

# Role of Lipoproteins in the Delivery of Lipids to Axons during Axonal Regeneration\*

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Nerve fiber elongation involves the input of lipids to the growing axons. Since cell bodies are often a great distance from the regenerating tips, alternative sources of lipids have been proposed. We previously demonstrated that axonal synthesis of phosphatidylcholine is required for axonal growth (Posse de Chaves, E., Vance, D. E., Campenot, R. B. and Vance, J. E. (1995) *J. Cell Biol.* 128, 913–918; Posse de Chaves, E., Vance, D. E., Campenot, R. B. and Vance, J. E. (1995) *Biochem. J.* 312, 411–417). In contrast, cholesterol is not made in axons. We now show that when compartmented cultures of rat sympathetic neurons are incubated with pravastatin, in the absence of exogenously supplied lipids, cholesterol synthesis is inhibited and axonal growth is impaired. The addition of cholesterol to the axons or cell bodies of neurons treated with this inhibitor restores normal axonal elongation. Similarly, a supply of cholesterol via lipoproteins restores normal axonal growth. In contrast, lipoproteins do not provide axons with sufficient phosphatidylcholine for normal elongation when axonal phosphatidylcholine synthesis is inhibited. Thus, our studies support the idea that during axonal regeneration lipoproteins can be taken up by axons from the microenvironment and supply sufficient cholesterol, but not phosphatidylcholine, for growth. We also show that neither apoE nor apoA-I within the lipoproteins is essential for axonal growth.

During nerve regeneration, large amounts of lipids are required for remyelination and expansion of axonal membranes. Synthesis of new myelin by Schwann cells in the regenerating peripheral nerve has been extensively studied (1–4). An interesting model for regeneration of injured peripheral nerve, involving apolipoprotein E (apoE)<sup>1</sup> and the coordinated storage and redistribution of cholesterol, has been proposed (5) and supported by experimental evidence (6). After peripheral nerve

injury, axon degeneration and myelin destruction proceed rapidly. The degenerating nerve is infiltrated by blood-derived macrophages that are responsible for clearing axonal and myelin debris (4, 7, 8). It has been proposed that most of the cholesterol, and possibly other key lipids released by degenerating axons and myelin, accumulate within Schwann cells and macrophages that remain in the area of degeneration. The salvaged cholesterol appears to be reutilized by the regenerating myelin membranes (4, 9, 10) and by neurons for axonal membrane regeneration. However, direct evidence for the reutilization of cholesterol by axons has not been provided.

After nerve injury, synthesis of several proteins is induced in the distal, but not the proximal, segment of the injured nerve (11). One protein in particular, apoE, is produced by infiltrating macrophages and accumulates to levels 100–200-fold greater than in uninjured nerve (12–15). Synthesis of apoE in neurons *per se* has not been detected.<sup>2</sup> It has been proposed that apoE, together with apoA-I, which enters the nerve from the circulation, play a central role in the reutilization of cholesterol (6, 16, 17). Current evidence suggests that cholesterol from the cellular and myelin debris is first stored in endoneurial macrophages and is subsequently secreted by the macrophages to form cholesterol-rich, apoE/A-I-containing lipoproteins. When regeneration begins, the proximal stump of the nerve sends out numerous neurites. The tips of the neurites, as well as Schwann cells, express high levels of LDL receptors, which have been postulated to participate in uptake of these lipoproteins (6, 17–19). More recently, studies on mice lacking functional apoE, apoA-I, or both apoE and apoA-I genes revealed that neither apoE nor apoA-I is required for nerve regeneration in the peripheral nervous system (20, 21). These data suggest that cholesterol reutilization by Schwann cells can occur in the absence of these apolipoproteins, favoring the idea that considerable redundancy is built into the lipoprotein-assisted process of cholesterol reutilization (20, 21).

Although the assumption has been that lipoprotein-derived cholesterol is used for nerve regeneration, the use of cholesterol from this source for axonal growth has not been directly demonstrated. Moreover, the fate of other lipoprotein-derived lipids, such as phospholipids, in relation to axonal regeneration is unknown.

The model proposed above for peripheral nerve regeneration by reutilization of exogenously supplied cholesterol departs from the generally accepted idea that nearly all membrane materials (*i.e.* proteins and lipids) required for axonal growth are synthesized in the cell bodies and transported by anterograde transport mechanisms into the axons, where these molecules are utilized for the assembly of new membranes (22, 23). It is now clear that axons of rat sympathetic neurons synthe-

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<sup>1</sup> The abbreviations used are: apo, apolipoprotein; HDL, high density lipoprotein; LDL, low density lipoprotein; PtdCho, phosphatidylcholine; VLDL, very low density lipoprotein.

<sup>2</sup> M. Bussière, and E. Posse de Chaves, unpublished results.

size significant amounts of the major phospholipids (PtdCho, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, and sphingomyelin) as well as fatty acids (24, 25). Of the total amount of PtdCho present in axons, at least 50% is made locally in the axons. Indeed, axonal synthesis of PtdCho is essential for normal axonal growth (26). In contrast, cholesterol synthesis does not occur in axons (25).

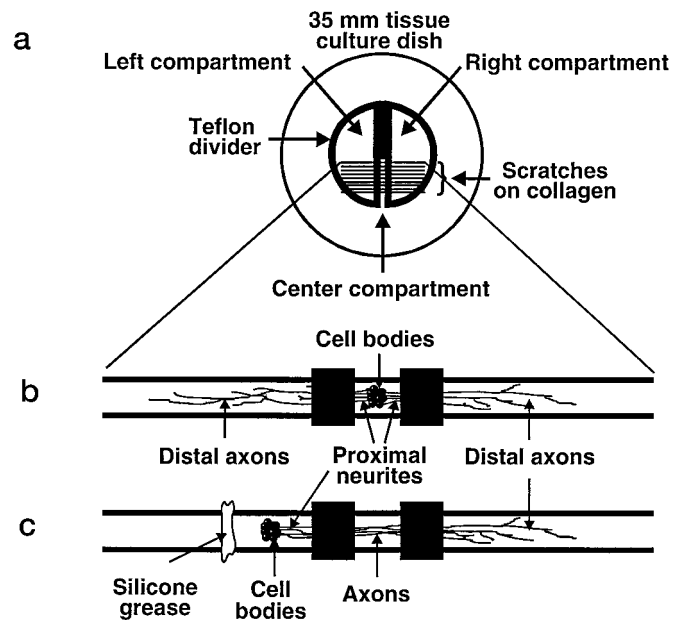
In the present study, we demonstrate that lipoproteins can be used by axons of rat sympathetic neurons as a source of cholesterol, but not PtdCho, for axonal elongation. We also show that the presence of apoE and/or apoA-I within the lipoproteins is not essential for axonal growth.

#### EXPERIMENTAL PROCEDURES

**Materials**—[1-<sup>14</sup>C]Acetic acid, sodium salt (specific activity 58 mCi/mmol) was purchased from Amersham Canada (Oakville, Ontario, Canada). Water-soluble cholesterol (methyl- $\beta$ -cyclodextrin-cholesterol) was provided by Sigma. Pravastatin was a gift from Dr. Shinji Yokoyama (University of Alberta). Thin layer chromatography plates (silica gel G) were obtained from BDH Chemicals (Edmonton, Alberta, Canada). Standard phospholipids and cholesterol were isolated from rat liver or purchased from Avanti Polar Lipids (Birmingham, AL). L15 medium without antibiotics was purchased from Life Technologies, Inc. Mouse 2.5S nerve growth factor was obtained from Cedarlane Laboratories Ltd. (Hornby, Ontario, Canada). Rat serum was provided by the University of Alberta Laboratory Animal Services. Polyclonal antibodies directed against human apoB were purchased from Boehringer Mannheim (Germany). Polyclonal antibodies directed against rat apoE and apoA-I were generated in rabbits and characterized in our laboratories by standard procedures (27). The reagents used for electrophoresis were supplied by Bio-Rad. Polyvinylidene difluoride membranes were from Millipore Corp. All other reagents were obtained from Sigma or Fisher.

**Preparation of Neuronal Cultures**—Procedures for growth of rat sympathetic neurons in compartmented cultures have been previously reported (28). Medium supplied to the compartment containing cell bodies (Fig. 1a) was supplemented with 2.5% rat serum, 1 mg/ml ascorbic acid, 10 mM cytosine arabinoside, and 10 ng/ml nerve growth factor. Cytosine arabinoside was added to prevent the growth of nonneuronal cells, resulting in cultures that were purely neuronal in gross appearance. In all growth experiments, delipidated rat serum (29) was used. Medium supplied to the axon-containing compartments contained 100 ng/ml nerve growth factor. After 6 days, cytosine arabinoside treatment was discontinued, and nerve growth factor was confined to the axon-containing compartments (30). Culture medium was changed every 3–6 days. Cells were cultured for 14 days prior to the start of experiments. For center-plated cultures, the center compartment contained cell bodies with some proximal axons; the left and right compartments each contained growing distal axons without cell bodies (Fig. 1b). Typically, the axons increased in length by  $\sim 1$  mm/day. In most experiments, as noted, cell bodies were plated in the left compartment, and the center and right compartments contained distal axons (Fig. 1c). When the neurons are plated in the left compartment, the center compartment is occupied by numerous distal axons that can be treated separately from cell bodies, and at the same time neurite elongation can be evaluated in the right compartment. One advantage of the three-compartment model for neuron culture is that metabolic events occurring in axons alone can be studied independently of those in cell bodies. This model is also applicable for studying axonal regeneration, since neurites can be mechanically removed from the side compartments and axonal extension can be accurately measured as neurites regenerate.

**Incorporation of [1-<sup>14</sup>C]Acetate into Cholesterol**—Neurons were plated in the center compartment of compartmented dishes and cultured for 14 days. The radioactive cholesterol precursor [1-<sup>14</sup>C]acetic acid (10  $\mu$ Ci/ml) was added to the cell body-containing compartment. After incubation periods of 1 day and 2 days, the radioactive medium was removed, the cells were washed twice with cold phosphate-buffered saline, and cellular material was harvested by the addition of methanol/water (1:1, v/v) to the center and side compartments separately. The lipids were extracted by the addition of chloroform to a final chloroform/methanol/water ratio of 2:1:1 (v/v) (31). The lipid samples were applied to thin layer chromatography plates, which were developed in the solvent system diisopropyl ether/acetic acid (96:4, v/v) using unlabeled cholesterol as carrier. The band corresponding to authentic cholesterol was scraped from the plate and radioactivity was measured. Radioactivity was normalized to total phospholipid mass measured in the same extract (32). To evaluate the effect of pravastatin on the incorporation



**FIG. 1. The three-compartment model for culture of rat sympathetic neurons.** Shown is a representation of a compartmented culture. *a* illustrates an entire culture, and *b* is an enlargement of a single track of a culture in which neurons are plated in the center compartment. Neurites extend to the left and right, under silicone grease barriers, and into the separate fluid environments of left and right compartments. *c* is an enlargement of a single track of a culture in which neurons are plated in the left compartment. Neurites cross under a silicone grease barrier, span the center compartment, cross under a second barrier, and extend into the right compartment.

of [1-<sup>14</sup>C]acetic acid into cholesterol, neurons (14 days old) cultured in 24-well dishes were incubated with medium containing [1-<sup>14</sup>C]acetic acid in the presence or absence of 50  $\mu$ M pravastatin. Pravastatin was dissolved in water to make a 10 mM stock solution, which was added to culture medium to give the desired final concentration of inhibitor. Extraction of lipids and isolation of cholesterol was performed as indicated for compartmented cultures.

**Separation and Analysis of Lipoproteins and Apolipoproteins**—VLDL, LDL, and HDL were isolated from human plasma by sequential ultracentrifugation on a benchtop Beckman TL-100 ultracentrifuge as described by Brousseau *et al.* (33), which allows the separation to be completed in 1 day. HDL3 and HDL2 subfractions were further separated by heparin-Sepharose affinity chromatography (34). The apolipoprotein content of the lipoprotein fractions was determined by sensitive Coomassie Blue staining (Sigma) and immunoblotting, using polyclonal antibodies directed against human apoB, rat apoE, and rat apoA-I. Proteins were separated by electrophoresis on 3–15% gradient polyacrylamide gels that contained 0.1% SDS and then transferred to polyvinylidene difluoride membranes for 12 h at 50 V, and immunoblotting was performed as described previously (27).

**Measurement of Axonal Extension**—For measurement of axonal extension, distal axons were mechanically removed from left and right compartments in the case of center-plated neurons, or from the right compartment for left-plated neurons, with a jet of sterile distilled water delivered with a syringe through a 22-gauge needle. The water was aspirated, and the wash was repeated twice followed by the addition of fresh culture medium. This procedure, termed axotomy, effectively removes all visible traces of axons from the side compartments. Neurite growth was measured as described previously (35).

#### RESULTS

**Inhibition of Cholesterol Synthesis in Cell Bodies Impairs Axonal Elongation**—In previous studies using the compartmented model for culture of neurons (25), we detected no cholesterol biosynthesis, as measured by incorporation of [1-<sup>14</sup>C]acetic acid into cholesterol, in axons of rat sympathetic neurons. We have now shown that under normal culture conditions cholesterol is synthesized in the cell body-containing compartment and efficiently transported into the distal axons. Rat

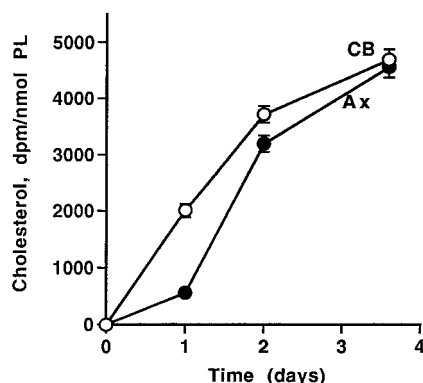


FIG. 2. **Cholesterol is synthesized in cell bodies and transported into axons.** Rat sympathetic neurons were maintained in compartmented cultures for 14 days. Medium containing 10  $\mu\text{Ci/ml}$  [ $^{14}\text{C}$ ]acetate was added to the central, cell body-containing compartment. At the indicated times, cellular material was harvested, lipids were extracted, and radioactivity was measured in cholesterol in the cell body-containing compartment (CB, open circles) and in the distal axon-containing compartments (left and right compartments) (Ax, solid circles). Data are dpm in cholesterol per nmol of total lipid phosphorous (PL) and are averages  $\pm$  S.D. of four separate cultures. The experiment was repeated twice with similar results.

sympathetic neurons (14 days old), cultured in three-compartment dishes, were incubated with [ $^{14}\text{C}$ ]acetate in the cell body-containing compartment alone. Radiolabeled cholesterol was isolated from the cell body-containing compartment and also from the left and right distal axon-containing compartments after time intervals of 1–3.5 days, and radioactivity was measured. Since the amount of neuronal material was small, we were unable to measure the mass of cholesterol. Instead, in this and other experiments, we related the radioactivity in cholesterol to the mass of cellular phospholipids. Fig. 2 shows that [ $^{14}\text{C}$ ]acetate had been efficiently incorporated into cholesterol in the cell body-containing compartment by 24 h, and some radiolabeled cholesterol had been transported into the distal axons. After 3.5 days, the amount of radiolabeled cholesterol in the distal axons corresponded to approximately 50% of total neuronal [ $^{14}\text{C}$ ]cholesterol present, and newly synthesized cholesterol had equilibrated throughout all compartments. This experiment implies that under these culture conditions cholesterol utilized for axonal membrane biosynthesis is derived from synthesis in the cell bodies, since no external source of cholesterol was provided to the axons.

We next determined if inhibition of cholesterol synthesis would inhibit axonal growth. Since in several cell types cholesterol synthesis is reduced by inhibitors of the rate-limiting enzyme of cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl-CoA reductase (36, 37), we examined the effect of one of these inhibitors, pravastatin, on cholesterol synthesis in rat sympathetic neurons. Neurons cultured in 24-well dishes for 14 days were incubated with [ $^{14}\text{C}$ ]acetate in the presence or absence of 50  $\mu\text{M}$  pravastatin. Fig. 3 shows that pravastatin had reduced the incorporation of [ $^{14}\text{C}$ ]acetate into cholesterol by approximately 83% at 24 h, demonstrating that cholesterol synthesis is inhibited by pravastatin in rat sympathetic neurons.

The response of neurite growth to the presence of pravastatin and the corresponding reduction of cholesterol biosynthesis was investigated. Neurons were plated in the center compartment of compartmented culture dishes for 14 days. Distal axons were removed from the side compartments by axotomy, and the neurons were subsequently incubated with 50  $\mu\text{M}$  pravastatin in either the center, cell body-containing compartment or the distal, axon-containing compartments. Axon extension was measured every day for the next 3.7 days (Fig. 4). Cultures that

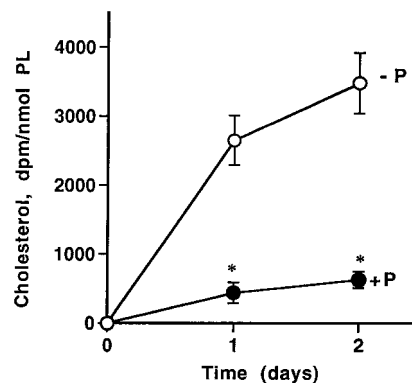


FIG. 3. **Pravastatin inhibits [ $^{14}\text{C}$ ]acetate incorporation into cholesterol.** Neurons (14 days old), cultured in 24-well dishes, were incubated with [ $^{14}\text{C}$ ]acetate in the presence (+ P, solid circles) or absence (– P, open circles) of 50  $\mu\text{M}$  pravastatin for the indicated times. Cells were collected, lipids were extracted, and cholesterol was isolated by thin layer chromatography. Data are dpm in cholesterol per nmol of total lipid phosphorous (PL) and are averages  $\pm$  S.D. of seven separate cultures. The experiment was repeated twice with similar results. Statistical significance of differences between groups was tested by Student's *t* test. The asterisk signifies statistical difference between cells cultured in the presence and absence of pravastatin  $p < 0.0005$ .

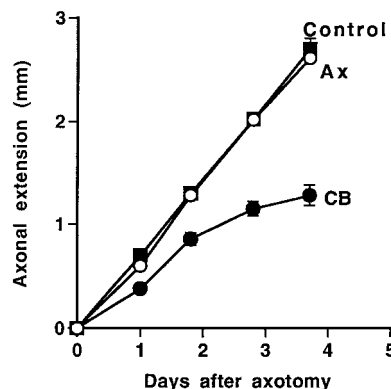
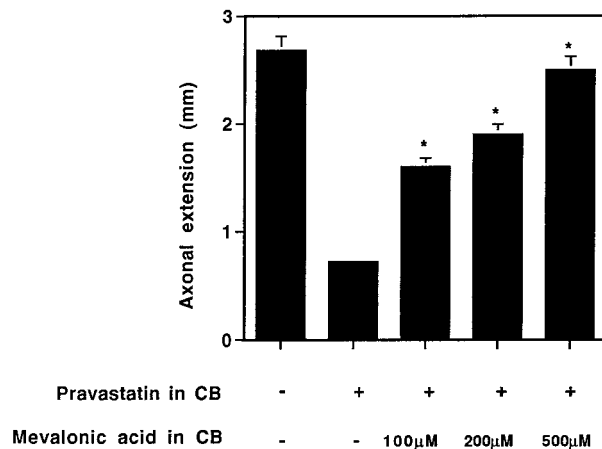


FIG. 4. **Inhibition of cholesterol biosynthesis impairs axonal elongation.** Neurons were plated in the center compartment of three-compartmented dishes and cultured for 14 days. At that time, cultures were axotomized, and 50  $\mu\text{M}$  pravastatin was supplied to the central, cell body-containing compartment alone (CB, solid circles) or to the side compartments containing distal axons alone (Ax, open circles). Control neurons (solid squares) were given medium without pravastatin throughout the experiment. Each point represents the mean  $\pm$  S.E. of axonal extension in 60–64 tracks. The experiment was repeated twice with similar results.

had been given pravastatin in the cell body-containing compartment alone had essentially stopped growing after 2.7 days. At all time points, axonal extension of pravastatin-treated neurons was statistically different from that of untreated neurons ( $p \leq 0.008$ ). As expected, since cholesterol synthesis is restricted to cell bodies, axons of cultures to which pravastatin had been added to the distal axon-containing compartments alone continued to elongate at the same rate as cells grown in the absence of pravastatin during the 3.7-day treatment period (Fig. 4). The effect of pravastatin on axonal growth was specifically related to the inhibition of cholesterol synthesis at the level of 3-hydroxy-3-methylglutaryl-CoA reductase, since the addition of mevalonic acid, the product of the reaction catalyzed by 3-hydroxy-3-methylglutaryl-CoA reductase, reversed the effect of pravastatin on axonal growth in a dose-dependent manner (Fig. 5). When mevalonic acid was added in the absence of pravastatin, axon growth was the same as in untreated cells (data not shown).

We conclude from these experiments that active cholesterol





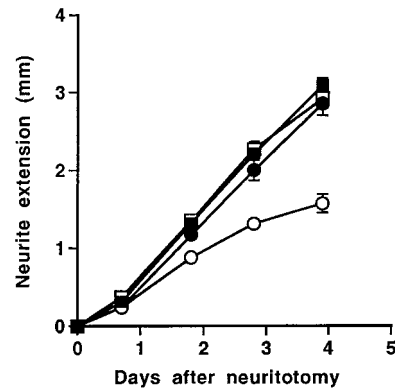
**FIG. 5. Mevalonic acid reverses the effect of pravastatin on axonal growth.** Neurons (14 days old) were cultured as indicated in the legend to Fig. 4. Axons in the side compartments were axotomized, and medium containing either pravastatin (50  $\mu$ M) alone or the indicated concentrations of mevalonic acid (100–500  $\mu$ M) with pravastatin was given to the cell body-containing compartment (CB). Control cultures were given medium lacking both pravastatin and mevalonic acid. Axonal extension was measured 3 days after axotomy. Results are means  $\pm$  S.E. of measurements from 60–64 tracks for each treatment. The experiment was repeated twice with similar results. The asterisk signifies the statistical difference between cultures treated with pravastatin in the presence or absence of mevalonic acid ( $p < 0.01$ ).

synthesis is required for normal axonal elongation of cultured rat sympathetic neurons and that synthesis of cholesterol in cell bodies supplies sufficient cholesterol for normal axonal extension.

**Cholesterol Can Be Supplied to Axons via Exogenous Delivery of Cholesterol or Lipoproteins**—Since in most cases in animals the tips of regenerating axons are localized at distances greatly removed from the cell bodies, cholesterol transport from the cell bodies to the regenerating sprout may not be the sole source of axonal cholesterol. An alternative scenario is that axons would “recycle” cholesterol salvaged from degeneration of both myelin and axons. We therefore investigated the ability of axons to utilize exogenous sources of cholesterol for elongation. Sympathetic neurons were plated in the left compartment of the three-compartment dishes (Fig. 1c) and allowed to grow for 14 days. Distal axons were removed from the right compartment, and the axons were allowed to regenerate under the following conditions. Incubation of neurons with pravastatin in the left, cell body-containing compartment impaired axonal extension, in agreement with the data shown in Figs. 4 and 5. Incubation of neurons with pravastatin and water-soluble cholesterol (cholesterol incorporated into  $\beta$ -cyclodextrin) in the cell body-containing compartment, or with pravastatin in the cell body-containing compartment and cholesterol in the axon-containing compartments (center and right compartments), allowed normal axonal growth (Fig. 6). (All values were not statistically different from controls at  $p < 0.1$ ). Cultures incubated without pravastatin in all three compartments were used as controls (Fig. 6). This experiment demonstrates that sympathetic neurons can use exogenous cholesterol added to either cell bodies or axons for axonal membrane biogenesis.

Cholesterol is normally delivered from the circulation to cells in the form of lipoproteins. The presence of lipoproteins has been demonstrated in the vicinity of regenerating and remyelinating nerves; however, no lipoprotein particles have been identified in noninjured nerves (6). These lipoproteins contain apoE and apoA-I as the major or exclusive apolipoproteins.

We investigated the ability of lipoproteins to provide cholesterol for axonal growth. Three different preparations of human

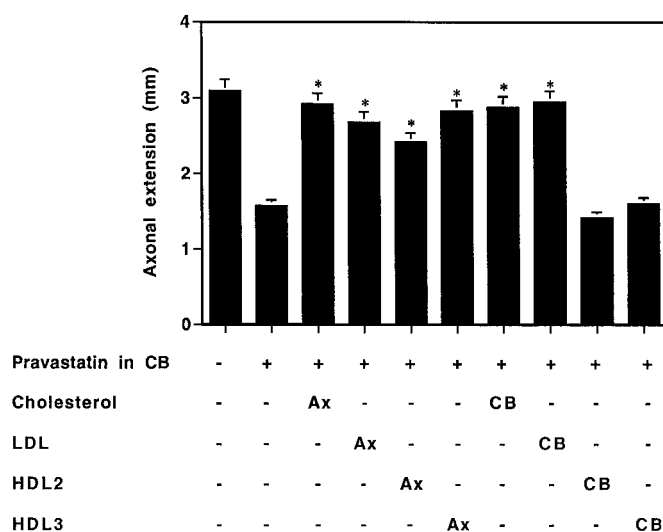


**FIG. 6. Exogenous cholesterol restores normal axonal growth.** Neurons were plated in the left compartments of compartmented dishes and allowed to grow for 14 days. Distal axons were removed from the right compartment, and medium containing one of the following was added: 50  $\mu$ M pravastatin in the cell body-containing compartment (open circles); 50  $\mu$ M pravastatin and 100  $\mu$ M cholesterol in the cell body-containing compartment (solid circles); 50  $\mu$ M pravastatin in the cell body-containing compartment and 100  $\mu$ M cholesterol in the center and right distal axon-containing compartments (open squares). Some cultures were incubated with medium lacking pravastatin and cholesterol in all compartments (solid squares). Axonal extension was measured at the indicated times. Data are averages  $\pm$  S.E. from measurements of 44–48 tracks. The experiment was repeated three times with similar results.

plasma lipoproteins were used as sources of cholesterol for axonal regeneration of cultured sympathetic neurons: LDL containing apoB; HDL2 containing apoE, apoA-I, and apoA-II; and HDL3 with apoA-I and apoA-II but with no apoE. The apoprotein content of the lipoproteins was verified by immunoblotting with anti-apoB, anti-apoA-I, and anti-apoE antibodies (data not shown). Sympathetic neurons were plated in the left compartment and allowed to grow for 14 days. At that time, the cells were axotomized, and cholesterol synthesis was inhibited by the addition of pravastatin to the cell body-containing compartment. To some cultures, lipoproteins were supplied to the distal axon-containing compartments alone, whereas to other cultures lipoproteins were added to only the cell body-containing compartment. The concentration of lipoproteins was normalized in terms of total cholesterol (100  $\mu$ g/ml of medium). A few cultures were given cholesterol (100  $\mu$ g/ml medium) for comparison. Axonal extension was measured after 4.2 days. In pravastatin-treated cultures, axonal extension was 54% less than in untreated cultures (Fig. 7). Cholesterol and all of the lipoproteins, when added to the distal axon-containing compartments, were able to overcome pravastatin inhibition of axonal elongation (Fig. 7), even LDL that lacked apoE and apoA-I. However, when given to the cell body-containing compartment alone (Fig. 7), only LDL, but not HDL2 or HDL3, was as effective as cholesterol in restoring neurite growth.

This experiment indicates that cholesterol supplied to the regenerating axons via exogenous delivery of lipoproteins satisfies the cholesterol requirement for axonal extension. Since HDL3 contains no apoE and LDL contains neither apoA-I nor apoE, we conclude that neither apoE nor apoA-I is required for delivery of lipoprotein cholesterol for growth of distal axons.

**Lipoproteins Do Not Provide Sufficient Phosphatidylcholine for Axonal Regeneration**—Since lipoproteins contain not only cholesterol but also phospholipids, we tested the hypothesis that lipoproteins can provide PtdCho for axonal growth. We have previously shown that when axonal PtdCho synthesis is inhibited by incubation of distal axons with choline-deficient medium (26) or with alkylphosphocholines (38), neurite growth is strongly impaired. Axonal growth is also inhibited during global choline deficiency. However, decreased PtdCho synthe-

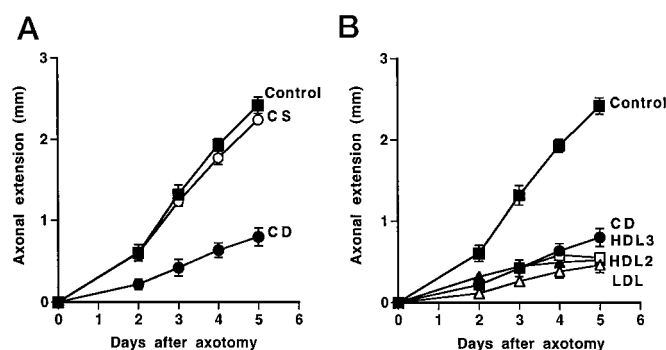


**FIG. 7. Reversal of pravastatin inhibition of axonal elongation by the addition of lipoproteins.** Neurons were cultured for 14 days as indicated in the legend to Fig. 6. After axotomy, the neurons were given 50  $\mu$ M pravastatin in the cell body-containing compartment. Simultaneously, either cholesterol (100  $\mu$ g/ml) or LDL, HDL2, or HDL3 (100  $\mu$ g of cholesterol/ml) was added to the center and right axon-containing compartments (Ax) or to the cell body-containing compartment (CB). Axonal extension was measured after 3.9 days. Data are means  $\pm$  S.E. of measurements of 45–48 tracks for each treatment. The statistical significance of the difference between groups was tested by Student's *t* test. \*, different from cultures treated with pravastatin alone ( $p \leq 0.05$ ). The experiment was repeated three times using different lipoprotein preparations, and similar results were obtained.

sis in the cell bodies does not inhibit axon extension (26). From these experiments, we concluded that PtdCho synthesis in distal axons, but not cell bodies, is required for normal axonal growth.

Neurons plated in the left compartment of three-compartment dishes were incubated with medium lacking choline in all three compartments for 3 days (preincubation). The axons in the right compartment were then axotomized and allowed to regenerate in the presence or absence of choline or PtdCho-containing lipoproteins. Axonal extension was measured every day for 5 days (Fig. 8). In cultures deprived of choline in all compartments, axonal growth was strongly diminished (Fig. 8A). All values were statistically different from those of control cultures incubated with choline throughout the experiment ( $p < 0.01$ ). However, when choline was added to the distal axon-containing compartments of cells that had been deprived of choline for 3 days, axonal growth resumed at a rate comparable with that of neurons that had been incubated with choline throughout the experiment, *i.e.* axonal extension of cultures incubated with choline was the same as for those supplemented with choline after 3 days of being choline-deficient ( $p > 0.5$ ). However, normal axonal elongation was not restored when any of the lipoprotein preparations (*i.e.* LDL, HDL2, or HDL3) was added to the compartment containing choline-deficient distal axons (Fig. 8B) ( $p > 0.5$  for all preparations at all time points). In these experiments, the lipoprotein concentration was adjusted to 100  $\mu$ g of cholesterol/ml of medium. Moreover, when the experiment was repeated with a 3-fold higher lipoprotein concentration, essentially the same results were obtained (data not shown). As expected, lipoproteins added to the cell body-containing compartment also failed to restore normal axonal growth (data not shown).

In many cell types, treatment with inhibitors of cholesterol synthesis (*e.g.* pravastatin) is known to up-regulate expression of LDL receptors (39). One possible explanation, therefore, for the failure of lipoproteins to restore normal axonal growth of



**FIG. 8. Lipoproteins do not provide PtdCho for axonal regeneration.** Left-plated neurons (14 days old) were incubated with medium lacking choline in all three compartments for 3 days. At that time, the cultures were axotomized and incubated with medium containing the following components: without choline in all three compartments (CD, solid circles); without choline in the cell body-containing compartment but with choline in the distal axon-containing compartments (CS, open circles); without choline in all compartments but with LDL (open triangles), HDL2 (open squares), or HDL3 (solid triangles) in distal axon-containing compartments; with choline in all compartments throughout the experiment (Control, solid squares). Data are the average  $\pm$  S.E. for measurements of 60–64 tracks for each treatment. The experiment was repeated three times with similar results.

choline-deficient neurons might be the presence of low levels of LDL receptors, which would result in an inefficient uptake of lipoproteins. We attempted to rule out this possibility by performing experiments parallel to those depicted in Fig. 8 except that the neurons were treated with pravastatin immediately after axotomy in an attempt to increase the number of LDL receptors. Axonal extension was measured 3 days after axotomy (Fig. 9). Choline-deficient neurons treated with pravastatin elongated very poorly compared with cells grown in choline-containing medium without pravastatin. However, if choline and cholesterol were given to the distal axon-containing compartments of choline-deficient neurons, together with pravastatin in the cell body-containing compartment, the neurons recovered normal axonal growth. On the other hand, neurons given lipoproteins (LDL or HDL) as a source of lipids did not resume normal axonal elongation.

These experiments imply that lipoproteins do not provide significant amounts of precursors of PtdCho synthesis or PtdCho itself for normal membrane biogenesis in axons.

#### DISCUSSION

In this paper, we show for the first time that regenerating rat sympathetic neurons can utilize cholesterol, but not PtdCho, from lipoproteins delivered to axons. The studies also show that synthesis of cholesterol in cell bodies is necessary and sufficient to sustain normal axonal growth of rat sympathetic neurons cultured in the absence of an exogenously added source of cholesterol.

We have previously demonstrated that axons of rat sympathetic neurons play an active role in providing lipids for axonal regeneration by synthesizing at least 50% of their own PtdCho. Indeed, synthesis of PtdCho by the axons is essential for axonal elongation, since PtdCho supplied by the cell bodies is not sufficient for optimal axonal growth (26). Cholesterol, on the other hand, is not synthesized in axons (25). In the adult animal, when axons are not elongating, the requirement for newly synthesized PtdCho for the maintenance of axonal membranes might be much higher than the requirement for cholesterol, since cholesterol is not catabolized within the cell and could be reutilized. During axonal regeneration, however, an input of cholesterol to the axons would be required. The observation that synthesis of PtdCho occurs in axons, whereas synthesis of cholesterol is restricted to cell bodies, suggested that

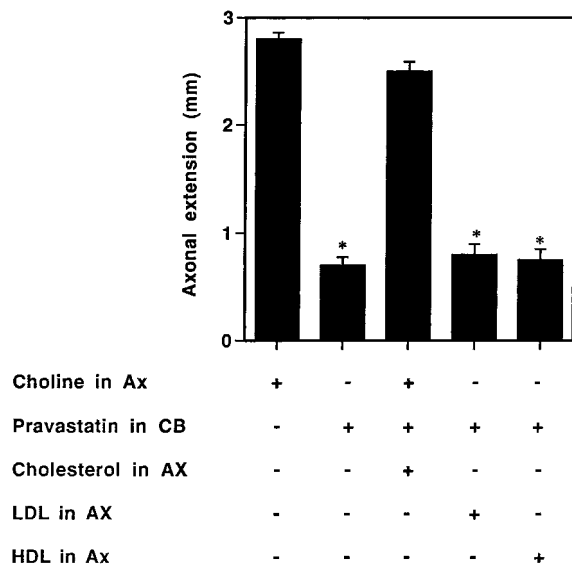


FIG. 9. **Lipoproteins do not restore normal axonal growth of choline-deficient neurons treated with pravastatin.** 14-day-old neurons cultured in left-plated cultures were incubated in medium lacking choline for 3 days. Distal axons were removed from the right compartment, and the neurons were given 50  $\mu$ M pravastatin in the cell body-containing compartment. Simultaneously, some cultures were given choline (11  $\mu$ M) and cholesterol (100  $\mu$ g/ml) or the lipoproteins LDL or HDL (100  $\mu$ g cholesterol/ml) in the distal axon-containing compartments. Cultures incubated without pravastatin in the cell body-containing compartment and with choline in the distal axon-containing compartments were used as controls. Axonal extension was measured 3 days after axotomy. Data are averages  $\pm$  S.E. for measurements of 40–48 tracks for each treatment. The statistical significance of the difference between groups was tested by Student's *t* test. \*, different from neurons given choline in the axons ( $p < 0.01$ ). The experiment was repeated twice with similar results.

*in vivo*, growth of axons might depend on an exogenous source of cholesterol, such as lipoproteins, whereas an exogenous source of PtdCho might not be utilized or required. The studies described herein support this hypothesis.

**Role of Lipoproteins in Supplying Cholesterol to Neurons—**Pheochromocytoma (PC12) cells, which can be stimulated to produce neurites *in vitro*, are able to take up apoE-containing lipoproteins via their growth cones (40). Consequently, lipids internalized by this mechanism have been postulated to be used in the assembly of new axonal membranes, providing an additional, local source of lipids for efficient axonal regeneration. However, lipoprotein-derived lipids have not been shown directly to be used for axonal extension. Boyles *et al.* (6) provided evidence for a novel lipid transport mechanism in normal and regenerating nerves. They suggested that after nerve injury, the concomitant rise in apoE production, the accumulation of apoE and apoA-I at sites of injury, the increased expression of LDL receptors in the denervated sciatic nerve during regeneration and remyelination, and the conservation of cholesterol within the degenerating nerve indicated that cholesterol was important for the robust membrane biogenesis required by the regenerating nerve. They also hypothesized that the rate of regeneration depended, in part, upon the efficiency of lipid transport from the external local environment to the elongating axon.

In support of the hypothesis that lipoproteins accumulating in injured peripheral nerves provide a vehicle for recycling cholesterol and cholesterol esters for membrane biogenesis during myelination and axon growth, Rothe and Müller (19) demonstrated that apoE-containing lipoproteins are taken up by Schwann cells and dorsal root ganglion neurons. These studies also showed that when dorsal root ganglion neurons were given

fluorescence-labeled endoneurial apoE-containing lipoproteins, the cell bodies, but not the neurites, were heavily labeled, suggesting that neurites did not participate in the uptake of lipoproteins. However, these authors did not evaluate the response of axonal growth or myelination to the addition of lipoproteins.

In contrast to the peripheral nervous system, the ability of neurons of the central nervous system to regenerate is very limited. However, presynaptic extensions from axons or terminals derived from undamaged neurons can proliferate as compensation for the loss of specific input. A mechanism allowing lipid redistribution during neuronal reinnervation in the central nervous system has been proposed, which involves the uptake of lipoproteins during synaptic remodeling (41).

The first direct evidence that apoE and/or apoE-containing lipoproteins modulate the outgrowth of neuronal processes was presented by Handelsmann *et al.* (42). Using rabbit dorsal root ganglia cells in culture, they showed that apoE facilitated the receptor-mediated uptake of the lipoprotein  $\beta$ -VLDL, resulting in an increase in the branching and in the length of neurites. The enhancement of neurite growth was attributed to both a direct effect of apoE and an effect of cholesterol in the  $\beta$ -VLDL. LDL receptors and the LDL receptor-related protein are both present in dorsal root ganglia neurons (41). The LDL receptor was found to be distributed on both the cell bodies and the neurites, whereas the LDL receptor-related protein was detected only on cell bodies. The distribution of these receptors in rat sympathetic neurons has not been reported.

We have now demonstrated that cholesterol, provided to axons of rat sympathetic neurons in the form of lipoproteins, can be used for biogenesis of new axonal membranes. It is noteworthy that the only source of cholesterol for these cultured neurons is synthesis in the cell bodies, since the rat serum used in these experiments was delipidated. Moreover, the medium bathing the distal axons contained no serum. The addition of cholesterol to the axons or cell bodies of neurons treated with pravastatin restored normal axonal elongation. Similarly, when human lipoproteins (LDL, HDL2, and HDL3) were supplied to the distal axons of neurons treated with pravastatin, normal axonal growth occurred.

Interestingly, growth of pravastatin-treated neurons recovered when LDL was added to either the cell body-containing compartment or to the compartments containing the distal axons. In contrast, normal growth did not resume when the cell body-containing compartment was given HDL2 or HDL3, whereas when these lipoproteins were added to distal axons, axonal growth was normalized. One possible explanation for these observations is that HDL or LDL or the cholesterol therein might be taken up by different mechanisms. For example, the differential expression of lipoprotein receptors (such as LDL receptor, the LDL receptor-related protein, and the recently described scavenger receptor, SR-B1 (43)) in different regions of the neurons might explain these findings, but to our knowledge the location of these receptors in sympathetic neurons has not yet been examined.

The presence of apoE and/or apoA-I within lipoproteins was proposed to be essential for the reutilization of myelin cholesterol within the regenerating nerve (6, 16, 17). Abnormalities in the level and metabolism of these apolipoproteins in the peripheral nerve have been correlated with a variety of peripheral neuropathies (44, 45). However, recent studies of mice lacking a functional apoE gene (20) or containing disrupted genes for both apoE and apoA-I (21) revealed that neither apoE nor apoA-I are required for nerve regeneration and that cholesterol reutilization in these animals continues to be mediated by endoneurial lipoproteins. In accordance with these findings,



we have shown that the presence of apoE and/or apoA-I in lipoproteins is not required for the recovery of normal axonal elongation in rat sympathetic neurons in which cholesterol biosynthesis has been inhibited. Indeed, LDL contains neither apoE nor apoA-I, yet it was able to deliver cholesterol to the axons. Moreover, HDL2 (which does not contain apoE) was as efficient a source of cholesterol as was HDL3 (which contains apoE).

**Role of Lipoproteins in Supplying Phosphatidylcholine to Neurons**—The phospholipid content of human lipoproteins ranges from 22% dry mass in LDL to 33 and 35% in HDL2 and HDL3, respectively (46, 47). However, our experiments demonstrated that lipoproteins were unable to provide the axons with sufficient PtdCho to elongate at a normal rate when PtdCho synthesis was inhibited by choline deficiency. At the lipoprotein concentrations used, the phospholipid concentrations were 120  $\mu$ M for LDL, 450  $\mu$ M for HDL2, and 484  $\mu$ M for HDL3. These concentrations were very high in terms of choline equivalents, since normal culture medium given to the neurons contains only 11  $\mu$ M choline. Even when the concentration of phospholipids in LDL in the distal axon-containing compartment was increased to 500  $\mu$ M, the same result was obtained. One explanation for these observations might be that lipoproteins are taken up by the axons by receptor-mediated endocytosis and retrogradely transported intact to the cell bodies where PtdCho would be hydrolyzed in lysosomes releasing choline in the cell bodies. We have recently demonstrated that choline provided to cell bodies does not support neurite growth in the absence of PtdCho synthesis in the axons (26). Alternatively, PtdCho internalized from lipoproteins might not be hydrolyzed in lysosomes in the cell bodies, and the transport of PtdCho from cell bodies to axons might be a less efficient process than transport of cholesterol to axons. A third possibility might be that PtdCho from lipoproteins is selectively not taken up by the axons. However, preliminary experiments performed in our laboratories indicate that both apolipoproteins and cholesterol from LDL and HDL are internalized by the axons and transported to the cell bodies, favoring the idea that intact lipoproteins are taken up as holoparticles. Moreover, we found that lipoproteins were unable to satisfy the requirement for PtdCho in choline-deficient, pravastatin-treated neurons, suggesting that either the LDL receptor is not involved in the axonal uptake of lipoproteins or that treatment with pravastatin does not up-regulate LDL receptor expression in these neurons.

Other reports have also suggested that phospholipids and cholesterol are provided to regenerating neurons by different mechanisms. For example, Goodrum (48) reported that [ $^{14}$ C]acetate incorporation into cholesterol and the activity of 3-hydroxy-3-methylglutaryl-CoA reductase are depressed during nerve regeneration. Similarly, decreased cholesterol synthesis was observed during ongoing reactive synaptogenesis and terminal proliferation in the central nervous system (41). In contrast, [ $^{14}$ C]acetate incorporation into phospholipids was increased for several weeks after peripheral nerve injury, concomitant with an elevation of radiolabeled glycerol incorporation into PtdCho. These findings were interpreted as reflecting increased phospholipid synthesis associated with remyelination (48), although the data are also consistent with increased axonal phospholipid synthesis during axonal regeneration.

In conclusion, our data support the hypothesis that during axonal regeneration lipoproteins are taken up by the axons from the microenvironment and that the different components of these particles (cholesterol and cholesterol esters, phospholipids, and apolipoproteins) are differently processed within the neuron. We are actively investigating the destiny of lipid and

apoprotein components of both LDL and HDL after uptake by the axons. Understanding the mechanisms by which lipids are taken up and used by neurons is essential for understanding axonal regeneration in the peripheral nervous system. Moreover, knowledge of how lipids are supplied to neurons might be essential for understanding the ability of the nervous system to compensate for the neuronal loss associated with normal brain aging and neurological diseases such as Alzheimer's and Parkinson's disease.

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