The Akt-nitric oxide-cGMP pathway contributes to nerve growth factor-mediated neurite outgrowth in apolipoprotein E knockout mice

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Abstract

Apolipoprotein E deficient (apoE-/-) mice have peripheral sensory nerve defects, and a reduced and delayed response to noxious thermal stimuli. However, to date, no report has focused on the influence of apoE deficiency on calcitonin gene-related peptide (CGRP)-containing nerve fiber extensions. We have shown that the density of CGRP containing nerve fibers decreases in mesenteric arteries of apoE-/- mice when compared to wild-type mice. Here, we investigated whether apoE deficiency is involved in nerve growth factor (NGF)-induced CGRP containing nerve regeneration using apoE-/- mice. NGF-mediated CGRP-like immunoreactivity (LI)-neurite outgrowth in apoE-/- cultured dorsal root ganglia (DRG) cells was significantly lower than wild-type cultures. However, the level of NGF receptor mRNA in apoE-/- DRG cells was similar to wild-type mice. To clarify the mechanism of the impaired ability of NGF-mediated neurite outgrowth, we focused on the Akt-NO-cGMP pathway. Expression of phosphorylated Akt was significantly reduced in apoE-/- DRG. The NO donor, sodium nitroprusside or S-nitroso-N-acetylpenicillamine, did not affect NGF-mediated neurite outgrowth in apoE-/- cultured DRG cells. However, 8-Bromoguanosine 3', 5'-cyclic monophosphate sodium salt n-hydrate (8-Br-cGMP), a cGMP analog, induced NGF-mediated nerve facilitation similar to wild-type NGF-mediated neurite outgrowth levels. Furthermore, in apoE-/- DRG, soluble guanylate cyclase expression was significantly lower than wild-type DRG. These results suggest that in apoE-/- mice the Akt-NO-cGMP pathway is impaired, which may be caused by NGF-mediated CGRP-LI-neurite outgrowth defects.
Introduction

Apolipoprotein (apo) E, a major component of plasma lipoproteins, transports cholesterol to the central and peripheral nervous system (Boyles et al., 1985; Stoll et al., 1989). ApoE appears to play an important role in neuronal growth and repair in a variety of cultured neurons (Nathan et al., 2004). ApoE receptors, such as the low density lipoprotein receptor-related protein, mediate neurite outgrowth in primary cortical neurons and through activation of p44/42 mitogen-activated protein kinase (MAPK) (Quet et al., 2004). ApoE-deficient (apoE−/−) mice have a reduced number of unmyelinated axons within the sciatic nerve, and display reduced sensitivity to noxious thermal stimuli (Fullerton et al., 1998).

The present study was designed to investigate the functional contribution of apoE deficiency to pain thresholds, using behavior and immunohistochemical assays. In this study, we used calcitonin gene-related peptide (CGRP)-like immunoreactivity (LI) as a sensory nerve indicator, as CGRP plays an important role in nociception and is a major neurotransmitter.

Our previous study demonstrated that administration of nerve growth factor (NGF) resulted in a greater density of CGRP containing nerve fibers in nerve-injured rats (Hobara et al., 2006). Exogenous NGF increased CGRP expression in sensory neurons (Lindsay and Harmar, 1989) and in pathologic conditions (Supowit et al., 2001). Here, we focused on NGF receptor expression and its function in apoE−/− mice. To clarify NGF receptor function, we used primary cell cultures of dorsal root ganglia (DRG) neurons isolated from apoE−/− or wild-type mice, and assessed NGF-induced neurite outgrowth. The cell bodies of DRG cells contain sensory nerves, which are the main source of CGRP. In addition, we focused on the NGF signaling cascade. NGF signals through
tropomyosin-related kinase A (trkA), with activation of common intracellular signaling intermediates, including Ras, mitogen activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K) and the serine-threonine kinase Akt (Jones et al., 2003; Akassoglou, 2005).

A study has shown that Akt phosphorylation is able to induce nitric oxide (NO) production and cGMP accumulation (Cunha et al., 2010). Therefore, we investigated whether apoE deficiency affected NGF-mediated neurite outgrowth, particularly focusing on the NO-cGMP pathway.
Materials and Methods

Experimental animals

Adult male apoE⁻/⁻ mice (B6.129P2-Apoe tm1Unc, The Jackson Laboratory, Bar Harbor, USA; 10× backcrossed) and wild-type mice (n=30 for each group), were housed in the Animal Research Center of Okayama University of Science at a controlled ambient temperature of 22°C with 50 ± 10% relative humidity and with a 12-h light/12-h dark cycle (lights on at 8:00 AM). They were fed a normal chow diet and water ad libitum. All procedures were used in accordance with the institutional guidelines for animal research, which are equivalent to the Japanese Government Animal Protection and Management Law (No. 105) and the Japanese Government Notification on Feeding and Safekeeping of Animals (No. 6). Every effort was made to minimize the number of animals used and their suffering.

Hot Plate test

An animal was placed on an aluminum plate (45 × 30 cm) maintained at 55 ± 0.5°C. Mice were placed on the hot plate, and the time taken to either lick the fore or hind-paws, or jump off the hot plate was recorded as the latency time. The latency to respond was measured using a stopwatch. A cut-off time (30 s) was set to avoid tissue damage.

Immunohistochemical study

Animals were treated with a large dose of sodium pentobarbital (50 mg/kg, intraperitoneally). The superior mesenteric artery was cannulated with polyethylene tubing and Zamboni solution (2% (w/v) paraformaldehyde and 15% (w/v) picric acid in
0.15 M phosphate buffer; pH = 7.4) was infused. The mesenteric artery was removed
together with the intestine as described previously (Hobara et al., 2005, 2006). The third
branch of the mesenteric artery proximal to the intestine was removed and
immersion-fixed in Zamboni solution for 48 h. After fixation, the artery was repeatedly
rinsed in 10 mM phosphate-buffered saline (PBS; pH = 7.4), immersed in PBS
containing 0.5% (v/v) Triton-X-100 overnight, and incubated with PBS containing
normal goat serum (1: 100) for 60 min. The tissue was then incubated with rabbit
polyclonal anti-CGRP (Biogenesis Ltd., Oxford, UK) antiserum at a dilution of 1:300
for 72 h at 4°C. After incubation, the artery was washed in PBS and the sites of
antigen-antibody reaction were detected by incubation with fluorescein-5-isothiocyanate
(FITC)-labeled goat anti-rabbit IgG (diluted 1: 100) (ICN Pharmaceuticals, Inc., Aurora,
OH, USA) for 60 min. Thereafter, the artery was thoroughly washed in PBS, mounted
on a slide, cover-slipped with glycerol/PBS (2: 1 v/v) and observed under a confocal
laser scanning microscope (CLSM510, Carl Zeiss, Tokyo, Japan) in Okayama
University Medical School Central Research Laboratory.

**Immunohistochemical analysis**

The immunostaining density of CGRP-like immunoreactive (CGRP-LI) nerve
fibers was analyzed using a previously described method (Hobara et al., 2005, 2006).
For quantitative evaluation of CGRP-LI, confocal projection images of CGRP
immunostaining, which consisted of 8-10 overlapping images (0.1 µm scanning)
patched together, were magnified 20× and digitized as TIF images using a digital
camera system (Olympus SP-1000, Olympus, Tokyo, Japan) and imported into a
Windows XP computer (Toshiba, Tokyo, Japan). The stored digital images were
analyzed using image-processing software (Simple PCI; Compix Inc., Imaging Systems, Cranberry Township, PA, USA). The extraction of specific color and measured field commands were used to extract the CGRP-LI areas (which were stained green). Extraction of the signal was carried out using specific protocols based on the hue, lightness and saturation color parameters. A measured field of 100 μm × 100 μm (10000 μm², which contained the adventitia layer (including immunostained perivascular nerve fibers) was randomly selected on magnified images of the whole mount artery. The objective areas command was used to calculate the percentage of the CGRP-LI-positive area. The intensity of staining was estimated using a point-counting computer program and the background level was subtracted from the experimental value to yield the corrected intensity. The average density in three arteries was taken as the nerve density per animal.

**Primary Cultures of DRG cells and drug treatments**

DRG neurons were isolated from adult male apoE⁻/⁻ or wild-type mice using previously reported procedures (Komagiri and Kitamura, 2003). Animals were anesthetized with sodium pentobarbital intraperitoneally (50mg/kg). The spinal cord was isolated and the ganglia were dissected using microforceps. After removal of connective tissue, the spinal roots were cut close to the ganglia, rapidly replaced in collagenase (5 mg/mL for 30 min at 37°C; Sigma Aldrich Japan) followed by incubation for 30 min at 37°C with 1.25 mg/mL trypsin (Invitrogen Japan) and 2.5 mg/mL collagenase. Ganglia were resuspended in PBS, collected by centrifugation and mechanically dissociated by pipetting in Dulbecco’s modified Eagle’s medium (GIBCO Invitrogen Japan) containing 10% (v/v) fetal bovine serum (GIBCO Invitrogen Japan),
100 U/mL penicillin and 100 μg/mL streptomycin. The resulting cell suspension was plated on poly-L-lysine (10 μg/mL; Sigma Aldrich Japan)-coated 15 mm glass coverslips and maintained for 5 days at 37°C in a humidified incubator with 5% (v/v) CO₂ and air.

To examine neurite outgrowth of DRG cells, cells were treated in the absence or presence of NGF (100 ng/mL; Toyobo Co., LTD. Japan), sodium nitroprusside (SNP, 100 nM; Sigma Aldrich Japan), S-nitroso-N-acetylpenicillamine (SNAP, 100 μM; Sigma Aldrich Japan) and 8-Bromoguanosine 3', 5'-cyclic monophosphate sodium salt n-hydrate (8-Br-cGMP, 1 mM; Wako Pure Chemical Industries, Ltd. Japan). The drugs were added to cells at 2 days in vitro for 4 days and the medium was replaced every day.

**Immunocytochemistry**

Cells were fixed with 10% (v/v) formalin for 20 min, incubated with 0.5% (v/v) Triton-X-100 for 10 min and washed with PBS. Cells were incubated with 5% (v/v) normal goat serum containing 1% (w/v) bovine serum albumin (BSA) for 30 min at room temperature. Thereafter, cells were incubated with rabbit anti-CGRP (Biomol International, LP CA, USA) primary antibody diluted 1:500 in 1% (w/v) BSA solution for 60 min at room temperature. After washing cells were incubated for 60 min at room temperature with HRP-labeled goat anti-rabbit IgG (Santa Cruz Biotechnology, Inc. CA USA), diluted 1:300 in PBS. Following several washes in PBS, cells were incubated in diaminobenzidine solution. To quantify neurite outgrowth in neurons, images of randomly chosen fields of neuron cultures were obtained with a OLYMPUS IX50 camera (Olympus Co., Japan). To assess neurites numbers, a 120 μm diameter circle was drawn around the perikaryon of a single neuron and the number of CGRP-positive
neurites within the circle corresponded to the extent of neurite outgrowth (Kosacka et al., 2006).

**RNA Extraction**

Total RNA was extracted from the DRG (thoracic spinal cord Th1-12) using Trizol (Invitrogen, Tokyo, Japan) according to the manufacturer's specifications. At the end of the extraction, RNA samples were dissolved in diethyl pyrocarbonate (Sigma Aldrich, Tokyo, Japan) and water, and the optical density values of each sample were determined using an absorptiometer (Beckman Coulter, Inc. Tokyo, Japan). The relative amount of specific mRNA was determined by quantitative real-time polymerase chain reaction analysis (RT-PCR). The forward primer (5'-GTGATGGCAACCTCTACAGTAG-3') and reverse primer (5'-GGCCTCGTGGGTTAAGGAGTC-3') were used for PCR amplification of the trkA receptor to yield a 140-basepair (bp) product. The forward primer (5'-GGTCAGAAGGACTCCTATGTG-3') and reverse primer (5'-AGCAGCACAGGGTGCTCCTCA-3') were used for PCR amplification of beta actin, which served as an internal control to yield a 171-bp product. The PCR products were separated by electrophoresis using a 4% (v/v) polyacrylamide gel and visualized by ethidium bromide staining. Band images were captured using a digital camera (Canon Inc., Tokyo, Japan) and analyzed using a FluorchemTM8800 (Alpha Innotech, San Leandro, CA).

**Western blotting**

For western blot analysis, DRG were isolated from mice (thoracic spinal cord
Th1-12) and minced in PBS. Samples (20 μg) were loaded on 10% (w/v) SDS-polyacrylamide gels. Following transfer on to polyvinylidene fluoride (PVDF) membranes (Hybond P; GE Healthcare UK, Limited UK), membranes were blocked in Tris-buffered saline (TBS; pH = 7.6) containing 0.1% (v/v) Tween 20 (TBS-T) and 2.5% (v/v) membrane blocking reagent (GE Healthcare UK Limited) at room temperature for 1 h. Membranes were then probed overnight at 4°C with either rabbit anti-Phospho-Akt (ser473) polyclonal antibody (Cell signaling Technology Japan, K.K. 1:1000), rabbit anti-Akt polyclonal antibody (Cell signaling 1:1000) or rabbit anti-guanylate cyclase alpha subunit polyclonal antibody (Cayman Chemical, MA, 1:200) in blocking buffer (2.5% (v/v) blocking reagent in TBS-T). After membranes were washed four times in TBS-T, the membranes were incubated with goat anti-rabbit secondary antibody conjugate to horse radish peroxidase-linked IgG (Santa Cruz, 1:3000) in blocking buffer for 1 h at room temperature. The PVDF membranes were then washed four times with TBS-T and the bound antibodies were detected using a chemiluminescent substrate kit (Amersham Biosciences, Buckinghamshire, UK). Bands were analyzed by densitometry using FluorchemTM8800 (Alpha Innotech, San Leandro, CA) and the content of GAPDH, which was detected using the rabbit anti-GAPDH antibody (1:10000; SIGMA), was used as a control to ensure that the same amount of protein was loaded in each lane.

**Statistical Analysis**

All data are expressed as the mean ± S.E.M. Comparisons between two values were analyzed using a Student’s t test. Analysis of variance followed by Tukey’s test was used to determine statistical significance where appropriate. A P-value <0.05 was
considered statistically significant.
RESULTS

Response to Thermal Nociception

During the hot plate test, licking or jumping responses were considered to be a result of supraspinal sensory integration (Eddy and Leimbach, 1953). To test sensory performance at the supraspinal level, we measured the latency to respond when the hot plate was set at 55°C (Fig. 1). The cut off time was set at 30 s. A significant increased in latency was observed in apoE−/− mice compared with wild-type mice, indicating that apoE−/− mice have a reduced sensitivity to noxious thermal stimuli.

Changes in innervation of CGRP-LI nerve fibers in mesenteric arteries of wild-type or apoE−/− mice

Typical patterns of CGRP-LI nerve fibers in the small mesenteric arteries of apoE−/− and wild-type mice were observed (Fig. 2). In addition, the distal mesenteric artery was densely innervated by CGRP-LI nerve fibers (Fig. 2A and 2B). The density of CGRP-LI nerve fibers of apoE−/− mice was markedly decreased by approximately 50% compared to age-matched wild types.

Effect of NGF-mediated CGRP-LI on neurite outgrowth in DRG neurons isolated from wild-type and apoE−/− mice

We examined whether exogenous NGF could affect the neuronal sprouting response on wild-type and apoE−/− DRG cells. The immunostaining assay showed CGRP-LI-positive neurons and neurite outgrowth in wild-type and apoE−/− mice with or without NGF treatment (Fig. 3A, 3B, 3C and 3D). In wild-type mice, exogenous NGF significantly induced neurite outgrowth accompanied by formation of networks.
compared with non-treatment cells (Fig. 3B). However, in apoE<sup>−/−</sup> DRG, NGF-mediated neurite outgrowth was significantly lower when compared to wild-type mice NGF treatment DRG cells (Fig. 3E). To assess attenuation of NGF-mediated sprouting in apoE<sup>−/−</sup> mice, NGF high affinity receptor (trkA) levels were measured by RT-PCR in wild-type and apoE<sup>−/−</sup> DRG cells. Typical bands of trkA and actin were observed (Fig. 4A) and there was no difference between wild-type and apoE<sup>−/−</sup> mice (Fig. 4B).

**Expression of phosphorylated Akt in wild-type and apoE<sup>−/−</sup> DRG neurons**

Because we found decreased NGF-mediated neurite outgrowth in apoE<sup>−/−</sup> DRG cells with no effect on trkA mRNA levels, we hypothesized that apoE<sup>−/−</sup> mice had an impaired NGF signaling cascade. Therefore, we focused on the PI3K-Akt pathway, which is considered a major pathway mediating neuronal survival (Brunet et al., 2001). To understand the affect of Akt in neurite outgrowth, phosphorylated Akt and Akt expression was examined by western blot analysis in wild-type and apoE<sup>−/−</sup> DRG cells. In apoE<sup>−/−</sup> DRG, phosphorylated Akt expression was significantly lower when compared to wild-type mice (Fig. 5).

**Effect of the NO donor and cGMP analog on NGF-mediated neurite outgrowth in cultured apoE<sup>−/−</sup> DRG neurons**

We focused on the Akt-NO-cGMP cascade. According to a previous report (Yamazaki et al., 2005), NO induced neurite outgrowth in PC12h cells. To investigate whether exogenously induced NO/cGMP could affect the NGF signaling pathway in cultured apoE<sup>−/−</sup> DRG neurons, we treated these cells with an NO donor or a membrane-permeable cGMP analog, and examined nerve extension in the presence of
NGF (100 ng/mL). The NO donors, SNP (100 nM) and SNAP (10 μM), did not affect NGF-induced neurite outgrowth (Fig. 6). However, 8-Br cGMP (1 mM) slightly increased neurite outgrowth by NGF, to an extent similar to NGF-mediated neurite outgrowth in cultured wild-type DRG.

**Expression of soluble guanylate cyclase in wild-type and apoE<sup>−/−</sup> DRG neurons**

We hypothesized that cGMP depletion was involved in the attenuation of NGF-mediated neurite outgrowth in cultured apoE<sup>−/−</sup> DRG. To confirm this hypothesis, we examined the expression of soluble guanylate cyclase by western blot analysis in wild-type and apoE<sup>−/−</sup> DRG cells. Our results showed that expression of soluble guanylate cyclase in apoE<sup>−/−</sup> mice was much smaller than wild-type mice (Fig. 7).
DISCUSSION

In this study, we have demonstrated that apoE–/– mice have peripheral sensory nerve defects, delayed responses to noxious thermal stimuli and reduced density of CGRP-LI nerve fibers in mesenteric arteries. Furthermore, NGF-mediated CGRP-LI neurite outgrowth was attenuated in apoE+/− DRG cultures, but there was no change in the level of NGF receptor mRNA between wild-type and apoE+/− mice. The expression of phosphorylated Akt and soluble guanylate cyclase was decreased in apoE+/− DRG neurons. In apoE+/− DRG cultures, NO donors (SNP or SNAP) did not influence neurite outgrowth following NGF treatment. However, 8-Br-cGMP induced greater NGF-mediated neurite outgrowth. Taken together, these results support the hypothesis that apoE deficiency induces nerve defects, most likely through the reduction of soluble guanylate cyclase.

ApoE is known to modify the risk of onset of several chronic neurological diseases, such as Alzheimer’s disease. Other studies suggest a direct relationship between apoE and neurite outgrowth (Holtzman et al., 1995; Nathan et al., 2002). ApoE receptors, such as low-density lipoprotein receptor-related protein, promote neurite outgrowth through activation of p44/42 MAPK, leading to cyclic AMP response element binding protein transcriptional regulation in primary mouse cortical neurons (Qiu et al., 2004). In the present study, we found that apoE deficient mice had a significant reduction in NGF-mediated neurite outgrowth. These results correspond with studies that suggest apoE can be used for membrane synthesis and lead to new nerve growth in central nervous system neurons and peripheral nerves (Ignatius et al., 1986; LeBlanc et al., 1990; Masliah et al., 1996). From these reports, we hypothesized that apoE+/− mice have less NGF or NGF receptors (trkA). However, we found no change in
mRNA levels of trkA in the DRG of apoE−/− and wild-type mice. Another study has reported that in apoE−/− mice, the cutaneous NGF amount or level of trkA expression did not change in apoE−/− mice compared with wild-type mice (Maysinger et al., 2008). These results indicate that the NGF signaling pathways in apoE−/− mice are dysfunctional. The signaling pathways responsible for NGF receptors include PI3K, protein kinase C and MAPK (Sofroniew et al., 2001). In the present study, we focused on PI3K/Akt-NO-cGMP, as PI3K/Akt signaling has been shown to be involved in survival of neuronal cells, including PC12 cells and sympathetic neurons (Yao and Cooper, 1995; Crowder and Freeman, 1998). Activation of Akt by phosphorylation of PI3K induces multifunctional regulation of apoptotic cell death and cell growth. In embryonic hippocampal cultures, activation of the NO/cGMP signaling pathway plays a major role in the regulation of trkA phosphorylation (Culmsee et al., 2005). In human coronary artery smooth muscle cell cultures, stimulating PI3K-Akt enhances NO production via type I nNOS (Han et al., 2007). We observed that treatment with SNP or SNAP, NO analogs, did not influence neurite outgrowth following NGF treatment, but 8-Br-cGMP induced more neurite outgrowth. Furthermore, phosho Akt and soluble guanylate cyclase expression were decreased in apoE−/− DRG neurons. These findings indicate that NO analogs do not influence NGF-mediated neurite outgrowth, indicating that not only Akt, but also down regulation of soluble guanylate cyclase plays a critical role in nerve dysfunction of apoE−/− mice.

Previous studies have reported that apoE deficiency results in impaired function of sensory nerves (Fullerton et al., 1998; Pola et al., 2003). ApoE−/− mice exhibited a reduction of unmyelinated axons within the sciatic nerves (Fullerton et al., 1998), and immunoreactivity with the neuronal marker PGP9.5 was reduced compared
with wild-type mice (Pola et al., 2003). In agreement with these findings, our results also showed that apoE−/− mice demonstrate a delayed response to noxious thermal stimuli, and that the amount of CGRP-LI containing nerve fibers was reduced when compared to wild-type mice. It is well known that NGF regulates CGRP expression through the ERK pathway in a variety of neurons. CGRP gene expression in PC12 cells is activated by NGF, which is mediated by the p42/p44 MAPK pathway (Freeland et al., 2000). In primary trigeminal neurons, MAPK stimulates the CGRP enhancer (Durham and Russo, 2003). In contrast, apoE receptors can signal through the p44/42 MAPK pathway to mediate neurite outgrowth (Qiu et al., 2004). However, it has not been demonstrated if apoE regulates CGRP expression through the MAPK-ERK pathways. In this study, we first demonstrated that the density of CGRP-LI nerve fibers is reduced in mesenteric arteries of apoE−/− mice. Although we did not measure expression of MAPK in apoE−/− mice, our data indicates that the other cascade of NGF signaling, the Akt-NO-cGMP pathway, has a critical role in regeneration of CGRP-LI sensory nerves.

In conclusion, our findings demonstrate that apoE−/− mice have sensory nerve defects, and that CGRP-LI nerve remodeling causes a decrease in nociceptive responses. In cultured DRG neurons from apoE−/− mice there is a decrease in NGF-mediated CGRP-LI neurite outgrowth and a reduction in the Akt-NO-cGMP signaling cascade. In addition, soluble guanylate cyclase plays an important role in processing nerve extension.
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Authorship Contributions

Participated in research design: Hashikawa-Hobara.

Conducted experiments: Hashikawa-Hobara, Hashikawa.

Contributed new reagents or analytic tools: Hashikawa-Hobara, Hashikawa, Yutani, Zamami, Jin, Takatori, Mio.

Performed data analysis: Hashikawa-Hobara, Hashikawa.

Wrote or contributed to the writing of the manuscript: Hashikawa-Hobara, Kawasaki.
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Footnotes

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Figure Legends

Figure 1. Nociceptive response of wild-type and apoE⁻/⁻ mice in the hot plate test. The hot plate test revealed that the latency was significantly increased in apoE⁻/⁻ mice (n = 9) compared with wild-type mice (n = 5). Each bar indicates the mean ± S.E.M. t=5.01, df=13.

Figure 2. Representative confocal laser images (A and B) showing changes in the innervation of calcitonin gene-related peptide (CGRP)-like immunoreactive (LI)-containing nerve fibers in mesenteric arteries of wild-type (A) and apoE⁻/⁻ (B) mice and bar graphs showing changes in the density (C) of CGRP-LI-containing nerve fibers. A horizontal bar in the right lower corner of each panel indicates 100 µm. Each bar indicates mean ± S.E.M. of 4 experiments. t=5.01, df=48.

Figure 3. Effect of NGF-mediated neurite outgrowth in primary cell cultures of DRG neurons isolated from wild-type and apoE⁻/⁻ mice. (A-D) Representative images showing neurite outgrowth of CGRP-immunopositive neurons in wild-type and apoE⁻/⁻ DRG cells with or without NGF treatment. Wild-type DRG cells were grown for 4 days in medium alone (wild-type control) (A), in medium with NGF (100 ng/mL) (B). ApoE⁻/⁻ DRG cells were grown for 4 days in medium alone (apoE⁻/⁻ control) (C), in medium with NGF (D). Bar graph shows effect of treatment with or without NGF on synapse formation in wild-type and apoE⁻/⁻ DRG cells (E). Arrows indicate CGRP-immunopositive neurons. A horizontal bar in the left lower corner of each panel indicates 100 µm. Each bar indicates the mean ± S.E.M. of five independent experiments. * P<0.05 vs. wild-type control. † P<0.05 vs. apoE⁻/⁻ control. F=22.
Figure 4. Level of NGF receptor (trkA) in DRG of wild-type and apoE<sup>−/−</sup> mice. (A) Typical bands show expression of NGF receptor (trkA) and Actin mRNA in the dorsal root ganglia. (B) Levels of trkA and Actin mRNA were determined by RT-PCR analysis. Each bar indicates the mean ± S.E.M. of three (wild-type mice) or four (apoE<sup>−/−</sup> mice) independent experiments. t=1.40, df=6.

Figure 5. Western blot analysis of pAkt expression in DRG isolated from wild-type and apoE<sup>−/−</sup> mice. (A) Typical bands showing expression of pAkt and Akt protein in DRG. (B) The ordinate indicates fold increase over wild-type mice values. Each bar indicates the mean ± S.E.M. of eight (wild-type mice) or six (apoE<sup>−/−</sup> mice) independent experiments. t=2.63, df=17.

Figure 6. The NO-cGMP pathway is involved in NGF-mediated neurite outgrowth in cultured apoE<sup>−/−</sup> DRG cells. Primary apoE<sup>−/−</sup> DRG neuron cultures were incubated with NGF (100 ng/mL) alone, NGF + NO donor (SNP 100 nM), NGF + NO donor (SNAP 100 µM) or NGF + the membrane-permeable cGMP analog (8-Br-cGMP; 1 mM) for 4 days. Bar graph represents the change in mean neurite length after treatment with each drug. Each bar indicates the mean ± S.E.M. of three independent experiments. F=8.68.

Figure 7. Western blot analysis of soluble guanylate cyclase expression in DRG isolated from wild-type and apoE<sup>−/−</sup> mice. (A) Typical bands showing expression of soluble guanylate cyclase and GAPDH protein in DRG. (B) The ordinate indicates fold increase over wild-type mice values. Each bar indicates the mean ± S.E.M. of eight (wild-type
mice) or six (apoE\textsuperscript{+/-} mice) independent experiments. t=1.90, df=20.
Figure 1

Comparison of latency (seconds) between wild-type and apoE−/− mice. The data show a statistically significant difference (p < 0.05) with the apoE−/− mice having a higher latency compared to the wild-type group. The sample sizes are n = 5 for wild-type and n = 9 for apoE−/−.
Figure 2

A  
Wild type

B  
ApoE⁻/⁻

C  
Density of CGRP-LI nerves  
(fold increase over wild type value)

wild type  
(n = 4)  

 apoE⁻/⁻  
(n = 4)  

p < 0.05
Figure 3

A. wild-type control

B. wild-type NGF

C. apoE⁻/⁻ control

D. apoE⁻/⁻ NGF

E. Bar graph showing the number of neurites per circle for control and NGF-treated wild-type and apoE⁻/⁻ conditions. The graph indicates a significant difference (p<0.05) between the treatments.
Figure 5

A

[p-Akt (60 kDa)
Akt (60 kDa)]

B

\[ \text{pAkt/Akt} \]

\[ \begin{array}{c}
\text{wild-type} \\
\text{apoE}^{-/-}
\end{array} \]

\[ \begin{array}{c}
(n = 8) \\
(n = 6)
\end{array} \]

\[ P < 0.05 \]
Figure 7

A

soluble guanylate cyclase (77~82 kDa)
GAPDH (~36 kDa)

B

![Bar chart showing soluble guanylate cyclase/GAPDH ratio for wild-type and apoE−/− mice.](chart)

- P<0.05

wild-type (n = 8)
apoE−/− (n = 6)