Phospholipid Metabolism and Protein Phosphorylation in Sciatic Nerve From Genetically Diabetic (*db/db*) Mouse

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The incorporation of [^{32}P]orthophosphate into phospholipids and proteins of sciatic nerve from genetically diabetic (*db/db*) and littermate control (*db/m*) C57BL/KsJ mice was studied. Nerves from animals of ages 12, 16, 22, 26, and 38 wk were incubated in vitro. Among phospholipids, the uptake of isotope into phosphatidic acid was higher at nearly all ages examined. Phosphorylation of several proteins, including the major myelin glycoprotein, P_0, and the small myelin basic proteins P_r + P_2, was significantly enhanced in nerves from both 12- and 38-wk-old diabetic mice. The altered pattern of protein phosphorylation, but not that of phospholipid metabolism, was similar to changes observed in sciatic nerve from streptozocin-induced diabetic rats. The relationship of the results to reported levels of *myo*-inositol, sorbitol, and Na^+-K^+-ATPase activity and to functional abnormalities in nerves of *db/db* mice is discussed. The findings suggest that caution should be exercised in reaching conclusions concerning which biochemical alterations observed in different animal models of diabetic neuropathy are invariably associated with the development of this disorder. *Diabetes* 37:1703–1707, 1988

Diabetic neuropathy in humans is characterized by electrophysiological and morphological alterations of peripheral nerves. In efforts to understand the molecular mechanism underlying this derangement, several potential animal models of the human disease have been studied. Streptozocin-induced diabetes (STZ-D) in the rat, which displays characteristics of the insulin-dependent (type I) form of the disease, is one such model and is associated with a significant reduction in motor nerve conduction velocity, increased content of sorbitol, decreased levels of *myo*-inositol, and diminished Na^+-K^+-ATPase activity in peripheral nerves (1). We have demonstrated the occurrence of alterations in the metabolism of polyphosphoinositides and in protein phosphorylation in nerves from STZ-D animals (2,3). Another widely studied model is the mutant diabetic (*db/db*) mouse, which displays an autosomal-recessive trait characterized by symptoms resembling non-insulin-dependent (type II) diabetes that appear spontaneously beginning at 5 wk of age. The affected animals develop distal symmetrical peripheral neuropathy with impairment of motor nerve conduction velocity, reportedly accompanied by axonal atrophy. Characteristics include hyperglycemia, obesity, and, early in the disease, high insulin levels (4–7). To determine whether metabolic abnormalities observed in the STZ-D rat were also characteristic of the *db/db* mouse, we investigated phospholipid metabolism and protein phosphorylation in sciatic nerves of these animals.

MATERIALS AND METHODS

Genetically diabetic (*db/db*) and heterozygous littermate control (*db/m*) C57BL/KsJ mice of both sexes between 5 and 6 wk of age were obtained from The Jackson Laboratories (Bar Harbor, ME). Groups of *db/db* and *db/m* mice were used for metabolic studies at 12, 16, 22, 26, and 38 wk of age. Animals were killed by decapitation, and blood was collected for serum glucose measurement.

Sciatic nerves were dissected from the sciatic notch to the popliteal fossa, and care was taken to remove nearly identical lengths. The tissue was incubated in 300 μl Krebs-Ringer bicarbonate buffer (pH 7.4), which contained 5.5 mM glucose and 50–100 μCi [^{32}P]orthophosphate. Each tube was flushed with 95% O_2/5% CO_2, capped, and incubated for 2 h at 37°C. After incubation, nerves were removed from the medium and washed four times with cold saline solution.

Phospholipids were extracted, separated by thin-layer

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TABLE 1
Body weight and serum glucose content of genetically diabetic mice

	Age (wk)				
	12	16	22	26	38
Weight (g)					
db/m	23.2 ± 0.4	25.3 ± 0.5	26.4 ± 1.3	25.5 ± 0.4	26.2 ± 1.4
db/db	46.7 ± 1.2	40.7 ± 1.2	54.5 ± 2.4	50.1 ± 1.5	56.7 ± 1.6
Serum glucose (mg/dl)					
db/m	125 ± 15	141 ± 5	160 ± 20	164 ± 8	125 ± 7
db/db	394 ± 42	653 ± 11	319 ± 27	361 ± 23	393 ± 27

Values are average ± SE for 6 animals. All results from *db/db* animals are different from lean controls (*db/m*) at $P < .01$.

chromatography, and measured for radioactivity in each band as previously described (2). Total lipid phosphorus was determined by the method of Bartlett (8). When protein phosphorylation was also examined, the contralateral nerve was homogenized in 150 μl of 1% sodium dodecyl sulfate (SDS) solution in an all-glass homogenizer. Separation and quantification of phosphate incorporated into proteins was carried out by SDS–polyacrylamide slab gel electrophoresis, and ^{32}P incorporation into individual protein bands was quantified as previously described (3).

Results were expressed as either picomoles or nanomoles of ^{32}P incorporated per mass of protein or total lipid phosphorus, respectively, as determined from the specific activity of [^{32}P]orthophosphate in the incubation medium. The percent distribution of label among phospholipid classes was also calculated. Statistical significance between means was tested with Student's *t* test.

RESULTS

General characteristics of diabetic animals. The body weights of *db/db* mice were approximately twice those of lean littermate controls at all ages studied (Table 1). Serum glucose levels of all diabetic animals were also significantly elevated. The lipid phosphorus content per nerve did not change with age between 12 and 38 wk. The averages ±SE for normal and spontaneously diabetic mice (*db/m*, 589 ± 36 nmol/nerve; *db/db*, 543 ± 40 nmol/nerve) were not significantly different from each other.

Incorporation of ^{32}P into nerve phospholipids of *db/db* mice. The amount of isotope incorporated into phospholipids is shown in Table 2. Compared with normal animals, the quantity of isotope taken up into phosphatidylinositol-4-phosphate (PIP) was decreased in 16- and 26-wk-old *db/db* mice but was not significantly changed at other ages. No alterations in incorporation of radioactivity into either phosphatidylinositol-4,5-bisphosphate (PIP_2) or phosphatidylinositol were observed. Among other phospholipids, a significant increase in isotope incorporated into phosphatidic acid occurred at nearly every age. The labeling of phosphatidylethanolamine was also higher than controls in nerves of 16-, 22-, and 38-wk-old diabetic mice, whereas entry of ^{32}P into phosphatidylcholine exhibited no change.

The overall incorporation of ^{32}P into lipids varied somewhat from nerve to nerve as well as between nerves from animals of different ages. In an effort to overcome this variability, data were expressed as percentages of isotope present in

TABLE 2
Incorporation of ^{32}P into phospholipids of sciatic nerves from *db/m* and *db/db* mice

Phospholipid	Age (wk)				
	12	16	22	26	38
Phosphatidylinositol-4,5-bisphosphate					
db/m	128 ± 16	385 ± 26	428 ± 20	248 ± 34	225 ± 21
db/db	145 ± 11	334 ± 16	484 ± 30	239 ± 16	263 ± 25
Phosphatidylinositol-4-phosphate					
db/m	100 ± 7	150 ± 7	212 ± 15	118 ± 5	136 ± 13
db/db	113 ± 7	120 ± 8*	195 ± 10	88 ± 9†	122 ± 9
Phosphatidylinositol					
db/m	96 ± 6	140 ± 7	173 ± 9	126 ± 8	96 ± 6
db/db	102 ± 4	140 ± 5	188 ± 10	107 ± 4	102 ± 7
Phosphatidic acid					
db/m	76 ± 5	122 ± 2	143 ± 10	92 ± 8	100 ± 7
db/db	106 ± 5*	136 ± 10	200 ± 12*	118 ± 7†	134 ± 14†
Phosphatidylcholine					
db/m	238 ± 14	284 ± 16	349 ± 26	232 ± 9	179 ± 11
db/db	264 ± 7	288 ± 6	409 ± 28	225 ± 9	214 ± 14
Phosphatidylethanolamine					
db/m	24 ± 2	39 ± 3	39 ± 4	23 ± 1	19 ± 1
db/db	24 ± 2	47 ± 3†	55 ± 4*	25 ± 1	26 ± 2†

Results are expressed as nanomoles of ^{32}P incorporated per millimole of lipid phosphorus. Each value represents the mean ± SE for 6 animals.
*$P < .01$, †$P < .05$.

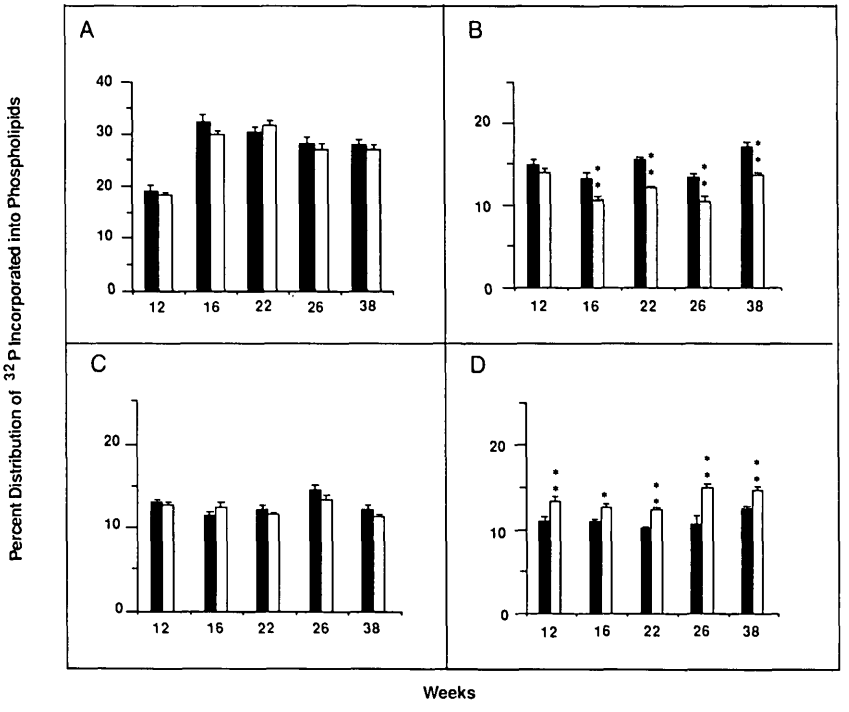

FIG. 1. Distribution of ^{32}P incorporated into sciatic nerve phospholipids (*A*, phosphatidylinositol-4,5-bisphosphate; *B*, phosphatidylinositol-4-phosphate; *C*, phosphatidylinositol; *D*, phosphatidic acid) from *db/m* and *db/db* mice as function of age. Incubations and determination of isotope incorporation into individual phospholipids were carried out as described in MATERIALS AND METHODS. Results are expressed as percentages of isotope present in each phospholipid ± SE for 6 animals. *Solid bars, db/m; open bars, db/db.* *$P < .05$; **$P < .01$.

each phospholipid. When this was done, the distribution of label among phospholipid classes was similar at each age (Fig. 1), except at 12 wk, when the proportion of radioactivity was lower in PIP_2 and higher in phosphatidylcholine (data not shown). For diabetic nerve, a significant decline was evident in the proportion of label in PIP from ≥16 wk, and an increase in the fraction of radioactivity in phosphatidic acid was apparent at all ages.

Phosphorylation of *db/db* mouse sciatic nerve proteins. The phosphorylation of nerve proteins was investigated in control and diabetic mice 12 and 38 wk old. Similar to rat nerve (3), the principal labeled proteins were unidentified 180,000- and 38,000-M_r bands; P_0, the major myelin glycoprotein; and the myelin basic proteins P_1 and $P_r + P_2$. When developed gels were stained with fast green FCF (Bio-Rad, Richmond, CA; 3), there were no visible differences in the pattern of stained proteins obtained from control and diabetic mouse nerve (data not shown). The amount of ^{32}P incorporated into all three major myelin proteins was significantly elevated in nerves from 12- and 38-wk-old diabetic mice, with the exception of P_1 at 38 wk (Table 3). At the older age, there was also a significant increase in the percentage of isotope accounted for by both P_0 (*db/m*, 23.3 ± 1.4; *db/db*, 27.2 ± 1.0; $P < .05$) and $P_r + P_2$ (*db/m*, 16.9 ± 1.3; *db/db*, 21.5 ± 0.9; $P < .01$).

DISCUSSION

At all ages examined, the pattern of ^{32}P incorporation into mouse nerve phospholipids closely resembled that exhibited by rat nerve (2). The reason why a smaller fraction of isotope was present in PIP_2 in nerves from 12-wk-old animals is unknown, but this observation was also made in another experiment with animals of this age. Several factors could affect the status of this metabolically active phospholipid, including the activity of enzymes that synthesize and degrade PIP_2 and the rate of synthesis of [γ-^{32}P]ATP, which supplies the phosphate groups for formation of the polyphosphoinositides and for synthesis of phosphatidic acid from 1,2-diacylglycerol.

Nerves from the *db/db* mouse displayed a distinctive pattern of alterations in phospholipid metabolism. The most consistent was an elevated incorporation of isotope into phosphatidic acid. A decline in the labeling of PIP was also evident at some ages. These changes were more apparent when the data were expressed as the percent distribution of radioactivity among phospholipids. The abnormalities in phospholipid metabolism in *db/db* mouse nerve were unlike those previously described for sciatic nerve from the STZ-D rat, in which increased incorporation of ^{32}P into PIP_2 is a prominent feature.

TABLE 3
Phosphorylation of proteins in sciatic nerves from 12- and 38-wk-old *db/m* and *db/db* mice

Protein	Age (wk) 12	38
180,000-M_r band		
db/m	11 ± 3	4 ± 1
db/db	18 ± 1*	8 ± 1†
P_0		
db/m	34 ± 7	43 ± 7
db/db	52 ± 4*	73 ± 6†
P_1		
db/m	10 ± 2	14 ± 4
db/db	16 ± 1†	17 ± 1
$P_r + P_2$		
db/m	20 ± 4	39 ± 8
db/db	34 ± 2†	58 ± 6*

Results are expressed as micromoles of ^{32}P incorporated per milligram of protein. Each value represents the mean ± SE for 6 animals. P_0, myelin glycoprotein; $P_r + P_2$, small myelin basic proteins. *$P < .05$, †$P < .01$.

The interpretation of metabolic data obtained with a heterogeneous tissue preparation such as diabetic mouse nerve is complicated by several considerations. These include the rate of ^{32}P uptake from the medium into the tissue and the regulation of ATP formation and its utilization. In addition, measurements of isotope incorporation into the phospholipids of whole nerve provide only a composite value, which does not take into account the possible presence of multiple metabolic pools of each phospholipid within structural elements of the tissue.

Available evidence indeed suggests the existence of distinct myelin and extramyelin pools of polyphosphoinositides in brain (9,10), and logically would be applicable in nerve as well. Recent findings also indicate that an appreciable portion of ^{32}P incorporated into phosphatidic acid in brain slices is recovered in purified myelin and is the outcome of the phosphorylation of 1,2-diacylglycerol rather than de novo synthesis of this phospholipid (10). A substantial fraction of ^{32}P-labeled phosphatidic acid is also present in myelin isolated from rat sciatic nerve that has been incubated with [^{32}P]orthophosphate (J. Lowery, L.N.B.-M., and J.E., unpublished observations). Assuming that stimulated phosphatidic acid labeling in *db/db* mouse nerve occurred partly or wholly in myelin, perhaps a larger or more accessible pool of diacylglycerol would be present at that site to serve as a substrate for phosphorylation. Although the turnover of 1,2-diacylglycerol in sciatic nerve has been demonstrated (11), virtually nothing is known concerning either the level or molecular-species composition of diacylglycerol in nerve, and this information will be important in identifying the likely phospholipid precursors that could generate the neutral lipid by means of phospholipase C–mediated degradation.

Greater availability of diacylglycerol should also tend to stimulate protein kinase C. Because the basic proteins and glycoprotein P_0 are both substrates for an endogenous myelin protein kinase C (12–14), the increased phosphorylation of these proteins observed in *db/db* mouse nerve, a phenomenon previously documented for STZ-D rat nerve (3,15), could result from activation of this enzyme. In support of this hypothesis, the increment in the percentage of ^{32}P incorporated into nerve proteins accounted for by P_0 observed in mouse nerve was also evident in diabetic rat nerve (3); in the latter tissue, the incorporation was not additive with the increase in phosphorylation elicited by phorbol dibutyrate (15), a well-known stimulator of protein kinase C activity. An alternative explanation for the rise in protein phosphorylation, i.e., an elevated specific activity of ATP, is unlikely because the polyphosphoinositides would be expected to exhibit enhanced labeling as well.

The development of diabetic neuropathy in the *db/db* mouse has been divided into two periods (4,7). The early, or metabolic, stage is considered to last until animals are ~16 wk of age and is characterized by hyperinsulinemia and a decrease in motor nerve conduction velocity that occurs almost simultaneously with the elevation of serum glucose levels detected in 5- to 6-wk-old mice. Insulin can partially restore the conduction defect. In the subsequent late, or neuronal, stage, insulin levels are nearly normal, and animals do not respond to the hormone. During this period, sciatic nerve shows morphometric alterations (4,16) and a reduction in the slow axoplasmic transport of acetylcholinesterase (17) and norepinephrine (18) concomitant with a decrease in the slow component *a* of cytoskeletal protein axonal transport (19).

In seeking to relate the metabolic changes we detected to the biochemical events underlying the development of deranged mouse nerve function, we found that the alterations in phospholipid metabolism and protein phosphorylation persisted through both stages, thus suggesting that the metabolic abnormalities are more likely to be the consequence of sustained hyperglycemia than high insulin levels. Moreover, in *db/db* mouse nerve, several of the other alterations documented in nerves of both the STZ-D rat and the genetically diabetic BB rat strain either do not occur or are not well established. In particular, sorbitol does not appear to be elevated, implying a lack of aldose reductase activity; Na^+-K^+-ATPase is not depressed; and whether *myo*-inositol is significantly reduced is equivocal (20–22).

Taken together, these biochemical findings are consistent with the existence of a link between the manifestation of increased sorbitol content, depressed *myo*-inositol levels, and enhanced PIP_2 turnover, all of which are present in the STZ-D rat and apparently either absent or much less pronounced in the *db/db* mouse. At least in the *db/db* model, functional deficits considered characteristic of diabetic neuropathy, e.g., reduced conduction velocity and impaired axonal transport, can develop without these accompanying compositional and metabolic changes. The possibility that the observed biochemical differences among the several animal models may be related to distinct pathogenetic mechanisms for diabetic neuropathy that arise as a consequence of type I and type II diabetes can be excluded because we have found that nerves from the Wistar fatty diabetic rat, another promising model for type II diabetes (23), display the same pattern of changes in sorbitol and inositol content, phosphoinositide metabolism, and protein phosphorylation as does the STZ-D rat (L.N.B.-M., J. Lowery, R.G. Peterson, and J.E., unpublished observations). Thus, further work will be required to pinpoint the biochemical abnormalities that are essential to the development of experimental diabetic neuropathy in all available animal models.

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REFERENCES

1. Sharma AK, Thomas PK: Animal models: pathology and pathophysiology. In *Diabetic Neuropathy*. Dyck PJ, Thomas PK, Asbury AK, Winegrad AI, Porte D, Eds. Philadelphia, PA, Saunders, 1987, p. 237–52
2. Bell ME, Peterson RG, Eichberg J: Metabolism of phospholipids in peripheral nerve from rats with chronic streptozotocin-induced diabetes: increased turnover of phosphatidylinositol-4,5-bisphosphate. *J Neurochem* 39:192–200, 1982
3. Schrama LH, Berti-Mattera LN, Eichberg J: Altered protein phosphorylation in sciatic nerve from rats with streptozocin-induced diabetes. *Diabetes* 36:1254–60, 1987
4. Robertson DM, Sima AAF: Diabetic neuropathy in the mutant mouse C57BL/Ks (*db/db*): a morphometric study. *Diabetes* 29:60–67, 1980
5. Sima AAF, Robertson DM: Peripheral neuropathy in the diabetic mutant mouse: an ultrastructural study. *Lab Invest* 40:627–32, 1979
6. Moore SA, Peterson RG, Felten DL, Cartwright TR, O'Connor BL: Reduced sensory and motor conduction velocity in 25 week old diabetic C57BL/Ks (*db/db*) mice. *Exp Neurol* 70:548–55, 1980
7. Norido F, Canella R, Zanoni R, Gorio A: Development of diabetic neuropathy in the C57BL/Ks (*db/db*) mouse and its treatment with gangliosides. *Exp Neurol* 83:221–32, 1984
8. Bartlett GR: Colorimetric assay methods for free and phosphorylated glyceric acids. *J Biol Chem* 234:466–68, 1959

9. Gonzales-Sastre F, Eichberg J, Hauser G: Metabolic pools of polyphosphoinositides in rat brain. *Biochim Biophys Acta* 248:96–104, 1971
10. Kahn DW, Morell P: Phosphatidic acid and phosphoinositide turnover in myelin and its stimulation by acetylcholine. *J Neurochem* 50:1542–50, 1988
11. Yao J: Metabolic turnover of fatty acids and acylglycerols in rat sciatic nerve. *J Neurochem* 45:589–95, 1985
12. Scott Turner R, Jen Chou CH, Kibler RF, Kuo JF: Basic protein in myelin is phosphorylated by endogenous phospholipid-sensitive Ca^{2+}-dependent protein kinase. *J Neurochem* 39:1397–1404, 1982
13. Murray N, Steck AJ: Activation of myelin protein kinase by diacylglycerol and 4β-phorbol 12-myristate 13-acetate. *J Neurochem* 46:1655–57, 1986
14. Brunden KR, Poduslo JF: A phorbol-sensitive kinase catalyzes the phosphorylation of P_0 glycoprotein in myelin. *J Neurochem* 49:1863–72, 1987
15. Eichberg J, Berti-Mattera LN, Schrama LH, Lin C-J, Lowery J, Rowe-Rendleman C, Zhu X, Peterson RG: Phosphoinositide metabolism, protein phosphorylation and the pathogenesis of diabetic neuropathy. In *Phospholipid Research and the Nervous System. Biochemical and Molecular Pathology*. Bazan NG, Horrocks LA, Toffano G, Eds. Padua, Italy, Livania. In press
16. Sharma AK, Thomas PK, Gabriel TG, Stolinski C, Dockery P, Hollins GW: Peripheral nerve abnormalities in the diabetic mutant mouse. *Diabetes* 32:1152–61, 1983
17. Vitadello M, Couraud JY, Hassig R, Gorio A, Di Giamberdino L: Axonal transport of acetylcholinesterase in the diabetic mutant mouse. *Exp Neurol* 82:143–47, 1983
18. Giachetti A: Axoplasmic transport of noradrenaline in the sciatic nerves of spontaneously diabetic mice. *Diabetologia* 16:1–4, 1979
19. Vitadello M, Filliatreau G, Dupont JL, Hassig R, Gorio A, Di Giamberdino L: Altered transport of cytoskeletal proteins in the mutant diabetic mouse. *J Neurochem* 45:860–68, 1985
20. Gillon KRW, Hawthorne JN: Sorbitol, inositol and nerve conduction in diabetes. *Life Sci* 32:1943–47, 1983
21. Bianchi R, Boccasavia E, Vitadello M, Schiavinato A, Gorio A: Sciatic nerve ATPase activity is unaffected in diabetic mutant mice C57BL/Ks (*db*/*db*) mice. *Diabetes* 36:1082–85, 1987
22. Whiteley SJ, Tomlinson DR: Motor nerve conduction velocity and nerve polyols in mice with short-term genetic or streptozotocin-induced diabetes. *Exp Neurol* 89:314–21, 1985
23. Peterson RG, Sharma AK, Little LA, Neil M-A, Potter CG, Eichberg J: Peripheral nerve abnormalities in Wistar fatty diabetic rats. In *Lessons From Animal Diabetes II. Proceedings of the 2nd International Workshop*. Shafrir E, Renold AE, Eds. London, Libbey, p. 488–91, 1988