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Original Scientific Paper

Novel 57 kDa Glycoprotein in the Sera of Humans under Stress[#]

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Serum glycoproteins from individuals under chronic stress (prisoners of war from detention camps, n = 30) were analyzed by the Western-blot method applying a set of five different lectins (DSA, GNA, MAA, PNA and SNA). Glycoprotein patterns of samples from the stressed group revealed significant changes if compared to the control sera (from apparently non-stressed volunteers): both galactose-specific DSA lectin and sialic acid-specific SNA lectin recognized a novel nonconsitutional glycoprotein with an apparent size of 57 kDa in the stress group, which was completely absent in the control group. In addition, concentration of a 45 kDa SNA reactive glycoprotein increased more than 500 fold. The high incidence of disturbed patterns of glycoproteins in the sera of stressed subjects (95%) points to their important role in stress response and qualifies them for widespread testing as potential diagnostic markers of the stress syndrome.

INTRODUCTION

A fundamental function of all living organisms is the maintenance of homeostasis in the rapidly changing conditions and different levels of environmental stress. In single-cell organisms, a major manifestation of this function is the heat shock response.[1] Heat shock proteins help restore homeostasis at the intracellular level following injury, such as that caused

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by transient periods of hyperthermia, or by intoxication with heavy metals, ethanol, sulfhydryl reagents, amino acid analogues and several other metabolic poisons.[2,3] Heat shock response is universal, and has been observed in every organism in which it has been sought, from bacteria to higher plants and vertebrates.[4] It seems to be essential for survival of all organisms, and its conservation during the course of evolution is striking. The sequence homology between human and bacterial heat shock proteins is as high as 50%.[4] However, their main role seems to be in the intracellular transport, refolding, and elimination of damaged proteins, and their involvement in a more complex defense reaction to stress, specific to higher organisms, is questionable.[3,5]

According to Seyle,[6] after exposure to stress, an animal passes through the following three phases: (i) alarm when the organism is not capable of resisting the stress; (ii) resistance when the body returns to normal functioning; and (iii) exhaustion when the resistance is lost. The first phases is regulated on a hormonal level, while the second and the third phase represent biochemical and physiological changes. A variety of different biotic and physical factors result in the same alterations (7).

Stress and depressive symptoms have been associated with the development and course of many human diseases, from simple virus infections to cancer.[8–10] Many of these diseases can be associated with the stress induced decrease in immune response, and a notable number of studies have addressed this problem.[11,12] Significant effects have been observed in stress-induced suppression of natural killer cytotoxicity,[13] and production of specific antibodies.[14] One study even showed a very clear correlation between stress and development of the common cold in humans upon artificial infection with virus.[15] However, all these studies reported only about observable phenomena and, in most cases, the authors did not even try to investigate these effects on a biochemical level.

Glycoproteins play an important role in many vital processes, including blastocyst implantation, cell-cell, and cell-matrix interactions in prenatal development, and immune response.[16] As mentioned above, stress-induced adverse effects have been detected in all these processes. However, only a few studies have been undertaken to evaluate the changes of specific glycoproteins as a consequence of stress exposure.[17–22]

In this study, we have analyzed the effects of imprisonment on serum glycoproteins using a set of five specific lectins (listed in Materials and Methods).

MATERIALS AND METHODS

Materials

5-Bromo-4-chloro-3-indolylphosphate (BCIP), *p*-nitro blue tetrazolium (NBT), bovine serum albumin, anti-HSP70 monoclonal antibody, and alkaline phosphatase conjugated anti-mouse IgG were purchased from Sigma

(St. Louis, MO), Immobilon PVDF membrane from Millipore (Bedford, MA), digoxigenin-labeled GNA, PNA, SNA, MAA and DSA lectins, and alkaline phosphatase-conjugated anti-digoxigenin $F(ab)_2$ fragments from Boehringer Mannheim (Mannheim).

Samples

Individuals (n = 30) that have been imprisoned for at least 90 days in Serbian concentration camps were used as an example of high intensity chronic stress. Life conditions in Serbian concentration camps are reported to have been harsh[23,24] and a number of medical,[25] psychological[26] and immunological[27] problems have been reported among detainees. For this study, 30 detainees (age 19–45) were selected randomly from the group of 309 volunteers with no apparent infectious or other disease detected by medical examination. Serums were donated willingly by the detainees within three days after their release from camps and stored at –20 °C until analyzed. The control group (n = 9) of sera was taken from apparently healthy volunteers.

Electrophoresis and blotting

Serum proteins were separated electrophoretically in 12% SDS-polyacrylamide slab gels as described by Laemmli.[28] After electrophoresis, proteins were transferred onto Immobilon PVDF membrane in a semi-dry apparatus (Pharmacia, Sweden) according to Towbin.[29] After blotting membranes were blocked overnight with 3% BSA, and developed with digoxigenin labeled lectins (GNA lectin from *Galantus nivalis*, SNA lectin from *Sambucus nigra*, MAA lectin from *Maackia amurensis*, DSA lectin from *Datura stramonium* and PNA lectin from peanut) in the following dilutions: 1:1000 for SNA, GNA and DSA lectins, 1:200 for MAA lectin and 1:100 for PNA lectin. The formed glycoprotein-lectin complexes were detected with anti-digoxigenin $F(ab)_2$ fragments conjugated to alkaline phosphatase and visualized with 0.02 mg/ml BCIP and 0.04 mg/ml NBT in 50 mM Tris/HCl, pH = 9.5, 100 mM NaCl, 5 mM $MgCl_2$.

Amounts of individual glycoproteins were calculated by scanning and integrating spots on the membrane. Integration was performed in the GelScan XL software (Pharmacia) by fitting Gaussian curves into scanning results on the basis of horizontal background. Intensities of individual glycoprotein bands were expressed in relative units as defined in the GelScan software (area units).

Protein concentrations were determined using the biuret method.[30]

RESULTS

Lectin-binding (glycoprotein) patterns were analyzed in sera from individuals under high-intensity chronic stress and compared to those of sera originating from apparently non-stressed volunteers. A set of five lectins with different specificities (Table I) was used.

TABLE I

Specificity of lectins

Lectin	Source	Specificity
GNA	*Galanthus nivalis*	Man-α(1,3), Man-α(1,6), or Man-α(1,3)-Man
SNA	*Sambucus nigra*	Sia-α(2,6)-Gal
MAA	*Maackia amurensis*	Sia-α(2,3)-Gal
PNA	Peanut	Gal-β(1,3)-GalNAc
DSA	*Datura stramonium*	Gal-β(1,4)-GlcNAc

Galactose-specific PNA, mannose-specific GNA, and sialic acid-specific MAA lectins did not show any significant differences in lectin binding patterns between the sera from stressed and control individuals. On the contrary, sera from stressed individuals revealed that glycoproteins reactive with SNA and DSA lectins underwent significant alterations in comparison to control sera. Normal lectin-binding patterns for Gal-β(1,4)-GlcNAc specific DSA lectin are shown in Figure 1a,b. Only one major glycoprotein band, of an apparent mass of 70 kDa, is visible. DSA lectin-binding patterns of sera from individuals belonging to the stress group are shown in Figure 1c–g. Besides the 70 kDa band, there is another clearly visible glycoprotein with an apparent mass of 57 kDa. GP57 was not found in only one out of 30 stressed individuals analyzed (Figure 1d), but it was completely absent from all the control sera examined.

Analysis of serum glycoproteins using Sia-α(2,6)-Gal specific SNA lectin also revealed significant differences between stressed (a–b) and non-stressed (c–g) individuals (Figure 2). Though SNA lectin recognizes more glycoproteins than DSA lectin, two distinct changes are clearly visible. The most impressive one is the appearance of an additional 57 kDa glycoprotein, which probably corresponds to the above mentioned DSA-reactive stress induced glycoprotein of the same size. Comparison to the normal lectin-binding pattern also showed a significant increase in the amount of glycoprotein of an approximate mass of 45 kDa in stressed samples.

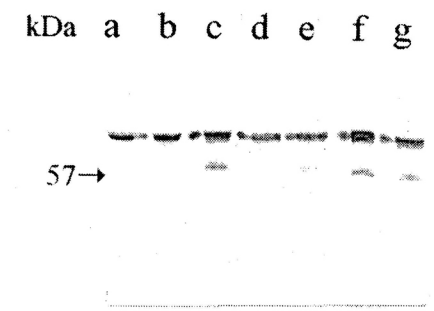

Figure 1. Stress induced changes of lectin binding patterns in sera from control individuals (a,b) and individuals under stress (c–g). Serum proteins (50 μg of total protein) were separated on 12% SDS PAGE and transferred to Immobilon PVDF membrane. Glycoproteins were detected using DSA lectin, as described in *Materials and Methods*. The major alteration was the appearance of a novel 57 kDa glycoprotein band. Serum analyzed in lane *d* is from the only stressed individual that had no GP57.

Hypothetical activation of the heat-shock response, caused by intensive stress or physical injuries, was tested using the anti-HSP70 monoclonal antibodies. Serum proteins were separated on SDS PAGE and analyzed following an analogous procedure to that with lectins, but applying anti-HSP70 monoclonal antibody, and anti-mouse IgG conjugated to alkaline phosphatase. Although some sera revealed the presence of the heat-shock protein 70, there was no correlation between the appearance of HSP70 in serum and exposure to stress (data not shown).

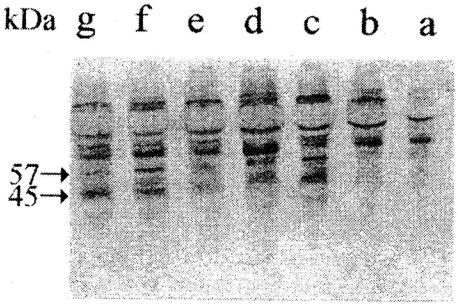

Figure 2. Stress induced changes of lectin binding patterns in sera from control individuals (a,b) and individuals under stress (c–g). Serum proteins (50 μg of total protein) were separated on 12% SDS PAGE and transferred to Immobilon PVDF membrane. Glycoproteins were detected using SNA lectin, as described in *Materials and Methods*. Two major alterations were detected: (i) appearance of a novel 57 kDa band, which most probably corresponds to 57 kDa glycoprotein reactive with DSA lectin; and (ii) a 500-fold increase in 45 kDa glycoprotein concentration.

DISCUSSION

In the presented study, we have analyzed glycoprotein patterns in sera from normal individuals and from individuals exposed to chronic stress of high intensity. Individuals who were considered to be under stress have spent at least 90 days in concentration camps, being exposed to complex inescapable stress including food deprivation, intensive fear, and possibly maltreatment. In a recently reported study on a similar population,[31] significant effects of stress were found on a number of immunological and endocrinological parameters. To investigate and describe the biochemical response of human organism to this kind of stress, we have undertaken extensive analysis of a large number of parameters. Here, we have reported changes in serum glycoproteins which seem to be a manifestation of a stress response activation.

Two major changes were found to be present in sera after exposure to stress: (i) appearance of a novel glycoprotein with a mass of 57 kDa; and (ii) increase in the amount of one glycoprotein with a mass of 45 kDa (Figures 1,2). The 57 kDa stress-induced glycoprotein was recognized by DSA lectin (specific to galactose) and by SNA lectin (specific to sialic acid) while it was not recognized by GNA, PNA and MAA lectins. Our unpublished results (data not shown) indicate that GP57 can also be found in sera of individuals who were exposed to war-related activities but spared malnutrition, physical injuries *etc.*, confirming its association with stress, and not with other consequences of detention in concentration camps.

On the basis of these results, it is possible to identify some segments of the carbohydrate part of the glycoprotein. It seems that the stress induced 57 kDa glycoprotein contains branched oligosaccharides with at least two different termini. One terminus is characterized by galactose, possibly bound by $\beta(1,4)$ glycosidic link to *N*-acetyl-glucosamine, but certainly not to N-acetyl-galactosamine. Another terminus possesses sialic acid, probably bound to galactose through $\alpha(2,6)$ glycosidic link. Lack of recognition of the glycoprotein by mannose specific GNA implies that it does not contain terminal mannose residues and, consequently, does not belong to the group of high-mannose glycoproteins.

Another alteration that was found to be present in sera after stress experience is the noticeable increase of SNA reactive 45 kDa glycoprotein. Though it was also present in sera of apparently non-stressed individuals, the amount of this glycoprotein in sera of stressed individuals is substantially increased (Figure 2).

There is no doubt that the glycoprotein pattern of stressed sera is highly disturbed in comparison to the control samples. However the attributed molecular masses of the relevant glycoproteins should be considered only within the context of the experimental procedures applied, since glycopro-

tein shows a rather unpredictable, though molecule specific, electrophoretic behaviour due to the sugar moiety. The question to be answered by further experiments is whether GP57 corresponds to a completely unknown glycoprotein, or its novelty originates from a change in the carbohydrate part of the molecule, as already reported for several glycoproteins.[32]

The exact role of the 45 kDa and 57 kDa stress-glycoproteins is not known. However, they probably do not belong to the family of heat-shock proteins. The main manifestations of the heat shock response is the appearance of HSP70. Since we were unable to demonstrate its presence, it is highly improbable that there was an activation of the heat shock response. Even if the 57 kDa stress induced glycoprotein belongs to heat-shock proteins, it is activated independently in this model of stress. It is more probable that 57 kDa stress glycoproteins represents a part of the acute phase response. The acute phase response is generally activated by a lower level stimuli than the heat-shock,[33] and indeed we have found increased concentrations of several acute-phase proteins in stressed sera. Whatever the complete function of these glycoproteins will prove to be, they seem to be the first reliable biochemical marker of the stress syndrome.

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SAŽETAK

Glikoprotein mase 57 kDa u serumu ljudi izloženih stresu

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Metodom Western-blot, primijenivši pet različitih lektina (DSA, GNA, MAA, PNA i SNA), analizirani su serumski proteini u uzorcima ispitanika s kroničnim stresom (zatvorenici ratnih logora, $n = 30$) i uspoređeni s glikoproteinskim sastavom kontrolnih seruma zdravih dobrovoljaca odgovarajuće dobi i spola ($n = 9$). DSA i SNA lektin upozorili su na pojavu novog, nekonstitutivnog glikoproteina, prividne mase 57 kDa, koji se pojavljuje samo u uzorcima s kroničnim stresom. U istim uzorcima koncentracija glikoproteina mase 45 kDa povećana je 500 puta u odnosu na kontrolne uzorke. Pojava značajnih i specifičnih promjena u sastavu serumskih glikoproteina u stresu upućuje na njihovu važnu ulogu u odgovoru organizma na stres, a njihovo dijagnostičko značenje vrijedno je testirati na znatno većem broju uzoraka radi primjene u laboratorijskoj dijagnostici.