Lipoic Acid Synthase (LASY): A Novel Role in Inflammation, Mitochondrial Function and Insulin Resistance

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**ABSTRACT**

*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 knock down 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 to age and sex-matched controls. *In vitro*, LASY mRNA levels were decreased by the pro-inflammatory cytokine TNFα and high glucose. Down-regulation 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 down-regulation of LASY induced a significant loss of mitochondrial membrane potential, and decreased insulin-stimulated glucose uptake in skeletal muscle cells. In endothelial cells, down-regulation of LASY aggravated the inflammatory response which manifested as increase in both basal and TNF-α induced expression of the pro-inflammatory 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.
Type 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, 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 reactive oxygen species (ROS) in the mitochondria, thereby neutralizing the ROS before they inflict damage upon cellular molecules. Lipoic acid (LA) 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). LA is also an essential co-factor of mitochondrial enzyme complexes involved in oxidative metabolism. Exogenous LA, by virtue of its antioxidant effect, has been shown to be beneficial in many metabolic and vascular diseases (2-7). Endogenously, LA is synthesized from octanoic acid by the action of lipoic acid synthase (LASY). Previously, synthesis of LA 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 LA was made quite recently, when a mouse homolog of LASY was identified (8). Mammalian LASY contains a putative mitochondria targeting sequence at the N-terminus and is mainly localized in mitochondria (8). Thus LASY is ideally positioned to generate LA at the site of action, namely, mitochondria. Although the pharmacological effects of LA have been explored in many studies, the importance of endogenous LA 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 down regulated in diabetes and inflammation. Down regulation of LASY resulted in decreased endogenous LA levels. The data that we have presented suggests that down regulation of LASY, and the resultant decrease in endogenous LA would cause redox imbalance leading to inflammation and mitochondrial dysfunction, two important hallmarks of diabetes.

METHODS AND MATERIALS

Reagents: Unless otherwise stated, reagents were purchased from Sigma-Aldrich Chemical Co. (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 2-4 upon arrival. All animals were allowed to feed ad libitum on a regular chow diet (Laboratory rodent diet, 5001, LabDiet, Inc.). Characteristics of the animals used in the study are outlined in the supplementary table)

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 following euthanization of the animals.
Cell growth and differentiation: Human skeletal muscle myoblasts (HSMM) (Cambrex) were grown and differentiated according to Cambrex’s protocol. Human microvascular endothelial cells (HMVEC) and Human aortic endothelial cells (HAEC) (Cambrex) were grown in HMVEC-MV / HMVEC growth media (Cambrex) until approximately 80% confluent for treatments or transfections.

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

Knock-down studies: Knock-down of the LASY gene was achieved by RNA interference (RNAi). The siRNA duplex for LASY knock down were custom designed and synthesized by Dharmacon, Inc. The negative control for the siRNA LASY knock-down experiment consisted of a non-target (control siRNA) oligo (Dharmacon). Differentiated cells were treated with siRNA oligos (10 nM) and TransIT-KO reagent (Miriuris Corp.) for 24-48 hrs at 37°C/CO2.

Overexpression studies: The human LASY gene was amplified from HAEC by Oligo dT priming with Superscript first strand synthesis system (Invitrogen Corp.). The amplification product was cloned into the pBKCMV vector. Presence of insert in the correct orientation was verified by PCR and sequencing.

Transfections: Transfections were done using Targefect reagents (Targeting Systems, Inc.) using the manufacturer’s protocol. Transfections were done in 100 mm plates for all assays. Transfections were allowed to proceed for 24 hours, after which, the cells were re-plated into 96-well or 24-well plates and incubated for 18 hrs before treatments.

Glucose Uptake assay: The glucose uptake assay in HSMM was performed by adapting the protocol described in reference 9, with modifications as described in the figure legends.

Cell treatments: For HSMM, 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, Inc.

MCP-1 ELISA: MCP-1 ELISA was carried out using Quantikine Human MCP-1 kit as described by the manufacturer (R&D Systems, Inc.).

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, Inc. 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 PDC (E2 subunit) (Bovine heart) was purchased from Globozymes. Secondary, HRP-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, Inc.) as described by the manufacturer.

Glutathione (GSH) assay: To determine intracellular levels of reduced glutathione
Lipoic acid synthase (GSH), cells were lysed with protein lysis buffer 48 hrs 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 Co.

Superoxide Dismutase (SOD) assay: SOD activity was determined by using a SOD assay kit from Cayman Chemical Co. 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 6 well plates for the SOD assay, and analyzed for SOD activity 48 hours after initiation of LASY knock-down.

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

RESULTS

The LASY gene is down-regulated 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 IR in type 2 diabetes. LASY gene expression is significantly down regulated in both skeletal muscle (P<0.02) and adipose tissue (P<0.004) of db/db mice compared to their normoglycemic, heterozygous (db/+) counterparts and normal control mice (Figure 1A). Interestingly, there was no decrease in LASY mRNA levels in the liver of db/db mice compared to the db/+ or non-diabetic mice. There was a small increase that was insignificant. Down regulation of LASY in adipose tissue of db/db mice translated to a significant decrease in endogenous LA levels (Figure 1C). The anti-LA antibodies used in the blot detected LA bound to the E2 subunits of the Pyruvate Dehydrogenase Complex (PDC) and alpha-Ketoglutarate Dehydrogenase (KDH), two key mitochondrial enzymes that use LA as a cofactor (P < 0.007, P<0.005 for LA-PDC-E2 and LA-KDH-E2, respectively) compared to db/+ mice (Figure 1C). This suggests that in diabetic mice such as db/db, decrease in LASY results in a decrease endogenous LA 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 to their lean counterparts (Figure 1B, ob/ob, P<0.0001, Zucker fa/fa, P<0.03).

LASY expression in vitro is down regulated by conditions that exist in diabetes: Hyperglycemia, and increased secretion of inflammatory cytokines are two hallmarks of diabetes. To understand the regulation of LASY, HMVEC were subjected to treatment with medium containing TNFα or high glucose (Figure 2). Treatment with glucose (25 mM) led to a significant down-regulation (P<0.02) in LASY mRNA levels in endothelial cells. Intermediary levels of glucose (11 mM ) or an osmotic control (D-mannitol) did not significantly affect LASY expression (Supplementary figure 1). Treatment of HSMM with 25 mM glucose also reduced LASY mRNA levels significantly (Figure 2, P<0.02). Treatment of both endothelial cells and skeletal muscle cells with TNFα significantly decreased LASY mRNA levels (Figure 2, P<0.001). Thus, consistent with the animal data which suggests reduction in LASY in diabetes, LASY appears to be susceptible to down-regulation by hyperglycemia and inflammatory cytokines.

Inhibition of LASY expression decreases mitochondrial LA levels: We knocked down
the LASY gene in HSMM using the siRNA-based gene silencing approach. Using this approach we were able to get 51% knock down of the LASY gene after 48 hours (Figure 3A). As shown in Figure 3B, the knock down was specific to LASY since expression of a housekeeping gene such as GAPDH was not affected. Consistent with the real time PCR data, knock down of LASY resulted in a significant reduction in LASY protein levels ($P < 0.009$). We then studied the effect of LASY knock down on endogenous LA levels by using Western blot analysis. As shown in figure 3D, knock down of LASY did not affect total levels of E2 protein (Figure 3E). Thus knock down of LASY directly translates to a decrease in LA associated with E2 subunit of PDC, without affecting levels of E2 protein. These results are consistent with our in vivo results in which down regulation of LASY in tissues translated to decrease in LA levels (Figure 1C).

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

**Knock down of LASY results in decreased activities of superoxide dismutase and catalase and increased superoxide anion levels**: To further investigate the effect of LASY knock down on the cellular antioxidant defense network, we determined the activities of two critical antioxidant enzymes, Catalase (CAT) and Superoxide Dismutase (SOD) in HSMM following knock down of LASY. As shown in Figure 4, knock down of LASY results in a significant decrease in the activities of catalase (Figure 4 B), mitochondrial and cytosolic SOD (Figure 4 C and D). Treatment with 500 µM LA restored the activities of these enzymes (Figure 4 B, C,D). There was a concentration dependent effect between 100 µM and 500 µM of LA. Based on the decrease in SOD activity we hypothesized that there would be an increase in superoxide anion levels with LASY knock down. This turned out to be true since there was a significant increase in superoxide anion levels both in basal and TNFα-treated conditions (Figure 4 E). Treatment with LA (500 µM) did not decrease the superoxide anion levels. Instead, there was a significant increase in superoxide anion levels with LA treatment in both control and LASY siRNA treated samples, compared to the TNFα-treated levels. The difference between control and LASY siRNA treated samples was also significant, suggesting that LA treatment worsens the TNFα-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 IR, which causes reduced glucose uptake by skeletal muscle. Based on our observation that LASY expression is down regulated in the skeletal muscle of insulin-resistant animals, we studied the effect of reduced LASY expression on glucose uptake in skeletal muscle cells. We knocked down the LASY gene by siRNA based gene silencing and studied the effect of the knock down on $[^3]$H]-2-deoxy glucose uptake in skeletal muscle cells. As in our earlier experiments, we obtained a substantial knock-down (64%) with the LASY siRNA oligo compared to the control siRNA oligo in HSMM cells (data not shown). Decreased
Lipoic acid synthase

LASY expression in skeletal muscle cells significantly reduced insulin-stimulated glucose uptake (Figure 5A) \( (P<0.01) \). There was a small, insignificant decrease in basal glucose uptake (Figure 5A). These data demonstrate that down regulation of LASY has a contributory role on IR in skeletal muscle.

**Reduced LASY expression decreases mitochondrial membrane potential in human skeletal muscle cells:** Since decline in mitochondrial function is one of the causes of IR in diabetes, we tested if decrease in LASY would affect mitochondrial function. We studied the effect of siRNA-based knock down of LASY 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 Methods section). Reduced LASY expression was accompanied by a significant decrease \( (P<0.003) \) in the ratio of red to green fluorescence of JC-1 dye indicating that LASY knock down affects mitochondrial membrane potential (Figure 5B).

**Inhibition of LASY expression increases nuclear translocation of NFκB:** NFκB 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 NFκB into the nucleus is a key event in the activation of NFκB. We investigated if inhibition of LASY has an effect on TNF-α-induced nuclear translocation of the p65 subunit of NFκB. Western blot analysis of nuclear extracts showed that siRNA based knock down of LASY increased nuclear translocation of the NFκB p65 subunit compared to the control following 8 hours of treatment with TNF (Figure 6C, \( p<0.002 \)). Nuclear levels of NFκB p65 subunit decreased after 18 hours of TNF treatment, with no significant differences between control and LASY at this time point (Figure 6C).

**Overexpression of LASY alleviates the inflammatory response:** We next studied the effect of over expressing 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 HMVEC, and the transfected cells were treated with proinflammatory agents such as TNFα or AGE. LASY mRNA levels were increased approximately three fold in cells transfected with pBKCMV-LASY compared to mock (pBKCMV)-transfected cells (Figure 7A). Cells that over expressed LASY showed a significant decrease in TNFα-stimulated secretion of MCP-1 compared to mock-transfected cells (Figure 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 over expressed LASY. As seen with
TNF-α treatment, LASY over expression caused a significant decrease in AGE-induced secretion of MCP-1 (Figure 7B, P<0.005). These results suggest that increased expression of LASY alleviates inflammation. *Treatment with alpha-lipoic acid (ALA) increases LASY expression, and has beneficial effects on inflammation and mitochondrial membrane potential.* Real time PCR studies revealed that treatment of cells with LA (500 µM) up regulates LASY expression by about 3.5 fold (Figure 8 A, P<0.003). Lower concentrations of LA (100 µM) did not increase LASY expression. The effects of LA on LASY gene expression correlates with its anti-inflammatory effect in TNFα-induced inflammation (Figure 8B, P<0.005) and its beneficial effects on mitochondrial membrane potential (Figure 8C, P<0.0001).

**DISCUSSION**

Our studies demonstrate for the first time that LASY, a mitochondrial enzyme responsible for synthesis of LA, a potent antioxidant, is down regulated in animal models of type 2 diabetes and obesity. Down regulation 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 demonstrates that treatment of endothelial cells and skeletal muscle cells with high glucose and TNF-α decreases LASY expression. Data from our *in vitro* gene silencing studies reveal that knock down 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 knock down 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 H₂O₂ and water. This hypothesis turned out to be true since there was a significant increase in the intracellular superoxide anions under basal and TNFα-treated conditions following LASY knock down. Thus down regulation of LASY results in an overall disturbance in the antioxidant defense network, tipping the balance towards oxidative stress. Treatment with exogenous LA 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 LA treatment in both the control and LASY siRNA treated samples in response to TNFα treatment. This may be due to the pro-oxidant activity of LA which 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 knock down 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 upon cellular macromolecules. Since 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 knock down studies could be a result of damage caused by ROS. In addition, accumulated ROS can activate inflammatory pathways...
such as the NFκB pathway which can lead to increased expression of inflammatory cytokines. We found that LASY knock down in endothelial cells increases TNF-α-induced NFκB translocation into the nucleus. Our data suggests that knock down of LASY and the consequent accumulation of ROS triggers the activation of NFκB, resulting in increased expression of NFκB-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 following knock down 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 following LASY knock down 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.

Down regulation 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 down regulation of the LASY gene in subjects with diabetes compared to age and sex-matched healthy individuals (data not shown).

Our studies demonstrate that deficiency of LASY results in a decrease in mitochondrial LA levels. Using antibodies that recognize protein-bound LA, we have shown that knock down of LASY reduces LA associated with two key enzyme complexes, namely PDC and KDH. Thus deficiency of LASY directly affects levels of LA within the mitochondria. The correlation between LASY expression and mitochondrial LA is also evident in the adipose tissue of db/db mice, where deficiency of LASY results in decrease in levels of LA (Figure 1C).

Reduced mitochondrial LA 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. Since endogenous LA is a potent antioxidant, it is conceivable that decrease in endogenous LA could cause perturbations in the endogenous antioxidant system. Supplementation with exogenous LA was able to completely restore SOD and CAT activities in a dose dependent manner. This suggests that exogenously administered LA can compensate for the deficiency of LASY. Interestingly, our data shows that exogenously administered LA increases the expression of LASY (at 500 µM), suggesting that, exogenously administered LA promotes its own generation. Thus some of the known beneficial effects of LA administration could be attributed to an indirect effect of endogenous LA generation by increasing LASY levels.

Although LA is a critical cofactor of PDC, knock down 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 figure 3). The lack of effect of LASY deficiency on PDC activity is in agreement with the data from in vivo LASY knock down studies in which PDC activity was reported to be normal in the livers from Lias+/− mice (12). Conceivably, due to the critical function of PDC in glucose
metabolism, there are compensatory mechanisms that ensure that PDC activity is not affected by LA deficiency. However, it is possible that deficiency of LA may affect other enzymes/pathways that we haven’t included in this study.

In conclusion, our data identifies a novel role for LASY in inflammation, insulin resistance and mitochondrial dysfunction. Our data demonstrates 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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FIGURE LEGENDS

Figure 1. LASY mRNA and LA levels in obese, diabetic animal models compared to 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). Figure 1 A: For db/db versus db/+, *P<0.02, **P<0.004. Figure 1 B: for ob/ob versus C57, ***P<0.0001. For fa/fa versus lean, ** P<0.03 C, Endogenous LA levels in adipose tissue (visceral fat pads) of db/db mice compared to 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±SD. P values were calculated using 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.

Figure 2. LASY mRNA levels in human endothelial and skeletal muscle cells after treatment with pro-inflammatory agents. Human microvascular endothelial cells (HMVEC) and human skeletal muscle cells (HSMM) were used in the experiment. Concentrations of TNF-α used for stimulation of the two cell types was based on the MCP-1 responses; HSMM required a higher concentration of TNF-α to secrete detectable amounts of MCP-1. HMVEC were treated with 0.05 ng/ml of TNF-α; HSMM were treated with 1 ng/ml of TNF-α. Glucose concentration for both cell types: 25 mM. Controls and treatments were done for 18 hours in low- serum (1% FBS) media. Fold changes were calculated, and expressed as percent of untreated control cells. P values: ** P<0.001, * P< 0.02. Results represent means±SE of three independent experiments. P values were calculated using Student’s t test (one-tailed, unpaired).

Figure 3. Effect of siRNA-based LASY knock down on LASY expression and endogenous lipoic acid (LA) levels in HSMM. **A**, LASY mRNA levels in HSMM treated with a LASY siRNA oligo or control (scrambled) siRNA oligo. **B**, GAPDH mRNA levels in HSMM treated with LASY siRNA oligo or control (scrambled) siRNA oligo. Ct values from real time PCR assays for LASY 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 HSMM treated with LASY siRNA oligo (L) or scrambled control oligo (C) using monoclonal anti-mouse antibodies directed to a peptide epitope of recombinant LASY protein (Novus Biologicals, Inc.). Cells were grown in 24-well plates. Following gene knock-down, cells were lysed with protein lysis buffer (0.5% SDS, 50 mM Tris-HCl, pH 8.0, 1X protease inhibitor cocktail, Sigma). Protein concentration was determined using the MicroBCA™ kit (Pierce). Equivalent amounts of protein were analyzed by SDS-PAGE (NuPage pre-cast gels). Western blotting protocols were done as described by the antibody manufacturer. Bands were quantitated by densitometry, using the Total Lab™ software. 1:500 dilution of the antibody detected an approximately 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<0.009 for LASY siRNA versus Control siRNA.

**D**, Western blot analysis of cell lysates from HSMM treated with LASY siRNA oligos (L) or scrambled control oligo (C) using polyclonal anti-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 LA Western blot represent LA bound to E2 subunit of PDC (upper band, LA-PDC-E2) or α-KGD (lower band, LA-KDH-E2). Quantitation of LA-PDC-E2 and LA-KDH-E2 bands in control and LASY 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 Student’s t test (one-tailed, unpaired). * *P<0.01 LASY siRNA versus Control siRNA for LA-PDC-E2 and LA-KDH-E2.

**E**, Western blot analysis of cell lysates from HSMM 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).

**Figure 4. Effect of LASY knock down on the antioxidant system in HSMM.**

**A**, Effect of LASY knock down on intracellular reduced glutathione levels. Intracellular GSH levels in HSMM 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 represents means ± 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, C, D**, Effect of siRNA-based LASY knock down on activities of catalase (B), Mitochondrial superoxide dismutase (Mn-SOD) (C) and cytosolic SOD(Cu-Zn-SOD) (D). Knock down was done as described in the text. Treatments with LA were done as described in the earlier section, twenty-four hours after knock down.

**B**, Effect of LASY knock down on catalase activity in HSMM. One unit is defined as the amount of enzyme that will cause the formation of 1.0 nmol of 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±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, D**, Effect of LASY knock down on SOD activity in HSMM. 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 10,000Xg for 15 minutes 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±SD of three independent experiments. SOD enzyme activity was determined in duplicate. Significance was calculated using 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 LASY siRNA+ LA500 versus LASY siRNA.
Lipoic acid synthase

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 knock-down on superoxide anion levels. Superoxide anion levels were quantitated using a commercially kit (Sigma Aldrich, Inc.), 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 α (1 ng/ml, 18 hours) 24 hours 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 minutes to 20 minutes). Values at peak intensity are shown. Data represent means+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 α-treated samples (control siRNA versus LASY siRNA); P<0.004 for control siRNA+TNF versus control siRNA+TNF+LA; P<0.002 for LASY siRNA+TNF versus LASY siRNA+TNF+LA; P<0.03 for control siRNA+TNF+LA versus LASY siRNA+TNF+LA.

Figure 5. Effect of LASY knock down on glucose uptake and mitochondrial membrane potential.

A. Effect of siRNA-based knock-down of LASY gene expression on insulin- stimulated glucose uptake in HSMM. Knock down of LASY was done as described in the Methods section. Cells were washed with DMEM containing 1% BSA and incubated in this medium for 18 hrs. Cells were washed with transport medium (20 mmol/l HEPES, pH 7.4, 140 mol/l NaCl, 5 mmol/l MgCl₂, 1 mmol/l CaCl₂ and 0.1% (w/v) BSA. Next, cells were incubated in transport medium with or without insulin (1 µmol/l) for 30 mins at 37°C/CO₂. This was followed by incubation in transport buffer containing 10 µmol/l, 2-Deoxy-D-[2, 6-³H] glucose (1.0 µCi/ml) for 30 mins at 37°C. Reactions were stopped by aspirating the media and washing cells with PBS containing 20mmol/l D-glucose at 4°C. Cells were lysed in 0.5% (w/v) 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 pmoles/min/mg of protein. Cells were stimulated with 1 µM/l insulin for 30 m in transport buffer as described in reference 9. Data represent means+SE of three independent experiments.  P values were calculated using Student’s t test (one-tailed, unpaired). *P<0.01 for LASY siRNA versus Control siRNA for insulin-treated cells. Difference between LASY siRNA and Control siRNA for basal, unstimulated cells is not significant.

B. Effect of siRNA-based LASY knock down on mitochondrial membrane potential in HSMM. Knock down was done as described in the text. Mitochondrial membrane potential was determined using the JC-1 assay. Following knock-down, cells were washed with 1X PBS and incubated with JC-1 dye as described in the JC-1 assay kit (Biotium, Inc.), for 30 mins at 37°C. Cells were washed twice with 1X PBS, trypsinized and transferred to 96-well PCR tube plates. Red and green fluorescence was measured using the Envision fluorescent plate reader (Perkin Elmer). The ratio of red to green fluorescence for the experimental was calculated and expressed as a percent of the control. Results are expressed as ratio of red to green fluorescence of JC-1. Data represents are means + SD of three independent experiments.  P values were calculated using Student’s t test (one-tailed, unpaired) *P<0.005 for LASY siRNA versus control siRNA.
Figure 6. Effect of knock down of the LASY gene on inflammation in HMVEC

A, LASY mRNA levels in HMVEC treated with a LASY siRNA oligo or control (scrambled) siRNA oligo; knock down was done for 48 hours. Ct values from real time PCR assay for LASY was corrected for Ct values from Actin. Fold changes were calculated as percent of fold change of control; P<0.001 for LASY siRNA versus control siRNA.

B, MCP-1 levels in control siRNA and LASY siRNA-treated cells under basal, and TNF-α-treated, conditions. TNF-α (0.05 ng/ml) treatment was done in 1% serum medium after 24 hours of siRNA transfection. Duration of treatment with TNF-α was 18 hrs. Treatment supernatants were used in an ELISA to determine MCP-1 levels expressed as pg/ml. Data represent means+SD of three independent experiments. P values were calculated using Student’s t test (one-tailed, unpaired). *P<0.05 for LASY siRNA versus control siRNA (Basal). **P<0.02 for LASY siRNA versus control siRNA (TNF-α-treated).

C, Western blot analysis of NFκB p65 subunit levels in nuclear extracts from cells treated with either control (scrambled) siRNA or LASY siRNA. Nuclear extracts were prepared from cells that were treated with TNF-α (0.05 ng/ml) 8 hours or 18 hours. Treatments were done as described in the previous section. A commercially available kit (Panomics, Inc.) 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-NFκB p65 subunit antibodies diluted 1:200 (Santa Cruz Biotechnology, Inc.) to monitor NFκB translocation. Results represent means+SE of three independent experiments. P values were calculated using Student’s t test (one-tailed, unpaired). *P<0.02 for LASY siRNA versus control siRNA for 8 hour time point.

Figure 7: Overexpression of LASY in HMVEC and effect of pro-inflammatory agents on MCP-1 expression in HMVECs over-expressing the LASY gene.

A, LASY mRNA levels in cells transfected with either the mock plasmid (pBKCMV) or pBKCMV-LASY. Transfections were done for 48 hours. LASY expression was monitored by real time PCR. Fold changes are expressed as percent of 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-α and AGE were done for 18 hrs in 1% serum medium. Concentrations used were as follows: TNF-α: 0.05 ng/ml; AGE: 300 μg/ml. MCP-1 levels were determined by ELISA, and expressed as percent of mock control. Data represent means+SD of three independent experiments. P values were calculated using Student’s t test (one-tailed, unpaired). *P<0.01 for TNF-α-treated and **P<0.005 for AGE treated cells respectively (LASY versus Mock).

Figure 8: Improvement in mitochondrial function and decrease in inflammation by exogenous LA correlates with LASY induction.

A, LASY gene expression in HSMM after treatment with 100 μM and 500 μM of LA. (±)-α-Lipoic acid was used in this experiment. Fold change is expressed as percent of untreated cells. Data represent means + SD of three independent experiments; *P<0.003 for LA500-treated cells versus untreated.

B, Expression of MCP-1 in HSMM treated with 100μM or 500μM of LA in response to TNF (1 ng/ml) treatment. Cells were treated with 0, 100 or 500 μM of LA for 1 hour prior to addition of 1 ng/ml of TNF-α. Cell treatments were allowed to proceed for 18 hours. MCP-1 expression was
determined by real time PCR. Data represent means ± SD of three independent experiments; *$P<0.005$ for TNF+LA500-treated cells versus TNF-treated cells.

C, Mitochondrial membrane potential in HSMM treated with 100 μM and 500 μM of LA was determined using the JC-1 assay. Results are expressed as ratio of red to green fluorescence of JC-1. Data is the average of three independent experiments. $P$ values were calculated using Student’s $t$ test (one-tailed, unpaired). *$P<0.0001$ (figure 8 c) for LA500-treated cells versus untreated cells.
Lipoic acid synthase

Figure 1
Figure 1 C
Figure 2
Lipoic acid synthase

Figure 3 A,B,C
Figure 3 D,E
Lipoic acid synthase

![Graph A: GSH levels](image)

![Graph B: Catalase activity](image)

![Graph C: Mn-SOD activity](image)

![Graph D: Cu/Zn-SOD activity](image)

Figure 4 A,B,C,D
Figure 4 E
Lipoic acid synthase

Figure 5 A,B
Figure 6 A, B
Figure 6 C
Figure 7 A, B
Lipoic acid synthase

Figure 8 A, B, C
Lipoic acid synthase