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Comparative Analysis of Tryptophan Oxygenase Activity and Glucocorticoid Receptor under the Influence of Insulin

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Summary

This investigation addresses the interaction of insulin (INS) and glucocorticoid (GC) signaling in the hepatic regulation of tryptophan oxygenase (TO) enzyme activity in the rat. Male Wistar rats (200-250 g b.w) received an injection of the different doses of INS (10, 25, 50, 70 and 100 μg/200 g b.w., i.p.) and were used for experiments 3 h and 18 h after INS administration. This study shows that maximum of TO activity was found at dose of 50 μg of INS with peak increases observed at 3 h and 18 h after injection of INS, while INS had no effect on TO activity in adrenalectomized rats. The analysis of INS effects on glucocorticoid receptor-complex (GC/GR complex) stability shows that complexes from INS-treated rats are less stable than those from control animals. In addition, INS-stimulated stability of glucocorticoid receptor (GR) protein was significantly increased from the controls. Furthermore, the results show that GC/GR complexes from INS-treated rats could be activated and accumulated at higher rate in cell nuclei of control animals. These data support the involvement of INS in modulation of GC signaling pathway which mediates, in part, the activity of TO.

Key words

Glucocorticoid • Glucocorticoid receptor • Stability of glucocorticoid-receptor complex • Insulin • Tryptophan oxygenase

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Introduction

Various hormones, such as insulin (INS) and glucocorticoids (GC), elicit their effects on the activity of the same enzymes in spite of the differences in their chemical nature and the specificity in the molecular mechanism of their action on the common target cells (Hirota *et al.* 1985, Schubart 1986). The consequences of transmission and transduction of hormone signals to their target cells comprise the regulation of gene expression involved in concomitant modification of cell function. All of these effects INS and GC are realized through hormone binding to specific receptors (White and Kahn 1994, Schaaf and Cidlowski 2002, Schoneveld *et al.* 2004).

The first step in INS action is the binding of INS to the specific plasma membrane receptors (IR). At the molecular level, INS binding to its transmembrane IR stimulates the intrinsic tyrosine kinase activity of the receptor, which then phosphorylates target protein such as family of IR substrate proteins (De Meyts 2004). This triggers the activities of downstream effectors molecules comprised by two major kinase cascades, the phosphatidylinositol 3-kinase and mitogen-activated protein (MAP) kinase pathways, which mediate the metabolic and growth-promotion function of INS respectively (Whitehead *et al.* 2000).

It is generally accepted that molecular mechanism of GC action includes binding of the GC to the specific cytoplasmic receptor, i.e. glucocorticoid-receptor (GR), activation of the GR and transport of the glucocorticoid receptor-complex (GC/GR complex) into the nucleus (Cidlowski and Munck 1979, Schoneveld *et*

al. 2004). In this way, GR becomes ligand activated transcription factor, known to regulate gene expression and cell functions (Schoneveld *et al.* 2004).

In mammals, most of the total tryptophan is degraded through the kynurenine pathway in the liver. The first enzyme of tryptophan oxidation is tryptophan oxygenase (TO; EC.1.13.11.11) (Comings *et al.* 1995). Expression of TO is an interesting model system for studying the control mechanism of gene expression, since the enzyme is expressed in a tissue-specific fashion, and its activity is a subject to hormonal control (Danesch *et al.* 1987). In addition to rapid effects of GC and INS to modify hepatic glucose output by regulating gluconeogenesis at the level of enzymes directly involved in this process, e.g. phosphoenolpyruvate carboxykinase (PEPCK), these agents also affect gluconeogenesis through altered synthesis of certain enzymes of amino acid metabolism (Exton 1987). TO and tyrosine amino-transferase are also regulated by GC and INS in part at the level of specific messenger RNA synthesis (Danesch *et al.* 1987, Schubart 1986).

The specific effects of steroids on target gene and also their influence on the cell-specific transcriptional modulation could be insured through synergistic action of these hormones and their receptors with other transcriptional factors. It has been reported that INS is an essential component for the transcriptional regulation of liver-specific genes in combination with GC (Pan and Koontz 1995). However, the molecular mechanism(s) of multihormonal regulation of cell processes as well as the levels at which the integration of hormonal effects occurs in target cells are not completely understood. In order to gain more information on the participation of INS in transduction of GC signals in the regulation of cell functions, the possible interactions between INS and GC in the regulation of TO activity *via* rat liver GC signaling pathway have been studied. This investigation has been based on hypothesis that INS enhances the ability of GC to increase TO activity by interfering with signaling through GR in the rat liver.

Methods

Chemicals

1,2,4 (n) [^{3}H]-triamcinolone acetonide ([^{3}H]TA) specific activity 32 Ci/mmol was obtained from Amersham (Amersham International, UK). Porcine insulin was obtained from ICN-Galenika, Belgrade, Serbia & Montenegro. All other chemicals were obtained from Sigma (Sigma, St. Louis, USA).

Animals and treatment

Male Wistar rats (2-2.5 months old; 200-250 g b.w.) were kept at 22 °C, with a 12/12 h light-dark schedule (rat chow and water *ad libitum*) and for some experiments were bilaterally adrenalectomized under Nesdonal anesthesia 5 days prior to the experiment. Animals were divided into two experimental groups (3 rats per group): control (CONT) and insulin (INS)-treated. After an overnight fast, animals were injected intraperitoneally with different doses of INS (10, 25, 50, 70 and 100 μg/200 g b.w.) and sacrificed at different time (3, 6, 12, 18 and 24 h) after INS administration. The CONT animals were used after an overnight fast, received 0.14 M NaCl, kept at room temperature and sacrificed along with their INS-treated counterparts. Experimental protocols were approved by local Ethical Committee and they were in compliance with „*Good Laboratory Animal Practice*".

Tryptophan oxygenase assay

Tryptophan oxygenase activity was assayed in crude liver homogenates essentially described previously (Hirota *et al.* 1981, Knox and Auerbach 1955). One unit of TO activity was defined as the amount of kynurenine/ h/g of dry weight of liver homogenates at 25 ^{o}C.

Preparation of hepatic cytosol and nuclei

The animals were killed by cervical dislocation and livers were perfused *in situ* with ice cold saline through portal vein. The livers were quickly removed, weighed, minced and homogenized with Potter-Elvejhem homogenizer in 2 volumes (w/v) of a 50 mM Tris buffer, pH 7.55, containing 0.25 M sucrose, 25 mM KCl, 10 mM $MgCl_2$ and 0.1 mM PMSF. The homogenate was centrifuged at 800 x g for 10 min, at 4 ^{o}C. To obtain the cytosolic fraction the supernatant was centrifuged at 105000 x g for 90 min. The upper lipid layer was aspirated and clear cytosol was used for GR analysis (Beato and Feigelson 1972, Schoneweld *et al.* 2004). For isolation of nuclei, the crude nuclear pellet, obtained after the first centrifugation, was resuspended in a cold buffer, pH 7.2, containing 2.2 M sucrose, 5 mM Tris-HCl, 5 mM $CaCl_2$, layered over the same cold solution and centrifuged at 24000 rpm for 60 min at 4 ^{o}C in a Beckman SW 27 rotor. The resultant pellet of nuclei was washed three times with a buffer containing 5 mM Tris-HCl and 1 mM $MgCl_2$, pH 7.9 and kept in the same

buffer supplemented with 25 % glycerol at 4 °C until use (Chauveau *et al.* 1956).

Stability of GR and GC/GR complex

The unliganded GR in the freshly prepared rat liver cytosol was first subjected to the activation at 23 °C for different time period (0, 15, 30, 60, 120, and 180 min). Cytosol aliquots were then incubated with 20 nM [^{3}H] TA for 2 h at 4 °C to form GC/GR complexes. For determining the rate of dissociation of GC/GR complexes at 23 °C, a series of tubes containing aliquots of cytosol were first complexed with 20 nM [^{3}H]TA at 4 °C for 2 h. After charcoal treatment a 1000-fold excess of radio inert TA was added to each tube and samples were incubated at 23 °C for different time intervals (0, 15, 30, 60, 120, and 180 min). At the end of each time period, samples were treated with dextran-coated charcoal in order to determine the amount of bound radioactivity (Isenovic *et al.* 2006a).

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

Complexes formed during incubation of the cytosolic fraction with 20 nM of [^{3}H]TA at 4 °C for 18 h were activated by exposure to 25 °C for 30 min (Parchman and Litwack 1977), and then samples were treated with dextran-coated charcoal (3.75 % Norit A and 0.375 % dextran T-500 in buffer for homogenization) to separate bound and free hormones. Samples were then centrifuged at 2000 x g for 10 min at 4 °C and the clear supernatants were kept at 4 °C until use. For the translocation assay purified nuclei were incubated 30 min at 4 °C with the same volumes of activated or nonactivated hormone-receptor complexes. Following incubation the samples were washed three times with buffer containing 5 mM Tris-HCl and 1 mM $MgCl_2$, pH 7.9, and centrifuged each time for 10 min at 800 x g. The final pellets were then resuspended in buffer containing 0.01 M Tris-HCl, 0.3 M NaCl, 1 mM β-mercaptoethanol, 1 mM EDTA, 0.5 mM $MgCl_2$, pH 8.0, and mixed at 10 min intervals for 30 min at 4 °C. The suspensions were centrifuged 20 min at 12000 x g and the resulting supernatant was used for determination of radioactivity and protein concentration. The extracted radioactivity was measured and expressed as disintegrations per minute per milligram of protein. In some experiments, the pellet, referred as the nuclear fraction insoluble in 0.3 M NaCl, was extracted overnight with absolute ethanol and centrifuged at 12000 x g for 20 min. The extracted radioactivity was measured and expressed as disintegrations per minute per milligram of pelleted DNA (Hirota *et al.* 1985, Schubart 1986).

Determination of protein and DNA

Protein content was determined by the method of Lowry (1951) using bovine serum albumin as a standard. DNA content was measured by the method of Burton (1956).

Measurement of radioactivity

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

Statistical analysis

Values are expressed as mean ± SE with n values representing the number of experiments. Each experiment was performed five times using 3 rats per group. Statistical significance was evaluated with Student's T-test or ANOVA (Moore *et al.* 1951). A value of $p<0.05$ was considered significant (compared to control values if it is not otherwise specified).

Results

Figure 1 presents group of data comparing the effect of different doses of INS (10, 25, 50, 70 and 100 μg/200 g b.wt. i.p.) injected for 3 h into rats, on TO activity. It can be seen that a dose of 50 μg of INS induced maximum TO activity. This increase in TO activity was 97 % because mean TO activity increased from control 1.22 ±0.13 μmol to 2.40±0.23 μmol following the 50 μg of INS injection.

Figure 2 demonstrates the effect of the 50 μg of INS on enzyme activity as a function of time after INS injection. These increases in TO activity were 96 % (at 3 h) and 60 % (at 18 h) of control.

It should, however, be noted that INS injected into bilaterally adrenalectomized rats, decreases TO activity compare to sham-operated rats (Isenovic *et al.* 2006a).

Figure 3 illustrates the effects of INS effects on GC/GR complex (panel A) and GR protein stability (panel B). GC/GR complexes formed from INS-treated rats are less stable than those from control animals, whereas injection of INS significantly stimulated stability

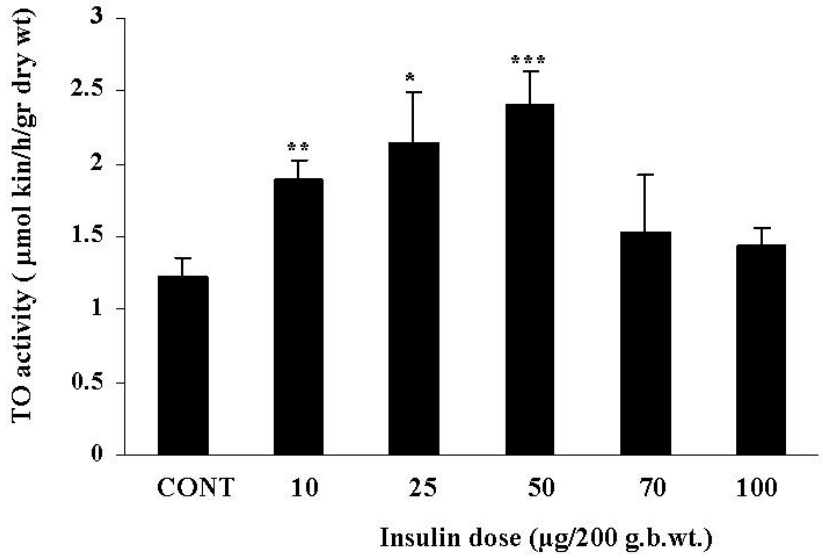

Fig. 1. Dose-dependent effects of INS on TO activity determined 3 h after administration of different doses of INS (10, 25, 50, 70 and 100 µg/200 g b.w.) as described in section Material and Methods. The enzyme activity is presented as amount of kynurenine/h/g of dry weight of rat liver homogenate. Each bar represents the mean ± S.E.M. of 5 experiments. ***p<0.001, **p<0.01, *p<0.05 INS *vs.* CONT. CONT indicates control; INS indicates insulin treatment for 3 h.

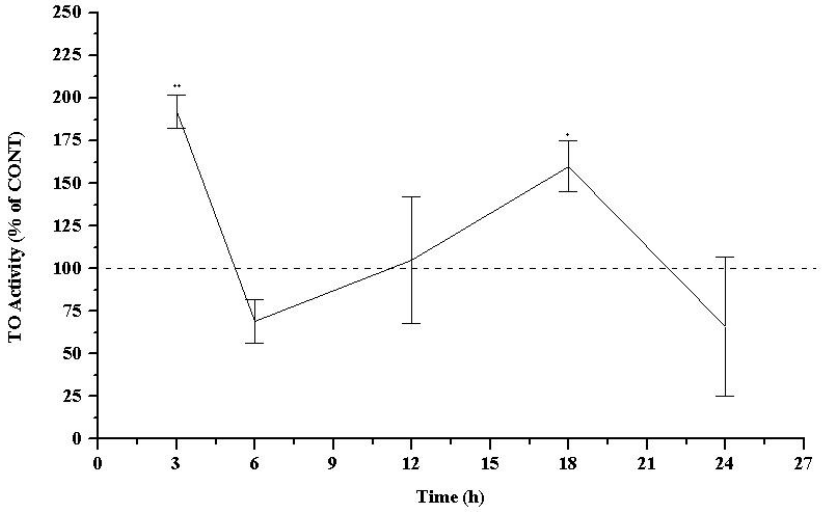

Fig. 2. Time-dependent effects of INS on TO activity determined after INS administration (50 µg/200 g b.w.) at different time intervals (3, 6, 12, 18 and 24 h) as described in section Material and Methods. The enzyme activity is presented as % increase *vs.* CONT. Each bar represents the mean ± S.E.M. of 5 experiments. **p<0.01; *p<0.05. CONT indicates control; INS indicates insulin (50 µg/200 g b.w.)

of GR protein.

Figure 4 exemplifies the effects of INS on nuclear translocation of *in vitro* formed ^{3}H-triamcinolone acetonide (^{3}HTA-R) complexes. It can be seen that different level of translocated ^{3}HTA-R complexes has been observed in isolated nuclei after administration of INS, implying that liver cytosol ^{3}HTA-R complexes from INS-treated rats could be activated by heat and translocated into isolated nuclei from the same livers at lower rate than those from the controls.

Discussion

In this study we have examined: 1) the effects of INS on TO activity, 2) the involvement of the adrenal gland in the effects of INS on TO activity and 3) the effects of INS on GR and GC/GR complex stability and nuclear translocation. The present study demonstrated that the maximum TO activity was found at a dose of 50 µg of INS (Fig. 1). These findings are of interest because it is well accepted that INS induced the activity of TO in cultured rat hepatocytes (Schubart 1986). In addition, we demonstrated that the peak of increase in TO activity was observed at 3 h and at 18 h of exposure to INS (Fig. 2). These findings highlight key differences in mechanisms underlying TO induction following stimulation by INS. One mechanism (peak at 3 h) could be explained by tryptophan accumulation in hepatocytes as a result of INS effects on the increased amino acid transport from extra hepatic tissues (Goldstein *et al.* 1962, Kim and Miller 1969), whereas the other (peak at 18 h) could reflect INS effects on the enzyme synthesis. In addition, Niimi *et al.* (1983) and Schubart (1986) demonstrated that regulation of TO activity by various hormones is due to a change in its translatable mRNA. However, the change of the mRNA can be explained by

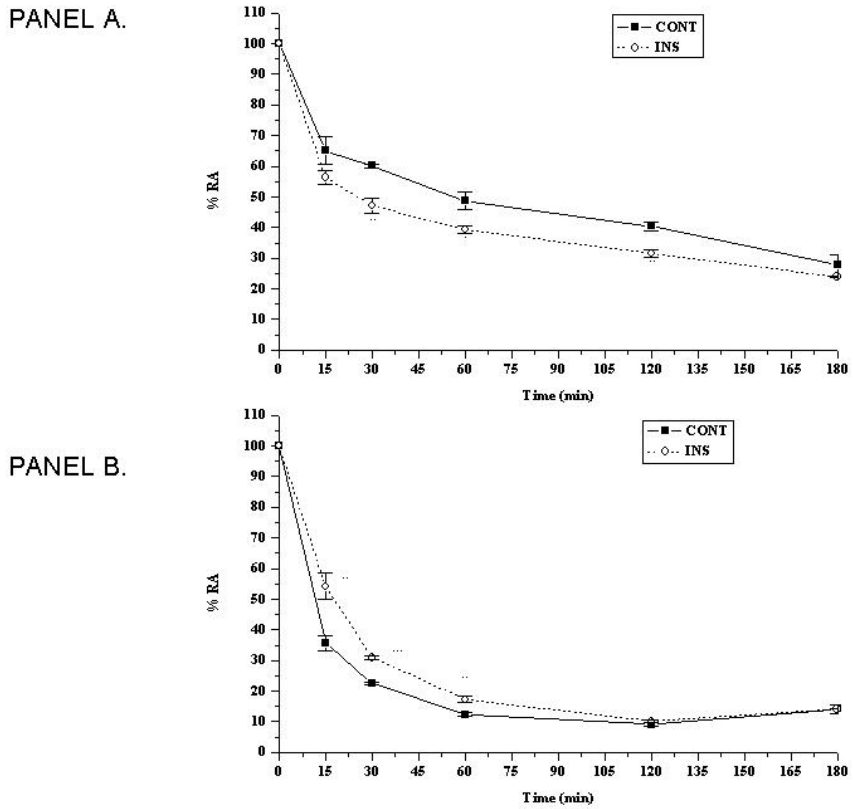

Fig. 3. Effects of INS on GC/GR complex (A) and GR stability (B) **A.** Aliquots of rat liver cytosol from CONT and INS-treated animals, were incubated with 20 nM [^{3}H]TA for 2 h at 4 °C. After charcoal treatment a 1000-fold excess of unlabeled TA was added to each tube and samples were incubated at 23 °C for different time periods shown. After the incubation, the mixtures were treated with charcoal in order to determine the amount of bound radioactivity. The results present % of bound radioactive [^{3}H]TA. Each bar represents the mean ± S.E.M. of 5 experiments. **p<0.01, *p<0.05.
B. Series of tubes was containing aliquots of rat liver cytosol from control and INS-treated animals were first incubated in the absence of steroid at 23 °C for time period shown. At the end of each time, cytosols were incubated with 20 nM [^{3}H]TA for 2 h at 4 °C for determination of bound radioactivity, as described in Materials and Methods. The results represent the mean ± S.E.M. of 5 experiments are expressed as % of bound radioactive [^{3}H]TA. ***p<0.001, **p<0.01. CONT indicates control, INS indicates insulin (50 μg/200 g b.w.) for 3 h; [^{3}H] TA indicates triamcinolone acetonide.

change in either its transcription or stability. Using this cDNA, Nakamura *et al.* (1987) measured the effects of hormones on the amount of TO mRNA by dot-blot hybridization and the relative rate of transcription of the TO gene by nuclear run-off with its cDNA as a probe. Their results indicated that changes in TO mRNA level were due to changes in regulation of its transcription. Since administration of INS to intact rats leads to elevation of TO activity (Isenovic *et al.* 2006a,b), it is possible to assume that INS provokes the interaction of unliganded GR with GC response elements (GREs) and in this way INS contributes to the enhancement of TO transcription (Pan and Koontz 1995). However, most of the INS-stimulated TO activity in rat liver was inhibitable by adrenalectomy (Isenovic *et al.* 2006a), suggesting that INS stimulation of the TO was largely dependent of GC presence. Taken together, these findings support our idea that 1) INS and GC may act in parallel signaling pathways, both of which are necessary for the regulation of TO, and 2) there is a cross-talk between INS and GC where INS is activated first, which is further resulting in the activation and/or induction of synthesis of GR. If induction or activation of GR is needed for the action of INS, this would explain why INS fails to induce TO after adrenalectomy (Isenovic *et al*. 2006a).

If the main effect of INS is mediated by GR, it is probably directed to the induction of TO gene transcription through GC response elements in this gene (Danesch *et al.* 1987). There is also a possibility that INS-stimulated amino acid uptake in liver cells induces TO activity by substrate induction (Fehlmann *et al.* 1979). Finally, it is well known that direct INS effect on TO mRNA exists (Niimi *et al.* 1983). The consequence of the increased tryptophan catabolism, i.e. TO activity, could be a stimulation of gluconeogenesis and glucose production in the liver (Exton 1987).

It must also be noted that our previous study provided evidence for the stimulatory effect of INS on GR protein expression (Isenovic *et al.* 1993, 2006a) as well as on functional properties of GR (Isenovic *et al.* 2006a), and indicated a role of INS in GR regulation. The present study indicates that INS injection significantly increased GC/GR complex dissociation (Fig. 3A) and GR protein stability (Fig. 3B). Furthermore, INS-stimulated GR stability is in conjunction with decreased GC/GR complex translocation (Fig. 4). These changes of GC/GR complexes stability under INS action could result from increased nuclear translocation of the receptor or from its posttranslational modifications such as phosphorylation/ dephosphorylation which is proposed to maintain the receptor in a conformation state necessary for ligand binding activity (Weigel 1996). Phosphorylation of GR

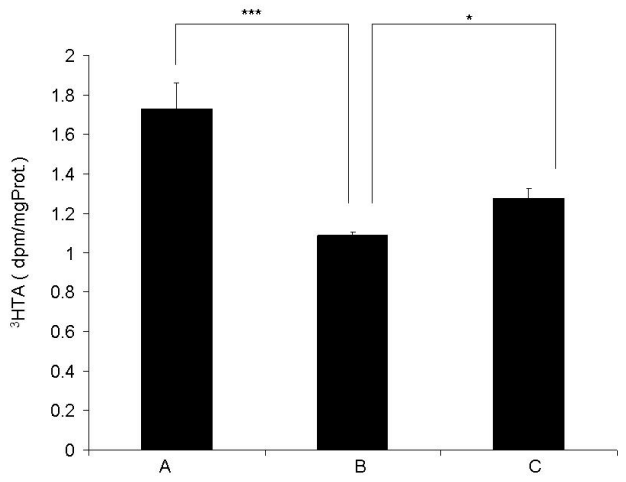

Fig. 4. The effect of INS on nuclear translocation of [^{3}H] TA-R complexes. Isolated liver nuclei from control (A) and INS (B) treated rats were incubated with cytosol ^{3}HTA-R complexes prepared from liver of the respective group of animals and (C) control nuclei were incubated with ^{3}HTA-R complexes obtained from liver cytosol following INS treatment as described in section Material and Methods. The extracted radioactivity was measured and expressed as disintegrations per minute per milligram of protein (dpm/mg Prot.). Each bar represents the mean ± S.E.M. of 5 experiments. ***p<0.001 and *p<0.05. CONT indicates control. INS indicates insulin (50 μg/200 g b.w.) for 3 h; [^{3}H] TA indicates triamcinolone acetonide.

upon the activation of receptor complex, which could be modulated by protein hormones *via* kinase activity of their membrane receptors, might provide a molecular basis for the interaction and integration of multiple effects and response of common target cells to INS and GC hormone action (Kuiper and Brinckmann 1994, O'Malley 1995). In addition, the presented results show that administration of INS induced changes in distribution of CG/GR complexes between cytosol and nuclei of the liver cells (Fig. 4).

These data (Fig. 4) together with our earlier results (Isenovic *et al.* 1993, 2006a,b) suggest that INS treatment causes the modification(s) of GR at the level of interaction with nuclear components. On the basis of the presented results it could be concluded that the activation and translocation of GC/GR complexes into the nuclei might be one of the possible sites where the interaction of the GC and INS is achieved in regulation of cell processes.

In summary, consistent evidence is emerging that TO activity and functional properties of GR are altered by INS injection. Based on these findings, we suggest that INS treatment might activate the GC signaling pathway, participating in the regulation of TO activity in the rat liver.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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