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

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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 2 h 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.

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

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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 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 consisting of 5% CO_2 and 95% O_2 in 300 μl 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 Ca^{2+} and Mg^{2+} (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 Ca^{2+} and Mg^{2+} (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-cm^2 flasks at a density of $1.0–1.5 \times 10^7$ cells/10 ml of medium and incubated at 37 °C in a humidified atmosphere containing 5% CO_2/95% O_2. 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 flasks were 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-cm^2 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.