

# Lipoic Acid Synthase (LASY)

## A Novel Role in Inflammation, Mitochondrial Function, and Insulin Resistance

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**OBJECTIVE**—Lipoic acid synthase (LASY) is the enzyme that is involved in the endogenous synthesis of lipoic acid, a potent mitochondrial antioxidant. The aim of this study was to study the role of LASY in type 2 diabetes.

**RESEARCH DESIGN AND METHODS**—We studied expression of LASY in animal models of type 2 diabetes. We also looked at regulation of LASY in vitro under conditions that exist in diabetes. Additionally, we looked at effects of LASY knockdown on cellular antioxidant status, inflammation, mitochondrial function, and insulin-stimulated glucose uptake.

**RESULTS**—LASY expression is significantly reduced in tissues from animal models of diabetes and obesity compared with age- and sex-matched controls. In vitro, LASY mRNA levels were decreased by the proinflammatory cytokine tumor necrosis factor (TNF)- $\alpha$  and high glucose. Downregulation of the LASY gene by RNA interference (RNAi) reduced endogenous levels of lipoic acid, and the activities of critical components of the antioxidant defense network, increasing oxidative stress. Treatment with exogenous lipoic acid compensated for some of these defects. RNAi-mediated downregulation of LASY induced a significant loss of mitochondrial membrane potential and decreased insulin-stimulated glucose uptake in skeletal muscle cells. In endothelial cells, downregulation of LASY aggravated the inflammatory response that manifested as an increase in both basal and TNF- $\alpha$ -induced expression of the proinflammatory cytokine, monocyte chemoattractant protein-1 (MCP-1). Overexpression of the LASY gene ameliorated the inflammatory response.

**CONCLUSIONS**—Deficiency of LASY results in an overall disturbance in the antioxidant defense network, leading to increased inflammation, insulin resistance, and mitochondrial dysfunction. *Diabetes* 58:600–608, 2009

**T**ype 2 diabetes is the most prevalent chronic metabolic disease in the world. In the past decade, considerable evidence has accumulated implicating oxidative stress as a key factor that accelerates the onset and progression of type 2 diabetes. Chronic oxidative stress causes inflammation and mitochondrial dysfunction and culminates in insulin resistance, which ultimately progresses to diabetes. Oxidative

stress also promotes cellular dysfunction and damage, leading to the development of secondary complications of diabetes. The underlying cause of redox imbalance is a deficiency in the endogenous antioxidant network. This deficiency would result in an inability to combat excessive amounts of reactive oxygen species (ROS) and tip the balance in favor of oxidative stress.

Redox balance is maintained by an antioxidant defense network within mitochondria, consisting of stress-responsive enzymes such as superoxide dismutase (SOD), catalase and reduced glutathione (GSH), and antioxidants. The antioxidant defense network is activated in response to excessive production of ROS in the mitochondria, thereby neutralizing the ROS before they inflict damage on cellular molecules. Lipoic acid is a potent mitochondrial antioxidant that plays a central role in establishing and maintaining the antioxidant defense network by effectively scavenging ROS and regenerating critical antioxidants (1). Lipoic acid is also an essential cofactor of mitochondrial enzyme complexes involved in oxidative metabolism. Exogenous lipoic acid, by virtue of its antioxidant effect, has been shown to be beneficial in many metabolic and vascular diseases (2–7). Endogenously, lipoic acid is synthesized from octanoic acid by the action of LASY. Previously, synthesis of lipoic acid was believed to be an exclusively prokaryotic phenomenon, and existence of LASY in higher organisms was unknown. The discovery that mammalian cells are capable of synthesizing lipoic acid was made quite recently, when a mouse homolog of LASY was identified (8). Mammalian LASY contains a putative mitochondria targeting sequence at the NH<sub>2</sub>-terminus and is mainly localized in mitochondria (8). Thus, LASY is ideally positioned to generate lipoic acid at the site of action, namely, mitochondria. Although the pharmacological effects of lipoic acid have been explored in many studies, the importance of endogenous lipoic acid is largely unknown. In this study, we explored the role of LASY in diabetes and inflammation. Our data show for the first time that LASY is downregulated in diabetes and inflammation. Downregulation of LASY resulted in decreased endogenous lipoic acid levels. The data that we have presented suggest that downregulation of LASY, and the resultant decrease in endogenous lipoic acid, would cause redox imbalance leading to inflammation and mitochondrial dysfunction, two important hallmarks of diabetes.

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### RESEARCH DESIGN AND METHODS

**Reagents.** Unless otherwise stated, reagents were purchased from Sigma-Aldrich (St. Louis, MO).

**Rodents, diets, and housing.** Rodents for in vivo studies were purchased from Jackson or Charles River Laboratories. Animals were housed in groups of two to four upon arrival. All animals were allowed to feed ad libitum on a regular diet (Laboratory Rodent Diet, 5001, LabDiet, Inc.). Characteristics of the animals used in the study are outlined in the Supplementary Table, found in an online-only appendix at <http://dx.doi.org/10.2337/db08-0473>.

**Animal tissue collection.** Blood for glucose determination was collected by orbital bleed. Skeletal muscle (soleus), adipose tissue (visceral fat pad), and liver sections were collected after euthanization of the animals.

**Cell growth and differentiation.** Human skeletal muscle myoblasts (HSMs) (Cambrex) were grown and differentiated according to Cambrex's protocol. Human microvascular endothelial cells (HMVECs) and human aortic endothelial cells (Cambrex) were grown in HMVEC-MV/HMVEC growth media (Cambrex) until ~80% confluent for treatments or transfections.

**Real-time PCR.** LASY real-time quantitative PCR was performed using LASY primers and the Brilliant SYBR Green one-step RT-PCR kit (Stratagene). Oligonucleotide primers for human LASY were designed using real-time PCR primer design software and were custom-made (Supplementary Methods). For GAPDH and Actin real-time PCR, validated primer sets were purchased from SuperArray.

Amplifications were performed using the Mx3000P instrument (Stratagene) according to the manufacturer's instructions. Values obtained for the genes of interest (LASY, GAPDH) were normalized to values for a housekeeping gene, Actin.

**Knockdown studies.** Knockdown of the LASY gene was achieved by RNA interference (RNAi).

The siRNA duplex for LASY knockdown was custom-designed and synthesized by Dharmacon. The negative control for the siRNA LASY knockdown experiment consisted of a nontarget (control siRNA) oligo (Dharmacon). Differentiated cells were treated with siRNA oligos (10 nmol/l) and TransIT-KO reagent (Mirius) for 24–48 h at 37°C/CO<sub>2</sub>.

**Overexpression studies.** The human LASY gene was amplified from human aortic endothelial cells by Oligo dT priming with the Superscript first-strand synthesis system (Invitrogen). The amplification product was cloned into the pBKCVM vector. Presence of insert in the correct orientation was verified by PCR and sequencing.

**Transfections.** Transfections were done using Targefect reagents (Targeting Systems) using the manufacturer's protocol. Transfections were done in 100-mm plates for all assays. Transfections were allowed to proceed for 24 h, after which the cells were replated into 96- or 24-well plates and incubated for 18 h before treatments.

**Glucose uptake assay.** The glucose uptake assay in HSMs was performed by adapting the protocol described by Maddux et al. (9), with modifications as described in the figure legends.

**Cell treatments.** For HSMs, treatments with compounds or cytokines and growth factors were done on differentiated cells. Cell treatments were done as described in the figure legends.

**RNA isolation.** RNA was isolated from cultured cells using the Absolutely RNA 96 microprep kit (Stratagene) following the manufacturer's protocol. RNA from tissue samples was isolated using tissue-specific kits from Qiagen.

**Monocyte chemoattractant protein-1 enzyme-linked immunosorbent assay.** Monocyte chemoattractant protein-1 (MCP-1) enzyme-linked immunosorbent assay (ELISA) was carried out using the Quantikine Human MCP-1 kit as described by the manufacturer (R&D Systems).

**Western blot analysis and antibodies.** Western blot was done as described in the figure legends. Monoclonal mouse anti-LASY antibodies were purchased from Novus Biologicals. Polyclonal anti-rabbit lipoic acid antibodies were purchased from Calbiochem. Monoclonal mouse antibodies specific for PDH (E2/E3 subunits) were purchased from Molecular Probes. Purified pyruvate dehydrogenase complex (PDC) (E2 subunit) (bovine heart) was purchased from Globozymes. Secondary, horseradish peroxidase-conjugated IgG (H+L) antibodies were purchased from Zymed labs.

**Detection of mitochondrial membrane potential.** Changes in mitochondrial membrane potential were determined using a JC-1 assay kit (Biotium) as described by the manufacturer.

**Glutathione assay.** To determine intracellular levels of reduced glutathione (GSH), cells were lysed with protein lysis buffer 48 h after initiation of LASY knockdown. GSH levels were determined using the Glutathione assay kit (Biochain) following the manufacturer's instructions. Data were expressed per milligram protein determined by the MicroBCA technique.

**Catalase assay.** Catalase (CAT) activity was determined using a CAT assay kit purchased from Cayman Chemical.

**SOD assay.** SOD activity was determined by using a SOD assay kit from Cayman Chemical. The assay was used to measure total SOD (Cu/Zn-, Mn-, and Fe-SOD) as well as cytosolic and mitochondrial SOD activities in the samples. Cells were grown in six-well plates for the SOD assay and analyzed for SOD activity 48 h after initiation of LASY knockdown.

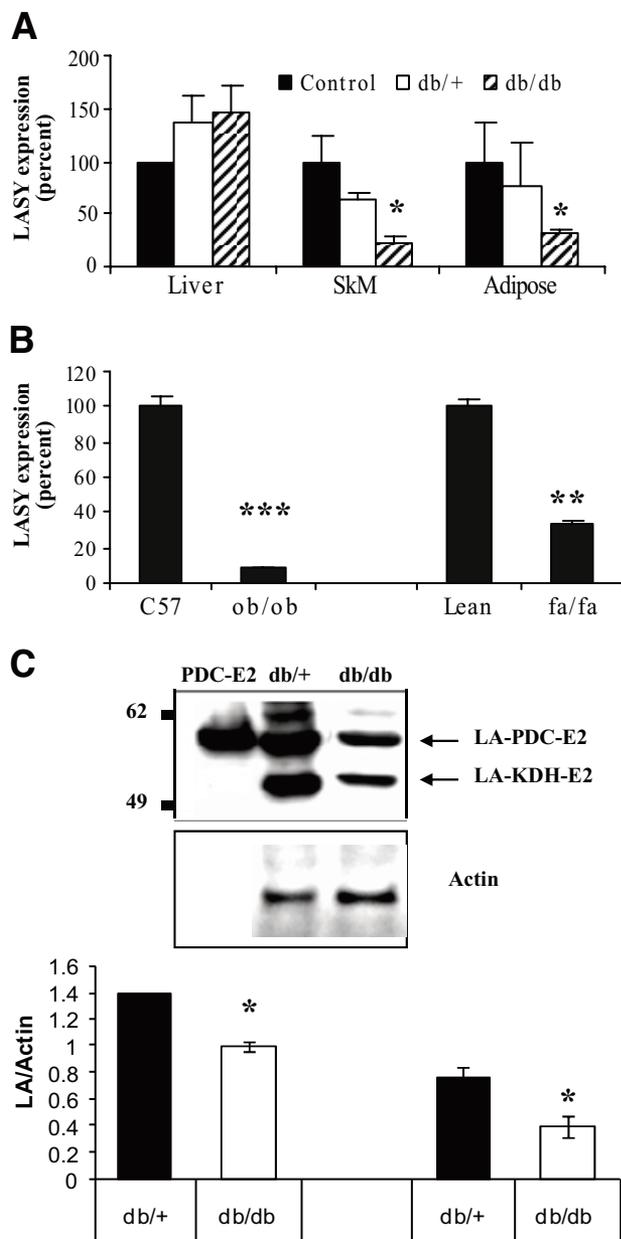
**Superoxide assay.** Production of superoxide anion was measured with a commercially available kit (Sigma-Aldrich). The kit uses a chemiluminescent method to measure the oxidation of luminol by superoxide anions.

## RESULTS

**The LASY gene is downregulated in animal models of diabetes and obesity.** To understand the role of LASY in diabetes, we first determined LASY expression in a mouse model of type 2 diabetes (*db/db*). Skeletal muscle and adipose tissue are key players that contribute to insulin resistance in type 2 diabetes. LASY gene expression is significantly downregulated in both skeletal muscle ( $P < 0.02$ ) and adipose tissue ( $P < 0.004$ ) of *db/db* mice compared with their normoglycemic heterozygous (*db/+*) counterparts and normal control mice (Fig. 1A). Interestingly, there was no decrease in LASY mRNA levels in the liver of *db/db* mice compared with the *db/+* or nondiabetic mice. There was a small increase that was insignificant. Downregulation of LASY in adipose tissue of *db/db* mice translated to a significant decrease in endogenous lipoic acid levels (Fig. 1C). The anti-lipoic acid antibodies used in the blot detected lipoic acid bound to the E2 subunits of PDC and  $\alpha$ -ketoglutarate dehydrogenase (KDH), two key mitochondrial enzymes that use lipoic acid as a cofactor ( $P < 0.007$ ,  $P < 0.005$ , for lipoic acid-PDC-E2 and lipoic acid-KDH-E2, respectively) compared with *db/+* mice (Fig. 1C). This suggests that in diabetic mice such as *db/db*, decrease in LASY results in a decrease in endogenous lipoic acid levels. We also studied LASY mRNA levels in adipose tissue from two other animal models of obesity, the *ob/ob* mouse model and Zucker *fa/fa* rat model. As seen in the case of the *db/db* mice, LASY expression was significantly reduced in these obese models compared with their lean counterparts (Fig. 1B, *ob/ob*,  $P < 0.0001$ , Zucker *fa/fa*,  $P < 0.03$ ).

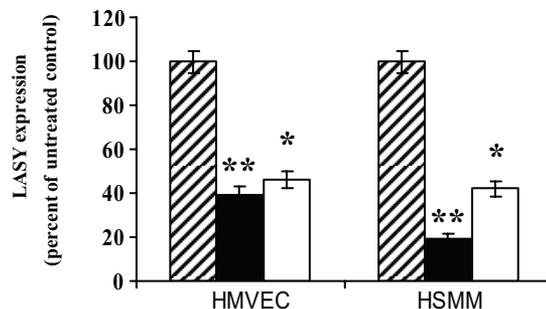
**LASY expression in vitro is downregulated by conditions that exist in diabetes.** Hyperglycemia and increased secretion of inflammatory cytokines are two hallmarks of diabetes. To understand the regulation of LASY, HMVECs were subjected to treatment with medium containing TNF- $\alpha$  or high glucose (Fig. 2). Treatment with glucose (25 mmol/l) led to a significant downregulation ( $P < 0.02$ ) in LASY mRNA levels in endothelial cells. Intermediary levels of glucose (11 mmol/l) or an osmotic control (D-mannitol) did not significantly affect LASY expression (Supplementary Fig. 1, found in the online appendix). Treatment of HSMs with 25 mmol/l glucose also reduced LASY mRNA levels significantly (Fig. 2,  $P < 0.02$ ). Treatment of both endothelial cells and skeletal muscle cells with TNF- $\alpha$  significantly decreased LASY mRNA levels (Fig. 2,  $P < 0.001$ ). Thus, consistent with the animal data, which suggests reduction in LASY in diabetes, LASY appears to be susceptible to downregulation by hyperglycemia and inflammatory cytokines.

**Inhibition of LASY expression decreases mitochondrial lipoic acid levels.** We knocked down the LASY gene in HSMs using the siRNA-based gene silencing approach. Using this approach, we were able to get 51% knockdown of the LASY gene after 48 h (Fig. 3A). As shown in Fig. 3B, the knockdown was specific to LASY, since expression of a housekeeping gene such as GAPDH was not affected. Consistent with the real-time PCR data, knockdown of LASY resulted in a significant reduction in LASY protein levels (Fig. 3C,  $P < 0.009$ ). We then studied the effect of LASY knockdown on endogenous lipoic acid levels by using Western blot analysis. As shown in Fig. 3D, knockdown of LASY resulted in a significant decrease in lipoic acid that is associated with PDC and KDH ( $P < 0.01$ ). However,



**FIG. 1.** LASY mRNA and liponic acid (LA) levels in obese diabetic animal models compared with normal controls. **A:** LASY mRNA levels in skeletal muscle, adipose, and liver tissues from *db/db* mice. **B:** LASY mRNA levels in adipose tissue of *ob/ob* mice and Zucker *fa/fa* rats.  $n = 3$  in each group. Ct values obtained from LASY primers were corrected for Ct values from Actin primers. Fold changes were calculated and expressed as a percent of the fold change of control(s).  $P$  values were calculated using Student's  $t$  test (one-tailed, unpaired). **A:** For *db/db* versus *db/+*,  $*P < 0.02$  for skeletal muscle (SkM),  $*P < 0.004$  for adipose tissue. **B:** For *ob/ob* versus C57,  $***P < 0.0001$ . For *fa/fa* versus lean,  $**P < 0.03$ . **C:** Endogenous liponic acid levels in adipose tissue (visceral fat pads) of *db/db* mice compared with *db/+* mice.  $n = 3$  in each group. Western blot (top panel): Upper and lower bands represent LA-PDC-E2 and LA-KDH-E2, respectively. Middle panel shows Actin. Quantitation of the WB for LA-PDC-E2 and LA-KDH-E2 is shown in the bottom panel. Results are means  $\pm$  SD.  $P$  values were calculated using the Student's  $t$  test (one-tailed, unpaired).  $*P < 0.007$  and  $P < 0.005$  for *db/db* versus *db/+* for LA-PDC and LA-KDH, respectively.

knockdown of LASY did not affect total levels of E2 protein (Fig. 3E). Thus, knockdown of LASY directly translates to a decrease in liponic acid associated with the E2 subunit of PDC, without affecting levels of E2 protein. These results are consistent with our *in vivo* results in which downregulation of LASY in tissues translated to a decrease in liponic acid levels (Fig. 1C).

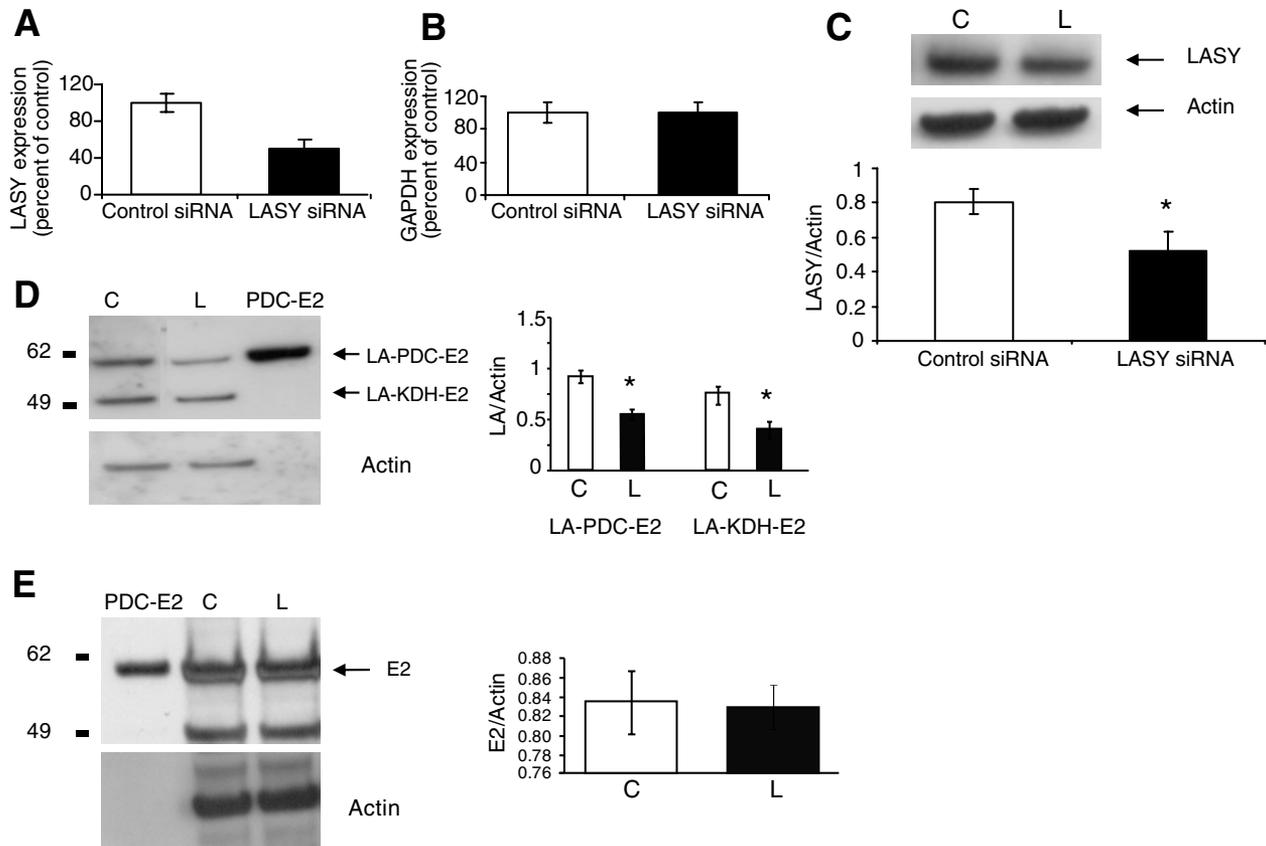


**FIG. 2.** LASY mRNA levels in human endothelial and skeletal muscle cells after treatment with pro-inflammatory agents. HMVECs and HSMs were used in the experiment. Concentrations of TNF- $\alpha$  used for stimulation of the two cell types were based on the MCP-1 responses; HSMs required a higher concentration of TNF- $\alpha$  to secrete detectable amounts of MCP-1. HMVECs were treated with 0.05 ng/ml of TNF- $\alpha$ ; HSMs were treated with 1 ng/ml of TNF- $\alpha$ . Glucose concentration for both cell types: 25 mmol/l. Controls and treatments were done for 18 h in low-serum (1% fetal bovine serum) media. Fold changes were calculated and expressed as percent of untreated control cells.  $**P < 0.001$ ,  $*P < 0.02$ . Results represent means  $\pm$  SE of three independent experiments.  $P$  values were calculated using Student's  $t$  test (one-tailed, unpaired). ▨, Untreated control; ■, TNF; □, glucose.

**Knockdown of LASY decreases intracellular GSH levels in human skeletal muscle cells.** The antioxidant properties of liponic acid facilitate the regeneration of other antioxidants such as reduced GSH, which in turn protects cells from oxidative damage (1). We hypothesized that reduction in the levels of liponic acid due to LASY knockdown would result in a decrease in intracellular GSH levels. We studied the GSH levels in HSMs in response to LASY knockdown. Compared with the control siRNA oligo, there was a significant decrease ( $P < 0.005$ ) in GSH levels in cells treated with LASY siRNA (Fig. 4A).

**Knockdown of LASY results in decreased activities of superoxide dismutase and catalase and increased superoxide anion levels.** To further investigate the effect of LASY knockdown on the cellular antioxidant defense network, we determined the activities of two critical antioxidant enzymes, CAT and SOD, in HSMs after knockdown of LASY. As shown in Fig. 4, knockdown of LASY results in a significant decrease in the activities of catalase (Fig. 4B) and mitochondrial and cytosolic SOD (Fig. 4C and D). Treatment with 500  $\mu$ mol/l liponic acid restored the activities of these enzymes (Fig. 4B–D). There was a concentration-dependent effect between 100 and 500  $\mu$ mol/l of liponic acid. Based on the decrease in SOD activity, we hypothesized that there would be an increase in superoxide anion levels with LASY knockdown. This turned out to be true, since there was a significant increase in superoxide anion levels both in basal and TNF- $\alpha$ -treated conditions (Fig. 4E). Treatment with liponic acid (500  $\mu$ mol/l) did not decrease the superoxide anion levels. Instead, there was a significant increase in superoxide anion levels with liponic acid treatment in both control and LASY siRNA-treated samples, compared with the TNF- $\alpha$ -treated levels. The difference between control and LASY siRNA-treated samples was also significant, suggesting that liponic acid treatment worsens the TNF- $\alpha$ -induced accumulation of superoxide when LASY is deficient.

**Reduced LASY expression results in decreased glucose uptake in skeletal muscle cells.** One of the factors contributing to hyperglycemia is insulin resistance, which causes reduced glucose uptake by skeletal muscle. Based on our observation that LASY expression is downregulated in the skeletal muscle of insulin-resistant animals, we studied



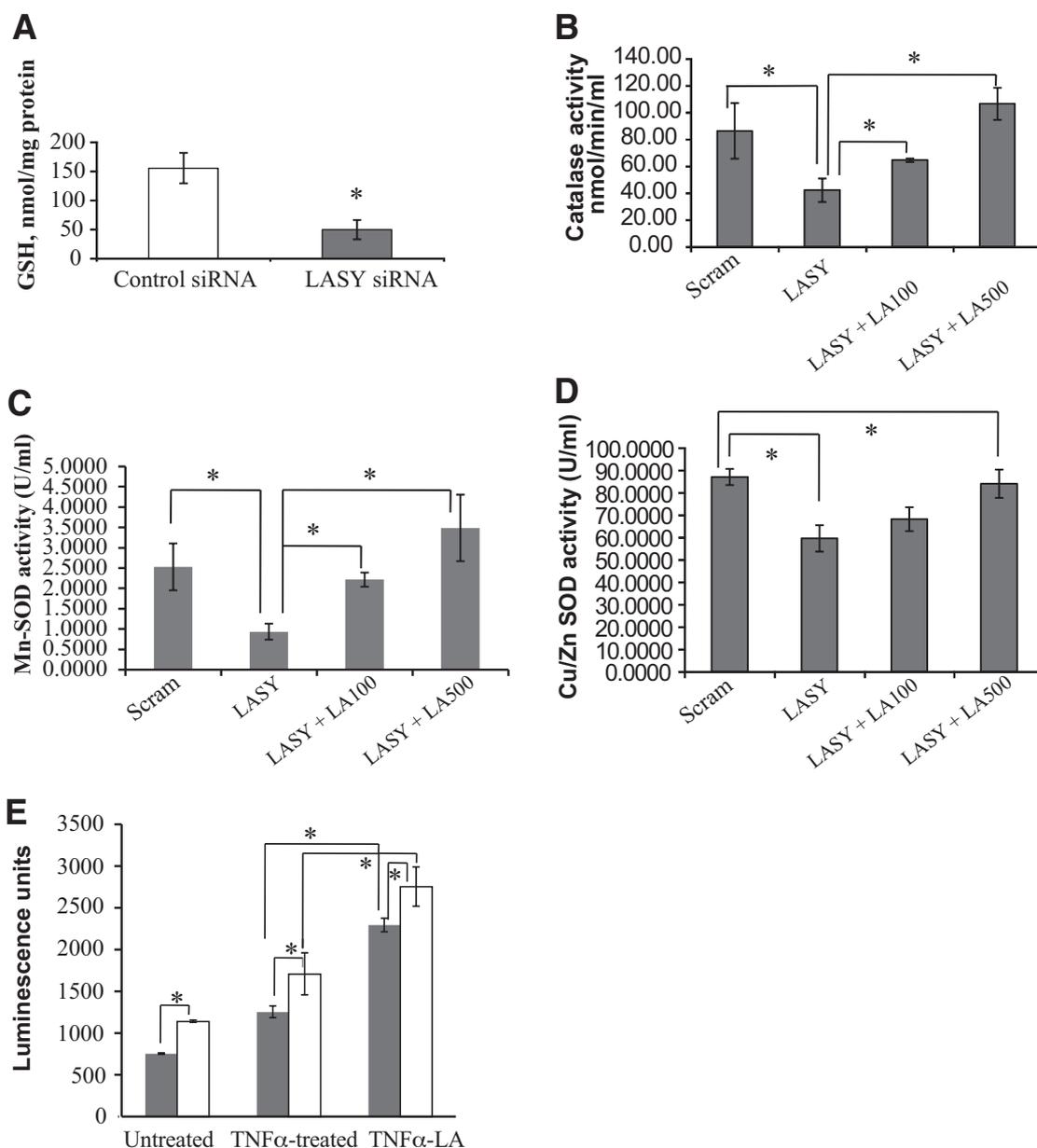
**FIG. 3.** Effect of siRNA-based LASYS knockdown on LASYS expression and endogenous lipioic acid (LA) levels in HSMMS. **A:** LASYS mRNA levels in HSMMS treated with a LASYS siRNA oligo or control (scrambled) siRNA oligo. **B:** GAPDH mRNA levels in HSMMS treated with LASYS siRNA oligo or control (scrambled) siRNA oligo. Ct values from real-time PCR assays for LASYS and GAPDH were corrected for Ct values from Actin. Fold changes were calculated as percent of fold change of control. **C:** Western blot analysis of cell lysates from HSMMS treated with LASYS siRNA oligo (L) or scrambled control oligo (C) using monoclonal anti-mouse antibodies directed to a peptide epitope of recombinant LASYS protein (Novus Biologicals). Cells were grown in 24-well plates. After gene knockdown, cells were lysed with protein lysis buffer (0.5% SDS, 50 mmol/l Tris-HCl, pH 8.0, 1× protease inhibitor cocktail, Sigma). Protein concentration was determined using the MicroBCA kit (Pierce). Equivalent amounts of protein were analyzed by SDS-PAGE (NuPage precast gels). Western blotting protocols were done as described by the antibody manufacturer. Bands were quantitated by densitometry, using the Total Lab software. A 1:500 dilution of the antibody detected an ~55-kDa band (*upper panel*). Actin is shown in the *middle panel*. Quantitation of the blot is shown in the *lower panel*. Results represent means ± SE of three independent experiments. *P* values were calculated using the Student's *t* test (one-tailed, unpaired). \**P* < 0.009 for LASYS siRNA versus Control siRNA. **D:** Western blot analysis of cell lysates from HSMMS treated with LASYS siRNA oligos (L) or scrambled control oligo (C) using polyclonal anti-lipioic acid (LA) rabbit antibodies directed to E2 subunit of PDC or KDH (above) or Actin antibodies (below). Purified PDC (E2 subunit) from bovine heart (GloboZymes) was run on the gel alongside cell lysates. The two bands in lipioic acid Western blot represent lipioic acid bound to E2 subunit of PDC (*upper band*, LA-PDC-E2) or  $\alpha$ -KGD (*lower band*, LA-KDH-E2). Quantitation of LA-PDC-E2 and LA-KDH-E2 bands in control and LASYS siRNA lanes is shown to the right; values are normalized to Actin values. Results are means ± SE of three independent experiments. *P* values were calculated using the Student's *t* test (one-tailed, unpaired). \**P* < 0.01, LASYS siRNA versus control siRNA for LA-PDC-E2 and LA-KDH-E2. **E:** Western blot analysis of cell lysates from HSMMS treated with monoclonal antibodies specific for pyruvate dehydrogenase (E2/E3 components of PDC) (Molecular Probes). The top band represents E2 subunit of PDC. Purified PDC (E2 subunit) was run on the gel alongside cell lysates. Quantitation of E2 band is shown to the right. Values were normalized to values for Actin (*bottom panel*).

the effect of reduced LASYS expression on glucose uptake in skeletal muscle cells. We knocked down the LASYS gene by siRNA-based gene silencing and studied the effect of the knockdown on [<sup>3</sup>H]-2-deoxy glucose uptake in skeletal muscle cells. As in our earlier experiments, we obtained a substantial knockdown (64%) with the LASYS siRNA oligo compared with the control siRNA oligo in HSMMS cells (data not shown). Decreased LASYS expression in skeletal muscle cells significantly reduced insulin-stimulated glucose uptake (Fig. 5A) (*P* < 0.01). There was a small, insignificant decrease in basal glucose uptake (Fig. 5A). These data demonstrate that downregulation of LASYS has a contributory role on insulin resistance in skeletal muscle.

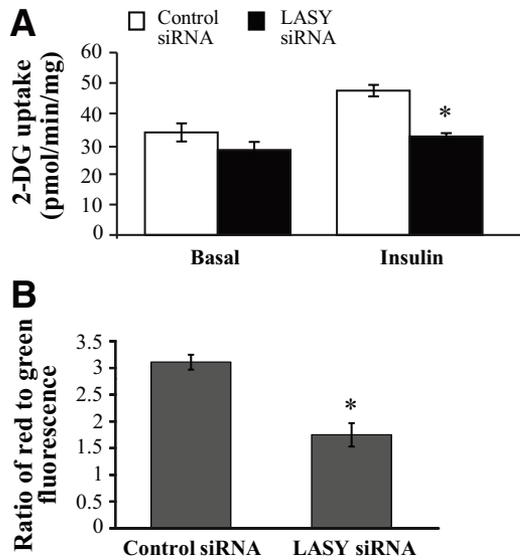
**Reduced LASYS expression decreases mitochondrial membrane potential in human skeletal muscle cells.** Because a decline in mitochondrial function is one of the causes of insulin resistance in diabetes, we tested if a decrease in LASYS would affect mitochondrial function. We studied the effect of siRNA-based knockdown of LASYS on

mitochondrial membrane potential. Mitochondrial membrane potential was measured in terms of the ratio of red to green fluorescence of a cationic dye, JC-1 (see RESEARCH DESIGN AND METHODS). Reduced LASYS expression was accompanied by a significant decrease (*P* < 0.003) in the ratio of red to green fluorescence of JC-1 dye, indicating that LASYS knockdown affects mitochondrial membrane potential (Fig. 5B).

**Inhibition of LASYS exacerbates inflammation.** Increase in inflammation and consequent endothelial cell dysfunction, which are two other hallmarks of diabetes, play a contributory role in atherosclerosis. We tested if knocking down the LASYS gene would affect the inflammatory response in endothelial cells. Using the RNAi approach, we were able to get 88% knockdown of LASYS in HMVECs (Fig. 6A, *P* < 0.001). Concomitant with LASYS reduction, basal secretion of MCP-1 in HMVECs with reduced expression of LASYS was significantly increased (Fig. 6B, *P* < 0.05). Treatment with TNF- $\alpha$  further elevated

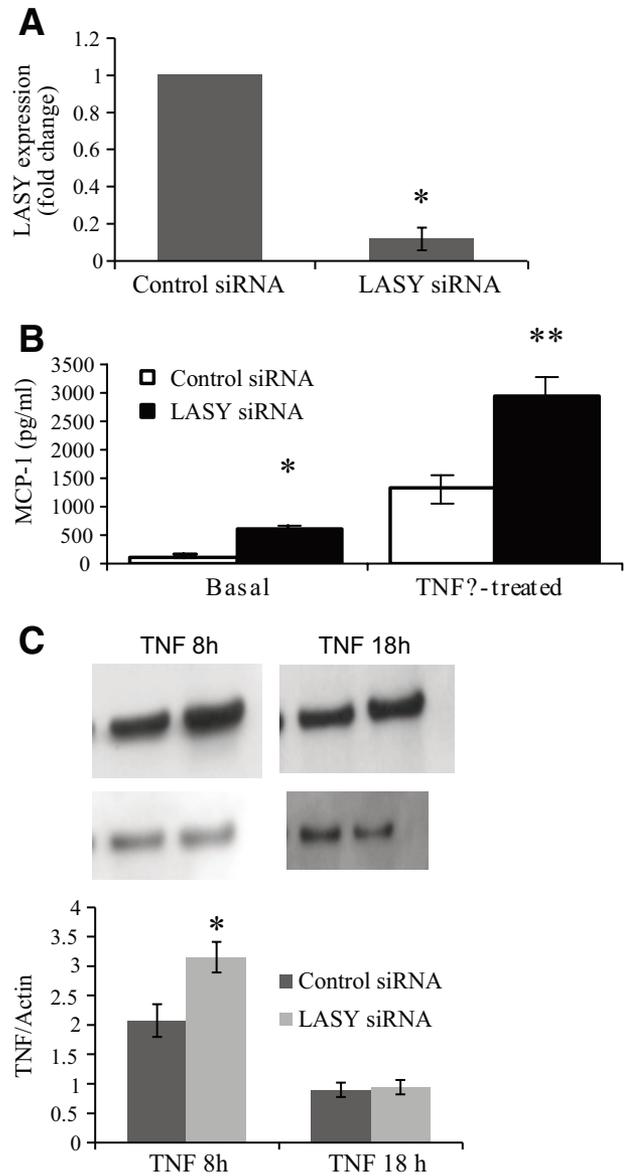


**FIG. 4.** Effect of LASY knockdown on the antioxidant system in HSMMs. **A:** Effect of LASY knockdown on intracellular reduced glutathione levels. Intracellular GSH levels in HSMMs treated with LASY or control siRNA oligos was quantitated using the GSH assay as described in the text. Results for GSH are corrected for amount of protein. Data represent the means  $\pm$  SD of three independent experiments. *P* values were calculated using Student's *t* test (one-tailed, unpaired). \**P* < 0.003 for LASY siRNA versus control siRNA. **B–D:** Effect of siRNA-based LASY knockdown on activities of catalase (**B**), mitochondrial superoxide dismutase (Mn-SOD) (**C**), and cytosolic SOD (Cu-Zn-SOD) (**D**). Knockdown was done as described in the text. Treatments with lipoic acid were done as described in the earlier section, 24 h after knockdown. **B:** Effect of LASY knockdown on catalase activity in HSMMs. One unit is defined as the amount of enzyme that will cause the formation of 1.0 nmol formaldehyde per minute at 25°C. Catalase activity was determined using a kit from Cayman Chemical. The standard curve was generated using a formaldehyde standard. Data represent means  $\pm$  SD of three independent experiments. Catalase enzyme activity was determined in duplicate. Significance was calculated using Student's *t* test (one-tailed, unpaired). *P* < 0.01 for LASY siRNA versus control siRNA; *P* < 0.006 for LASY siRNA+LA100 versus LASY siRNA; *P* < 0.008 for LASY siRNA+LA500 versus LASY siRNA. **C and D:** Effect of LASY knockdown on SOD activity in HSMMs. One unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. SOD activity was determined using a kit from Cayman Chemical. To separate cytosolic (Cu/Zn) and mitochondrial (Mn) SOD, cell lysate supernatants (after homogenization) were centrifuged at 10,000*g* for 15 min at 4°C to pellet mitochondrial SOD. The supernatant contained cytosolic SOD. The standard curve for the SOD assay was generated using a quality-controlled SOD standard. Data represent means  $\pm$  SD of three independent experiments. SOD enzyme activity was determined in duplicate. Significance was calculated using the Student's *t* test (one-tailed, unpaired). Mitochondrial SOD: *P* < 0.0005 for LASY siRNA versus control siRNA; *P* < 0.005 for LASY siRNA+LA100 versus LASY siRNA; *P* < 0.003 for LASYsiRNA+LA500 versus LASY siRNA. Cytosolic SOD: *P* < 0.0001 for LASY siRNA versus control siRNA; *P* < 0.0007 for LASY siRNA+LA500 versus LASY siRNA. **E:** Effect of LASY knockdown on superoxide anion levels. Superoxide anion levels were quantitated using a commercially available kit (Sigma-Aldrich), which measures the oxidation of luminol by superoxide anions by chemiluminescence. Cells that were transfected with either control siRNA or LASY siRNA were treated with TNF- $\alpha$  (1 ng/ml, 18 h) 24 h after transfection. The manufacturer's protocol was followed for harvest of the cells and the assay. Luminescence intensity was measured at various time points (5–20 min). Values at peak intensity are shown. Data represent means  $\pm$  SD of three independent experiments. Assay was done in triplicate. Significance was calculated using Student's *t* test (one-tailed, unpaired). *P* < 0.003 for untreated samples (control siRNA versus LASY siRNA); *P* < 0.03 for TNF- $\alpha$ -treated samples (control siRNA versus LASY siRNA); *P* < 0.004 for control siRNA+TNF versus control siRNA+TNF+lipoic acid; *P* < 0.002 for LASY siRNA+TNF versus LASYsiRNA+TNF+lipoic acid; *P* < 0.03 for control siRNA+TNF+lipoic acid versus LASYsiRNA+TNF+lipoic acid.  $\blacksquare$ , Control siRNA;  $\square$ , LASY siRNA.

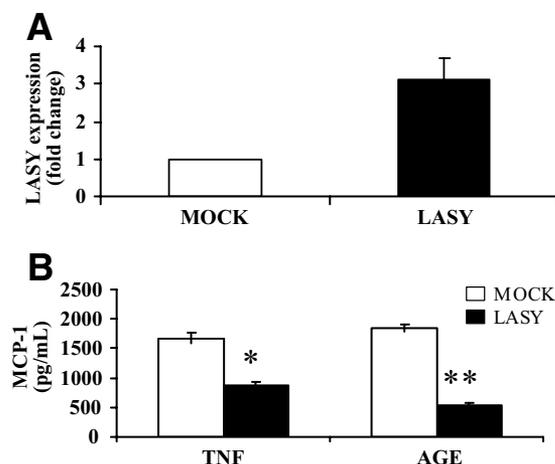


**FIG. 5.** Effect of LASYS knockdown on glucose uptake and mitochondrial membrane potential. **A:** Effect of siRNA-based knockdown of LASYS gene expression on insulin-stimulated glucose uptake in HSMMs. Knockdown of LASYS was done as described in RESEARCH DESIGN AND METHODS. Cells were washed with DMEM containing 1% BSA and incubated in this medium for 18 h. Cells were washed with transport medium (20 mmol/l HEPES, pH 7.4, 140 mol/l NaCl, 5 mmol/l MgCl<sub>2</sub>, 1 mmol/l CaCl<sub>2</sub>, and 0.1% (wt/vol) BSA. Next, cells were incubated in transport medium with or without insulin (1  $\mu$ mol/l) for 30 min at 37°C/CO<sub>2</sub>. This was followed by incubation in transport buffer containing 10  $\mu$ mol/l 2-deoxy-D-[2,6-<sup>3</sup>H]glucose (1.0  $\mu$ Ci/ml) for 30 min at 37°C. Reactions were stopped by aspirating the media and washing cells with PBS containing 20 mmol/l D-glucose at 4°C. Cells were lysed in 0.5% (wt/vol) SDS, and radioactivity was measured by liquid scintillation counting. Data were expressed per milligram of protein, determined by using the bicinchoninic acid (BCA) method (Pierce Chemical Company). Glucose uptake values under basal (unstimulated) and insulin-stimulated conditions are expressed as picomoles per minute per milligram protein. Cells were stimulated with 1  $\mu$ mol/l insulin for 30 min in transport buffer, as described by Maddux et al. (9). Data represent means  $\pm$  SE of three independent experiments. *P* values were calculated using Student's *t* test (one-tailed, unpaired). \**P* < 0.01 for LASYS siRNA versus control siRNA for insulin-treated cells. Difference between LASYS siRNA and control siRNA for basal unstimulated cells is not significant. **B:** Effect of siRNA-based LASYS knockdown on mitochondrial membrane potential in HSMMs. Knockdown was done as described in the text. Mitochondrial membrane potential was determined using the JC-1 assay. After knockdown, cells were washed with 1 $\times$  PBS and incubated with JC-1 dye as described in the JC-1 assay kit (Biotium) for 30 min at 37°C. Cells were washed twice with 1 $\times$  PBS, trypsinized, and transferred to 96-well PCR tube plates. Red and green fluorescence was measured using the Envision fluorescent plate reader (Perkin Elmer). Results are expressed as ratio of red to green fluorescence of JC-1. Data represent means  $\pm$  SD of three independent experiments. *P* values were calculated using the Student's *t* test (one-tailed, unpaired). \**P* < 0.005 for LASYS siRNA versus control siRNA.

the levels of MCP-1 (Fig. 6B, *P* < 0.02). These data demonstrate an inverse relationship between LASYS expression levels and secretion of MCP-1, suggesting that inhibition of LASYS expression increases inflammation. **Inhibition of LASYS expression increases nuclear translocation of nuclear factor- $\kappa$ B.** Nuclear factor (NF)- $\kappa$ B is a key transcription factor that is involved in the transcription of over 150 known genes, including genes encoding inflammatory markers such as MCP-1. Translocation of NF- $\kappa$ B into the nucleus is a key event in the activation of NF- $\kappa$ B. We investigated if inhibition of LASYS has an effect on TNF- $\alpha$ -induced nuclear translocation of the p65 subunit of NF- $\kappa$ B. Western blot analysis of nuclear extracts showed that siRNA-based knockdown of LASYS increased nuclear translocation of the NF- $\kappa$ B p65 subunit compared with the control after 8 h of treatment with TNF



**FIG. 6.** Effect of knockdown of the LASYS gene on inflammation in HMVECs. **A:** LASYS mRNA levels in HMVECs treated with a LASYS siRNA oligo or control (scrambled) siRNA oligo; knockdown was done for 48 h. Ct values from real-time PCR assay for LASYS was corrected for Ct values from Actin. Fold change in LASYS expression is shown relative to control; \**P* < 0.001 for LASYS siRNA versus control siRNA. **B:** MCP-1 levels in control siRNA and LASYS siRNA-treated cells under basal and TNF- $\alpha$ -treated conditions. TNF- $\alpha$  (0.05 ng/ml) treatment was done in 1% serum medium after 24 h of siRNA transfection. Duration of treatment with TNF- $\alpha$  was 18 h. Treatment supernatants were used in an ELISA to determine MCP-1 levels expressed as picograms per milliliter. Data represent means  $\pm$  SD of three independent experiments. *P* values were calculated using Student's *t* test (one-tailed, unpaired). \**P* < 0.05 for LASYS siRNA versus control siRNA (basal). \*\**P* < 0.02 for LASYS siRNA versus control siRNA (TNF- $\alpha$ -treated). **C:** Western blot analysis of NF- $\kappa$ B p65 subunit levels in nuclear extracts from cells treated with either control (scrambled) siRNA or LASYS siRNA. Nuclear extracts were prepared from cells that were treated with TNF- $\alpha$  (0.05 ng/ml) for 8 or 18 h. Treatments were done as described in the previous section. A commercially available kit (Panomics) was used for the nuclear extracts. Protein concentrations were determined by microBCA method as described previously. Nuclear extracts were analyzed by Western blotting using polyclonal anti-NF- $\kappa$ B p65 subunit antibodies diluted 1:200 (Santa Cruz Biotechnology) to monitor NF- $\kappa$ B translocation. Results represent means  $\pm$  SE of three independent experiments. *P* values were calculated using Student's *t* test (one-tailed, unpaired). \**P* < 0.002 for LASYS siRNA versus control siRNA for the 8-h time point.



**FIG. 7.** Overexpression of LASY in HMVECs and effect of proinflammatory agents on MCP-1 expression in HMVECs overexpressing the LASY gene. **A:** LASY mRNA levels in cells transfected with either the mock plasmid (pBKCMV) or pBKCMV-LASY. Transfections were done for 48 h. LASY expression was monitored by real-time PCR. Fold changes are expressed relative to Mock (pBKCMV)-treated control. Ct values obtained from LASY primers were corrected for Ct values from Actin primers. **B:** MCP-1 levels in supernatants from cells transfected with either mock (pBKCMV) or pBKCMV-LASY plasmid. Treatments with TNF- $\alpha$  and AGE were done for 18 h in 1% serum medium. Concentrations used were as follows: TNF- $\alpha$ : 0.05 ng/ml; AGE: 300  $\mu$ g/ml. MCP-1 levels were determined by ELISA and expressed as pg/ml. Data represent means  $\pm$  SD of three independent experiments. *P* values were calculated using the Student's *t* test (one-tailed, unpaired). \**P* < 0.01 for TNF- $\alpha$ -treated cells and \*\**P* < 0.005 for AGE-treated cells, respectively (LASY versus Mock).

(Fig. 6C, *P* < 0.002). Nuclear levels of NF- $\kappa$ B p65 subunit decreased after 18 h of TNF treatment, with no significant differences between control and LASY at this time point (Fig. 6C).

**Overexpression of LASY alleviates the inflammatory response.** We next studied the effect of overexpressing LASY in endothelial cells, to confirm that LASY does indeed protect cells against inflammatory insults. The LASY gene was cloned into pBK-CMV, so that its expression was under the control of the strong cytomegalovirus (CMV) promoter. The resulting construct (pBKCMV-LASY) was transfected into HMVECs, and the transfected cells were treated with proinflammatory agents such as TNF- $\alpha$  or AGE. LASY mRNA levels were increased approximately threefold in cells transfected with pBKCMV-LASY compared with mock (pBKCMV)-transfected cells (Fig. 7A). Cells that overexpressed LASY showed a significant decrease in TNF- $\alpha$ -stimulated secretion of MCP-1 compared with mock-transfected cells (Fig. 7B, *P* < 0.01). AGEs generated by hyperglycemia are implicated in the development of inflammation and atherosclerosis (10). We looked at the effect of AGE treatment on MCP-1 levels in cells that overexpressed LASY. As seen with TNF- $\alpha$  treatment, LASY overexpression caused a significant decrease in AGE-induced secretion of MCP-1 (Fig. 7B, *P* < 0.005). These results suggest that increased expression of LASY alleviates inflammation.

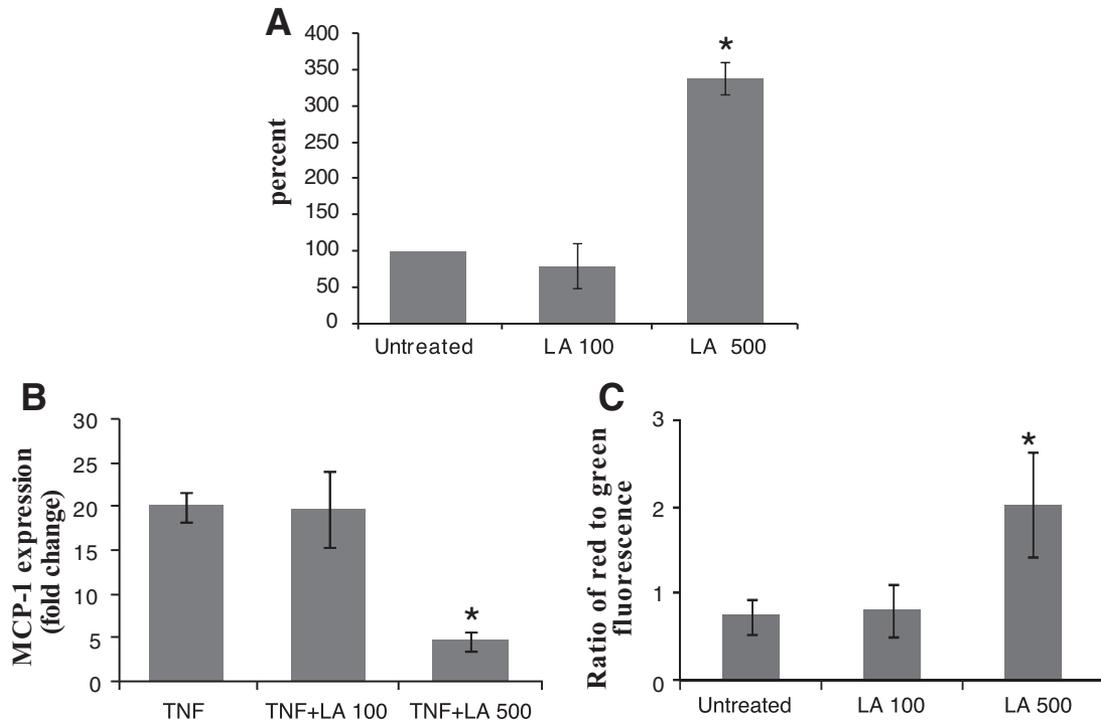
**Treatment with  $\alpha$ -lipoic acid increases LASY expression and has beneficial effects on inflammation and mitochondrial membrane potential.** Real-time PCR studies revealed that treatment of cells with lipoic acid (500  $\mu$ mol/l) upregulates LASY expression by  $\sim$ 3.5-fold (Fig. 8A, *P* < 0.003). Lower concentrations of lipoic acid (100  $\mu$ mol/l) did not increase LASY expression. The effects of lipoic acid on LASY gene expression correlates with its

anti-inflammatory effect in TNF- $\alpha$ -induced inflammation (Fig. 8B, *P* < 0.005) and its beneficial effects on mitochondrial membrane potential (Fig. 8C, *P* < 0.0001).

## DISCUSSION

Our studies demonstrate for the first time that LASY, a mitochondrial enzyme responsible for synthesis of lipoic acid, a potent antioxidant, is downregulated in animal models of type 2 diabetes and obesity. Downregulation of LASY in leptin/leptin receptor-deficient models could be a secondary effect of hyperglycemia and increased cytokine expression in these models. Our in vitro data demonstrate that treatment of endothelial and skeletal muscle cells with high glucose and TNF- $\alpha$  decreases LASY expression. Data from our in vitro gene silencing studies reveal that knockdown of LASY has detrimental effects on various aspects of cellular function. One of them is the disruption of the antioxidant defense system. We have shown that knockdown of LASY results in a significant decrease in the activities of two critical antioxidant enzymes, SOD and CAT, which are involved in maintaining intracellular redox balance. Deficiency of LASY reduced activities of both mitochondrial and cytosolic SOD. The net effect of reduction in SOD and CAT activities would be increased accumulation of reactive species such as superoxide anion, a highly reactive species that would have been normally detoxified by SOD and CAT in a sequential manner to H<sub>2</sub>O<sub>2</sub> and water. This hypothesis turned out to be true, since there was a significant increase in the intracellular superoxide anions under basal and TNF- $\alpha$ -treated conditions after LASY knockdown. Thus, downregulation of LASY results in an overall disturbance in the antioxidant defense network, tipping the balance toward oxidative stress. Treatment with exogenous lipoic acid restored the activities of SOD and catalase but was unable to reduce the superoxide anion levels. In our experiment, we saw a significant increase in superoxide anion levels with lipoic acid treatment in both the control and LASY siRNA-treated samples in response to TNF- $\alpha$  treatment. This may be due to the pro-oxidant activity of lipoic acid that has been reported in the literature (11). Disturbance in the endogenous antioxidant network is reflected in decreased GSH levels, suggesting inability to regenerate reduced glutathione. Our in vitro data showing that knockdown of LASY results in decreased GSH levels is consistent with published literature. A significant decrease in erythrocyte GSH levels has been reported in mice that are heterozygous for disruption of the LASY gene (12).

Accumulation of excessive amounts of ROS such as superoxide anion could directly inflict damage on cellular macromolecules. Because mitochondria are a major site of ROS generation, accumulated ROS could cause damage to the mitochondrial membrane, resulting in its inability to maintain a gradient. This could then lead to mitochondrial dysfunction. The decrease in mitochondrial membrane potential that we have demonstrated in our in vitro LASY knockdown studies could be a result of damage caused by ROS. In addition, accumulated ROS can activate inflammatory pathways such as the NF- $\kappa$ B pathway, which can lead to increased expression of inflammatory cytokines. We found that LASY knockdown in endothelial cells increases TNF- $\alpha$ -induced NF- $\kappa$ B translocation into the nucleus. Our data suggest that knockdown of LASY and the consequent accumulation of ROS triggers the activation of NF- $\kappa$ B, resulting in increased expression of NF- $\kappa$ B-



**FIG. 8.** Improvement in mitochondrial function and decrease in inflammation by exogenous lipoic acid correlates with LASY induction. **A:** LASY gene expression in HSMMs after treatment with 100 and 500  $\mu\text{mol/l}$  of LA. ( $\pm$ )- $\alpha$ -Lipoic acid was used in this experiment. Fold change is expressed as percent of untreated cells. Data represent means  $\pm$  SD of three independent experiments. \* $P < 0.003$  for LA500-treated cells versus untreated. **B:** Expression of MCP-1 in HSMMs treated with 100 or 500  $\mu\text{mol/l}$  of lipoic acid in response to TNF (1 ng/ml) treatment. Cells were treated with 0, 100, or 500  $\mu\text{mol/l}$  of lipoic acid for 1 h before addition of 1 ng/ml of TNF- $\alpha$ . Cell treatments were allowed to proceed for 18 h. MCP-1 expression was determined by real-time PCR. Fold change is shown relative to untreated cells. Data represent means  $\pm$  SD of three independent experiments. \* $P < 0.005$  for TNF+LA500-treated cells versus TNF-treated cells. **C:** Mitochondrial membrane potential in HSMMs treated with 100 and 500  $\mu\text{mol/l}$  lipoic acid was determined using the JC-1 assay. Results are expressed as the ratio of red to green fluorescence of JC-1. Data are the average of three independent experiments.  $P$  values were calculated using Student's  $t$  test (one-tailed, unpaired). \* $P < 0.0001$  (C) for LA500-treated cells versus untreated cells.

regulated inflammatory genes such as MCP-1. Increase in circulating inflammatory cytokines and mitochondrial dysfunction contribute to insulin resistance, which could account for the decrease in glucose uptake in skeletal muscle after knockdown of LASY. It has been suggested that accumulation of ROS could be interpreted by the cell as an imbalance between substrate availability and oxidative capacity to which decreased insulin signaling (and thus decreased glucose uptake) would be an appropriate response (13). Decreased insulin signaling due to ROS has been attributed to decreased levels of phosphorylated Akt accompanied by an increase in phosphorylated JNK (13,14).

Increased secretion of inflammatory cytokines, mitochondrial dysfunction, and decreased glucose uptake after LASY knockdown could all contribute to further deterioration of hyperglycemia and associated metabolic abnormalities in animal models of diabetes and obesity. In *db/db*, *fa/fa*, and *ob/ob* models, which are already deficient in leptin signaling, deficiency in LASY would therefore accelerate progression of disease.

Downregulation of LASY is not a phenomenon restricted to animal models of diabetes. We studied blood samples from human subjects with type 2 diabetes for LASY expression by real-time PCR. These studies revealed significant downregulation of the LASY gene in subjects with diabetes compared with age- and sex-matched healthy individuals (data not shown).

Our studies demonstrate that deficiency of LASY results in a decrease in mitochondrial lipoic acid levels. Using antibodies that recognize protein-bound lipoic acid, we

have shown that knockdown of LASY reduces lipoic acid associated with two key enzyme complexes, namely PDC and KDH. Thus, deficiency of LASY directly affects levels of lipoic acid within the mitochondria. The correlation between LASY expression and mitochondrial lipoic acid is also evident in the adipose tissue of *db/db* mice, where deficiency of LASY results in a decrease in levels of lipoic acid (Fig. 1C).

Reduced mitochondrial lipoic acid levels due to LASY deficiency could be the predominant cause of the disturbances in the antioxidant defense system manifested as reduced SOD and CAT activities, increased superoxide anion levels, and decreased intracellular GSH. Because endogenous lipoic acid is a potent antioxidant, it is conceivable that a decrease in endogenous lipoic acid could cause perturbations in the endogenous antioxidant system. Supplementation with exogenous lipoic acid was able to completely restore SOD and CAT activities in a dose-dependent manner. This suggests that exogenously administered lipoic acid can compensate for the deficiency of LASY. Interestingly, our data show that exogenously administered lipoic acid increases the expression of LASY (at 500  $\mu\text{mol/l}$ ), suggesting that exogenously administered lipoic acid promotes its own generation. Thus, some of the known beneficial effects of lipoic acid administration could be attributed to an indirect effect of endogenous lipoic acid generation by increasing LASY levels.

Although lipoic acid is a critical cofactor of PDC, knockdown of LASY did not appear to have an immediate effect on the phosphorylation state of PDC, which is inversely related to its activity (15) (Supplementary Fig. 3).

The lack of effect of LASY deficiency on PDC activity is in agreement with the data from in vivo LASY knockdown studies in which PDC activity was reported to be normal in the livers from *Lias*<sup>+/-</sup> mice (12). Conceivably, because of the critical function of PDC in glucose metabolism, there are compensatory mechanisms that ensure that PDC activity is not affected by lipoic acid deficiency. However, it is possible that deficiency of lipoic acid may affect other enzymes/pathways that we have not included in this study.

In conclusion, our data identify a novel role for LASY in inflammation, insulin resistance, and mitochondrial dysfunction. Our data demonstrate that LASY plays a critical role in the maintenance of the endogenous antioxidant defense network, deficiency of which can aggravate the metabolic abnormalities associated with type 2 diabetes.

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