ALS/Lt: A New Type 2 Diabetes Mouse Model Associated With Low Free Radical Scavenging Potential

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Outbred CD-1 mice carry a spectrum of genetic susceptibilities for obesity and type 2 diabetes. ALS is an inbred strain with low antioxidant defenses produced by inbreeding CD-1 mice, with selection for susceptibility to alloxan, a generator of highly reactive oxygen free radicals and a potent β-cell toxin. The objective of this study was to determine if the low ability to diffuse free radical stress would contribute to spontaneous type 2 diabetes development in alloxan-untreated males. Indeed, both hyperinsulinemia and impaired glucose tolerance developed spontaneously between 6 and 8 weeks of age in alloxan-untreated males. Further aging was accompanied by increases in body mass, progressively more severe hyperinsulinemia, and development of overt hyperglycemia. Transition from impaired glucose tolerance to overt hyperglycemia correlated with a decreased ratio of reduced to oxidized glutathione. Evidence that the increased oxidative burden elicited the type 2 diabetes syndrome was obtained by the systemic elevation of the antioxidative capacity through daily administration of R-lipoic acid. R-lipoic acid (30 mg/kg) prevented hyperglycemia, reduced insulin levels, and increased free radical scavenging potential. This mouse model with reduced ability to diffuse free radical stress is of obvious interest because free radical–mediated damage is implicated in the pathogenesis and complications of both type 1 and type 2 diabetes. *Diabetes* 53 (Suppl. 1):S125–S129, 2004

Outbred CD-1 mice (a commercial stock of ICR mice) carry a spectrum of genetic susceptibilities for obesity and impaired glucose tolerance (IGT), both risk factors for the development of type 2 diabetes. Homozygous fixation of potential type 2 diabetes susceptibility genes is reflected in male mice of two ICR-derived inbred strains: NSY and NON (1,2). Both of these inbred strains develop IGT, but neither makes the transition into chronic nonfasting hyperglycemia. ALS (alloxan [AL]-susceptible) mice were derived from outbred CD-1 mice by selection for sensitivity to AL-induced diabetes, with concomitant selection for an AL-resistant (ALR) line (3). Alloxan, a chemical with structural similarities to glucose and a potent generator of free radicals, is a selective β-cell toxin because of the high affinity of AL for GLUT2 transporters. Interestingly, the differential AL sensitivity of the ALS/Lt and ALR/Lt strains correlated with differential ability to dissipate free radical stress (4). The ALS/Lt strain has significantly decreased global free radical defenses compared with the ALR/Lt strain, which reciprocally shows a constitutive elevation in systemic antioxidative capacity (4,5). Although ALS mice were selected for high susceptibility to AL-induced insulin-requiring diabetes, this selection has in fact produced a mouse strain with high type 2 diabetes predisposition (6).

Initially, the type 2 diabetes predisposition of ALS was recognized by congenic analysis of the yellow mutation (A^y) at the agouti locus on chromosome 2 (6). The incidence of diabetes in ALS-A^y males and females at 24 weeks of age was 100 and 60%, respectively. Mortality was high under these conditions, with only 50% of ALS-A^y males surviving to 50 weeks. Hyperinsulinemia appeared early, and pancreatic β-cell function in males was virtually abolished by 24 weeks, as evidenced by absence of circulating insulin. In contrast, ALR-A^y mice exhibited comparable increases in body weight, yet a significantly lower frequency of glycosuria (6).

The high frequency of A^y-mediated type 2 diabetes in ALS mice, coupled with this strain's low global antioxidant defenses, suggested that ALS/Lt (the substrain maintained at The Jackson Laboratory) might differ from the closely related NON/Lt strain in transiting from IGT to overt type 2 diabetes. In this article, we demonstrate that application of diabetogenic stress mediated through diet causes ALS males to transit into chronic nonfasting hyperglycemia. This diabetogenic stress entailed increased oxidative damage as evidenced by chronic treatment with the antioxidant R-lipoic acid (LA). LA treatment prevented hyperglycemia and hyperinsulinemia while concomitantly increasing plasma concentrations of reduced glutathione (GSH), a reflection of increased redox potential.

RESEARCH DESIGN AND METHODS

Mice. ALR/Lt and ALS/Lt mice used in this study were bred and maintained in the animal research facility at The Jackson Laboratory (Bar Harbor, ME). This animal facility is a specific pathogen-free vivarium. All mice were allowed free access to food (autoclaved diet NIH-31, 4% or 11% fat; Purina, St. Louis, MO) and acidified drinking water.

Glucose tolerance test. Intraperitoneal glucose tolerance tests were performed by administration of 2.5 mg/kg of D-glucose (Sigma, St. Louis, MO) in PBS to mice that were fasted for 16 h. Blood samples were removed before the glucose administration and then at 30, 90, and 120 min after injection.

Plasma glucose and insulin measurements. Plasma glucose was determined from retroorbital sinus blood collected in heparinized capillary tubes. Capillary tubes were centrifuged in a hematocrit centrifuge, and plasma was frozen until assayed. Plasma glucose values were determined using a Beck-

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AL, alloxan; GSH, glutathione; IGT, impaired glucose tolerance; LA, R-lipoic acid; ROS, reactive oxygen species.

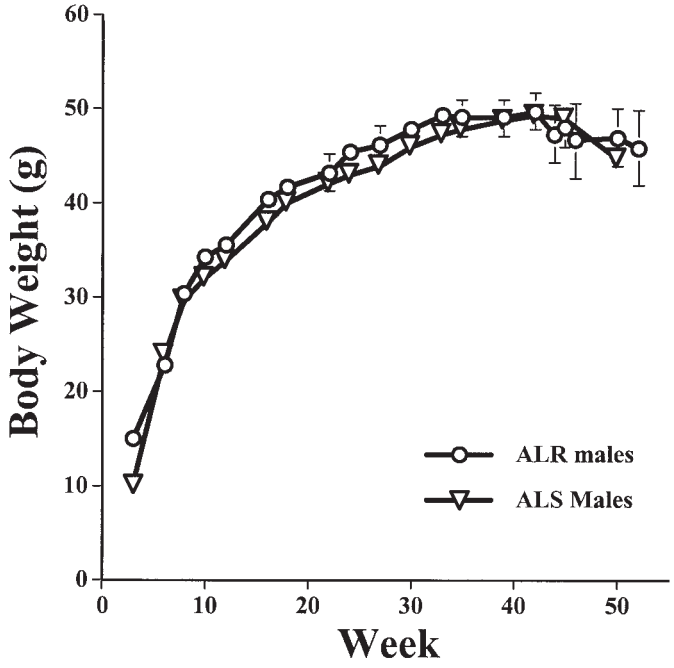

FIG. 1. ALR/Lt and ALS/Lt males show equivalent rates of body weight gain.

man Glucose Analyzer II (Beckman Instruments, Fullerton, CA). Insulin concentrations were obtained using an insulin radioimmunoassay with rat insulin standards (Linco, St. Louis, MO).

Preparation and administration of AL and insulin. ALS males and females at 10 weeks of age were weighed before AL treatment. Alloxan monohydrate (Sigma) was diluted in sterile PBS immediately before intravenous (tail vein) administration at a concentration of 52 mg/kg. Mice were bled from the retroorbital sinus via heparinized capillary tubes before AL administration and on days 1 and 7 after AL. On day 7, ALS mice of both sexes received 1 unit of Regular Iletin II porcine insulin intraperitoneally (Eli Lilly, Indianapolis, IN). One hour after the insulin injection, a blood sample was collected.

Determination of reduced GSH. Plasma was assayed directly for GSH content. Assays for reduced GSH were performed in COSTAR flat-bottom 96-well microtiter plates using the Bioxytech GSH-400 colorimetric assay for GSH (OXIS Health Products, Portland, OR), with a sample volume of 4 μl in a total volume of 200 μl. Supernatants were neutralized by the presence of 176 μl 200 mmol/l potassium phosphate buffer (pH 7.8) in this mixture. The 4-chloro-1-methyl-7-trifluromethyl-quinolinium methylsulfate reacts with only the reduced form of mercaptans. The second step involves a β-elimination reaction forming a chromophoric thione. Absorbance of the GSH-specific thione was read at 400 nm in a SpectraMax Microplate Spectrophotometer with SOFTmax PRO for Macintosh (Molecular Devices, Sunnyvale, CA).

LA administration. ALS males were singly housed and allowed to feed ad libitum on an 11% fat diet. LA (Astra Zenica, Södertälje, Sweden) was dissolved in 120 mmol/l Tris base (pH 8.0) (Sigma), and the pH was adjusted to 7.4 with NaOH. Starting at 10 weeks of age, mice received 30 mg/kg LA or vehicle alone daily via intraperitoneal injection over the 8-week experimental period. Mice were weighted and bled at 10, 14, and 18 weeks of age. Dual-emission X-ray absorptiometry was performed at the end of the 8-week trial period (18 weeks of age), as described previously (7).

Statistical analyses. All values reported are means ± SE, calculated from duplicate measures with an n of at least 6. Significance was determined with a one-way ANOVA using SuperANOVA for the Macintosh (Abacus Concepts, Berkeley, CA).

RESULTS

Body weight. As reported previously (8), body weight gain in ALS and ALR males is equivalent from weaning until 52 weeks of age (Fig. 1).

Glucose intolerance appears early in ALS males. Administration of an intraperitoneal glucose tolerance test demonstrated the glucose intolerance of AL-untreated ALS males as early as 6 weeks of age (Fig. 2). This intolerance to glucose further decreased with age. Further, the fasting plasma glucose levels of AL-untreated ALS males also increased with age from 88 ± 4 mg/dl (6 weeks) to 143 ± 10 mg/dl (50 weeks), correlating with IGT.

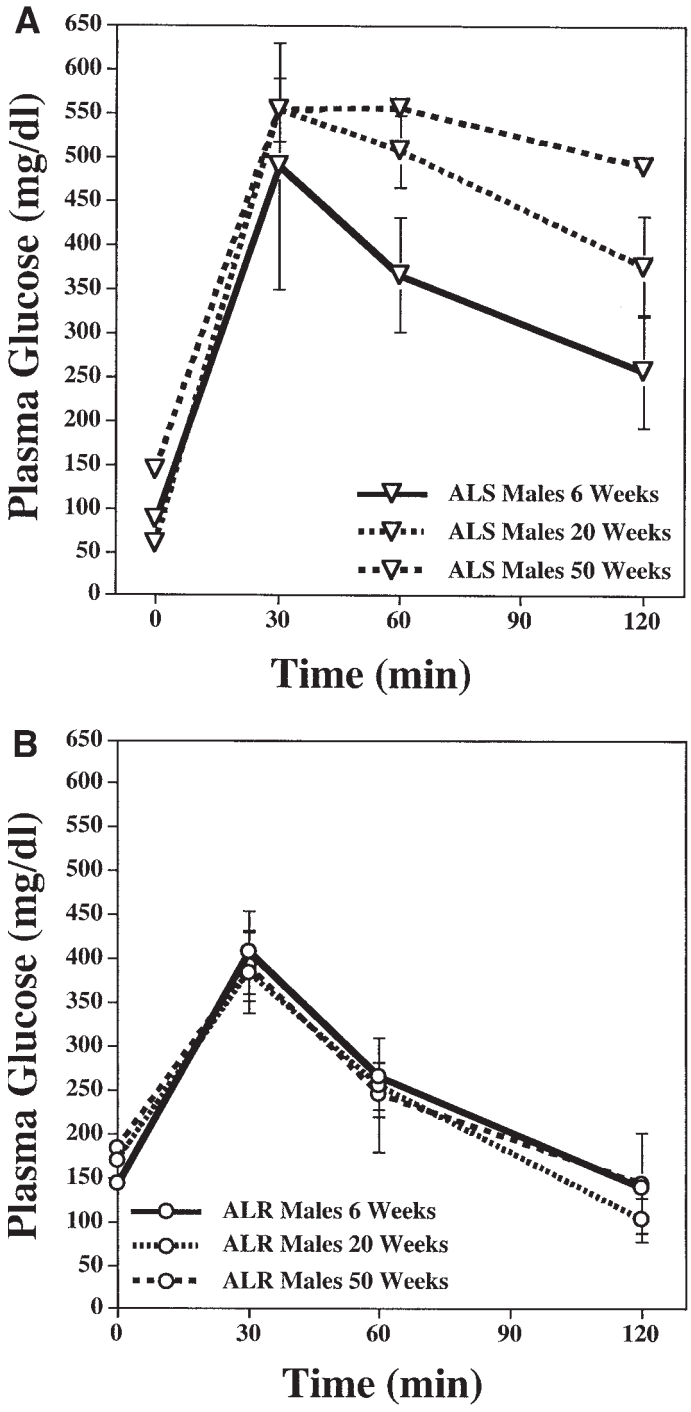

FIG. 2. IGT develops early in ALS/Lt males and worsens with age (*A*). In comparison, males of the closely related ALR/Lt strain do not exhibit glucose intolerance (*B*).

Insulin resistance in ALS males. ALS mice respond to a relatively low AL concentration (47–49 mg/kg) with development of insulin-dependent diabetes within 24 h after administration (3). One of the unusual features of this chemically induced diabetes is the relationship between

plasma insulin and glucose. In rodents, AL induces massive β-cell necrosis and release of insulin stores within hours after administration, producing an immediate hyperinsulinemia that, in turn, elicits an initial drop in blood glucose levels before hyperglycemia is detected. In AL-treated ALS/Lt males, the expected immediate hyperinsulinemia was not accompanied by transient hypoglycemia, but rather by hyperglycemia (Fig. 3*A*). Indeed, unlike ALS/Lt females, ALS/Lt males made diabetic at 10 weeks of age by an AL dose of 52 mg/kg i.v. failed to respond to insulin therapy (Fig. 3*A*). Plasma glucose values in hyperglycemic males receiving 1 unit of porcine insulin (~35.7 units/kg i.p.) fell from 784 ± 54 to 564 ± 77 mg/dl in 60 min. This minimal reduction was significantly different when compared with the decrease of 574 ± 108 to 165 ± 104 mg/dl in insulin-treated ALS/Lt females. Clearly, this was suggestive of an underlying insulin resistance in males. Longitudinal measurements of plasma insulin in AL-untreated ALS/Lt males maintained on a 6% fat diet confirmed underlying defects in glucose homeostasis associated with a progressively more severe insulin resistance (Fig. 3*B*). In accord with the marked IGT, males as young as 6 weeks of age exhibited plasma insulin levels that were moderately increased (4.7 ± 0.7 ng/ml) and progressively increased with age (14.5 ± 1.4 ng/ml by 12 weeks; 40 ± 15 ng/ml by 20 weeks). In comparison, ALR/Lt males exhibited plasma insulin levels at the upper end of a normal spectrum.

LA treatment of ALS males inhibits the onset of type 2 diabetes. Although ALS males present the type 2 diabetes phenotypes of IGT, hyperinsulinemia, and insulin resistance, the transition to clinical type 2 diabetes represented a threshold phenomenon. This is similar to type 2 diabetes frequencies in other polygenic models of type 2 diabetes. To determine if the reduction in free radical scavenging potential was associated with the onset of diabetes, mice were placed on 11% fat, singly housed and injected daily with either 30 mg/kg of LA or vehicle. LA significantly improved type 2 diabetes–linked phenotypes (body weight [Fig. 4*A*] and plasma glucose [Fig. 4*B*]). Further, the administration of LA had positive effects not only on body weight but also on adiposity. In controls, body weight increased from 46 ± 1.4 to 55 ± 2 g over the 8-week trial, compared with an increase of only 3 g (47 ± 4 to 50 ± 1 [$P < 0.05$]) in LA-treated ALS males. Final adiposity measure (% fat) of the untreated controls was 24.4 ± 1.2% (total fat mass 13.42 ± 0.66 g) compared with 18 ± 0.8% (total fat mass 9 ± 0.4 g [$P < 0.05$]). This difference in body weight can almost be totally explained by the difference in body lipid content as measured by dual-emission X-ray absorptiometry.

Plasma glucose increases were measured in controls, from 166 ± 8 mg/dl at the initiation of the dietary stress to 308 ± 27 mg/dl after 8 weeks, whereas LA-treated mice did not change (155 ± 7 [10 weeks] and 160 ± 6 [18 weeks] [$P < 0.05$]) when exposed to the dietary stress. Plasma insulin levels under this stress attained exceedingly high (and variable) concentrations, reaching 212 ± 131 ng/ml after 8 weeks, compared with 6.5 ± 0.6 ng/ml in LA-treated ALS males ($P < 0.05$). Further, administration of LA elevated circulating reduced GSH, a marker of free radical scavenging potential (Fig. 4*C*). In untreated males, a

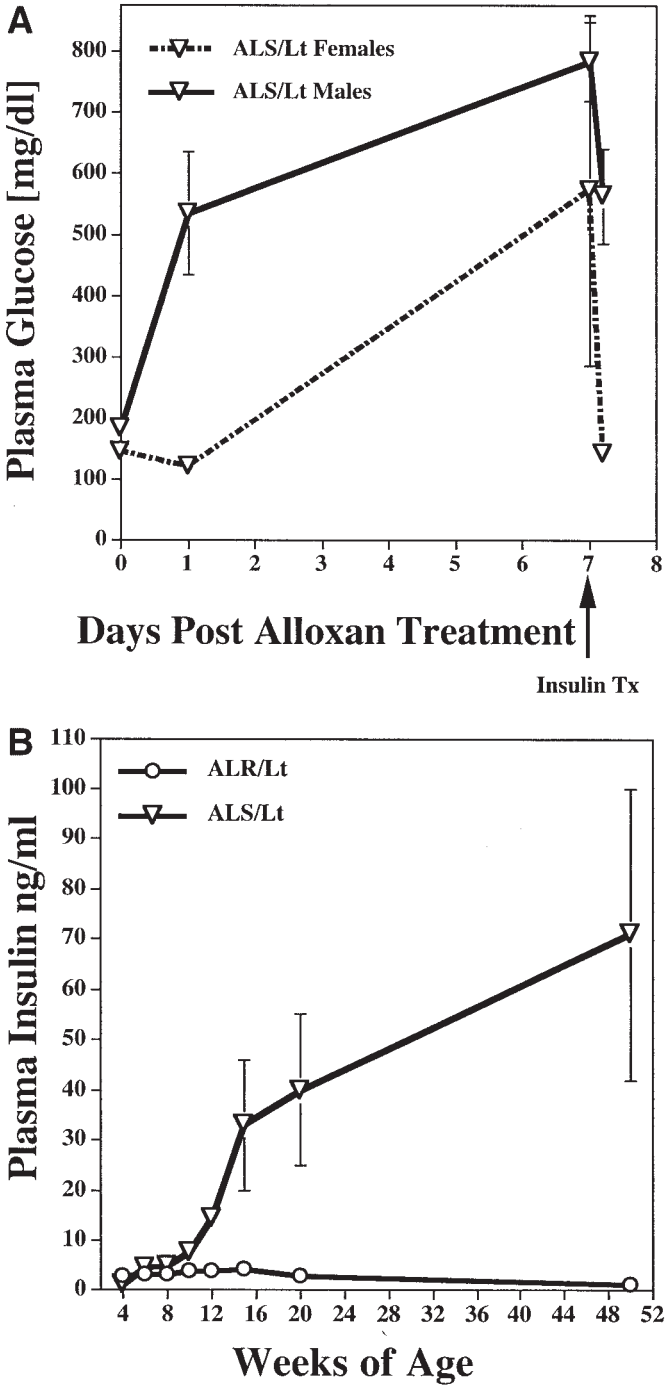

FIG. 3. ALS/Lt males exhibit insulin resistance. *A*: Alloxan-treated ALS males show strong resistance to insulin. Alloxan-treated (52 mg/kg) ALS males (solid line) and females (broken line) were treated with 1 unit porcine insulin 7 days after AL administration. One hour later, a blood sample was drawn to test plasma glucose. Figure 3*A* is the only experiment included in this study where mice were treated with AL. *B*: ALS/Lt males spontaneously develop hyperinsulinemia and present with insulin resistance as early as 10 weeks of age. Plasma insulin levels were monitored in unmanipulated ALS/Lt and ALR/Lt males from 4 to 52 weeks of age.

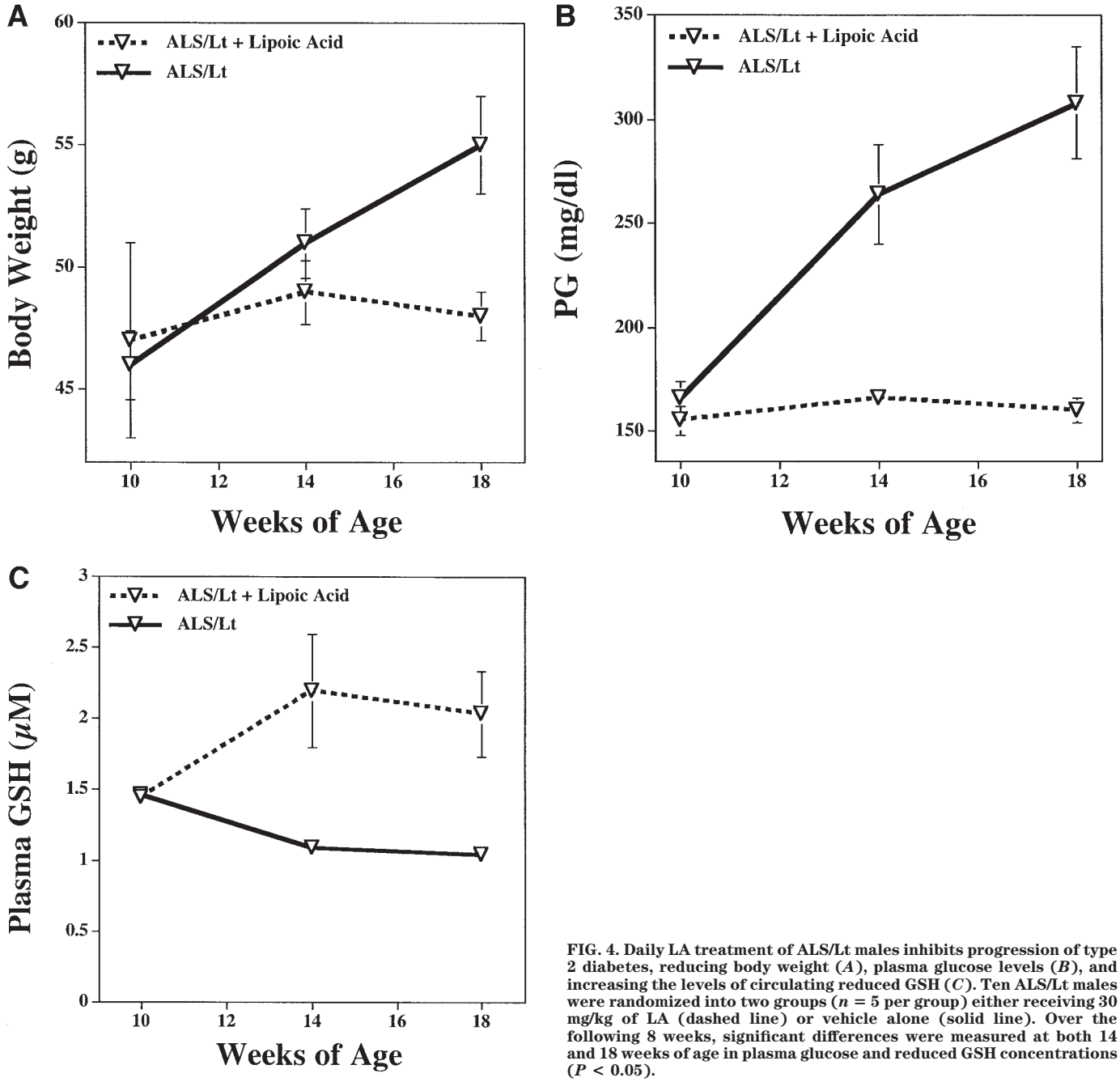

FIG. 4. Daily LA treatment of ALS/Lt males inhibits progression of type 2 diabetes, reducing body weight (*A*), plasma glucose levels (*B*), and increasing the levels of circulating reduced GSH (*C*). Ten ALS/Lt males were randomized into two groups ($n = 5$ per group) either receiving 30 mg/kg of LA (dashed line) or vehicle alone (solid line). Over the following 8 weeks, significant differences were measured at both 14 and 18 weeks of age in plasma glucose and reduced GSH concentrations ($P < 0.05$).

significant decrease in GSH from 1.47 ± 0.07 to 1.05 ± 0.05 μmol/l ($P < 0.05$) was measured, whereas in LA-treated animals, plasma GSH increased from 1.45 ± 0.05 to 2.04 ± 0.3 μmol/l after 8 weeks of daily LA injections.

DISCUSSION

Free radical stress has been implicated in the pathogenesis of both type 1 and type 2 diabetes (9–11). The pathological changes associated with diabetic hyperglycemia entail increased production of reactive oxygen species (ROS). Indeed, the ALR/Lt strain, selected for elevated systemic free radical dissipation, contains genes capable of suppressing autoimmune type 1 diabetes mediated by the immune system of the closely related NOD/Lt strain (12). ALS/Lt males, when compared with ALR/Lt males, show significantly lower ratios of reduced to oxidized GSH as well as an increase in circulating lipid peroxides (4,5). These ratios decrease with age. Because development of type 2 diabetes was inhibited by administration of the antioxidant LA, we conclude that cumulative free radical damage was an important contributor to diabetes pathogenesis. LA manipulation markedly reduced a number of type 2 diabetes–related subphenotypes, including hyperinsulinemia, increased adiposity, and hyperglycemia.

As noted in the introduction, the CD-1 (ICR) outbred parental stock reflects a spectrum of susceptibility for glucose intolerance disorders. Inbreeding ICR mice produced the NOD and NON strains known, respectively, for type 1 diabetes and pre–type 2 diabetes phenotypes. Similarly, CD-1 inbreeding produced reciprocally high or low antioxidant defenses, translating into strong diabetes resistance (ALR) or susceptibility type 2 diabetes (ALS).

Interestingly, just as ALR is closely related to NOD, ALS is a close genetic relative of NON. Both ALS and NON share a rare major histocompatibility complex haplotype ($H2^{nb1}$) and a less common Thy1^a T-cell allotype. Whereas these two strain share identity at numerous genetic markers as well as IGT in males, the ALS but not NON strain, is capable of making the transition to clinical type 2 diabetes. Differences in genes regulating systemic redox balance may well be central to the difference in partial versus strong type 2 diabetes susceptibility in these two strains.

Reports have established and discussed that redox balance, at the β-cell level and in insulin-responsive tissues, may be critical in maintenance of a normoglycemic state (13–17). Our previous work has shown that, when compared with the ALR/Lt strain, ALS/Lt exhibits reduced free radical defenses in the periphery and at the pancreatic islet level (5,18). Because LA protects ALS/Lt males from transitioning into hyperinsulinemia and clinical diabetes, the clear suggestion is that free radical-induced tissue damage is critical to the events that precipitate type 2 diabetes onset (Fig. 4). LA-induced increases in circulating GSH correlated with improved type 2 diabetes outcomes (Fig. 4*C*). It appears that LA increases the sensitivity of insulin-responsive tissues while reducing the insulin output of β-cells. In non–LA-treated controls, plasma insulin values increased over the 8-week observation period. Male ALS/Lt mice receiving daily LA treatment experienced only a modest increase in circulating levels. The increase in insulin, and presumably insulin resistance, correlated with an increase in plasma glucose levels over the course of the study (Fig. 4*B*).

ROS, a normal byproduct of cellular processes such as mitochondrial respiration, can directly cause damage to DNA proteins and lipids but can further activate stress response pathways. Recent works have implicated that these pathways can lead to insulin resistance and impaired insulin release (19–23). The current report introduces the ALS mouse as a new mouse model of type 2 diabetes with reduced systemic ability to diffuse ROS. In ALS/Lt males, a progressive increase in oxidant burden is associated with dietary fat–induced type 2 diabetes development, as shown by administration of the antioxidant LA. Administration of LA significantly increased circulating GSH in ALS males and markedly reduced hyperinsulinemia, improved glucose tolerance, and prevented the dietary-induced type 2 diabetes. The association of heightened free radical stress to glucose intolerance and hyperinsulinemia in ALS/Lt males provides a valuable system to analyze the relationship between ROS and the development of type 2 diabetes.

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