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Altered neuronal and endothelial nitric oxide synthase expression in the bladder and urethra of cyclophosphamide-treated rats

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ABSTRACT

Increased nitric oxide (NO) production seems to play a key role in cyclophosphamide (CYP)-induced cystitis, although the underlying mechanisms and the relative involvement of the different NO synthase (NOS) isoforms remain to be elucidated. Moreover, the role of the urethra in this process is also unclear. In this study, we have analyzed the changes in the expression and distribution of the inducible (iNOS), endothelial (eNOS) and neuronal (nNOS) isoforms of NOS, and the alterations in nerve-mediated contractility in the bladder and urethra of CYP-treated rats. Accordingly, Wistar rats were treated with 150 mg kg^{-1} CYP for 4 (acute treatment) or 48 h (intermediate treatment), or with 70 mg kg^{-1} CYP every 3 days for 10 days (chronic treatment), and the changes in protein expression were assessed by immunohistofluorescence and in Western blots, while mRNA expression was assessed by conventional and quantitative PCR. Similarly, nerve-mediated contractility was analyzed *in vitro*. Unexpectedly, no iNOS expression was detected in CYP-treated animals, while a transient downregulation of nNOS expression and a progressive upregulation of eNOS was observed, although the eNOS accumulated was not in the active phosphorylated form. Qualitative changes in mRNA expression were also observed in the bladder and urethra, although contractility only diminished in the bladder and this change was not dependent on NOS activity. These findings suggest that spatiotemporal alterations in NO production by constitutive NOS may be involved in the pathogenicity of CYP. Further studies will be necessary to understand the contribution of eNOS to the increases in NO associated with bladder inflammation, or that of free radicals.

1. Introduction

Haemorrhagic cystitis is a common clinical complication of cyclophosphamide (CYP) therapy, a treatment used in chemotherapy for solid tumours and in conditioning for hematopoietic cell transplantation [1,2]. Inflammation of the urinary bladder can produce irritative lower urinary tract symptoms, as well as life-threatening complications and severe hemorrhage. In experimental animals, mainly rats, CYP-induced cystitis is a common model of bladder inflammation in which symptoms of an overactive bladder develop [2–4]. Among the many mediators implicated in CYP-induced cystitis, the overproduction of nitric oxide (NO) is considered to play a key role [1,2,5–8]. This increased NO production is often assumed to result from the expression of the inducible isoform of NO synthase (iNOS) [2,7–9], while the production of NO by the two constitutive NOS isoforms, endothelial (eNOS) and neural (nNOS), has attracted little attention. Constitutive NOS activity can produce controlled amounts of NO, mainly from the endothelium and nerve structures [10]. However, changes in NO release from these different sources may alter efferent contractile and/or afferent sensory functions, affecting distinct target cells including smooth muscle cells, sensory nerves, urothelial cells and interstitial cells.

CYP-induced cystitis is thought to be triggered by the accumulation of its hepatic metabolite, acrolein, in the urine. Acrolein is a

Abbreviations: ACh, acetylcholine chloride; AVP, arginine vasopressin; CYP, cyclophosphamide; DAPI, 4′,6-diamino-2-phenylindole dihydrochloride; EFS, electrical field stimulation; H&E, haematoxylin and eosin; ir, immunoreactivity or immunoreaction; NA, noradrenaline; L-NOARG, N^G-nitro-L-arginine; LPS, lipopolysaccharides from *E. coli* 026:B6; NO, nitric oxide; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase; PB, phosphate buffer; p-eNOS, phosphorylated eNOS; RT-PCR, real time PCR; SNC, S-nitroso-L-cysteine.

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highly reactive aldehyde that can initiate an epithelial inflammatory process which spreads to the whole bladder wall [1]. Accordingly, the urethra would be briefly exposed to acrolein during micturition, although the contribution of urethral inflammation to bladder overactivity induced by CYP remains unexplored. In the present study, we investigated the changes in the expression (both protein and mRNA) and distribution (by immunohistofluorescence) of iNOS, eNOS and nNOS in the bladder and urethra of rats subjected to acute, intermediate or chronic CYP treatment. Since eNOS may be further activated by phosphorylation at Ser1177 [11], the distribution of phosphorylated eNOS (p-eNOS) was also assessed. Finally, the possible alterations of nerve-mediated excitatory and inhibitory contractility provoked by CYP were investigated *in vitro* using bladder and urethral preparations. As a result, we observed prominent inflammatory reactions in the urethra and bladder, although contractility was only modified in the bladder. A surprising lack of iNOS expression was observed in both organs in response to CYP treatment, while nNOS and eNOS expression diminished and augmented, respectively. The possible implications of these changes in CYP-induced cystitis are discussed.

2. Materials and methods

2.1. Drugs

Acetylcholine chloride (ACh), atropine sulphate, cyclophosphamide monohydrate (CYP), guanethidine monosulphate, lipopolysaccharides from *Escherichia coli* 026:B6 (LPS), noradrenaline bitartrate salt (NA), D-tubocurarine hydrochloride, N^G-nitro-L-arginine (L-NOARG) and [Arg^8] vasopressin acetate salt (AVP) were all obtained from Sigma Chemie GMbH (Steinheim, Germany). All drugs were dissolved in distilled water, except for CYP and LPS, which were dissolved in NaCl (0.9%). Solutions were stored at −20 °C and the drugs were diluted to working concentrations in 0.9% NaCl. Solutions of S-nitrocysteine (SNC) were prepared by adding sodium nitrite (100 mM) to the equivalent volume of a solution containing 250 mM HCl, 1 mM EDTA and 100 mM L-cysteine. SNC concentrations were determined spectrophotometrically (Shimadzu UV-1601 UV–visible spectrophotometer; Shimadzu, Tokyo, Japan), assuming a molar absorption coefficient of $\varepsilon_{544} = 16.6\ M^{-1}\ cm^{-1}$. Working dilutions were prepared in deoxygenated distilled water immediately before use, storing the solutions on ice and in the dark.

2.2. CYP treatment

Haemorrhagic cystitis was induced by CYP administration to 80 adult female Wistar rats (200–250 g). Rats were housed individually and they were maintained under standard laboratory conditions (12 h light/dark cycle), with *ad libitum* access to food and water. CYP was administered according to the following regimes: (i) acute treatment: 150 mg kg^{-1} i.p., 4 h before sacrifice; (ii) intermediate treatment: 150 mg kg^{-1} i.p., 48 h before sacrifice; or (iii) chronic treatment: 70 mg kg^{-1} i.p., administered every 3 days for 10 days. Control animals (20 in total) received a corresponding volume of saline (0.9%) alone instead of CYP. Furthermore, as a positive control for the up-regulation of iNOS, LPS was injected according to the following regimes (4 animals each): (i) acute treatment: 16 mg kg^{-1} i.p., 6 h before sacrifice; or (ii) intermediate treatment: 16 mg kg^{-1} i.p., 48 h before sacrifice, as described previously [12]. All procedures were approved by the Ethical Committee of the Complutense University and performed in accordance with European guidelines (EC Directive 2010/63/EU).

2.3. Voiding frequency test

Micturition frequency was analysed in all rats as described previously [13]. Drinking water was removed 1 h before testing and the rats were left in cages lined with filter paper for 30 min. A UV light source was used to visualize and trace the urine spots on the filter paper, and the total number of urine spots and those of small diameter (<0.5 cm) was counted and expressed as the number of voids per hour.

2.4. Histology and immunofluorescence

Animals were anesthetized (40 mg kg^{-1} ketamine + 5 mg kg^{-1} xylazine, i.p.) and then subjected to cardiac perfusion with heparinised 0.1 M phosphate buffer (PB), followed by 4% paraformaldehyde in PB for 30 min. The lower urinary tract was removed from the rats and tissue samples (5 × 5 mm) were obtained from the mid-detrusor, the trigone or the proximal urethra. In LPS treated rats, samples from the liver, spleen, lungs, bladder and urethra were obtained. The tissues were then processed as described previously [14]. Briefly, they were fixed in ice-cold 4% paraformaldehyde in 0.1 M PB (pH 7.0) for 30 min and cryoprotected with increasing concentrations of sucrose in ice-cold 4% paraformaldehyde (10% for 90 min followed by 20% for 120 min). The tissues were then incubated overnight at 4 °C in 30% sucrose in PB, snap-frozen in liquid nitrogen-cooled isopentane and stored at −80 °C for up to 15 days. The tissues were embedded in Tissue-Tek OTC compound and cryostat sections transverse to the mucosal surface (7 μm: CM1850 UV, Leica Microsystems, Barcelona, Spain) were recovered on poly-L-lysine coated slides. The slides were air-dried at room temperature for 24 h and then processed directly or stored at −80 °C for no more than 30 days.

The sections were stained with haematoxylin and eosin (H&E) and two different researchers blind to the treatment evaluated the histological damage. Accordingly, the lesions (oedema, haemorrhage, vascular congestion, cell infiltration and epithelial damage, including epithelial denudation or proliferation) were classified as non-existent (0), mild (1), moderate (2) or severe (3). Immunofluorescence was performed as described previously [14] using the antibodies listed in Table 1. Briefly, sections were washed with PB (3 × 5 min) and incubated for 1 h in PB containing 3% normal donkey serum (Chemicon International, Temecula, CA, USA) and 0.3% Triton X-100. The sections were incubated for 24 h in a humidified chamber at 4 °C with the primary antibody diluted in PB with 2% normal donkey serum and 0.3% Triton X-100, and for 2 h at room temperature in the dark with the secondary antibodies. After washing with PB (3 times, 10 min each), the nuclei were counterstained for 20–30 min with 4′,6-diamino-2-phenylindole (DAPI, 10.9 mM; Sigma–Aldrich) and the sections were then mounted with Prolong Gold® antifade reagent (Molecular Probes, Eugene, OR, USA). In all cases, negative controls from which the primary antibody was omitted were run in parallel. The sections were visualized on an Axioplan 2 fluorescence microscope (Carl Zeiss Microimaging, Göttingen, Germany) and photographed with a Spot-2 digital camera (Diagnostic Instruments Sterling Heights, MI, USA). Images were stored digitally as 12-bit images using MetaMorph 6.1 software (MDS Analytical Technologies, Toronto, ON, Canada) and the intensity of immunofluorescence was quantified by measuring the proportion of the selected area that exceeded the threshold value [14]. For each experimental group, this measurement was made on 5–7 randomly selected areas of tissue per animal, taken from at least 4 animals and for at least 3 different staining procedures performed on different days. Images at 20× magnification were obtained at a constant time exposure to permit direct comparison and a threshold was established to subtract background immunoreactivity (ir). The proportion of the