Cranial irradiation modulates hypothalamic-pituitary-adrenal axis activity and corticosteroid receptor expression in the hippocampus of juvenile rat

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Abstract. Glucocorticoids, essential for normal hypothalamic-pituitary-adrenal (HPA) axis activity, exert their action on the hippocampus through two types of corticosteroid receptors: the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). Recent studies report that exposure of juvenile rats to cranial irradiation adversely affects HPA axis stability leading to its activation along with radiation-induced inflammation. This study was aimed to examine the acute effects of radiation on HPA axis activity and hippocampal corticosteroid receptor expression in 18-day-old rats. Since immobilization was part of irradiation procedure, both irradiated and sham-irradiated animals were exposed to this unavoidable stress. Our results demonstrate that the irradiated rats exhibited different pattern of corticosteroid receptor expression and hormone levels compared to respective controls. These differences included upregulation of GR protein in the hippocampus with a concomitant elevation of GR mRNA and an increase in circulating level of corticosterone. In addition, the expression of MR, both at the level of protein and gene expression, was not altered. Taken together, this study demonstrates that cranial irradiation in juvenile rats leads to enhanced HPA axis activity and increased relative GR/MR ratio in hippocampus. The present paper intends to show that neuroendocrine response of normal brain tissue to localized irradiation comprise both activation of HPA axis and altered corticosteroid receptor balance, probably as consequence of innate immune activation.

Key words: HPA axis — Corticosteroid receptor — Irradiation — Hippocampus

Introduction

Cranial radiotherapy (CRT) is widely used not only to treat patients with primary and secondary brain tumors, but also as prophylaxis, to prevent brain metastases development and central nervous system (CNS) involvement in hematological malignancies (Gibbs et al. 2006). However, damage to surrounding normal tissue constitutes a major problem, and CRT is associated with both acute and long-lasting severe side effects. Children treated for brain tumors with radiotherapy are at risk of developing endocrine deficits when the hypothalamus-pituitary axis falls within the fields of radiation, which may resul in growth hormone deficiency and adrenal dysfunction (Schmiegelow et al. 2003; Spoudeas et al. 2003; Darzy and Shalet 2005). Animal models may therefore be useful in assessing the neuroendocrine response following CNS irradiation, in a more controlled and reproducible way than is possible in the clinic.

The initial response of the brain to irradiation involves expression of inflammatory mediators (tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), inter-cellular adhesion molecule 1 (ICAM-1)), which are probably responsible for clinically observed early symptoms of CRT (Hong et al. 1995; Van de Meeren et al. 2001). Concomitantly with the radiation-induced inflammation, hypothalamic-pituitary-adrenal (HPA) axis is activated in early response phase, as reported by a number of studies. In these studies, HPA axis hyperactivity has been documented by increased level of plasma cortisol in patients (Girinsky et al. 1994), increased

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corticosterone (CORT) level in mice (Van de Meeren et al. 2001) and rats (Lebaron-Jacobs et al. 2004) and enlargement of adrenal glands after irradiation (Lebaron-Jacobs et al. 2004). This acute activation of HPA axis most likely contributes to the neuroprotection against oxidative stress and inflammatory reaction caused by irradiation.

One of the prime neuronal targets for glucocorticoid action is the hippocampus (De Kloet et al. 1998), which comprises two types of corticosteroid receptors: the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). GR and MR are two closely-related members of the steroid receptor family of transcription factors that bind common ligands (CORT and cortisol) with different affinity in the brain. High-affinity MR in the hippocampus mediates non-stress circadian fluctuation of glucocorticoids and is primarily activational. The low-affinity GR can be activated additionally to MR only when CORT levels are high, such as the circadian peak or during stress (De Kloet et al. 1998). Thus, CORT action *via* MR exerts a tonic, permissive influence on hippocampus-associated functions, whereas activation of GR in this brain region mediates feedback actions aimed to terminate stress-induced HPA activation (De Kloet et al. 1998). An imbalance in GR/MR-mediated actions underlies behavioral deficits and neuroendocrine disturbances, increasing vulnerability for stress-related brain disorders (De Kloet 2000).

Recently, we showed in juvenile rats that radiation-induced late response is characterized by hyposuppressive state of the HPA axis that is associated with a decrease in GR/MR ratio (Velickovic et al. 2008). Therefore, we designed the present studies to determine i) whether cranial irradiation modulates the HPA axis activity in acute response phase and ii) whether cranial irradiation could modify the efficiency of glucocorticoid feedback on the HPA axis through changes in the expression of GR and MR. For this purpose, main product of HPA axis activity, CORT, was measured in serum of irradiated rats and appropriate sham-irradiated controls using ELISA assay. In addition, GR and MR mRNA and protein in the hippocampus were determined at various time intervals after radiation treatment. The experiments were performed on previously established animal model for CNS prophylactic therapy in children with acute lymphoblastic leukemia (ALL) (Mullenix et al. 1990).

Materials and Methods

Animals

Experiments were conducted on 18-days-old male Wistar rats, weighing 32.45 ± 0.71 g, bred at the Institute of Nuclear Sciences "Vinča". The animals were maintained in the animal room on a 12 : 12 h light/dark cycle (lights on: 7:00 a.m.– 7:00 p.m.), under constant temperature (22°C) and humidity (55 ± 5%). The animals had free access to food-commercial rat pellets (provided by Veterinary Institute Subotica, Serbia), and water. All procedures were approved by the Ethics Committee of the Serbian Association for the Use of Animals in Research and Education.

Irradiation procedure

Previously established animal model for CNS prophylactic therapy of childhood ALL (Mullenix et al. 1990) was used in this study. At the age of 18 days (the day of birth taken as day 0), animals were divided into two groups: sham-irradiated (sham-IR) controls and irradiated (IR) animals. Since irradiated animals were immobilized for 1 h during irradiation procedure (Bayer and Peters 1977), the sham-irradiated controls were treated equally, except for being exposed to the source of radiation. Immobilization was performed by placing the animal on the 2.5-cm thick polystyrene bar and fixation with medical plaster tape so that the animal was unable to move. The heads of the IR rats were exposed to a single 10 Gy dose of γ-rays using ^{60}Co-source (Laboratory for Radiation Chemistry and Physics, Institute of Nuclear Sciences "Vinča"). This dose approximated a biological equivalent to the clinical dose of 24 Gy given in 12–14 fractions in CNS prophylactic therapy of childhood ALL (Schunior et al. 1990). In order to minimize radiation exposure of the abdomen or adrenals, the bodies of the animals were protected by a 5-cm lead blocks with a 2.5-cm thick polystyrene bar. Scattered radiation to the shielded parts of the body was measured using Fricke dosimeter. Radiation was administered in the morning (9:00 to 10:00 a.m.), with a source-skin distance of 31 cm and dose rate 0.32 Gy/min. The entire procedure was completed within 32 min, four animals being irradiated at a time. After completing irradiation procedure, the irradiated and appropriate sham-irradiated animals were sacrificed by decapitation in the following time intervals: 1, 2, 4, 8 and 24 h (in 11:00, 12:00, 14:00, 18:00 h and next day in 11:00 h). In order to evaluate the effects of immobilization stress on activity of HPA axis, in an additional experiment we included as third group: non-immobilized (control) animals. They were sacrified by decapitation in following time points: in 11:00, 12:00 and 14:00 (correspond to 1 h, 2 h and 4 h post-irradiation intervals). Each group was consisting of 8–9 animals.

CORT assay

After decapitation, trunk blood was rapidly collected and the sera, obtained by centrifugation for 15 min at 2500 × *g*, were kept at –20°C until assayed. Serum obtained from each

individual animal was used for CORT measurement and the results are presented as mean ± SEM (n = 7–8 animals). The level of serum CORT was measured in group of non-immobilized (control), sham-irrradiated and irradiated animals.

CORT levels were measured by ELISA assay (REF AC-14F1; Immunodiagnostics Systems, UK). The standards and samples were measured in duplicate, all in one assay. The difference between the pairs was less than 6%. The ELISA plates were read at 450 and 650 nm on the WALLAC 1420-Victor2 Multilabel Counter (LKB, UK).

Tissue collection

After blood collections, brains were quickly removed and placed on ice for immediate dissection of the hippocampus. For each group, 3–5 hippocampi were pooled, and either placed in cold buffer (4°C) and homogenized within 10 min after collection, or rapidly frozen in liquid nitrogen and stored at –70°C for subsequent processing.

RNA extraction and reverse transcription

RNA was isolated by a modified single step method based on acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi 1987). Hippocampi were homogenized in 1 ml of denaturing solution per 100 mg of tissue (4 mol/l guanidinium thiocyanate, 0.5% sarcosyl, 25 mol/l sodium citrate, pH 7.0 and 0.1 mol/l 2-mercaptoethanol) in glass-teflon homogenizer. Sequentially, 0.05 ml 2 mol/l sodium acetate, pH 4.0, 0.5 ml phenol (water saturated) and 0.1 ml of chloroform-isoamyl alcohol mixture (49 : 1) were added to the homogenate, with thorough mixing after the addition of each reagent. Samples were centrifuged at 10,000 × g for 20 min at 4°C. The aqueous phase was mixed with 1 ml of isopropanol and kept at –20°C overnight to precipitate RNA. Pelleted (10,000 × g, 20 min, 4°C) RNA was dissolved in 0.3 ml denaturing solution, and precipitated once more with 1 vol. of isopropanol at –20°C for 1 h. After centrifugation (10,000 × g, 10 min, 4°C), the RNA pellet was resuspended in 75% ethanol, centrifuged (7500 × g, 5 min, 4°C), dried on air and dissolved in 50–100 µl 0.1% diethyl pyrocarbonate water.

For the synthesis of cDNAs, First Strand cDNA Synthesis Kit (#K1612; Fermentas, Lithuania) was used by manufacturer's instructions.

Semi-quantitative PCR

cDNAs were amplified using primers designed for the amplification of MR and GR together with a housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1). For polymerase chain reaction (PCR), appropriate dilutions of cDNA samples representing 200 ng total RNA were mixed with PCR buffer containing 10 mmol/l deoxyribonucleotide triphosphate, 2.1 mmol/l $MgCl_2$, 0.25 µmol/l primers for GR or MR, 0.125 µmol/l primers for GAPDH, and 2U Taq polymerase in a total volume of 25 µl. cDNAs were amplified in a Techne thermocycler (UK) for 30 cycles (GR) or 28 cycles (MR) using the following conditions: denaturation 94°C/1 min; annealing 57°C/45 s (GR) or 59°C/45 s (MR), extension 72°C/45 s; final extension 72°C/5 min. PCR products were electrophoresed on 2% agarose gels together with a DNA Molecular Weight Marker X, 0.07–12.2 kbp, (Boehringer Mannheim, Germany) and visualized under UV light using ethidium bromide. The intensity of PCR products were measured with an image analysis system GelDoc 1000 (BioRad, CA, USA) and expressed in arbitrary units (count). The arbitrary units related to the MR or GR amplification products were divided by that corresponding to the GAPDH product obtained in same PCR reaction. All the experiments were replicated three times, each time with the new groups of sham-irradiated and irradiated animals, each group consisting of 3–5 animals.

Preparation of cytosols

Cytosols were prepared from the pools of 3–5 hippocampi. Briefly, the tissue was homogenized with a glass-teflon homogenizer in 2 vol. (w/v) of ice-cold buffer A (0.25 mol/l sucrose, 15 mmol/l Tris-HCl, pH 7.9, 16 mmol/l KCl, 15 mmol/l NaCl, 5 mmol/l EDTA, 1 mmol/l EGTA, 0.15 mmol/l spermine and 0.15 mmol/l spermidine) and supplemented with 1 mmol/l DTT and the following protease inhibitors: 0.1 mmol/l phenylmethanesulphonyl fluoride, 2 µg/ml leupeptin, 5 µg/ml aprotinin, 5 µg/ml antipain. After centrifugation (10 min, 2000 × g, 4°C), the resulting supernatants were further processed to generate cytosol extracts. Supernatant was centrifuged 60 min, at 105,000 × g, at 4°C and the final supernatant was aliquotted and stored at –70°C. Protein

Table 1. Primer sequences used in RT-PCR analysis

Product size	Primer sequence[a]	Gene position[b]
GR	TGCAAACCTCAATAGGTCGACCG	509–532
(522 bp)	TAAACTGGGCCCAGTTTCTCTTGC	1007–1030
MR	AGCTCTTCTGTTAGCAGCCCGCTG	1044–1067
(472 bp)	CTGAAGTGGCATAGCTGAAGGCT	1492–1515
GAPDH	AAGGTGAAGGTCGGAGTCAACG	8–29
(332 bp)	GGCAGAGATGATGACCCTTTTGGC	362–339

[a] the upper and lower primers of each amplification pair represent the 5'- and 3'-primers, respectively (the 3'-primers are antisense); [b] nucleotides corresponding to the amplified sequences.

concentration was determined by the modified method of Lowry (Markwell et al. 1978) using bovine serum albumin as a standard.

Western blot analysis

Samples containing 20 μg protein were mixed with Laemmli's sample buffer, boiled for 5 min and loaded on 8% polyacrylamide gels. For Western blot analysis, the samples intended to be directly compared were always run on the same gel and GR and MR proteins were simultaneously detected on same membranes, however, the samples for 24 h time point, both irradiated and respective sham-irradiated control, were analyzed on another gel due to limited number of wells. Cruz Marker™ Molecular Weight Standards (Santa Cruz Biotechnology, USA), consisting of 6 bands (132, 90, 55, 43, 34 and 23 kDa), were used as molecular mass references. Separated proteins were transferred to polyvinylidene fluoride membranes (Amersham Biosciences, USA). The blots were blocked in 5% (w/v) nonfat dry milk in TBST (Tris-buffered saline, 0.1% Tween-20) (50 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl, 0.1% Tween-20) for 2 h. After extensive washing, membranes were incubated overnight (4°C) with primary antibodies (rabbit polyclonal anti-GR, PA1-511A, 1 : 1000, Affinity Bioreagents (USA); mouse monoclonal anti-MR, MA1-620, 1 : 1000, Affinity Bioreagents; goat polyclonal anti-β-actin, C-11, 1 : 2500, Santa Cruz Biotechnology). Membranes were subsequently washed in TBST and incubated for 2 h with peroxidase-conjugated anti-rabbit or anti-mouse immunoglobuline antibodies (Santa Cruz Biotechnology, 1 : 5000), and subsequently with anti-goat immunoglobuline antibodies (Santa Cruz Biotechnology, 1 : 10,000). Immunopositive bands were visualized by enhanced chemiluminiscent method (Cell Signaling, USA). β-actin was used as an equal load control. The optical density (OD) of bands visible on light-sensitive films (Fuji, Japan) was measured using image analysis system (ImageJ). Background OD levels were subtracted from the OD of each individual immunoreactive band. All the experiments were replicated three times, each time with the new groups of sham-irradiated and irradiated animals, each group consisting of 3–5 animals.

Statistics

Data were expressed as means ± SEM. One-way ANOVA followed by Tukey *post hoc* test was used to determine statistical significance of measured parameters with respect to the control. All interactions between factors were assesed using two-way ANOVA test followed by Tukey *post hoc* test. Differences were considered as significant at $p < 0.05$.

Results

HPA axis activity after cranial irradiation

In order to assess the acute effects of irradiation on responsiveness of the juvenile HPA axis, serum CORT levels were measured in the sera of 18-day-old animals, both irradiated (IR) and sham-irradiated (sham-IR). Since immobilization was unavoidable part of irradiation procedure, and both irradiated and sham-irradiated animals were exposed to immobilization stress in duration of 1 h, in an additional experiment we included non-immobilized (control) animals and measured the level of CORT in the serum. We found major effects of immobilization stress 1 h after treatment (Fig. 1, control (1 h) vs. sham-irradiated (1 h), $p < 0.001$), while 2 h and 4 h after treatment there was no difference between control (non-immobilized) and sham-irradiated (immobilized) animals (Fig. 1). Two way ANOVA, followed by Tukey *post hoc* test, showed that interaction between immobilization and irradiation was significant in 1 h time point (Fig. 2, $F = 40.288$, $p < 0.001$). However, no interaction between immobilization and irradiation was observed in 2 h and 4 h time points (Fig. 2, $F = 0.185$, p = n.s. (not significant)).

Cranial irradiation led to increased activity of juvenile HPA axis, as CORT level was raised by 60% 2 h after irradiation, compared to sham-irradiated control (Fig. 2, IR_{2h} vs. sham-IR_{2h}, $p < 0.05$). It is important to remark that the observed enhancement of serum CORT is foremost the effect of cranial irradiation than result of additive effect

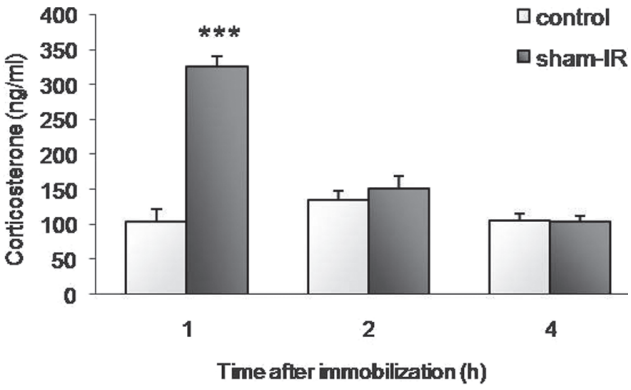

Figure 1. Effect of unavoidable immobilization stress on HPA axis activity in sham-irradiated animals. Serum corticosterone levels were determined in the blood of 18-day-old animals, which were untreated (control) or immobilized for 1 h during irradiation procedure, but not exposed to source of radiation (sham-IR) and sacrificed in different time intervals following treatment (1, 2 and 4 h). The results are expressed as the mean ± SEM ($n = 7–8$ animals per experimental group). Statistical analysis was done by comparing sham-IR group to the corresponding control group using one way ANOVA. * $p < 0.001$, sham-IR_{1h} vs. control (1 h).

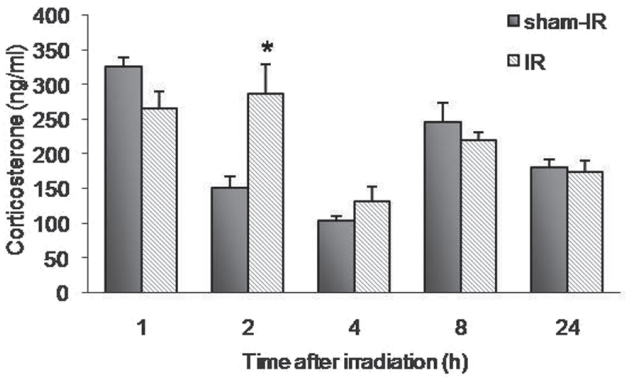

Figure 2. Activity of the HPA axis after cranial irradiation. Serum corticosterone levels were determined in the blood of 18-day-old animals, which were irradiated (IR) or sham-irradiated (sham-IR) and sacrificed in different time intervals following treatment (1, 2, 4, 8 and 24 h). The results are expressed as the mean ± SEM (n = 7–8 animals per experimental group). Statistical analysis was done by comparing IR to the corresponding sham-IR group using one way ANOVA. * $p < 0.05$, IR_{2h} vs. sham-IR_{2h}.

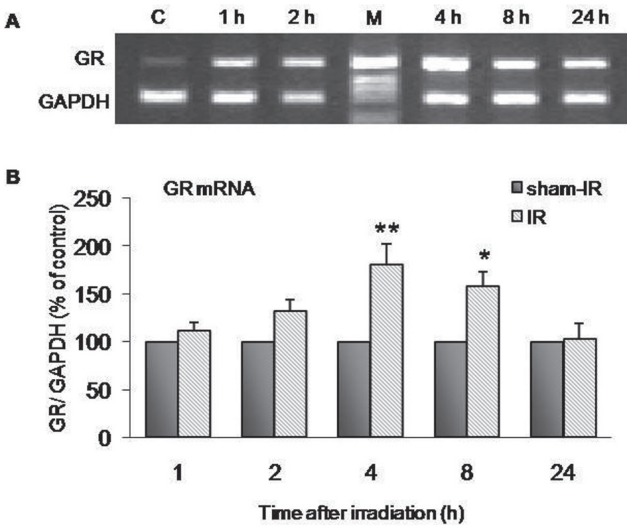

Figure 3. The effect of cranial irradiation on GR mRNA levels in the rat hippocampus. Panel A: The level of GR mRNA was examined by RT-PCR in the hippocampi of sham-irradiated (sham-IR) and irradiated (IR) rats in different time intervals after treatment (1, 2, 4, 8 and 24 h). C, appropriate sham-irradiated animals; M, DNA molecular weight marker. Panel B: Relative mRNA levels of GR were normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA level in the same samples. Each sample is a pool of 3–5 animals belonging to the same experimental group. The relative GR mRNA levels are expressed as percent of control (sham-IR rats). Data represent the means ± SEM from 3 independent experiments. Statistical analysis was done by comparing IR group to the corresponding sham-IR group using one way ANOVA. * $p < 0.05$, significantly different from sham-irradiated rats; ** $p < 0.01$, significantly different from sham-IR rats.

of immobilization and irradiation, given that there is no interaction between immobilization and irradiation in 2 h post-irradiation interval. This radiation-mediated inducement is time-limited and CORT returns to basal level within 4 h (Fig. 2).

GR and MR mRNA expression in the hippocampus

The acute effects of irradiation on the GR and MR mRNA levels were examined in the rat hippocampus of 18-day-old animals (Fig. 3 and Fig. 4). Cranial irradiation of 10 Gy led to time-dependent increase in GR mRNA level, compared to sham-irradiated control, with maximum achieved 4 h after radiation treatment (Fig. 3B, F = 4.655, $p < 0.01$). On the other hand, MR mRNA level was not significantly different between the two groups of animals (Fig. 4B, F = 0.668, p = n.s.).

GR and MR protein expression in the hippocampus

To examine possible influence of cranial irradiation on level of GR and MR proteins, the two receptors were detected by semi-quantitative Western blotting in cytosolic extracts of the rat hippocampus. As seen in Fig. 5, cytosolic GR steady-

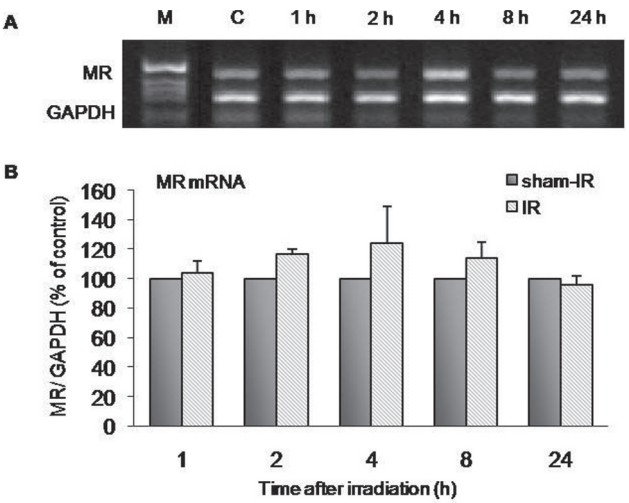

Figure 4. The effect of cranial irradiation on MR mRNA levels in the rat hippocampus. Panel A: The level of MR mRNAs was examined by RT-PCR in the hippocampi of sham-irradiated (sham-IR) and irradiated (IR) rats in different time intervals after treatment (1, 2, 4, 8 and 24 h). C, appropriate sham-irradiated animals; M, DNA molecular weight marker. Panel B: Relative mRNA levels of MR were normalized to GAPDH mRNA level in the same samples. Each sample is a pool of 3–5 animals belonging to the same experimental group. The relative MR mRNA levels are expressed as percent of control. Data represent the means ± SEM from 3 independent experiments.

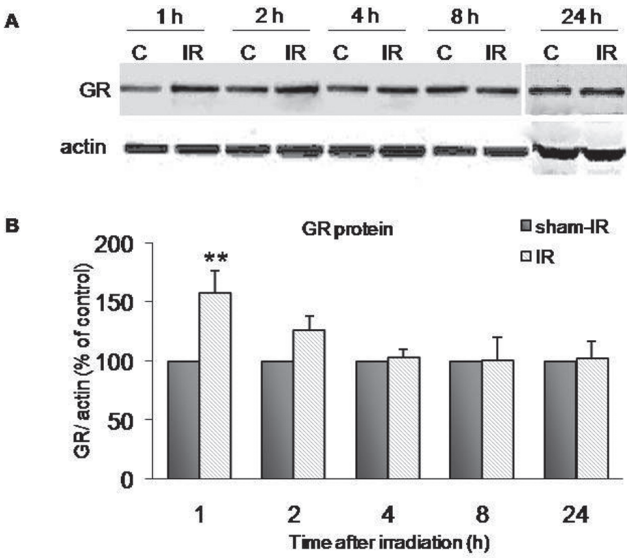

Figure 5. GR protein level in the cytosolic extract of rat hippocampus after cranial irradiation. Panel A: Western blot analysis of cytosolic GR protein in hippocampi of sham-irradiated (sham-IR) and irradiated (IR) rats in different time intervals after treatment (1, 2, 4, 8 and 24 h). C, appropriate sham-irradiated animals. Panel B: The relative abundance of GR protein is quantified by densitometry and normalized against the level of β-actin in the same sample. Each sample is a pool of 3–5 animals belonging to the same experimental group. The relative GR protein levels are expressed as percent of control (sham-IR rats). The values represent the mean ± SEM. from three independent experiments. Statistical analysis was done by comparing IR group to the corresponding sham-IR group using one way ANOVA. ** $p < 0.01$, significantly different from sham-IR rats.

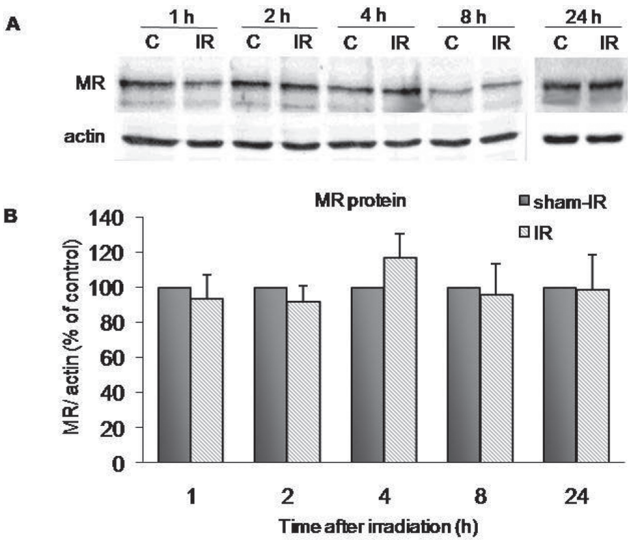

Figure 6. MR protein level in the cytosolic extract of rat hippocampus after cranial irradiation. Panel A: Western blot analysis of cytosolic MR protein in hippocampi of sham-irradiated (sham-IR) and irradiated (IR) rats in different time intervals after treatment (1, 2, 4, 8 and 24 h). C, appropriate sham-irradiated animals. Panel B: The relative abundance of MR protein is quantified by densitometry and normalized against the level of β-actin in the same sample. Each sample is a pool of 3–5 animals belonging to the same experimental group. The relative MR protein levels are expressed as percent of control. The values represent the mean ± SEM from three independent experiments.

state level was significantly increased in irradiated rats compared to sham-irradiated animals (Fig. 5B, F = 3.360, $p < 0.05$); increment observed in 1 h post-irradiation time declined to basal level after 4 h. In contrast to GR protein level, cytosolic MR protein level remained unchanged in the rat hippocampus after irradiation (Fig. 6B, F = 0.642, p = n.s.).

Deregulation of GR/MR balance in hippocampal neurons appears critical for neuronal excitability, stress responsiveness and behavioral adaptability (De Kloet et al. 1998). Direct comparison between the GR and MR mRNA and protein levels in the hippocampus revealed radiation-induced increase in GR/MR ratio (Fig. 7), suggesting that cranial irradiation is accompanied by a shift in GR/MR balance. Increment of GR protein above MR protein was observed in 1 h and 2 h post-irradiation time (Fig. 7, $GR_{1h\ WB}$ vs. $MR_{1h\ WB}$, $p < 0.05$), due to upregulation of GR while expression of MR was unaltered. At the level of gene expression, increase in relative GR/MR mRNA ratio was significant only in 8 h post-irradiation time, although GR mRNA is maintained at high level in comparisons to MR mRNA all through examined time interval (Fig. 7, $GR_{8h\ PCR}$ vs. $MR_{8h\ PCR}$, $p < 0.05$).

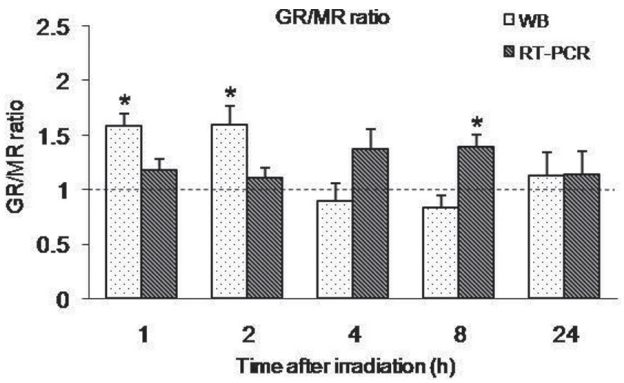

Figure 7. The GR/MR mRNA and protein ratio in the hippocampus after cranial irradiation. The levels of GR and MR mRNAs and proteins in the hippocampus of sham irradiated (sham-IR) and irradiated (IR) rats were determined by RT-PCR (Figs. 3 and 4) and Western blot (Figs. 5 and 6), respectively, and presented as their relative ratio. If GR/MR relative ratio is above or under 1 (marked as a dash line) the GR/MR balance are shifted to one direction or the other. The values are the means ± SEM from three independent experiments. * $p < 0.05$, significantly different from sham-IR rats; WB, Western blot.

Discussion

The goal of the present study was to examine, *in vivo*, the acute effects of cranial irradiation on HPA axis activity and corticosteroid receptors (GR and MR) expression in hippocampus of 18-day-old Wistar rats under unavoidable stress. We analyzed the radiation-response effects after a single dose of 10 Gy, a dose equivalent to those used for CNS prophylactic therapy in children with ALL (Schunior et al. 1990). In this study cranial irradiation led to acute activation of HPA axis, characterized by an enhancement of serum CORT level 2 h after radiation treatment, compared to sham-irradiated control (Fig. 2). This inducement is time-limited and CORT returns to normal level within 4 h (Fig. 2). Observed increase in serum CORT is consistent with a previous report of acute response of HPA axis 30 min to 6 h after radiation exposure (Van de Meeren et al. 2001; Lebaron-Jacobs et al. 2004). In patients, single dose (10 Gy) of total body irradiation also induced the activation of HPA axis, manifested as concomitant increase in cortisol and adrenocorticotropic hormone (ACTH) (Girinsky et al. 1994). Although we can generally conclude that an activation of HPA axis is a consequence of radiation, this response has not been fully characterized. Many studies have provided evidence that acute effects of ionizing irradiation involve release of inflammatory mediators, associated with changes in microvasculature, activation of microglia and neurogenesis (Girinsky et al. 1994; Hong et al. 1995; Van de Meeren et al. 2001; Monje et al. 2003). Cranial irradiation induces significant increases in TNF-α, IL-1β and IL-1α mRNA in the brain (Hong et al. 1995), increase in IL-6 in plasma or increase in IL-1β protein in whole brain (Van de Meeren et al. 2001). Increasing data indicate that activation of innate immune responses in brain after cranial irradiation may contribute to altered regulation of neuroendocrine system, especially activity of HPA axis (Miller et al. 2008). This hypothesis is plausible because several studies have demonstrated the potent action of cytokines (IL-1, IL-6, TNF-α) directly on the HPA axis activity, and consequently release of glucocorticoids (Turnbull and Rivier 1999; Dunn 2000). Prostaglandins are also involved in the increase in plasma ACTH and CORT levels induced by radiation, as shown by study of Kandasamy et al. (1995). More recently, chronic oxidative stress has been suggested to contribute to the progression of radiation-induced inflammatory reactions (Raju et al. 1999; Tofilon and Russell 2002), thus leading to further activation of HPA axis trough negative action of glucocorticoids on nuclear factor-kappa B activity (Ramdas and Harmon 1998). Systemically, by activation of the HPA axis, an excessive release of glucocorticoids stimulates an important negative feedback mechanism, which protects the organism from an overshoot of proinflammatory cytokines and other tissue-damaging products after irradiation (Turnbull and Rivier 1999; De Bosscher et al. 2003).

Hippocampus plays the major inhibitory role on HPA axis activity and the sensitivity of the HPA axis to glucocorticoid feedback suppression depends on activation of two corticosteroid receptors: GR and MR (Feldman and Weidenfeld 1999). Whereas MRs maintain neuronal homeostasis and limit the disturbance by stress, GRs insure an efficient negative feedback action on the HPA axis. If the MR/GR activation ratio is shifted, the control of glucocorticoids on neuronal excitability, neuroendocrine reactivity and behavior will change (De Kloet et al. 1998). Therefore, we examined the effects of irradiation on the hippocampal glucocorticoid and mineralocorticoid receptors, both at the level of protein and mRNA. Western blot revealed that GR protein is significantly increased in the cytosolic fraction 1 h after irradiation (Fig. 5), although it returns to normal level within an 4 h postirradiation time. Accumulation of GR protein in cytosol after irradiation could be result of action of proinflammatory cytokine such as interleukin-1α (IL-1α), which interfere with GR shuttling from the cytoplasm to the nucleus, thus leading to cytoplasmic retention of activated GR (Pariante et al. 1999). The observed rise of circulating CORT level 2 h after irradiation probably restore the nucleo-cytoplasmic shuttling (Nishi and Kawata 2006), hence decreasing its level in cytosolic fraction. Parallel to GR protein, GR mRNA in hippocampus was increased with time following irradiation (Fig. 3). However, effects at the mRNA level with time have been more pronounced, implying that transcriptional changes are not readily translated into increases in protein expression. The change in GR expression is a cumulative result of a number of distinct steps, including receptor assembly, phosphorylation, interactions with heat shock proteins, and regulation by transcription factors (Pariante et al. 1999).

Contrary to GR, neither MR protein (Fig. 6) nor MR mRNA (Fig. 4) were affected by irradiation, since these high-affinity receptors are responsible for maintenance of basal circadian HPA axis activity and are principally activated under basal level of CORT (De Kloet et al. 1998).

Overall, these data emphasizes that expression profile of corticosteroid receptors in juvenile rat hippocampus is specifically regulated in acute radiation-response phase. The relative GR/MR ratio is disturbed in hippocampus following CRT, showing the selective enhancement in GR relative to MR (Fig. 7), which leads to enhanced HPA axis activation after irradiation. These results are in accordance with "MR/GR balance hypothesis" (De Kloet et al. 1994), which states that IL-1 secretion, which is generally associated with radiation treatment, appears to change the MR/GR balance. The change in receptor balance induced by IL-1 leads to a condition characterized by deficient MRs and increased stimulation of GRs. Since hippocampus conveys inhibitory influences over the HPA axis, increase in the amount of GR versus MR is thought to produce limbic disinhibition

and enhanced HPA activation (De Kloet et al. 1994). This hypothesis was confirmed by other authors, reporting radiation-induced raise of proinflamatory cytokines IL-1β in brain altogether with increase in plasma CORT (Van de Meeren et al. 2001).

Our previous study analyzed late response of neuroendocrine system of juvenile rats after cranial irradiation, characterized by hyposuppressive state of the HPA axis, decreased relative GR/MR ratio and reduced functioning of glucocorticoid negative feedback (Velickovic et al. 2008). On the other side, early radiation response phase is distinguished by acute activation of HPA axis and increased relative GR/MR ratio. Therefore, this study suggest that early neuroendocrine response after cranial irradiation comprise an alteration of both HPA axis activity and corticosteroid receptor expression, probably as adaptive mechanism which protects the organism from an overshoot of proinflammatory cytokines and other tissue-damaging products after irradiation.

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