Glycation and inactivation of sorbitol dehydrogenase in normal and diabetic rats

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Sorbitol dehydrogenase (SDH) is involved in the polyol pathway, which plays an important role in the pathogenesis of diabetic complications. We have measured the tissue distributions of SDH mRNA, both the immunoreactive enzyme levels and the enzyme activity. SDH mRNA was especially abundant in liver, kidney and testis. Both the activity and enzyme content are high in liver and kidney but not in testis. The discrepancy between mRNA and immunoreactive enzyme levels and the activity of SDH observed in testis was also seen in livers of streptozotocin-

induced diabetic rats. SDH was found to exist in both glycosylated and non-glycosylated forms, with larger amounts of the glycosylated protein in the diabetic liver. Moreover, after incubation of purified enzyme with glucose or fructose, its activity was markedly decreased. These results indicate that glycation causes a decrease in SDH activity in liver under diabetic conditions. The same post-transcriptional event might occur to decrease the activity of SDH in testis in normal animals.

INTRODUCTION

Sorbitol dehydrogenase (SDH; NAD⁺ oxidoreductase, EC 1.1.1.14), is a member of the polyol pathway, which is important in the development of such diabetic complications as cataract, neuropathy, retinopathy and nephropathy [1]. As a member of the alcohol dehydrogenase superfamily [1] it has been studied mainly from the enzymological point of view. The characteristics of aldose reductase (AR; NADP⁺ 1-oxidoreductase, EC 1.1.1.21), the other enzyme in the polyol pathway, has been extensively studied but the role of SDH in diabetic conditions has been almost ignored. The tissue distribution of SDH mRNA in normal rats has been determined [2]. Comparisons of SDH and AR mRNAs and the activities of the corresponding enzymes in kidney, brain, testis and muscle of normal and diabetic rats have also been made [3]. For a more precise understanding of the polyol pathway in diabetes it is necessary to know the variations in the tissue distribution of SDH and its mRNA induced by this condition. In the present studies the tissue levels of the mRNA, the specific protein and enzymic activity have been determined in some tissues of diabetic and normal rats.

MATERIALS AND METHODS

Experimental animals

Diabetes mellitus was induced by injection of streptozotocin (60 mg/kg) in 0.1 M sodium citrate, pH 4.5, to 7-week-old male Wistar rats (SLC Japan) weighing 200 ± 20 g (mean ± S.E.M.). The duration of diabetes was 28–33 weeks. Rats were fed with MM-5 chow (Clea Japan) and given water *ad lib*. They were killed after an overnight fast, a group of five age-matched animals being used as controls. For determination of the normal tissue distribution of enzymes, 9–15-week-old male Wistar rats were used. Their body weights and blood glucose levels were 287 ± 9.8 g and 128 ± 1.9 mg per 100 ml (*n* = 7, means ± S.E.M.) respectively. In the experiments on diabetes, five rats were treated with streptozotocin and five age-matched animals were used as controls. Blood glucose levels and body weights of the diabetic

rats were 359 ± 15 mg per 100 ml and 188 ± 13 g, and for the controls 130 ± 8 mg per 100 ml and 579 ± 15 g respectively. Diabetic rats had only little visceral and subcutaneous fat tissue compared with normal rats. After dissection, tissues were immediately frozen in liquid nitrogen, and stored at –180 °C until used. Use of animals was authorized within the guidelines of the Declaration of Helsinki and the principles in the care and use of animals [3a].

Cloning of rat SDH cDNA

Two oligonucleotide primers, 5'-GATGAATTCTGCAAGATCGG-3' and 5'-TTGATCATCTCGGGGCCCAT-3', corresponding to rat SDH cDNA sequences [4], were used in a PCR with cDNAs obtained by reverse transcription of total rat liver RNA. The amplified cDNA fragment (535 bp) was used to screen a rat kidney cDNA library. The longest clone initiated at the *Eco*RI site located at approx. 330 bp downstream of the Met initiator and was subcloned into the Bluescript KS[–] vector. The approx. 970 bp *Eco*RI–*Xba*I DNA fragment was used as a probe.

Northern blotting analysis

Total RNA was extracted from various tissues by the acid/guanidinium thiocyanate/phenol/chloroform method of Chomczynski and Sacchi [5]. RNA samples were denatured in the presence of formaldehyde and formamide, separated by electrophoresis in a 1% (w/v) agarose/formaldehyde gel, and transferred to a Zeta-probe membrane (Bio-Rad) by capillary action. The filter was hybridized with ³²P-labelled rat SDH cDNA at 42 °C in hybridization buffer [6]. The filter was washed at 55 °C with 2 × SSC (where SSC is 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0) and 0.1% SDS for 30 min, then twice with 0.2 × SSC and 0.1% SDS for 30 min, and then exposed to X-ray film (Kodak) with an intensifying screen at –80 °C for 1–7 days.

Abbreviations used: AR, aldose reductase; SDH, sorbitol dehydrogenase.

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SDH activity assay

SDH activity was measured spectrophotometrically by a slight modification of the procedure of Maret and Auld [7]. Tissues were homogenized in five volumes of cold 20 mM sodium phosphate buffer, pH 6.5, containing 2 mM MgCl_2 , 5 mM benzamidine hydrochloride and 5 μM *p*-amidinophenylmethanesulphonyl fluoride hydrochloride. After centrifugation at 100 000 *g* for 60 min at 4 °C, the reduction of NAD was measured spectrophotometrically at 340 nm with D-sorbitol (Nacalai Tesque, Kyoto, Japan) as the substrate. The specific activity was expressed as units/min per mg of protein. One unit of activity was defined as the amount of enzyme needed to oxidize 1 μmol of substrate per min under initial velocity conditions. Protein was measured by the Bradford method [8] with BSA as the standard.

Purification of SDH from rat liver

SDH was purified by a modified method described previously for human liver [7]. Briefly, rat livers (200 g) were homogenized in 200 ml of 20 mM sodium phosphate, pH 6.5, containing 2 mM MgCl_2 . After ultracentrifugation at 100 000 *g* for 60 min at 4 °C, the supernatant was passed through a Green B-Sepharose CL-4B column. A pool of the active fraction was applied to a Green A-Sepharose CL-4B column and the enzymically active protein was eluted with a gradient of 0–0.5 M NaCl. After ammonium sulphate fractionation (45–70 % saturation), chromatography was performed on a CM-cellulose column. The active fractions were then fractionated on an FPLC column of Mono S (Pharmacia). Gel filtration on a TSK 3000W was performed to remove minor contaminants from the enzyme fraction.

Antibodies

A polyclonal antibody against SDH for immunoblot analysis was raised in rabbits by injecting 100 μg of rat liver SDH purified by the method of Maret and Auld [7]. The antiserum was obtained after the final booster injection. Anti-hexitol-lysine IgGs were used as previously described [9].

Immunoblotting analysis

For Western blot analysis, protein samples (40 μg) prepared by centrifugation of the tissue homogenates at 100 000 *g* for 60 min at 4 °C were subjected to SDS/PAGE [10 % (w/v) gel] by the method of Laemmli [10]. One of duplicate gels was stained with Coomassie Brilliant Blue and the other was transferred to a nitrocellulose membrane under semi-dry conditions with the use of a Semi-phor TE-70, a Semi-dry transfer unit (Hoefer). Non-specific proteins on the membrane were blocked by incubation with 5 % (w/v) skimmed milk for 12 h at 4 °C. After three 10 min washes, the membranes in Tris-buffered saline, pH 7.6, containing 2.5 % (w/v) BSA were reacted with a 1:500 dilution of the rabbit antiserum. After three 10 min washes to remove non-specifically bound proteins, the membranes were incubated for 2 h with peroxidase-conjugated goat anti-rabbit IgG (Organon Teknica, West Chester, NC, U.S.A.). The membranes were then washed again as above and reacted with 4-chloro-1-naphthol as substrate (Wako Pure Chemical Industries). Stained areas were quantified by densitometry and the protein level of each tissue was expressed as a proportion of the density of the stomach extract. Immunoblot detection of glycosylated proteins with a polyclonal hexitol-lysine antibody was performed by the method of Myint et al. [9]. Purified and of glycosylated SDH (1 μg) was subjected to SDS/PAGE (10 % gel) by the method of Laemmli [10]. After electrophoresis the gels were transferred to

poly(vinylidene difluoride) transfer membranes (Millipore, Bedford, MA, U.S.A.), and the transferred protein was reduced with 0.1 M sodium borohydride in 0.01 M NaOH [9].

Immunoprecipitation of SDH

SDH was immunoprecipitated from rat liver, kidney and testis homogenates that had been adjusted to a protein content of 8.1 mg/ml in 200 μl by incubation with 4 μl of anti-SDH IgG at 4 °C for 2 h. The samples were then incubated with 5 μl of Protein A–Trisacryl (50 % suspension) (Pierce) for 2 h at 4 °C. The suspensions were analysed by SDS/PAGE (10 % gel) [10]. The gels were then subjected to immunoblotting on poly(vinylidene difluoride) membranes.

Glycation of SDH *in vitro*

Purified SDH protein (80 μg) was dissolved in 400 μl of 50 mM sodium phosphate buffer, pH 7.4, and loaded on a Chelex-100 column (Bio-Rad) containing 150 mM NaCl and 0.025 % sodium azide in a 1.5 ml tube. The solutions were incubated with various concentrations of glucose or fructose at 37 °C for various periods. SDH activities were measured at the indicated times. The remainder of each sample was frozen, stored at –30 °C and used for immunoblotting.

Statistical analysis

Data were analysed by Student's *t*-test and results are expressed as means $\pm$ S.E.M.

RESULTS

SDH mRNA levels in normal rats

To evaluate the role of the polyol pathway we investigated the expression of SDH mRNAs in various tissues. Large amounts were found in testis, liver, and kidney but relatively low levels in stomach, brain, lung and spleen (Figure 1). In skeletal muscle the mRNA level was very low and below detectable levels.

SDH levels in normal rats

A rabbit antiserum was used to determine the contents of SDH in various tissues by immunoblot analysis. The antiserum bound

Figure 1 Northern blot analysis of SDH and AR in various tissues of normal rats

RNA (15 μg) prepared from various tissues was analysed. Hybridizations were performed with cDNAs encoding SDH.

Figure 2 Purity of rat SDH and specificity of the antiserum

Standards (lane 1), 40 μ g of protein from rat liver homogenate (lane 2) and 1 μ g of purified SDH (lane 3) were separated by SDS/PAGE. Proteins were stained with Coomassie Brilliant Blue (A) and analysed by immunoblotting (B).

Figure 3 Immunoblot analyses of SDH and enzymic activities in tissues of normal rats

Proteins (40 μ g) from various tissues were analyzed with antiserum against SDH. Sk., skeletal.

Table 1 Levels of SDH proteins and enzymic activities in normal rat tissues

Proteins (40 μ g) from various tissues were analysed by immunoblotting with antiserum against SDH as shown in Figure 3. Data from quantitative densitometry are expressed as means $\pm$ S.E.M. for seven experiments. SDH activities were also determined for the same samples.

Tissue	SDH activity (m-units/mg of protein)	Immunoreactive SDH contents, relative to stomach
Liver	13.9 $\pm$ 3.9	3.3 $\pm$ 0.4
Kidney	12.1 $\pm$ 1.4	2.1 $\pm$ 0.2
Testis	3.1 $\pm$ 0.6	0.8 $\pm$ 0.1
Skeletal muscle	0.6 $\pm$ 0.3	0.2 $\pm$ 0.1
Stomach	4.8 $\pm$ 1.5	1.0
Lung	5.0 $\pm$ 4.2	0.5 $\pm$ 0.2
Brain	3.5 $\pm$ 1.5	0.6 $\pm$ 0.1
Spleen	0.7 $\pm$ 0.2	0.6 $\pm$ 0.2

only to the 39 kDa band in rat liver homogenate and to purified SDH (Figure 2), indicating that the antiserum was specific for SDH. The immunoreactive 39 kDa protein was found in every rat tissue examined (Figure 3). We also determined the enzymic activity of SDH in the cytosolic fraction of normal rat tissues (Table 1). These activities were consistent with the levels of protein determined by the immunoblot analysis except for spleen, in which part of the SDH protein may exist in an inactive form.

Figure 4 Comparison of SDH mRNA and protein levels in liver, kidney and testis of control and diabetic rats

SDH mRNA (A) and protein (B) levels in control (N) and diabetic (D) rats were examined by Northern and Western blot analysis.

Table 2 Comparison of SDH protein levels and enzyme activities in liver, kidney and testis of control and diabetic rats

SDH protein and activities in diabetic and control rats were measured as described in Table 1. Data are means $\pm$ S.E.M. for five rats in normal and diabetic groups. Statistical significance: * $P < 0.001$ compared with normal rats.

	SDH activity (m-units/mg of protein)		Immunoreactive SDH contents (arbitrary units)	
	Normal	Diabetic	Normal	Diabetic
Liver	18.4 $\pm$ 4.5	7.2 $\pm$ 1.4*	4.7 $\pm$ 0.7	1.8 $\pm$ 0.2*
Kidney	6.4 $\pm$ 1.1	7.7 $\pm$ 3.6	2.4 $\pm$ 0.3	3.0 $\pm$ 0.5
Testis	6.6 $\pm$ 2.3	4.7 $\pm$ 1.3	0.6 $\pm$ 0.2	0.6 $\pm$ 0.2

Comparison of levels of SDH mRNA, protein and specific activities in tissues of diabetic and control rats

Although SDH mRNA levels were increased in the diabetic liver (Figure 4A), protein levels and activity of SDH were decreased (Figure 4B and Table 2). In liver, the SDH activity in the diabetic group was less than half of that in the control group (7.2 compared with 18.4 m-units/mg). In kidney and testis, SDH mRNA and protein levels were unchanged in the diabetic group (Figure 4A and Table 2).

Detection of glycosylated SDH *in vivo*

Soluble fractions from both control and diabetic rat livers were subjected to immunoprecipitation with SDH antibody followed by separation by SDS/PAGE. The blots were examined by reactivities with anti-hexitol-lysine IgG (Figure 5B). Although no significant differences were observed in the levels of precipitated SDH protein in the samples (Figure 5A), a glycosylated form of SDH was more prominent in diabetic liver than in the liver from controls (Figure 5B). A small amount of the glycosylated form of SDH was also observed in purified SDH samples (Figure 5B),

Figure 5 Identification of SDH (A) and glycated SDH (B) in liver from normal (N) and diabetic (D) rats

SDH was immunoprecipitated with anti-SDH IgG from homogenate of 0.2 g of rat liver, and proteins were separated by SDS/PAGE [10% (w/v) gel]. Samples were stained with Coomassie Brilliant Blue (A) and with anti-hexitol-lysine IgG (B).

Figure 6 Incubation of SDH with fructose and glucose

SDH was incubated with various concentrations of fructose (A) and glucose (B). The relative enzyme activities are shown. The SDH protein was subjected to SDS/PAGE (10% gel) and the glycated protein was detected with a hexitol-lysine antibody by immunoblotting (C).

suggesting that SDH undergoes glycation *in vivo*. The amount of glycated form increased markedly after incubation with glucose.

Degradation and inactivation of SDH by glycation

Incubation of rat liver SDH with 0–0.1 M fructose or glucose for 3 and 7 days at 37 °C resulted in a time-dependent decrease in enzymic activity. SDS/PAGE showed a corresponding decrease in the intensity of the SDH (Figure 6). Because neither fragmentation nor cross-linking of the protein caused by glycation was observed on the gel, the decrease in intensity will have been caused by a decrease in the staining ability of Coomassie Brilliant Blue for the glycated proteins [11]. The specific activities of SDH during glycation decreased in a time- and dose-dependent manner. Incubation with fructose, a more strongly glycating sugar than glucose [12,13], resulted in a more rapid decrease in specific activity. Western blot analyses with hexitol-lysine antibody after incubation of SDH with 100 mM glucose clearly demonstrated the appearance of the glycated enzyme (Figure 6C).

DISCUSSION

This work was undertaken to investigate the expression and activities of SDH in normal and diabetic rats as part of continuing studies on polyol metabolism. The activity and protein levels of SDH were highest in liver (Table 1) and consistent with a previous report [1]. The function of liver SDH is to metabolize sorbitol that is absorbed in the jejunum and ileum by means of passive diffusion and then transferred to the portal vein [14].

The expression of SDH mRNA was highest in testis, but the level of enzyme, as well as its activity, was not consistent with the level of the mRNA. There are several ways in which this discrepancy could be explained. Protein degradation by proteolytic enzymes such as proteasome [12] might be faster in testis, although the turnover rate of the SDH is not known at present. The other possibility is that SDH might undergo some post-translational modification that results in an accelerated degradation. The activity of the polyol pathway is supposed to be augmented in testis, because sperm can utilize fructose as the sole energy source. However, the straight-chain forms are present in much higher concentration in the case of fructose and therefore amino groups in proteins and other compounds react more rapidly with fructose than glucose in the glycation reaction [13,15]. This modification is known to decrease the activities of some enzymes such as Cu,Zn-superoxide dismutase [16,17], aldehyde reductase [18], alcohol dehydrogenase [19], Na,K-ATPase [20] and carbonic anhydrase [21]. The glycated sites of SDH are still unknown although a major site of horse liver alcohol dehydrogenase, one of the same superfamily as SDH, has already been determined [19]. A glycated site of SDH might be similar to horse liver alcohol dehydrogenase at Lys-210. The functional sites of SDH, probably Lys-319 and Lys-369 [22], were glycated, leading to a decrease in its activity.

Reactive oxygen species, especially hydroxyl radicals produced from the Amadori product, have been shown to cleave peptide bonds by Fenton reaction with transition-metal ions [23]. Because the amount of glycated SDH increases in diabetic rat liver (Figure 5) and the enzyme is inactivated by incubation with glucose or fructose (Figure 6), it is conceivable that SDH is glycated *in vivo* by fructose or glucose and inactivated. After this modification, degradation of SDH might be facilitated by a scavenging system such as proteasome. The discrepancy between mRNA and enzyme levels of SDH in testis might relate to a relatively high amount of fructose in this tissue [24].

Recent observations on an SDH inhibitor suggested that an increased oxidation of sorbitol to fructose is closely linked to vascular dysfunction [25]. In peripheral nerve, kidney, vascular and ocular tissues pathological changes are hallmarks of diabetic complications. Our studies demonstrate for the first time that SDH protein, as well as its activity in liver, decreased under hyperglycaemic conditions whereas the mRNA levels were increased. Because glucose uptake by liver is not dependent on insulin, the glycation reaction would be enhanced in liver by hyperglycaemia [9], and fructose levels would also be increased. Incubation of SDH with glucose and fructose lowered both the enzyme activity and the intensity of the protein bands stained by Coomassie Brilliant Blue (Figure 6). Because the colour intensity of the interaction of Coomassie Brilliant Blue with glycated proteins is lowered [11], a part of the decrease in the band intensity of Figure 6 might be caused by this mechanism. The elevated levels of fructose under diabetic conditions would accelerate the glycation of SDH *in vivo* and contribute to the decrease seen in the diabetic liver.

In conclusion we have demonstrated an augmented glycation of SDH under diabetic conditions *in vivo* and a decrease in SDH activity by glycation *in vitro*. The discrepancy between protein and mRNA levels in testis and diabetic liver might be explained by a glycation reaction with glucose and fructose. Activation of the polyol pathway and consequent elevation of the fructose levels might accelerate the glycation reaction.

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