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ALTERATION OF GLUCOCORTICOID RECEPTOR SUBCELLULAR DISTRIBUTION BY HYPERTHERMIC STRESS

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Abstract - The aim of the present study was to examine intracellular redistribution of the glucocorticoid receptor (GR) in rat liver cells during a 24-h time period after exposure of the animals to 41°C whole body hyperthermic stress. The level of the receptor protein in the cytoplasmic and nuclear compartments was measured by immunoblotting procedures applied to both crude cytosol and immunopurified GR, as well as by immunocytochemical analyses applied to both paraffin-embedded liver sections and unfixed nuclear smears. All the experimental approaches employed in the study provided similar results, demonstrating that the transient stress-related decline of the cytoplasmic GR observed during the first five hours after exposure of the animals to whole-body hyperthermic stress is accompanied by enhanced nuclear accumulation of the receptor. The study can contribute to a better understanding of the influence of stress on the glucocorticoid signal transduction pathway.

Key words: Glucocorticoid receptor, hyperthermic stress, intracellular localization

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INTRODUCTION

The glucocorticoid receptor (GR) is a member of the nuclear receptor superfamily and functions as an inducible transcription factor activated by hormone binding. Its subcellular distribution, as an issue crucial for better understanding of molecular and cellular mechanisms underlying glucocorticoid hormone action, has been studied for many years, but has still remained controversial. There are studies demonstrating that unliganded GR, in contrast to other nuclear receptors, is predominantly localized within the cytoplasm of target cells (Pickard and Yamamoto, 1987; Sackey *et al.,* 1996), its migration to the nucleus being provoked by hormone binding. However, other studies provided data in support of a prevailing nuclear localization of the receptor (Brink *et al.,* 1992), its equilibrated distribution between the cytoplasm and the nucleus (Wikström *et al.,* 1987; Haché *et al.,* 1999), or its continuous shuttling between the two compartments (Madan and DeFranco, 1993). It is believed that the observed differences in GR intracellular localization derive from different cell types and/or cell cycle stages, as well as from varying experimental procedures.

Glucocorticoid hormones play an indispensable role in the organismal response to stress. This fact, together with evidence indicating that hormone-free GR exists in target cells in the form of multiprotein complexes with heat shock proteins (Pratt and Toft, 1997), led researchers to explore possible connection between glucocorticoid hormone action and heat shock response. Studies along these lines were performed predominantly on cells grown in culture and have revealed that heat stress produces serious disturbances at the most important steps controlling the receptor's function. For example, heat shock treatment of a variety of mammalian cell lines was shown to result in a loss of GR-binding capacity (Vedeckis *et al.,* 1989; Sanchez, 1992), which was attributed to an increased rate of GR degradation (Vedeckis *et al.,* 1989) or to stimulated nuclear translocation of the receptor (Sanchez, 1992). Moreover, enhancement of hormone-free GR transactivation activity was demonstrated in CHO cells subjected to heat or chemical stress (Shen *et al.,* 1993). These two kinds of stress were also shown to potentiate the effect of dexam-

ethasone on GR-mediated gene transcription in mouse L929 cells stably transfected with MMTV-CAT reporter plasmid (H u *et al.*, 1996). Similar observations were reported for other steroid receptors. Thus, increased nuclear accumulation of hormone-free estrogen receptor was noticed after heat treatment of rat uterine cells (C a m p b e l l and S w a n s o n, 1989), and enhancement of hormone-dependent progesterone receptor transactivation activity in human T47D breast cancer cells was observed after heat or chemical shock (E d w a r d s *et al.,* 1992). Although molecular mechanisms underlying the heat shock potentiation effect remain to be elucidated, this phenomenon is usually linked with stress-induced increase in nuclear accumulation of the receptors.

In contrast to studies on cells grown in culture, *in vivo* studies on the effects of stress on steroid receptor action have remained limited. Results from our laboratory showed that both 41°C whole-body hyperthermic stress (M a t i ć *et al.,* 1989, 1995) and intoxication by heavy metals (D u n đ e r s k i *et al.,* 1992) cause a considerable reduction of rat liver GR hormone-binding capacity, which could rather be attributed to stimulated nuclear import of the receptor than to its faster degradation. Our previous immunoblot analyses of the GR in rat liver revealed a significant decrease in its cytoplasmic level in response to both hyperthermic (Č v o r o *et al.*, 1998) and chemical (D u n đ e r s k i *et al.,* 2000; B r k lj a č i ć *et al.,* 2004) stress. However, Western blot analysis of the receptor in the liver nuclei failed to provide unambiguous data, probably because of low efficiency of the receptor immunopurification from the nuclear extracts.

In the present study, we performed immunoblotting and immunostaining of paraffin-embedded tissue sections and unfixed smears of isolated nuclei to examine intracellular redistribution of GR in the rat liver during a 24-h time period after exposure of the animals to 41°C whole body hyperthermic stress. The results demonstrate that the stress-related decline of the cytoplasmic GR observed during the first five hours after exposure of the animals to whole-body hyperthermic stress is accompanied by enhanced accumulation of the receptor in the nuclei.

MATERIALS AND METHODS

Chemicals

Mouse monoclonal anti-GR antibody BuGR2 was obtained from Affinity BioReagents (Golden, CO, USA), biotinylated goat anti-mouse IgG and horseradish peroxidase-conjugated streptavidin from StressGen (Victoria, British Columbia, Canada), [^{125}I]-conjugated sheep anti-mouse IgG from Amersham (Amersham International, UK), and diaminobenzidine (Sigma Fast^{TM} DAB) and pre-immune mouse IgG from Sigma Chemical Co (St. Louis, MO, USA).

Animals and treatment

Male Wistar rats (2-2.5 months old; 200-250 g b.w.) were reared under standard laboratory conditions (22°C, 12:12 h light-dark cycle). Hyperthermic stress was achieved by placing the rats, anesthetized with Nesdonal (4.6 mg/100 g b.w., *i.p.*; Specia, Paris, France), in a ventilated and humidified chamber set at 44°C. Rectal temperature, continually monitored by a digital thermometer, reached 41°C in about 45 min, and was maintained at 41°C for additional 15 min. The animals were then allowed to recover at room temperature for 0, 2, 5, 12, or 24 h, as indicated. The control (unstressed) animals were Nesdonal-anesthetized and kept at room temperature. When appropriate, dexamethasone (ICN-Galenika, Belgrade, Serbia) was *i.p.* administered in a dose of 5 mg/kg b.w. 4 h before death. All interventions were performed following the appropriate European laws and regulations on the care and protection of animals.

Preparation of cytosol and nuclei

After removal of the small lobes, the livers were perfused *in situ* with ice cold 0.14 M NaCl and homogenates from at least four animals per experimental group were prepared in 2 vol. (w/v) of 50 mM Tris buffer, pH 7.55, containing 0.25 M sucrose, 25 mM KCl, and 10 mM $MgCl_2$. The homogenates were centrifuged first for 10 min at 6000g and 4°C, then for 1 h at 105000g and 4°C. The supernatants, referred to as cytosols, were stored in liquid nitrogen until use. Protein content of the cytosols was determined as described by L o w r y *et al.* (1951). Pellets obtained after the first centrifugation were used for preparation of purified nuclei (C h a u v e a u *et al.,* 1956).

Immunopurification of cytosolic GR

Immunoadsorbent was prepared according to C z a r *et al.* by rotating (30 min, 4°C) 8 ml portions of Protein A-Sepharose pellets with 1 mg of BuGR2 anti-GR antibody in 300 ml of TEGM buffer (10 mM Tes, pH 7.6 at 0°C containing 50 mM NaCl, 4 mM EDTA, 10% glycerol, and 20 mM Na-molybdate). After addition of 200 ml

cytosol aliquots (4 mg cytosol protein) to each tube, the rotation was continued for another 2 h at 4°C. The immune pellets were washed three times by suspension in 1 ml of TEGM buffer and centrifugation. For non-immune controls, the pre-immune mouse IgG was used instead of BuGR2. The immunoadsorbed proteins were extracted by boiling in ^{2X}SDS-sample buffer and subjected to SDS-PAGE.

SDS-PAGE and Western blotting

Proteins were electrophoretically separated through 10% SDS-polyacrylamide gels at 4°C, according to L a e m m l i (1970). Myosin (205 kDa), b-galactosidase (116 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa) were run as molecular mass references. Western transfer of proteins from the gels to nitrocellulose membranes was performed in 25 mM Tris buffer, pH 8.3, containing 192 mM glycine and 20% (v/v) methanol at 35 V overnight. Unbound sites on the membranes were blocked by 20 mM Tris buffer, pH 7.4, containing 150 mM NaCl, 0.125% BSA, and 0.1% Tween 20. The GR was detected by rocking the membranes (16 h, 4°C) in transfer buffer with 1 mg/ml BuGR2 antibody. Subsequently, the blots were incubated with [^{125}I]-labeled counter-antibody, washed, dried, and placed on [^{125}I] sensitive storage phosphor screens (Molecular Dynamics, USA). After exposure, the screens were scanned (PhosphorImager, Molecular Dynamics, USA) and images analyzed by computerized densitometry (Image Quant software, Molecular Dynamics, v.3.3.).

Immunohistochemistry

For immunohistological analyses, small lobes of the livers were fixed with 3.5% formaldehyde and embedded in paraffin. Serial 5 μm sections were deparaffinized in xylene and rehydrated through a series of decreasing ethanol concentrations followed by distilled water. The sections were successively treated in a humid chamber at room temperature with: 0.2 % Triton X-100 in TBS (200 mM Tris, 1.5 M NaCl, pH 7.4; 15 min), 1% H_2O_2 in methanol (20 min), and 2% BSA in TBS (20 min), and then incubated for 1 h with BuGR2 antibody (dilution 1:200). Negative (non-immune) controls were prepared by omitting the primary antibody. After washing with TBS (3 x 15 min), the sections were transferred for 1 h into biotinylated secondary antibody and washed again by the same procedure. The subsequent incubation with streptavidine-peroxidase lasted 45 min at room temperature. Freshly prepared DAB solution was used as the chromogen. Counterstaining of the sections with methyl green was followed by dehydration through an ethanol series, clearing in xylene, and mounting in DPX. Light microscope examination was performed on a Leica (Germany) microscope. The experiments were replicated six times independently.

Nuclear smear preparation and immunocytochemistry

Smears of isolated liver nuclei were made on glass slides coated with gelatin and air-dried for 1 h at room temperature. The protocol for immunocytochemical staining of nuclear smears was the same as for tissue sections. For assessing the quality, preservation and morphological integrity of the nuclei, the smears were stained with 1% toluidine blue solution.

RESULTS

The level and intracellular redistribution of GR in the liver of rats exposed to 41°C whole body hyperthermic stress were determined by both immunoblotting and immunocytochemical procedures. When the concentration of GR immunopurified from hepatic cytosol by BuGR2 monoclonal antibody was followed for 24 h after exposure of the animals to the stress, a pronounced decrease was observed immediately after the stress (Fig. 1). Two hours later the level of the receptor dropped to only about 10% of the level in hepatic cytosol of unstressed animals. At the end of a 5-h recovery period, the GR level increased to about 60% of that in the unstressed control, while 12 h after the stress almost complete restoration of the receptor level was achieved.

A very similar pattern of hyperthermia-induced changes of the GR level was observed when the receptor level was determined in hepatic cytosol by immunoblotting without previous immunoadsorption. A 90% decrease of GR concentration was noticed shortly after the stress and partial restoration was evident 5 h after the stress (Fig. 2).

The influence of hyperthermic stress on intracellular redistribution of GR was followed by immunocytochemical staining of the receptor using BuGR2 antibody (Fig. 3). In the control (unstressed) group of animals, a predominant cytoplasmic localization of the receptor was observed. On the contrary, in dexamethasone-administered animals serving as positive controls, the receptor was localized almost exclusively in the nuclei. Exposure

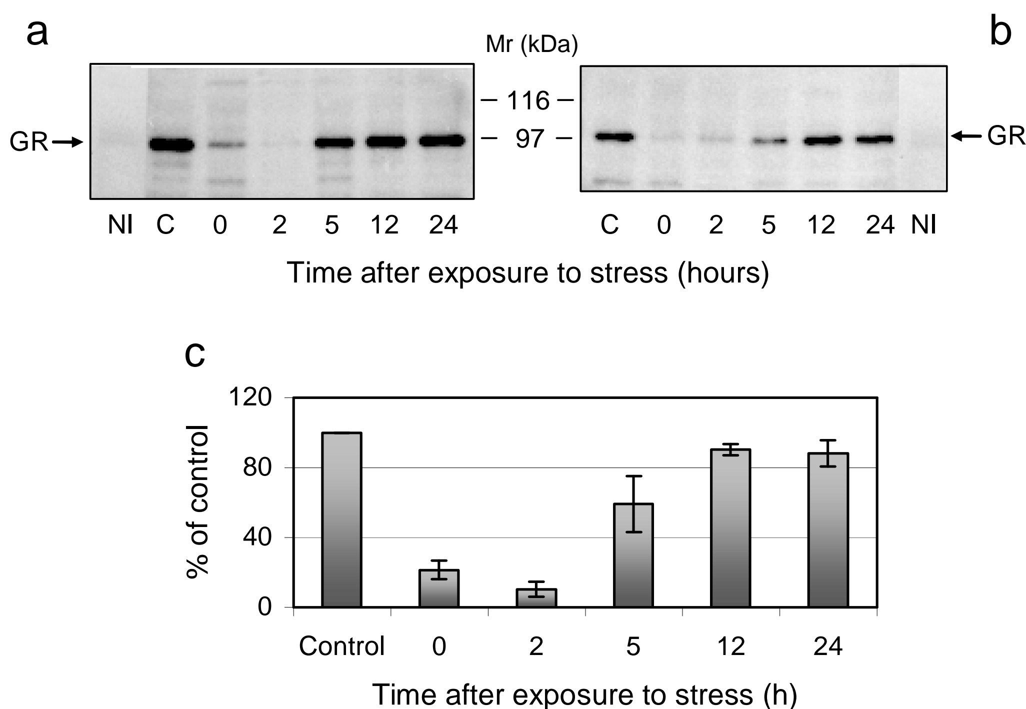

Fig. 1. Amount of the GR immunopurified from rat liver cytosol after exposure of the animals to 41°C whole body hyperthermic stress. (a) and (b): GR was immunoadsorbed to Protein A-Sepharose from 200 ml liver cytosol (4 mg protein) by BuGR2 antibody. After SDS-PAGE and Western immunoblotting GR was detected using BuGR2 as primary antibody followed by ^{125}I-labeled secondary antibody. Two representative scans obtained by PhosphorImaging are shown. Lane NI: nonimmune control (preimmune mouse IgG used instead of BuGR2 for immunoadsorption); lane C: unstressed control. The position of GR is indicated by the arrow. (c): Data obtained after quantification of immunospecific bands by ImageQuant software. The percentages of unstressed controls are displayed. The values represent the means ± S.E. from four independent experiments.

of the animals to hyperthermic stress led to a rapid shift of immunopositivity from the cytoplasm to the nucleus, which was evident immediately after the stress and persisted for at least 2 h afterwards. At the end of a 5-h recovery period, a decrease of immunopositivity in the nuclei, accompanied by its increase in the cytoplasm, was noticed. Such a redistribution continued during the whole examined period after the stress, so that 24 h after the treatment the receptor distribution within the cell resembled that seen in the control group. Exactly the same pattern of receptor intracellular redistribution in untreated, dexamethasone-administered and hyperthermia-exposed animals was observed when the smears of isolated liver nuclei were immunostained by BuGR2 anti-GR antibody (Fig. 3, insets).

DISCUSSION

The GR has two nuclear localization signals. The first one (NL1) is located within the DNA-binding domain, while the second (NL2), which mediates hormonal control of receptor localization, is in the ligand binding domain (Picard and Yamamoto, 1987). A wide range of stressful signals, including heat stress, modulate the circulating level of glucocorticoids, influencing the activity and intracellular localization of the GR. The purpose of this study was to examine heat stress-induced alterations in GR subcellular distribution under conditions that resemble a physiological situation as much as possible. For that purpose, physiologically relevant whole-body hyperthermia was applied as a stressful insult and the GR level was measured in both the cytoplasm and the nuclei of rat liver cells.

Subcellular distribution of the GR and the control mechanisms implicated are not yet fully understood. In most cells and tissues studied so far, GR was found in both the cytoplasm and the nucleus, its shift to the nuclear compartment being of the utmost importance for transduction of the glucocorticoid signal to the cellular genome. Both cytological and biochemical approaches have provided evidence for GR association with components of the cytoskeleton, which unquestionbly play a key role

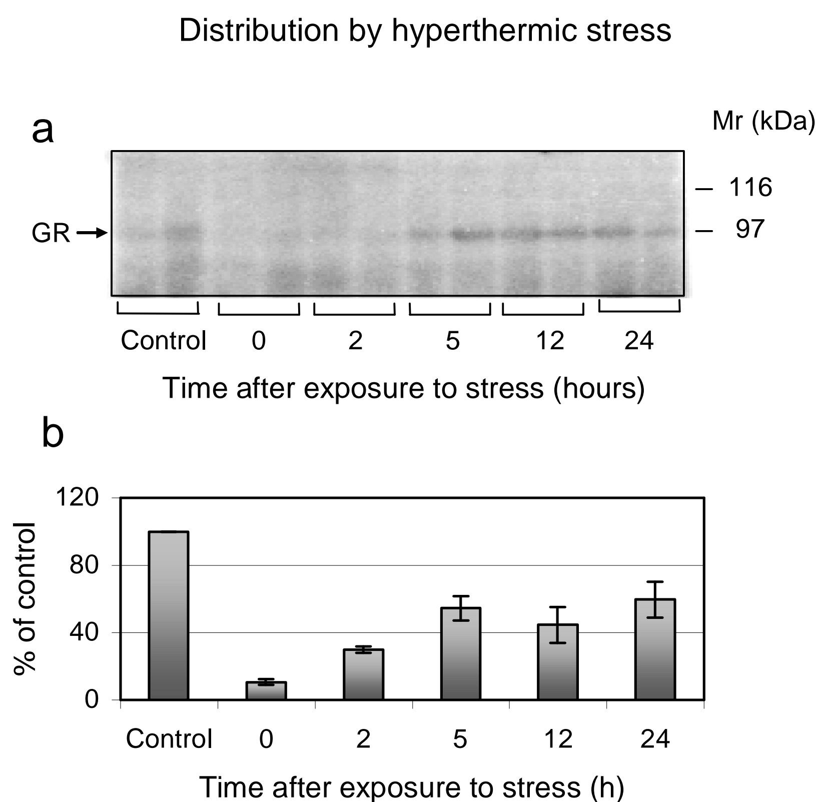

Fig. 2. Level of GR in the rat liver cytosol after exposure of the animals to 41°C whole body hyperthermic stress. (a): Liver cytosol proteins (120 mg) from unstressed rats (control) and rats sacrificed at indicated time intervals after exposure to the stress were resolved by SDS-PAGE and transferred to nitrocellulose membrane. The GR was detected using BuGR2 as primary antibody followed by 125I-labeled secondary antibody. The representative scan obtained by PhosphorImaging is shown. The samples deriving from two independent experiments are presented. (b): Data obtained after quantification of immunospecific bands by ImageQuant software. The percentages of unstressed controls are displayed. The values represent the means ± S.E. from four independent experiments.

in intracellular shuttling of the receptor (Pratt *et al.,* 2004). However, both approaches suffer from serious limitations as for as measurements of the GR level in the two cellular compartments are concerned. Thus, it has been documented that the amount of the receptor measured in the cytosol and the nuclei greatly depends on the procedure applied for preparation of the cellular fractions (Martin and Sheridan, 1980). On the other hand, tissue fixation procedures may affect relative nuclear to cytoplasmic distribution of the receptor (Yamashita, 2001). In this study, we applied both immunoblotting and immunocytochemical procedures in order to compare the two sets of data on GR intracellular redistribution under conditions of hyperthermic stress. Moreover, in an attempt to avoid tissue fixation as a potential source of artifacts, we also used unfixed nuclear smears to examine the nuclear level of the GR.

The results presented in this paper confirm our previous assumption that 41°C whole-body hyperthermic stress induces a transient GR loss from the cytoplasm and stimulates its nuclear accumulation (Matić *et al.,* 1995). Hyperthermia-related decrease of the GR level in the cytosolic fraction of rat liver cells was evidenced by immunoblotting (before and after GR immunopurification) and by immunocytochemical analysis of both tissue sections and unfixed nuclear smears. The immunocytochemical approaches applied herein also provide evidence for a hyperthermia-induced shift of the receptor from the cytoplasm to the nuclei, since decrease in immunopositivity accumulated in the cytoplasm was accompanied by its increase in the nuclei. These results are consistent with previous studies on GR intracellular localization after heat stress, which showed that this stress applied to both cell cultures (Sanchez, 1992) or the whole organism (Matić *et al.,* 1989, 1995; Čvoro

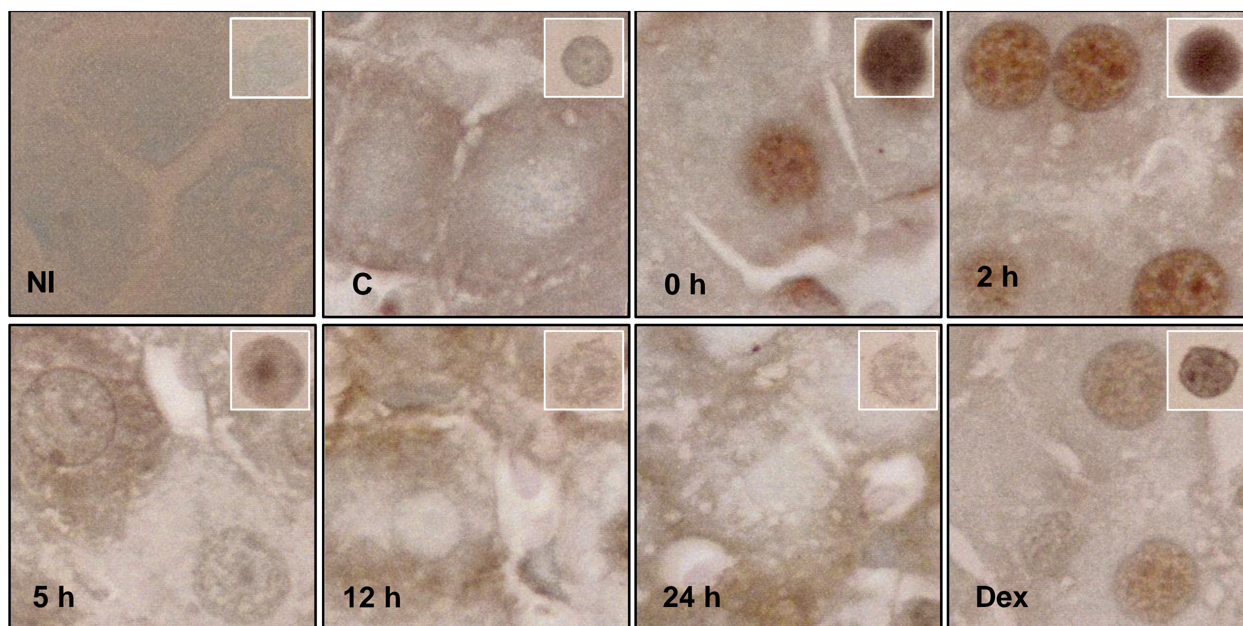

Fig. 3. Immunocytochemical detection of GR in rat hepatocytes after 41°C whole-body hyperthermic stress. Paraffin-embedded liver sections and nuclear smears were prepared from unstressed control rats (C) and rats sacrificed at indicated time intervals after exposure to the stress. The GR was detected by immunocytochemical staining with monoclonal BuGR2 antibody followed by biotinylated secondary antibody. Representative nuclei from unfixed nuclear smears are shown in insets. The non-immune probe is designated NI, while the sample deriving from an animal given dexamethasone is designated Dex. The experiments were repeated six times independently. Magnification: liver sections – x100; nuclear smears – x100, orig.

et al., 1998) led to considerable loss of cytosolic GR binding capacity. This effect of heat stress coincided with decrease in the amount of GR protein in the cytosolic fraction and its increase in the nuclei, whereas the total cellular concentration of the receptor seemed to remain unchanged. Similar reduction of the glucocorticoid binding capacity and GR protein level in the cytosol was observed during chemical stresses, such as cadmium or arsenite intoxication (S i m o n s *et al.,* 1990; D u n đ e r s k i *et al.,* 1992).

It is interesting to note that the increase in the GR nuclear level noticed 0 h and 2 h after exposure to the stress was much larger than that observed after dexamethasone administration, which is a well-known signal provoking receptor shift to the nucleus. This finding is consistent with the previously described heat shock potentiation effect (H u *et al.,* 1996) and suggests that stress-induced increase in the nuclear level of GR could result not only from its translocation to the nucleus induced by an elevated level of endogenous glucocorticoids, but also from hormone-independent translocation stimulated by stress. In support of such an assumption, it has been documented by H a c h é *et al.* (1999) that unliganded, cytoplasmic GR associated with heat shock proteins (Hsps) may constitutively shuttle between the nucleus and the cytoplasm, existing in a dynamic equilibrium between the two compartments. In addition, our previous study (Č v o r o *et al.,* 1998) showing that 41°C whole-body hyperthermic stress stimulates association of rat liver cytosolic GR with Hsp70 suggested that this chaperone may be linked with intracellular transportation of the receptor. It has been shown that Hsp70 is required for nuclear import of a number of proteins (S h i and T h o m a s, 1992) and, moreover, that it can be found only within GR heterocomplexes originating from cells in which unliganded GR is localized in the nucleus, but not from those in which it occurs in the cytoplasm (S a n c h e z *et al.,* 1990).

Owing to the fact that several different experimental approaches applied in this study led to very similar observations on hyperthermic stress-related alterations in GR intracellular localization, the results can contribute to a better understanding of stress-related disturbances in the glucocorticoid signal transduction pathway.

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ПРОМЕНЕ УНУТАРЋЕЛИЈСКЕ ЛОКАЛИЗАЦИЈЕ ГЛУКОКОРТИКОИДНОГ РЕЦЕПТОРА ИЗАЗВАНЕ ХИПЕРТЕРМИЈСКИМ СТРЕСОМ

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Циљ ове студије био је да се испита унутарћелијска редистрибуција глукокортикоидног рецептора (GR) у ћелијама јетре пацова током 24-часовног временског периода после излагања животиња хипертермијском стресу од 41°C. Заступљеност рецепторног протеина у цитоплазматичној и једарној фракцији одређивана је техником имуноблотинга примењеном на укупни цитосол и на пречишћени GR, као и имуноцитохемијском анализом примењеном на парафинске пресеке и на размазе нефиксираних, изолованих једара. Сви експериментални приступи коришћени у овом раду дали су сличне резултате који показују да је пролазно смањење цитоплазматичног GR, запажено током првих 5 часова након излагања животиња хипертермијском стресу, праћено повећаном акумулацијом рецептора у једрима. Ова студија би могла да допринесе бољем разумевању утицаја стреса на пут преноса глукокортикоидног сигнала кроз ћелију.