Experimental Neurology 239 (2013) 102–110

Contents lists available at SciVerse ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Apolipoprotein E knockout as the basis for mouse models of dyslipidemia-induced neuropathy

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ARTICLE INFO

Article history:
Received 19 July 2012
Revised 24 September 2012
Accepted 1 October 2012
Available online 8 October 2012

Keywords:
Peripheral neuropathy
Dyslipidemia
Diabetes
Apolipoprotein E
Apolipoprotein B48
Lipid profile
Mouse

ABSTRACT

Dyslipidemia has been identified as an important pathogenic risk factor for diabetic neuropathy, but current animal models do not adequately reproduce the lipid profile observed in human diabetics (increased triglycerides with an elevated LDL-cholesterol and reduced HDL-cholesterol). High fat feeding of mice produces hyperlipidemia, but mice are resistant to increases in the LDL to HDL ratio, reducing the potential for peripheral lipid deposits to impact neuropathy, as is postulated to occur in human subjects. Genetic manipulations provide an alternative approach to reproducing a neuropathic plasma lipid profile. Based on findings from the atherosclerosis literature, we began with knockout of ApoE. Since knockout of ApoE alone only partially mimics the human diabetic lipid profile, we examined the impact of its combination with a well-characterized model of type 2 diabetes exhibiting neuropathy, the db/db mouse. We added further gene manipulations to increase hyperlipidemia by using mice with both ApoE and ApoB48 knockout on the ob/+ (leptin mutation) mice. In all of these models, we found that either the db/db or ob/ob genotypes had increased body weight, hyperlipidemia, hyperglycemia, and evidence of neuropathy compared with the control groups (db/+ or ob/+, respectively). We found that ApoE knockout combined with leptin receptor knockout produced a lipid profile most closely modeling human dyslipidemia that promotes neuropathy. ApoE knockout combined with additional ApoB48 and leptin knockout produced similar changes of smaller magnitude, but, notably, an increase in HDL-cholesterol. Our data suggest that the overall effects of ApoE knockout, either directly upon nerve structure and function or indirectly on lipid metabolism, are insufficient to significantly alter the course of diabetic neuropathy. Although these models ultimately do not deliver optimal lipid profiles for translational diabetic neuropathy research, they do present glycemic and lipid profile properties of value for future therapeutic investigations.

Introduction

Dyslipidemia is now recognized as an independent risk factor for the development of neuropathy in patients with diabetes (Ansquer et al., 2009; Vincent et al., 2009b). Lipid profiles are commonly abnormal early in the course of type 2 diabetes (high levels of plasma triglycerides, elevated very-low-density cholesterol (VLDL-C) and low-density lipoprotein cholesterol (LDL-C), and decreased high-density lipoprotein cholesterol (HDL-C)), and correlate with the onset of neuropathic symptoms (Clemens et al., 2004). Approximately 60% of prediabetic patients with impaired glucose tolerance display painful small nerve fiber neuropathy (Singleton et al., 2001). Furthermore, hypertriglyceridemia is epidemiologically associated with electrophysiological evidence of non-symptomatic neuropathy (Drory et al., 1999; Kassem et al., 2005). These findings have prompted us to establish mouse models with varying profiles of dyslipidemia and glycemia in order to confirm the role and explore the mechanisms of lipid-induced peripheral nerve injury.

Apolipoprotein E (ApoE) is a 34 kDa glycoprotein constituent of lipoproteins. It facilitates the transport of lipids between the sites of synthesis or absorption to the sites of utilization or excretion (Mahley, 1988). ApoE plays a key role in the local redistribution of lipids within tissues both during normal homeostasis and during injury and repair (Donahue and Johanson, 2008; Zhang et al., 2011). In addition to its role in lipid metabolism, ApoE is proposed to have important functions within the central and peripheral nervous systems in maintaining neuronal health and survival.

For these reasons, ApoE knockout mice have been used in dyslipidemia studies (Poirier, 2000). Commercially available ApoE knockout mice on the C57BL/6 background demonstrate decreased clearance of remnant lipoproteins that leads to hypercholesterolemia and hypertriglyceridemia (Buzello et al., 2003). Despite the abnormalities in plasma lipid profiles, these mice exhibit normal fasting glycemic and insulin levels (Trauner et al., 2010). In the sciatic nerves

Abbreviations: DN, diabetic neuropathy; IENFD, intraepidermal nerve fiber density; NCV, nerve conduction velocity.

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http://dx.doi.org/10.1016/j.expneurol.2012.10.002

of ApoE knockout mice, there were fewer unmyelinated nerve fibers and morphological abnormalities in the axons (Fullerton et al., 1998). These defects resulted in reduced sensitivity to noxious thermal stimuli in the feet and tail.

Evidence for peripheral nerve deficits in ApoE knockout mice led to the proposal that lack of ApoE would exacerbate the development of diabetic microvascular complications, including neuropathy, i.e., that dyslipidemia and hyperglycemia together aggravate the onset of diabetes complications. In support of this concept, ApoE knockout mice crossed with mice lacking the leptin receptor (db/db mice) have increased retinopathy compared with mice lacking either of these proteins alone (Barile et al., 2005).

Plasma cholesterol in mice is primarily carried in ApoB48-containing lipoprotein particles (Lloyd et al., 2008). Humans do not produce ApoB48; ApoB100 predominates, with high levels of ApoB100-containing LDL associated with atherosclerosis (Lloyd et al., 2008). Loss of ApoB48 in mice leads to increased levels of circulating ApoB100-expressing LDLs, more closely resembling lipid profiles in humans (Lloyd et al., 2008). With the goal of developing improved mouse models of diabetic neuropathy by developing mice with hyperglycemia, hyperlipidemia and a lipoprotein profile modeling that in humans, we examined the impact of ApoE knockout combined with leptin receptor mutation (db/db) on lipid profiles, glycemic control, and extent of neuropathy at 24 weeks of age. We added further gene manipulations to increase dyslipidemia by utilizing mice with both ApoE and ApoB48 knockout in the ob/+ (leptin mutation) mouse (Lloyd et al., 2008). Despite dramatic changes in lipid profiles, we find small differences in neuropathy parameters between these apolipoprotein knockout models, and generally no robust effect of increasing obesity upon the degree of neuropathy.

Materials and methods

Generation of mouse genotypes

ApoE knockout mice on the C57BL/6 background and heterozygous db+mice on the C57BLKS background were obtained from Jackson Laboratories. These mice were crossed and F1 offspring carrying the $ApoE^{+/-}$ $db/^{+}$ were bred to generate F2 offspring. These were backcrossed for another generation, and the resulting F3 $ApoE^{-/-}$ $db/^{+}$, $ApoE^{-/-}$ db/db, $ApoE^{+/+}$ $db/^{+}$, and $ApoE^{+/+}$ db/db mice were included in the study (8 mice for each genotype). Triple knockout $ApoE^{-/-}$ $ApoB^{100only}/ob/^{+}$ mice were generated by Dr. Murielle Veniant and colleagues (Lloyd et al., 2008) and bred to obtain $ApoE^{-/-}$ $ApoB^{100only}/ob/^{+}$ and $ApoE^{-/-}$ $ApoB^{100only}$/ob/ob littermates for this study (10 mice for each genotype). Male mice only were used in all experiments.

Mice were housed in a pathogen-free environment and cared for following the University of Michigan Committee on the Care and Use of Animals guidelines. Mice were weaned at 4 weeks of age and fed a standard rodent chow. All data were collected at 24 weeks of age.

Phenotypic measures

Final body weight was measured using standard laboratory scales. Following a 6 hour fast, one drop of tail blood was analyzed using a standard glucometer (One Touch Profile, LIFESCAN, Inc. Milpitas, CA). Two drops of tail blood were mixed with 10 μL 50 μM EDTA for measurement of glycated hemobglobin using the Helena Laboratories Test Kit, Glyco-Tek Affinity Column Method (Helena Laboratories Corp., Beaumont, TX).

Assessment of neuropathy

Hind paw withdrawal and tail flick latency to heat stimulus were recorded electronically per our previous studies (Lee et al., 1990; Sullivan et al., 2008; Vincent et al., 2009a) (6 mice per group). In the ApoE only knockout and db/+ and db/db mice, sural and sciatic nerve conduction velocities (NCV) were measured under 30/0.75 mg/kg ketamine/acepromazine IP anesthetic while maintained at 32–34 °C using a heating pad (Kern et al., 2009; Sullivan et al., 2008) (8 mice per group). In 2010, we found that NCV measurements were significantly improved using isofluorane anesthetic (Oh et al., 2010) and this method was used when we obtained the triple knockout mice (8 mice per group). Intraepidermal nerve fiber density (IENFD) in footpads were measured as previously described (Kern et al., 2009; Sullivan et al., 2008) (6 mice per group).

Terminal metabolic phenotyping

Mice were euthanized using an overdose of sodium pentobarbital. Blood was collected from the superior vena cava and mixed with a final concentration of 50 μM EDTA. The blood was centrifuged at 800*g* for 10 min at 4 °C, and plasma (supernatant) collected. A 50 μL sample from each mouse was collected for total cholesterol and triglyceride measurements using a colorimetric kit (Diagnostic Chemicals Ltd.). The remaining plasma samples from 3 mice per group were pooled and plasma lipoproteins were separated by FPLC. The levels of triglycerides and cholesterol in each fraction were measured using colorimetric kits (Diagnostic Chemicals Ltd.). Lipid measurements were performed at the Mouse Metabolomics Phenotyping Core at the University of Washington (McMillen et al., 2005).

Data analysis

Data analysis was performed using GraphPad Prism 5.0. When performing comparisons across more than two groups, a one-way ANOVA test was performed and Tukey's multiple point comparison post-test *p* values presented. If the Bartlett's test for equal variances was significant, a non-parametric Kruskal–Wallis test was performed and Dunn's post-test *p* values are presented. If the multiple group comparison did not reach significance, the directed hypothesis that leptin/leptin receptor and ApoE knockout mice develop complications was tested using an unpaired *t*-test. If the f-test to compare variances was significant, Welch's correction was performed and the *t*-test re-run. Due to modification of methods, nerve conduction velocity data from the triple knockout model was analyzed using the unpaired *t*-test, as described.

Results

Final body weight

The weights of the mice are shown in Fig. 1A. In mice that were wild-type for ApoE, there was a significant increase in weight between db/+ and db/db mice ($p<0.05$), reflecting the hyperphagia in mice lacking leptin receptor. This weight increase resulting from loss of leptin signaling was blunted in the $ApoE^{-/-}$ mice. In the mice lacking both ApoE and ApoB48, the ob/+ mice showed no weight abnormality but the ob/ob littermates were significantly heavier than any of the db/db groups ($p<0.001$).

Glycemic control

Similar to previous studies in db mouse models, fasting blood glucose was significantly elevated in db/db compared with db/+ littermates (Fig. 1B). There was no difference between mice expressing normal (wt) ApoE and ApoE knockouts in the db mice. The increase in hyperglycemia between ob/+ and ob/ob mice in the group lacking both ApoE and ApoB48 was largely eliminated and there was no significant difference between the $ApoE^{-/-}/ApoB^{100only}/ob/+$ and $ApoE^{-/-}/ApoB^{100only}$/ob/ob groups. Despite no significant difference in fasting glucose, there was a significant increase in glycated hemoglobin