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EFFECT OF ACUTE ADRENALECTOMY ON RAT LIVER GLUCOCORTICOID RECEPTOR

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Abstract - In order to improve current clinical treatment of human hypocortisolism, it is necessary to understand molecular aspects of this pathophysiology. In this study liver tissues from male Wistar rats were used as an experimental model to study structural and functional properties of glucocorticoid receptor (GR) in the absence of glucocorticoid hormones (GC). Results show that acute adrenalectomy (ADX) significantly increases the number of GR binding sites and GR protein content. In addition, acute ADX stimulates increase in stability of the GR, decrease in stability of the glucocorticoid-receptor complex (G-R), and changes in accumulation of the G-R complex in nuclei and its cellular distribution.

Key words: Adrenalectomy, glucocorticoid receptor, number of binding sites, receptor protein content, stability of glucocorticoid-receptor complex, nuclear translocation.

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INTRODUCTION

Adrenal insufficiency or hypocortisolism, also referred to as Addison's disease, affects about one in 100,000 people, and in 90 percent of cases the adrenal cortex has been destroyed. As a result, both glucocorticoid (cortisol) and mineralocorticoid (aldosterone) hormones are often lacking. Consequently, numerous vital functions of cortisol are compromised, causing symptoms of Addisonian crisis, which if left untreated can be fatal. Throughout the body, cortisol exerts its functions through binding to its intracellular receptor, called the glucocorticoid receptor (GR). In order to understand cellular pathophysiology in hypocortisolism and adjust its treatment, it is necessary to gain insight into molecular changes of the GR receptor.

The cortisol receptor or GR belongs to the superfamily of steroid/thyroid/retinoic acid receptor proteins that function as ligand-dependent transcription factors (Yamamoto, 1985; Evans, 1988; Carson–Jurica *et al.*, 1990). Like other members of this family, the GR consist of an amino-terminal trans-activation domain, a central DNA-binding domain, and a carboxy-terminus that contains the hormone-binding domain as well as sequences important for interacting with heat shock proteins (hsp) (Dalman *et al.*, 1991), nuclear translocation (Picard and Yamamoto, 1987), receptor dimerization (Dahlman-Wright *et al.*, 1992), and trans-activation (Hollenberg and Evans, 1988). In the absence of a ligand, the GR resides in the cytoplasm of cells as a multiprotein complex consisting of the receptor polypeptide, two molecules of hsp90, and several additional proteins (Pratt, 1993; Webster *et al.*, 1994). The GR undergoes a change in conformation upon hormone binding, resulting in the dissociation of hsp90 and the other associated proteins. The activated GR then translocates into the nucleus, where it binds to glucocorticoid receptor-responsive elements (GREs) located in the promoter regions of target genes (Yamamoto, 1985). The GR thereafter interacts with the basal transcription machinery and either positively or negatively regulates expression of the linked gene, depending on the GRE sequence and promoter context (Yamamoto, 1985; Webster and Cidlowski, 1999). In addition, the activated GR can also modulate gene expression apart from DNA binding by physically interacting with other transcription factors (Jonat *et al.*, 1990; Yang Yen *et al.*, 1990; Schule *et al.*, 1990; Scheinman *et al.*, 1995). The presence of functional receptors is therefore a prerequisite for the biological response to GC actions. In most systems, although not in all (Philippa

and K i n g , 1987), a relationship between GC concentration and the degree of biological response has been demonstrated. For instance, early studies (B l o o m *et al.*, 1980; M a y o and P a l m i t e r, 1981) indicated a relationship between nuclear GC binding and the effect of GC, implying a limiting nature of the GR in the cellular response to hormones. V a n d e r b i l t *et al.*, (1987) studied an initially receptor-negative cell line after stable transfection with GR expression vector and showed that the magnitude of several transcriptional responses elected by GR are approximately proportional to the number of GR molecules expressed in this cell line. This suggests that small changes of GR concentration will be reflected in parallel changes in cellular sensitivity towards GC.

In the present study, adrenalectomized male Wistar rats (ADX) were used as an animal model to study intracellular effects of hypocorticism. The level of GR protein expression and GR functional properties were followed in a major GC target tissue, rat liver, before and after acute ADX. The results indicate that the GR binding properties, GR protein level, GR and G-R complex stability, and GR nuclear translocation were affected by acute ADX.

MATERIALS AND METHODS

Chemicals

1, 2, 4 (n) [^{3}H] Triamcinolone acetonide ([^{3}H] TA), with specific activity of 32 Ci/mmol, was obtained from Amersham (Amersham International, UK). Unlabeled TA and dextran were purchased from Sigma Chemical Co. (Sigma, St. Louis, MO, USA). Monoclonal anti-rat glucocorticoid receptor antibody Ab-2 (GR32L, Clone BuGR2) was obtained from Oncogene Research Product (San Diego, CA, USA). All other chemicals were obtained from Sigma.

Animals and treatment

Male Wistar rats (2–2.5 months old; 200–250 g b.m.) were maintained at 22°C, with a 12/12 h light-dark schedule (free access to food and water or saline for sham and ADX rats, respectively) and bilaterally adrenalectomized under Tiopental (50 mg/ml) anesthesia 3 or 18 h prior to the experiment. Animals were divided into the following experimental groups (three rats per group): control (sham operated) rats; and rats, adrenalectomized for 3 or 18 h. Experimental protocols were approved by the local ethical committee and were in compliance with the guidelines set forth in Good Laboratory Animal Practice.

Preparation of rat liver cytosol and isolation of nuclei

The rats were sacrificed at a fixed time of day to avoid any possible circadian variations. Livers were rapidly excised after perfusion with cold 0.14 mol/l NaCl through the portal vein, cleared of connective tissues, and placed on ice. All further procedures, including isolation of nuclei, were carried out at 4^{o}C on the same day, as we described previously (R i b a r a c – S t e p i ć *et al.*, 2005; I s e n o v i ć *et al.*, 2006).

Parameters of GR binding

Binding parameters such as the number of binding sites (N) and the appropriate equilibrium dissociation constant (Kd) were obtained as previously described (R i b a r a c – S t e p i ć *et al.*, 2005; I s e n o v i ć *et al.*, 2006) and expressed as fmol/mg of protein and nmol/l, respectively, according to Scatchard analysis (S c a t c h a r d , 1949). The binding parameters were calculated using regression analysis of Scatchard plots by computer program (L e a k e and H a b i b, 1987).

Determination of GR protein content

Cytosol fractions of liver were resolved on 7.5% SDS-polyacrylamide gels using a Mini-Protean II Electrophoresis cell (BioRad, Hercules, CA, USA) according to L a e m m l i (1970) and transferred to anitrocellulose membrane as we described previously (I s e n o v i ć *et al.*, 1993). Following incubation with a blocking solution, the nitrocellulose membranes were incubated with a monoclonal anti GR antibody (in a dilution of 1 : 500) for 2 h at room temperature, washed and incubated with a secondary anti-mouse antibody (in a dilution of 1 : 2500) coupled with alkaline phosphatase (antimouse IgG-ALP, BioRad) for 2 h at room temperature. The immunoprecipitates were visualized by incubation of membranes with ALP substrates (bromochloroindolyl phosphate and nitrobluetetrazolium chloride), and GR was quantified using a Pharmacia LKB-Ultrascan XL densitometer and the Origin 3.5 program.

Determination of thermal stability of the GR and G-R complex

Thermal stability of the GR was analyzed in freshly prepared rat liver cytosol (Z a k u l a and M o u d g i l, 1991; I s e n o v i ć *et al.*, 2006). The unliganded GR was

first subjected to thermal activation at 25°C for 2 h. Cytosol aliquots were then incubated with 20 nmol/l [^{3}H] TA for 2 h at 4°C to form steroid-receptor complexes. In order to determine the rate of dissociation of G-R complex at 25°C, a series of tubes containing aliquots of cytosol were first complexed with 20 nmol/L [^{3}H] TA at 4°C for 2 h (P a r c h m a n and L i t w a c k, 1977). After charcoal treatment, a 1000-fold excess of radioinert TA was added to each tube and samples were incubated at 25°C for 3 h. At the end of incubation, samples were treated with DCC in order to determine the amount of bound radioactivity.

Translocation of [^{3}H] TA-receptor complexes into isolated nuclei

Complexes formed during incubation of the cytosolic fraction with 20 nmol/l of [^{3}H] TA at 4^{o}C for 18 h were activated by exposure to 25^{o}C for 30 min as we described previously (R i b a r a c – S t e p i ć *et al.,* 2005; I s e n o v i ć *et al.,* 2006). The extracted radioactivity was measured and expressed as decays per minute per milligram of protein.

Determination of protein and DNA

Protein content was determined by the Lowry method (L o w r y *et al.,* 1951) using bovine serum albumin as a standard. The content of DNA was measured by the Burton method (B u r t o n, 1956).

Measurement of radioactivity

For radioactivity measurements, samples were directly introduced into 3 ml of Optiphase Hisafe scintillation cocktail and counted in a 1219 Rackbeta liquid scintillation counter (LKB) at an efficiency of 30% with automatic cpm/dpm calculation.

Statistical analysis

Values are expressed as the mean ± SE with n values representing the number of experiments. Statistical significance was evaluated with Student's *t*-test or by ANOVA analysis (M o o r e *et al.,* 1951). A value of $p < 0.05$ was considered significant (compared to control values).

RESULTS

To investigate the effect of acute ADX on GR binding parameters and GR protein content, rats were bilaterally adrenalectomized 3 and 18 h before sacrifice. The results show that Kd values are in the range of physiological values for GR in all experimental groups (ranging from 0.655-1.73 nmol/l for sham controls and from 1.026-2.55 nmol/l for ADX). Thus, neither 3 nor 18 h afterwards did ADX significantly affect the values of Kd. An increase by a factor 1.5 in the amount of specific hormone binding sites (Fig. 1A) was observed at 3 and 18 h after acute ADX. The number of hormone binding sites (N) increased already 3 h after the operation (153%; $p < 0.01$) and remained at the increased level (1.5-fold) until 18 h following ADX ($p < 0.05$) (Fig. 1A). Results obtained by Western blot analysis (Figs.1B and 1C) showed that increase in N occurred through significant increase in the GR protein level at 3 and 18 h after acute ADX. The respective numerical values were 167% ($p < 0.01$) and 166% ($p < 0.01$). Thus, results of both measurement techniques show significant increase of GR concentration as a consequence of acute ADX.

We have shown (Fig. 1A) that acute ADX induces changes in N and the GR protein level (Figs. 1B and 1C). We now questioned whether acute ADX is also involved in regulation of stability of the formed G-R complexes and cytoplasmic GR *in vitro*. Analysis of ADX effects on G-R complex stability showed that the G-R complexes in acute ADX rats were less stable than those from sham operated animals (Fig. 2A). This decrease in G-R complex stability was statistically significant (3 h: 65±4%, $p > 0.01$; and 18 h: 64±4%, $p > 0.05$) (Fig. 2A). However, the given reduction in G-R complex stability occurred through significant increase in stability of the GR 3 h ($p < 0.001$) and 18 h ($p < 0.001$) after acute ADX (Fig. 2B). Taken together, these data indicate that stability of the rat liver GR and that of its G-R complexes were influenced by acute ADX.

Because the aforementioned results (Fig. 2) indicate that stability of G-R complexes (Fig. 2A) and GR (Fig. 2B) are altered by acute ADX, we further examined the functionality of G-R complexes by analyzing nuclear translocation of the formed cytosol [^{3}H] TA-R complex (Fig. 3). For these experiments, purified liver nuclei from sham and ADX rats were incubated with heat activated cytosol [^{3}H] TA-R complexes prepared from liver of the same group of animals (Figs. 3A and 3B) or sham nuclei were incubated with [^{3}H] TA-R complexes prepared from acute ADX rats (Figs. 3C and 3D). The presented results show (Figs. 3A and 3B) that ADX led to higher transformation of the steroid-receptor complex into an activated form, suggesting that acute ADX induces changes in dis-

tribution of G-R complexes between cytosol and nuclei. In addition, the results show (Figs. 3C and 3D) that [^{3}H] TA-R complexes from ADX rat liver are accumulated at a lower rate in cell nuclei then in the liver of sham animals (Figs. 3C and 3D).

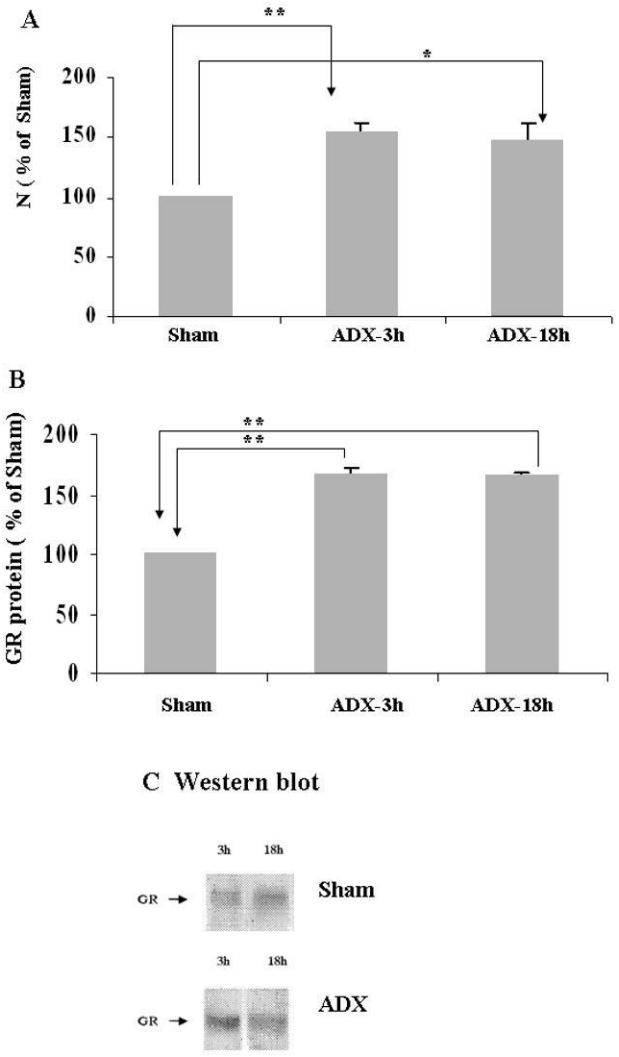

Fig. 1. Number of specific GC binding sites (N) and GR content in acute adrenalectomized rats (ADX).
The N (A) and relative content of GR protein (B) were analyzed in the liver of sham-operated (Sham) and acute ADX rats 3 and 18 h after the operation by the Scatchard and Western blot methods, respectively, as described in section Materials and Methods. The N was expressed as % of changes *vs.* the sham control. Each bar represents the mean ± SE, n = 4 experiments. **p < 0.001; *p < 0.05. Representative immunoblot (C). The designation GR indicates glucocorticoid receptor protein.

DISCUSSION

Glucocorticoid hormones are essential for life, their functions including regulation of the metabolism of carbohydrates, proteins, and lipids; suppression of inflammatory and immunological responses; and suppression of the hypothalamic-pituitary-adrenal axis. These hormones are widely used for treatment of medical problems such as Addison's disease, allergy, arthritis, blood disorders, vascular disorders, eye ailments, gastrointestinal and pulmonary diseases, and skin conditions, aa well as in organ transplantation. Because of their extensive use and the fact that they can have serious side effects (B a x t e r and T y r e l l, 1987), it is of crucial importance to understand how these hormones regulate cellular functions and investigate the molecular aspects of their effects in particular physiological or pathological conditions. It is generally accepted that the molecular mechanism of GC action involves its binding to the specific cytoplasmatic receptor (*i.e.*, the GR), G-R complex activation, and transport into the nucleus (Y a m a m o t o, 1985). In this way, the GR becomes a ligand-activated transcription factor that is known to regulate gene expression and various cell functions (Y a m a m o t o, 1985). The results presented in this study show increase in the GR protein level in rat liver cytosol at both 3 and 18 h after GC removal by ADX (Figs. 1B and 1C). These findings are in agreement with those reported by Beato (B e a t o *et al.*, 1973) and by B o e r and O d d a s (1979) who also found increased cytosolic GR levels in rat liver after ADX. This could be a consequence of increase in the GR mRNA level after ADX (F r e e m a n *et al.*, 2004). The increased number of GC binding sites after ADX observed in our study (Fig. 1A) followed closely increase in GR protein content, whereas the Kd remained unchanged. Our observations are in agreement with results previously reported by other authors (G r e g o r y *et al.*, 1976; S v e c *et al.*, 1989). Autoregulation of steroid receptors has been indicated by several investigators using ligand binding assays. For instance, the presence of GC has been reported to cause a down-regulation of GR. It has been found that GC administration is accompanied by a decrease in cellular GR mRNA (O k r e t *et al.*, 1986). However, adrenalectomy gives rise to an up-regulation of the cellular GR concentration, increasing receptor mRNA (F r e e m a n *et al.*, 2004). The GR has also been shown to recognize specific sequences within a fragment of 3' nontranslated GR cDNA (O k r e t *et al.*, 1986).

Our attempt to measure the stability of formed G-R

complexes revealed that the stability of G-R complexes was lower in acute ADX rat liver cells (Fig. 2A). These changes of G-R complexes could result from their degradation as well as from posttranslational modifications such as phosphorylation/dephosphorylation, which is presumed to maintain the receptor in a conformational state necessary for ligand-binding activity (O r t i *et al.*, 1993; S h e r m a n and G o l d b e r g, 1994; Č v o r o *et al.*, 1999). As ADX rats lack endogenous GC, differences in stability of the receptor complex formed with synthetic glucocorticoid *in vitro* may result from an altered phosphorylation state of GR protein or from changes of inhibitor action (D a h m e r *et al.*, 1985; O k a m o t o and I s o h a s h i, 2000).

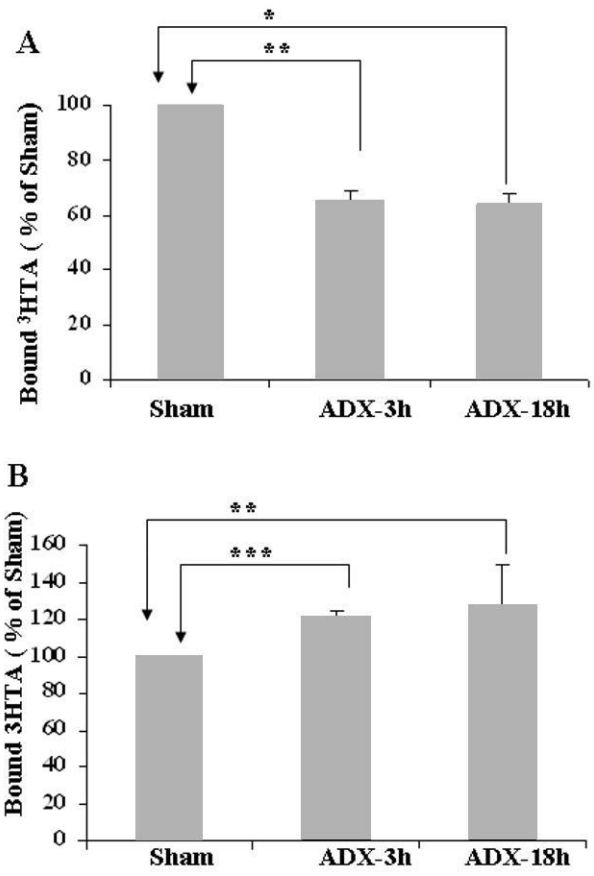

Fig. 2. Stability of G-R complexes (A) and GR (B) in rat liver of acute adrenalectomized rats (ADX).
(A) ADX and sham operated (Sham) rat liver cytosols were incubated for 2 h at 4°C with 20 nmol/l of [^{3}H] TA. After charcoal treatment and addition of an excess of unlabeled TA, samples were incubated at 25°C for an additional 2 h as described in Materials and Methods. The results present G-R complex stability and are expressed as % of the control value. (B) Liver cytosols from ADX and sham operated rats were first incubated in the absence of steroid at 25°C for 3 h. At the end of incubation, ligand-free cytosols were incubated with 20 nmol/l of [^{3}H] TA for 2 h at 4°C for determination of bound radioactivity. The results present % of bound radioactive [^{3}H] TA as compared to the sham value. Each bar represents the mean ± SE, n = 4 experiments. ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$.

In order to determine whether the observed adrenalectomy-induced changes in GR concentration and G-R complex stability are due to reduced translocation, we also indirectly examined changes in translocation of the cytosol receptor complex to nuclear components. As seen from Figs. 3A and 3B, the results show that liver cytosol [^{3}H] TA-R complexes from ADX rats can be translocated more efficiently than those from sham-operated animals. In comparing the specific binding of G-R complexes from ADX animals to the nuclei of the same group, significant differences ($p < 0.001$, $p < 0.05$) of translocation were found in relation to sham-operated rats. These differences may reflect an ADX-induced enhancement of the G-R complex sensitivity to activation. Nuclear binding studies also revealed that the complexes from both groups of ADX animals exhibit higher affinity for nuclei isolated from the ADX rats than for nuclei isolated from the control rats (Figs. 3C and 3D). Since this effect of ADX was detected by nuclear binding assay, it can be assumed that ADX affects very important functional properties of the receptor, i.e., the activation of unactivated complexes. This study presents evidence for lower stability of the G-R complex and its increased nuclear accumulation upon exposure to ADX. Adrenalectomy influences not only the G-R complex and GR stability in rat liver, but also interaction of the translocated GR with the nucleus.

In summary, results of this investigation suggest that the lack of GC induces changes in GR properties. This evidence implies that mechanisms of GR regulation involve modulation of hormone signals transduction through changes in the number and functionality of GR proteins. Thus, the results presented in this work point to considerable complexity of biochemical events involved in the regulation of GR in different pathophysiological conditions such as a low level of GC. Since they indicate that acute ADX induces changes in cellular mechanisms responsible for increase of GR content, these parameters should be considered in therapy of Adisson's disease.

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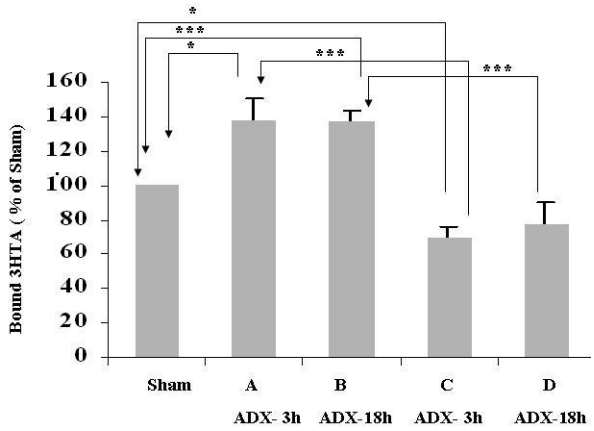

Fig. 3. The effect of acute adrenalectomy (ADX) on nuclear translocation of [^{3}H] TA-R complexes in rats.
Isolated liver nuclei from sham and ADX rats were incubated with cytosol [^{3}H]TA-R complexes prepared from liver of the same group of animals (A, B) or sham nuclei were incubated with [^{3}H]TA-R complexes obtained from ADX animals 3 and 18 h after ADX (C, D) as described in Material and Methods. The results are presented as specific binding of radioactive [3H] TA-R complexes to the nuclei and expressed as % of sham. Each bar represents the mean ± SE, n = 4. ***p < 0.001, **p < 0.01.

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ЕФЕКАТ АДРЕНАЛЕКТОМИЈЕ НА ГЛУКОКОРТИКОИДНИ РЕЦЕПТОР У ЈЕТРИ ПАЦОВА

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У циљу побољшања постојећег третмана смањеног нивоа кортизола код људи, неопходно је проучавање молекуларних механизама који леже у основи овог патофизиолошког стања. Са циљем да се изуче структурна и функционална својства глукокортикоидног рецептора (GR) у овом раду, као модел систем, коришћена је јетра мужјака пацова соја Wistar. Добијени резултати показују да акутна адреналектомија доводи до значајног повећања броја везујућих места GR и његовог протеинског садржаја. Осим тога, у условима смањеног нивоа глукокортикоида долази до повећања стабилности GR и смањења стабилности глукокортикоид-рецептор комплекса (G-R), као и промена у ћелијској дистрибуцији и транслокацији G-R комплекса у једра ћелија јетре.