Lipoprotein lipase is expressed in rat sciatic nerve and regulated in response to crush injury

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Abstract **Male adult Sprague-Dawley rats were subjected to unilateral crush injury, and expression of LPL protein and mRNA were assessed as a function of time post-crush. LPL activity increased in the distal portion of the injured nerve by Day 4 post-crush, after which LPL activity gradually returned to normal levels. Conversely, quantification of LPL mRNA by reverse transcription-polymerase chain reaction demonstrated unchanged or decreased LPL mRNA in the distal nerve. Immunohistochemical analysis of LPL protein expression using an anti-rat LPL antibody revealed that LPL protein is present throughout the endoneurium of the sciatic nerve and increases in abundance following crush injury. The possibility that infiltrating macrophages are responsible for the increase in LPL protein levels in the crush injured nerve was addressed by immunohistochemical staining for ED-1, a differentiated macrophage marker protein. ED-1 was minimally present in the uninjured nerve and was detected at Day 4 post-crush, suggesting that the increase in LPL protein and activity that occurs following crush injury is at least partly derived from macrophages. These data suggest a role for LPL in the response of peripheral nerves to crush injury, possibly in order to facilitate reutilization of lipids from degenerating myelin.**—Huey, P. U., K. C. Waugh, J. Etienne, and R. H. Eckel. **Lipoprotein lipase is expressed in rat sciatic nerve and regulated in response to crush injury.** ***J. Lipid Res.*** **2002.** 43: **19–25.**

Supplementary key words myelin • peripheral nerve • ED-1 • Schwann cell • macrophage

LPL is a multifunctional enzyme produced by several tissues including adipose tissue and skeletal and cardiac muscle (1, 2). In these tissues, it is rate-limiting for the hydrolysis of the triglyceride core of the circulating triglyceride-rich lipoproteins, chylomicrons and VLDL. The reaction products, fatty acids and monoacylglycerol, are in part taken up by the tissues locally where they are processed in a tissue-specific manner, e.g., stored as neutral lipids (triglycerides or cholesteryl esters) in adipose tissue, or oxidized in muscle.

LPL is also present throughout the nervous system, including the brain, spinal cord, and peripheral nerve (1, 3-5). In the brain, LPL mRNA is found in dentate granule cells, as well as the CA1, CA2, and CA3 cells in the hippocampus, pyramidal cells in the cortex, and Purkinje cells in the cerebellum; however, the lipase protein is distributed on endothelial surfaces throughout the brain (4, 5). In the spinal cord, both the mRNA and protein are localized in tracts, areas where nerve cell bodies would not be expected (5); this observation suggested that LPL in the spinal cord is made by glial cells rather than neurons.

We have previously demonstrated that LPL is expressed by immortalized Schwann cells in vitro and functions to allow utilization of exogenous triacylglycerol for de novo lipid biosynthesis (6). We hypothesized that expression of LPL in the peripheral nerve may function to help maintain myelin lipid and to allow lipid reutilization from the myelin lipids degraded during injury-induced nerve degeneration. In this study, we investigate the expression of LPL in the rat sciatic nerve and examine its response to crush injury.

MATERIALS AND METHODS

Animals and nerve injury procedure

Adult male Sprague-Dawley rats (200–250 g) were anesthetized with an intraperitoneal injection of 12 mg/kg body weight xylazine and 80 mg/kg body weight ketamine. An incision was made in the lateral surface of the right thigh to expose the sciatic nerve, which was crushed for 10 s with a pair of fine forceps approximately 1 cm distal to the sciatic notch. The incision was closed with Autoclip staples, and the animals allowed to recover with free access to food and to water containing 0.64 mg/ml acetaminophen for the first 48 hours postoperative. Thereafter, the

Abbreviations: apo, apolipoprotein; KRP, Krebs-Ringer phosphate; RT-PCR, reverse transcription-polymerase chain reaction.

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rats were allowed chow and untreated water ad libitum. This crush injury protocol causes an immediate reduction of normal limb function, as evidenced by an inability to uncurl the toes and a reduced range of motion of the entire leg; these effects gradually wane over the time course investigated here as the nerve heals and mobility is restored.

LPL activity in proximal and distal segments of crush-injured nerve

On each of the indicated days post-crush, three rats were sacrificed by an overdose of sodium pentobarbital (>100 mg/kg body weight injected i.p.) and the left (uninjured) and right (injured) sciatic nerves were removed and placed in Krebs-Ringer phosphate (KRP) buffer on ice. The portions of the injured nerves proximal and distal to the crush sites were minced into ~1-mm pieces and incubated in KRP containing 13.3 μg/ml heparin at 37°C for 45 min to release LPL. Approximately equal-sized portions of the contralateral uninjured nerves were treated likewise. Duplicate aliquots of the supernatants were assayed for LPL activity as described (6), and data are expressed as nEq of FFA released per min per g nerve tissue.

LPL mRNA in proximal and distal nerve segments

Portions of the injured and contralateral nerves proximal and distal to the crush sites were isolated as above and homogenized in TRIzol reagent (Life Technologies). Total RNA was isolated according to the manufacturer's instructions. 1 μg of total RNA was subjected to reverse transcription-polymerase chain reaction (RT-PCR) quantification of LPL utilizing primers specific for LPL and 18S ribosomal RNA as described (6). Data are expressed as the relative ratio of LPL mRNA:18S rRNA in each segment.

Immunohistochemistry of LPL and ED-1 protein in sciatic nerve sections

Adult male Sprague-Dawley rats were anesthetized and subjected to unilateral sciatic nerve crush injury as described above. After the indicated times, rats were anesthetized with an overdose of sodium pentobarbital and perfused with ~150 ml PBS through the apex of the heart to flush out blood. The rats were then perfused with 250–300 ml cold 4% paraformaldehyde in PBS as described (5). The injured and uninjured sciatic nerves were removed and frozen in OCT embedding medium under liquid nitrogen, then transferred to −80°C prior to cryosectioning. Ten μm transverse and longitudinal sections of injured and uninjured nerves were obtained using a Minitome Plus cryotome (Triangle Biomedical Services, Raleigh, NC) and allowed to air-dry onto glass slides. The sections were rinsed with PBS to remove excess OCT and blocked overnight with 5% normal rabbit serum (Vector Laboratories) plus 1 mg/ml bovine serum albumin in PBS (BSA/PBS) at 4°C. For detection of LPL protein, the sections were incubated overnight at 4°C with purified IgG obtained from a goat anti-rat LPL antiserum (7) and diluted 1:40 in BSA/PBS. Parallel slides were incubated with purified IgG from goat preimmune serum as a control. The slides were rinsed six times with BSA/PBS and then incubated with biotinylated rabbit anti-goat IgG (Vector Laboratories, 5 μg/ml in BSA/PBS) overnight at 4°C. The sections were then rinsed three times with BSA/PBS and three times with PBS, then incubated with fluorescein-linked avidin D (Vector Laboratories, 5 μg/ml in PBS) for 2 h at RT in the dark. Following extensive rinsing with PBS, the slides were coverslipped using VectaShield fluorescence mounting medium (Vector Laboratories) and photographed using an Olympus DP-10 digital camera mounted on an Olympus BH-2 fluorescence microscope at an excitation wavelength of 490 nm.

To determine if the LPL signal coincided with macrophage infiltration at the injury site, nerve sections were obtained and blocked as above, then incubated with a mouse monoclonal antibody against rat ED-1 antigen (Serotec Ltd., 10 μg/ml in BSA/PBS) overnight at 4°C. Parallel slides were incubated with mouse IgG (Vector Laboratories, 10 μg/ml in BSA/PBS) as a control. After rinsing as above, the sections were incubated with a biotinylated horse anti-mouse IgG (Vector Laboratories, 5 μg/ml in BSA/PBS), followed by rhodamine-linked avidin D (Vector Laboratories, 5 μg/ml in PBS). After extensive rinsing, the slides were coverslipped and photographed as above at an excitation wavelength of 545 nm.

Statistical analyses

Unpaired two-tailed *t*-tests were used to assess differences between LPL activity and mRNA in injured and uninjured nerve segments and differences between LPL mRNA levels between proximal and distal nerve segments. Significant differences were assumed at $P < 0.05$.

RESULTS

The response of sciatic nerve LPL activity to crush injury differed markedly between the portions of the nerve proximal and distal to the crush site. No difference in LPL activity was observed in the nerve segments proximal to the crush site compared with uninjured nerves over the time course studied (**Fig. 1**, top panel). Although there was a tendency toward higher activity in the injured proximal nerve on Day 4, it was not significant (injured: 15.59, 6.23, and 5.32 nEq FFA/min/g tissue; uninjured: 2.83, 5.87, and 3.05 nEq FFA/min/g tissue, $P = 0.21$). However, in the distal segment, an initial drop in LPL activity was observed on Day 2 following crush injury. Thereafter, LPL activity increased in the distal portion and remained elevated until at least Day 21, after which activity levels returned to the levels observed in the uninjured nerve (Fig. 1, bottom panel).

Quantification of LPL mRNA was carried out by RT-PCR using 18S rRNA as an internal standard. LPL mRNA was increased approximately twofold in the proximal segments of crush-injured nerves at Day 4 post-injury ($P = 0.006$, data not shown); no statistically significant differences were demonstrated between injured and uninjured proximal nerve segments at the other time points examined. In contrast, distal segments of crush-injured nerves tended to have lower LPL mRNA levels throughout the time course of recovery until at least Day 35 post-crush (data not shown). When LPL mRNA levels in injured nerve segments were normalized to the levels in the corresponding uninjured nerve segment, the proximal segments displayed values approximating unity for all time points except Day 4 as noted above. Conversely, LPL mRNA levels in the distal segments were reduced to between 30% and 60% of uninjured distal segment values; however, with the exception of Day 4 ($P = 0.008$), there were no significant differences between normalized distal nerve LPL mRNA levels and those of the corresponding proximal segments (**Fig. 2**).

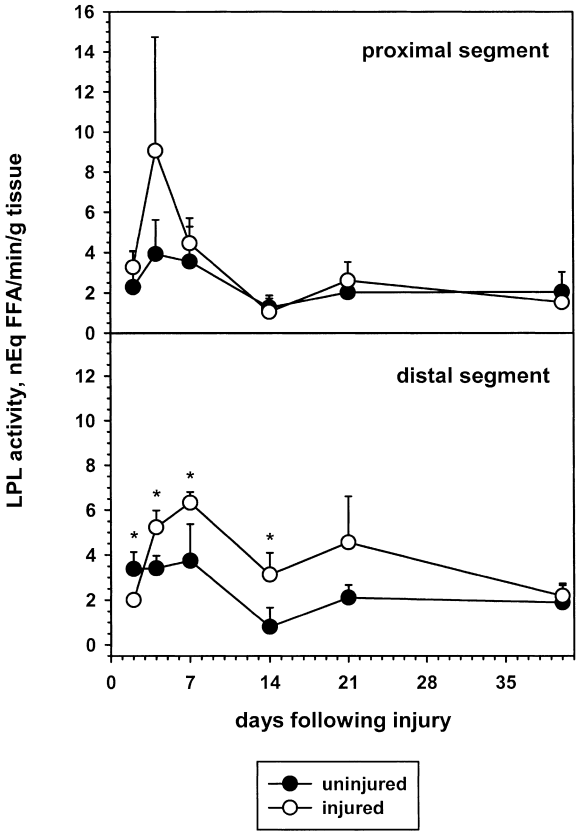

Fig. 1. LPL activity in proximal and distal segments of crush-injured rat sciatic nerve. Heparin-releasable LPL activity was measured in the nerve segments proximal (upper panel) and distal (lower panel) to the crush site of the injured nerve and in a mid-thigh point of uninjured nerve as described in Materials and Methods. Each point represents the average LPL activity ± SD of nerve segments isolated from three rats at each time point. * $P < 0.05$.

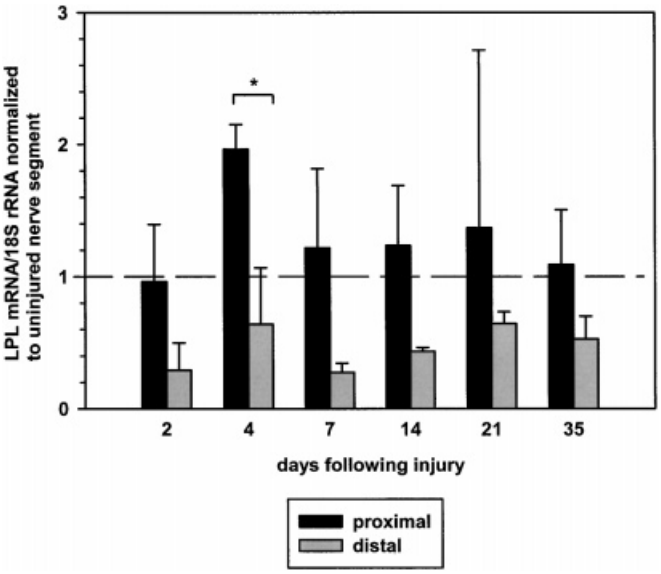

Fig. 2. LPL mRNA in injured proximal and distal nerve segments normalized to uninjured levels. LPL mRNA and 18S ribosomal RNA from nerve segments proximal and distal to the crush site of the injured nerves or at a mid-thigh point of the uninjured nerves were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) as described in Materials and Methods and quantified using digital image analysis of the ethidium bromide-stained gel. The LPL mRNA/18S rRNA value from each injured nerve segment was normalized to the value from the corresponding uninjured nerve segment for each rat. Each bar represents the average ± SD for three rats at each time point. * $P < 0.05$.

LPL protein was visualized in transverse and longitudinal sections of injured and uninjured rat sciatic nerve by fluorescence immunohistochemistry at various time points post-crush (**Fig. 3**). Control cross sections incubated with preimmune serum showed essentially no staining (Fig. 3A). Cross sections of uninjured sciatic nerve immunostained for LPL protein demonstrated general endoneurial fluorescence, with periaxonal and perivascular staining evident in the uninjured nerve. This localization of LPL protein is consistent with its synthesis by Schwann cells, macrophages, and/or fibroblasts within the endoneurium. Crush injury resulted in a disintegration of the normally ordered endoneurial structure at and distal to the crush site, followed by a marked increase in endoneurial fluorescence by Day 4. Longitudinal sections of crush-injured sciatic nerve demonstrated a similar pattern of expression, with increased LPL immunostaining evident as early as Day 2. A second experiment examining LPL protein expression at later time points post-injury showed persistent structural damage distal to the crush site up to Day 21 post-injury (Fig. 3B) and continued elevation of LPL-associated fluorescence until at least Day 7 post-crush.

The observation that LPL activity increased following crush injury in a time course coincident with the expected infiltration of macrophages led us to investigate whether macrophages might be the source of increased LPL in the injured nerve. Transverse and longitudinal sections of crush-injured sciatic nerve were incubated with a mouse monoclonal antibody against ED-1, a lysosomal and cell-surface marker that is expressed by tissue macrophages and peripheral blood granulocytes. Control sections were incubated with nonimmune mouse IgG. Staining for ED-1 showed little expression in either longitudinal or transverse sections on Day 2 post-injury, whereas Day 4 post-injury sections demonstrated highly localized ED-1 immunoreactivity at and distal to the crush site (**Fig. 4**).

DISCUSSION

Crush injury to a peripheral nerve induces a series of responses known collectively as Wallerian degeneration (8, 9). Myelin lipid and protein synthesis in the nerve portion distal to the injury site ceases as the preexisting myelin begins to degrade. HMG-CoA reductase, the rate-limiting step in cholesterol biosynthesis, is also downregulated as cholesterol released from the degrading myelin sheath is reutilized for new myelin synthesis (10). Axonal components also degenerate, and Schwann cells de-differentiate and proliferate in response to axonal and myelin debris.

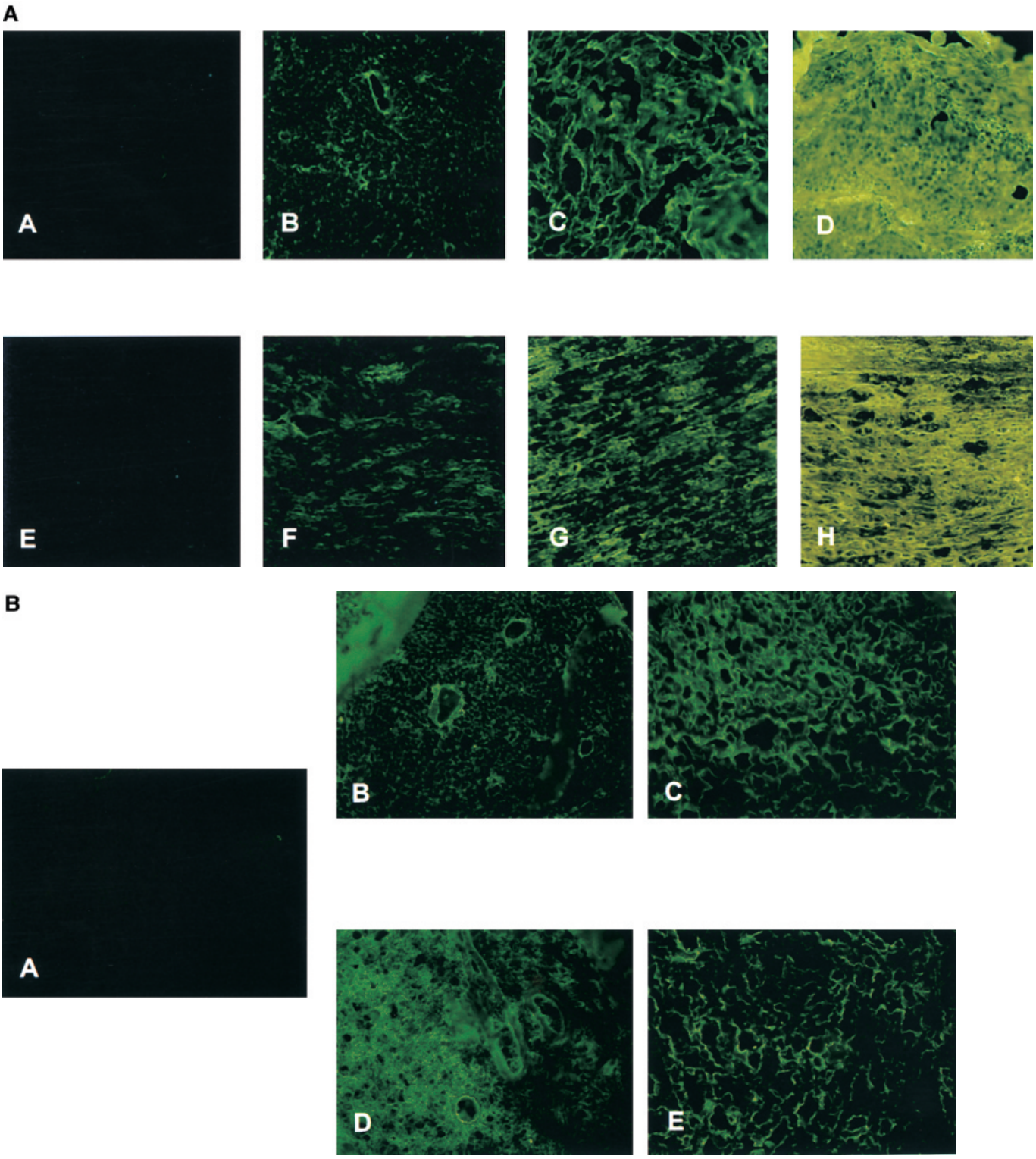

Fig. 3. Immunohistochemistry of LPL in sciatic nerve sections. Ten μm sections of sciatic nerve were incubated with purified IgG from either goat anti-rat LPL antiserum or goat preimmune serum, followed by biotinylated rabbit anti-goat IgG and avidin-fluorescein as described in Materials and Methods. A: Transverse (panels A–D) and longitudinal (panels E–H) sections from uninjured nerve incubated with preimmune goat antiserum (panels A and E) or anti-LPL antiserum (panels B and F), and sections obtained at the crush site of injured nerves on Day 2 (panels C and G) and Day 4 (panels D and H) incubated with anti-LPL antiserum. B: Transverse sections obtained just distal to the crush site from a second, longer-term experiment. Panel A, uninjured nerve incubated with preimmune serum; panels B–E, uninjured nerve (panel B) and injured nerve obtained on Days 2 (panel C), 7 (panel D), and 21 (panel E) incubated with anti-LPL antiserum.

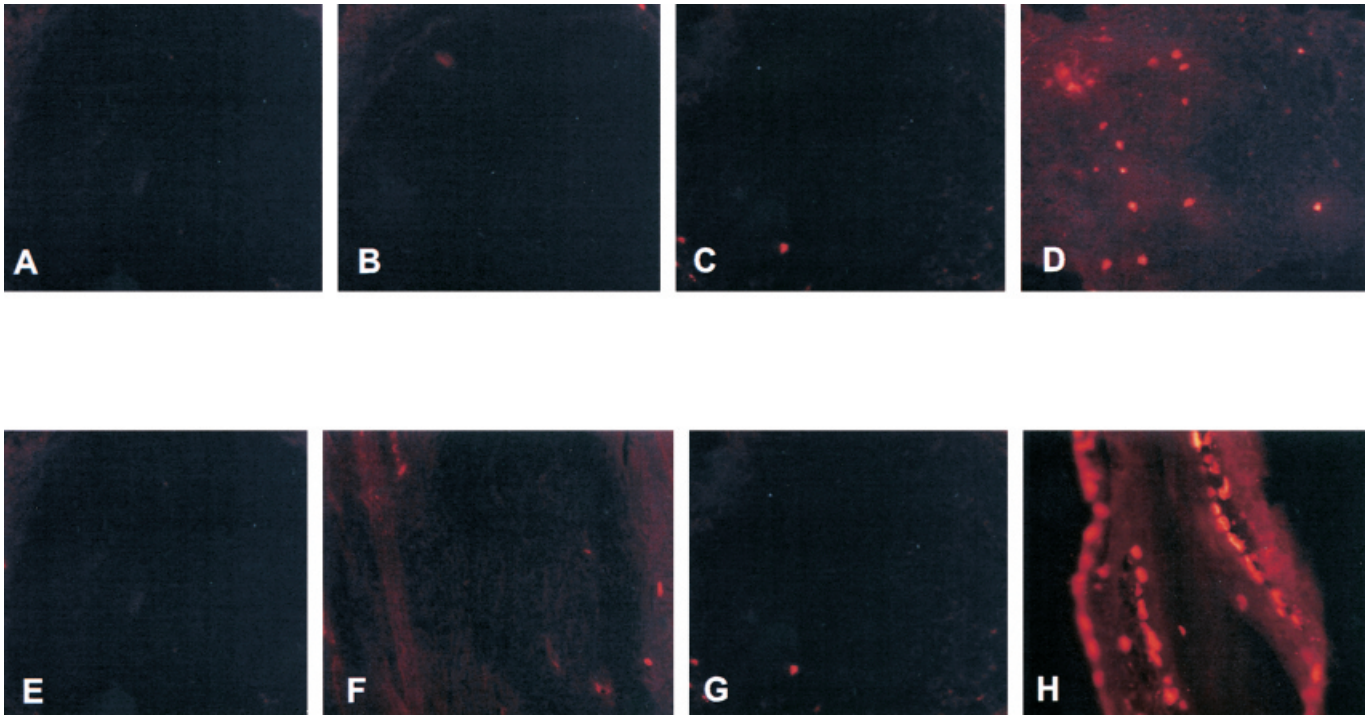

Fig. 4. Immunohistochemistry of ED-1 in sciatic nerve sections. Ten μm sections of sciatic nerve were incubated either with a mouse monoclonal antibody against rat ED-1 or with nonimmune mouse IgG, followed by biotinylated horse anti-mouse IgG and avidin-rhodamine as described in Materials and Methods. Transverse (panels A–D) and longitudinal (panels E–H) sections from uninjured nerve incubated with nonimmune mouse IgG (A and E) or anti-ED-1 antibody (B and F), and sections obtained at the crush site of injured nerves on Day 2 (C and G) and Day 4 (D and H) incubated with anti-ED-1 antibody.

Infiltration of macrophages into the distal stump also occurs soon after injury, and both macrophages and Schwann cells serve to scavenge myelin lipids from the degrading distal end. The lipids released by myelin degradation, primarily cholesterol and fatty acids derived from phospholipids, are actively reutilized as the proximal end regenerates.

Non-neuronal cells (macrophages and nonmyelinating Schwann cells) associated with the injured nerve secrete a protein (11, 12) that was identified as apolipoprotein (apo) E (13, 14). The identification of LDL receptors on the surface of neurites in vitro (15) and regenerating axons in vivo (16) suggested that the regenerating nerve takes up locally synthesized lipoprotein particles, composed of myelin lipids from the degenerating distal end, by LDL receptor-mediated binding to apoE. Indeed, an autoradiographic study directly demonstrated the recycling of myelin cholesterol into apoE-containing lipoproteins in the crush-injured nerve (17). The identification on the neuronal cell surface of the LDL receptor-related protein (LRP), which mediates uptake of β-VLDL enriched in apoE, further implicated apoE as a vital component of the regeneration process (18). The generation of apoE-null mice that display peripheral nerve abnormalities supports the involvement of apoE in the normal maintenance of nerve integrity (19). However, these mice as well as double "knockout" mice expressing neither apoE nor apoA-I are able to regenerate their peripheral nerves after crush injury (20, 21). Thus, other mechanisms exist in the peripheral nerve to facilitate provision of degraded myelin lipids to the regenerating nerve.

One such mechanism may be uptake via LPL. We have previously shown that LPL mRNA and cell-surface activity are expressed by cultured Schwann cells isolated from rat peripheral nerve. Inhibiting LPL activity with a polyclonal anti-LPL antiserum inhibited the cells' ability to utilize exogenous triacylglycerol-derived FFA for incorporation into newly synthesized phospholipids, triacylglycerol, and cholesteryl esters (6). Based on these results, we hypothesized that LPL in the nerve functions in maintenance of nerve myelin and may be involved in the recycling of myelin lipids that occurs upon nerve injury. In this study, we found that LPL mRNA and active protein are present in the normal, uninjured sciatic nerve. This finding supports the theory that LPL is involved in the maintenance of normal nerve structure and/or function, perhaps by facilitating FFA uptake and utilization by nerve cells. The specific cell types within the peripheral nerve that would benefit from the presence of LPL remain unknown. However, our previous in vitro results suggest that Schwann cells in the nerve in vivo may depend upon LPL for lipid uptake and normal maintenance of the myelin sheath. It is also possible that fibroblasts, satellite cells, and even the neuron itself may utilize FFA released by LPL activity.

Macrophages normally infiltrate a crush-injured nerve within 4 days following injury and remain associated with the injured nerve until at least 3 weeks post-injury, during which time they scavenge the lipids released by degrading

myelin. They also synthesize and secrete apoE, which associates with lipids to form lipoproteins that may be taken up by receptor-dependent or -independent means. We hypothesized that this process is also a potential source of endoneurial LPL in the injured nerve. To investigate whether macrophage infiltration and the increase in nerve LPL activity coincide, we incubated sections of injured nerve with a mouse monoclonal antibody against ED-1, a marker for differentiated myeloid cells that corresponds to the human CD68 antigen. We observed positive staining of macrophages within the distal portion of the injured nerve at Day 4 post-crush, whereas little signal was observed in Day 2. LPL activity in the distal portion of the crush-injured nerve was similarly elevated starting from Day 4 until Day 21 post-injury, after which it declined to uninjured nerve levels by Day 40. These observations suggest that macrophages contribute to endoneurial LPL activity beginning at Day 4 post-injury and continue to do so until the nerve heals. Interestingly, LPL activity in the distal segment was significantly decreased at Day 2 post-crush, a time point when we observed no decrease, and even a slight increase (Fig. 3) in endoneurial LPL protein levels. This result may suggest some form of regulation of endoneurial LPL activity. Alternatively, our LPL activity measurements may not represent active endoneurial LPL protein alone, as adherent adipose tissue may contaminate the nerve preparations, particularly of the injured distal segment.

Our previous results in cultured Schwann cells indicated that cell-surface LPL activity increases as a function of time in culture, possibly related to cell density but apparently not to LPL mRNA levels, which remained constant over time. In the present study, we examined the expression of LPL mRNA in the proximal and distal segments of crush-injured sciatic nerve. For all time points examined except Day 4, no changes in LPL mRNA were observed in the proximal segment, in agreement with the lack of difference in LPL activity. However, in the distal segment, where LPL activity and protein were increased at Day 4 and beyond, a trend of lower LPL mRNA levels was observed up until at least Day 35 post-injury. The regulation of LPL in the injured nerve may therefore be relatively complex, possibly due to the large number of cell types within the endoneurium both before and after crush injury. In addition, the observed effects of crush injury on LPL activity, protein, and mRNA levels may be related to components of a general inflammatory response other than infiltrating macrophages. We are currently attempting to address the differences in LPL mRNA expression between de-differentiated Schwann cells, actively myelinating Schwann cells, endoneurial fibroblasts, resident endoneurial macrophages, infiltrating macrophages, and the neuron itself using in situ hybridization in sections of crush-injured and normal sciatic nerve.

The LPL activity measurements obtained in this study were performed in vitro in the presence of an exogenous radiolabeled triolein substrate and human plasma as a source of apoC-II, an activator of LPL activity. In the case of the crush-injured nerve, apoC-II-containing lipoproteins are likely able to infiltrate the damaged tissue along with circulating macrophages; this influx of apoC-II may in part account for the increase in LPL activity observed in the injured sciatic nerve. It is of considerable interest whether apoC-II is available to the normal uninjured nerve to activate LPL. We are not aware of any reports documenting the presence of apoC-II in the peripheral nerve, and it may be that the function of LPL in the normal nerve is primarily structural rather than enzymatic. Catalytically inactive LPL can facilitate lipoprotein uptake by binding to proteoglycans, the LDL receptor (22), the LDL receptor-related protein (LRP) (23), gp330 (24), and the VLDL receptor (25), potentially through interactions with apoB (26). Nonenzymatic interactions of this type may play a role in myelin formation in the peripheral nerve by facilitating lipoprotein particle uptake and lipid utilization. We are currently investigating this possibility by examining animal models with deficient LPL activity in the sciatic nerve to determine the structural versus enzymatic role(s) of peripheral nerve LPL.

The authors gratefully acknowledge the excellent assistance of Ms. Tere Marcell, Mr. David Pennington, and the staff of the UCHSC Center for Laboratory Animal Care. This work was supported by NIH grant DK42286 (to RHE).

Manuscript received 23 April 2001 and in revised form 25 September 2001.

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