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THE EFFECT OF NUTRITIONAL STATUS ON THE MAGNITUDE OF ACUTE PHASE PROTEIN RESPONSE IN FEMALE RATS

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In response to inflammation, infection and tissue injury, the sequence of events known as acute-phase (AP) response is induced and a number of plasma proteins, called acute-phase proteins (APPs) are synthesized in the liver, as a part of the host defence mechanism. Any condition which impairs acute phase protein response (APPR) and hence organism's ability to restore homeostasis may limit its survival in various stress circumstances. Malnutrition is well-known causes of several metabolic, immune and neuroendocrine dysfunctions and this study was aimed at investigating its effect on the magnitude of normal APPR and in relation to turpentine injury. Malnutrition in female Wistar rats was induced by food restriction during six weeks period until they riched and kept body weight to 50% of that of ad libitum fed mates. Results of rocket immunoelectrophoresis revealed that malnutrition alone or in conjunction with turpentine was capable of elevating an expression of

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one of APPs, haptoglobin (Hp). Based on the studies of liver-specific regulatory proteins that bind to hormone regulatory elements of Hp gene and thus determine its expression we found that malnutrition probably modulated Hp response by changing the expression of active members of C/EBP, STAT and NF-kB transcription factor families.

Key words: malnutrition, haptoglobin, C/EBP, STAT, NF-kB, acute phase protein response

INTRODUCTION

Following injury and infection, various immunological, inflammatory and metabolic changes occur to promote repair and healing. These changes are mediated by a number of endogenous factors, including cytokines, hormones and growth factors (Lowry 1992; Baumann and Gauldie, 1994). Early activation of the cytokine network through interleukin-6 (IL-6), IL-1β and tumor necrosis factor (TNF-α), is of particular importance in inducing the APPR (SMITH *et al.*, 1992;. BAUMANN and GAULDIE, 1994). These proteins, synthesized in the liver, play widespread roles in limiting tissue damage and aiding recovery (MACKIEWICZ, 1997) and any condition which impairs APPR may limit survival from injury .

Some studies have indicated that the host ability to respond to and recover from various stress stimuli depends of age and/or nutritional status. It was found that obesity (TSCHOP and HEIMAN, 2001) and malnutrition affects reproductive, adypocite, metabolic and neuroendocrine functions (PERALTA *et al.*, 2002; TAPPY *et al.*, 2000). GIOVAMBATISTA *et al.* (2000) have shown that malnutrition induced basal hypoglycemia, hypotrigliceridemia, hypoleptinemia, hypercorticosteronemia and enhanced adrenal glucocorticoid content in female rats. Hepatic stimulation of APP synthesis is incorporated in the complex interchange of proinflammatory cytokines and glucocorticoids resulting in a specific assembly of induced transcription factors on the APP gene regulatory elements (MACKIEWICZ, 1997). Therefore, through modulation of immune and neuroendocrine functions malnutrition could change gene expression of certain liver-specific regulatory proteins, which would increase or decrease their steady state level in the cell and thus affect the total amount of their transcriptional activity that is available for regulating the expression of target genes. Roesler (2001) has already reported that transactivating capacity of C/EBPs (CCAAT enhancer binding proteins), a family of transcription factors with important roles in constitutive regulation of APPR and energy metabolism, can be modulated by different nutrients.

This study attempts to provide insight into the mechanism whereby malnutrition alters magnitude of APPR in basal condition and in relation to acute injury, with the focus on a APP haptoglobin (Hp) and liver enriched transcription factors involved in the regulation of its gene activity. Hp is considered to be one of the major APPs synthesized in rat hepatocytes during AP response due to its pleiotropic activities mostly related to the binding and clearance of hemoglobin, inhibition of superoxide production and stimulation of angiogenesis (MACKIEWICZ,

1997). The full expression of its gene is mediated by proximal promoter element which contains elements responsive to glucocorticoids, IL-1 and IL-6 (MARINKOVIĆ and BAUMANN, 1990). Induction level of Hp is variable and highly dependent upon the nature of inflammatory stimulus (ŠEVALJEVIĆ *et al.*, 1988; BAUMANN *et al.*, 2000). Since turpentine oil causes sterile tissue injury through a confined but strong local inflammation followed by systemic acute phase reaction, here we used this inflammatory stimuli to evaluate the mode of undernourished rats response to injury.

MATERIAL AND METHODS

The experiments were performed on female Wistar rats at the age of 1 and 2.5 months. Animals were maintained at constant temperature (22±2°C) with 12-hr light-dark cycle and fed standard laboratory chow (a product of D. D. Veterinarski zavod, Subotica, Serbia and Montenegro). Tap water was available *ad libitum*. One-month-old female rats (n=15) were placed in individual cages and fed daily during 6 weeks with an amount of chow equivalent to 50% of the normal food intake until they riched and keep body weight to about 50% of that of *ad libitum* fed age-mates (n=15). Undernourished (UN) animals were weighed weekly and the amount of food provided was adjusted individually to maintain the weight.

To induce APPR, UN and *ad libitum* fed female rats aged 2.5-months, termed well-nourished (WN) controls, received cubcutaneous injection of turpentine oil (250µl) in the dorsal lumbar region. Each experimental series included 5 animals per treatment. Animals were sacrificed by cervical dislocation at 12- and 24-hr after turpentine tretment when maximal Hp gene transcription and serum level were established (ŠEVALJEVIĆ *et al.* 1989). The serum level of Hp was measured by rocket immunoelectrophoresis according to BAUMANN (1988). For the Hp determination a polyspecific antibody to human Hp was used (SIGMA-ALDRICH Inc), which was cross-reactive with rat Hp. Relative concentration of Hp was established by quantification of the areas under the respective immunoprecipitation peaks and expressed as a relative increase in relation to the initial control values that were taken as 100%.

Liver nuclear protein extracts (NEs) were isolated from the untreated and the 12-hr turpentine treated WN/UN rats, following the procedure elaborated by GORSKI *et al.* (1986). Protein concentration in NEs was determined by the method of Bradford. For Western immunoblot assay rat liver NEs were separated by 10% SDS-PAGE, transferred to Hybond P membranes (Amersham, England) and probed with rabbit polyclonal antibodies (Santa Cruz Biochemicals Inc., California, USA) specific to C/EBPα (14AA, dilution 1:1000), C/EBPβ (C-19, dilution 1:1000), STAT3 (H-190, dilution 1:1000), and NF-kB p65 (C-20, dilution 1:1000). Immunoreactive proteins were detected according to the procedure recommended by the supplier of ECL Western immunoblot kit RPN 2108 (Amersham, England). Each filter was consecutively reprobed with all used antibodies according to the reprobing protocol.

RESULTS AND DISCUSSION

We studied the effects of malnutrition on magnitude of normal APPR and in relation to turpentine injury. This approach was validated by comparing serum protein levels of Hp between the 2.5 month old female rats fed *ad libitum* (WN) or exposed to food restriction during six weeks period (UN). Data on their body and liver weights are summarized in Table 1. As expected, food restriction kept body weight of UN rats to about 50% of WN females. Total liver weights of UN females were significantly lower than those of their WN controls ($p<0.01$) suggesting that the synthetic and/or secretion function of the liver was changed.

Table 1. - Characteristics of the well-nourished (WN) and undernourished (UN) female rats: the effect of food restriction on body weight and liver weight

	WN	UN
Body weight, g	211 ± 10	110 ± 6
Liver weight, g	7.55 ± 0.48	3.37 ± 0.29
Liver weight, %body weight	3.58 ± 0.35	3.06* ± 0.21

The values represent the mean SD (n = 15)
* $p<0.01$ compared with lean WN control

Comparison of the serum levels of Hp between WN and UN rats (Fig. 1) revealed a significant increase in the basal level of the Hp in UN rats (172%). When UN females were treated with turpentine, the relative level of serum Hp was much higher (879%) than after the same treatment of WN controls (541%) suggesting that the effects of turpentine treatment were potentiated by malnutrition. This data imply that liver cells were probably sensitizing by malnutrition which made them more susceptible to the action of a second inflammatory stressor, turpentine. This may be due to changes in expression profile of liver regulatory transcription factors involved in the regulation of Hp gene expression.

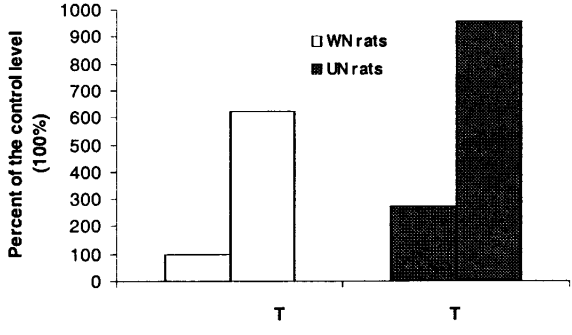

Fig. 1. - Changes in the relative Hp level in the serum of WN and UN Wistar female rats after 24h of turpentine treatment (T). Data are expressed as the percentages of initial control values of WN rats that were taken as 100%

Recent studies on the AP induction of Hp, which occurs within 24 h of exposure to turpentine, have indicated that transcriptional induction of rat Hp gene is primarily regulated by few positive IL-6 responsive regulatory elements (IL6-RE) and their cognate binding transcription factors, C/EBPs (α, β, δ) and STAT 3 (Signal transducer and activators of transcription) (GRIGOROV *et al.*, 1998; GRIGOROV *et al.*, 2000; MILOSAVLJEVIĆ *et al.*, 2003). Its known that expression and biological activity of these factors are modified under physiopathological conditions during liver regeneration, toxic stimuli, inflammation or malignancy (POLI, 1998; FERRINI *et al.* 2001; FRANK, 1999). Therefore, to evaluate the mechanism of transcriptional induction of Hp gene during malnutrition, we have investigated whether inducible forms of C/EBP and STAT3 proteins are expressed. Nuclear expression profile of the C/EBPα , C/EBPβ , and STAT 3 in the livers of WN and UN rats before and after turpentine treatment was obtained by Western immunoblot analysis with specific anti-C/EBP/STAT3 antibody.

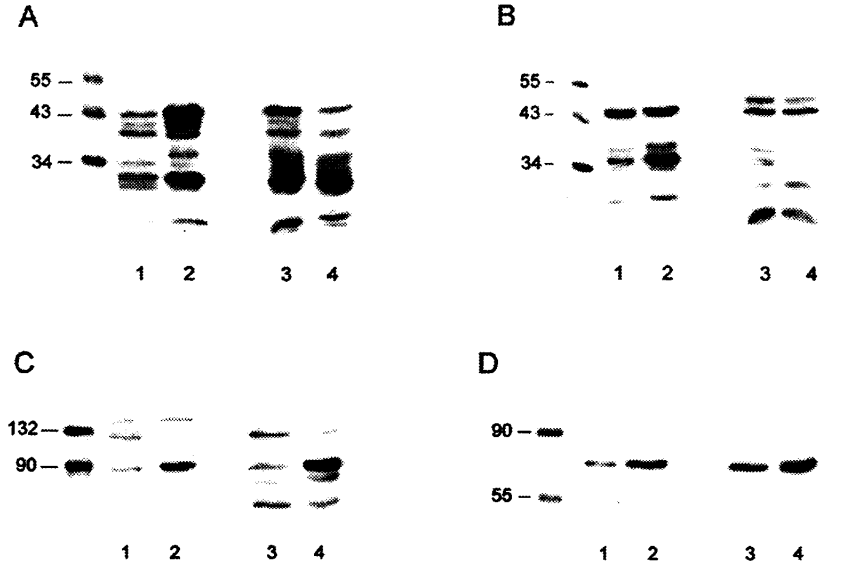

Fig. 2. - Western immunoblot analysis of the levels of C/EBPα (A), C/EBPβ (B), STAT 3 (C) and NF-kB p65 proteins in the liver NEs of WN and UN rats in basal condition and in response to turpentine treatment. Liver NEs were subjected to 10% SDS-PAGE and subsequent ECL-Western immunoblot analysis with anti-specific antibody as described in Material and Methods. Lanes 1 and 2 contain NEs from the livers of untreated rats while lanes 3 and 4 contain NEs from the livers of turpentine-treated rats. The molecular weight standards in kD are shown on the left

Both C/EBPα and C/EBPβ mRNAs were found to code for several smaller protein isoforms with distinct regulatory and physiological functions (OSSIPOW *et al.* 1993; AN *et al.* 1996; HSIEH *et al.* 1998). Anti-C/EBPα antibody recognized several different isoforms of C/EBPα protein with the molecular masses ranging from 28 to 42 kD in the liver NEs of control WN rats (Fig. 2A,

lane 1). Apart from 34 kD-C/EBPα, amount of other C/EBPα isoforms increased after turpenine treatment (Fig. 2A, lane 2) in relation to the control and two more C/EBPα isoforms were identified at the 35 kD and 14 kD positions. Similar profile of C/EBPα isoforms such as in NEs of turpentine treated WN rats were obtained in the NEs of UN rats before (Fig 2A, lane 3) and after turpentine treatment (Fig. 2A, lane 4). Anti-C/EBPβ antibody revealed extensive changes in C/EBPβ expression profile in UN rats. Amount of 35kD-C/EBPβ isoforms, also known as LAP (liver activatory protein), increased significantly after turpenine treatment of WN rats (Fig. 2B, lane 2), while it was absent in NEs of UN rats (Fig. 2B, lanes 3 and 4). 20kD-C/EBPβ isoform known as LIP (liver inhibitory protein) appeared in turpentine treated WN rats as well as in the NEs of UN rats.

In the liver NEs of WN control rats anti-STAT3 antibody (Fig. 2C, lane 1) recognized low level of constitutively active 91kD-STAT3 isoform also known as acute phase response factor (APRF) amount of which increased significantly in response to turpentine (Fig. 2C, lane 2). Appearence of inducible 86kD-STAT3 (Ripperger *et al.*, 1995) isoform and a small increase of amount of 91kD-STAT3 in the NEs of UN rats (Fig. 2C, lane 3) was significantly potentiated after turpentine treatment (Fig. 2C, lane 4). This suggests that malnutrition is able to activate and acumulate APRF required for induction of Hp gene transcription as well as some other APPs (GRIGOROV *et al.*, 2000; RIPPERGER *et al.*, 1995).

The NF-kB transcription factors consist of dimeric proteins of the Rel homology family p50 and p65 known to mediate directly or indirectly the transcriptional activation of a variety of cytokine-induced genes involved in inflammatory responses (MCKAY and CIDLOWSKY, 1999). Since inducible members of C/EBPs and STATs have been shown to interact preferably with NF-kB p65 it is likely that persistent Hp expression during malnutrition reflects changes in basal expression of this protein as well. Western immunoblot analysis by using anti-p65 NF-kB antibody showed different amounts of p65 NF-kB in the liver NEs of UN rats as well as in turpentine treated WN and UN rats. High expression of this protein in UN rat livers (Fig. 2D, lane 3) was significantly potentiated by turpentine treatment (Fig.2D, lane 4). This increase was more pronaunced than after same treatment of WN controls (Fig. 2D, lane 2).

The present study provide evidence that malnutrition is a chronic inflammatory condition that leads to activation of some aspects of APPR by influencing liver metabolism and transcription rates of proinflammatory genes. Persistent high level of serum Hp during malnitrution probably results from constitutive expression of the inflammatory transcription factors C/EBPα, β and STAT3, as well as p65 NF-kB which assembly is required from transient acute and therefore chronic inflammatory conditions. In conclusion, together with the reports of other authors, our results support the involvement of the nutritional status in the host ability to respond to and recover from various stress stimuli.

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REFERENCES

AN, M. R., HSIEH, C. C., REISNER, P. D., RABEK, J. P., SCOTT, S. G., KUNINGER, D. T. and PAPACONSTANTINOU, J. (1996): Evidence for posttranscriptional regulation of C/EBPα and C/EBPβ isoform expression during the lipopolysaccharide-mediated acute-phase response. -Mol. Cell. Biol. 16: 2295-2306.

BAUMANN, H., (1988): Electrophoretic analysis of acute-phase proteins.-Methods Enzymol. 163: 566-594.

BAUMANN, H. J. and GAULDIE, J. (1994): The acute phase response.- Immunol. Today 15: 74-88.

BAUMANN, H., WANG, Y., RICHARDS, C. D., JONES, C. A., BLACK, T. A. and GROSS, K. W. (2000): Endotoxin-induced renal inflammatory response.- J. Biol. Chem. 275: 22014-22019.

FERRINI, J. B., RODRIGUES, E., DULIC, V., PICHARD-GARCIA, L, FABE, J. M., BLANC, P. and MAUREL, P. (2001): Expression and DNA binding activity of C/EBPα and C/EBPβ in human liver and differentiates primary hepatocytes.- J. Hepatology 3: 170-177.

FRANK, D. A. (1999): STAT signaling in the pathogenesis and treatment of cancer.- Molecular Medicine 5: 432-456.

GIOVAMBATISTA, A., CHISARI, A. N., CORRO, L., GAILLARD, R. C. and SPINEDI, E. (2000): Metabolic, neuroendocrine and immune functions in basal conditions and during the acute phase response to endotoxic shock in undernourished rats. -Neuroimmunomodulation 7: 92-98.

GORSKI, K., CARNEIRO, M. and SCHIBLER U. (1986): Tissue specific in vitro transcription from the mouse albumin promoter. - Cell 47: 767-776.

GRIGOROV, I., MILOSAVLJEVIĆ, T., CVETKOVIĆ, I. and PETROVIĆ, M. (1998): Participation of two isoforms of C/EBP transcription factor in the acute-phase regulation of the rat haptoglobin gene.- Cell Biol. Int. 22: 685-693.

GRIGOROV, I., LAZIĆ, T., CVETKOVIĆ, I., MILOSAVLJEVIĆ, T. and PETROVIĆ, M. (2000): STAT3 involvement in the acute phase-related expression of the rat haptoglobin gene. - Mol. Biol. Rep. 27: 81-86.

HSIEH, C. C., XIONG, W., XIE, Q., RABEK, J. P., SCOTT, S.G., AN, M. R., REISNER, P. D., KUNINGER, D. T. and PAPACONSTANTINOU, J. (1998): Effects of age on the posttranscriptional regulation of CCAAT/enhancer binding protein β and CCAAT/enhancer binding protein isoform synthesis in control and LPS-treated livers.- Mol. Cell. Biol. 9: 1479-1494.

LOWRY, S. F. (1992): Modulating the metabolic response to injury and infection. - Proc. Nut. Soc. 51: 267-277.

MACKIEWICZ, A. (1997): Acute phase proteins and transformed cells. A Review.- Int. Rev. Cytol. 170: 225-300.

MARINKOVIĆ, S. and BAUMANN, H. (1990): Structure, hormonal regulation, identification of the interleukin-6 and dexamethasone-responsive element of the rat haptoglobin gene.- Mol. Cell Biol. 10: 1573-1583.

MCKAY, L. I. and CIDLOWSKI, J. A. (1999): Molecular control of immune/inflammatory responses:Interactions between nuclear factor-kB and steroid receptor-signaling pathways.- Endocrine Reviews 20: 435-459.

MILOSAVLJEVIĆ, T., LAZIĆ, T., USKOKOVIĆ, A., PETROVIĆ, M. and GRIGOROV, I. (2003): Expression of the rat haptoglobin gene is mediated by isoforms of C/EBPα, -β and -δ proteins.- Gen. Physiol. Biophys. 22: 181-190.

OSSIPOW, V. P., DESCOMBES, P. and SCHIBLER, U. (1993): CCAAT/enhancer binding protein mRNA is translated into multiple proteins with different transcription activation potentials.- Proc. Natl. Acad. Sci. USA 90: 8219-8223.

PERALTA, S., CARRASCOSA, J. M., GALLARDO, N., ROS, M. and ARRIBAS, C. (2002): Ageing increases SOCS-3 expression in rat hypothalamus: effects of food restriction. -Biochem. Biophys. Res. Commun. 296: 425-428.

POLI, V. (1998) The role of C/EBP isoforms in the control of inflammatory and native immunity functions.- J. Biol. Chem. 273:, 29279-29282.

RIPPERGER, J. A., FRITZ, S., RICHTER, K., HOCKE, G. M., LOTTSPEICH, F. and FEY, G. H. (1995): Transcription factors STAT3 and STAT5B are present in rat liver late inan acute phase response and bind interleukin-6 response elements. - J. Biol. Chem. 270: 29998-30006.

ROESLER, W. J. (2001): The role of C/EBP in nutrient and hormonal regulation of gene expression. - Annu. Rev. Nutr. 21: 141-165.

SMITH, W. B., GAMBLE, J. R. and VADAS, M. A. (1992): Cytokines in the inflammatory response. - Interferons Cytokines 21: 26-30.

ŠEVALJEVIĆ, LJ., GLIBETIĆ, M., POZNANOVIĆ, G., PETROVIĆ, M., MATIĆ, S. and PANTELIĆ, D. (1988): Thermal-injury induced expression of acute phase proteins in rat liver-Burns 14: 280-286.

ŠEVALJEVIĆ, LJ., PETROVIĆ, M., BOGOJEVIĆ, D., SAVIĆ, J. and PANTELIĆ, D. (1989): Acute phase response to scalding: changes in serum properties and acute phase proteinconcentrations.- Cyrc. Shock 28: 293-307.

TSCHOP, M. and HEIMAN, M. L. (2001): Rodent obesity models: An overview.- Exp. Clin. Endocrinol. Diabetes 109: 307-319.

TAPPY, L., BERGER, M. M. and CHIOLERO, R. L. (2000): Nutrition and stress.- Ann. Med. Interne (Paris). 151: 584-593.

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EFEKAT NUTRITIVNOG STATUSA NA NIVO AKUTNOG FAZNOG ODGOVORA PROTEINA KOD ŽENKI PACOVA

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Izvod

U odgovoru na inflamaciju, infekciju i povredu tkiva, kao sastavni deo odbrambenih mehanizama u organizmu se pokreće sled događaja poznat kao akutno-fazni odgovor (AFO) u okviru koga se u jetri sintetiše grupa proteina plazme označena kao akutno fazni proteini (AFP). Svako stanje koje narušava akutno fazni proteinski odgovor organizma (AFPO), a time i njegovu sposobnost da ponovo uspostavi homeostazu može da deluje ograničavajuće na njegovo preživljavanje u uslovima različitih vrsta stresa. Izgladnjivanje je dobro poznat uzrok nekoliko metaboličkih, imunih i neuroendokrinih poremećaja te je ovaj rad imao za cilj da ispita uticaj neuhranjenosti na nivo normalnog AFPO-a i onog izazvanog akutnom povredom. Ženke pacova soja Wistar su izgladnjivane tokom perioda od šest nedelja sve do dostizanja telesne mase koja predstavlja 50% telesne mase ženki pacova koje su se u istom periodu hranile normalno. Rezultati "roket" imunoelektroforeze su pokazali da je izgladnjivanje, samo ili u sprezi sa terpentinskom povredom dovelo do uvećanja ekspresije jednog od AFP-a, haptoglobina (Hp). Izučavanjem za jetru specifičnih regulatornih proteina koji se vezuju za hormon regulatorne elemente gena Hp i time određuju nivo njegove ekspresije, zaključili smo da izgladnjivanje najverovatnije moduliše odgovor Hp-a tako što menja ekspresiju aktivnih clanova C/EBP, STAT and NF-kB familija transkripcionih faktora.

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