Corticosterone differentially regulates *bax*, *bcl-2* and *bcl*-x mRNA levels in the rat hippocampus

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Abstract

It has previously been shown that adrenalectomy (ADX) produces apoptosis in the granule cell of the dentate gyrus (DG), and that this effect is prevented by corticosterone replacement. Thus, we have investigated how this phenomenon takes place in rat hippocampus using in situ hybridization. The expression of the pro-apoptotic gene *bax* was measured in the pyramidal cell fields and in the DG. After 5 days of ADX, there was a significant increase in *bax* mRNA levels in the suprapyramidal layer of the DG, an effect prevented by corticosterone replacement. The mRNA of the anti-apoptotic *bcl-2* gene was expressed in CA3 and DG. ADX increased *bcl-2* mRNA levels, but only in the suprapyramidal layer of the DG, an effect that was prevented by corticosterone administration. It is concluded that the up-regulation of *bax* may explain the apoptosis observed in DG after ADX, while the *bcl-2* induction may correspond to a compensatory mechanism protecting the cells from death.

Keywords: Hippocampus; Corticoids; Adrenalectomy; Apoptosis; *bax*; *bcl-2*

Corticoids are known to exert specific effects on neuronal survival in the hippocampus. Thus, rats with chronic overexposure to corticosterone displayed cell death in the pyramidal layer of the CA3 hippocampal subfield [11]. However, several investigations [13,14,16] including our studies [4] have demonstrated that adrenalectomy (ADX) produces apoptosis in the granule cell of the dentate gyrus (DG), and that this effect is prevented by corticosterone replacement [4,13,14]. These results suggest a selective trophic influence of adrenal steroids on granular cell survival. In agreement, it has been shown that corticoids modulate the expression of neurotrophic factors in the CNS [1,9]. It is also possible that corticoids influence cell viability via modulation of the *bcl-2* gene family, which plays a role in determining whether a cell will or will not undergo apoptosis. The *bcl-2* gene family includes both pro-apoptotic and anti-apoptotic members that enhance or block cell death [5]. Thus, the *bax* gene generates a protein (BAX), which is known to promote apoptosis in mammalian cells [10], while the genes *bcl-2* and *bcl-x* generate the proteins BCL-2 and BCL-X_L, which neutralize the actions of BAX [10,17]. We have recently demonstrated, using the RT-PCR technique, that ADX reduces the expression of *bcl-2* and *bax* mRNA in whole hippocampus, and that both effects are prevented by corticosterone administration [4]. Thus, the main purpose of this paper is to investigate if corticosterone induces changes in the expression of the *bcl-2* gene family in hippocampus subfields, which could explain the role of corticosterone in cell viability.

Adult male Sprague–Dawley rats (190–220 g) underwent ADX and were maintained as previously reported [4]. A group of ADX animals received a subcutaneous implantation of a 20 mg corticosterone/80 mg cholesterol pellet for 100 g rat weight, while another group received pellets made of 100% of cholesterol (sham rats). After 5 days, the animals were anesthetized and a blood sample was taken for corticosterone determination [4], and then rapidly perfused with a fixative as previously described [4]. Coronal 18 μm thick sections were cut from the frozen fixed brains, and processed for in situ hybridization. Plasmids containing *bax* (85–408 pb), *bcl-2* (254–792 pb) and *bcl-x* (74–780

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pb) cDNA fragments generated by RT-PCR [4] were linearized by digestion with *Sal*I, *Apa*I and *Bam*H1, respectively. cRNA probes were transcribed at 37 °C for 2 h using Sp6 polymerase (*bcl-2* and *bcl-x*) or T7 polymerase (*bax*) using the Digoxigenin RNA labeling kit (Roche, Molecular Biochemicals, Mannhein, Germany). Brain sections were hybridized for 16 h at 54 °C in 50% formamide, 1 mM DTT, 61.5 mM Tris–HCl (pH 7.5), 60 mM NaCl, 4 mM EDTA, 0.2% lauryl-sarcosine, 0.2% DEPC, 10 μg/ml salmon sperm DNA and 20–50 ng digoxigenin-cRNA in 1× Denhart's solution. The following controls were included: (i) no digoxigenin-cRNA; and (ii) a 10-fold excess of cRNA without digoxigenin labeling. After treatment with ribonuclease A (20 μg/ml) for 30 min at 37 °C, the sections were desalted in standard saline citrate (SCC) and finally washed in SCC 0.1× for 45 min at 54 °C. The sections were blocked in 0.1 M Tris–HCl (pH 7.5), 0.1 M NaCl, 20 mM $MgCl_2$ (TA buffer) with 1 mg/ml BSA and 0.001% Triton X-100 for 30 min at room temperature and incubated for 2 h with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (1:2000 dilution; Roche, Molecular Biochemicals, Mannhein, Germany). Sections were washed in TA buffer and incubated with phosphatase substrate nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M Tris–HCl (pH 9.5), 0.1 M NaCl, and 5.0 mM $MgCl_2$ for 5–6 h at room temperature. Slices were dehydrated and mounted with ENTELLAN (Merck, Darmstadt, Germany). For semiquantitative analysis, densitometric measurements of each subfield of the hippocampus were analyzed using computer-assisted image analysis UN-SCAN-IT software (Silk Scientific Inc. Orem, UT, USA). The low hybridization signal in the CA1 stratum radiatum was considered as indicating background, and subtracted from the optical density values for the hippocampal cell layers. The data are expressed as optical density (pixels) and for each animal the values represent the average of measurements from four to five brain sections. Data were analyzed by one-way ANOVA followed by the Tukey post-hoc test.

In situ hybridization was used to detect changes in hippocampal *bax*, *bcl*-x and *bcl*-2 mRNA levels after ADX and corticosterone replacement. After 5 days of ADX, we observed a reduction in corticosterone serum levels below the limit of detection in the RIA assay (<0.3 μg/dl). ADX rats implanted with a corticosterone/cholesterol pellet had 50% (7.8 ± 2.4 μg/dl) of the hormone level observed in sham-operated animals (15.9 ± 3.0 μg/dl). In sham rats, a positive hybridization for *bax* mRNA was observed in pyramidal cells and granular cells of the DG, with a similar intensity (Fig. 1A,D). The ADX procedure failed to produce any change in the intensity of *bax* hybridization in CA1 and in the infrapyramidal layer of the DG (Fig. 1B,D). In CA3, ADX produced a minor, but non-significant increase in *bax* mRNA levels, but, interestingly, corticosterone produced a significant decrease in *bax* mRNA levels, as compared to ADX alone. However, the ADX procedure produced a 40% increase ($P < 0.01$) in the level of the pro-apoptotic *bax* in the suprapyramidal layer of the DG (Fig. 1B,D). This effect was prevented by corticosterone administration (Fig. 1C,D).

The *bcl*-x gene generates long and short transcripts which encode two proteins with anti-apoptotic and pro-apoptotic activity, respectively [2]. The riboprobe used in this study hybridizes both transcripts, but due to their relative abundance in whole hippocampus [4], the hybridization observed probably represents the large transcript. The levels of *bcl*-x mRNA were higher in CA3 and in the suprapyramidal layer of DG than in CA1 ($P < 0.01$) (Fig. 2). ADX did not cause changes of *bcl-x* mRNA levels in any region of the hippocampus (Fig. 2). Nevertheless, the hormone replacement to ADX animals reduced the levels of *bcl-x* mRNA in the suprapyramidal layer of the DG (Fig. 2).

There was a strong *bcl-2* mRNA expression in CA3 and DG, but that was low in the CA1 subfield compared to CA3 ($P < 0.05$) (Fig. 3A,D). However, ADX increased the *bcl-2* mRNA levels in CA1 with respect to sham animals

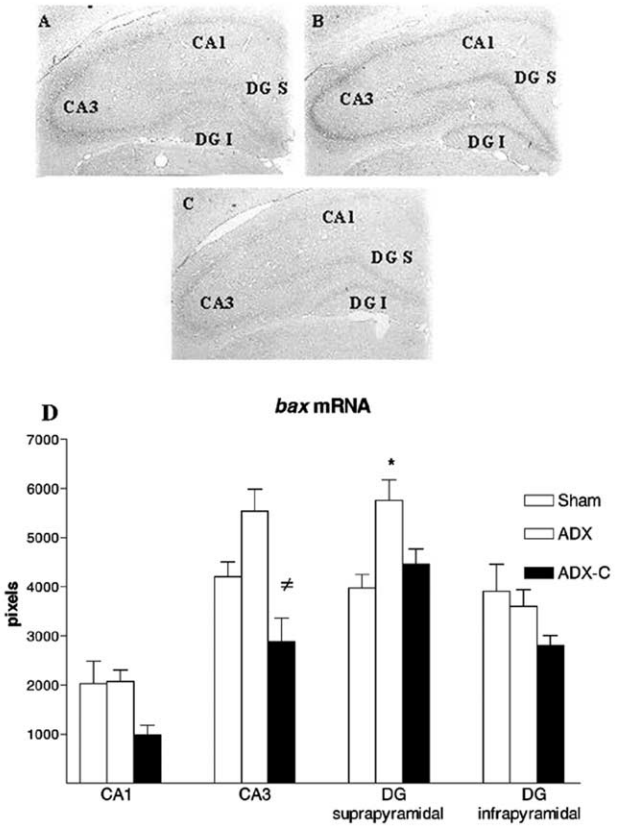

Fig. 1. Effect of ADX and corticosterone replacement on *bax* mRNA levels in hippocampus. The microphotographs illustrate the expression of *bax* in hippocampus of (A) sham, (B) ADX and (C) ADX animals following corticosterone replacement (ADX-C). Scale bar: (A–C) 5 μm. Suprapyramidal (S) and infrapyramidal (I) blades of DG are denoted. (D) Semiquantitative analysis of ADX and ADX-C effects on *bax* mRNA levels in the hippocampal subfields was expressed as specific hybridization measured in pixels. Data are the mean ± SEM. *$P < 0.01$ vs. sham; ≠$P < 0.01$ vs. ADX.

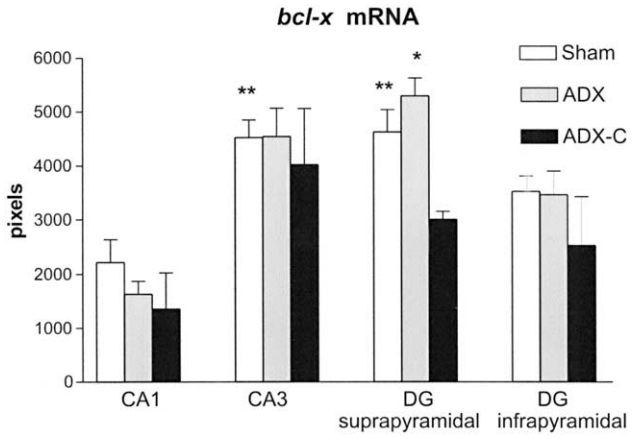

Fig. 2. Effect of ADX and corticosterone replacement on *bcl-X* mRNA levels in hippocampus. The semiquantitative analysis of *bcl-X* expression in different areas of hippocampus (CA1, CA3 and suprapyramidal and infrapyramidal blades of DG) was expressed as specific hybridization measured in pixels. Data are the mean ± SEM. $^{**}P < 0.01$ vs. CA1; $^{*}P < 0.05$ vs. ADX.

($P < 0.05$), an effect that was not affected by corticosterone replacement (Fig. 3). Also, ADX increased the *bcl-2* mRNA in the suprapyramidal layer of the DG, which was partially prevented by corticosterone (Fig. 3B,D). In contrast, ADX or corticosterone administration did not produce any effect on *bcl-2* mRNA levels in CA3 and in the infrapyramidal layer of DG (Fig. 3D).

This study shows that *bax*, *bcl-2* and *bcl-x* mRNAs are topographically distributed in the hippocampus, and that their expression is modified by ADX and/or corticosterone administration. The present results strongly suggests that corticosterone down-regulates the expression of both pro-apoptotic *bax* and anti-apoptotic *bcl-2* genes in the hippocampus, mainly in the DG region.

The effect of corticosterone may be produced by two receptors, i.e. the mineralocorticoid (MR) and the glucocorticoid (GR) receptors. The MR binds corticosterone and aldosterone with high affinity, while the GR binds corticosterone with low affinity [3]. Although it is not clear whether the effects shown here are mediated by MR and/or GR receptors, it is important to keep in mind that the hormone levels obtained with corticosterone pellets are enough to activate MR and perhaps to partially activate GR receptors [15].

The pyramidal neurons of CA3 are vulnerable to high corticosterone levels [11]. In contrast, reduction of corticosterone by the ADX surgery induces only apoptosis in the granular cells of the DG [4,13,14,16]. This evidence suggests that there are intrinsic differences in the types and levels of various gene products present in those neurons, which may influence their ultimate sensitivity to cell death mediated by high or very low corticosterone levels. The mRNA of the anti-apoptotic gene *bcl-2* had low expression in the CA1 subfield, an area resistant to corticosterone changes. However, in ADX animals there was an induction of *bcl-2* expression, which was not prevented by corticosterone replacement. The levels of *bcl-x* mRNA and the pro-apoptotic *bax* mRNA in CA1 were corticosterone independent. Similarly, in the CA3 subfield the *bcl-2* and *bcl*-x mRNA levels were also independent of corticosterone levels. The mRNA of the cell death inducer gene *bax* was expressed in the CA3 subfield, and appeared to be enhanced by ADX, although no statistical significance was achieved. Furthermore, corticosterone administration produced a significant decrease of *bax* mRNA to levels below those observed following ADX only.

We have demonstrated that after ADX the pro-apoptotic *bax* mRNA is induced in the suprapyramidal layer of DG, an effect prevented by corticosterone pellets. The up-regulation of *bax* may explain the apoptosis observed in DG after ADX [4]. Interestingly, it has been reported that ADX induces *p53* [12] which acts as a transcriptional factor stimulating *bax* gene expression [8], making it possible that the increase in *bax* mRNA is related to *p53* activation induced by ADX.

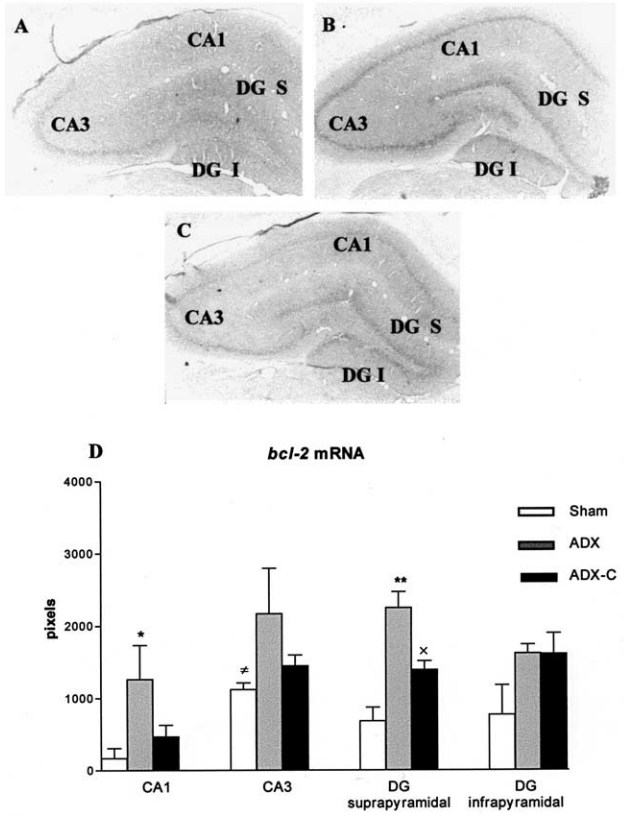

Fig. 3. Effect of ADX and corticosterone replacement on *bcl-2* mRNA levels in hippocampus. Microphotographs illustrate the expression of *bcl-2* mRNA in hippocampus of (A) sham, (B) ADX and (C) ADX animals following corticosterone replacement (ADX-C). Scale bar: (A–C) 5 μm. Suprapyramidal (S) and infrapyramidal (I) blades of DG are denoted. The semiquantitative analysis of *bcl-2* expression in different areas of hippocampus (CA1, CA3 and DG) was expressed as specific hybridization measured in pixels. $\neq P < 0.05$ vs. CA1; $^{*}P < 0.05$ vs. sham; $^{**}P < 0.01$ vs. sham; $\times P < 0.05$ vs. ADX.

It has been demonstrated by in situ hybridization that *bax* levels are not modified in any hippocampal subfield studied after MR blockade [7]. Furthermore, we have seen in preliminary studies that ADX animals with corticosterone replacement in the drinking water (serum levels, 2–7 μg/dl) did not prevent the *bax* mRNA increase in DG. These results suggest the participation of GR receptors, acting as repressors of *bax* expression, and hence GR stimulation may be important for granule cell survival. In agreement, ADX-induced granule cell death is associated with a loss of GR immunoreactivity, supporting the relevance of GR receptors in the maintenance of DG neurons [6]. However, we might consider that corticosterone administered in drinking water to ADX animals does not reduce *bax* mRNA but instead prevents DG apoptosis [4]. The protective action of low levels of corticoids in DG is probably mediated by the induction of *bcl-2*.

The *bcl-2* mRNA levels were increased in the suprapyramidal layer of DG following ADX, perhaps indicating a compensatory mechanism to prevent further neuronal death. Interestingly, we have recently observed that while the increase of *bcl-2* mRNA levels could be prevented by corticosterone pellets, low levels of the hormone delivered in the drinking water could induce a further increase in *bcl-2* mRNA levels in the CA1 and DG regions (in preparation). These preliminary findings could explain the protection against apoptosis in DG observed under low doses of corticosterone. Indeed, it is well known that BCL-2 neutralizes BAX actions [10,17]. Also, these results suggest the participation of MR in the control of *bcl-2* mRNA levels. It has been reported that the MR antagonist, spironolactone, reduces the *bcl-2* mRNA content, but only in the CA1 and CA3 subfields, indicating that in these regions the *bcl-2* gene is positively regulated by MR [7]. As a whole, it can be suggested that MR up-regulates the *bcl-2* expression in CA1 and CA3 subfields, while GR down-regulates the *bcl-2* and *bax* expression in DG. These results suggest that corticosterone, via modulation of *bax* and *bcl-2* mRNA, maintains cell viability in hippocampus.

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