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# Targeting of reactive isolevuglandins in mitochondrial dysfunction and inflammation

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ABSTRACT

Inflammation is a major cause of morbidity and mortality in Western societies. Despite use of multiple drugs, both chronic and acute inflammation still represent major health burdens. Inflammation produces highly reactive dicarbonyl lipid peroxidation products such as isolevuglandins which covalently modify and cross-link proteins via lysine residues. Mitochondrial dysfunction has been associated with inflammation; however, its molecular mechanisms and pathophysiological role are still obscure. We hypothesized that inflammation-induced isolevuglandins contribute to mitochondrial dysfunction and mortality. To test this hypothesis, we have (a) investigated the mitochondrial dysfunction in response to synthetic 15-$E_2$-isolevuglandin (IsoLG) and its adducts; (b) developed a new mitochondria-targeted scavenger of isolevuglandins by conjugating 2-hydroxybenzylamine to the lipophilic cation triphenylphosphonium, (4-(4-aminomethyl)-3-hydroxyphenoxy)butyl)-triphenylphosphonium (mito2HOBA); (c) tested if mito2HOBA protects from mitochondrial dysfunction and mortality using a lipopolysaccharide model of inflammation. Acute exposure to either IsoLG or IsoLG adducts with lysine, ethanolamine or phosphatidylethanolamine inhibits mitochondrial respiration and attenuates Complex I activity. Complex II function was much more resistant to IsoLG. We confirmed that mito2HOBA markedly accumulates in isolated mitochondria and it is highly reactive with IsoLGs. To test the role of mitochondrial IsoLGs, we studied the therapeutic potential of mito2HOBA in lipopolysaccharide mouse model of sepsis. Mito2HOBA supplementation in drinking water (0.1 g/L) to lipopolysaccharide treated mice increased survival by 3-fold, improved complex I-mediated respiration, and histopathological analyses supported mito2HOBA-mediated protection of renal cortex from cell injury. These data support the role of mitochondrial IsoLG in mitochondrial dysfunction and inflammation. We conclude that reducing mitochondrial IsoLGs may be a promising therapeutic target in inflammation and conditions associated with mitochondrial oxidative stress and dysfunction.

## 1. Introduction

Inflammation is implicated in many diseases that represent major causes of morbidity and mortality in Western societies including cardiovascular diseases, acute kidney injury, and lung and heart failure, [1]. Despite use of multiple drugs, both chronic and acute inflammation still represent major health burdens. In the recent years, it has become clear that oxidative stress plays an important role in pathophysiology of many conditions associated with inflammation such as cardiovascular diseases and sepsis [1,2]. Increased lipid peroxidation [3] has been shown in hypertension, atherosclerosis and sepsis, using the clinically validated marker F2-isoprostanes [4]. Lipid peroxidation via the isoprostane pathway produces a family of highly reactive γ-ketoaldehydes, the isolevuglandins (IsoLGs) [5], which rapidly react with primary amines and causes cellular dysfunction [6]. IsoLGs covalently modify and cross-link proteins by reacting with their lysine residues, and this modification can directly inhibit enzymatic functions, induce inflammation and cause cytotoxic effects [6,7]. IsoLGs have been implicated in pro-inflammatory dendritic and T cell activation in hypertension [8]. Acute treatment of isolated mitochondria with IsoLG

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**Abbreviations**

IsoLG isolevuglandins (aka isoketals)
PE phosphatidylethanolamine
15-$E_2$-IsoLG 15-$E_2$ stereoisomer of isolevuglandins
15-$E_2$-IsoLG-PE adduct of PE with 15-$E_2$-IsoLG stereoisomer
2HOBA 2-hydroxybenzylamine
4HOBA non-scavenger analog of 2HOBA, 4-hydroxybenzylamine
mito2HOBA conjugate of 2-hydroxybenzylamine to lipophilic cation triphenylphosphonium, (4-(4-aminomethyl)-3-hydroxyphenoxy)butyl)-triphenylphosphonium
mitoTEMPO (2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl) triphenylphosphonium
mPTP mitochondrial permeability transition pore
PUFA polyunsaturated fatty acids
ROS reactive oxygen species
LPS Lipopolysaccharide
WT wild-type C57BL/6J mice
SMP submitochondrial particles

disrupts mitochondrial respiration and promotes mitochondrial permeability transition pore (mPTP) opening [9], however, the role of mitochondrial IsoLGs in pathological conditions has not been investigated.

Sepsis causes devastating multi-organ failure such as acute kidney injury [10] which is linked with increased oxidative stress and mitochondrial dysfunction. Levels of IsoLG adducts are elevated in experimental models of sepsis [11]. Inflammation has been associated with mitochondrial dysfunction; however, its pathophysiological role and molecular mechanisms are still obscure. Mitochondria are one of the major sources of free radicals [12] that can potentially produce IsoLG. Given the potential highly injurious nature of IsoLGs, we propose that inflammation-induced IsoLGs plays an important role in mitochondrial dysfunction.

Although oxidative stress is common in multiple pathological conditions [13], antioxidant therapy is not currently available and common antioxidants like ascorbate and vitamin E are ineffective in the clinical studies [14]. These agents unlikely reach important sites of free radical production such as mitochondria. Furthermore, antioxidants can potentially interfere with redox signaling increasing inflammation and tissue injury due to increased cytokine production and suppression of Nrf2 signaling [15,16].

Oxidative stress in aging and inflammation results in increased peroxidative damages to polyunsaturated fatty acids (PUFA). The cause of the isoprostane type of lipid peroxidation remained unclear. It has been proposed that autoxidation of PUFA can be initiated by perhydroxyl radical ($HO_2^{\bullet}$) [17,18], a protonated form of superoxide radical, which is produced in mitochondria. Hypoxia and acidification of a tissue increase production of $HO_2^{\bullet}$ [17]. We propose that accumulation of the oxidatively damaged mitochondrial phospholipids and IsoLG adducts is the result of PUFA oxidation by mitochondrial $HO_2^{\bullet}$. The $HO_2^{\bullet}$ hypothesis of the isoprostane lipid peroxidation agrees with the known fact that classical antioxidants are ineffective in prevention of this type oxidative stress and aging [19]. Isoprostane lipid peroxidation produces racemic mixture of various forms of isoprostanes and isolevuglandins [5]. Some of the isoprostanes can be responsible for initiation of the inflammatory responses directly [4,20] while reactive IsoLG produces cytotoxic and immunogenic IsoLG-lactam adducts [7].

Production of IsoLG is one of the common downstream products of oxidative stress [7,21] and scavenging IsoLG with 2-hydroxybenzylamine (which is not an antioxidant) reduces endothelial dysfunction, diminishes fibrosis and attenuates hypertension [8,22]. Therapies specifically targeted at mitochondria represent promising strategies to reduce target-organ-damage [23,24]. We hypothesized that mitochondrial targeting of the IsoLG scavenger 2-hydroxybenzylamine [8], by conjugating it with the lipophilic cation triphenylphosphonium [25], would reduce mitochondrial dysfunction and attenuate mortality associated with sepsis. In this work, we studied (a) mitochondrial dysfunction in response to synthetic IsoLG and its adducts, (b) developed new mitochondria-targeted IsoLG scavenger mito2HOBA, and (c) tested if mito2HOBA protects from mitochondrial dysfunction and mortality in lipopolysaccharide (LPS) model of sepsis.

## 2. Materials and methods

### 2.1. Reagents

LPS was obtained from Sigma (St Louis, MO). 2-Hydroxybenzylamine (2HOBA) and its non-scavenger analog 4-hydroxybenzylamine (4HOBA) were prepared as described previously [26]. 15-$E_2$-IsoLG was synthesized by the method of Amarnath et al. [27] and kept in DMSO stock solution at −80 °C. All other reagents were from Sigma (St Louis, MO).

### 2.2. Animal experiments

All experimental procedures were approved by Vanderbilt and Mercer Institutional Animal Care and Use Committees. The use of LPS is well-established model of bacterial sepsis in rodents (1–3). The concentrations of LPS that can be used to induce sepsis in mice depend on many factors (source of LPS, age/size, and strains of animals, desired time of response, target of interest, etc.) and may vary between different manufactures [28]. To test the protective properties of mito2-HOBA, we used LPS from *E. coli* O111:B4 (Sigma L8274) in our studies. The lot of LPS tested in preliminary studies had a $LD_{50}$ of 25 μg/g at 24 h post-injection. The same lot of LPS was used throughout entire study.

Forty C57BL/6J 3-month old mice were equally divided in four groups: Sham (Control), LPS-injected mice (LPS), mice supplemented with mito2HOBA (0.1 g/Liter) in the drinking water (mito2HOBA), and LPS-injected mice pretreated with mito2HOBA in the drinking water (LPS + mito2HOBA). Sepsis-induced mortality among animals was used to evaluate the protective role of mito2HOBA. Mortality was assessed regularly several times per day for three consecutive days. In additional experiments mice were sacrificed after 24 h of LPS injection to analyze mitochondrial complex I and complex II activities.

### 2.3. Mitochondrial studies

All procedures for mitochondrial isolation, respiration analysis and respiratory chain enzymology have been previously described [29,30]. Mitochondrial complex I and complex II activities were evaluated after 24 h of LPS injection as described above. Mitochondria were isolated from 12 to 14-week-old male C57BL6/6J mouse kidneys. For respiration studies, electrons were entered at either complex I (glutamate + malate as substrate) or complex II (succinate as substrate). Mitochondria in some organs, e.g. brain, oxidize up to 50% of pyruvate and glutamate via transamination to α-ketoglutarate and further conversion to succinate [31]. Since kidney mitochondria are much less studied than mitochondria from other organs, we used the specific inhibitor of Complex II malonate (5 mM) to evaluate the alternative pathways for glutamate oxidation. The Complex II mediated respiration was defined as malonate-inhibited oxygen consumption while Complex I specific respiration was defined as malonate resistant oxygen consumption.

Respiration rates were measured using Fluorescence Lifetime Micro

Oxygen Monitoring System (Instech Laboratories, Inc.). Two oxygen consumption rate measurements were performed for each substrate and each run included additions of 0.24 mg/ml protein, ADP (125 μM) to stimulate state III and subsequent state IV respiration. OXPHOS specific enzyme activities in submitochondrial particles (SMP) were measured using a Varian Cary 300 Bio UV/Vis spectrophotometer with temperature controlling cell holder. Briefly, SMP were prepared by sonication of isolated organelles. Complex I activity was monitored in triplicate samples as the reduction of 10 μM decylubiquinone at 272 nm by 15 μg of mitochondrial protein with 40 μM NADH. Using this method, 90–100% of the total complex I activity is sensitive to rotenone inhibition. Complex II activity was measured by monitoring the absorbance at 600 nm during the oxidation of 50 μM DCPIP as artificial electron acceptor by 65 μM ubiquinone in presence of 2 mM KCN and 2 μg/ml rotenone and antimycin A.

### 2.4. Analysis of complex I and complex II mediated respiration in kidney mitochondria

In order to define specific alterations in the mitochondrial respiration in LPS model of sepsis and test the potential protection by mito2HOBA we have adopted the Seahorse protocol for mitochondrial studies [32] in the presence of mitochondrial substrates Glutamate + Malate (GM) or succinate. To define the specific role of complex I mediated respiration we performed measurements in the presence of complex II inhibitor malonate (5 mM). We have measured basal respiration in the presence of mitochondria plus substrates, coupled respiration after addition of ADP (2 mM), proton leak following addition of oligomycin A (2.5 μM), uncoupled respiration after supplementation of CCCP (1 μM), and non-mitochondrial respiration with mixture of antimycin A and rotenone (1 μM) [32]. Mitochondrial studies where independently verified in two labs using Oroboros O2k high-resolution respirometry and Fluorescence Lifetime Micro Oxygen Monitoring System (Instech Laboratories, Inc). Kidney mitochondria were isolated from control sham mice, LPS-injected mice (25 μg/g, 16 h post-injection), mito2HOBA supplemented mice (0.1 g/Liter drinking water, 4 days), or mito2HOBA plus LPS (0.1 g/Liter mito2HOBA for 3 days plus LPS injection). One kidney was used for mitochondrial studies and second kidney was used for histopathological studies.

### 2.5. Kidney histological analyses

Kidneys were harvested from mice and placed immediately in 10% formalin. Following fixation, kidneys were washed with saline, placed in 70% ethanol, and processed in the following sequence: 70% ethanol; 80% ethanol; 95% ethanol; 100% ethanol; 100% xylene. Then kidneys were embedded in POLY/Fin paraffin (ThermoFisher). Five-μm sections were cut using a Leitz 1512 microtome and were mounted on glass slides. Sections were stained with hematoxylin and eosin and were viewed using an Olympus IX70 microscope. Images were captured with a Jenoptix Progress C12 digital camera. Histopathological scores of kidneys were measured as follows: (0) No tubular injury; (1) < 10% tubules injured; (2) 10–25% tubules injured; (3) 25–50% tubules injured; (4) 50–75% tubules injured; (5) > 75% tubules injured [33].

### 2.6. Synthesis of mitochondria-targeted IsoLG scavenger mito2HOBA (Fig. figs1)

Cesium carbonate (4.9 g, 15 mmol) was added to 2,4-dihydroxybenzaldehyde (4.2 g, 30 mmol) and 1,4-dibromobutane (6.6 g, 30 mmol) in acetonitrile (50 ml). The mixture was heated at 80 °C under argon for 5 h, cooled and added to 1 M phosphate buffer, pH 7 (30 ml), ice and $KH_2PO_4$ (2 g) with mixing. The solid was removed by filtration and the filtrate was extracted with ethyl acetate. Purification by column (silica, 9:1 hexane-ethyl acetate) yielded 4-(4-bromobutoxy)-2-hydroxybenzaldehyde (4.1 g, 50%). It was mixed with triphenylphosphene (4.2 g) in toluene (75 ml) and refluxed under argon for 15 h. The pink solid was purified by flash chromatography (0–10% methanol in dichloromethane to get 4-(4-formyl-3-hydroxyphenoxy)butyl)triphenylphosphonium bromide (4.8 g, 60%). The aldehyde was converted to oxime by stirring with $NH_2OH{\cdot}HCl$ (0.63 g) and $CH_3CO_2Na$ (0.74 g) in ethanol (40 ml) for 1 h. The crude product (5.6 g) was dissolved in

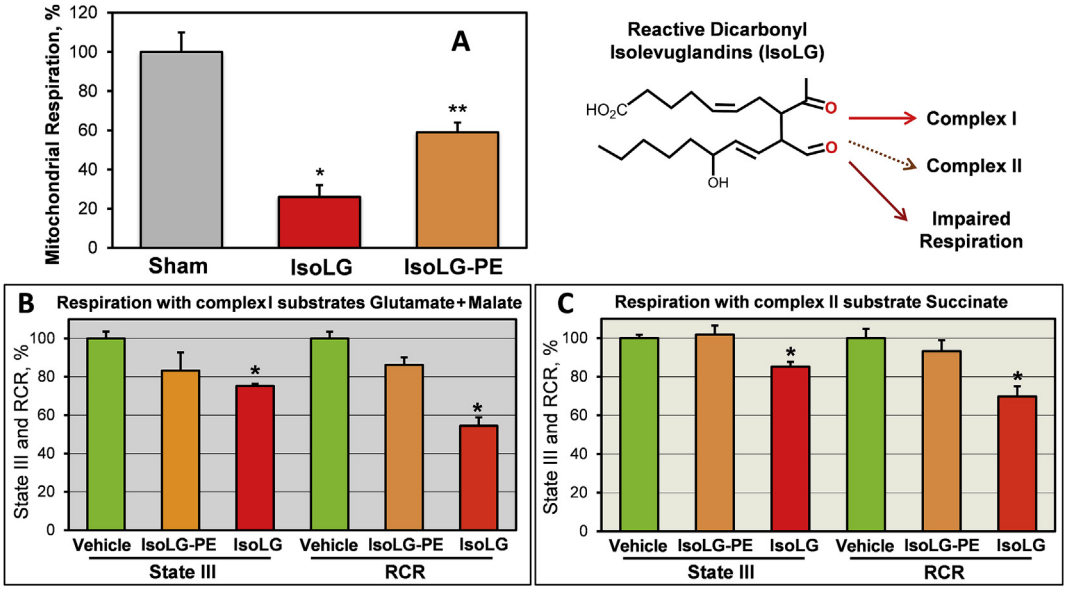


**Fig. 1.** Acute treatment with IsoLG or IsoLG-PE impairs mitochondrial respiration. (A) Intact mouse kidney mitochondria were incubated (5 min) with ethanol as vehicle (Sham), IsoLG (20 μM) or IsoLG-PE (20 μM) and then diluted with respiration buffer 20-fold prior to addition of Glutamate and Malate, ADP (50 μM) and measurements of oxygen consumption in State III. *P < 0.001 vs Sham, **P < 0.03 vs IsoLG. (B) Oxygen consumption in the presence of complex I substrates Glutamate + Malate and ADP (State III) and Respiratory Control Ratio (State III/State IV, %) in intact kidney mitochondria treated with vehicle, IsoLG (1.5 μM) or IsoLG-PE (1.5 μM) in the respiration chamber. (C) State III respiration and Respiratory Control Ratio (State III/State IV, %) in presence of complex II substrate Succinate and ADP following addition of vehicle, IsoLG (1.5 μM) or IsoLG-PE (1.5 μM) in the respiration chamber. Data are expressed as mean ± STD (N = 4–6). *P < 0.01 vs Vehicle.

acetic acid (60 ml). Zinc powder (6 g) was added and the suspension was heated in a water bath (60 °C) for 1 h. The mixture was cooled, filtered through Celite. The filtrate was evaporated and co-evaporated with toluene (3 × 10 ml) and ethanol (15 mL). The residue was heated in hot 2-propanol (200 ml), filtered and cooled to obtain pure mito2HOBA; 3.0 g; MS *m/z* 456 ($M^+$).

*Statistics*. Data were analyzed using the Student-Neuman-Keuls post-hoc test and analysis of variance (ANOVA). P levels < 0.05 were considered significant.

## 3. Results

### 3.1. Isolevuglandins impaired mitochondrial respiration

Complex I is a key component of mitochondrial oxidative phosphorylation. Inactivation of complex I can lead to reduced ATP production and tissue damage. Addition of IsoLG to cells produces both protein-adducts and IsoLG-phosphatidylethanolamine adducts (IsoLG-PE) which can independently contribute to mitochondrial dysfunction. We tested whether IsoLG or IsoLG-PE could contribute to mitochondrial dysfunction. Five-minute treatment of isolated mitochondria with 15-$E_2$-IsoLG-PE (20 μM) inhibited state 3 respiration by 41% while similar doses of 15-$E_2$-IsoLG reduced state 3 respiration by 74% in the presence of complex I substrates glutamate + malate (Fig. 1A). These data support the potential role of IsoLG-PE and IsoLG-protein adducts in mitochondrial dysfunction. To further define the potential targets of IsoLG in mitochondria, we studied the effect of IsoLG and IsoLG-PE on complex I- and complex II-mediated respiration. Acute addition of a low dose of 15-$E_2$-IsoLG (0.5 μM) significantly attenuated complex I-mediated respiration, but complex II respiration was much less affected (Fig. 1B and C). Treatment of intact mitochondria with a low dose of 15-$E_2$-IsoLG-PE partially diminished complex I respiration but did not affect complex II respiration. These data directly demonstrate the impairment of mitochondrial respiration by IsoLG and IsoLG-PE.

### 3.2. IsoLG and IsoLG adducts inhibit complex I activity

We have hypothesized that IsoLG can directly affect the complex I and complex II activity. To test this hypothesis, we studied the activity of complex I and complex II in mitochondrial lysates treated with a bolus of IsoLG. It was found that IsoLG causes robust complex I inactivation by 74% while complex II activity was inhibited by only 21% (Fig. 2A). These data showed that complex I respiration is particularly sensitive to IsoLG.

As shown above, both IsoLG and IsoLG-PE diminish complex I-mediated respiration, therefore, complex I could be affected by IsoLG directly or inhibited by low-molecular IsoLG adducts. We tested the inhibition of complex I by IsoLG adducts compared with bolus IsoLG. Supplementation with IsoLG modified-ethanolamine (IsoLG-ETN), modified L-Lysine (IsoLG-Lys), or modified-PE (IsoLG-PE) inhibited complex I activity by more than 80%, similar to the effect of bolus IsoLG (Fig. 2B). Interestingly, IsoLG modified spermine (IsoLG-spermine) did not affect complex I, suggesting that natural poly-amines could potentially protect complex I from IsoLG mediated inhibition. These data demonstrate that complex I is directly inhibited by low-molecular IsoLG adducts such as IsoLG-Lys and IsoLG-PE; thus, these adducts may mediate the impairment of complex I induced by direct addition of IsoLG. These data directly confirm that IsoLG-mediated inhibition of complex I contribute to mitochondrial dysfunction.

### 3.3. Mitochondria-targeted IsoLG scavenger mito2HOBA

To test the hypothesis that specific scavenging of IsoLG in mitochondria improves mitochondrial function, we developed a

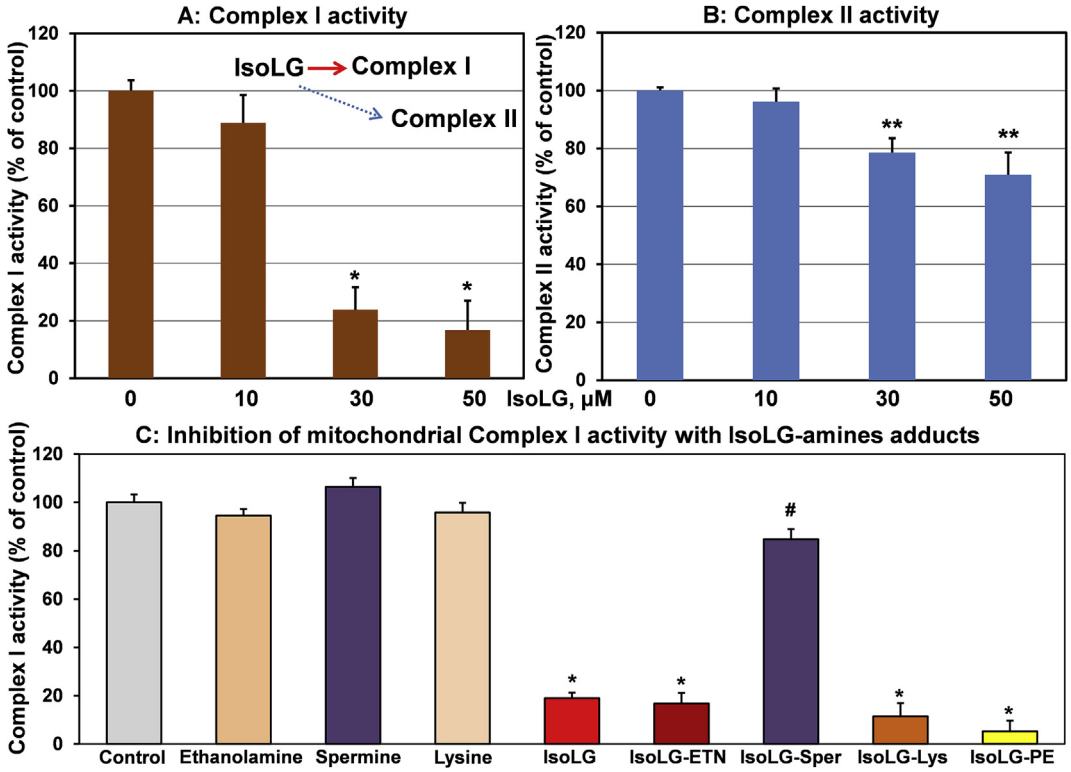


**Fig. 2.** Inhibition of complex I and complex II activity by IsoLG and IsoLG-adducts. (A, B) Mouse kidney mitochondrial lysate was incubated with DMSO (vehicle) or IsoLG for 5-minites prior to analysis of complex I or complex II activity as described previously [29,30]. *P < 0.01 vs Sham. **P < 0.05 vs Sham. (C) Mouse kidney mitochondrial lysate was acutely treated with DMSO (Control), ethanolamine, spermine, L-lysine, IsoLG (1.5 μM) or IsoLG modified-ethanolamine (IsoLG-ETN, 1.5 μM), -spermine (IsoLG-Sper, 1.5 μM), L-lysine (IsoLG-Lys, 1.5 μM), -phosphatidylethanolamine (IsoLG-PE, 1.5 μM) prior to measurements of complex I activity expressed as a % of Control (100%). Data are expressed as mean ± STD (N = 3–6). *P < 0.001 vs Sham, #P < 0.01 vs IsoLG.

mitochondrial-targeted IsoLG scavenger, mito2HOBA, by conjugating the lipophilic cation triphenylphosphonium to 2-hydroxybenzylamine (Fig. 3). The membrane potential of mitochondria within living cells is negative inside (−150 mV). As this membrane potential is much higher than in other organelles within cells, lipophilic cations such as triphenylphosphonium selectively accumulate within mitochondria [34]. Molecules conjugated to triphenylphosphonium are therefore targeted to the mitochondria [25]. For example, mitoTEMPO is concentrated within the mitochondrial matrix by more than a five hundred-fold [35,36].

Mito2HOBA is a water-soluble compound which can be supplied in the media and provided to animals in the drinking water. In our animal experiments, mito2HOBA was well tolerated in doses from 0.1 to 0.3 g/L. Mass spectrometric analysis of kidney and heart mitochondria isolated from mice that received mito2HOBA in their drinking water (0.1 g/L) for 5-days confirmed that mito2HOBA predominantly accumulated in the mitochondrial fraction (by 80%) at μM levels. Likewise, incubation of isolated mitochondria with mito2HOBA (0.1 μM) causes robust accumulation of mito2HOBA in the mitochondrial pellet by 400 to 600-fold (Fig. 3).

In order to confirm the IsoLG scavenging properties of mito2HOBA, we studied its reaction with the IsoLG analog 4-oxopentanal as we described previously [26]. Mito2HOBA was highly reactive with 4-oxopentanal with a reaction rate constant that was about 50% of 2HOBA itself (Supplemental Fig. 1S). The slightly reduced reaction rate may be due to steric hindrance by the bulky triphenylphosphonium group. The overall rate of IsoLG scavenging in physiological conditions depends both on the rate constant and the local concentration of the scavenger (V = k*[mito2HOBA]*[IsoLG]). Of note, 2HOBA analogs do not scavenge oxidants such as $O_2^{\cdot}$ and peroxynitrite [8]. We hypothesized that supplementation with mito2HOBA at low submicromolar level would result in low cytoplasmic level but significant mitochondrial accumulation (Fig. 3) as we had previously described for mitochondria-targeted mitoTEMPO [36]. This will result in low level of mito2HOBA in cytoplasm but high mitochondrial accumulation leading to specific scavenging of IsoLG in mitochondria (Fig. 3).

### 3.4. Complex I- and complex II-mediated kidney respiration in LPS and mito2HOBA treated mice

The kidney has a high demand for energy and renal mitochondria can potentially oxidize glutamate via transamination to α-ketoglutarate and further conversion to succinate [31]. We analyzed mitochondrial respiration in the presence of Glutamate + Malate (GM), succinate and used Complex II inhibitor malonate to measure the specific Complex I mediated respiration. Malonate inhibited 58% of glutamate-driven respiration (Fig. 4A) supporting the metabolic plasticity of renal mitochondria. Interestingly, LPS injection reduced both GM- and succinate-mediated respiration, and substantially diminished complex I-specific oxygen consumption in the presence of GM + malonate (Fig. 4B). LPS significantly increased mitochondrial protein leak with both substrates indicating uncoupling of mitochondrial respiration. Mito2HOBA alone slightly reduced succinate-driven respiration and improved complex I-specific oxygen consumption in the presence of GM + malonate (Fig. 4C). Furthermore, mito2HOBA supplementation significantly protected from LPS-induced impairment of GM-mediated respiration and complex I-specific oxygen consumption in the presence of GM + malonate but did not affect the succinate mediated respiration or mitochondrial protein leak (Fig. 4D).

### 3.5. Mito2HOBA reduces mitochondrial dysfunction and attenuates mortality in LPS model of sepsis

To test the role of IsoLG-mediated mitochondrial dysfunction we supplemented mice with novel mitochondria-targeted IsoLG scavenger mito2HOBA (0.1 g/L). Treatment with LPS (25 μg/g by body weight) caused severe mortality but treatment with mitochondria-targeted IsoLG scavenger mito2HOBA increased animal survival at 96 h post-injection by 3-fold (Fig. 5A). Additional studies showed that complex I/complex II activity ratio was markedly decreased in mitochondria isolated from the kidneys of LPS-treated mice compared to vehicle treated mice (Fig. 5B). Supplementation of mice with mito2HOBA completely preserved the complex I/complex II activity ratio even after LPS treatment. These data support the role of mitochondrial IsoLG in the mitochondrial dysfunction and mortality associated with sepsis.

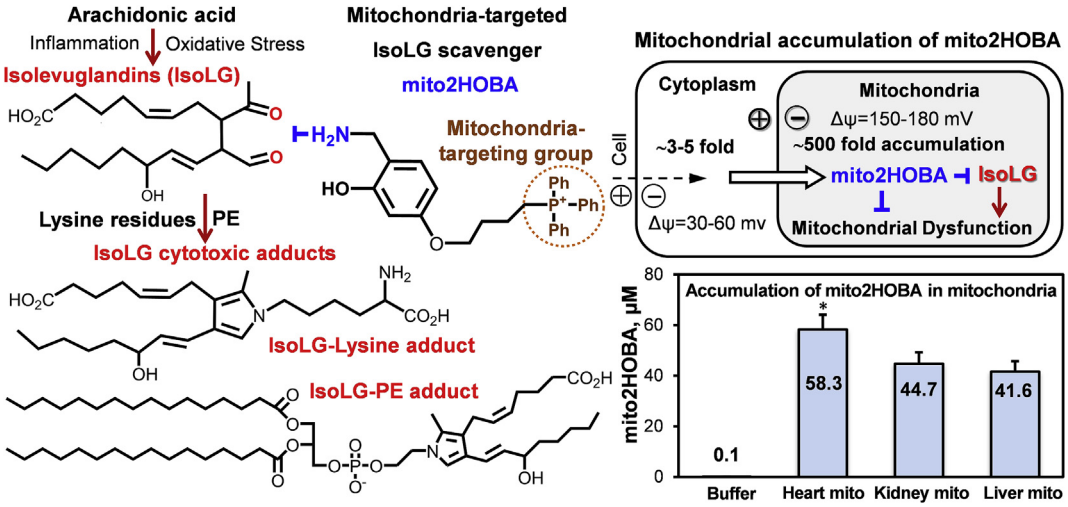


**Fig. 3.** Mitochondria-targeting of IsoLG scavenger mito2HOBA. Linking the 2-hydroxybenzylamine to lipophilic cation triphenylphosphonium directs mito2HOBA to mitochondria since its membrane potential is much higher than in other organelles within cells leading to selective accumulation within mitochondria [34]. Inflammation and oxidative stress oxidizes arachidonic acid to reactive IsoLG which react rapidly with protein lysine residues and phosphatidylethanolamine yielding cytotoxic IsoLG adducts [6]. Incubation of mito2HOBA (0.1 μM) with isolated mitochondria (1 mg/ml) causes robust accumulation of mito2HOBA in the mitochondrial fractions. Data are expressed as mean ± STD (N = 4). Mitochondria-targeted mito2HOBA can potentially reduce mitochondrial dysfunction by scavenging of IsoLG in the mitochondrial matrix.

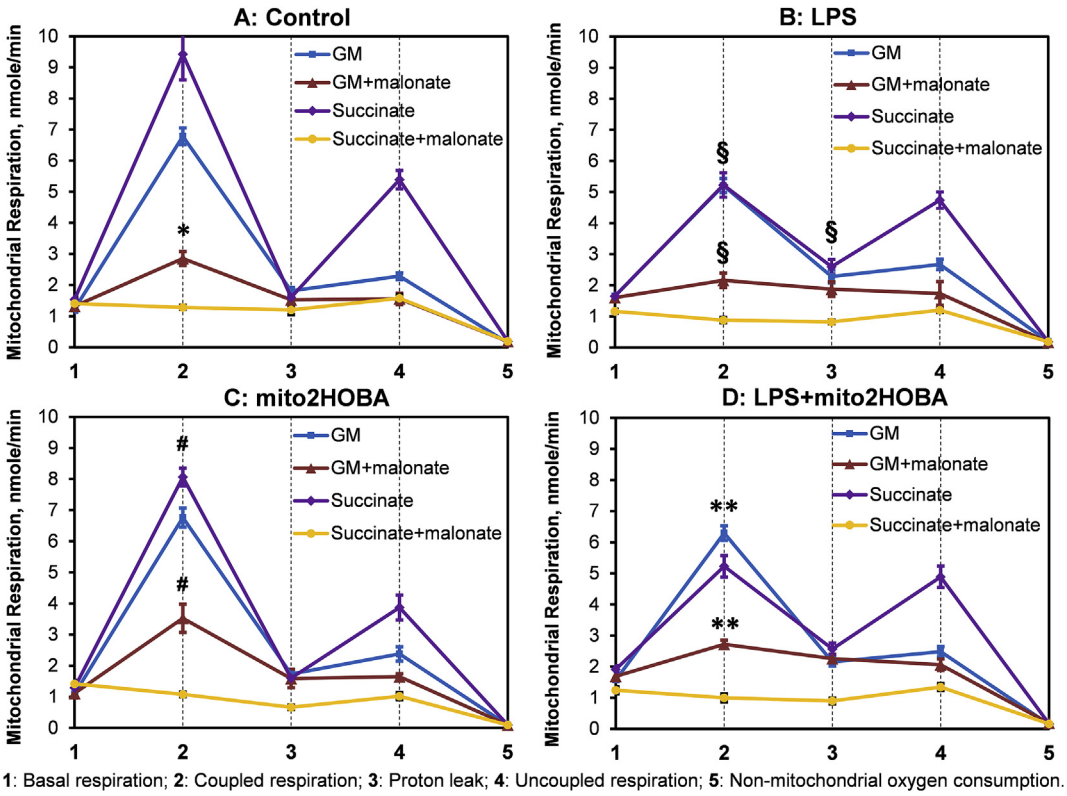


**Fig. 4.** Respiration of kidney mitochondria isolated from control sham, mito2HOBA-supplemented, LPS treated and LPS plus mito2HOBA mice. To study the mitochondrial function, the combination of glutamate and malate (GM) or succinate was used as substrates. Since glutamate can be converted via transamination to α-ketoglutarate and further to succinate [31], we used complex II inhibitor malonate to define specific complex I respiration. The basal respiration (1) was measured in mitochondria supplemented with respiratory substrates. Then ADP was added to measure the coupled respiration (2). The proton leak (3) was determined after addition of complex V inhibitor oligomycin A. The uncoupled respiration (4) was measured after supplementation with CCCP. Finally, antimycin A plus rotenone were added to assess non-mitochondrial respiration (5) as described previously [32]. Data are mean ± STD (n = 4–6). *P < 0.001 vs GM, #P < 0.01 vs Control, §P < 0.001 vs Control, **P < 0.01 vs LPS.

### 3.6. Mito2HOBA protects against LPS-induced renal injury

Histological analyses of kidneys were performed to provide visual evidence of mito2HOBA protection (Fig. 6). The cortex and medulla of control kidneys appeared normal with no evidence of injury. In contrast, kidneys of mice treated with LPS demonstrated significant cellular injury. Numerous areas of vacuolization and cellular degeneration (arrows) were identified in the cortex and medulla of kidneys from LPS-injected mice. In the medulla, numerous proximal tubules stained basophilic (arrowheads) suggesting alterations in intracellular metabolic processes. Similar to control mice, kidneys of mice supplemented with mito2HOBA appeared normal. A few small areas of cellular degeneration were scattered sparsely throughout the medulla (not pictured). When mice were treated with LPS plus mito2HOBA, the cortex appeared normal while cellular injury was evident in the renal medulla. Small areas of cellular degeneration and basophilic staining were observed throughout the medulla, but the extent and degree of injury was less than that in kidneys of mice treated with LPS alone. As shown quantitatively in Fig. 5C, mito2HOBA appeared to be protective against LPS-induced cellular injury.

## 4. Discussion

In this work we show that IsoLG or its stable adduct IsoLG-PE impair mitochondrial respiration, particularly complex I-mediated respiration in the presence of malate and glutamate (Fig. 1). Our experiments in mitochondrial lysates show that IsoLG and IsoLG adducts specifically inhibited complex I activity (Fig. 2). Furthermore, we showed that a mitochondria-targeted form of a known IsoLG scavenger 2HOBA (Fig. 3) markedly protected against renal injury and animal mortality in LPS-induced sepsis model (Figs. 5 and 6).

Impaired oxidative phosphorylation significantly contribute to organ damage in sepsis [37]. Mitochondrial dysfunction in sepsis was recently linked to complex I damage, and targeted protection of complex I was proposed as a treatment for sepsis [38]. Sepsis was previously shown to increase IsoLG production [11] and IsoLG can induce the mitochondrial permeability transition [9]. We hypothesized that IsoLG might also mediate the mitochondrial dysfunction found in sepsis. Indeed, our data indicates that complex I activity is particularly sensitive to IsoLG and IsoLG adducts suggesting that sepsis-induced IsoLG likely inhibits complex I activity to promote mitochondrial dysfunction. Consequently, this pathway may be important for the multi-organ failure induced by sepsis [39].

It is well known that with NADH and the NAD-dependent substrates the rate of respiration is limited by the FAD-dependent NADH dehydrogenase of Complex I. Therefore, all downstream sites of the respiratory chain remain oxidized and produce very little reactive oxygen species [40]. Organs requiring fast ATP production use oxidation of succinate produced via transamination of pyruvate or glutamate in order to accelerate the rates of mitochondrial respiration and ATP production. We have previously shown that brain mitochondria utilize this pathway, and now we show for the first time that kidney mitochondria also adopt to high energy demands by diverting the mitochondrial metabolite flux to succinate to feed the Complex II mediated respiration. Complex II is a much simpler protein and it has higher abundance compared to Complex I [41]. Interestingly, it is seemingly much less sensitive to the damaging effects of inflammation in the LPS model (Figs. 4 and 5). Meanwhile, it must be noted that higher

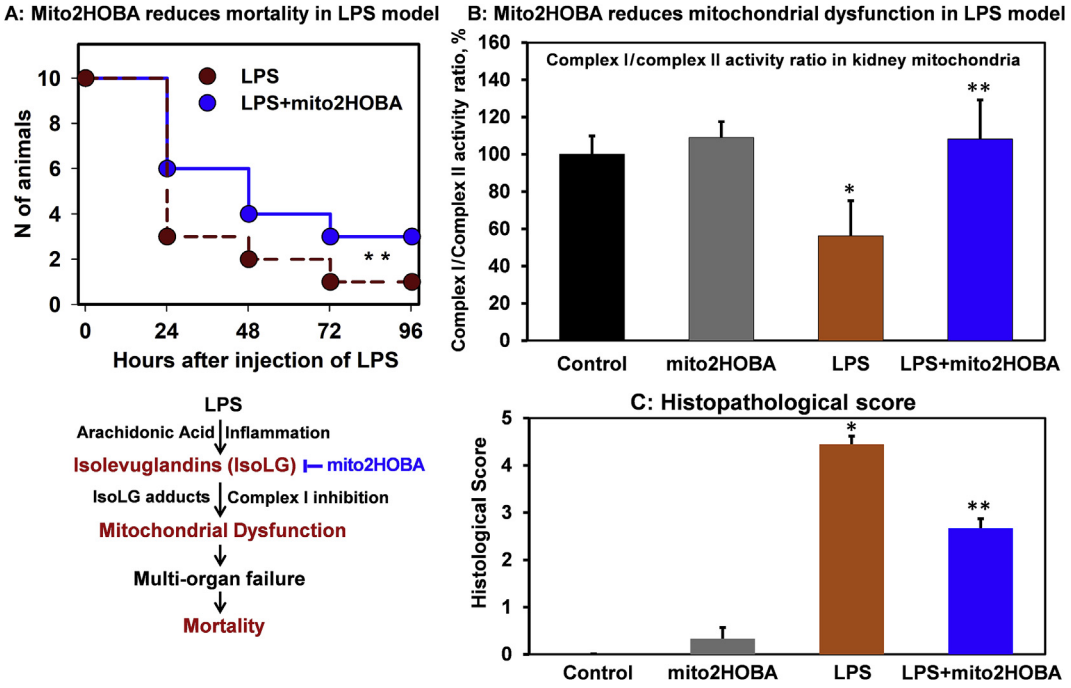


**Fig. 5.** Animal survival (A), Complex I/Complex II activity ratio (B) and (C) histopathological scores in Control, mito2HOBA-supplemented and LPS treated mice. Three months old C57BL/6J mice (25–28 g) were supplemented with mito2HOBA (drinking water, 0.1 g/L) for 72 h prior to LPS injection (25 μg/g). Complex I/Complex II activity ratio is expressed as a % compared to Control (100%). (C) Histopathological scores of renal injuries as described in methods section. Quantitative analysis of cellular injury shows that treatment with mito2HOBA leads to a significant reduction in cellular injury compared with treatment with LPS alone. Data are mean ± STD *P < 0.01 vs LPS (n = 6), *P < 0.01 vs LPS (n = 10).

respiration rate by Complex II has substantial pitfalls since it drives overproduction of mitochondrial reactive oxygen species via reverse electron transport [31,42]. The succinate mediated oxidant production contributes to brain and heart injury, and succinate-driven reverse electron transport has been proposed as a new therapeutic target [43,44]. Our data suggest that LPS induces switch from complex I to complex II respiration, however, this maladaptation can promote kidney damage and inflammation similarly to the previously reported succinate driven cardiac injury [44,45]. Analysis of specific substrate utilization showed significant decrease in complex I, II and IV and fatty acid-mediated respiration in kidney mitochondria in LPS-induced sepsis [46]. Our data suggest that mito2HOBA protects mitochondria and reduces cellular injury; however, the specific targets of mitochondrial IsoLG remain elusive. IsoLG can potentially attenuate functions of multiple mitochondrial complexes and diminish mitochondrial metabolism of fatty acids. Further studies are warranted to elucidate the specific pathophysiological role of mitochondrial IsoLG. Sepsis induces kidney inflammation, renal tubular cell injury, apoptosis and mitochondrial swelling [46,47] and treatment with mitochondria-targeted inhibitor of lipid oxidation, SS-31 [48], reduces sepsis-induced organ dysfunctions [49]. Our data support the protective effect of mito2HOBA from pathological alterations in sepsis [50].

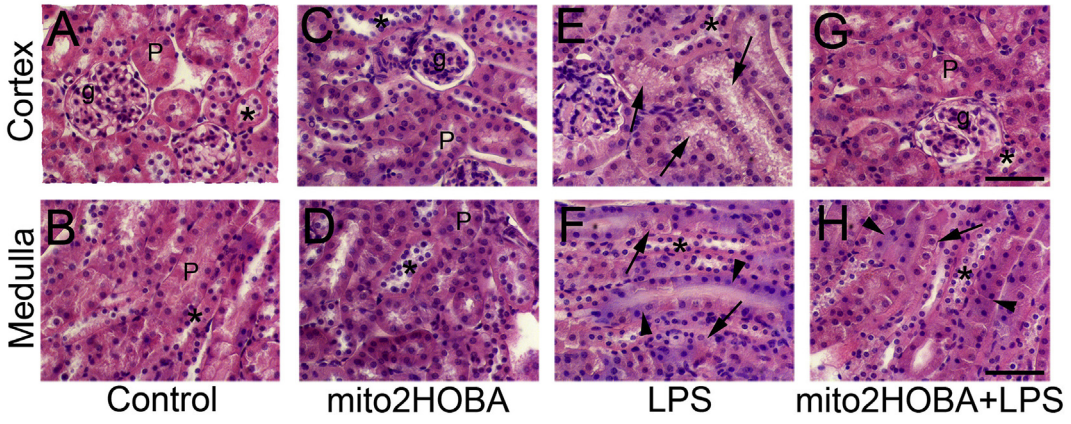


**Fig. 6.** Histological analysis of cellular injury in kidneys of control, mito2HOBA supplemented, LPS-injected and LPS + mito2HOBA-treated mice. Representative sections from control mice show normal glomeruli (g), proximal tubules (P), and distal tubules (*) in the cortex (A) and medulla (B). Sections of kidney from mice treated with mito2HOBA alone (C & D) were very similar to those from control mice. While most tubules appeared normal, a small number of proximal tubular cells in the medulla (D) showed slight evidence of cytoplasmic vacuolization. There was no evidence of injury in the cortex (C). In contrast, sections of kidney from mice treated with LPS show vacuolization and cellular degeneration (arrows) in the cortex (E) and medulla (F). In the medulla, numerous proximal tubular cells stained basophilic (arrowheads) suggesting alterations in intracellular metabolism. Distal tubules (*) appeared normal. When mice were treated with LPS and mito2HOBA, cellular injury was detected in medulla (H) while the cortex (G) appeared normal. The overall injury was greatly reduced compared with that of mice treated with LPS. In the medulla, small areas of cytoplasmic degeneration (arrows) and basophilic staining (arrowheads) were evident. Scale bar = 50 μm.

Mitochondrial dysfunction in sepsis was recently linked to complex I damage and targeting complex I was proposed in sepsis [38]. In this work we have demonstrated the potential role of IsoLGs and IsoLG adducts in inhibition of complex I and therapeutic effect of mito2HOBA on complex I function. We propose to target the mitochondrial IsoLGs to improve the mitochondrial respiration and rescue from mitochondrial dysfunction in conditions associated with inflammatory injury.

To test the potential role of IsoLG in inflammation-induced mitochondrial dysfunction, we developed the mitochondria-targeted IsoLG scavenger mito2HOBA. Driven by its lipophilic triphenylphosphonium moiety, mito2HOBA robustly accumulated in the mitochondria of multiple organs such as kidney, heart and liver. At 0.1 g/L in drinking water, mito2HOBA was well tolerated by mice. Mito2HOBA showed significant protective effects in the LPS model of sepsis. Indeed, mito2HOBA supplementation reduces animal immediate and extended mortality by 3-fold and mito2HOBA completely preserves complex I/complex II activity ratio in LPS-treated mice. Treatment with mito2HOBA also eliminated injury to tubules in the renal cortex and significantly reduced cellular degeneration and injury in medullary tubules. The rapid effect of mito2HOBA could potentially improve outcomes in the clinical settings, as it can give additional time for medical personnel to perform additional live-saving procedures for septic patients. Overall, these data support the role of mitochondrial IsoLG in mitochondrial dysfunction and therapeutic potential of mitochondria-targeted IsoLG scavenger mito2HOBA.

Our work also suggests that targeting mitochondrial IsoLG may be more effective than simply targeting mitochondrial ROS production. Kozlov and colleagues proposed that mitochondrial ROS accelerates inflammatory response and promotes the end-organ-damage, so targeting of mitochondrial ROS would be an effective treatment for inflammation [24]. Indeed, treatment of LPS-treated rats with the mitochondria-targeted antioxidants mitoTEMPO and SkQ1 reduced the expression of inducible nitric oxide synthase and diminished markers of organ damage [24]. However, these mitochondria-targeted antioxidants also increased the markers of organ damage at earlier time points suggesting the potential interference of the antioxidants with cell signaling needed to activate protective responses. Furthermore, recent studies of cecal ligation and puncture sepsis model also showed a lack of survival benefit of mitochondrial antioxidants [51]. Whether the apparent contradictory effects observed in these previous studies can be attributed to the specific model of sepsis, the animal species used, or the magnitude of the sepsis insult is unclear. Genetic ablation of NADPH oxidase in p47phox-deficient mice, a major non-mitochondrial source of ROS, exaggerated LPS-induced NF-κB activation, increased expression of proinflammatory cytokines in lungs, increased neutrophilic alveolitis and sustained greater lung injury compared with wild-type mice [15]. Of note, 2HOBA derivatives do not scavenge ROS such as superoxide and peroxynitrite [8], and, therefore, mito2HOBA will not interfere with cell redox signaling as many antioxidants described previously [51]. These data suggest potentially diverse roles of ROS in sepsis and the importance of targeting of specific cells and subcellular compartments such as mitochondria.

In this work we have tested the potential role of IsoLGs in mitochondrial dysfunction and mortality associated with sepsis using new mitochondria-targeted IsoLG scavenger mito2HOBA (see graphical Abstract and Fig. 5 scheme). We suggest that IsoLG can be produced by multiple enzymatic and non-enzymatic pathways [5,11], and that scavenging of mitochondrial IsoLG can specifically attenuate mitochondrial dysfunction and cell injury associated with inflammation. Interestingly, mitochondria-targeted antioxidants MitoQ and MitoE attenuate mitochondrial lipid peroxidation, reduce interleukin-6, improve mitochondrial function and diminish the markers of organ dysfunction in rat model of LPS-induced sepsis [52] which is in line with pathophysiological role of IsoLG produced by lipid peroxidation in isoprostane pathway [5]. Our work suggests the potential therapeutic benefit of specifically targeting mitochondrial IsoLGs.

## Disclosures

None.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2019.101300.

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