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ENHANCED SYNTHESIS AND SECRETION OF APOLIPOPROTEIN E FROM SCIATIC NERVES OF STREPTOZOTOCIN-INDUCED DIABETIC RATS AFTER INJURY

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To elucidate the pathogenesis of diabetic neuropathy, synthesis and secretion of apolipoprotein E (apo E) from sciatic nerves after injury was studied in normal and streptozotocin-induced diabetic rats. Seven, 14, 28, 45 and 59 days after making crush injury on sciatic nerves with concomitant administration of streptozotocin (50 mg/kg body weight), the nerves were taken out and incubated with [^{35}S]methionine. The [^{35}S]labeled apo E was precipitated with specific antiserum. The amounts of apo E secreted into medium by nerves of diabetic rats were 7 times greater than those of non-diabetic rats 7 days after injury. This enhanced secretion of apo E was relatively selective for this protein, since the ratio of the immunoprecipitable apo E to the TCA preciptitable protein in the medium increased in diabetic rats. Intriguing possibility deduced from these results is that the secretion of apo E is involved in the development of diabetic neuropathy.

Peripheral polyneuropathy is a common cause of disability in patients suffering from diabetes mellitus. This troublesome complication is presumed to result from a complex interplay between metabolic derangement and unidentified genetic factors. Recently, a new insight for pathogenesis of peripheral neuropathy has emerged from finding of a protein whose secretion and synthesis is greatly stimulated in nerve tissue after its injury (1,2). This protein was identified as apo E (3), which is a major constituent of plasma lipoproteins and functions both as a transporter of lipids and as a ligand for lipoprotein receptors. Since its expression after nerve injury is closely associated with nerve growth and degeneration, two roles of this apolipoprotein in nerve injury were suggested: first, removing by-products (especially cholesterol) formed during degeneration of nerves, and second, providing the regenerating nerves with lipids necessary for axon growth (4). On the other hand, we and others have demonstrated abnormality in the metabolism of plasma apo E in streptozotocin-induced diabetic rats (5,6). These findings suggest that

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apo E is involved in the development of diabetic polyneuropathy. In the present study, we investigated the apo E secretion from sciatic nerves in response to injury in streptozotocin-induced diabetic rats. To our knowledge, there have been no reports on the regulation of apo E secretion from peripheral nerves.

MATERIALS AND METHODS

Materials Adult male Sprague-Dawley rats weighing 180-220g were purchased from Nissayzai (Tokyo, Japan). Streptozotocin was obtained from Sigma (St. Louis, MO). [^{35}S]methionine was obtained from ICN (Irvine, CA). Methods for preparation of anti-rat apo E rabbit serum were described previously (7).

Animal experiments After an overnight fast, rats were anesthetized with pentobarbital, 35-40 mg/kg body weight and injected intravenously with streptozotocin at a dose of 50 mg/kg body weight. Then, left sciatic nerves were exposed in the hip and crushed with small forceps until the nerves were translucent. The rats were fed a standard chow until sacrifice.

After blood was collected for the determination of glucose, the sciatic nerve stumps distal to the crush site, or comparable pieces of sciatic nerve from contralateral legs were removed rapidly and rinsed in ice cold Dulbecco's phosphate buffered saline.

Each nerve segment was minced with scissors on ice cold plastic dish, and the nerve was incubated with 0.5 ml of Eagle's Minimum Essential Medium (MEM) containing 80 µCi of [^{35}S]methionine. The incubation was carried out for 12-18 hours at 37 °C under humidified atmosphere of 5% CO_2 and 95% air with vigorous shaking.

Sample preparation Proteins secreted by the minced nerves into the incubation medium were collected. The minced tissue was washed once with 0.5 ml of MEM and the wash was added to the incubation medium. The collected medium was centrifuged at 12,000 g for 5 min and the supernatant is termed as 'medium' fraction. The resulting pellets were homogenized in 1 ml of buffer A (10 mM Tris-HCl, pH 7.4 containing 2%(v/v) SDS, 5 mM EDTA, 1 mM dithiothreitol and 1 mM phenylmethysulfonylfluoride (PMSF)) using Dounce homogenizer and centrifuged at 12,000 g for 10 min. The resulting supernatant was termed as 'cell' component. 50%(v/v) trichloroacetic acid (TCA) solution was added to both the medium and the cell sample to give a final concentration of 10%(v/v) of TCA. After incubation on ice for 30 min, TCA precipitable proteins were collected by centrifugation at 12,000 g for 5 min and pellets were washed twice with diethyl-ether before counting.

0.4 ml of the medium was mixed with 1 ml of buffer B (25 mM Tris-HCl, pH7.4 containing 0.15 M NaCl, 0.5%(v/v) Nonidet P-40, 1 mM EDTA and 1 mM PMSF) containing 5 µl of anti-rat apo E rabbit serum (7), and incubated overnight at 4°C. 50 µl of protein A bacterial adsorbent (Bio-Yeda, Israel) solution was added to the mixture and incubated for another overnight on a shaker at 4°C. The mixture was centrifuged at 12,000 g for 5 min. The resulting pellets were washed four times with 1 ml of the buffer B without antiserum, dissolved in 100µl of buffer C (62.5 mM Tris-HCl, pH6.8 containing 2%(v/v) SDS and 5%(v/v) ß-mercaptoethanol) and boiled for 3 min. An aliquot of this solution was mixed with 10 ml of Aqualuma Plus (Lumac, Netherlands) and the radioactivity was measured by Packard Tri-Carb 460 (Packard Instrument Company, IL). Another aliquot was analysed by SDS/polyacrylamide gel electrophoresis in 10%(v/v) acrylamide/0.3%(v/v) bisacrylamide gels as described by Laemlli (8). After fluorography using EN^{3}HANCE (New England Nuclear, MA), the portion of gel presumed to contain apo E was soaked in 1 ml of 35%(v/v) hydrogen peroxide for overnight and then mixed with 10 ml of Aqualuma Plus for counting radioactivity.

Density gradfient ultracentrifugation The incubation medium was dialized against 5 l of dialysis buffer (2 mM sodium phosphate, pH 7.4 containing 0.15 M NaCl, 0.01%(v/v) EDTA and 0.01%(v/v) NaN_3) to remove free [^{35}S]methionine and then ultracentrifuged in a 40.3 rotor (Beckman Instruments, CA) at 38,000 rpm for 40 hr at 12°C, after its density

was adjusted to d = 1.21 g/ml by KBr. The top fraction was re-ultracentrifuged with discontinuous gradient of d = 1.006 to 1.210 g/ml in a SW 41 rotor (Beckman Instruments, CA) for 24 hr at 12°C as described by Redgrave et. al. (9). After the ultracentrifugation, the samples were fractionated into 24 fractions of 0.5 ml and 0.2 ml of each fraction was counted for the radioactivities. The hydrated density of each fraction was determined by its electric conductivity.

RESULTS

Apo E synthesis in the cells and its secretion into the medium Two weeks after the administration of streptozotocin to rats, body weights of the control rats increased from 254 ± 19 g to 336 ± 48 g (n = 4), while those of diabetic rats increased a little from 249 ± 10 g to 275 ± 34 g (n = 5). The bood glucose concentrations of diabetic rats and controls at the time of sacrifice were 493 ± 44 mg/dl and 170 ± 21 mg/dl, respectively.

The ratio of radioactivities of [^{35}S] incorporated into apo E immunoprecipitated with anti-rat apo E antiserum to total protein precipitated with TCA was 0.032 ± 0.007 and 0.022 ± 0.005 in the medium of crushed nerves from diabetic rats and controls, respectively, and that in the cells was 0.012 ± 0.001 and 0.0063 ± 0.001, respectively ($p < 0.001$). There was no difference between diabetic and control groups in total proteins secreted into the medium, while apo E secretion was significantly greater in diabetic rats than that in controls at $p < 0.05$. These findings suggest that the nerves obtained from rats treated with streptozotocin have enhanced synthesis and secretion of apo E selectively rather than concomitantly with total protein synthesis and secretion.

Characterization of secreted apo E Distribution of the hydrated density of the secreted apo E was studied by density gradient ultracentrifugation and the results were illustrated in Fig. 1. In the non-injured nerve, radioactivities increased slightly with an increase in density from 1.05 to 1.18 g/ml, while there was almost no radioactivity between the density of 1.01 and 1.05 g/ml. Appreciable radioactivities remained in the fractions of

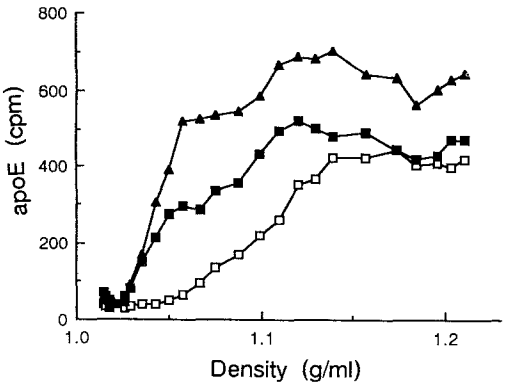

Figure 1. Density gradient ultracentrifugation of the medium incubated with rat sciatic nerves. Equal volume of medium of non-injured (□) and injured nerves (■) from non-diabetic control and that of injured nerves from streptozotocin-induced diabetic rats (▲) were combined in each group to give 1 ml of medium.

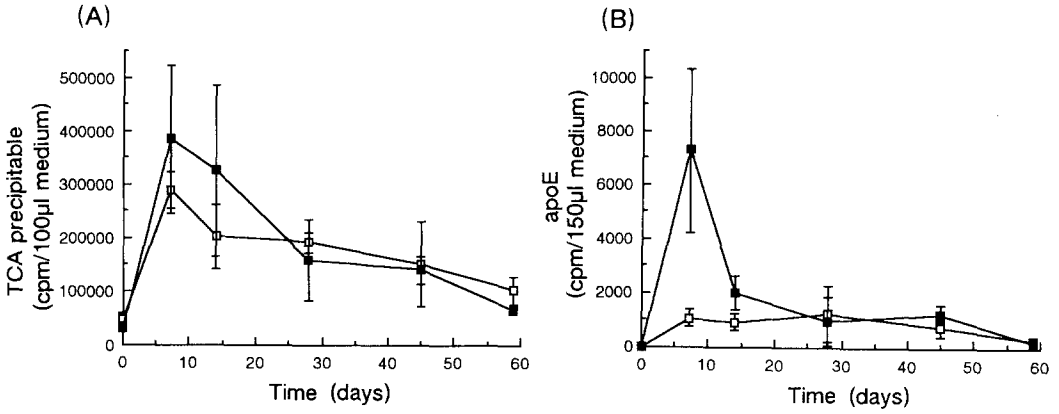

Figure 2. Changes of radioactivities in TCA precipitable proteins (A) and apo E (B) secreted into medium by sciatic nerves from streptozotocin-induced diabetic (■) and non-diabetic rats (□) after injury. The sciatic nerves of four sham-operated aminals were used as controls. At the time indicated in the abscissa before the sacrifice, nerves were crushed with or without administration of streptozotocin. Sacrifice and metabolic labeling of nerves of all rats were carried out at the same time. Values represent mean ± 1SD for each group.

heavier density (~ d = 1.20 g/ml). These radioactovities in the fractions of heavier density might represent free apo E dislodged from lipoprotein particles by ultracentrifugal force (7). In the injured nerves from non-diabetic rats, the increased radioactivities were found in the density between 1.05 and 1.125 g/ml. Treatment with streptozotocin induced overall increases in the radioactivities without any essential change of the distribution of apo E.

Time course of the effect of nerve injury on the secretion of apolipoprotein E into medium Time course experiment was carried out as a manner described in the legend of Fig. 2. In the control group, rats gained 38 ± 32 (n = 4), 108 ± 10 (n = 3), 223 ± 55 (n = 4), 260 ± 42 (n = 2) and 247 ± 69 g (n = 3) of body weight 7, 14, 28, 45 and 59 days after injury, respectively, while diabetic rats gained 11 ± 9 (n = 4), 0 ± 28 (n = 2), 32 ± 19 (n = 3), 113 ± 28 (n = 3) and 190 ± 35 g (n = 2), respectively.

Both the changes in TCA precipitable and immunoprecipitable radioactivities in the medium are shown in Fig. 2. In both control and diabetic rats, TCA precipitable radioactivity increased markedly on day 7, thereafter, decreased gradually, but even on day 45, the TCA precipitable radioactivities were significantly greater than those before injury ($p < 0.01$). There was no difference between the control and diabetic rats (Fig. 2A). The immunoprecipitable radioactivities for apo E were also significantly increased on day 7 and gradualy decreased both in control and in diabetic rats (Fig. 2B). In contrast to TCA precipitable radioactivities, the increase in the immunoprecipitable radioactivities for apo E in the diabetic rats was seven times and twice greater than that in control rats on day 7 and day 14, respectively ($p < 0.01$ and 0.05, respectively). Fig. 3 shows that the immunoprecipitation was specific for apo E.

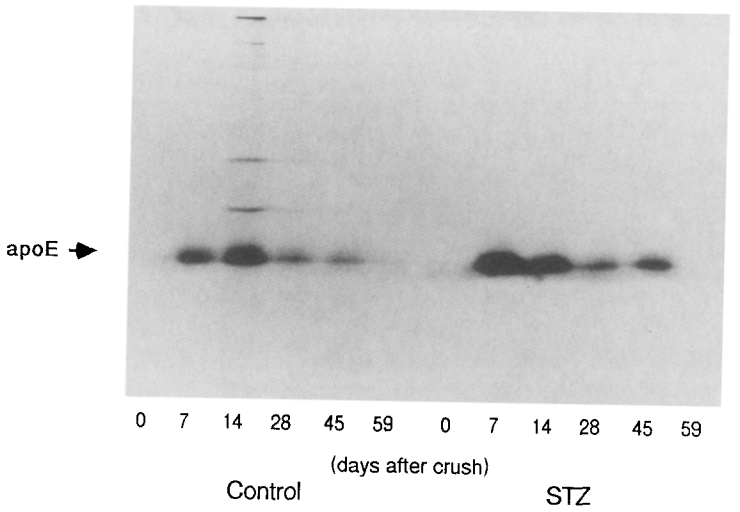

Figure 3. Immunoprecipitation of apo E. Nerves were incubated with [^{35}S]methionine and the medium was immunoprecipitated with anti apo E antiserum. The radioactivities of each sample was adjusted to give an equivalent values to the mean value of TCA precipitable radioactivities shown in Fig. 2A. Then the samples were combined in each group and subjected to SDS/polyacrylamide gel electrophoresis.

DISCUSSION

In the present study, we presented evidence that streptozotocin treatment enhances the apo E secretion from sciatic nerves in response to injury at an early stage and this effect was relatively selective for apo E. The secreted apo E into the medium from injured nerves of both non-diabetic and diabetic rats floated between hydrated density of 1.05 and 1.125 g/ml after density gradient ultracentrifugation. This indicated that apo E was secreted as a component of lipoproteins and the composition of the lipoproteins was very similar between the diabetic and non-diabetic rats. Thus the enhanced secretion of apo E by the injured nerves of diabetic rats is presumably associated with increased release of lipids from the nerve. Stimulation of the synthesis and secretion of apo E by streptozotocin treatment was observed only in the injured nerves. This suggests that streptozotocin treatment changes the response of nerve to injury, since we could not find the effects of the treatment in the non-injured nerves.

Some morphological studies on nerve injuries revealed increased activities of Schwann cells, appearance of macrophages, degeneration of axon and appearance of myelin debris during Wallerian degeneration (10, 11). Especially it has been reported that macrophages are able to synthesize and secrete apo E (12) and responsible for the production of apo E during nerve degeneration and regeneration (12). Therefore, if treatment of streptozotocin increses the activities and/or number of macrophages, the amounts of apo E secreted by the cells is expected to increase. There have been many studies showing that diabetic state induced by streptozotocin dramatically changes both physiological and anatomical characteristics of peripheral nerves (14-16). Our present

study disclosed one of the biochemical changes in peripheral nerves following streptozotocin administration, although the study could not exclude the possibility that the changes are due to the pharmacological effect of streptozotocin itself. However, the results of this study gives an intriguing implication that diabetes auguments the apo E secretion by nerve cells in response to injury and this contribute to the development of diabetic neuropathy. To our knowledge, there have been no reports on the regulation of apo E secretion from nerves so far. This study first provides us with evidence that apo E secretion from nerves can be regulated by either pharmacological agents or diabetic state.

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