Hydroxyurea induces vasopressin release and cytokine gene expression in the rat hypothalamus

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Received 1 March 2006; received in revised form 23 May 2006; accepted 7 June 2006

Abstract

We previously showed that the cytostatic drug hydroxyurea (HU) activates the hypothalamo–pituitary–adrenal (HPA) axis in intact rats, whereas it is lethal in rats with impaired HPA function. In these animals, HU toxicity is mediated by increased circulating levels of proinflammatory cytokines, whose secretion cannot be counteracted by glucocorticoids, suggesting that HPA activation blunts HU toxicity. Here we investigated the mechanisms through which HU activates the HPA axis, looking at the direct effects of the drug on the isolated hypothalamus. We found that HU significantly increases the release of arginine vasopressin but not that of corticotrophin-releasing hormone in short-term incubation experiments. The levels of arginine vasopressin are also increased in the hypothalamus and systemic circulation 2h after the in vivo administration of the drug. Furthermore, HU increased significantly the expression of interleukin-6 and, to a lesser extent, interleukin-1β in the hypothalamus. Interestingly, experiments with HU on primary cultures of rat microglia and astrocytes suggested that the increase in cytokine gene expression observed in hypothalamic explants is not accounted for by glial cells. Since both vasopressin and cytokines can activate the HPA axis, our present findings provide a reasonable explanation of the HPA activation elicited by HU in vivo in the rat.

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Keywords: Hydroxyurea; Arginine vasopressin; Corticotrophin-releasing hormone; Interleukin-6; Interleukin-1β; Hypothalamus; Microglia; Astrocytes; Rat

1. Introduction

Hydroxyurea (HU) is an old drug still largely used as an antileukemic agent and an inducer of fetal hemoglobin in sickle-cell disease (Navarra and Preziosi, 1999). We have previously shown that HU produces a marked, dose-related activation of adrenal secretion after single or repeated (5 days) oral administration in the rat. The large increase in corticosterone levels turned out to be protective towards acute HU toxicity, since the drug resulted rapidly lethal in hypophysectomized or adrenalectomized (ADX) rats. In these animals, replacement therapies with synthetic ACTH or corticosterone restored the ability to tolerate HU treatments (Vacca et al., 1984, 1985; Navarra et al., 1990). Such neuroendocrine and toxic profile of HU, i.e. activation of the hypothalamo–pituitary–adrenal (HPA) axis and increased toxicity in the absence of normal HPA function, suggested that the effects of HU could be mediated by increased systemic levels of certain proinflammatory cytokines, such as interleukin-1 (IL-1) and IL-6 or tumor necrosis factor (TNF) (Preziosi et al., 1992). In this regard, we found that HU strongly increased splenic expression of IL-1, -6 and TNF, taken as a marker of systemic cytokine production, in ADX but not in intact rats, thereby confirming the postulated mechanism of HU toxicity in animals with impaired HPA function (Navarra et al., 1997). However, this study also showed that peripheral cytokines do not mediate the stimulatory effects of HU on the HPA axis in intact rats, leaving the latter issue unanswered.

Therefore, in the present study, we investigated the mechanisms through which HU activates the HPA in the rat. Since the drug does not activate the HPA axis in hypophysectomized animals and it is able to cross the
blood–brain barrier (Navarra et al., 1990; Dogruel et al., 2003; Gwilt et al., 2003), we tested the hypothesis that HU acts directly at the hypothalamic level to promote HPA activation. To this purpose, we used isolated rat hypothalamic explants (Tringali et al., 2005) and evaluated the effects of HU on the production and release of neuropeptides involved in the control of HPA axis, namely corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP). In view of the fact that certain cytokines may also control HPA activation (Turnbull and Rivier, 1995), we also studied the effects of HU on proinflammatory cytokine gene expression in the hypothalamus. Since glial cells represent the main source of proinflammatory cytokines in the central nervous system, the effect of HU on primary cultures of rat microglia and astrocytes was also evaluated.

2. Materials and methods

2.1. Hypothalamic incubations

The use of animals for this experimental work has been approved by the Italian Ministry of Health (licensed authorization to P. Navarra). Male Wistar rats (200–250 g) were decapitated between 09.00 and 10.00 a.m. and the brains were rapidly removed. Hypothalami were dissected within their anatomical limits and divided longitudinally in two halves. The hypothalami were then incubated in a 24-well plate (one hypothalamus/well), at 37°C in a humidified atmosphere containing 5% CO2 and 95% O2 in 300 ml incubation medium, minimum essential medium (MEM) with glutamine and Earle’s salts, supplemented with 0.2% bovine serum albumin, 50 μg/ml ascorbic acid and 20 IU/ml aprotinin, pH 7.4. After a 60-min pre-incubation period (during which the medium was changed every 20 min), the medium was aspirated and replaced with fresh medium alone (control), or medium containing HU (Sigma Biochemical Co., St. Louis, MO). The latter was dissolved in sterile bidistilled water and further diluted in incubation medium. Hypothalamic tissues remain viable and functional during the timeframe of the experiments (1–3 h), as assessed by the lactate dehydrogenase assay for cell toxicity (Pozzoli et al., 2001).

At the end of experiments, hypothalami were weighed, and incubation media were collected and stored at −35°C until assays for CRH and AVP immunoreactivities. In order to measure intra-hypothalamic CRH and AVP, the hypothalami were snap-frozen and kept at −80°C until homogenization. The latter was performed in 1 ml of Tris–HCl 50 mM, pH 7.4, using a Teflon glass homogenizer. For RNA analysis, hypothalami were embedded in 2 ml of RNA Later™ solution (Ambion, Austin, TX) and kept at −20°C until RNA extraction.

2.2. In vivo experiments

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 h of light (06.00–18.00) followed by 12 h of dark and had free access to food pellets and water. On the day of the experiment, animals were treated between 09.00 and 10.00 a.m. with 800 mg/kg HU, or corresponding volumes of vehicle, both given by oral gavage, and were decapitated 2 h later. Trunk blood was collected for the measurement of plasma AVP levels; hypothalami were rapidly removed and stored at −35°C until processed for the measurement of intra-hypothalamic CRH and AVP immunoreactivities.

2.3. Primary cultures of astrocytes

Primary cultures of cortical rat astrocytes were obtained as described by McCarthy and de Vellis (1980), with some modifications introduced in our laboratory. In brief, 1- or 2-day-old Wistar rats were decapitated; the brains were rapidly removed under aseptic conditions and placed in phosphate-buffered saline with Ca2+ and Mg2+ (PBS-w) (Sigma Chemicals Co., St. Louis, MO) containing antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin; Sigma). Under a stereomicroscope, the meninges were carefully removed and the cortex was dissected. The tissue was cut into small fragments, digested with 0.125% trypsin (EuroClone Ltd., Pero, Milano, Italy) in 10 ml PBS without Ca2+ and Mg2+ (PBS-wo) with antibiotics as above, for 25 min at 37°C, and mechanically dissociated in Dulbecco’s MEM with Glutamax-I (DMEM; Gibco, Life Technologies, Paisley, Scotland) containing 10% heat-inactivated endotoxin-free fetal calf serum (FCS; Gibco, Gaithersbourg, MD) and antibiotics as above to obtain single cells.

The cells were seeded in 75-cm² flasks at a density of 1.0–1.5 × 10⁶ cells/ml of medium and incubated at 37°C in a humidified atmosphere containing 5% CO2/95% O2. The culture medium was changed within 24 h, and then twice a week until the astrocytes were grown to form a monolayer firmly attached to the bottom of the flask (8–9 days after dissection). At this time, the culture medium was replaced with PBS-wo and the flask was vigorously shaken to remove non-adherent cells, oligodendrocytes and microglia, grown on the astrocyte monolayer. Subsequently, astrocytes were detached from the flask by a 5-min 0.05% trypsin-EDTA treatment (Euroclone). Astrocytes obtained with this procedure were then sub-cultured twice every 7–9 days (i.e. the average time for the cells to reach confluence), the first time in 75-cm² flasks and the second time directly in 24-well plates used for experiments. Once the confluence was reached and 12 h before the beginning of experiments, cells were starved, i.e. medium was replaced with serum-free DMEM with Glutamax-I (Gibco), added with antibiotics and 0.2% bovine serum albumin (BSA, Sigma). The average total time from dissection to experiments was 28 days.
2.4. Primary cultures of microglia

Rat cortical microglial cells were obtained as described by Giulian and Baker (1986), with some modifications. In brief, 1- or 2-day-old Wistar rats were decapitated; the brains were rapidly removed under aseptic conditions and placed in PBS-w containing antibiotics as above. Under a stereomicroscope, the meninges were carefully removed and the cortex was dissected. The tissue was cut into small fragments, digested for 20 min at 37°C with 0.125% trypsin (Euroclone) in 10 ml PBS without Ca²⁺ and Mg²⁺ (PBS-wo) with antibodies as above, and for further 5 min at 37°C in the same medium as above, supplemented with 150 U/ml DNase I (Sigma); this step was followed by gentle syringing in DMEM with Glutamax-I (Gibco) containing 10% FCS (Gibco) and antibiotics as above, to obtain single cells.

The cells were cultured in a bright line counting chamber (Sigma), seeded at a density of 3 × 10⁶ cells/10 ml of medium/flask and incubated at 37°C in a humidified atmosphere containing 5% CO₂/95% O₂. The culture medium was changed within 24 h and then twice a week. After 11 days from dissection, microglia was detached from the astrocyte monolayer (see above) by gentle shaking in the same culture medium; the supernatant was then centrifuged at 1200 rpm for 10 min at 4°C, the medium discarded, the cells re-suspended in 5 ml of fresh medium and counted. Thereafter, the cells were plated in 48-well plastic plates at a density of 2 × 10⁵ cells/300 μl/well and incubated at 37°C in a humidified atmosphere containing 5% CO₂/95% O₂. Experiments were performed within 24 h from the last plating; at that time, culture medium was replaced with plain medium (controls) or with medium containing HU.

2.5. RNA isolation and RNase protection assay

Total RNA was extracted from homogenized tissue by the guanidine thiocyanate lysis method (Chomczynski and Sacchi, 1987). The yield of RNA was ranging between 45 and 55 μg/100 mg of wet tissue.

To measure mRNA levels of a number of cytokine genes, the RiboQuant multiprobe template set rCK-1 (Pharmingen, San Diego, CA, USA) containing cDNA templates for rat IL-1α, IL-1β, TNFβ, IL-3, IL-4, IL-5, IL-6, IL-10, TNFα, IL-2 and interferon-γ (IFNγ), as well as the housekeeping genes L32 and glyceraldehyde-3-phosphate dehydrogenase, was used. Nucleotide antisense riboprobes for the above-mentioned genes were synthesized using T7 RNA polymerase in the presence of [α-³²P]UTP (800 Ci/mmol). [³²P]RNA probes were extracted by phenol–chloroform and precipitated with ethanol.

RNase protection analyses were performed (Pozzoli et al., 1996) by hybridizing 25 μg total RNA per sample in 24 μl deionized formamide plus 6 μl hybridization buffer containing 3 x 10⁵ cpm of each riboprobe. A control sample of yeast tRNA was included in each assay as a negative control. After heating at 80°C, the samples were hybridized at 56°C for 15–18 h. Samples were then digested with RNase (40 μg/ml RNase A and 350 U/ml RNase T1) at room temperature for 60 min, followed by proteinase K treatment, phenol–chloroform extraction, and ethanol and ammonium acetate precipitation. Double-stranded RNase-protected fragments were resolved on a 5% polyacrylamide–8 M urea gel. After drying, gel was visualized by auto-radiography. Quantitative analysis was performed by using the ImageMaster® VDS and the Imagesystem software package (Amersham-Pharmacia Biotech, San Francisco, CA, USA). The intensity of protected cytokine fragments was normalized to the intensity of the protected L32 fragment of the same sample, and results reported as corrected arbitrary units.

2.6. CRH radioimmunoassay

CRH was measured by radioimmunoassay as previously described (Navarra et al., 1991), with modifications. A CRH antiserum (kindly gifted by Prof. R. Bernardini) and (2-¹²⁵I)-iodohystidyl[³²]CRH (Amersham-Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) were used. The detection limit of the assay was 1 pg/tube, with intra- and inter-assay coefficients of variation of 5% and 10%, respectively.

2.7. AVP assay

Samples of incubation media and homogenates (100 μl in both cases) were assayed for the presence of AVP using a commercially available AVP enzyme immunoassay kit (Assay Designs Inc., Ann Arbor, MI, USA), following the manufacturer’s directions. This kit does not cross-react with oxytocin, TRH, VIP, Leu- and Met-enkephalin, mesotocine, somatostatin, vasotocin and Arg-vasotocine, and desmopressin. The detection limit of the assay was 4 pg/ml and the EC₅₀ was 21.5 pg/ml.

Plasma AVP levels were measured by RIA as previously described (Mancuso et al., 1999).

2.8. Statistical analysis

Data were expressed as the mean ± 1 standard error of the mean (S.E.M.) of (n) replicates per group and analyzed by ANOVA followed by post-hoc Newman–Keul’s test for comparison between group means, or by Student’s t-test where appropriate. All data were analyzed using a Prism™ computer program (GraphPad, San Diego, CA, USA). Differences were considered significant if p < 0.05.

3. Results

Hypothalamic explants were incubated in vitro in presence of 1 mM HU, and the amount of CRH, the main ACTH secretagogue (Rivier et al., 1983), released in the incubation media was measured after short incubation times. As shown in Fig. 1A, HU did not affect CRH release both
after 1 and 3 h treatments. Moreover, the tissue content of CRH, which more closely reflects CRH biosynthesis, was also unaffected by HU treatment (data not shown). Similar to the in vitro experiments, HU did not modify in a significant manner hypothalamic CRH content when administered in vivo (Fig. 2, left panel), which rules out the possibility that in vivo generated metabolites of HU may exert a stimulatory effect on CRH release.

Fig. 1. The effects of HU on the production and release of CRH (panel A) and AVP (panel B) from rat hypothalamic explants. Two experiments, each carried out in triplicate (AVP) or in quadruplicate (CRH), were carried out; CRH and AVP levels were measured in the incubation media and in the hypothalamic tissues, and the results were expressed as the percent of peptide released over the total peptide content (intra-hypothalamic plus released) of each hypothalamus. Data are the means±1 S.E.M. of six (for AVP experiments) or eight (for CRH experiments) hypothalami per group. *: p<0.05 versus controls.

Fig. 2. The effects of HU on the hypothalamic content of CRH and AVP, as well as on plasma AVP levels in in vivo experiments. Data are expressed as pg/mg of wet tissue (hypothalamic tissues) or pg/ml (plasma); the means±1 S.E.M. of 16 (for hypothalamic measurements) or 8 (for plasma measurements) animals per group. * and **: p<0.05 and p<0.01 versus controls, respectively.

Fig. 3. The effects of HU on IL-1β (panel A) and IL-6 (panel B) mRNA levels in the rat hypothalamus. Hypothalamic explants were incubated with medium alone (controls) or medium containing 1 mM HU, and total RNA extracted. To quantify the effect of HU, the intensity of protected cytokine was normalized to the intensity of protected L32 housekeeping genes (see Materials and methods). Results are from three independent experiments performed in duplicate. * and **: p<0.05 and p<0.01 versus controls. A representative experiment is shown in the insert.
Despite not modifying CRH release, HU significantly increased (+54.3%, \( p < 0.05 \) vs. controls) the fraction of AVP released from the explants after 3h of incubation (Fig. 1B), suggesting that the activation of HPA following HU administration in vivo may be mediated by increased hypothalamic release of AVP. This finding was confirmed in subsequent in vivo experiments, where HU was able to elicit a significant increase in hypothalamic AVP content, which was accompanied by a marked increase in circulating AVP (Fig. 2, right panel).

In hypothalamic explants, HU also induced an increase in IL-1\( \beta \) gene expression, which reached statistical significance after 3h of exposure (Fig. 3A). A fairly higher increase (+200%) was elicited by HU on mRNA expression of IL-6 (Fig. 3B), whereas the gene expression of all of the other cytokines tested (see Methods) remained unaffected (data not shown).

Since in the brain IL-1\( \beta \) and IL-6 are mainly released by glial cells (McGeer and McGeer, 1995), we tested the effects of HU in primary cultures of rat cortical astrocytes and microglia. Both in astrocytes and microglia, HU induced IL-1\( \beta \) gene expression; however, such effect was delayed compared to that observed in hypothalamic explants, reaching statistical significance after 6h of incubation in the case of astrocytes and after 24h in the case of microglia (Fig. 4A and B). None of the other cytokines tested, notably including IL-6, showed changes higher than 5% compared to controls (data not shown).

4. Discussion

We have previously shown that in vivo treatment with HU activates the HPA in rats and that this effect may occur directly at the hypothalamic level. To test this hypothesis, we first studied the effect of HU on the release of CRH, the main ACTH secretagogue (Rivier et al., 1983), from hypothalamic explants in vitro. HU was added to the incubation medium at 1mM concentration. Such fixed concentration was adopted in all the in vitro experiments since it corresponds almost exactly to the circulating levels of HU (0.87 ±0.009mM, \( n = 6 \)) achieved 2h after the administration of 800mg/kg of the drug, a dose which consistently and significantly activates the HPA axis in vivo (Navarra et al., 1990). However, 1mM HU did not modify both CRH hypothalamic release and its tissue content; which led us to postulate that the putative HU stimulation of CRH release in vivo might be related to HU metabolites, in particular nitric oxide generated through non-enzymatic HU degradation in the presence of heme-proteins (Cokic et al., 2003; King, 2004).

Subsequently, we carried out an ex-vivo experiment, treating the animals with HU and dissecting the hypothalami 2h later, at the peak of circulating corticosterone levels (Navarra et al., 1990). Consistent with in vitro results, we did not observe significant changes in CRH hypothalamic content after in vivo HU administration; which finally excluded the involvement of CRH in mediating HU-induced activation of the HPA axis.

These negative results on CRH suggested that other important ACTH secretagogues, such as AVP (Bilezikjian and Vale, 1987; Antoni, 1993), could be involved. Interestingly, we found that HU increased AVP hypothalamic release after 3h incubation, a finding paralleled by significant increases in both intra-hypothalamic and circulating AVP levels in vivo. HU also induced the expression of proinflammatory cytokines, IL-6 and--to a lesser extent--IL-1\( \beta \); the latter effect does not seem to be related to HU
actions on glial cells. Since both AVP and the cytokines, IL-1β and -6, are known inducers of HPA activation (Turnbull and Rivier, 1995; Aguilera and Rababan-Diehl, 1993), the present in vivo and in vitro findings provide a reasonable answer to the main end-point of this study, i.e. to unravel the action mechanism(s) through which HU activates the HPA in vivo in intact rats. While the present findings point out to the hypothalamus as a primary site of action for HU activation of the HPA axis, we cannot rule out the alternative hypothesis that the drugs may act directly at the pituitary level to stimulate ACTH secretion. There are no reports in the literature on this topic and this issue remains unanswered at present.

Concerning the effects of HU on IL-1β and IL-6 gene expression within the hypothalamus, it is worth of note that: (i) the induction of IL-1β and IL-6 gene expression by HU does not appear to be related to HU interference with cell proliferation, since it is highly unlikely that any cell type within the hypothalamic fragment is proliferating during the experiments. Consistent with this notion is the assumption that the effects of HU on cytokine gene expression may not be accounted by the inhibition of ribonucleotide reductase, i.e. the biochemical mechanism underlying the cytostatic effects of HU (Navarra and Preziosi, 1999); (ii) findings in primary cultures of microglia and astrocytes would exclude that glia is a target for the effects of HU on IL-6 gene expression, thus suggesting alternative targets such as neurons or endothelial cells.

Both IL-1 and IL-6 were shown to be able to directly stimulate ACTH release from the anterior pituitary gland (Woloski et al., 1985), where IL-6 seems to act in a paracrine manner (Spangelo et al., 1991; Vankelecom et al., 1993). However, at the hypothalamic level, the HPA-activating effects of the cytokines are seemingly indirect, relying on downstream stimulation of primary ACTH secretagogues, CRH and AVP. In fact, both IL-1 and -6 elicit a potent stimulation of CRH and AVP release in this paradigm (Navarra et al., 1991; Yasin et al., 1994). However, in the present study, the increase in IL-1β and -6 gene expression elicited by HU was only associated to an effect on AVP- but not CRH-release. Interestingly, Ghorbel et coll. (2003) described groups of neurons in the supraoptic and paraventricular nuclei (SON and PVN, respectively) of the rat hypothalamus where AVP and IL-6 are largely co-localised; stress induced by dehydration strongly up-regulates IL-6 gene expression which accompanies AVP release from the posterior pituitary. Based on the above observations, these authors suggest that IL-6 can be envisioned as a cytokine-neurotransmitter in the hypothalamo-hypophyseal system. In this light, HU might act specifically on these neuronal populations in the SON and PVN to induce the simultaneous increase in IL-6 gene expression and AVP secretion, with no apparent involvement of glial cells. Such effect might well account for the activation of HPA induced by the drug in vivo in the rat.

Acknowledgements

We thank Dr. Cinzia Dello Russo for critical reading of the manuscript and helpful suggestions. We are very grateful to Prof. Cecilia Zuppi (Institute of Biochemistry and Clinical Biochemistry, Catholic University Medical School of Rome) for plasma AVP assays.

References
