Stress Causes Tissue-Specific Changes in the Sialyltransferase Activity

Sanja Dabelić, Mirna Flögel, Gordana Maravić, and Gordan Lauc*

University of Zagreb, Faculty of Pharmacy and Biochemistry, Department of Biochemistry and Molecular Biology, A. Kovačića 1, 10000 Zagreb, Croatia. Fax: +385 14 85 62 01. E-mail: glauc@pharma.hr

* Author for correspondence and reprint requests

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Numerous pathological conditions are associated with specific changes in glycosylation. Recent studies clearly demonstrated a link between stress and the development and course of many diseases. Biochemical mechanisms that link stress and diseases are still not fully understood, but there are some indications that changes in glycosylation are involved in this process.

Influence of acute and chronic psychological stress on protein sialylation as well as the activity of sialyltransferases, enzymes that synthesize sialoglycoproteins, has been studied on Fischer rats. Liver, spleen, kidney, skeletal muscle, heart, adrenal gland, serum, cerebellum, hippocampus, medulla oblongata and cortex have been analyzed. Statistically significant tissue- and type of stress-specific changes in total sialyltransferase (ST) activity were observed. Acute stress resulted in 39% increase of ST activity in liver and spleen, while at the same time there was 43% decrease in ST activity in cerebellum. In chronic stress, ST activity increased in spleen (93%) and decreased in liver (17%), cerebellum (38%) and hippocampus (64%). Western-blot analysis using *Maackia amurensis* and *Sambucus nigra* lectins did not reveal any difference in protein sialylation. The results of serum corticosterone analysis indicate that showed increase in acute stress and decrease in chronic stress are in good accordance with the hypothesis that corticosterone has a role in the regulation of liver ST activity.

Key words: Psychological Stress, Sialyltransferases, Sialoglycoproteins

Introduction

Glycosylation is a very important and abundant posttranslational modification of many biologically relevant molecules. Carbohydrates, when bound to proteins and lipids, serve as sophisticated information-carriers with distinct and subtle biological specificities mediating vital tasks. They steer many pathological phenomena, such as storage diseases, inflammation, cancer progression, or autoimmune diseases; their antigenicity is responsible for rejection of xenotransplantants (Crocker, 2002; Drickamer and Taylor, 1998; Hernandez and Baum, 2002).

A number of epidemiological and experimental studies conducted during the past years clearly demonstrated a link between stress and the development and course of many diseases, some of which also showed significant changes in glycosylation patterns (Black and Garbutt, 2002; Marsland *et al.*, 2002; Rabin, 2002). Our previous results (Barišić *et al.*, 1996; Dumić *et al.*, 2000b; Lauc and Flögel, 2000), as well as some studies from other groups (Henle *et al.*, 1998), support the hypothesis that glycosylation plays an important role in the stress response, but still molecular mechanisms of these processes are not known.

Negative charge of sialic acids, their bulky hydrophilic appearance and the capability to either mask underlying biological recognition sites or to form novel recognition sites, enable them to perform numerous important biological functions. Sialic acids influence ion transport, the proper arrangement and function of membrane receptors and the viscosity of mucins, protect proteins and glycan chains from enzymatic degradation and shield antigenic sites (Schauer, 2000). The latter effect may be of benefit for sialylated microorganisms or tumor cells, which are often over-sialylated (Georgopoulou and Breen, 1999). On the other hand, loss of sialic acid on normal cells, for example by microbial sialidases, may lead to premature cell death or to autoimmune diseases (Traving and Schauer, 1998).

The biosynthesis of sialylated oligosaccharide sequences is catalysed by a family of enzymes named sialyltransferase. The sialyltransferase family (EC 2.4.99.1) consists of more than 20 sialyltransferases. In general, they can be classified

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into three categories, α2,3 (ST3), α2,6 (ST6) and α2,8 (ST8), according to the regiochemistry of the resulting sialylated reaction products. Sialyltransferases are widely distributed among some microorganisms and higher animals and are localized in the Golgi apparatus of animal cells as type II transmembrane glycoproteins, but they can also be found in a soluble form as in the colostrums of goat, cow and man. The relative sialyltransferase activities and their intracellular localization influence the final pattern of sialylated compound expression as well as the glycosylation pattern of the whole cell (Traving and Schauer, 1998).

Materials and Methods

Materials

All chemicals used were of analytical grade. Protease inhibitors, Bicinchoninic Acid Protein Assay Kit, asialofetuin and trichloroacetic acid (TCA) were obtained from Sigma (St. Louis, MO, USA). Digoxigenin-labeled *Sambucus nigra* agglutinin (SNA), *Maackia amurensis* agglutinin (MAA) and anti-digoxigenin antibodies labeled with alkaline phosphatase were purchased from Boehringer Mannheim (now Roche Diagnostic). Immobilon poly-vinylidene-difluoride-membrane was from Millipore (Bedford, MA, USA), and bovine serum albumin from Roth (Karlsruhe, Germany). Cytidine-5-monophosphate-4,5,6,7,8,9-^{14}C-N-acetylneuraminic acid (CMP-^{14}C-NeuNAc) and tissue solubilizator NCS were obtained from Radiochemical Centre Amersham (Uppsala, Sweden) and ImmunoChem Double Antibody ^{125}I corticosterone RIA Kit from ICN Biomedicals (Costa Mesa, CA, USA).

Animals

Three groups of male Fischer rats, aged 6 months, altogether 40 animals, were used for this study. Five rats of the same experimental group were housed per cage. Two animal rooms were maintained at a constant temperature of 22 °C and a 12-h light/12-h dark schedule (time of illumination: 7 a.m. to 7 p.m.). Except during tests, food and water were freely available to the animals. The institutional ethical committee approved all animal handling and treatment protocols.

The control group (n = 15) was left undisturbed during the whole experiment. These animals were placed in an animal room separated from the other two experimental groups and didn't have any kind of contact with the other animals after the beginning of the experiment.

The acutely stressed group of animals (n = 10) was exposed to twenty 10-min electro-shocks (DC, 2.5 V/cm) during 10 min.

The chronically stressed group of animals (n = 15) was exposed alternately to immobilization, electro-shock or cold-water-swimming every day during 8 d. Immobilization was performed by placing animals in closed 750-cm^3 cylindrical Plexiglass tubes during 2 h. Electro-shocks were performed in the same way as for the acutely stressed group of animals. Cold-water swimming stress was performed by placing the animals in an open cylindrical Plexiglass tube (20 cm in diameter) filled with 1500 cm^3 of cold water (17 °C) during 10 min.

At the end of the stressing procedure, the animals were placed back in their cages, where they remained for the next 2 h, subsequent to sacrificing.

Tissue preparation

Just before sacrificing, the animals were ether-anesthetized, blood was taken from the arteria carotis communis and serum was separated by centrifugation. Following sacrifice, liver, spleen, adrenal glands, heart, kidneys, part of the skeletal muscle, medulla oblongata and brain were removed from the animals. The brains were then further dissected into three regions (hippocampus, cortex, and cerebellum), snap-frozen and maintained at – 70 °C until use. The tissues were homogenized in 10 volumes of ice-cold homogenization buffer [50 mM tris(hydroxymethyl)aminomethane buffer, pH 6.5, containing 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1% Triton X-100 and protease inhibitors], using a Teflon homogenizer, and the homogenized samples were stored in aliquots at – 70 °C prior to analysis. The protein concentration of the samples was determined by the method of bicinchoninic acid (Smith *et al.*, 1985).

Total sialyltransferase assay

Total tissue sialyltransferase activity was assayed using cytidine-5-monophosphate-4,5,6,7,8,9-^{14}C-N-acetylneuraminic acid (CMP-^{14}C-NeuNAc, specific activity 1×10^{13} Bq/mol) as the sugar donor and asialofetuin as the exogenous acceptor as described previously (Breen and Regan, 1986). All samples were analyzed in duplicate and are shown as average values.

Homogenized samples containing 1 mg of proteins were mixed with 1 mg of asialofetuin and 925 Bq CMP-^{14}C-NeuNAc in 0.1 M Tris/HCl, pH 6.8. Samples were incubated at 37 °C for 45 min. Reaction was terminated by the addition of 12% ice-cold trichloroacetic acid (TCA). After 30 min of incubation on ice, proteins were pelleted by centrifugation at 14,000 × *g* for 3 min, washed 3 times with 12% ice-cold TCA to remove radioactivity that was non-specifically bound and spun again at 14,000 × *g* for 3 min. The precipitate was solubilized during the night in tissue solubilizator NCS at 37 °C. Following the addition of Ecolite scintillation fluid, the radioactivity of each sample was determined by liquid scintillation counting for 2 min and the sialyltransferase enzyme activity was expressed as number of counts in min per mg of protein (dpm/mg protein). Boiled samples served as blanks. Differences were considered to be significant when p values calculated by t-test were less than 0.01.

SDS polyacrylamide gel electrophoresis

Denaturing SDS polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). The homogenized samples were mixed with the SDS-gel loading buffer (2:1), containing 50 mM Tris/HCl (pH 6.8), 0.3 M 2-mercaptoethanol, 2% SDS, 0.1% bromphenol blue and 10% glycerol, and heated at 95 °C for 5 min. Denaturated proteins (5 μg per lane) were loaded on the gel and separated using 8 V/cm for stacking (5% polyacrylamide) and 12 V/cm for the resolving (12% polyacrylamide) gel. Gels were run until the dye front reached the bottom of the resolving gel.

Sialylation analysis

Electroforetically separated proteins were semidry blotted onto Immobilon-P (PVDF) membranes according to Towbin *et al.* (1979). Current of 0.8 mA/cm^2 of gel area was applied and the blotting was stopped after 90 min. Blots were blocked overnight in TBS containing 3% bovine serum albumin.

α(2,3) and α(2,6) sialylation of glycoproteins was analyzed using digoxigenin-labeled MAA and SNA lectins, respectively. Blocked PVDF membranes (blots) were washed 3 × 10 min in TBS containing 0.1% Tween 20 (TBST), then blots were incubated with lectins in TBS buffer containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$, in dilutions 1:200 for MAA and 1:1000 for SNA. After 2 h incubation with lectins, blots were washed 3 × 10 min in TBST and incubated with anti-digoxigenin antibodies labeled with alkaline phosphatase (diluted 1:3000 in TBS) for 60 min at room temperature. Blots were washed 3 × 10 min in TBST and developed with 0.02 mg/ml 5-bromo-4-chloro-3-indolyl phosphate and 0.04 mg/ml nitroblue tetrazolium salt in 0.1 M Tris/HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5.

Corticosterone concentration in sera

Corticosterone concentrations in sera were determined by RIA method, using ImmunoChem Double Antibody ^{125}I corticosterone RIA Kit.

Results

We have used a non-primate animal model of acute and chronic stress to study effects of stress on the activity of sialyltransferases and the sialylation of proteins. Experiments were performed on male Fischer rats that were acutely stressed using electro-shocks. To avoid habituation, chronic stress was induced using a combination of immobilization, electro-shocks and cold-water-swimming stress. Plasma corticosterone levels were measured and found to obey the well-documented pattern of increase in acute stress, followed by a decline in chronically stressed animals (data not shown).

The level of basal sialyltransferase activity was measured in liver, kidney, spleen, skeletal muscle, heart muscle, adrenal gland, cerebellum, hippocampus, medulla oblongata and cortex (Fig. 1, upper panel). The highest sialyltransferase activity was found in liver, kidneys and spleen, while other tissues had less than 5% of the activity in the liver. In animals that were exposed to acute stress the sialyltransferase activity in both liver and spleen increased by 39% compared to control animals ($p < 0.01$). In the same time, the activity of sialyltransferases was a 43% decrease in cerebellum, while in kidneys, skeletal muscle, heart, adrenal glands, medulla oblongata and cortex were no significant changes (Fig. 1, lower panel).

Interestingly, prolonged stress had exactly the opposite effect on sialyltransferase activity in the liver than acute stress. While acute stress increased sialyltransferase activity in the liver, chronic stress decreased the activity of the same enzymes to 83%. The activity of sialyltransferase was also decreased in hippocampus (to 36% of control activity) and cerebellum (to 62% of control activity).

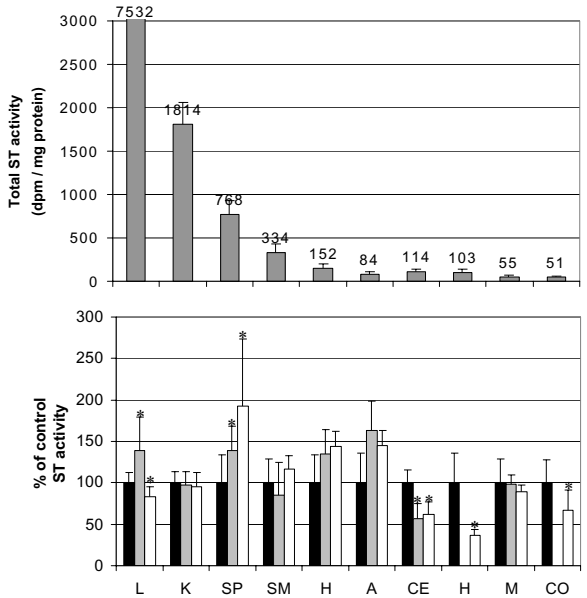

Fig 1. Sialyltransferase (ST) activity in different rat tissues. The level of sialyltransferase activity was measured in liver (L), kidney (K), spleen (SP), skeletal muscle (SM), heart muscle (H), adrenal gland (A), cerebellum (CE), hippocampus (H), medulla oblongata (M) and cortex (CO) as described in the Materials and Methods section. Average values (± standard deviation) for each tissue are shown. Total basal activity is shown on the upper panel. Effects of acute (gray bars) and chronic (white bars) stress are shown on the lower panel. Statistically significant differences ($p < 0.01$ compared to the controls) are marked with asterisk.

The only tissue where we found increased sialyltransferase activity after prolonged stress was spleen, where the activity nearly doubled (93% increase) (Fig. 1, lower panel).

Using SNA and MAA lectins we analyzed $\alpha(2,6)$ and $\alpha(2,3)$ sialylation of glycoproteins in liver, kidney, heart, skeletal muscle, adrenal gland, and medulla oblongata of the same animals. As expected, we did not observe any changes in sialylation in tissues where there were no changes in sialyltransferase activity, but we were also not able to identify any significant change in sialylation of liver and spleen glycoproteins despite the fact that significant changes of sialyltransferase activity were observed in these tissues (data not shown).

Discussion

Metabolic response to psychological stress is a very complex and demanding physiological process. Though it is highly important for survival in constantly changing environment, its excessive activation is associated with various detrimental effects (Marsland *et al.*, 2002; Rabin, 2002). Molecular mechanisms underlying the link between the response to stress and the development of disease appear to be exceedingly complex and are only partly understood. Though hormonal changes are key mediators of the physiological changes in stress, other factors appear to be decisive in the development of stress-associated disorders.

Stress alert is conveyed by hormonal signals throughout the body, yet a particular cell response to a hormonal signal is not determined by the signal itself, but by the molecular composition, energy content, and by the physiological role and current status of the target cell. Since the response to psychological stress is a function of a whole organism, we hypothesized that glycosylation is a suitable candidate for a role in this process. Our previous studies (Barišić *et al.*, 1996; Dumić *et al.*, 2000a, 2000c), as well as studies of others (Henle *et al.*, 1998), showed that stress affects glycosylation patterns of some glycoproteins and/or expression of their receptor-lectins. Due to the multiple crucial role of sialoglycoproteins in organisms, we have directed our investigation on potential changes in sialylation and on sialyltransferases, enzymes involved in that process. The aim of this study was to investigate whether and how stress influences the sialylation process in variety of tissues of different type and origin.

Sialyltransferase assay showed that the basal enzyme activity varied significantly between different tissues (Fig. 1, upper panel). The highest sialyltransferase activity was observed in liver, a metabolically very active organ, probably due to the production of many different glycoproteins. Sialyltransferase activity was 4-fold lower in kidney, about 10-fold lower in spleen and about 20-fold lower in other tissues, compared to the activity in liver.

The activity of sialyltransferases changed in a tissue-specific manner when rats were exposed to either acute or prolonged stress. For some time it is known that $\alpha(2,6)$ sialyltransferase is transcriptionally regulated by corticosteroids (Wang *et al.*, 1990), and tissue-specific effects of corticosteroid treatment have been reported (Coughlan *et al.*, 1996). The pattern of sialyltransferase activity observed in the liver followed the pattern of serum corticosterone, indicating that $\alpha(2,6)$ sialyltransferase is a dominant sialyltransferase in the liver

and it is under significant positive control of corticosteroids. However, sialyltransferases in other tissues did not follow this pattern indicating that other factors are involved in the control of their activity.

Despite the observed changes in total sialyltransferase activity, lectin-Western blot with MAA [which specifically recognizes α(2,3)-bound sialic acid and SNA [which specifically recognizes α(2,6)-bound sialic acid on glycoproteins] lectins did not reveal any changes in protein sialylation that can be associated with stress (data not shown). Although this is somewhat unexpected, similar effects have already been reported (Matsuura *et al.*, 1998; Recchi *et al.*, 1998) and are probably the consequence of multifactorial nature of the glycosylation process.

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