, which in turn activate the HPA axis through a stimulatory effect on CRH release. Alternatively, or additionally, HU might enhance the local synthesis of proinflammatory cytokines within the hypothalamus, because the drug very easily diffuses throughout all tissues, including the CNS (Morgan et al., 1986; Volpe et al., 1989).

Stimulation of the HPA axis, resulting in increased glucocorticoid secretion, represents a protective mechanism against damage arising from cytokine overshooting (Chrousos, 1995). In fact, glucocorticoids not only decrease the transcription rate and subsequent synthesis of several proinflammatory cytokines, including IL-1, IL-6 and TNF, but also suppress the expression of phospholipase A2, cyclo-oxygenase-2 and nitric oxide synthase-2 genes. The latter are, in contrast, induced by these cytokines, leading to the synthesis of mediators of inflammation that might play a role in cytokine toxicity (i.e., prostanooids, platelet-activating factor and nitric oxide) (Chrousos, 1995). Moreover, we and others (Daynes and Araneo, 1989) have shown that glucocorticoids enhance IL-4 gene expression in *ex vivo* and *in vitro* experiments. IL-4 is regarded as an anti-inflammatory cytokine, because it suppresses gene expression and synthesis of IL-1 (Essner et al., 1989; te Velde et al., 1990). Thus the lack of endogenous glucocorticoid secretion in ADX and HYX rats, *vis-à-vis* the increase in gene expression and synthesis of...
proinflammatory cytokines associated with HU treatment, may well account for the exacerbated toxicity of the drug. Conversely, increased CORT levels associated with HU treatments in intact rats would exert negative feedback on cytokine production, which affords tolerance to HU toxicity.

What relationship exists between the established mechanism of action of HU and its effects on cytokine gene expression in vivo? HU interferes with the formation of a tyrosyl free radical in the catalytic center of ribonucleoside diphosphate reductase, thereby inhibiting the reduction of ribonucleotides to deoxyribonucleotides. Reductive conversion of ribonucleotides is a rate-limiting step in DNA biosynthesis (Chabner et al., 1986; Nocentini, 1996). This mechanism of action accounts for the cell cycle-specificity of HU: the drug is active in the S phase, producing an arrest of proliferating cell populations in the G1/S phase of cell cycle, during which cells maintain their ability to synthesize RNA and proteins (Tomita and Plager, 1979). It is known that S phase synchronization is associated with an increase in the biosynthesis of several proteins; for example, a 3-fold increase in the number of glucocorticoid receptors was observed in the S phase compared with the G1 phase (Cidlowski and Cidlowski, 1982). A cell cycle-synchronizing agent, HU should then be able to enhance protein synthesis. In fact, HU treatments produced an increase in glucocorticoid receptor binding in cultured human lymphoblasts, an effect that did not appear to depend on the presence of glucocorticoids (Littlefield et al., 1986). Hydroxyurea was reported to potentiate the stimulatory effects of LPS and silica on IL-1 production by cultured THP-1 cells, a myelomonocytic line characterized by active cell cycle synchronization, because it was not observed in normal monocytes, which usually do not proliferate in vitro (Matsushima et al., 1986). The ability to stimulate IL-1 production in vitro appears to be a common feature of several cell cycle-specific anticancer drugs. In fact, increases in IL-1 mRNA expression and/or synthesis were also observed in cultured THP-1 monocytes after treatments with podophyllin and vinca alkaloids (Ferrua et al., 1990) or azacytidine (Kovacs et al., 1987) and in alveolar macrophages treated with bleomycin (Suwabe et al., 1988). By contrast, a cell cycle-nonspecific agent, cyclophosphamide, was shown to decrease both TNF and IL-1 synthesis (McBride et al., 1987).

These considerations lead to the conclusion that, at least as far as IL-1 is concerned, the effect of HU on gene expression might be part of a generalized up-regulation of mRNA and protein synthesis secondary to cell cycle synchronization. However, other considerations suggest that this explanation may be an oversimplification. In fact, proinflammatory cytokines are mostly released by macrophages (Dinarello, 1991), which are active in the S phase of the cell cycle dependent on ribonucleic acid and protein synthesis. Endocrinology 110: 1653–1682, 1982.

In conclusion, we have shown that HU selectively enhances gene expression of the proinflammatory cytokines, TNF, IL-6 and IL-1α, in experiments conducted in vivo in intact and ADX rats. Hydroxyurea also increases circulating TNF levels in these animals. The effects of HU on proinflammatory cytokines might account for both the rise in glucocorticoid secretion induced by the drug in intact rats and the dramatic increase in lethality observed in animals with disruptions of the HPA axis. These findings in experimental animals show the need for similar studies in humans; if such effects of HU are also observed in humans, the drug should be used cautiously in diseases, such as AIDS, where increased levels of circulating TNF and glucocorticoids might be detrimental to the patient.

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