Statins Decrease Expression of the Proinflammatory Neuropeptides Calcitonin Gene-Related Peptide and Substance P in Sensory Neurons

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ABSTRACT

Clinical and experimental observations suggest that statins may be useful for treating diseases presenting with predominant neurogenic inflammation, but the mechanism(s) mediating this potential therapeutic effect are poorly understood. In this study, we tested the hypothesis that statins act directly on sensory neurons to decrease expression of proinflammatory neuropeptides that trigger neurogenic inflammation, specifically calcitonin gene-related peptide (CGRP) and substance P. Reverse transcriptase-polymerase chain reaction, radioimmunoassay, and immunocytochemistry were used to quantify CGRP and substance P expression in dorsal root ganglia (DRG) harvested from adult male rats and in primary cultures of sensory neurons derived from embryonic rat DRG. Systemic administration of statins at pharmacologically relevant doses significantly reduced CGRP and substance P levels in DRG in vivo. In cultured sensory neurons, statins blocked bone morphogenetic protein (BMP)-induced CGRP and substance P expression and decreased expression of these neuropeptides in sensory neurons pretreated with BMPs. These effects were concentration-dependent and occurred independent of effects on cell survival or axon growth. Statin inhibition of neuropeptide expression was reversed by supplementation with mevalonate and cholesterol, but not isoprenoid precursors. BMPs signal via Smad activation, and cholesterol depletion by statins inhibited Smad1 phosphorylation and nuclear translocation. These findings identify a novel action of statins involving down-regulation of proinflammatory neuropeptide expression in sensory ganglia via cholesterol depletion and decreased Smad1 activation and suggest that statins may be effective in attenuating neurogenic inflammation.

Statins, a class of drugs that inhibit HMG-CoA reductase (EC 1.1.1.34), the rate-limiting enzyme in mevalonate synthesis, are widely used to protect against cerebrovascular (Alvarez-Sabín et al., 2007; Tunón et al., 2007; Zivin, 2007) and cardiovascular (Richardson and Vasko, 2002; Collins et al., 2004) disease. Recent clinical and experimental observations suggest that statins may also be efficacious in the treatment of diseases linked to neurogenic inflammation, specifically migraine headache (Liberopoulos and Mikhailidis, 2006), rheumatoid arthritis (Leung et al., 2003; Abeles and Pillinger, 2006; Gazi et al., 2007), and reactive airway diseases (McKay et al., 2004; Kim et al., 2007). The authors of these studies postulated that the therapeutic actions of statins in these diseases were attributable to their known vascular and anti-inflammatory effects (Leung et al., 2003; McKay et al., 2004; Abeles and Pillinger, 2006; Liberopoulos and Mikhailidis, 2006; Gazi et al., 2007; Kim et al., 2007), but whether statins also interfere directly with cellular and molecular mechanisms of neurogenic inflammation has not been addressed.

Neurogenic inflammation is mediated by the release of proinflammatory substances from primary sensory nerve terminals, which in turn act on target cells in the periphery, such as mast cells, immune cells, and vascular smooth mus-
ple to produce inflammation (Richardson and Vasko, 2002; Schaible et al., 2005). The small diameter capsaicin-sensitive sensory neurons are of major importance in the generation of neurogenic inflammation and, of the various substances released by these sensory neurons, the neuropeptides calcitonin gene-related peptide (CGRP) and substance P are primarily responsible for initiating inflammatory responses (Richardson and Vasko, 2002; Schaible et al., 2005). CGRP and substance P are also implicated in the pathophysiology of migraine headache (Geppetti et al., 2005; Durham, 2006; Unger, 2006), rheumatoid arthritis (O'Connor et al., 2004; Bring et al., 2005), and reactive airway diseases (Carr and Undem, 2001; Springer et al., 2003; Gronenberg et al., 2004; O'Connor et al., 2004), and pharmacological inhibition of CGRP synthesis or release is effective in alleviating migraine symptoms (Olesen et al., 2004; Fischer et al., 2005; Goadsby, 2005; Goadsby et al., 2006). Because of the prominent role of CGRP and substance P in neurogenic inflammation, we investigated the possibility that statins modulate the expression of these proinflammatory neuropeptides.

Materials and Methods

Materials. Statins were purchased from LKT Laboratories (St. Paul, MN); GGTI-298, FTI-277, and SB202190 were obtained from Calbiochem (La Jolla, CA); mevalonate, cholesterol, and geranylgeraniol were purchased from Sigma-Aldrich (St. Louis, MO); and recombinant human Activin A was obtained from PeproTech Inc. (Rocky Hill, NJ). Curis (Cambridge, MA) generously provided recombinant human bone morphogenetic proteins (BMPs).

Animals. All experiments involving animals were carried out in accordance with the Institute of Laboratory Animal Resources (1996) as adopted and promulgated by the United States National Institutes of Health and as approved by the State University of New York at Buffalo and Oregon Health & Science University Institutional Animal Care and Use Committees. Male (200 g) Holtzman rats (Harlan, Indianapolis, IN) were given lovastatin (LVS; 20 mg/kg/day i.p.) or an equal volume (200 µl) of vehicle (1:1 ethanol/proplylene glycol) daily for 14 days, or atorvastatin (20 mg/kg/day p.o.) or an equivalent amount of vehicle (20% sucrose) daily for 7 or 21 days. Animals were allowed water and food ad libitum, and daily weighing indicated no treatment effects on body weight. Rats were euthanized 24 h after the last treatment, and thoracolumbar dorsal root ganglia (DRG) were harvested, flash-frozen, and stored at ~80°C.

Tissue Culture. Postmitotic sensory neurons were dissociated from DRG of embryonic (14-5 days) Holtzmann rats as described previously (Kleitman et al., 1998), plated at a density of 50 neurons/mm² onto glass coverslips precoated with poly-d-lysine (100 µg/ml) and laminin (3 µg/ml), and maintained in serum-free medium supplemented with β-nerve growth factor (NGF; 100 ng/ml). To eliminate endogenous non-neuronal cells, the antimitic cytokine β-d-arabinofuranoside (0.6 µM) was added to the culture medium for 48 h beginning 12 h after plating. Experimental treatments were performed on day 3 in vitro.

Reverse Transcriptase-Polymerase Chain Reaction. Total RNA was extracted from DRG cultures using RNeasy (QIAGEN, Valencia, CA). The Access reverse transcriptase-polymerase chain reaction (RT-PCR) system (Promega, Madison, WI) was used to reverse-transcribe RNA (1 µg) and amplify the resulting cDNA using previously described primers specific for α-CGRP and glyceraldehyde 3-phosphate dehydrogenase (Doi et al., 2000). PCR products were resolved by 2% agarose gel electrophoresis, and optical densities of ethidium bromide-stained bands were quantified using Quantity One software (Bio-Rad, Hercules, CA).

Immunocytochemistry. DRG cultures fixed in 4% paraformaldehyde were reacted with antibodies specific for α-CGRP (1:2500; as adopted and promulgated by the United States National Institutes of Health and as approved by the State University of New York at Buffalo and Oregon Health & Science University Institutional Animal Care and Use Committees. Male (200 g) Holtzman rats (Harlan, Indianapolis, IN) were given lovastatin (LVS; 20 mg/kg/day i.p.) or an equal volume (200 µl) of vehicle (1:1 ethanol/proplylene glycol) daily for 14 days, or atorvastatin (20 mg/kg/day p.o.) or an equivalent amount of vehicle (20% sucrose) daily for 7 or 21 days. Animals were allowed water and food ad libitum, and daily weighing indicated no treatment effects on body weight. Rats were euthanized 24 h after the last treatment, and thoracolumbar dorsal root ganglia (DRG) were harvested, flash-frozen, and stored at ~80°C.

![Fig. 1. Statins reduce neuropeptide expression in DRG in vivo and in vitro. Administration of LVS (20 mg/kg/day i.p.) to adult male rats (200 g) for 14 days reduced CGRP (A) and substance P (B) levels in thoracolumbar DRG (8–10 ganglia per animal) relative to control animals treated with an equal volume (200 µl) of vehicle (1:1 ethanol/proplylene glycol) as determined by RIA. C, likewise, treatment with atorvastatin (20 mg/kg/day p.o.) for 7 or 21 days reduced DRG levels of CGRP relative to controls treated with an equivalent amount of vehicle (20% sucrose). RIA values were normalized to total protein content. D, DRG neurons were cultured in the absence or presence of BMP2 (30 ng/ml) for 6 days before addition of LVS (0.1 µM) for an additional 6 days. The percentage of CGRP-IR neurons was determined every 3 days throughout the treatment period (n = 80 fields per experimental condition). Data are presented as the mean ± S.E.M. *p < 0.02 versus vehicle control.](https://jpet.aspetjournals.org/content/1173-1178)
Statins Inhibit BMP-Induced CGRP and Substance P Expression in Cultured DRG Neurons. There is evidence that BMPs and the related transforming growth factor-β superfamily member, activin A, mediate the up-regulation of CGRP and substance P expression in response to injury or inflammation (Cruise et al., 2004; Xu et al., 2005). To determine whether statins block neuropeptide induction by BMPs, cultured embryonic rat DRG neurons were exposed to BMP2 and lovastatin simultaneously for 3 to 4 days before collecting total RNA or protein for analyses by RT-PCR or RIA, respectively. BMP2 increased CGRP mRNA by approximately 60% (Fig. 2, A and B) and CGRP protein by 3-fold (Fig. 2C) relative to control levels, and these increases were completely blocked by lovastatin at 0.1 μM (Fig. 2, A–C).

Immunocytochemical analyses of DRG cultures revealed that BMP2 and lovastatin altered the percentage of neurons expressing CGRP. When maintained in medium containing NGF at concentrations optimal for cell survival, approxim-
mately 10% of neurons in DRG cultures were immunoreactive for CGRP, and immunoreactivity was restricted to neuronal cell bodies (Fig. 3, A, B, and K). Consistent with previous reports (Ai et al., 1999; Patel et al., 2000), varying the NGF concentration did not alter the percentage of CGRP-immunoreactive neurons (data not shown), but adding BMP2 increased the percentage of CGRP-immunoreactive neurons by 3 to 4-fold (Fig. 3K). In BMP2-treated cultures, CGRP immunoreactivity was evident in not only neuronal cell bodies (Fig. 3, C and D) but also axons (Fig. 3, E and F). Lovastatin did not alter the percentage of CGRP-immunoreactive neurons in control cultures (Fig. 3, G, H, and K); however, lovastatin significantly decreased the percentage of CGRP-immunoreactive neurons in BMP2-treated cultures and completely eliminated axonal CGRP immunoreactivity (Fig. 3, I–K). Lovastatin effects on CGRP expression were concentration-dependent with an IC50 of approximately 50 nM (Fig. 3L). Atorvastatin, simvastatin, and mevastatin also inhibited BMP2-induced neuropeptide expression with similar efficacy and potency as lovastatin (Fig. 3M). Pravastatin, which is hydrophilic and poorly absorbed by nonhepatic cells (Corsini et al., 1999), was included as a negative control and found to have no effect on BMP2-induced neuropeptide expression. Lovastatin also blocked induction of CGRP expression by either BMP7 or activin (Fig. 2N). BMP2 also increased the percentage of substance P-immunoreactive neurons in a concentration-dependent manner with an EC50 of 3 ng/ml (Fig. 3O), and this effect was similarly inhibited by...
lovastatin (Fig. 3P) with an IC_{50} (∼50 nM) comparable with that observed for lovastatin effects on BMP2-induced CGRP expression.

Decreased CGRP Immunoreactivity in Statin-Treated DRG Cultures Is Not Due to Increased CGRP Release or Decreased Cell Viability. Although the above data strongly suggest that statins decrease synthesis of neuropeptides, an alternative possibility is that statin treatment stimulates release of neuropeptides. To address this, DRG cultures were treated with BMP2 (30 ng/ml) ± lovastatin (0.1 μM) in the absence or presence of tetrodotoxin (TTX) and MgCl₂ at concentrations previously shown to block neuropeptide release in primary neuronal cell cultures (Sun et al., 1992). TTX/MgCl₂ treatment did not influence BMP and lovastatin effects on CGRP immunoreactivity (Fig. 4A).

Statins at concentrations ≥1 μM are reported to adversely affect cell survival in cultured neurons (Tanaka et al., 2000); therefore, we examined cell survival in lovastatin-treated DRG cultures. At 1 μM, a concentration 20-fold higher than the IC_{50} (50 nM), lovastatin decreased cell survival as determined by neuron number and percentage of neurons with fragmented nuclei (Fig. 4, B and C). However, lovastatin concentrations <1 μM that significantly inhibited BMP2-induced CGRP expression (Fig. 3L) had no effect on either parameter of cell survival (Fig. 4, B and C). To further evaluate cell viability, DRG cultures were analyzed by Western blotting using antibodies specific for phosphorylated forms of NF-H and NF-M. These cytoskeletal proteins are predominantly expressed in axons, and their expression level is positively correlated with axonal growth (Guo et al., 1998). Treatment with BMP2 and lovastatin alone or in combination did not alter levels of these cytoskeletal proteins relative to control cultures (Fig. 4D).

Lovastatin Effects on CGRP Immunoreactivity Are Reversed by Supplementation with Mevalonate or Cholesterol, but Not Isoprenoids. Statins inhibit HMG-CoA reductase, the enzyme that catalyzes the synthesis of mevalonate, which is a necessary precursor for synthesis of cholesterol and the isoprenoids farnesyl- and geranylgeranylpyrophosphate (Fig. 5A). Thus, statins not only inhibit cholesterol synthesis but also deplete isoprenoids (Liao and Lauf, 2005). To determine whether inhibition of these metabolic pathways mediates statin effects on neuropeptide expression, we tested the ability of products downstream of HMG-CoA to reverse lovastatin effects on CGRP expression in DRG cultures. Supplementation of the culture medium with either mevalonate (Fig. 5B) or cholesterol (Fig. 5C) significantly reversed the inhibitory effects of lovastatin on BMP2-induced CGRP expression. In contrast, supplementation with the isoprenoid precursor geranylgeraniol had no effect on lovastatin inhibition of BMP2-induced CGRP expression (Fig. 5D). Consistent with this latter observation, BMP2-induced CGRP expression was not inhibited by either the geranylgeranyl transferase inhibitor FTI-277 (Fig. 5E).

Lovastatin Inhibits BMP2-Induced Smad1 Phosphorylation and Nuclear Translocation. The observation that statins blocked BMP and activin-induced neuropeptide expression but did not decrease CGRP or substance P expression below background levels observed in the absence of BMPs and activin suggested that statins interfered with BMP and activin signaling. BMPs and activin signal via p38 mitogen-activated protein (MAP) kinase or Smad1 (Nohe et al., 2004). Pharmacological inhibition of p38 MAP kinase signaling did not block BMP2-induced CGRP expression in DRG neurons (Fig. 6A). However, consistent with previous reports implicating Smad1 in BMP- and activin-induced CGRP expression in DRG neurons (Ai et al., 1999; Cruise et al., 2004), BMP2 caused a 7.5-fold increase in phosphorylated Smad1 (pSmad1) in DRG cultures (Fig. 6, B and C) and significantly increased nuclear localization of Smad1 (Fig. 6, D).
When added simultaneously with BMP2, lovastatin had no effect on BMP2 induction of pSmad1 or on the percentage of neurons with nuclear localization of Smad1 (data not shown). However, pretreatment with lovastatin for 24 h to deplete cells of mevalonate before acute BMP2 exposure significantly attenuated BMP2-induced pSmad1 (Fig. 6, B and C) and reduced the percentage of BMP-treated neurons with nuclear Smad1 (Fig. 6, D and E). The latter effect was significantly reversed by supplementation with cholesterol (20 μg/ml) during the 24-h lovastatin pretreatment (Fig. 6, D and E).

**Discussion**

Recent clinical and experimental observations suggest that statins may be useful in the treatment of diseases with a primary component of neurogenic inflammation (Leung et al., 2003; McKay et al., 2004; Abeles and Pillinger, 2006; Liberopoulos and Mikhailidis, 2006; Gazi et al., 2007; Kim et al., 2007). In this study, we describe a novel action of statins that may contribute to these therapeutic effects: their ability to decrease the expression of CGRP and substance P, the proinflammatory neuropeptides released by sensory neurons that trigger neurogenic inflammation. In support of this hypothesis, we observed that statins administered to adult male rats at doses previously reported to have anti-inflammatory effects (McKay et al., 2004; Paumelle et al., 2006) significantly decreased CGRP and substance P in DRG in vivo. These effects were observed in the absence of treatment-related effects on body weight with two different st-
atins dissolved in two different vehicles and administered by different routes, suggesting that the ability to alter neuropeptide expression is a specific and general pharmacological property of this class of drugs.

There is evidence that BMPs and activin mediate the up-regulation of CGRP and substance P in response to injury or inflammation (Cruise et al., 2004; Xu et al., 2005), and it was previously demonstrated that, in vitro, BMPs and activin increased the percentage of DRG neurons immunoreactive for CGRP (Ai et al., 1999). Our data extend these findings by demonstrating that BMP2 up-regulated substance P as well as CGRP expression in sensory neurons cultured from DRG. We also observed that statins blocked induction of CGRP and substance P when added simultaneously with BMPs or activin, and that they significantly decreased neuropeptide expression when added to DRG cultures pretreated with BMPs. These effects were not blocked by TTX/MgCl₂ and occurred at statin concentrations that had no effect on survival or growth.
of cultured DRG neurons, suggesting that statins act directly on DRG neurons to selectively alter neuropeptide synthesis independent of any altered electrical activity or decreased cell viability associated with statin treatment.

Consistent with our in vivo observations, analyses of different statins in cultured DRG neurons suggests that attenuation of neuropeptide expression is a generalized pharmacological property of lipophilic statins. Lovastatin, atorvastatin, mevastatin, and simvastatin all decreased CGRP expression in a concentration-dependent manner with similar efficacies and potencies, suggesting a common mechanism of action. In contrast, the hydrophilic statin pravastatin was inactive, probably reflecting the fact that it is poorly absorbed by neurons because they lack the membrane carrier protein necessary to transport pravastatin across the cell membrane (Liao and Laufs, 2005).

Maximal effects of the lipophilic statins were observed at 0.1 μM with an ED_{50} ~30 nM. These concentrations are similar to those required to inhibit HMG-CoA reductase or cholesterol synthesis in vivo (Black et al., 1998) and are within the range of steady-state serum levels of lovastatin (~0.1 μM) in humans being treated for hypercholesterolemia (Pan et al., 1990).

Inhibition of HMG-CoA reductase underlies the efficacy of statins in treating cardiovascular disease. In cultured DRG neurons, statin inhibition of BMP2-induced CGRP expression was reversed by supplementation with mevalonate, the product of this enzyme, indicating that this mechanism also mediates statin effects on neuropeptide expression in sensory neurons. Mevalonate is used in the synthesis of both cholesterol and isoprenoid precursors, and many of the anti-inflammatory effects of statins are mediated by inhibition of isoprenoid synthesis (Liao and Laufs, 2005). However, supplementation with isoprenoid precursors did not reverse statin effects on CGRP immunoreactivity in cultured DRG neurons, and two highly specific isoprenyl transferase inhibitors failed to mimic the statin effect. These findings strongly suggest that statin-mediated disruption of protein isoprenylation does not underlie statin effects on neuropeptide expression in sensory neurons.

In contrast, supplementation of the medium with cholesterol did reverse statin effects, supporting a sterol-sensitive mechanism for statin inhibition of neuropeptide expression. This finding is consistent with the localization of BMP receptors to cholesterol-rich lipid rafts (Hartung et al., 2006; Black et al., 1998) and are within the range of steady-state serum levels of lovastatin (~0.1 μM) in humans being treated for hypercholesterolemia (Pan et al., 1990).

References


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