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INTRANASAL DELIVERY OF INSULIN AND A NITRIC OXIDE SYNTHASE INHIBITOR IN AN EXPERIMENTAL MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

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Abstract—Amyotrophic lateral sclerosis (ALS) is a fatal neuromuscular disorder in which motor neurons may be targeted by oxidative and nitrergic stress without sufficient compensation by intrinsic support mechanisms. In this work, we addressed two key tenets of this hypothesis for the pathogenesis of ALS. Using superoxide dismutase (SOD) 1^{G93A} mice, we studied the impact of reduction of nitrergic stress within the CNS with the use of a broad spectrum nitric oxide synthase (NOS) inhibitor, NG-nitro-L-arginine methyl ester. A separate cohort of SOD1^{G93A} mice received direct insulin neurotrophic support, ligating receptors expressed upon motor neurons, to attempt protection against neuronal and functional motor dropout. For direct access, we used a novel form of intranasal delivery that provides peak concentration levels in the CNS within 1 h of delivery without systemic side effects at doses which previously rescued retrograde loss of motor axons after axotomy. To identify even minor impacts of these interventions on the outcome, we utilized an intensive program of serial behavioral and electrophysiological testing weekly, combined with endpoint quantitative morphometry and molecular analysis. This intensive evaluation enhanced our knowledge of the time course in SOD1^{G93A} mice and impact of the SOD1^{G93A} mutation upon motor neurons and their function. Neither intervention had even minimal impact upon slowing progression of disease in SOD1^{G93A} mice. Our data argue against significant roles for nitrergic stress in promoting motor neuron loss and the importance of alternative neurotrophic support mechanisms that might support motor neurons and prevent disease progression in SOD1^{G93A} mice.

Key words: amyotrophic lateral sclerosis, insulin, free oxygen radicals, endplate innervation, behavior testing, electrophysiology.

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by progressive death of motor neurons, leading to subsequent skeletal muscle denervation, weakness, and eventually paralysis. Approximately 10% of ALS cases are familial (fALS); of these, 10–20% have been linked to mutations in the superoxide dismutase (SOD) gene which leads to ALS in some mutation carriers, but without a well understood mechanism (Rosen, 1993) The most widely used model of fALS is the human mutant cytosolic Cu/Zn SOD1 mouse possessing a glycine to alanine conversion at the 93rd codon leading to SOD1 overexpression (SOD1^{G93A} mice) (Gurney et al., 1994; Pasinelli and Brown, 2006). These mice develop an ALS-like disease with initial symptoms including fine tremors and weakness developing in the hind limbs after 90 days of age, followed by severe hind-limb paralysis at ~120 days of age (Gurney et al., 1994). Delivery of neurotrophic molecules and genetic strategies to supplement neurotrophic support have been examined as possible therapeutic measures for ALS treatment in rodent models (Alisky and Davidson, 2000; Boillee and Cleveland, 2004; Federici and Boulis, 2006) with mixed results in animal and clinical studies (Kaspar et al., 2003; Lepore et al., 2007; Messi et al., 2007; Lai et al., 1997; Borasio et al., 1998). Insulin-like growth factor (IGF)-1, a pleiotropic neurotrophic factor, (Trejo et al., 2004) has particularly been studied for attempts to slow progression of ALS (Dodge et al., 2008; Lepore et al., 2007; Kaspar et al., 2003). Although viral delivery methods and direct intrathecal injections of IGF-1 proved beneficial in rodent models, unsatisfactory clinical results may have resulted from a lack of sustained or adequate delivery, and poor efficiency of protein delivery to motor neurons through systemic injections privy to systemic metabolism. Although it is doubtful that altered levels of CNS trophic factor signaling are the primary cause of ALS, loss of neurotrophic factors, altered receptor levels, and impaired functioning of their associated intracellular signal transduction pathways are associated with ALS and may contribute to progression of disease (Beck et al.,

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Abbreviations: ALS, amyotrophic lateral sclerosis; ChAT, choline acetyltransferase; CMAP, compound motor action potential; CREB, cAMP response element-binding protein; EDB, extensor digitorum brevis; fALS, familial amyotrophic lateral sclerosis; GluR1, glutamate receptor 1; GSK3β, glycogen synthase kinase 3β; IDE, insulin degrading enzyme; IGF-1, insulin-like growth factor-1; I-I, intranasal insulin; I-L, intranasal NG-nitro-L-arginine methyl ester; IR, insulin receptor; IRS, insulin receptor substrate; I-S, intranasal saline; IRβ, insulin receptor β subunit; L-NAME, NG-nitro-L-arginine methyl ester; MNCV, motor nerve conduction velocity; MUNE, motor unit number estimation; NeuN, neuronal nuclear marker; NFκB, nuclear factor κB; NMJ, neuromuscular junction; NO, nitric oxide; NOS, nitric oxide synthase; OCT, optimal cutting temperature; PBS, phosphate buffer solution; PI3K, phosphatidylinositol-3-OH kinase; qRT-PCR, quantitative real-time polymerase chain reaction; S-I, s.c. insulin; S-L, s.c. NG-nitro-L-arginine methyl ester; SOD, superoxide dismutase; S-S, s.c. saline; SYP, synaptophysin; TA, tibialis anterior.

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2001; Wilczak and de Keyser, 2005). Insulin, a cousin of IGF-1, is itself a potent neurotrophic factor for neuroregenerative support and neurodegenerative protection (Toth et al., 2006a,b) which acts through insulin receptors (IRs) or IGF-1 receptors (Recio-Pinto et al., 1986; Recio-Pinto and Ishii, 1988; Edbladh et al., 1994), sharing downstream signaling pathways with IGF-1 (White 2002). Ventral horn cells are immunoreactive for IRs (Sugimoto et al., 2002; Toth et al., 2006a) suggesting that insulin presence may be important for their sustenance.

In prior work examining an experimental type I diabetes model of diabetic peripheral neuropathy, long term replacement of insulin by intranasal delivery slowed progression of diabetic peripheral neuropathy, and prevented motor unit loss as well as end-plate denervation at skeletal muscle (Francis et al., 2008b). Intranasal drug delivery is a non-invasive, rapid, non-receptor mediated method for direct targeting of agents such as insulin and NG-nitro-L-arginine methyl ester (L-NAME) to the nervous system by bypassing the blood–brain barrier through olfactory and trigeminal neural pathways in rats, who have obligate nasal breathing leading to strong uptake of delivered substances at the olfactory epithelium (Thorne et al., 2004; Liu et al., 2004; Vig et al., 2006) and humans (Reger et al., 2006, 2008). We used this intervention in the SOD1^{G93A} mouse model of ALS, with daily intranasal insulin (I-I) delivery over a lifespan during performance of behavioral and electrophysiological testing for monitoring progression of motor neuron disease (MND). Nitrergic stress, involving nitric oxide (NO) toxicity, may be an important mechanism of motor neuron degeneration (Cassina et al., 2008; Ezzi et al., 2007; Wengenack et al., 2004), with NO elaborated by more than one isoform of nitric oxide synthase (NOS) including inducible NOS (iNOS) generated by microglia, or neuronal NOS (nNOS) upregulated in motor neurons in human ALS (Sasaki et al., 2001; Cassina et al., 2008; Ezzi et al., 2007; Wengenack et al., 2004). All three NOS isoforms can be inhibited by L-arginine analogues such as L-NAME (Sander et al., 1999; Frandsen et al., 2000). For this reason, we also delivered intranasal L-NAME, which has also been provided to humans (Sippel et al., 1999). We postulated that I-I and L-NAME delivery would slow progression of motor neuron disease in SOD1^{G93A} mice while limiting systemic side effects.

EXPERIMENTAL PROCEDURES

Animals

Transgenic mice used in this study were donated (Dr. T Gordon, University of Alberta, Edmonton, AB, Canada) and were bred from mice originally obtained from Jackson Laboratories, USA. These transgenic mice express a high copy number of the human SOD gene with a glycine to alanine base pair mutation at the 93rd codon of the cytosolic Cu/Zn SOD1 gene (SOD1^{G93A}; B6JSL-TgN (SOD1-G93A)) or a high copy number of normal human SOD1 gene (SOD1^{WT}; B6JSL-TgN (SOD1)). SOD1^{WT} mice do not develop any ALS-like disease (Gurney et al., 1994). The transgenic male SOD1^{G93A} and SOD1^{WT} mice were bred to non-transgenic B6JSL hybrid females. The resulting progeny were identified using standard quantitative real-time polymerase chain reaction (qRT-PCR) protocol for the human SOD1 (Rosen, 1993; Hegedus et al., 2007) performed on ear biopsies taken at the time of weaning (before 1 month of age). All mice were kept in standard animal housing with free access to food and standard rodent chow. All protocols were reviewed and approved by the University of Calgary Animal Care Committee using the Canadian Council of Animal Care guidelines or by the institutional animal care and use committee at Regions Hospital, St. Paul, MN, USA. All experiments conformed to international and local guidelines on the ethical use of animals. All attempts were made to minimize the number of animals used and their suffering during the entire protocol. Mice were anesthetized with pentobarbital (60 mg/kg) prior to all procedures. Only male mice were used to avoid ambiguity reported with gender-related differences in mean survival time of SOD1^{G93A} mice (Veldink et al., 2003).

Previous reports have indicated that male SOD1^{G93A} mice become symptomatic at approximately 90 days of age. We intended to determine the onset of loss of motor units *in vivo* by performing motor unit number estimation (MUNE) (described below) in the first cohort of mice studied. Once statistically significant differences in motor unit estimation, performed weekly, occurred between SOD1^{G93A} and SOD1^{WT}, then this time point was used as the starting point for later intervention studies. The time of 1 week after the loss of the righting reflex, used as an end point in many studies, in more than 50% of the first cohort of SOD1^{G93A} mice studied was used as an endpoint for behavioral, electrophysiological, and harvesting of tissues.

We studied a total of 18 male mice, nine SOD1^{G93A} and nine SOD1^{WT} mice, for the initial determination of measurable differences in MUNE. Following this, we used an additional 165 male mice, 79 SOD1^{G93A} and 86 SOD1^{WT} mice, for intervention studies. Initial weights were 18–25 g. At the age determined with the first cohort of SOD1^{G93A} and SOD1^{WT} mice, MUNE and behavior studies commenced for all mice. These second and subsequent cohorts of mice were divided into intervention cohort groups as demonstrated in Fig. 1.

Our sample size calculations assumed that placebo-treated SOD1^{G93A} mice live 130±12 days, a statistical power to detect the true intergroup difference was 90%, alpha was two-tailed at 0.05, and detection of 10% or greater difference in mean motor unit survival of treated SOD1^{G93A} mice. Using these assumptions, we required a minimum of 10 mice per treatment arm.

Whole blood glucose measurements were performed weekly in those intervention cohorts receiving intranasal insulin (I-I) or s.c. insulin (S-I) or their control cohort groups (intranasal saline (I-S) or s.c. saline (S-S) (OneTouch Ultra Meter, LifeScan Canada, Burnaby, BC, Canada). All animals were weighed monthly as well.

Animals were inspected twice daily, and examined for signs of depressed level of consciousness, ataxia, or general malaise. When such signs were identified, whole blood glucose testing was performed, with a measurement of <3.5 mmol/L defined to represent hypoglycemia. No intervention was to be performed at any time with regard to additional insulin, glucose or fluid delivery. In situations where the mouse was obviously ill, euthanasia was performed. In circumstances where severe hyperglycemia was found (>33 mmol/L) in an ill mouse, euthanasia was again performed.

Daily intranasal and S-I and L-NAME delivery studies

Daily I-I (Humulin R, Eli Lilly, Toronto, ON), I-S, S-I and S-S were administered to either SOD1^{G93A} or SOD1^{WT} mice as described in Fig. 1. With each mouse held in supine position with neck extended, a total of 24 μl containing either 0.87 U of insulin or equal volume 0.9% saline was provided by Eppendorf pipetter over alternating nares every 1 min as four drops of 6 μl each. S-I and S-S were also administered daily to either SOD1^{G93A} or SOD1^{WT} mice as described in Fig. 1.

Daily intranasal NG-nitro-L-arginine methyl ester (I-L) (Cedarlane, Burlington, ON), I-S, s.c. NG-nitro-L-arginine methyl ester (S-L) or S-S was also administered daily to either SOD1^{G93A} or