HHS Public Access

Author manuscript

Free Radic Biol Med. Author manuscript; available in PMC 2017 February 07.

Published in final edited form as:

Free Radic Biol Med. 2014 June ; 71: 362–367. doi:10.1016/j.freeradbiomed.2014.03.022.

Alpha-lipoic acid protects mitochondrial enzymes and attenuates lipopolysaccharide-induced hypothermia in mice

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Abstract

Hypothermia is a key symptom of sepsis, but the mechanism(s) leading to hypothermia during sepsis is largely unknown and thus no effective therapy is available for hypothermia. Therefore, it is important to investigate the mechanisms and develop effective therapeutic methods. Lipopolysaccharide (LPS)-induced hypothermia accompanied by excess nitric oxide (NO) production, lead to a reduction in energy production in wild type mice. However, mice lacking inducible nitric oxide synthase did not suffer from LPS-induced hypothermia, suggesting that hypothermia is associated with excess NO production during sepsis. This observation is supported by the treatment of wild type mice with α-lipoic acid (LA) in that it effectively attenuates LPS-induced hypothermia with decreased NO production. We also found that LA partially restored ATP production, and activities of the mitochondrial enzymes involved in energy metabolism, which were inhibited during sepsis. These data suggest that hypothermia is related to mitochondrial dysfunction, which is likely compromised by excess NO production and that LA administration attenuates hypothermia mainly by protecting mitochondrial enzymes from NO damage.

Keywords

Lipoic acid; antioxidant; mitochondria; body energy

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Author Disclosure Statement

The authors declare that no competing financial interests exist.

Introduction

Sepsis remains a critical problem resulting in high morbidity and mortality rates. In the United States alone, there are 751,000 estimated cases of sepsis every year with an overall mortality of 100,000 people [1]. The incidence of sepsis is expected to rise at a rate of 1.5% per year with total costs of $16.7 billion [2].

Both hyperthermia (body temperature >38 °C) and hypothermia (body temperature <36 °C) are key diagnostic criteria for sepsis [3]. Hypothermia results in an increased rate of wound infection, coagulopathy and other postoperative complications [4], and is particularly associated with severe infection and a higher mortality rate [5, 6]. The mechanism for hypothermia remains largely unknown. Many strategies have been proposed for the treatment of sepsis, but few noteworthy breakthroughs have been made during the past several decades [7]. Since there is no effective therapy to prevent hypothermia, it is also clinically important to develop effective methods for modulating hypothermia.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are involved in sepsis [8-10]. One of the characteristic features of sepsis is markedly increased levels of plasma nitric oxide (NO) [11]. NO plays an important role in oxidative phosphorylation [12], and it is well-known that excess NO causes inhibition of complexes I and IV in the electron transport chain in several ways [13-15]. Antioxidant therapy is a plausible adjunctive method for treatment of sepsis. Among antioxidants, α-lipoic acid (1, 2-dithiolane-3-pentanoic acid; LA) is a well-known potent antioxidant with anti-inflammatory activity [16-18]. LA is also a cofactor for several enzymes including pyruvate dehydrogenase complex (PDH; EC1.2.4.1 + EC 1.6.4.3 + EC 2.3.1.2) and α-ketoglutarate dehydrogenase complex (KDH; EC 1.2.4.2 + EC 2.3.1.61 + EC 1.8.1.4), two enzymes located in mitochondria and involved in energy production. LA has a therapeutic potential in mitochondria-related disorders [19, 20]. We have previously reported that a mouse embryo cannot survive without endogenous LA production, strongly suggesting that LA is essential for maintenance of normal levels of energy metabolism in addition to its antioxidant activity [21].

Lipopolysaccharide (LPS) administration may produce hypothermia in rodents [22, 23]. In the current study, we hypothesize that excess production of NO during sepsis inhibits some proteins in the Krebs cycle and the mitochondrial respiratory chain, leading to overall energy deficiency. To demonstrate this, we treated mice with LPS to induce hypothermia and studied molecular and physiological responses associated with this treatment. In this study, we found that LA increased mitochondrial antioxidant capacity, partially restored enzymatic activities in two mitochondrial enzymes, and ATP yield. These changes may contribute to the modification of hypothermia-associated sepsis mechanisms.

Materials and Methods

Materials

R-(+)-α-lipoic acid was from Toronto Chemical Research (Toronto, Canada), and Cytochrome C from Sigma Aldrich (St. Louis, MO).

LPS-induced endotoxic shock in mice

Three-month-old female C57BL/6J mice, and female *iNOS*-null mice were given intraperitoneally (i.p.) a dose (4 mg/kg body weight) of LPS (*E. Coli* 055:B5, Sigma, St. Louis), or saline as a control [18, 24]. Half of each of the LPS-treated and control mice received i.p. a single injection (40 mg/kg body weight) of LA 30 min before or after the LPS injection [18]. To help distinguish the effect of LA on hypothermia from other antioxidants, four LPS-treated mice were i.p. injected with ascorbate (pH 7.0 previously adjusted with NaOH; 5 mg/g of body weight) [25]. The dosage of LA was based on our experience and that of other investigators [26-29]. Body temperature was monitored for 4 hours after LPS injection using rectal temperature sensors (Physitemp Instruments Inc. Clifton, NJ). The experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of North Carolina and complied with the U. S. National Institutes of Health standards.

Nitrate/Nitrite (NOx) Assay

Plasma nitrate (NO_3^-) and nitrite (NO_2^-) levels, indicators of the production of NO, were measured after 4 hours of LPS injection using a colorimetric assay kit (Cayman Chemical, Ann Arbor, MI). The absorbance was read on a Biotek Synergy HT Multi-Mode Microplate Reader (Winooski, VT) using a wavelength of 540 nm.

Mitochondria isolation

Liver mitochondria were isolated following protocols already published [30, 31] with minor modifications. Mice were sacrificed by cervical dislocation and the liver was rapidly explanted from the peritoneal cavity. The liver was immersed in ice-cold isolation buffer [100 mM Tris– 3-(N-morpholino) propanesulfonic acid (MOPS, Sigma Aldrich), 10 mM of ethylene-bis (oxyethylenenitrilo) tetraacetic acid tetrasodium (EGTA, Sigma Aldrich)/Tris and 200 mM sucrose, pH 7.4] and chopped finely with scissors and then rinsed with the isolation buffer several times to remove traces of blood. Chopped tissue was homogenized in the buffer, with 12 strokes of a loose pestle in a glass Dounce. The homogenate was centrifuged for 10 min at 3,000 *g* at 4° C. Supernatants (including the pink salmon layer above the pellet) were collected in isolation buffer and then centrifuged for 10 min at 10,000 *g* at 4° C. The resulting pellets were finally re-suspended in ~1 ml isolation buffer and retained on ice until use (within 4 hours).

Determination of reduced glutathione (GSH) levels in mitochondria

Liver mitochondrial levels of reduced GSH were determined using a commercially kit (Cayman Chemical) following the manufacturer's instructions. The protein concentration of each sample was determined using a bicinchoninic acid assay (BCA; Thermo Scientific, Rockford, IL).

Determination of ATP production levels

ATP generation in LPS-treated liver homogenate was analyzed by a luciferase substrate assay (Invitrogen, Carlsbad, CA). Briefly, LPS-treated mice, in the presence or absence of LA, were sacrificed 4 hours after the LPS injection. The liver was homogenized, and the

homogenate was sonicated in ATP Assay Buffer provided by the company. After centrifugation at 12,000 *g* for 5 minutes at 4° C, the supernatant was removed and used for ATP measurement in an Orion Microplate Luminometer (Berthold Detection Systems, Titertek Instruments, Inc., Huntsville, AL). For calculations the amount of ATP was normalized by protein concentration as determined using the BCA method.

Alpha-ketoglutarate dehydrogenase and complex III activities

KDH activity was determined by monitoring NAD^+ reduction at 340 nm. Isolated liver mitochondria were suspended in medium containing 25 mM KH_2PO_4, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.1% (v/v) Triton X-100, 40 mM rotenone, 1.0 mM NAD^+, 0.2 mM thiamin pyrophosphate, and 0.1 mM Coenzyme A, pH 7. The mitochondria were sonicated in a water bath (Denville Scientific, South Plainfield, NJ) at room temperature for 1 min. A reaction mixture containing 5 mM $MgCl_2$, 40 mM rotenone, 0.1 mM Coenzyme A, 0.2 mM thymine pyrophosphate, and 1 mM NAD^+ were added to the mitochondrial medium to reach a final concentration of 0.4 mg mitochondrial protein/ml. The reaction was initiated by adding 2.5 mM α-ketoglutarate [32-34].

Complex III (ubiquinol-ferricytochrome C oxidoreductase; EC 1.10.2.2) activity was measured by following an increase in absorbance at 550 nm due to the reduction of ferricytochrome C [35, 36]. The reaction mixture consisted of 25 mM KH_2PO_4, pH 7.4, 5 mM $MgCl_2$, 2 mM KCN, 2.5 mg bovine serum albumin, 50 μM cytochrome C, and 4 mg rotenone. Mitochondrial protein (25 μg) was added and the reaction was initiated by the addition of 60 μM decylubiquinol; any increase in absorbance at 550 nm was monitored for 2 minutes. The assay was repeated, but with the inclusion of 5 μg antimycin A to the reaction mixture to determine antimycin A-insensitive activity. Antimycin A-sensitive complex III activity was determined using the initial rate as baseline. Enzyme activity was expressed as nmole of reduced cytochrome C/minute per mg of protein.

PGE_2 and cytokine analyses

The levels of prostaglandins E_2 (PGE_2) and cortisone were measured by an enzyme immunoassay kit (Cayman Chemical), according to the manufacturer's instructions. Plasma levels of interleukin-6 (IL-6) and tumor necrosis factor (TNF-α) in plasma were measured using a LINCO multiplex mouse cytokine kit (Linco Research, Inc., St. Charles, MO) on a Luminex 100 system, according to the manufactures' protocols (Luminex Corporation, Austin, TX).

Statistical analysis

All data are reported as means ± S.E.M. Values of $p<0.05$ were considered to be statistically significant (95% confidence limits). The effects of NO and LA were analyzed using ANOVA. Effects of fluctuation in body temperature were assessed by MANOVA by repeated measurements of each mouse's physiological conditions.

Results

α-Lipoic acid attenuates LPS-induced hypothermia mediated by decreasing excessive NO production during sepsis

Injection of LPS in C57BL/6 wild type (WT) mice induced a drop in body temperature from an initial 36.5 °C to a mean nadir of 28 °C during the 4 hours following LPS treatment (ΔT = − 8.5 °C) (Figure 1A). In contrast, a temperature drop in C57BL/6 mice lacking the inducible nitric oxide synthase (*iNOS*) was considerably attenuated (Figure 1A; ΔT = − 3.0 °C). Administering LA (40 mg/kg body weight) to WT mice 30 min prior to LPS injection protected them from hypothermia to a similar degree as untreated WT mice, but gave no further protection to the LPS-treated *iNOS* null mice (Figure 1). During the four hours of observation, the body temperature of the WT mice that were not treated with LPS remained unchanged (ΔT < 0.5 °C) and LA supplementation alone did not affect body temperature (data not shown). Supplementing with LA at 30 min post LPS injection was equally effective in preventing hypothermia as illustrated in Figure 1B. In addition, there was no significant attenuation of hypothermia in septic mice treated with ascorbate (Figure 1B).

Next, we measured changes in total plasma nitrate/nitrite (NOx) after LPS injection since the circulating levels of NOx represent overall NOS activity. At 4 hours post-injection, NOx levels were increased 11-fold in WT mice (Figure 2). LA markedly attenuated LPS-induced NOx increase in WT mice but not in *iNOS* null mice (Figure 2). LPS injection decreased *eNOS* (endothelial nitric oxide synthase) gene expression by 5% and increased *iNOS* gene expression by 1700% in WT mouse liver. On the other hand, administration of LA did not significantly change the expression of *eNOS* and *iNOS* at 4 hours after LPS injection (P = 0.15 and 0.43, respectively; data not shown). Taken together, these data suggest that iNOS-derived NO is a major source of the NOx responsible for hypothermia during sepsis, and that the thermoregulatory effect of LA is mediated through NO.

Effect of LA on ATP production, mitochondrial functions and redox status

To investigate whether partial restoration of the body temperature is due to sustained energy metabolism, we measured ATP production in the liver. At 4 hours post LPS injection, liver ATP content in WT mice was reduced by 40% compared with un-treated mice, but addition of LA completely blocked this reduction (Figure 3A). ATP level in LPS-treated $iNOS^{-/-}$ mice did not significantly drop compared with non-treated control, and LA supplementation did not increase ATP production (Figure 3B).

To evaluate whether administration of LA may compensate for possible loss of mitochondrial antioxidant capacity, we measured GSH levels in mitochondria. We observed that the liver mitochondrial GSH content was decreased by about 40% after LPS treatment (P<0.01) and LA-treated mice almost fully retained their original GSH levels (Figure 3C).

Complex III and KDH activities

Enzymatic assay of complex III in the mitochondria isolated from WT mouse liver demonstrates a 40% decrease in its activity at 4 hours post-injection of LPS, which is

attenuated in the presence of LA ($P<0.05$) (Figure 4A). Likewise, KDH activity dropped by about 50% while LA partially prevented this reduction ($P<0.01$) (Figure 4C). We also found that KDH activity in liver mitochondria was not significantly altered in septic mice treated with ascorbate (Figure 4C). These results suggest that ascorbate supplementation does not completely share the same mechanisms as LA in terms of hypothermia treatment. In addition, both complex III and KDH activity in the mitochondria isolated from *iNOS*$^{-/-}$ mouse liver did not significantly decrease after injection of LPS and addition of LA did not show any obvious effect for the enzyme activities (Figures 4B and 4D).

Examination of other possible regulatory factors for hypothermia

To explore other possible thermoregulatory mechanisms involving LA in addition to NO, we examined the effect of LA on PGE_2 and cytokines. Our results showed that PGE_2 levels in serum in all non-LPS treated mice were very low (with LA: 90 ± 12 pg/ml; without LA 85 ± 12 pg/ml). Serum PGE_2 levels were increased at 2 hours (687 ± 72 pg/ml), continued to rise at 4 hours (1,115 ± 55 pg/ml) and reached a plateau at 24 hours post LPS challenge (Figure 5A). Treatment with LA did not significantly change serum PGE_2 levels (At 2 hour: 654 ± 114 pg/ml; at 4 hours: 1,156 ± 65 pg/ml) (Figure 5A). Similarly, concentrations of serum TNF-α (Figure 5B) and IL-6 (Figure 5C) were markedly increased at 2 hours (TNF-α: 7.5 ± 0.98 ng/ml; IL-6: 25.4 ± 2.1 ng/ml) and 4 hours (TNF-α: 4.5 ± 0.32 ng/ml; IL-6: 20.4 ± 1.26 ng/ml) following LPS injection; but LA supplementation did not significantly affect their levels (TNF-α: 5.6 ± 1.1 ng/mg; IL-6: 26.2 ± 1.8 ng/ml at 2 hours; TNF-α: 3.9 ± 1.42 ng/ml; IL-6: 22.4 ± 2.1) at 4 hours; i.e. thermoregulation *via* LA neither directly affects levels of PGE_2 nor pro-inflammatory cytokines.

Discussion

In this study, we found that treatment with LA significantly attenuates LPS-induced hypothermia in septic mice. Thermoregulation by LA is likely associated with its ability to protect mitochondria by markedly decreasing NO production and increasing mitochondrial antioxidant capacity. Our data shows that LA treatment significantly decreased LPS-induced accumulation of the major nitric oxide end products (NO_3^- and NO_2^-) without significantly affecting *eNOS* and *iNOS* gene expression, suggesting that decrease of NO by LA is primarily through inhibiting NO biosynthesis. Excessive NO produced during sepsis is expected to be involved in impairment of mitochondrial enzymes. It is well-known that excessive NO interacts with the mitochondrial respiratory chain by inhibiting cytochrome c oxidase (complex IV) and NADH-ubiquinone oxidoreductase (complex I) [37-39]. We find that activities of two other enzymes, KDH and complex III, are also decreased by excessive NO in LPS-treated WT mice and the activities are partially restored by LA supplementation. Our study presents new RNS targets and extends the knowledge about the protective capabilities of LA. KDH is in a key regulatory step of the Krebs cycle [40], which determines the capacity of mitochondrial metabolism [41], and could play a vital role in the ROS-associated bioenergetic deficit [42]. Complex III is a central component of all primary energy transduction systems. It functions in maintaining the proton gradient, which provides about 30% of energy production [43]. Therefore, it is possible that the decrease in catalytic activities of the two enzymes contributes to decreased ATP production, leading to

hypothermia during sepsis, since mitochondria generate more than 90-95% of ATP in the body [44]. This assumption is supported by the observation in *iNOS*$^{-/-}$ mice in which body temperature does not significantly drop upon injection of LPS. In addition, ATP production, enzymatic activities of KDH and complex III in *iNOS*$^{-/-}$ mice neither significantly decrease after LPS injection, nor respond to LA supplementation. These data strongly suggest that excessive NO induced by LPS injection plays a critical role in hypothermia formation and a link may exist between NO levels and amount of ATP synthesis. On the other hand, since GSH is predominantly an intra-mitochondrial antioxidant [12] that protects mitochondrial complexes from NO-induced damage [45], a declined GSH level weakens mitochondrial antioxidant defense and deteriorates mitochondrial function. It has been observed that GSH decreases when NO production increases [38]. Our result showed that the decreased mitochondrial GSH levels were partially restored with LA treatment. Since both GSH and LA are thiol antioxidants, treatment with LA may spare consumption of GSH [46], and reduce formation of protein disulfide bridges [47]. In addition, LA can raise GSH levels by reducing cystine to cysteine, a GSH precursor [48]. However, it is not certain whether this reaction can take place under the oxidative stress conditions. The increased mitochondrial GSH levels by LA may provide protection from NO-induced damage to mitochondrial components related to energy production like KDH, and consequently attenuate hypothermia. It is known that KDH is sensitive to ROS/RNS such as hydrogen peroxide, peroxynitrite and nitric oxide [49, 50]. Thus, it is not surprising at the outcome that enzyme activity decreases during sepsis.

These data are also consistent with previous observations from our and other investigators. For instance, human endothelial cells exposed to serum from patients with sepsis have shown lower rates of mitochondrial respiration and significantly reduced ATP production than cells treated with serum from healthy volunteers [51, 52]. Likewise, *in vitro* studies showed mitochondrial complex inhibition by RNS with decreased ATP synthesis [52, 53]. Additionally, mitochondrial damage develops into energetic failure during sepsis, leading to cell death and organ failure [54, 55]. Moreover, sepsis-associated oxidative stress can be effectively quenched by LA treatment [18, 56]. Our previous data showed that LA in hepatocytes decreased noticeably in LPS treated mice [18]. At this point it is not clear whether decreasing LA levels affect the availability of the cofactor for normal KDH function. The fact that KDH activity does not change with LA supplementation in LPS-treated *iNOS*$^{-/-}$ mice indicates that KDH activity under these conditions is not dependent on the cofactor. Earlier reports have shown that LA administration improved the activities of KDH by improving the co-factor availability [57, 58]; however, these observations were made in aged animals, and they cannot be applied to studies in animals as young as those used in our studies.

Evidence shows that LA and ascorbate are powerful antioxidants that catalyze denitrosylation [59, 60]. However, ascorbate fails to prevent hypothermia. Thermoregulatory properties of LA may be related to its unique structure and physical/chemical characteristics. We believe that the presence of a thiol group in LA could result in better protection of mitochondria [61].

Since hypothermia may be a reversible process, we extended the time of observation for body temperature from 4 hours to 24 hours to probe possible thermo-regulative mechanisms of LA. We found that low body temperature persisted for 24 hours post LPS injection and that the highest level of plasma NO occurred at 24 hours in septic mice. The thermoregulatory effect of LA weakened over time and body temperature increased <1°C 6-8 hours after LPS injection, suggesting that extended exposure to NO could lead to irreversible inhibition of mitochondrial function, which is difficult to attenuate with a one-time treatment of LA. Repeated treatment or a higher dose may be required to overcome this situation.

Acknowledgments

This project was supported by HL42630 and HL087946 (N.M.), by UNC University Research Council Grants, and in part by DK056350 to the UNC-CH Nutrition Obesity Research Center (X.Yi.).

The authors thank Dr. Rosaline Coleman for her insightful suggestions.

The manuscript has been reviewed by the US Environmental Protection Agency, NHEERL and approved for publication. The authors would like to thank Drs. P.R. Kodavanti and J. Royland for their invaluable suggestions. Approval does not signify that the contents reflect the views of the US EPA, nor that the mention of trade names or commercial products could constitute endorsement or recommendation for use.

Abbreviations

iNOS	inducible Nitric Oxide Synthase
eNOS	endothelial Nitric Oxide Synthase
IL-6	interleukin-6
KDH	α-ketoglutarate dehydrogenase complex
LA	α-lipoic acid
LPS	lipopolysaccharide
NO	nitric oxide
GSH	glutathione
TNF-α	tumor necrosis factor alpha
WT	wild type
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species

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Highlights

- α-lipoic acid (LA) significantly attenuates hypothermia.
- LA restores enzymatic activities inhibited by excessive nitric oxide.
- Our results should provide a new strategy for effective treatment of sepsis.

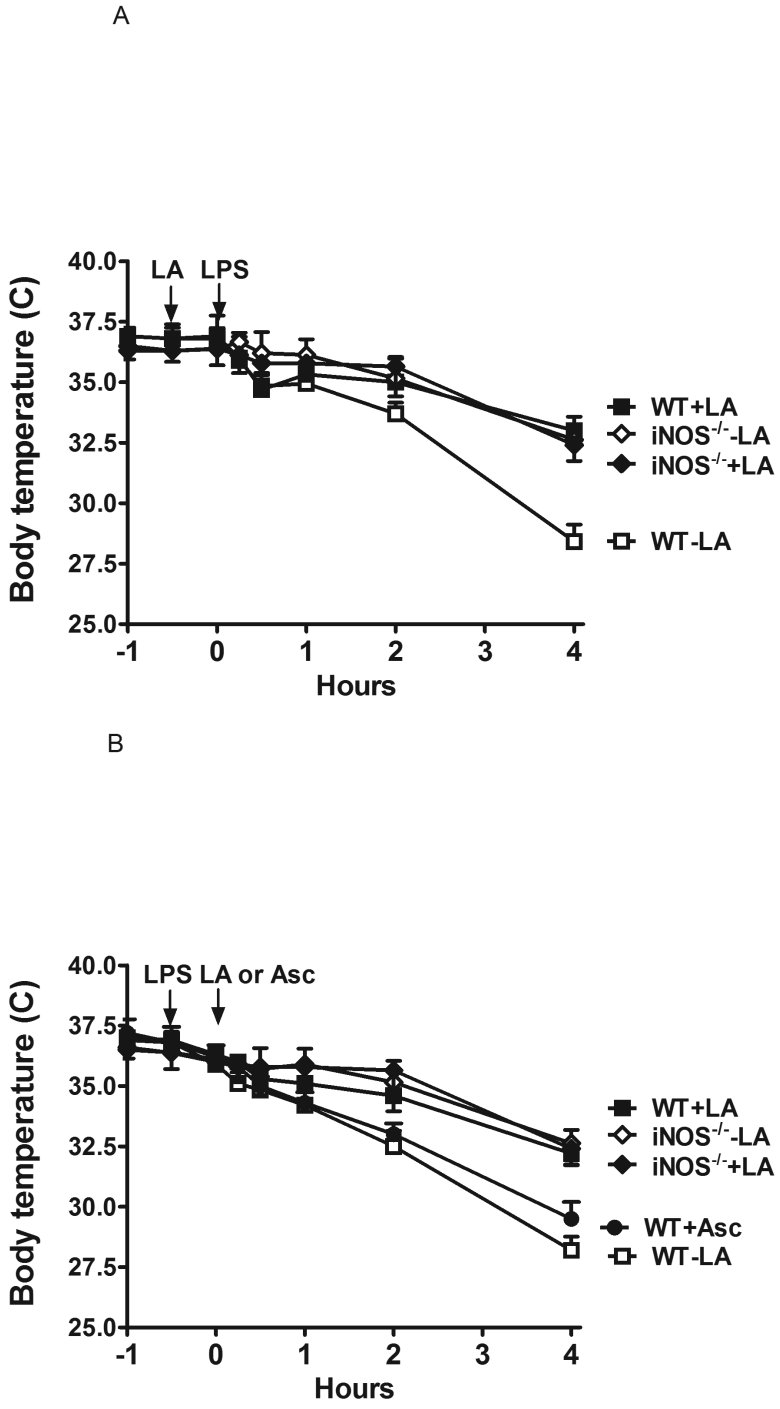

Figure 1.

Time course of hypothermia in wild type (WT) and *iNOS*$^{-/-}$ mice induced by lipopolysaccharides (LPS). A). α-lipoic acid (LA) was given at 30 min prior to LPS injection. Animal number: WT+LA=17; WT-LA=14; *iNOS*$^{-/-}$+LA=10; *iNOS*$^{-/-}$-LA=10. B). LA or ascorbate were administered at 30 min after LPS injection. Data are expressed as means ± SEM. Animal number: WT+LA=12; WT-LA=13; *iNOS*$^{-/-}$+LA=10; *iNOS*$^{-/-}$-LA=10; and WT+ascrobate=4.

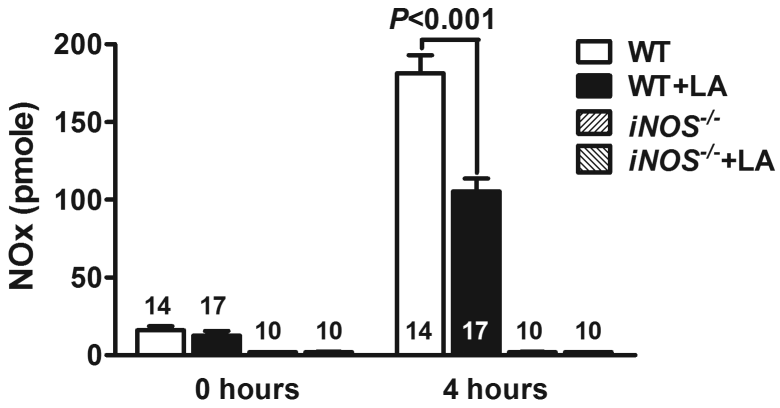

Figure 2.

Total plasma nitrate/nitrite (NOx) immediately before, and 4 hours after LPS injection in septic mice. Values are the mean ± SEM. Statistical analysis are by one-way ANOVA.

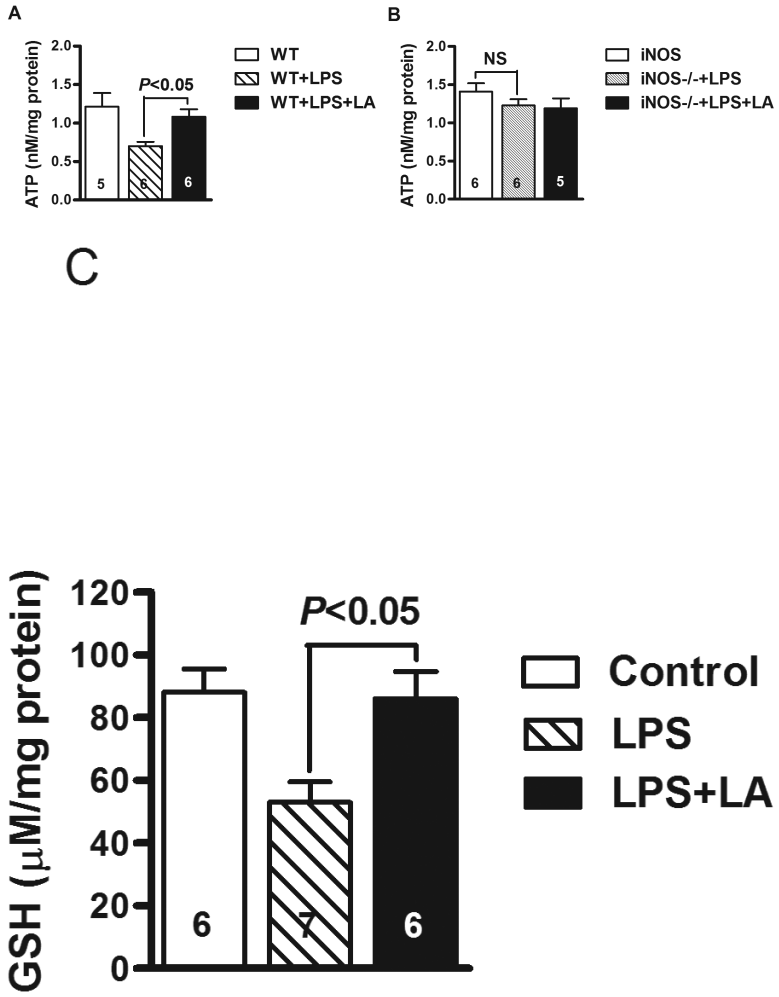

Figure 3.

Effects of LA on energy production during sepsis. ATP content in the livers of the WT (A) and *iNOS*$^{-/-}$ mice (B) at 4 hours following LPS treatment. Control mice were injected with PBS. (C) Glutathione (GSH) levels in mitochondria isolated from liver of LPS-treated mice. All results are expressed as mean ± SEM. The numbers inside the bars indicate the number of animals used for each measurement.

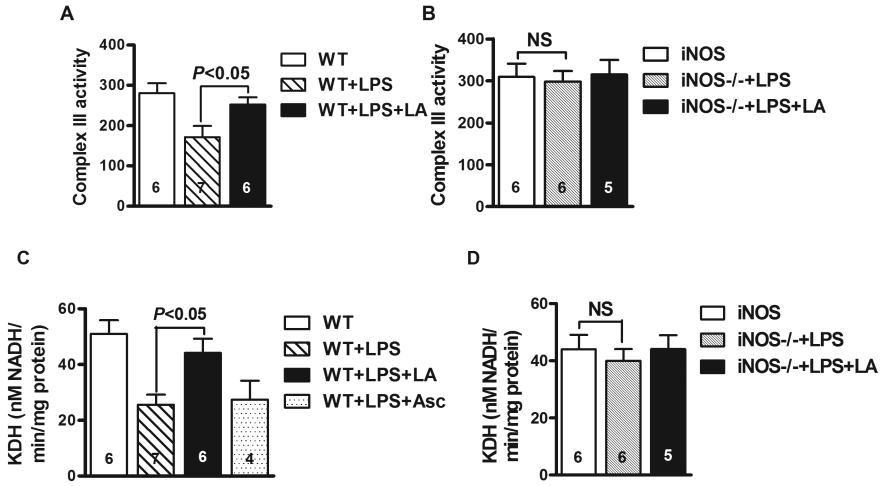

Figure 4.

Activities of complex III and alpha-ketoglutarate dehydrogenase (KDH) in mitochondria isolated from liver of LPS-treated WT mice (A and C) and *iNOS*$^{-/-}$ mice (B and D) in the presence or absence of LA or ascorbate. (A and B) Complex III activity was expressed as nmole of reduced cytochrome C/minute per mg of protein. (C and D) KDH activity was determined by monitoring NAD^+ reduction at 340 nm. The activities of two enzymes shown are the means ± SEM.

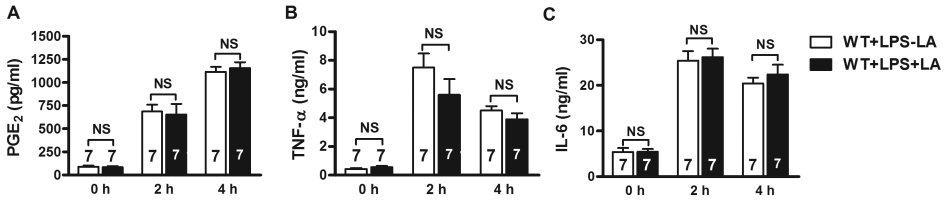

Figure 5.

Serum concentrations of (A) prostaglandin E2 (PGE_2), (B) tumor necrosis factor alpha (TNF-α), and (C) interleukin-6 (IL-6) at 2 hours and 4 hours after intraperitoneal injection of LPS in mice supplemented with LA compared to controls. Error bars represent SEM.