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Alterations of protein mono-ADP-ribosylation and diabetic neuropathy: a novel pharmacological approach

Alfredo Gorio *, Maria Lucia Donadoni, Cristina Finco, Fabio Borella, Anna Maria Di Giulio

Laboratory for Research on Pharmacology of Neurodegenerative Disorders, Department of Medical Pharmacology, University of Milano, Via Vanvitelli 32, 20129 Milano, Italy

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Abstract

This study monitored the extranuclear endogenous mono-ADP-ribosylation of proteins. At least 10 proteins were ADP-ribosylated in a crude extract from control superior cervical ganglia, and 7 were labeled in control dorsal root ganglia; whereas in the diabetic rat the extent of labeling was reduced. These data suggest that proteins of peripheral ganglia are excessively ADP-ribosylated in vivo. Treatment of diabetic animals with silybin, a flavonoid with ADP-ribosyltransferase inhibitory activity, did not affect hyperglycemia, but prevented the alterations in the extent of mono-ADP-ribosylation of proteins. This effect was associated with the prevention of substance P-like immunoreactivity loss in the sciatic nerve. In the membrane fraction of sciatic nerve Schwann cells, at least 9 proteins were ADP-ribosylated, in diabetic rats the extent of labeling was increased. A comparable increase involving the same proteins was triggered by chronic nerve injury and by corticosteroid treatment. Silybin treatment of diabetic rats prevented such an increase. We propose that the inhibition of excessive protein mono-ADP-ribosylation by silybin prevented the onset of diabetic neuropathy, while the silybin effect on mono-ADP-ribosylation of Schwann cells is likely indirect and secondary to the improvement of diabetic axonopathy.

Keywords: Protein mono-ADP-ribosylation; Diabetes; Nerve lesion; Corticosterone

1. Introduction

Diabetes mellitus is a common disease with frequent complications affecting peripheral nerves, these alterations include reduction in nerve conduction velocity, axon atrophy and axon dwindling with changes in axonal transport of specific axonal constituents (Brown and Asbury, 1984; Norido et al., 1984; Vitadello et al., 1985; Medori et al., 1988). Sensory nerves are particularly affected by diabetes, their involvement constitutes an early correlate of diabetic neuropathy (Brown et al., 1980; Sima et al., 1983). Substance P is a neuropeptide present in small unmyelinated sensory fibers and is most affected by diabetes, with reduced axonal transport (Lembeck, 1953; Brimijoin et al., 1980; Robinson et al., 1987; Di Giulio et al., 1989, 1995), and, at later stages, reduced peptide nerve content (Di Giulio et al., 1985). Diabetic neuropathy is characterized by several biochemical alterations, including a reduction in myo-inositol content, reduced Na^+,K^+-ATPase activity and abnormalities in polyphosphoinositides metabolism (Green et al., 1989; Kim et al., 1991). The observed decrease of Na-pump activity has been correlated with abnormalities in protein kinase C activity in diabetic nerves (Lattimer et al., 1989; Kim et al., 1991). In addition, previous results obtained in our laboratory, as well as in the present study, show that protein mono-ADP-ribosylation might be one of the biochemical changes abnormally triggered by diabetes and capable of causing peripheral diabetic neuropathy (Gorio et al., 1995; Donadoni et al., 1995). The α-subunits of G-proteins are selectively ADP-ribosylated by cholera and pertussis toxins (Stryer and Bourne, 1986; Graziano and Gilman, 1987; Lander et al., 1993), however, endogenous mono-ADP-ribosyltransferases have been described in many biological tissues. These enzymes regulate the transfer of ADP-ribose from NAD to receptive proteins in vivo and in the absence of cholera and pertussis toxins (Duman et al., 1991; Matsuyama and Tsuyama, 1991; Williams et al., 1992). This post-translational modification affects several membrane and cytoplasmic proteins (Duman et al., 1991), G-proteins have been shown as a potential target (Jacquemin et al., 1986; Matsuyama and

* Corresponding author.

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Tsuyama, 1991), suggesting that mono-ADP-ribosylation might be a modulatory mechanism for receptor signal transduction. We had observed that Gi/Go function is impaired in striatum and retina of diabetic rats; such a reduced function is correlated with reduced pertussis toxin-mediated labeling and normal protein expression (Abbracchio et al., 1989; Finco et al., 1992). The reduced incorporation of ADP-ribose suggested that there is a higher concentration of endogenously ADP-ribosylated proteins in the retina and striatum of diabetic animals than of controls. Two studies have shown that such a process might take place; at least 6 proteins are ADP-ribosylated endogenously in the rat retina, but in diabetic rats only two retina proteins, 85 K and 36 K, are barely labeled in vitro, suggesting that endogenous ADP-ribosylation is enhanced in diabetes (Gorio et al., 1995; Donadoni et al., 1995). Treatment of diabetic animals with insulin normalizes Gi/Go functions in the retina (Abbracchio et al., 1991) and also normalizes mono-ADP-ribosylation of a 39 K protein (Gorio et al., 1995) identifiable as the α-subunit of a G-protein (Pozdnyakov et al., 1993). We have also evaluated the effects of treating diabetic animals with silybin, a flavonoid with clear inhibitory activity on mono-ADP-ribosylation (Donadoni et al., 1995; Banasik et al., 1992). The treatment partially prevents both the excessive mono-ADP-ribosylation of retina proteins and the onset of diabetic peripheral neuropathy assessed as substance P axonal transport (Donadoni et al., 1995). Protein ADP hydrolases cleave ADP-ribose from ADP-ribosylated proteins, promoting recovery of the original activity, i.e. actin regains its property to form microfilaments (Takada et al., 1994). Therefore, ADP-ribosylation of proteins may represent a reversible way to inhibit specific proteins causing cellular derangements as in diabetes. In this study, we have evaluated the extent of mono-ADP-ribosylation of proteins in the sympathetic and dorsal root ganglia, and in the sciatic nerve.

2. Materials and methods

2.1. Animals and sample preparation

Male albino Sprague-Dawley rats (Charles River, Calco, Como, Italy) weighing 240–260 g were used. Diabetes was induced with a single s.c. injection of 100 mg/kg alloxan (Sigma Chemical, St. Louis, MO, USA) dissolved in citrate-phosphate buffer at pH 4.5 (Gorio et al., 1992). One week after alloxan injection we established the onset of diabetes by determining plasma glucose levels (GOP PAP test, Boehringer, Mannheim, Germany). Diabetic rats with glycemia below 450 mg/dl plasma did not enter the study. Diabetes was monitored further during the experimental period by systematic checking of glycosuria (Test Tape; Ely Lilly, Indianapolis, IN, USA) and body growth. The final values for glycemia and body weight were taken at the time of killing. Starting 7 days after diabetes induction, a group of diabetic animals was treated with silybin (Istituto Biochimico Italiano, Milano, Italy) at a dose of 50 mg/kg given in the drinking water (Donadoni et al., 1995). Stable overnight solubility of silybin was obtained by microabsorption in β-cyclodextrin. The silibyn : β-cyclodextrin molar ratio is 1 : 2.5. We monitored the water consumption (ml) daily. At 1 week after diabetes induction the average water intake of control and diabetic rats was 55 ± 10 and 132 ± 16, respectively, at 6 weeks 73 ± 9 and 190 ± 12, at 12 weeks 85 ± 11 and 235 ± 17. The correct dose of drug, weekly adjusted for the weight, was supplied in 50 ml of drinking water to control animals throughout the experimental period, and in 100 ml up to 6 weeks and 150 ml up to the end to the diabetics. The drug was supplied overnight from 17:00 to 08:00 h, at that time the drug-containing water had been fully swallowed and was replaced by drug-free water. Drug treatment was terminated 48 h before killing. Controls consisted of age- and weight-matched animals receiving overnight β-cyclodextrin alone (280 mg/kg) or silybin. Animals were maintained under standard conditions with ad libitum access to water and chow. No effect of silybin on water consumption was ever observed. The rats were killed 14 weeks after diabetes induction by deep sodium pentobarbital anesthesia (35 mg/kg) followed by decapitation. Superior cervical ganglia, L4 and L5 dorsal root ganglia, and both sciatic nerves were removed quickly, cleaned, snap-frozen in liquid nitrogen and stored at −80°C until protein ADP-ribosylation and substance P radioimmunoassay were performed. The data on diabetes reported here are derived from 2 consecutive sets of experiments and a total of 80 animals were used.

In a second series of experiments, 12 control rats weighing 250 g were anesthetized with sodium pentobarbital (35 mg/kg), then following established procedures, the left sciatic nerve was exposed at the mid thigh and resected to prevent regeneration (Gorio et al., 1980, 1983). Six of the lesioned and 6 of the unlesioned rats were treated with corticosterone-21 acetate as described by Duman et al. (1991). Corticosterone was supplied by s.c. implantation of a 100-mg pellet of the steroid under anesthesia as above. The controls underwent identical surgery but without lesioning of the nerve and without implantation of the steroid pellet. Seven days later, the animals were deep-anesthetized, decapitated and the distal stump of the lesioned nerve and the contralateral intact nerve were dissected and processed as described above until ADP-ribosylation assay.

2.2. Preparation of cellular fractions

Tissue homogenization was carried out by means of a polytron (Ultra Turrax) in 5 volumes of homogenization buffer consisting of: 25 mM Tris-HCl, pH 7.4; 0.4 mM phenylmethylsulfonylfluoride; 1 mM benzamidine-HCl; 0.4 mM sodium azide; 0.4 mM EDTA; 0.4 mM EGTA; and

0.25 M sucrose (Tanaka et al., 1989). The homogenate was centrifuged at 1500 × *g* for 10 min at 4°C. The pellet was homogenized again and recentrifuged as above. The supernatants of both centrifugations were combined and used as crude extracts, i.e. a cell fraction without nuclei. Crude extract was further centrifuged at 100 000 × *g* for 60 min. The 100 000 × *g* pellet was resuspended in homogenization buffer containing 0.1% Triton X-100 and was recentrifuged at 100 000 × *g* for 60 min. The resulting supernatant was used as the particulate or membrane-rich fraction. Protein content was evaluated for aliquots of each fraction (Lowry et al., 1951).

2.3. Assay of mono-ADP-ribosylation

We performed ADP-ribosylation on aliquots of the different fractions containing 20 or 40 μg of protein (Duman et al., 1991). The total volume of 200 μl of incubation medium contained 25 μM [^{32}P]NAD and 50 mM sodium acetate buffer, pH 6.0, with 10 mM thymidine. The reaction was allowed to run for 30 min at 30°C, then was stopped by the addition of 1% of deoxycholic acid with cold 10% trichloroacetic acid. Acid precipitation was promoted by keeping the samples on ice for 45 min and the insoluble precipitates were centrifuged at 1300 × *g*, washed with cold 95% ethanol and air-dried. This procedure allows for the complete elimination of free unbound [^{32}P]NAD (Pozdnyakov et al., 1993). The pellet was resuspended with a 1 : 1 solution of denaturation buffer (5% dithiothreitol, 5 mM urea and 100 mM Tris-HCl at pH 8.0) and Laemmli buffer (0.0625 M Tris-HCl, 2% sodium dodecyl sulfate, 10% glycerol, 5% 2-mercaptoethanol and 0.05% blue bromophenol). The samples were then denatured at 95°C for 4 min and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by using 12.5% gel (Laemmli, 1970) followed by autoradiography. The autoradiograms were analyzed using the NIH IMAGE 1.44 program written by Wayne Rasband and a Macintosh computer (available from Internet by anonymous ftp from zippy.nih.gov). The data were expressed as arbitrary units (pixel) and represent the densitometric analysis of each single band.

2.4. Substance P radioimmunoassay

Frozen nerve samples (16 per experimental group) were homogenized as above in polypropylene tubes containing ice-cold 1 M acetic acid in a 1 : 10 ratio (w : v). Protein determination (Lowry et al., 1951) was performed using an aliquot of the homogenate, whereas substance P radioimmunoassay was performed on the other aliquot according to commonly used techniques (Di Giulio et al., 1985, 1989).

2.5. Data analysis

The data were expressed as means ± S.D. and were evaluated statistically with the Student's *t*-test. The differences between means (calculated as *P* values) were considered statistically significant when *P* values were < 0.05.

Each value shown represents the evaluation of the analysis of 4 specimens for the ganglia and of at least 6 specimens for the nerves. Each ganglion specimen was a pool of 2 and 4 ganglia for the superior cervical ganglion and dorsal root ganglia, respectively.

3. Results

3.1. Body weight and plasma glucose levels

Diabetes deeply affected body weight and plasma glucose levels. Both parameters were evaluated in all experimental animals. Untreated and silybin-treated controls gained weight throughout the experimental period, whereas the growth rate of treated and untreated diabetic rats was about 10% of control values. The body weight (grams) was 548 ± 8.9 and 536 ± 6.2 for untreated and treated controls, respectively, 295 ± 7.1 and 288 ± 9.2 for untreated and treated diabetics, respectively. The plasma glucose levels were particularly elevated in treated (496 ± 9 mg/dl) and untreated (485 ± 7.9 mg/dl) diabetics. Silybin did not affect the plasma glucose levels in control rats either, with 106 ± 0.9 mg/dl in untreated and 100.6 ± 3.8 mg/dl in silybin-treated controls.

3.2. Protein mono-ADP-ribosylation

Protein mono-ADP-ribosylation was studied in the absence of exogenous toxins in cell nuclei-free crude extract

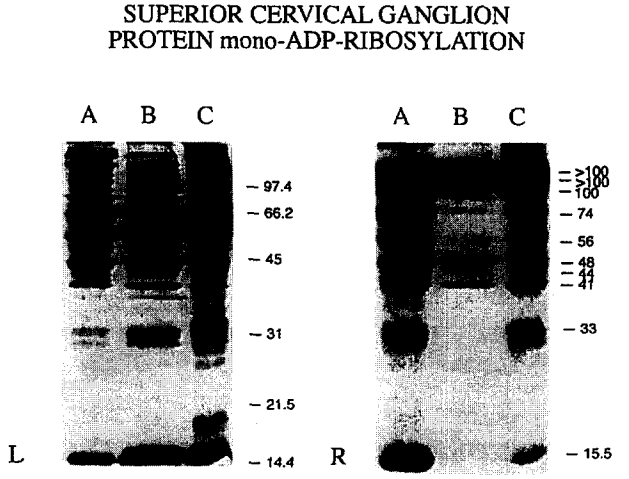

Fig. 1. Superior cervical ganglia crude extract proteins were identified by gel electrophoresis and Coomassie blue staining (L) and autoradiography after mono-ADP-ribosylation assay (R). Ganglionic preparations were collected from control (A), diabetic (B) and silybin-treated diabetic (C) rats. In diabetic preparations, the extent of protein mono-ADP-ribosylation was reduced markedly for 74 K, 56 K, 48 K, 44 K and 15.5 K proteins. Treatment with silybin markedly improved the labeling of these proteins (C).

Table 1
Quantitative evaluation of autoradiographic protein bands from mono-ADP-ribosylation in superior cervical ganglia samples (crude extract fraction) (see Fig. 1)

M. weight	Control	Diabetic	Diabetic treated
> 100 K	1064±31	1315±12 ***	1381±76
> 100 K	805±30	834±26	853±14
100 K	454±39	333±27 **	645±26 °°°
74 K	1589±31	212±18 ***	845± 7 °°°
56 K	1781±35	613±16 ***	957±22 °°°
48 K	1085±14	643±23	814±13
44 K	692± 5	547±35 **	691±13
41 K	624±15	547±22	635± 9
33 K	261±16	123±14 **	192±11 °°
15.5 K	2721±88	340± 6 ***	1196±39 °°°

The data are means ± S.D. of the analysis of 4 specimens. Diabetic vs. control: ** $P < 0.01$; *** $P < 0.001$. Diabetic treated vs. diabetic: °° $P < 0.01$; °°° $P < 0.001$.

Table 2
Quantitative evaluation of autoradiographic protein bands from mono-ADP-ribosylation in dorsal root ganglia samples (crude extract fraction) (see Fig. 2)

M. weight	Control	Diabetic	Diabetic treated
> 100 K	3603±131	4178±56 **	4224±103
> 100 K	852± 55	1451±31 ***	1444± 29
74 K	636± 23	873±32 **	1340± 13 °°°
48 K	122± 3	222±15 **	448± 14 °°°
44 K	202± 14	139± 7 **	199± 7 °°
41 K	146± 4	345± 8 ***	547± 17 °°
38 K	659± 3	806±26 **	2631± 91 °°°

The data are means ± S.D. of the analysis of 4 specimens. Diabetic vs. control: ** $P < 0.01$; *** $P < 0.001$. Diabetic treated vs. diabetic: °° $P < 0.01$; °°° $P < 0.001$.

and membrane-rich fractions of control and of treated and untreated 14-week diabetic rats. We have previously reported that treatment of control rats with silybin gave results similar to controls, so that the pattern and extent of protein mono-ADP-ribosylation were not affected (Donadoni et al., 1995). Superior cervical ganglia Coomassie blue-stained proteins separated by electrophoresis (Fig. 1L) and the respective autoradiograms (Fig. 1R) from control, untreated and silybin-treated diabetic rats are shown. The incubation of control superior cervical ganglion crude extracts (A) with [^{32}P]NAD promoted a measurable extent of labeling in 10 proteins of the controls; the molecular weight of ADP-ribosylated proteins was > 100 K, > 100 K, 100 K, 74 K, 56 K, 48 K, 44 K, 41 K, 33 K, 15.5 K (Fig. 1R). In the superior cervical ganglion of untreated diabetic rats (B), the extent of labeling was markedly reduced in 5 bands, 74 K, 56 K, 48 K, 44 K, 15.5 K (Fig. 1R). The quantification of the differences is shown in Table 1. The in vivo inhibition of endogenous mono-ADP-ribosylation by silybin treatment of diabetic rats (C) allowed a partial restoration of protein labeling in crude extract from the superior cervical ganglion of diabetic rats (Fig. 1R). The in vitro [^{32}P]NAD-mediated labeling was improved in almost any protein band when diabetic rats were treated with silybin (Table 1). The improvement ranged from 35% to 100% and was highly significant (Table 1). In the dorsal root ganglia crude extract preparations (Fig. 2L) from untreated control rats (A), there were 7 proteins capable of incorporating labeled ADP-ribose, > 100 K, > 100 K, 74 K, 48 K, 44 K, 41 K, 38 K (Fig. 2R). The quantification of the autoradiogram is shown in Table 2. The extent of labeling of the proteins in diabetic rat preparations (B) was slightly changed, some proteins incorporate more ADP-ribose as also shown by

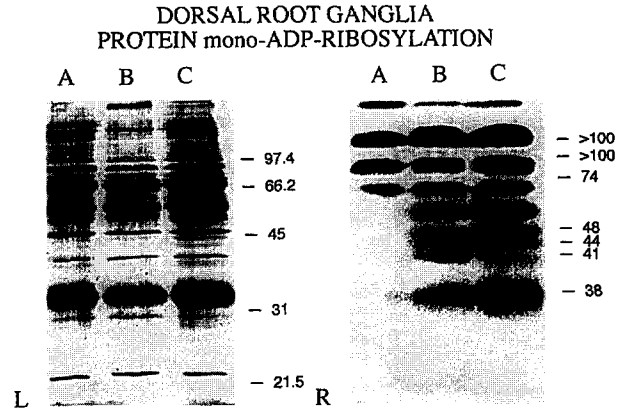

Fig. 2. L4 and L5 dorsal root ganglia crude extract proteins were identified by gel electrophoresis and Coomassie blue staining (L) and autoradiography (R), from control (A), diabetic (B) and silybin-treated diabetic (C) rats. Seven proteins were mono-ADP-ribosylated in controls (R). In diabetic rats, there was a slight increase as also shown by the quantification (Table 2). The in vivo inhibition of endogenous mono-ADP-ribosylation with silybin (C) promoted a marked increase of labeling of several proteins.

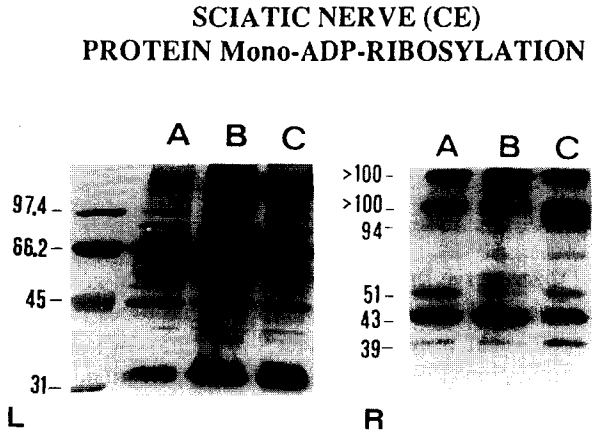

Fig. 3. Sciatic nerve crude extract proteins were identified by gel electrophoresis and Coomassie blue staining (L) and autoradiography (R), from control (A), diabetic (B) and silybin-treated diabetic (C) rats. Several protein bands were mono-ADP-ribosylated (R) in controls. In untreated diabetics, labeling increased in two bands, the lower > 100 K and 43 K, and decreased in two other bands, 94 K and 51 K, but the treatment with silybin restored a normal extent of ADP-ribosylation in most proteins.

Table 3
Quantitative evaluation of autoradiographic protein bands from mono-ADP-ribosylation in sciatic nerve samples (crude fraction fraction) (see Fig. 3)

M. weight	Control	Diabetic	Diabetic treated
> 100 K	4834 ± 108	5577 ± 54 **	5657 ± 60
> 100 K	5224 ± 415	6444 ± 44 ***	4770 ± 83 °°°
94 K	1880 ± 38	357 ± 11 ***	719 ± 33 °°°
51 K	3038 ± 64	1862 ± 40 ***	1821 ± 16
43 K	5078 ± 62	7299 ± 8 ***	4638 ± 60 °°°
39 K	643 ± 23	924 ± 25 ***	1086 ± 50

The data are means ± S.D. of the analysis of 6 specimens. Diabetic vs. control: ** $P < 0.01$; *** $P < 0.001$. Diabetic treated vs. diabetic: °°° $P < 0.001$.

Table 4
Quantitative evaluation of autoradiographic protein bands from mono-ADP-ribosylation in sciatic nerve samples (P fraction) (see Fig. 4)

M. weight	Control	Diabetic	Diabetic treated
> 100 K	4312 ± 58	4324 ± 51	4610 ± 39
95 K	3134 ± 141	2640 ± 14 **	3278 ± 21 °°°
82 K	889 ± 16	907 ± 19	2515 ± 13 °°°
70 K	939 ± 6	1059 ± 13 *	2298 ± 24 °°°
51 K	226 ± 3	1703 ± 37 ***	182 ± 11 °°°
43 K	293 ± 7	2895 ± 38 ***	278 ± 12 °°°
38 K	209 ± 17	908 ± 12 ***	164 ± 14 °°°
33 K	1248 ± 26	4333 ± 75 ***	1451 ± 24 °°°
30 K	245 ± 7	563 ± 23 ***	99 ± 6 °°°

The data are means ± S.D. of the analysis of 6 specimens. Diabetic vs. control: * $P < 0.05$; *** $P < 0.001$. Diabetic treated vs. diabetic: °°° $P < 0.001$.

the quantification. The in vivo inhibition of endogenous ADP-ribosylation in diabetic animals (C) promoted an increase of labeling of several proteins, 74 K, 48 K, 41 K and 38 K (Table 2). The effect was most evident for the 38 K. Due to their small size, the ganglia supplied tissue sufficient for testing the crude extract fraction only and each preparation consisted of a pool of 4 ganglia.

Sciatic nerve crude extract Coomassie blue-stained proteins separated by electrophoresis (Fig. 3L) and the respective autoradiograms (Fig. 3R) from control, untreated and silybin-treated diabetic rats are shown. The pattern of protein mono-ADP-ribosylation shows that at least 6 proteins were labeled in the controls, with molecular weights > 100 K, > 100 K, 94 K, 51 K, 43 K and 39 K. In the sciatic nerve of untreated diabetic rats (B), labeling was increased in two bands, the lower > 100 K and 43 K, and decreased in two other bands, 94 K and 51 K. Treatment of diabetic animals with silybin restored the normal extent of labeling for the lower > 100 K, 94 K and 43 K proteins, and no effect was observed on the 51 K. The quantification of the differences is shown in Table 3. In the sciatic nerve membrane-rich fraction of control rats (Fig. 4L), there were 9 labeled proteins (Fig. 4R); however, good labeling was present only in the > 100 K, 95 K, 82 K, 70 K and 33 K proteins. In untreated diabetics the labeling of the other bands, 51 K, 43 K, 38 K, 30 K and 33 K, increased markedly. The treatment of diabetics with silybin normalized the extent of ADP-ribosylation to one similar to the control values except for 82 K and 70 K proteins. The quantification of these data is shown in Table 4.

The ADP-ribosylation assay of the membrane-rich fraction of sciatic nerves from control and lesioned rats with or without corticosteroid treatment is shown in Fig. 5. The autoradiogram shows that the number of labeled proteins

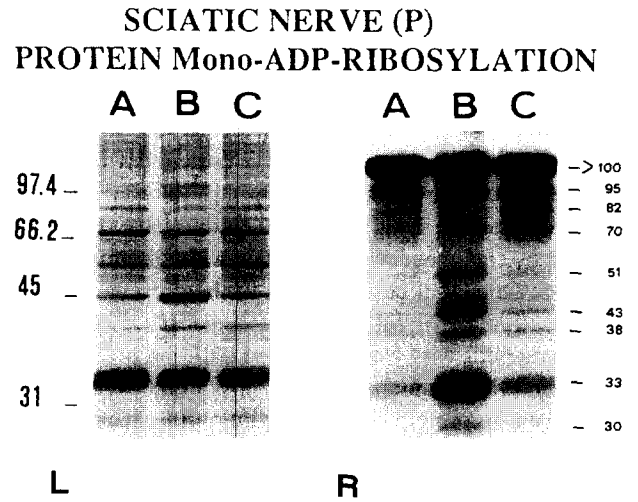

Fig. 4. Sciatic nerve membrane-rich fraction proteins were identified by gel electrophoresis and Coomassie blue staining (L) and autoradiography after mono-ADP-ribosylationassay (R). Sciatic nerve preparations were collected from control (A), diabetic (B) and silybin-treated diabetic (C) rats. At least 9 proteins were labeled in controls but, in diabetics, the extent of protein mono-ADP-ribosylation was increased in several proteins (Table 4); treatment of diabetics with silybin restored the normal extent of protein labeling.

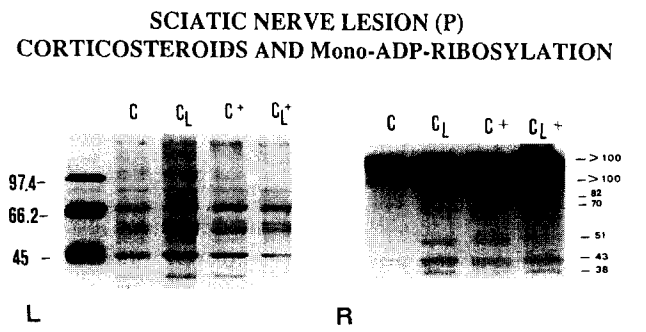

Fig. 5. Membrane-rich fractions from unlesioned sciatic nerve (C) and from the non-regenerating distal stump (CL) and with corticosterone treatment (C+ and CL+). The proteins were identified by gel electrophoresis and Coomassie blue staining (L) and autoradiography after mono-ADP-ribosylation assay (R). At 7 days after axotomy, the distal stump lacked the axonal part that was fully degenerated and contained only Schwann cells. The distal stump of the lesioned nerve displayed the same increase of protein ADP-ribosylation as in diabetics, and corticosterone treatment had the same effect as well. The lack of some protein bands (see Fig. 4) might have been due to the much younger age of these animals, the chronic diabetics being 14 weeks older.

Table 5
Quantitative evaluation of autoradiographic protein bands from mono-ADP-ribosylation in sciatic nerve samples (P fraction) (see Fig. 5)

M. weight	C	CL	C+	CL+
> 100 K	7954±80	7570±76 *	7150±66 °°	7305±89
> 100 K	3636±26	5610±96 ***	4039±54 °°	6312±48 §§§
82 K	309±14	2345±25 ***	2217±29 °°°	4563±64 §§§
70 K	370±14	2557±32 ***	2443±32 °°°	4339±26 §§§
51 K	328± 8	1064±26 ***	1230±16 °°°	1042±20
43 K	677±24	1664±67 ***	1179±26 °°°	22209±78 §§
38 K	282±26	1064± 7 ***	627±31 °°	1002±13

The data are means ± S.D. of the analysis of 6 specimens. C, control; CL, control with nerve lesion; C+, control + corticosteroids; CL+, control with nerve lesion + corticosteroids. CL vs. C: * $P < 0.05$; *** $P < 0.001$. C+ vs. C: °° $P < 0.01$; °°° $P < 0.001$. CL+ vs. CL: §§ $P < 0.01$; §§§ $P < 0.001$.

increased markedly after injury, giving a pattern of labeling similar to that of the diabetic nerve; corticosteroid treatment also increased the extent of labeling as expected from the Duman et al. (1991) report and the effect on ADP-ribosylation was quantitatively similar to that of injury. It is remarkable that diabetes, injury and corticosteroids all increased the ADP-ribosylation of the same proteins. The quantification of such labeling is shown in Table 5.

3.3. Substance P-like immunoreactivity assay

The mean levels of substance P-like immunoreactivity were markedly decreased in the sciatic nerve of 14-week diabetic rats; in the controls the content was 2.5 ± 0.11 ng/mg protein, whereas in diabetics it decreased to 0.9 ± 0.05. Treatment of diabetic rats with silybin prevented the decrease of substance P-like immunoreactivity that was 2.2 ± 0.13. The difference between control and untreated diabetics and between treated and untreated diabetics was significant with level of $P < 0.01$. At least 8 animals per group were used.

4. Discussion

The results of this study confirmed previous results that showed alterations of protein mono-ADP-ribosylation in experimental diabetes and suggested that such protein post-translational modification might be involved in the mechanisms leading to the onset of diabetic neuropathy. The treatment of diabetic rats with silybin, an inhibitor of protein mono-ADP-ribosylation, prevented both these biochemical changes and the marked reduction of substance P in the sciatic nerve of chronic diabetic rats. Diabetes was induced by treating rats with alloxan, which is toxic to pancreatic β-cells by causing nuclear poly-ADP-ribosylation of proteins (Uchigata et al., 1982). Following the degeneration of pancreatic β-cells (Dunn et al., 1943), the animals develop hyperglycemia, then a peripheral neuropathy not related to direct toxic effects of alloxan on the nervous system (Eliasson, 1964). The treatment with silybin was begun a week after alloxan injection to avoid any interference of the drug with diabetes induction. We showed that in the retina of diabetic animals there is an abnormally high level of endogenous protein mono-ADP-ribosylation of proteins that prevents in vitro ADP-ribosylation under our testing conditions (Gorio et al., 1995; Donadoni et al., 1995). We proposed that, in diabetes, ADP-ribosyltransferase activity is higher than normal, increasing the level of endogenous mono-ADP-ribosylation of selected proteins and that, as a consequence, a number of neuronal dysfunctions might exist, causing diabetic neuropathy. We also proposed that neurons with cell bodies placed outside the blood-brain barrier might be more exposed to the effects of hyperglycemia. This hypothesis would explain the higher susceptibility of sympathetic and sensory nervous system to diabetes. By preventing the excessive protein mono-ADP-ribosylation with silybin, we had also prevented the drop of substance P-like immunoreactivity axonal transport.

This work has shown that, in the superior cervical ganglion, 10 proteins were sensitive to endogenous mono-ADP-ribosylation. In diabetic rat preparations, the extent of protein labeling according to the ADP-ribosylation assay was reduced markedly in 5 proteins, being particularly evident for the 74 K, 56 K, 48 K, 44 K and 15.5 K. The treatment of diabetic rats with silybin did not affect hyperglycemia and the reduced body weight, whereas the extent of protein mono-ADP-ribosylation was partially normalized. In the dorsal root ganglia, 7 proteins were in vitro labeled and the quantitation of labeling in diabetic ganglia showed small differences from the controls. However, the in vivo treatment with silybin promoted an increase of labeling in several proteins, with the effect very evident for the 38 K protein. This protein might be related to the 39 K retina membrane-bound protein described by Pozdnyakov et al. (1993) as the α-subunit of G-proteins. This subunit in diabetic retina was shown to be less labeled by ADP-ribose under our testing conditions, and to have its labeling restored by treatment with insulin or with silybin (Gorio et al., 1995; Donadoni et al., 1995). In the present study, the positive effect of silybin treatment on peripheral ganglia of chronic diabetic rats was associated with the prevention of substance P-like immunoreactivity loss in the sciatic nerve. These data on protein ADP-ribosylation in peripheral ganglia are in agreement with earlier data on the retina (Gorio et al., 1995; Donadoni et al., 1995). The changes in protein mono-ADP-ribosylation were quite different in the sciatic nerve of diabetic rats. We observed that, in the membrane-rich fraction, there were 9 proteins which were labeled according to the in vitro assay and that diabetes markedly increased the labeling of at least 5 proteins, 51 K, 43 K, 30 K and 33 K. To understand whether the increased labeling was due to the axonal part or to the

Schwann cells of the sciatic nerve, we lesioned some control rats by resecting the sciatic nerve and 7 days later we analyzed the distal stump that was now free of axons and enriched in Schwann cells. We observed that denervated Schwann cells showed the same increase of labeled proteins as in the diabetic nerves. Therefore, it is apparent that such labeling in diabetics was likely due to Schwann cells that react to diabetes differently from neuronal cell bodies. In addition, these proteins were sensitive to corticosteroid treatment. Intact sciatic nerve treated with corticosterone showed a pattern of protein mono-ADP-ribosylation similar to that of lesioned or diabetic nerves. Duman et al. (1991) had shown that in vivo corticosteroid treatment increases in vitro labeling of receptive proteins by [^{32}P]ADP-ribose. Therefore, we would suggest that injury and diabetes trigger some reactive processes in Schwann cells that bring about a higher incorporation of ADP-ribose under our testing conditions. It is well known that both conditions activate Schwann cells (Gorio, 1993; Conti et al., 1993). Silybin treatment was capable of preventing such changes in the diabetic nerve membrane-rich fraction. Silybin is a good mono-ADP-ribosyltransferase inhibitor. The in vitro test had shown that silybin at a concentration of 1 μM inhibited ADP-ribosylation of most proteins, and in vivo inhibition of excessive ADP-ribosylation brought about a normal extent of labeling (Donadoni et al., 1995). The effect on the membrane-rich fraction of the sciatic nerve of diabetic animals cannot be explained by this type of pharmacological activity, because the greater labeling observed in diabetes is reduced by silybin. We would explain such an effect as secondary to the beneficial effects of the treatment on the neuropathy, as indicated by the positive effects upon substance P-like immunoreactivity nerve levels or axonal transport previously reported. It is well known that, in diabetic neuropathy, alterations of Schwann cells that are secondary to the axonopathy are seen (Brown and Asbury, 1984). It is, therefore, likely that the positive effect of silybin treatment on Schwann cells derives from the improvement of the diabetic axonopathy.

In conclusion, this study has provided further information about alterations of protein mono-ADP-ribosylation and diabetic neuropathy. In the cell body of peripheral and retina (Donadoni et al., 1995) neurons of diabetic rats, endogenous protein mono-ADP-ribosylation is increased and treatment with a selective inhibitor of this enzymatic activity restores a normal extent of protein mono-ADP-ribosylation and prevents diabetic neuropathy assessed as axonal transport (Donadoni et al., 1995) and metabolism of substance P-like immunoreactivity.

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