

6-2018

Effect of Putative MitokATP Channel Blocker on Lipopolysaccharide-induced Effects in Human Umbilical Vein Endothelial Cells

Indranie Ramsaroop

Philadelphia College of Osteopathic Medicine

Follow this and additional works at: <https://digitalcommons.pcom.edu/biomed>



Part of the [Medicine and Health Sciences Commons](#)

Recommended Citation

Ramsaroop, Indranie, "Effect of Putative MitokATP Channel Blocker on Lipopolysaccharide-induced Effects in Human Umbilical Vein Endothelial Cells" (2018). *PCOM Biomedical Studies Student Scholarship*. 145.

<https://digitalcommons.pcom.edu/biomed/145>

This Thesis is brought to you for free and open access by the Student Dissertations, Theses and Papers at DigitalCommons@PCOM. It has been accepted for inclusion in PCOM Biomedical Studies Student Scholarship by an authorized administrator of DigitalCommons@PCOM. For more information, please contact library@pcom.edu.

**EFFECT OF PUTATIVE MITO_KATP CHANNEL BLOCKER ON
LIPOPOLYSACCHARIDE-INDUCED EFFECTS IN HUMAN UMBILICAL VEIN
ENDOTHELIAL CELLS**

Indranie Ramsaroop

A Thesis Presented to Philadelphia College of Osteopathic Medicine – Georgia

In Partial Fulfillment

For the Degree of

Master of Science

In

Biomedical Sciences

Philadelphia College of Osteopathic Medicine – Georgia, June 2018

This thesis has been presented to and accepted by the Associate Dean for Curriculum and Research Office of Philadelphia College of Osteopathic Medicine – Georgia in partial fulfillment of the requirement for the degree of Master of Science in Biomedical Sciences.

We the undersigned duly appointed committee have read and examined this manuscript and certify that it is adequate in scope and quality as a thesis for this master's degree. We approve the content of the thesis to be submitted for processing and acceptance.

Avadhesh C. Sharma, Pharm. D., Ph.D.
Thesis Advisor
Chair and Professor
Department of Pharmaceutical Sciences

Date of Signature

Rangaiah Shashidharamurthy, Ph.D.
Co-Advisor
Associate Professor
Department of Pharmaceutical Sciences

Date of Signature

Srujana Rayalam, Ph.D.
Assistant Professor
Department of Pharmaceutical Sciences

Date of Signature

Richard E. White, Ph.D., FAHA
Professor of Neuroscience, Physiology and Pharmacology
Associate Director, Biomedical Sciences Program
Department of Basic Science

Date of Signature

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude towards Dr. Avadhesh Sharma and Dr. Rangaiah Shashidharamurthy for their mentorship throughout this study. In addition, I would like to thank my thesis committee members Dr. Richard White and Dr. Srujana Rayalam for their support and expertise on this project.

I would also like to thank the GA-PCOM Department of Biomedical Sciences and Department of Pharmaceutical Sciences for the opportunity and resources to conduct this study.

ABSTRACT

Heart failure and high mortality rates are associated with severe sepsis. Recently, focus has been on mitochondrial ultrastructural changes and function due to sepsis and sepsis-related pathologies. Chopra et al. (2011) has provided data showing the involvement of the mitochondrial-mediated intrinsic apoptosis cascade in regulating adult rat ventricular myocyte (ARVM) dysfunction in sepsis. Experimental endotoxemia has shown mitochondrial membrane potential collapse, membrane permeability changes and release of cytochrome C to be indicators of mitochondrial dysfunction. Outer mitochondrial membrane (OMM) permeability is controlled by Bax translocation, resulting Voltage Dependent Anion Channels (VDACs) pores in the OMM and activation of mitoK_{ATP} channels in the inner mitochondrial membrane. Endotoxins can induce mitochondrial damage and there is a correlation between the severity of sepsis and mitochondrial damage and dysfunction. Since the progression of sepsis occurs from vascular dysfunction to organ dysfunction, in the current study we examined mitochondrial dysfunction in endotoxic HUVEC cells. This study has provided evidence for 5HD, a putative mitoK_{ATP} blocker, in the preservation of $\Delta\Psi_m$ in endotoxic HUVEC. Additionally, 5HD appears to prevent Apaf-1 expression and reduce H₂O₂ generation in endotoxic HUVEC.

TABLE OF CONTENTS

Acknowledgements	i
Abstract	ii
List of Figures	iv
Abbreviations	v
Introduction	
Mitochondria as an Energy Source for Cells	1
Mitochondria: Significance in Pathogenic Disorders	2
Apoptosis and the Role of Mitochondria	6
Oxygen Free Radicals and Apoptosis.....	11
NO and Apoptosis.....	15
Endotoxemia or Sepsis and Apoptosis	16
MitoK _{ATP} Channels and Apoptosis	18
Aims and Objectives	22
Materials and Methods	
Chemical Preparations.....	24
Cell Culture	25
Treatment	25
Determination of Cell Viability	26
Determination of Mitochondrial Membrane Potential	26
Immunoblot Analysis.....	27
Assays	29
Results	
Effect of LPS (50, 100 and 1000 ng/mL) on HUVEC viability.....	31
Effect of 5HD on HUVEC viability	31
Effect of 5HD on LPS-induced effect on nitrite levels	34
The effect of 5HD in LPS-induced effect on cytosolic Bax, Cytochrome C and Apaf-1 proteins	36
Effect of 5HD on LPS-induced mitochondrial membrane potential alterations in HUVEC.....	40
The effect of 5HD on LPS-induced increase in cytosolic catalase	42
Discussion	43
Conclusions	47
References	48

LIST OF FIGURES

Figure 1	Dose-dependent effect of LPS (50, 100 and 1000 ng/mL) on human umbilical vein endothelial cell (HUVEC) viability at 16 hours.	29
Figure 2	Dose-dependent effect of LPS (50, 100 and 1000 ng/mL) on human umbilical vein endothelial cell (HUVEC) viability at 24 hours.	30
Figure 3	Dose-dependent effect of 5-hydroxydecanoic acid (5HD) on human umbilical vein endothelial cell (HUVEC) viability at 24 hours.	30
Figure 4	Effects of LPS, 5HD and LPS + 5HD on nitrite release in HUVEC.	31
Figure 5	Effect of 5HD on the LPS-induced response on Bax.	32
Figure 6	Effect of 5HD on the LPS-induced response on Cytochrome C.	33
Figure 7	Effect of 5HD on the LPS-induced response on protein levels of Apaf1.	34
Figure 8	Effect of LPS, 5HD and LPS + 5HD on mitochondrial membrane potential ($\Delta\Psi_m$) in HUVEC.	35
Figure 9	Effect of LPS, 5HD and LPS + 5HD on mitochondrial membrane potential ($\Delta\Psi_m$) in HUVEC.	36
Figure 10	Effect of 5HD on LPS-induced increase in Catalase.	37

ABBREVIATIONS

$\Delta\Psi_M$	Mitochondrial membrane potential
4-HNE	4-hydroxynonenal
5HD	5-hydroxydecanoic acid
ALDH2	Aldehyde dehydrogenase 2
Apaf-1	Apoptotic protease activating factor-1
ANT	Adenine nucleotide translocator
ARVM	Adult rat ventricular myocyte
Bak	Bcl-2 homologous antagonist killer
Bax	BCL2-Associated X Protein
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
CLP	Cecal ligation and puncture
dATP	Deoxyadenosine triphosphate
ETC	Electron transport chain
FADH ₂	Flavin adenine dinucleotide
GLUT1, 4	Glucose transporter 1, 4
H ₂ O ₂	Hydrogen peroxide
HIF-1 α	Hypoxia-inducible factor-1 α
HUVEC	Human Umbilical Vein Endothelial Cell
IFN- γ	Interferon-gamma
IL-6	Interleukin-6
LPS	Lipopolysaccharide
MDA	Malondialdehyde
MitoK _{ATP}	Mitochondrial ATP-dependent potassium channel
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Norepinephrine
NO	Nitric oxide
(e/i/n) NOS	(endothelial/inducible/neuronal) Nitric oxide synthase
OMM	Outer mitochondrial membrane
ROS	Reactive oxygen species
SOD1, 2	Superoxide dismutase1, 2
SUR	Sulfonylurea receptor
TLR4	Toll-like receptor 4
TNF- α	Tumor necrosis factor alpha
UCP1	Uncoupling protein 1
VDAC	Voltage-dependent anion channel

INTRODUCTION

Mitochondria: An Energy Source for Cells

Mitochondria serve as a significant source of energy for cells due to the considerable amounts of ATP produced during aerobic respiration. Mitochondria facilitate the use of oxygen by cells to generate large quantities of ATP compared to lower amounts under anaerobic conditions. Energy sources such as carbohydrates, lipids and proteins are broken down through the process of glycolysis which produces two molecules of ATP per one event of glycolysis under anaerobic conditions and lactic acid accumulation (5). However, under aerobic states, cells further oxidize these energy sources through the citric acid cycle to generate electron carriers such as NADPH and FADH₂. Mitochondria enable the use of these electron carriers to produce over thirty ATP molecules through the process of oxidative phosphorylation.

An electron transport chain (ETC) also exists along the inner mitochondrial membrane (IMM). The ETC serves to generate ATP through electron donation from electron carriers NADH and FADH₂ and formation of a proton gradient across the IMM (34). Four protein complexes form the ETC to shuttle electrons donated by NADH and FADH₂, three of which also transport protons across the IMM into the intermembrane space: complexes I, III and IV (34), (44). Transport of electrons from NADH and FADH₂ to ubiquinone, or coenzyme Q, is accomplished by complex I (NADH/ubiquinone oxidoreductase) and complex II,

respectively. Electrons are then transported from ubiquinone to complex III and from complex III to IV by cytochrome C to ultimately reduce molecular oxygen to water. Protons are simultaneously transferred with electron transport from the mitochondrial matrix to the intermembrane space by complex I, III and IV. It is the influx of protons from the intermembrane space into the mitochondrial matrix through ATP synthase which provides the energy for ATP production by mitochondria. This ATP can either remain in the mitochondrial matrix or be transported into the intermembrane space by an adenine nucleotide translocator (ANT) and into the cytoplasm by a voltage-dependent anion channel (VDAC) located in the outer mitochondrial membrane (OMM) (44),(5).

Mitochondria: Significance in Pathogenic Disorders

Mitochondrial dysfunction contributes to several physiological disorders due to the essential role in energy supply. Normal mitochondrial activity produces harmful compounds and the balance between generation and neutralization of these compounds is fundamental. Mechanisms exist in mitochondria to maintain stability in the presence of various by-products; however, distinct pathologies can have deleterious effects on these mechanisms. However, mitochondria also have many other functions in addition to energy production. For example, specific mitochondrial enzymes exist to act as defense mechanisms which are also susceptible to disruption. Defects in mitochondrial function and associated

pathologies can cause oxidative stress and metabolic disorders in addition to a diminished supply of ATP.

Mitochondria and Diabetic Cardiomyopathy

Diabetic cardiomyopathy can be characterized by left ventricular dysfunction and hypertrophy in diabetic individuals (69). Cardiac contractility diminishes due to the influence of pathological morphological and functional changes which occur in the heart in diabetic cardiomyopathy. Zhang *et al.* have provided evidence of mitochondrial ultrastructural alterations in mitochondria isolated from cardiac tissue of a murine model of type 1 diabetes, as well as demonstrated disrupted mitochondrial membrane potential ($\Delta\Psi_M$) in cardiomyocytes exposed to high levels of glucose (71). Moreover, it is mitochondria-derived ATP through oxidative phosphorylation which supplies the majority of the energy required for cardiac function (22). Up to 70% of the total ATP received is used for contraction of cardiac myocytes alone (22). The bioenergetics of cardiac physiology requires a substantial and consistent supply of ATP which is limited in diabetic individuals. Insulin is responsible for the insertion of glucose transporters, GLUT1 and GLUT4, into cardiac cell membranes. Expression of GLUT4 is known to be reduced in individuals with diabetes (69). Hyperglycemia persists and ATP production is decreased due to the lack of glucose uptake by cardiac cells in these individuals (69). The fact that

fatty acids remain the primary source of ATP generation by cardiac myocytes also allows for mitochondrial dysfunction to be recognized in diabetic cardiomyopathy as an abnormal buildup of lipids is characteristic of this condition (22), (25). Evidently, mitochondria remain a crucial organelle for normal cardiac function taking into consideration the energetic demands of this organ. Pathological conditions which lead to mitochondrial dysfunction and alterations in morphology can alter the normal heart function.

Reactive Oxygen Species and Related Pathologies

For mitochondria to meet energetic demands, a steady supply of oxygen is necessary to accept electrons at the termination of the electron transport chain. Mitochondrial oxygen consumption under non-pathological conditions results in the production of reactive oxygen species (ROS) which, if neutralized, remain unproblematic. However, mitochondrial dysfunction and subsequent ROS generation have been linked to obesity and insulin resistance (6), (44). Uncoupling protein 1 (UCP1) uncouples ATP synthesis from the proton gradient generated during oxidative phosphorylation to facilitate the clearance of ROS (6). UCP1 is located in the inner mitochondrial membrane and mitochondrial membrane disruption may eliminate its activity in ROS disposal. Moreover, the balance between generation and removal of ROS through mitochondrial uncoupling can be disturbed in the event of excessive nutrient consumption and

overabundant substrate availability for ATP generation through mitochondria (6), (44). Consequently, oxidative damage and cell death can occur (6). Excessive nutrient availability for prolonged periods of time can also lead to insulin resistance (6), (44). The essential activities of mitochondria are also accompanied by the generation of destructive by-products; however, mitochondrial mechanisms exist to control the damaging effects. Chronic pathological conditions may abolish these mechanisms and lead to cellular damage and death.

Aldehyde Dehydrogenase 2 Deficiency

Aldehyde dehydrogenase 2 (ALDH2) is a mitochondrial enzyme which exists in the matrix and acts during the rate-limiting step of ethanol metabolism to convert acetaldehyde to acetic acid (11). Due to the hydrophilic nature of acetaldehyde, accumulation of this compound from defects in ALDH2 causes adverse reactions in the cell following passage through the lipid bilayers (11), (19), (2). Consequently, ALDH2 participates in defense against damage from oxidative stress. For example, ALDH2 has a crucial function in neutralizing the highly reactive aldehyde products of lipid peroxidation, 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) (11), (19), (2). Deficiency in mitochondrial aldehyde dehydrogenase has also been linked to several pathologies. Neuron cell death and reduced cognitive ability have been presented as the results of reactive aldehyde accumulation in the brain (36). Particularly, 4-HNE has been implicated in Alzheimer's disease (36). It has been suggested that ALDH2, which

acts to convert the reactive products of lipid peroxidation to less injurious compounds, plays a key role in neuroprotection (19). Indeed, upregulation of ALDH2 may be considered a marker of improved cognitive function in animal models of Alzheimer's disease (36). Further, D'Souza *et al.* have shown cognitive deterioration in an age-dependent manner in a murine ALDH2 knockout model (19). ALDH2 is also present in greater quantities in highly metabolic organs which require plenty of oxidative phosphorylation, such as the heart and brain indicating the need for protection from oxidative damage (11). Clearly, proper mitochondrial function is necessary beyond the energetic demands for ATP generation. Enzymes contained in these organelles provide a vital defensive function as evidence shows that deleterious effects result from deficits in mitochondrial aldehyde dehydrogenase activity.

Apoptosis and the Role of Mitochondria

Apoptosis is a form of cellular death which follows a specific and genetically controlled procedure. The systematic activation of various proteins results in destruction of the cell which can be recognized by characteristic changes in cell morphology as well as protein expression. Two forms of apoptosis exist: the intrinsic and extrinsic pathways. The extrinsic path of apoptosis requires the activation of death receptors at the surface of cells (23).

Mitochondria serve as the conduit through which the stress-induced intrinsic pathway of apoptosis is carried out. Bak and Bax, pro-apoptotic members of the Bcl-2 protein family, are inserted into the mitochondrial membrane to form the mitochondrial permeability transition pore (64). Due to this disruption in the mitochondrial membrane, cytochrome C is subsequently released from the intermembrane space into the cytoplasm. Oligomerization of typically monomeric apoptotic protease activating factor-1 (Apaf-1) occurs following hydrolysis of dATP to dADP (54). Apaf-1 functions to bind released cytochrome C in the cytoplasm to form the apoptosome. This structure, composed of seven Apaf-1 units and cytochrome C function to cleave procaspase-9 into its active form, caspase-9. Activated caspase-9 proceeds to activate caspase-3 through cleavage from the inactive procaspase-3 to result in destruction of the cell (54). Degradation of the cell involves various processes such as DNA fragmentation, shrinkage of the cell and protrusion of the plasma membrane. These morphological changes are characteristic indicators of a cell undergoing apoptosis through the mitochondrial-mediated pathway.

Mitochondrial Outer Membrane Permeabilization

Bax and Bak are Bcl-2 (B-cell lymphoma protein-2) family members and form pores in the mitochondrial outer membrane in response to an apoptotic stimulus through the intrinsic pathway (21), (72), (38). Homo-oligomerization occurs once

Bax and Bak are activated and the BH3 (Bcl-2 homology) domain of one molecule will interact with the hydrophobic groove of another activated molecule (21). Bax, in monomeric form, primarily remains in the cytosol prior to the apoptotic signal after which Bax translocates to the outer mitochondrial membrane (OMM) to insert its transmembrane domain (21), (72). This contrasts with Bak which perpetually remains anchored to the OMM. Bax and Bak regulation mechanisms are unclear, however it is believed that these proteins are detained in the cytosol by pro-survival proteins prior to their activation by an apoptotic stimulus (21).

Voltage-dependent anion channels (VDACs) are considered the most predominant outer mitochondrial membrane proteins responsible for ion and metabolite flux ranging from 1.5-5 kDa in size (3), (43). Adenine nucleotide translocase (ANT), a prevalent inner mitochondrial membrane protein, functions in ADP import into the matrix and ATP export eventually to the cytosol (40). The OMM pore has been previously thought to include VDACs and ANT (21), (3). Evidence is lacking and inconsistent for a necessary role of VDACs in mitochondrial permeability pore formation and function during apoptosis (3), (43). Although, a regulatory role for ANT has been implicated with proposed pro- and anti-apoptotic isoforms (3), (40), (8). Ultimately, the pores created in the mitochondrial membrane are primarily established by Bcl-2 proteins and function to release cytochrome C into the cytosol from the intermembrane space.

Cytochrome C and the Apoptosome

Cytochrome C in healthy cells is a key component of the electron transport chain and participates in substantial amounts of ATP generation. Yet, the resulting mitochondrial dysfunction, apoptosome formation and caspase cascade activation from cytochrome C release commits the cell to death (21), (72). Cardiolipin (CL), an inner mitochondrial membrane (IMM) lipid, is known to anchor cytochrome C to the IMM (38), (65). Accordingly, CL oxidation is thought to lead to the release of cytochrome C from the IMM so that it may be transported from the intermembrane space into the cytosol (38), (65), (73). Further, data from Zhong *et al.* proposes that modulation of CL oxidation is a mechanism used by cancer cells to evade apoptosis (73). Experimental evidence by Zhang *et al.* also suggests that cytochrome C release into the cytosol requires contact with Bax, interactions with the pore residues and can influence the pore structure to facilitate release (72). Evidently, this systematic process of cell death is controlled at multiple levels.

Constitutive, but low levels of Apaf-1 are present in cells, however, expression can be increased by transcription factors E2F transcription factor 1 (E2F1) and tumor-suppressor protein (p53) (54), (33). E2F1 phosphorylation, stabilization, subsequent activation of APAF1 genes and apoptosis are known to occur following DNA damage or serum starvation (33). The nucleotide-binding oligomerization domain (NOD) of Apaf-1 facilitates binding between multiple units of Apaf-1 to form the heptameric apoptosome structure (54), (65). The WD40

(tryptophan-aspartate) repeats domain (WRD) possesses a regulatory function as the binding of cytochrome C to this domain causes a conformational change that allows procaspase-9 attachment (54), (65). Multiple Apaf-1 isoforms exist ranging from 133-142 kDa with caspase recruitment domains (CARDs) located at their N-terminus responsible for binding procaspase-9 (54). It is known that dATP/ATP is necessary for Apaf-1 oligomerization, however, cytochrome C binding is not dependent on the presence of dATP/ATP (54), (65). The apoptosome provides the scaffold which permits the cleavage of procaspase-9 to active caspase-9 (54), (65).

The Caspase Cascade and Cell Death

Caspases are cysteine-aspartic acid proteases which mainly cleave at the C-terminal end of aspartate residues (30). In particular, caspase-3 and caspase-7 are further classified as apoptotic effector caspases (30). Procaspases are the inactive zymogen forms of caspases which are present in cells prior to cleavage to active forms (30). Certain procaspases are expressed as monomers and later form dimers after activation, or as dimers which require cleavage within the linker region, nevertheless, active caspases ultimately function as dimers (30).

Apoptosis due to caspases is not limited to the intrinsic pathway as the extrinsic pathway of cell death is also carried out by caspase-8, caspase-3 and caspase-7

(30). Further, caspases can cause cell death through mechanisms other than apoptosis. For example, the binding of LPS to inflammatory caspase-4 and caspase-11, in response to bacterial infections, can result in activation, oligomerization and inflammatory cell death (30).

Once bound to the apoptosome, procaspase-9 cleaves itself in order to activate procaspase-3 and procaspase-7 (54), (9). It has been proposed that up to four molecules of procaspase-9 attach to the apoptosome (54). Bretnall *et al.* have proposed distinct roles for caspase-9, caspase-3 and caspase-7 in the intrinsic apoptosis pathway (9). The data implies additional functions for caspase-9 such as mitochondrial remodeling (9). Also, caspase-3 may inhibit the production of reactive oxygen species as well as being the primary effector caspase and caspase-7 may be responsible for cleavage of ECM proteins and cell detachment (9). Nevertheless, the resulting effects of the caspase cascade on the cell include shrinkage of the cell, DNA fragmentation, membrane blebbing and exposure of phosphatidylserines to the extracellular environment (30), (9). This controlled process of cell death ensures un-injurious degradation and removal of damaged or unwanted cells.

Oxygen Free Radicals and Apoptosis

Oxygen free radicals exist as types of highly reactive oxygen-containing molecules due to the possession of an unpaired electron. Similarly, reactive

oxygen species (ROS) exist as a class of highly reactive oxygen-containing compounds with roles in multiple cellular functions including cell death and can include superoxide (O_2^-) and hydrogen peroxide (H_2O_2) (47). Routine cellular activities, such as aerobic respiration, can result in the production of ROS.

Further, nitric oxide synthase (NOS) can contribute as an endogenous source of ROS under conditions of reduced availability of tetrahydrobiopterin or L-arginine and ultimately generates OH^\bullet and NO_2^\bullet (47), (4). Hypoxic conditions additionally serve as a cause of mitochondrial production of ROS (47). ROS maintain the ability to both stimulate and inhibit cell death (47), (4).

Aerobic Respiration and ROS Production

Mitochondria house an electron transport chain (ETC) which coincides with its purpose of serving as the major source of energy for cells. Remarkably, it is the ETC which also provides the major source of ROS in mammalian cells (4), (51). Electron "leak" in pairs from electron carriers forms H_2O_2 and a single electron leak forms superoxide (60). Complex III (cytochrome C oxidoreductase) and complex IV (cytochrome C oxidase) are the sites of superoxide production in the ETC (51), (60). Although mitochondrial respiration is the major source of ROS, the amount produced is not sufficient to cause devastating effects on the cell in non-disease states.

Hypoxia and ROS Production via Mitochondria

Mitochondrial production of ROS functions in normal cell signaling when generated at low concentrations (27). However, a deficiency in oxygen available to cells is suggested to result in mitochondrial ROS production by complexes I and III (27), (59), (74). Superoxide appears to be the primary ROS generated by complex III under hypoxic conditions and ROS from complex III are thought to activate hypoxia-inducible factor-1 α (HIF-1 α) (27), (74). HIF-1 plays a protective role for cells experiencing hypoxic conditions and is known to execute a number of functions in response to low oxygen levels in attempts to maintain cell survival (10). However, Shvetsova *et al.* have provided evidence which shows that HIF-1 α activation is dependent on the proper functioning of mitochondria (74).

ROS and Apoptosis

Production of ROS, if not equally countered by their catabolism, can result in accumulation which leads to oxidative stress (47). This excessive quantity of ROS can react with biomolecules to cause cellular and DNA damage resulting in cell death. Evidence exists to support the role of hydrogen peroxide (H₂O₂) contributing to oxidative stress and ultimately signaling apoptosis through the intrinsic pathway (51), (39). Liu *et al.* have shown that H₂O₂ can induce cellular morphological defects, oxidative stress, increased levels of Bax, disruption of $\Delta\Psi_m$ and apoptosis (39). Additionally, Bcl-2 is known to be involved in inhibition

of cell death through apoptosis (7). Kern *et al.* have provided evidence for the role of Bcl-2 in prevention of endothelial superoxide (O_2^-) production in a murine model of Bcl-2 overexpression (32). Further, support exists for a reduction in apoptosis in a model of mitochondrial superoxide dismutase (SOD2) expression by adenovirus transfection (7). Together, the present evidence suggests that oxidative stress, attributable to H_2O_2 and O_2^- , bring about apoptosis through the mitochondrial-mediated pathway.

Mechanisms do exist to regulate the production of ROS. For example, the availability of necessary cofactors, receptor activation and cell signaling cascades which result in ROS production can be controlled (47). Furthermore, enzymes exist to neutralize harmful oxygen free radicals. Superoxide dismutase (SOD) functions to convert O_2^- into H_2O_2 which can be further broken down into water and oxygen by the enzyme catalase. Mn-dependent SOD (SOD2) exists in the mitochondrial matrix and functions to protect mitochondria from the effects of O_2^- . However, few researchers have observed SOD2 decrease in response to oxidative stress induced by H_2O_2 (39).

Nitric Oxide (NO) and Apoptosis

Infection and the Immune Response

Invasion by foreign microbes or toxins elicit standard responses by the immune system which are categorized as innate and adaptive. Adaptive immunity includes specific T-cell and B-cell responses which require more time to occur and last for longer periods compared to innate immune responses (48). Innate immunity involves an initial swift response to invading pathogens and toxins due to recognition of foreign molecular structures (48). Release of inflammatory mediators is a well-established innate immune reaction (48), (50). Macrophages, derived from monocyte differentiation, function in the innate immune response to eliminate invading microbes through nitric oxide (NO) release and oxidative damage (48). Apoptosis can occur because of infection by many bacterial species despite efforts by the immune system to control and eliminate pathogens (50). Bacterial toxins, such as lipopolysaccharide (LPS) derived from gram negative species, are also known elicit inflammation due to its recognition by Toll-like receptor 4 (TLR4) (12). Pro-inflammatory factors, such as tumor necrosis factor alpha (TNF- α), interferon-gamma (IFN- γ), interleukin-6 (IL-6) and LPS can lead to the generation of NO by inducible nitric oxide synthase (iNOS) (61). Macrophage release of NO in response to LPS has been well-characterized as well as the cytotoxicity of high exogenous NO levels (46), (62).

Role of NO in the induction of apoptosis

There exist three enzymatic sources of NO production by nitric oxide synthases including neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) (61), (62). NO production by nNOS and eNOS from L-arginine remains a physiological necessity, however, iNOS production of NO is known to occur under inflammatory conditions (61), (62). In addition to its role in maintaining normal physiological functions, NO is involved in inducing mitochondria-mediated cell death (13). Snyder *et al.* has provided experimental data implicating that NO induces the formation and subsequent insertion of Bax/Bak oligomers into the outer mitochondrial membrane to result in cytochrome C release and apoptosis (61).

Endotoxemia or Sepsis and Apoptosis

Sepsis and Apoptosis

Sepsis occurs as the result of a widespread inflammatory reaction to infection. This condition consists of two stages: hyperdynamic and hypodynamic, also referred to as the warm and cold stages of sepsis, respectively. The initial hyperdynamic stage involves warm and perfused extremities due to high cardiac output and low peripheral resistance of blood vessels. However, the cardiovascular system is unable to maintain this perfused stage which can lead to a decline to the hypodynamic stage (15), (55). When this occurs, organ

perfusion is dramatically reduced. A patient in this stage of sepsis will experience cold extremities due to the effects of inflammation on vascular permeability and subsequent lack of perfusion.

Data from Chopra *et al.* has provided evidence to show that adult rat ventricular myocytes (ARVMs) undergo apoptosis (14). The evidence implicates the involvement of the intrinsic pathway of apoptosis due to the increased levels of Bax and cytochrome C as well as morphological changes in mitochondria such as swelling and membrane deformity (46),(68). Further, progression to apoptosis in sepsis has been well-documented in the pathophysiology of sepsis (13), (67).

Endotoxemia and Apoptosis

Endotoxemia is defined as the presence of endotoxins in the blood, for example, lipopolysaccharide (LPS) derived from the outer membranes of gram-negative bacteria. Consequences of endotoxemia include metabolic endotoxemia defined as endotoxins in the blood derived from intestinal bacteria, the development of inflammation, shock, or death (37), (20). The induction of endotoxemia under experimental conditions can be utilized as a model system for studying pharmacological interventions for septic patients (31). According to Kamisoglu *et al.*, the pathophysiology of sepsis and endotoxemia share similar metabolic profiles and the metabolic distinctions between endotoxemia and sepsis increase with time (31). These findings suggest that pharmacological

treatments evaluated during experimental endotoxemia should be administered as early as possible during the onset and progression of sepsis in patients.

Present data also provides evidence for the ability of LPS to induce apoptosis in hepatocytes at 1000 ng/mL (49). In addition to cell death, LPS has been observed to produce dissipated $\Delta\Psi_m$, reduced levels of ATP and mitochondrial cytochrome C (49). Moreover, Wang *et al.* have demonstrated the capacity for LPS to induce apoptosis and caspase-3 activity in adult rat ventricular myocyte (ARVM) at concentrations as low as 10 ng/mL (66). Further, this study also demonstrated a greatly enhanced effect of LPS on disrupted $\Delta\Psi_m$, mitochondrial Bax translocation and cytochrome C release in combination with β_1 -adrenergic receptor activation in cardiomyocytes (66). Together, these experimental data support the capacity for LPS-induced endotoxemia to cause mitochondrial dysfunction and activation of the intrinsic apoptosis cascade - effects which can be augmented by β -adrenergic stimulation and affect multiple cell types. However, the degree of these effects appears to vary with LPS concentration.

MitoK_{ATP} Channels and Apoptosis

Potassium ATP (K_{ATP}) channel structure consists of 4 subunits each of Kir and SUR proteins. The aqueous pore through which potassium ions flow out of the cell is formed by the Kir subunits. The SUR subunits exhibit pharmacological functions in that they contain binding sites for various agents which affect ion flow

through the channel. Ion transport through these channels can be regulated by varying ATP and nucleotide diphosphate concentrations. During the physiological state, mitoK_{ATP} channels function to transport potassium ions into the matrix to maintain volume. Low levels of ATP can activate the flow of potassium ions through these channels and increased flow of potassium out of the cell leads to hyperpolarization of the cell membrane. In mitochondria, the opening of these channels leads to depolarization of the mitochondrial membrane (68).

Matrix Volume and Cytochrome C Release

Mitochondrial swelling due to increased matrix volume has been shown to result in cytochrome C release without the need for increased mitochondrial membrane permeability (28). Evidence also exists to suggest that mitochondrial swelling due to K⁺ influx through mitoK_{ATP} channels can rupture the OMM and permit cytochrome C release (18). Moreover, recent data has shown that valinomycin, an ionophore specific to K⁺, can induce mitochondrial swelling and release of cytochrome C (41). Consequently, blockage of mitoK_{ATP} channels by 5HD, a mitoK_{ATP} channel blocker, may reduce matrix swelling and cytochrome C release, thereby reducing the extent of cell death under the intrinsic pathway of apoptosis.

MitoK_{ATP} Channels and Mitochondrial Membrane Potential ($\Delta\Psi_m$)

Dissipation of mitochondrial membrane potential ($\Delta\Psi_m$) has been considered an early event in apoptosis and also necessary for cytochrome C release (63). Likewise, collapse of $\Delta\Psi_m$ is linked to the progression of cell death and is a conventional parameter used for detection and measurement of apoptosis (63). Reduced mitochondrial membrane potential is a known result of mitoK_{ATP} channel opening (42). Accordingly, prevention of mitoK_{ATP} channel activity may limit the degree of $\Delta\Psi_m$ disruption and reduce the magnitude of cell death.

Reactive Oxygen Species

MitoK_{ATP} channel opening has been found to lead to increases in mitochondrial generation of ROS, particularly superoxide and hydrogen peroxide (18), (42), (70). This effect has also been proposed as a mechanism of cardioprotection from prolonged ischemic damage through ROS signaling pathways - an effect which can be induced by the mitoK_{ATP} channel opener diazoxide and inhibited by 5-HD (18), (70). However, the effects of ROS signaling are concentration-dependent and oxidative stress is known to cause cell death involving the intrinsic apoptosis pathway (17). Consequently, it can be suggested that under the conditions of sepsis or endotoxemia, mitoK_{ATP} channel opening may be detrimental to the survival of patients experiencing myocardial

dysfunction. Rather, blockage of these channels may prove more beneficial to the condition as a method of preserving cardiomyocyte function.

AIMS AND OBJECTIVES

Vascular dysfunction, end-organ failure and high mortality rates are associated with endotoxemia, sepsis and septic shock. Recently, focus has been on mitochondrial ultrastructural changes and function due to sepsis and sepsis-related pathologies. Data from Sharma's laboratory indicated involvement of mitochondrial-mediated intrinsic apoptosis cascade in regulating ARVM dysfunction in sepsis. Experimental endotoxemia has shown mitochondrial membrane potential collapse, membrane permeability changes and release of cytochrome C as indicators of mitochondrial dysfunction. Outer mitochondrial membrane (OMM) permeability is controlled by Bax translocation, resulting Voltage Dependent Anion Channels (VDACs) pores in the OMM and activation of mitoK_{ATP} channels in the inner mitochondrial membrane. Endotoxins can induce mitochondrial damage and there is a correlation between the severity of sepsis and mitochondrial damage and dysfunction. The data from our laboratory indicate that 5HD treatment that blocks mitoK_{ATP} channels could increase survival in septic rats and increase contractility in septic cardiomyocytes. The progression of sepsis is known to involve vascular dysfunction. Therefore, we hypothesize that inhibition of mitoK_{ATP} channels by 5HD restores altered mitochondrial membrane permeability in human umbilical vein endothelial cells due to LPS-induced endotoxemia. To investigate this hypothesis the following specific aims are developed.

Specific Aim 1. To determine time-dependent and dose-dependent effects of LPS and 5HD on mitochondrial dysfunction in HUVECs

Prior to testing the restorative properties of 5HD (5-hydroxydecanoic acid) on mitochondrial membranes in endotoxic human umbilical vein endothelial cells (HUVEC), it is necessary to determine the time- and dose-dependent effects of 5HD and lipopolysaccharide (LPS) on HUVEC viability. Cell viability assays have been conducted using Presto blue dye to measure metabolic activity of the cells following treatment with LPS and 5HD at increasing concentrations.

Specific Aim 2. To determine if 5HD modulates LPS-induced a) mitochondrial dysfunction b) NO and catalase levels and c) proapoptotic markers such as Bax, Cytochrome C and Apaf-1 in HUVECs

The effect of 5HD on LPS-induced alterations of mitochondrial membrane potential (measure of mitochondrial membrane integrity) in HUVECs can be determined. Macrophages serve as a positive control as the ability of LPS to decrease viability of these cells has been well characterized. Mitochondrial membrane potential will be assessed using JC-1 cationic dye. NO and catalase levels were measured by colorimetric assay and levels of proapoptotic proteins were measured using immunoblot procedures.

Materials and Methods

Chemical Preparations

Chemical compounds were purchased from Sigma-Aldrich (St. Louis, MO) and prepared in sterile deionized water. Treatments were prepared in warm Medium 200 (Gibco™; Thermo Fisher Scientific, Grand Island, NY) at the required concentrations. All drugs and treatments were prepared under sterile conditions and vortexed prior to usage.

5-Hydroxydecanoic Acid (5HD)

5-hydroxydecanoic acid sodium salt (MW; 210.25 g/mol) was prepared at a 100,000 μM stock concentration. The 100,000 μM stock was further diluted to a working concentration of 1000 μM in sterile deionized water. 1000 μM concentration aliquots were prepared and stored at -80°C for future use.

Lipopolysaccharide (LPS)

Lipopolysaccharide was prepared at a 100,000 ng/mL stock concentration. The 100,000 ng/mL stock was further serially diluted to 10,000 ng/mL and then to a working concentration of 1000 ng/mL in sterile deionized water. 1000 ng/mL concentration aliquots were prepared and stored at -80°C for future use.

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were purchased from ATCC (Manassas, VA) and cultured in Medium 200 supplemented with low serum growth supplement (LSGS) (Gibco™; Thermo Fisher Scientific, Grand Island, NY) containing 10 µg/mL heparin, 1 µg/mL hydrocortisone, 10 ng/mL human epidermal growth factor, 3 ng/mL basic fibroblast growth factor, 2% FBS and 1% penicillin and streptomycin (46). Cells were incubated at 37°C, 5% CO₂ and 80% humidity. Medium was exchanged every other day and cells were passaged at 95% confluency in trypsin plus 0.25% EDTA. Cells were cultured and maintained in T175 cell culture flasks and treated at 75-80% confluency for cytotoxicity assays and 90-95% confluency for all other assays.

Treatment

Cells were passaged with trypsin plus 0.25% EDTA, counted using the trypan blue cell counting method and cell suspension was plated on cell culture plates at volumes and seeding densities based on specifications necessary for each experiment. Treatments were prepared based on cell culture plate well volume and concentration of drug required per well from 1000 µM or 1000 ng/mL working concentrations. A master mix was prepared and calculated for based on the number of plated wells to be treated and the volume of each well. All drugs were prepared in Medium 200 warmed in a 37°C water bath and under sterile conditions, vortexed and mixed during treatment preparation as well as prior to

addition to cells. HUVEC model of endotoxemia was induced by treatment of cells with LPS 100 ng/mL.

Determination of Cell Viability

HUVECs were cultured on 48-well plates and seeded at a density of 70,000 cells per well. The cells were treated with LPS for 16 and 24 hours with the following dosages: 50 ng/mL, 100 ng/mL, 500 ng/mL, 1000 ng/mL, 5000 ng/mL and LPS 100 ng/mL plus 5HD 50 μ M. Cells were treated with 5HD at the following dosages: 50 μ M, 100 μ M, 200 μ M and 500 μ M. Cells were treated at 75-80% confluency. Cells were incubated for 1 hour with PrestobluTM Cell Viability Reagent (Molecular Probes; Invitrogen, Carlsbad, CA) and absorbance was measured using Biotek Synergy HT microplate reader. Cell viability was quantified based on absorbance of metabolically active cells at 570 nM.

Determination of Mitochondrial Membrane Potential

JC-1 Fluorescent Imaging (13)

JC-1 assay kit was purchased from Molecular Probes (Life Technologies; Thermo Fisher, Grand Island, NY). Performance of the JC-1 cationic dye assay was adapted from vendor directions. HUVECs were cultured and plated on 48-well plates at 60,000 cells per well and treated at 90% confluency with the following treatment groups: LPS 100 ng/mL, 5HD 50 μ M and LPS plus 5HD.

Assays were performed at 2, 4, 6, 16 and 24 hours. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP), a mitochondrial membrane depolarizer, was added 5 minutes prior to addition of JC-1 dye at a final concentration of 50 μM as per vendor guidelines to serve as a positive control for JC-1 fluorescence activity. Treatment groups and positive controls were incubated with 5 μL of 200 μM JC-1 cationic dye for 30 minutes and washed with 10X PBS. Cells were imaged using EVOS FL Cell Imaging System (Thermo Fisher Scientific, Grand Island, NY) at 20X magnification.

Immunoblot Analysis (13)

HUVECs were seeded at a density of 2×10^6 cells per well on 6-well cell culture plates and treated with LPS 100 ng/mL, 5HD 50 μM and LPS plus 5HD for 16 hours. Cells were lysed with 100 μL RIPA buffer and incubated on ice for 5 minutes. The collected lysate remained on ice for 10 minutes prior to centrifugation at 13.3g and 4°C for 10 minutes. Protein quantification of collected lysate was performed using Pierce™ BCA Protein Assay Kit (Thermo Fisher, USA) according to vendor directions. 40 μg of protein sample was loaded into each sample well of gel. Gels were run at 150V for 1 hour prior to transfer to PVDF membrane. Membranes were incubated with primary antibody overnight at 4°C on a rocker and secondary antibody for 45 minutes at room temperature on a rocker. Primary antibody was prepared in 5 mL Odyssey® Blocking Buffer (PBS)

(Li-COR, USA), 5 μ L Tween 20 and 1 μ L mouse monoclonal β -actin antibody (Santa Cruz). All primary antibodies were prepared at a 1:1000 concentration. Membranes were imaged using Li-COR Odyssey® CLx Imaging System (Lincoln, NE) for 7 minutes exposure time per membrane. Bands were quantified using Li-COR Image Studio™ Lite (Lincoln, NE). All samples were run in triplicate (N = 3).

Apoptotic Protease Activating Factor 1 (Apaf1)

Samples were run on 10-well 7.5% SDS gels prepared according to directions adapted from Northern Arizona University Lab Protocols. Rabbit polyclonal anti-Apaf1 antibody was purchased from Abcam (Cambridge, MA). PVDF membranes were incubated with 1 μ L anti-rabbit and 1 μ L anti-mouse secondary antibodies.

Cytochrome C and Bax

Samples were run on 10-well 4-20% gels purchased from Bio-Rad (Hercules, CA). Sheep polyclonal anti-Cytochrome C and rabbit monoclonal anti-Bax antibodies were purchased from Abcam (Cambridge, MA). PVDF membranes for Bax quantification was incubated with 1 μ L anti-mouse plus 1 μ L anti-rabbit. PVDF membranes for Cytochrome C quantification were incubated with 1 μ L anti-sheep purchased from Abcam (Cambridge, MA) and 1 μ L anti-mouse secondary antibodies.

Assays

Enzyme-Linked Immunosorbent Assays

Catalase

HUVECs were seeded at a density of 2×10^6 cells per well on 6-well cell culture plates and treated with LPS 100 ng/mL, 5HD 50 μ M and LPS plus 5HD for 16 hours. Following treatment, cells were lysed and protein was extracted for catalase quantification using Abcam Human Catalase ELISA Kit (Cambridge, MA) adapted from manufacturer directions. HUVEC lysate was centrifuged at 13.3 g for 20 minutes at 4°C and the collected supernatant was immediately assayed. Samples were incubated with capture and detection antibody cocktail out of direct light and at room temperature for 1 hour and 15 minutes on a plate shaker at 150 rpm. Optical density (OD) was recorded at 450 nM using Biotek Synergy HT microplate reader. Samples were assayed as three sets for each treatment group and run in duplicates (N = 6).

Griess Reagent Assay (56)

HUVECs supernatant was collected from prior treatments with LPS 100 ng/mL, 5HD 50 μ M and LPS plus 5HD for 2, 4, 6, 16 and 24 hours and used for quantification of nitrite release using Promega Griess Reagent System (Madison, WI). Samples were run in three sets in duplicates (N = 6) on a 96-well plate according to manufacturer protocol. Samples were incubated for 10 minutes at room temperature and out of direct light with sulfanilamide. Samples were then

incubated for 10 minutes at room temperature and out of direct light with N-1-napthylethylenediamine dihydrochloride (NED). Absorbance was measured at 520 nM using Biotek Synergy HT microplate reader.

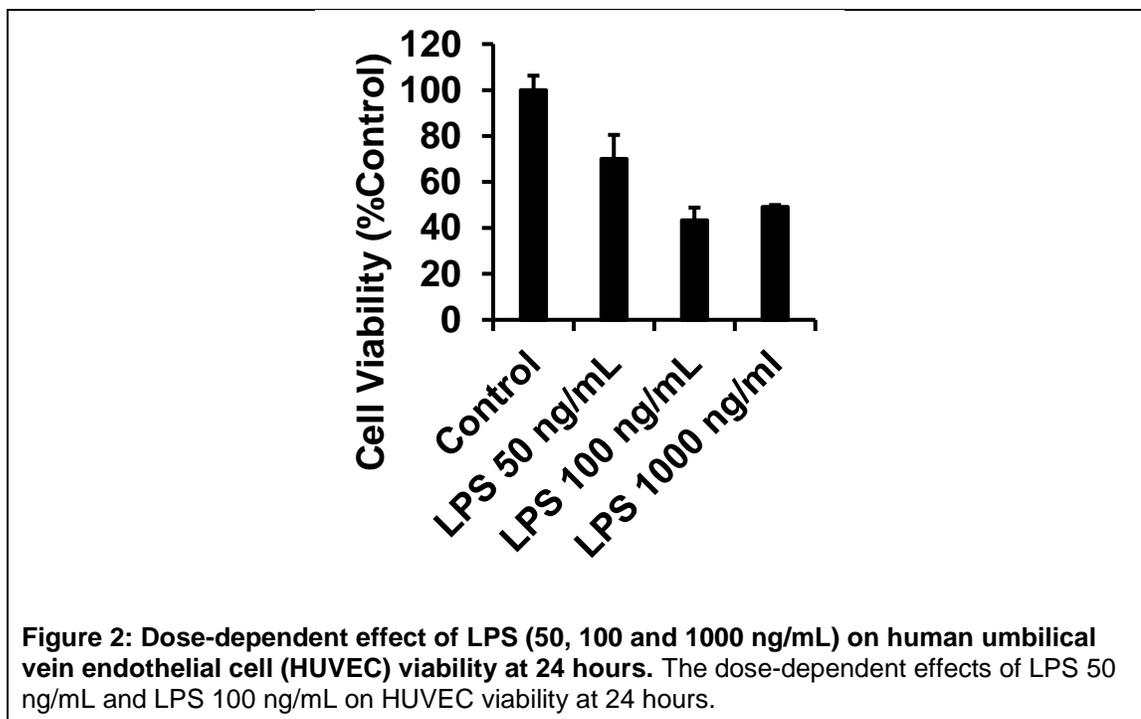
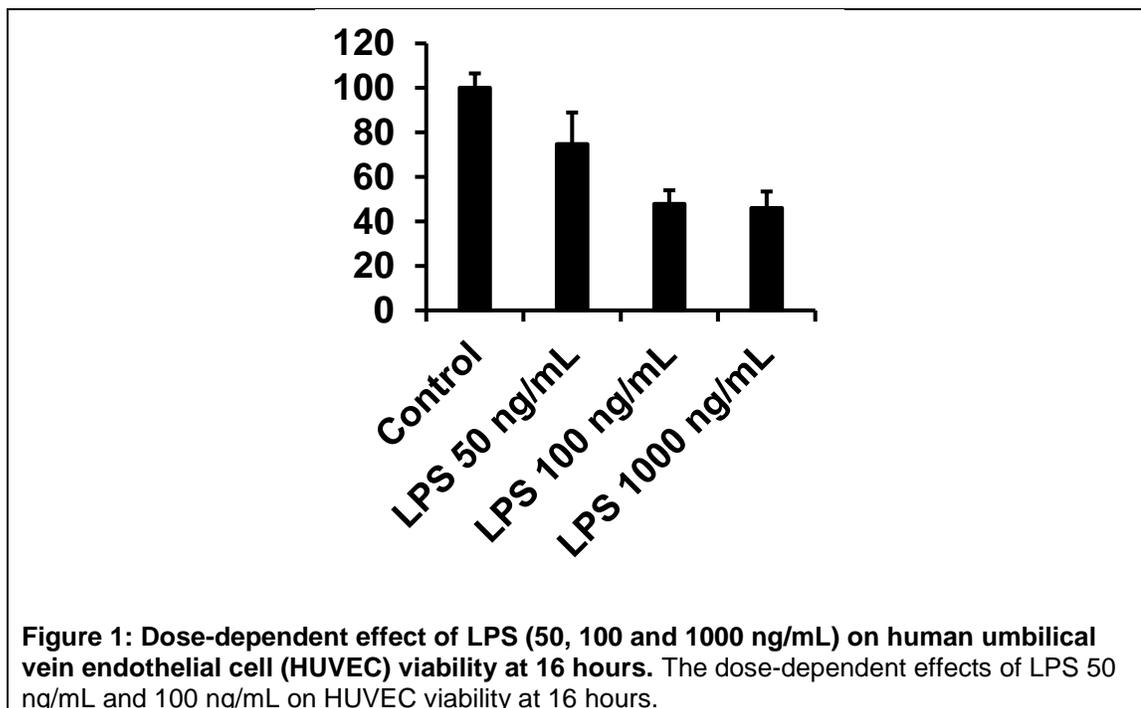
Results

Effect of LPS (50, 100 and 1000 ng/mL) on HUVEC viability

The effect of LPS at increasing concentrations, i.e., 50, 100 and 1000 ng/mL was observed in HUVEC. With increasing concentration, a decrease in HUVEC viability was observed in cells treated with LPS 50 ng/mL, 100 ng/mL and 1000 ng/mL for both 16 (Fig. 1) and 24 (Fig. 2) hours. This suggests that LPS decreases cell viability in dose-dependent and time-dependent manner.

Effect of 5HD on HUVEC viability

5HD, a putative mitoK_{ATP} blocker, produces a decrease in viability in HUVEC treated with 5HD 50 μ M, 100 μ M and 200 μ M for 24 hours. Significant reductions in HUVEC viability were observed at the aforementioned concentrations with the higher concentrations of 100 and 200 μ M producing similar significant reductions in cell viability. This data suggests that 5HD reduces HUVEC viability at increasing concentrations of the compound (Fig. 3).



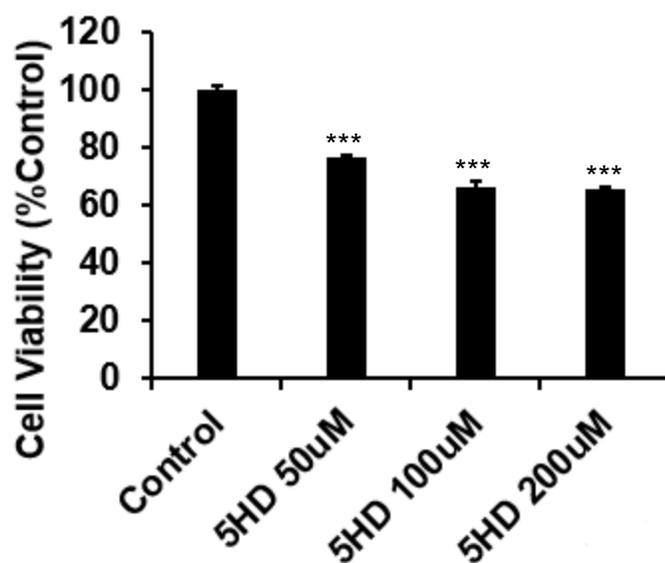
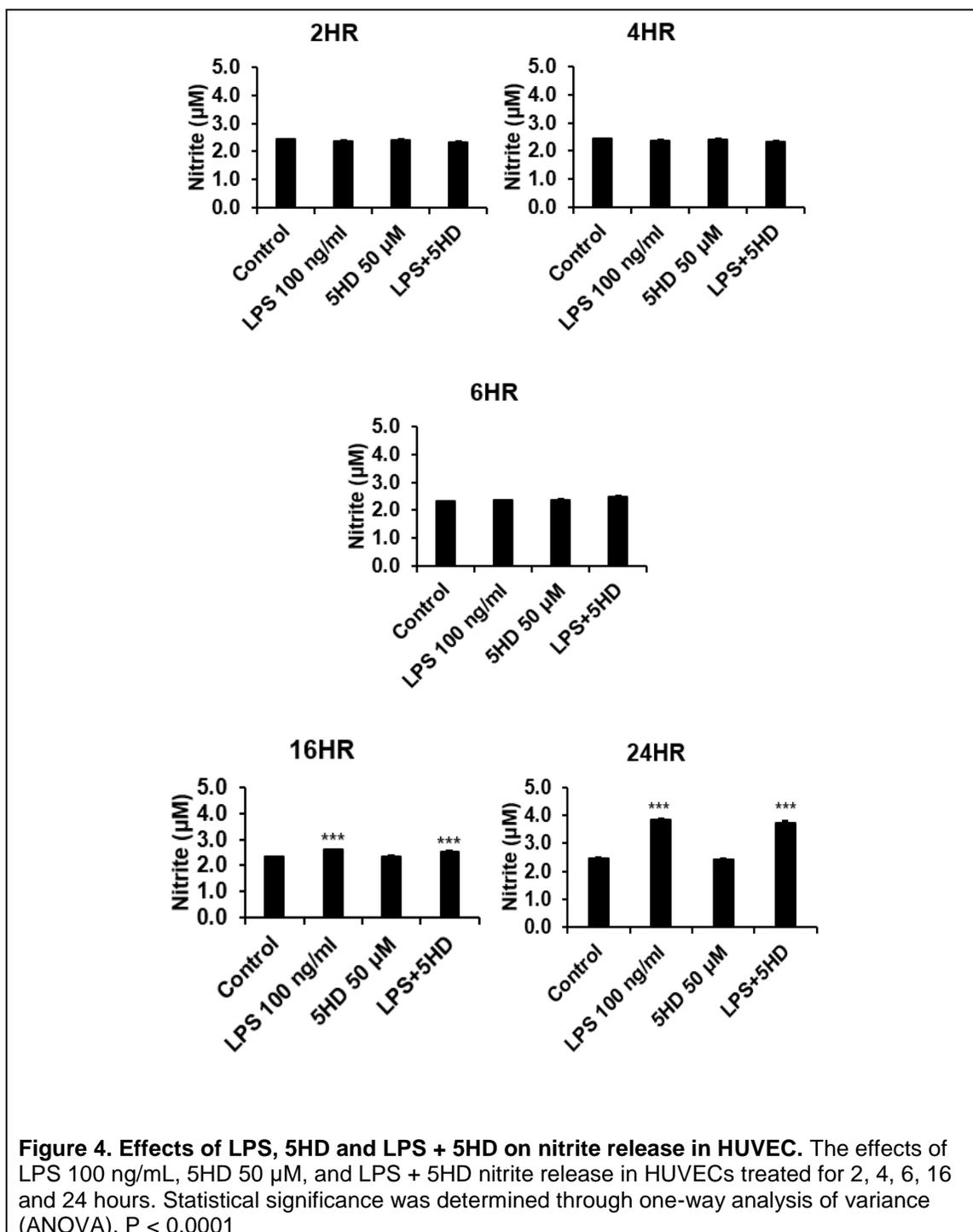


Figure 3: Dose-dependent effect of 5-hydroxydecanoic acid (5HD) on human umbilical vein endothelial cell (HUVEC) viability at 24 hours. The dose-dependent effects of 5HD at 50 µM, 100 µM and 200 µM on HUVEC viability at 24 hours. Statistical significance was determined through one-way analysis of variance (ANOVA). $P < 0.0001$

Effect of 5HD on LPS-induced effect on nitrite levels

The time-dependent effect of 5HD on LPS-induced effects on nitrite release was measured in HUVEC treated at 2, 4, 6, 16 and 24 hours. HUVEC treated with LPS alone showed significant increases in nitrite release following treatment for 16 and 24 hours. 5HD (50 μ M) in the presence of LPS at 100 ng/mL did not affect the LPS-induced elevated levels of nitrite at both 16 and 24-hour. This suggests that 5HD does not diminish the LPS-induced nitrite release in HUVEC (Fig. 4).



The Effect of 5HD on LPS-induced effect on cytosolic Bax, Cytochrome C and Apaf-1 proteins

The effect of 5HD (50 μ M) in the presence and absence of LPS (100 ng/mL) was observed using Western blot at 16 hours post treatment. LPS did not significantly alter protein levels of cytosolic Bax and Cytochrome C. Also, 5HD did not alter the effect of LPS on Bax and Cytochrome C levels (Fig. 5 and Fig 6, respectively).

LPS produces a significant decrease in the levels of Apaf-1 (Fig. 7). Treatment with 5HD significantly reduced levels of Apaf-1 compared to control (alone group).

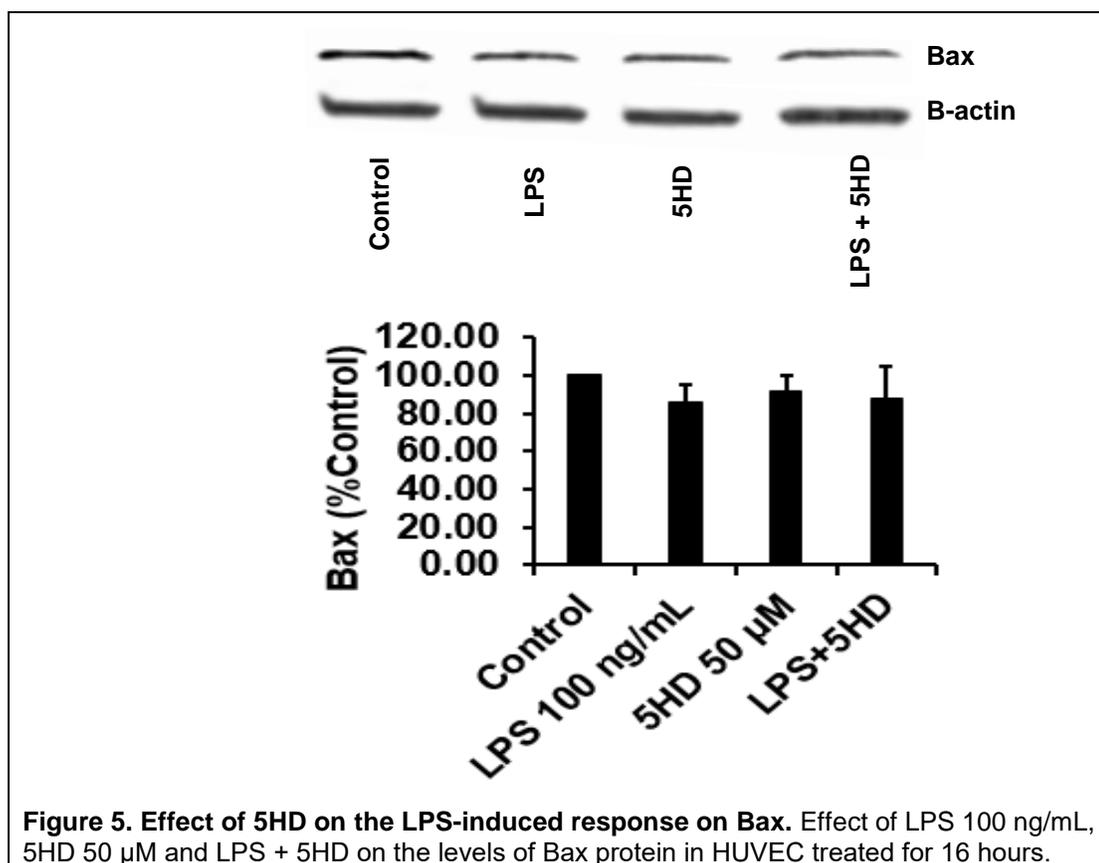
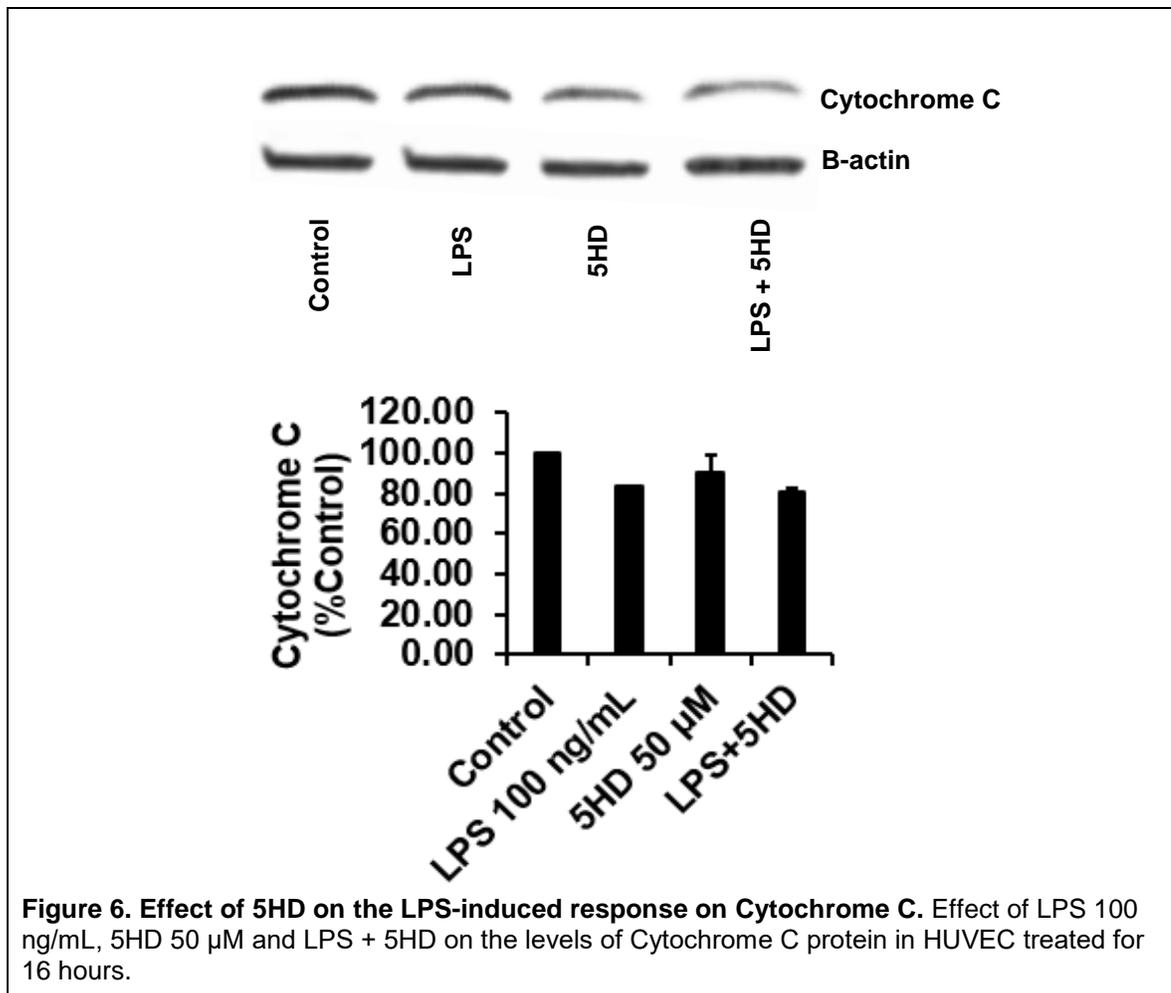
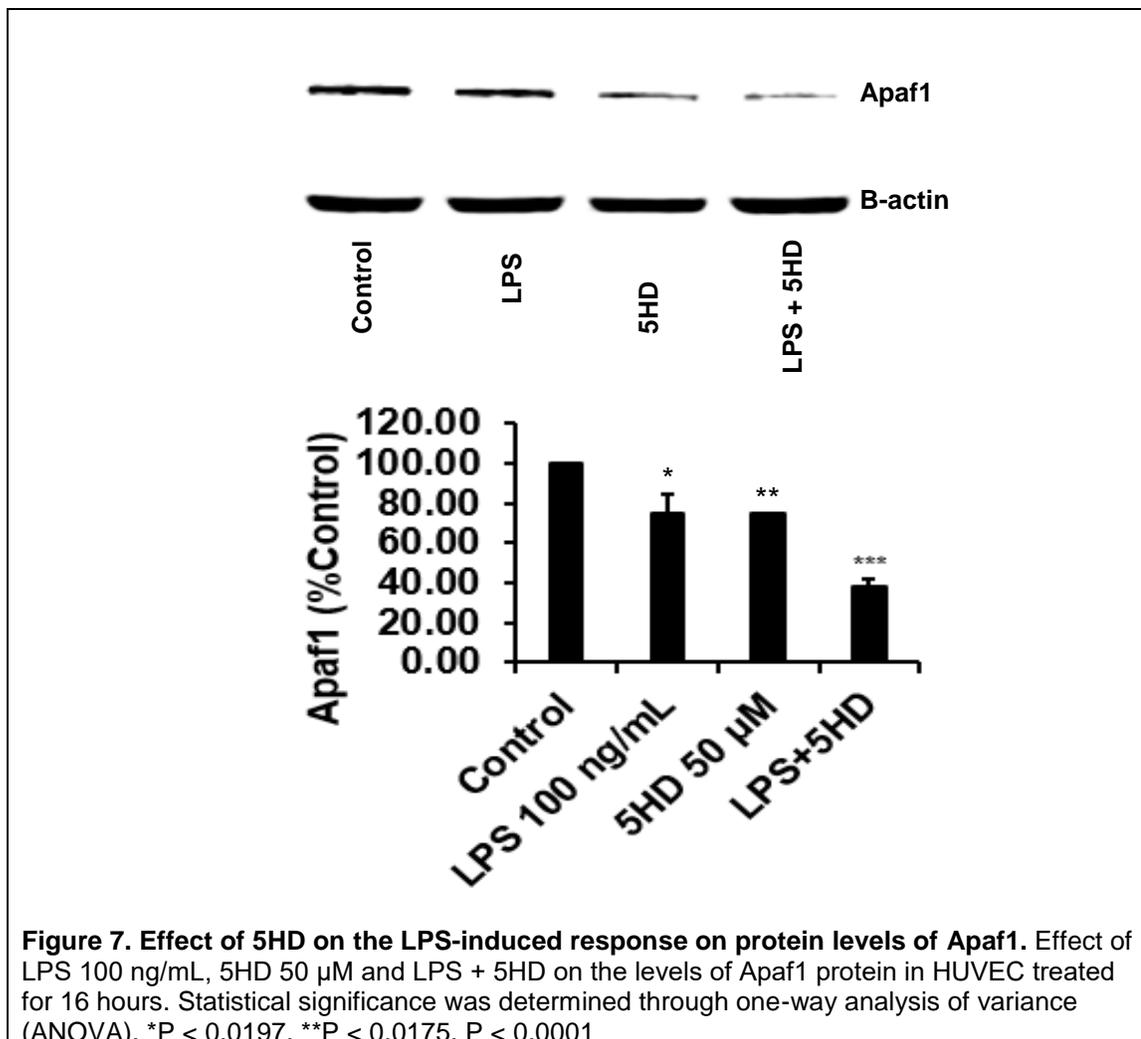


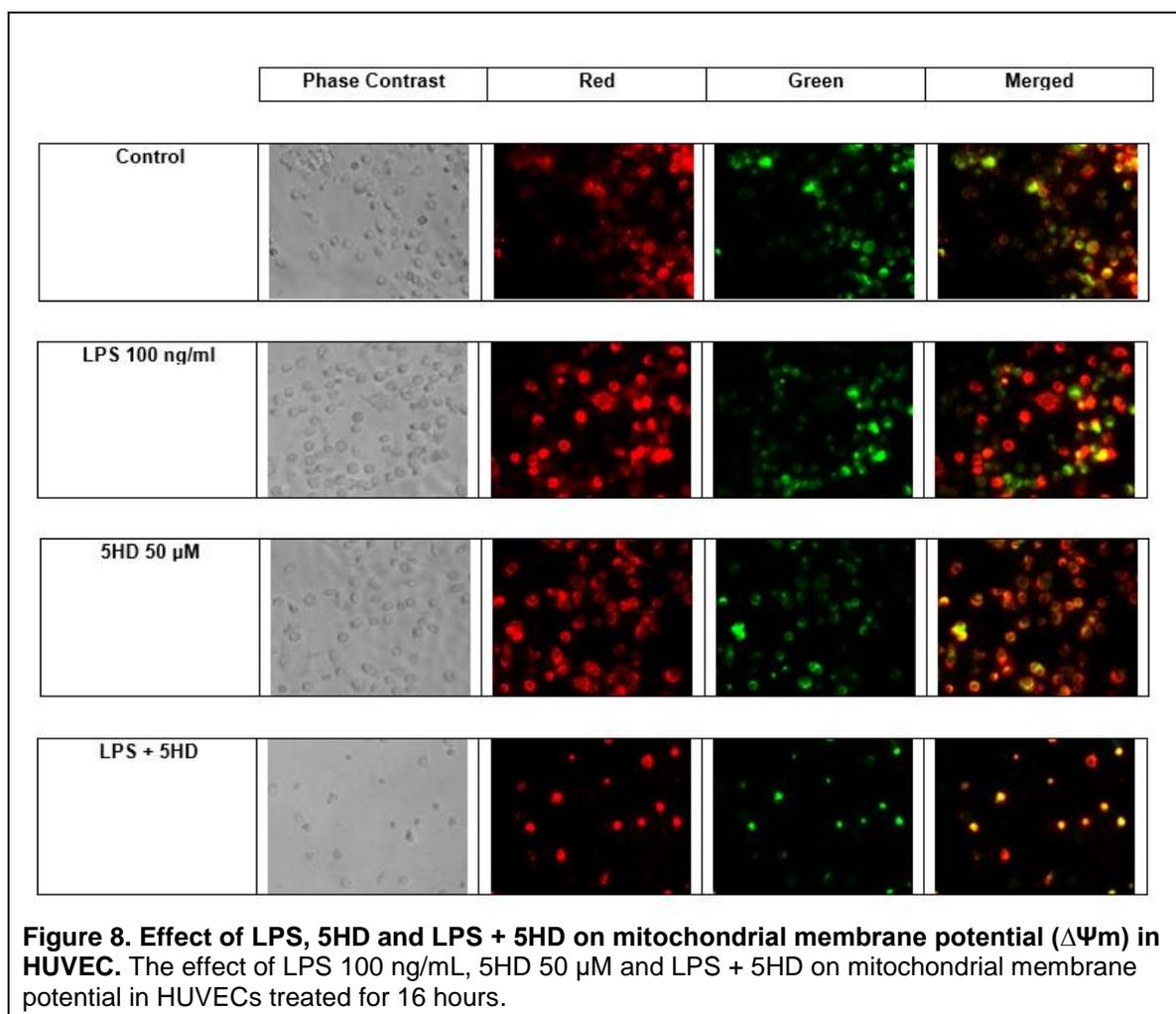
Figure 5. Effect of 5HD on the LPS-induced response on Bax. Effect of LPS 100 ng/mL, 5HD 50 μ M and LPS + 5HD on the levels of Bax protein in HUVEC treated for 16 hours.

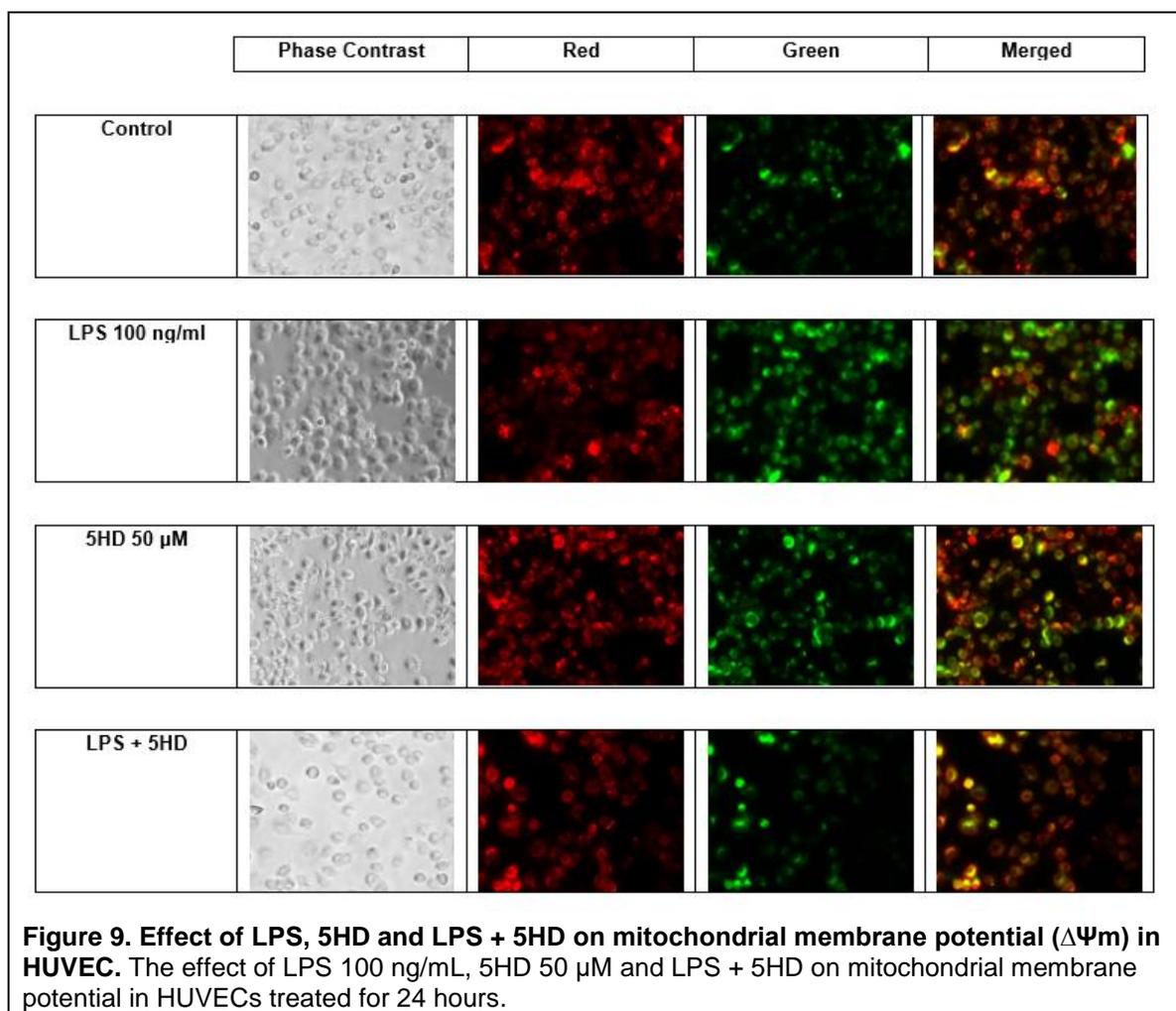




Effect of 5HD on LPS-induced mitochondrial membrane potential alterations in HUVEC

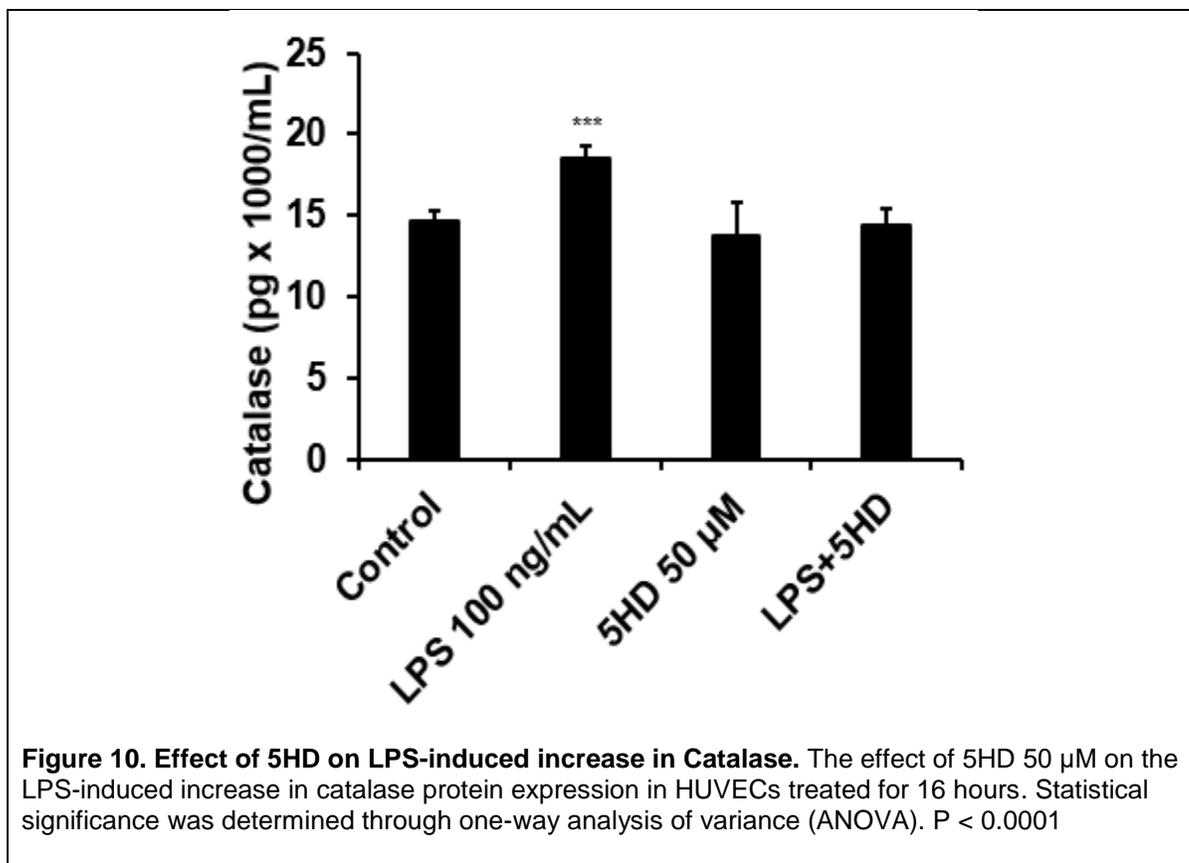
The effect of 5HD on mitochondrial membrane integrity in LPS-treated HUVEC was visualized at 16 and 24 hours by JC-1 assay. Mitochondrial membrane disruption is evident as green fluorescence in LPS treatment groups at both 16 and 24 hours indicating the presence of cytoplasmic JC-1 monomers (Fig. 8 and 9).





The effect of 5HD on LPS-induced increase in cytosolic catalase

LPS-induced endotoxemia is known to lead to the production of reactive oxygen species and ultimately oxidative stress (27). To determine whether 5HD plays a role in protection from oxidative damage in endotoxic HUVEC, catalase levels were quantified through ELISA following 16-hour treatment. LPS-treated HUVEC showed a significant increase in catalase expression. A high level of catalase in response to LPS was blocked by co-treatment with 5HD (Fig. 10) suggesting that 5HD offers endotoxic cells protection from the generation of reactive oxygen species.



DISCUSSION

Sepsis, septic shock and endotoxemia have been shown to produce vascular perfusion dysfunction in experimental models (1, 52, 53, 57). Likewise, in the current *in vitro* model of endotoxemia in HUVEC, a 25 and 30% decrease in cell viability after 16 and 24 hours post LPS treatment was observed. Additionally, a 51% decrease after 24-hour treatment with LPS 1000 ng/mL was observed suggesting a time- and dose-dependent decrease in cell viability in HUVECs. Similar data has been reported by other investigators (46, 58).

It is well-established that LPS activates the toll-like receptor 4 (TLR-4) signaling pathway (26), (16). The TLR-4 pathway ultimately activates NF- κ B nuclear translocation which can go on to induce the transcription of pro-inflammatory molecules and iNOS (26). Choy *et al.* have recently demonstrated that LPS acts through the TLR-4 pathway to induce apoptosis in HUVEC as well as increases inducible nitric oxide synthase (iNOS) levels (16). Additionally, nitrite can be used to generate nitric oxide (NO) and can, therefore, also be used to indirectly quantify levels of NO production (35). Experimental evidence shows that LPS treatment increases nitrite levels in HUVEC in a time-dependent manner. However, it appears that 5HD does not diminish the LPS-induced nitrite release in HUVEC. These data suggest that the protective effects of 5HD likely occur downstream of initial inflammatory events in response to LPS.

LPS-induced endotoxemia is known to lead to the production of reactive oxygen species and ultimately oxidative stress (61). MitoK_{ATP} channel opening is also known to lead to the production of ROS, an effect which can be opposed by blockage of the channel by 5HD (18). Prevention of mitoK_{ATP} channel activity by 5HD could reduce the generation of ROS from mitoK_{ATP} channels and therefore suppress the need for antioxidant enzyme expression. Data show that LPS treatment increases catalase levels. Low levels of ROS are known to be typically generated as a result of oxidative phosphorylation which may be involved in the catalase levels observed in the control group. 5HD treatment alone resulted in catalase levels similar to the control group suggesting that the protective effects of 5HD are limited to HUVEC in endotoxemia rather than healthy cells. However, treatment of HUVEC with LPS induced an increase in the levels of H₂O₂ as indicated by the greater catalase levels. In contrast, 5HD reduced this LPS-induced increase in catalase expression. These results suggest that 5HD may offer protection to endotoxic cells from oxidative damage by preventing the generation of excessive H₂O₂ thereby reducing the need for catalase activity. Since, in the current study, 5HD did not alter LPS-induced elevated NO levels, it appears the free radical scavenging effect could be mediated through cytosolic catalase and may have no or negligible effects from superoxide dismutase. Further studies are needed to establish this stipulation.

LPS has been shown to induce cell death directly in several *in vitro* models such as bovine endothelial cells and HUVECs (45),(29). Shioiri *et al.*,

(2009) reported that LPS causes activation of caspase-3 and 7, decreases cell viability and produces cytotoxicity. In the current study, we observed that LPS failed to produce an increase in cytosolic Bax and cytochrome C levels. This result is most likely due to the fact that LPS causes activation of caspase-3 which rapidly releases to the supernatant (58). In addition, cytochrome C is released from the intermembrane space rather than expressed and Bax exists primarily in the cytosol prior to mitochondrial relocation and oligomerization following an apoptotic stimulus (21), (72). Moreover, 5HD reduces Apaf-1 expression due to LPS-induced endotoxemia in HUVEC. These data suggest that 5HD treatment can prevent Apaf-1 expression above normal baseline levels in endotoxic HUVEC which may prevent apoptosome formation. However more studies are warranted to confirm this speculation.

To elucidate the role of the intrinsic apoptotic cascade mediated via mitochondria, we also studied role of 5HD in mitochondrial membrane potential alterations, an indicator for mitochondrial integrity, during endotoxemia in HUVECs. Preservation of mitochondrial membrane potential ($\Delta\Psi_m$) requires intact mitochondrial membranes. Mitochondrial membrane permeabilization due to Bax insertion and pore formation which facilitates cytochrome C release is known to occur under the intrinsic pathway (24). Pore formation can impede oxidative phosphorylation and the resulting ATP depletion may activate mitoK_{ATP} channels. MitoK_{ATP} channel opening leads to influx of potassium ions, matrix swelling and depolarization which are known to occur in apoptosis. The increase

in matrix volume due to this channel opening has also been shown to disrupt outer mitochondrial membrane integrity and contribute to cytochrome C release (28), (18). 5HD appears to alleviate the LPS-induced mitochondrial membrane potential alterations in endotoxic HUVEC at both 16 and 24 hours. HUVEC treated with 5HD alone for 16 hours exhibited more red than green fluorescence compared to 24-hour treatment which shows substantially more green than red. This abundance of cytoplasmic JC-1 monomers at 24 hours suggests that prolonged exposure to 5HD may disrupt $\Delta\Psi_m$ in healthy HUVEC. Greater red fluorescence is noticeable following LPS and 5HD co-treatment which indicates more JC-1 aggregates within mitochondria with intact $\Delta\Psi_m$ compared to LPS alone at 16 and 24 hours. Considerable green color is not obvious in LPS group at 16 hours compared to 24 hours indicating that LPS in a time-dependent manner produced the degree of $\Delta\Psi_m$, an effect which was reversed by inhibition of mitoK_{ATP} channels. The data supports our earlier observation in ARVM, where 5HD, a mitoK_{ATP} channel blocker, increased viability of septic cardiomyocytes and survival of septic animals (13).

CONCLUSIONS

The current study provides evidence for the role of 5HD, a putative mitoK_{ATP} blocker, in preserving $\Delta\Psi_m$ in endotoxic cells. 5HD may exert a protective effect on endotoxic HUVEC by reducing the magnitude of cell death as this compound appears to play a role in preventing Apaf-1 expression and maintaining $\Delta\Psi_m$ in endotoxic HUVEC which may reduce cytochrome C release. Further protection from cell death arises from the ability of 5HD to reduce H₂O₂ generation in endotoxic HUVEC.

REFERENCES

1. **Alden KJ, Motew SJ, Sharma AC, and Ferguson JL.** Effect of aminoguanidine on plasma nitric oxide by-products and blood flow during chronic peritoneal sepsis. *Shock* 9: 289-295, 1998.
2. **Anderson EJ, Katunga LA, and Willis MS.** Mitochondria as a source and target of lipid peroxidation products in healthy and diseased heart. *Clin Exp Pharmacol Physiol* 39: 179-193, 2012.
3. **Baines CP.** The molecular composition of the mitochondrial permeability transition pore. *J Mol Cell Cardiol* 46: 850-857, 2009.
4. **Bhattacharyya A, Chattopadhyay R, Mitra S, and Crowe SE.** Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. *Physiol Rev* 94: 329-354, 2014.
5. **Bonora M, Patergnani S, Rimessi A, De Marchi E, Suski JM, Bononi A, Giorgi C, Marchi S, Missiroli S, Poletti F, Wieckowski MR, and Pinton P.** ATP synthesis and storage. *Purinergic Signal* 8: 343-357, 2012.
6. **Bournat JC, and Brown CW.** Mitochondrial dysfunction in obesity. *Curr Opin Endocrinol Diabetes Obes* 17: 446-452, 2010.
7. **Brand MD.** Mitochondrial generation of superoxide and hydrogen peroxide as the source of mitochondrial redox signaling. *Free Radic Biol Med* 100: 14-31, 2016.

8. **Brenner C, Subramaniam K, Pertuiset C, and Pervaiz S.** Adenine nucleotide translocase family: four isoforms for apoptosis modulation in cancer. *Oncogene* 30: 883-895, 2011.
9. **Brentnall M, Rodriguez-Menocal L, De Guevara RL, Cepero E, and Boise LH.** Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis. *BMC Cell Biol* 14: 32, 2013.
10. **Chaplin DD.** Overview of the immune response. *J Allergy Clin Immunol* 125: S3-23, 2010.
11. **Chen CH, Ferreira JC, Gross ER, and Mochly-Rosen D.** Targeting aldehyde dehydrogenase 2: new therapeutic opportunities. *Physiol Rev* 94: 1-34, 2014.
12. **Chi DS, Qui M, Krishnaswamy G, Li C, and Stone W.** Regulation of nitric oxide production from macrophages by lipopolysaccharide and catecholamines. *Nitric Oxide* 8: 127-132, 2003.
13. **Chopra M, Golden HB, Mullapudi S, Dowhan W, Dostal DE, and Sharma AC.** Modulation of myocardial mitochondrial mechanisms during severe polymicrobial sepsis in the rat. *PLoS One* 6: e21285, 2011.
14. **Chopra M, and Sharma AC.** Contractile response of norepinephrine is modulated by caspase-3 in adult rat ventricular myocytes isolated from septic rat heart. *Pharmacol Res* 60: 303-313, 2009.
15. **Chopra M, and Sharma AC.** Distinct cardiodynamic and molecular characteristics during early and late stages of sepsis-induced myocardial dysfunction. *Life Sci* 81: 306-316, 2007.

16. **Choy KW, Lau YS, Murugan D, Vanhoutte PM, and Mustafa MR.** Paeonol Attenuates LPS-Induced Endothelial Dysfunction and Apoptosis by Inhibiting BMP4 and TLR4 Signaling Simultaneously but Independently. *J Pharmacol Exp Ther* 364: 420-432, 2018.
17. **Circu ML, and Aw TY.** Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radic Biol Med* 48: 749-762, 2010.
18. **Costa AD, Quinlan CL, Andrukhiv A, West IC, Jaburek M, and Garlid KD.** The direct physiological effects of mitoK(ATP) opening on heart mitochondria. *Am J Physiol Heart Circ Physiol* 290: H406-415, 2006.
19. **D'Souza Y, Elharram A, Soon-Shiong R, Andrew RD, and Bennett BM.** Characterization of Aldh2 (-/-) mice as an age-related model of cognitive impairment and Alzheimer's disease. *Mol Brain* 8: 27, 2015.
20. **de Punder K, and Pruijboom L.** Stress induces endotoxemia and low-grade inflammation by increasing barrier permeability. *Front Immunol* 6: 223, 2015.
21. **Dewson G, and Kluck RM.** Mechanisms by which Bak and Bax permeabilise mitochondria during apoptosis. *J Cell Sci* 122: 2801-2808, 2009.
22. **Doenst T, Nguyen TD, and Abel ED.** Cardiac metabolism in heart failure: implications beyond ATP production. *Circ Res* 113: 709-724, 2013.
23. **Falschlehner C, Schaefer U, and Walczak H.** Following TRAIL's path in the immune system. *Immunology* 127: 145-154, 2009.

24. **Flores-Romero H, Garcia-Porras M, and Basanez G.** Membrane insertion of the BAX core, but not latch domain, drives apoptotic pore formation. *Sci Rep* 7: 16259, 2017.
25. **Galloway CA, and Yoon Y.** Mitochondrial dynamics in diabetic cardiomyopathy. *Antioxid Redox Signal* 22: 1545-1562, 2015.
26. **Garcia Bueno B, Caso JR, Madrigal JL, and Leza JC.** Innate immune receptor Toll-like receptor 4 signalling in neuropsychiatric diseases. *Neurosci Biobehav Rev* 64: 134-147, 2016.
27. **Gillespie MN, Al-Mehdi AB, and McMurtry IF.** Mitochondria in hypoxic pulmonary vasoconstriction: potential importance of compartmentalized reactive oxygen species signaling. *Am J Respir Crit Care Med* 187: 338-340, 2013.
28. **Gogvadze V, Robertson JD, Enoksson M, Zhivotovsky B, and Orrenius S.** Mitochondrial cytochrome c release may occur by volume-dependent mechanisms not involving permeability transition. *Biochem J* 378: 213-217, 2004.
29. **Hu X, Yee E, Harlan JM, Wong F, and Karsan A.** Lipopolysaccharide induces the antiapoptotic molecules, A1 and A20, in microvascular endothelial cells. *Blood* 92: 2759-2765, 1998.
30. **Julien O, and Wells JA.** Caspases and their substrates. *Cell Death Differ* 24: 1380-1389, 2017.
31. **Kamisoglu K, Haimovich B, Calvano SE, Coyle SM, Corbett SA, Langlely RJ, Kingsmore SF, and Androulakis IP.** Human metabolic response

to systemic inflammation: assessment of the concordance between experimental endotoxemia and clinical cases of sepsis/SIRS. *Crit Care* 19: 71, 2015.

32. **Kern TS, Du Y, Miller CM, Hatala DA, and Levin LA.** Overexpression of Bcl-2 in vascular endothelium inhibits the microvascular lesions of diabetic retinopathy. *Am J Pathol* 176: 2550-2558, 2010.

33. **Kumari A, Iwasaki T, Pyndiah S, Cassimere EK, Palani CD, and Sakamuro D.** Regulation of E2F1-induced apoptosis by poly(ADP-ribosyl)ation. *Cell Death Differ* 22: 311-322, 2015.

34. **Leipnitz G, Mohsen AW, Karunanidhi A, Seminotti B, Roginskaya VY, Markantone DM, Grings M, Mihalik SJ, Wipf P, Van Houten B, and Vockley J.** Evaluation of mitochondrial bioenergetics, dynamics, endoplasmic reticulum-mitochondria crosstalk, and reactive oxygen species in fibroblasts from patients with complex I deficiency. *Sci Rep* 8: 1165, 2018.

35. **Li H, Cui H, Kundu TK, Alzawahra W, and Zweier JL.** Nitric oxide production from nitrite occurs primarily in tissues not in the blood: critical role of xanthine oxidase and aldehyde oxidase. *J Biol Chem* 283: 17855-17863, 2008.

36. **Li M, Zhang P, Wei HJ, Li MH, Zou W, Li X, Gu HF, and Tang XQ.** Hydrogen Sulfide Ameliorates Homocysteine-Induced Cognitive Dysfunction by Inhibition of Reactive Aldehydes Involving Upregulation of ALDH2. *Int J Neuropsychopharmacol* 20: 305-315, 2017.

37. **Liang H, Hussey SE, Sanchez-Avila A, Tantiwong P, and Musi N.** Effect of lipopolysaccharide on inflammation and insulin action in human muscle. *PLoS One* 8: e63983, 2013.

38. **Liu C, Stonestrom AJ, Christian T, Yong J, Takase R, Hou YM, and Yang X.** Molecular Basis and Consequences of the Cytochrome c-tRNA Interaction. *J Biol Chem* 291: 10426-10436, 2016.
39. **Liu XR, Cao L, Li T, Chen LL, Yu YY, Huang WJ, Liu L, and Tan XQ.** Propofol attenuates H₂O₂-induced oxidative stress and apoptosis via the mitochondria- and ER-mediated pathways in neonatal rat cardiomyocytes. *Apoptosis* 22: 639-646, 2017.
40. **Liu Y, and Chen XJ.** Adenine nucleotide translocase, mitochondrial stress, and degenerative cell death. *Oxid Med Cell Longev* 2013: 146860, 2013.
41. **Lofrumento DD, La Piana G, Abbrescia DI, Palmitessa V, La Pesa V, Marzulli D, and Lofrumento NE.** Valinomycin induced energy-dependent mitochondrial swelling, cytochrome c release, cytosolic NADH/cytochrome c oxidation and apoptosis. *Apoptosis* 16: 1004-1013, 2011.
42. **Malinska D, Mirandola SR, and Kunz WS.** Mitochondrial potassium channels and reactive oxygen species. *FEBS Lett* 584: 2043-2048, 2010.
43. **McCommis KS, and Baines CP.** The role of VDAC in cell death: friend or foe? *Biochim Biophys Acta* 1818: 1444-1450, 2012.
44. **McMurray F, Patten DA, and Harper ME.** Reactive Oxygen Species and Oxidative Stress in Obesity-Recent Findings and Empirical Approaches. *Obesity (Silver Spring)* 24: 2301-2310, 2016.
45. **Meyrick B, Hoover R, Jones MR, Berry LC, Jr., and Brigham KL.** In vitro effects of endotoxin on bovine and sheep lung microvascular and pulmonary artery endothelial cells. *J Cell Physiol* 138: 165-174, 1989.

46. **Mula RV, Machiah D, Holland L, Wang X, Parihar H, Sharma AC, Selvaraj P, and Shashidharamurthy R.** Immune Complex-Induced, Nitric Oxide-Mediated Vascular Endothelial Cell Death by Phagocytes Is Prevented with Decoy Fcγ Receptors. *PLoS One* 11: e0153620, 2016.
47. **Nathan C, and Cunningham-Bussel A.** Beyond oxidative stress: an immunologist's guide to reactive oxygen species. *Nat Rev Immunol* 13: 349-361, 2013.
48. **Parthasarathy G, and Philipp MT.** Review: apoptotic mechanisms in bacterial infections of the central nervous system. *Front Immunol* 3: 306, 2012.
49. **Raza H, John A, and Shafarin J.** Potentiation of LPS-Induced Apoptotic Cell Death in Human Hepatoma HepG2 Cells by Aspirin via ROS and Mitochondrial Dysfunction: Protection by N-Acetyl Cysteine. *PLoS One* 11: e0159750, 2016.
50. **Rosadini CV, and Kagan JC.** Early innate immune responses to bacterial LPS. *Curr Opin Immunol* 44: 14-19, 2017.
51. **Ryter SW, Kim HP, Hoetzel A, Park JW, Nakahira K, Wang X, and Choi AM.** Mechanisms of cell death in oxidative stress. *Antioxid Redox Signal* 9: 49-89, 2007.
52. **Sam AD, 2nd, Sharma AC, Law WR, and Ferguson JL.** Splanchnic vascular control during sepsis and endotoxemia. *Front Biosci* 2: e72-92, 1997.
53. **Sam AD, 2nd, Sharma AC, Rice AN, Ferguson JL, and Law WR.** Adenosine and nitric oxide regulate regional vascular resistance via

interdependent and independent mechanisms during sepsis. *Crit Care Med* 28: 1931-1939, 2000.

54. **Shakeri R, Kheirollahi A, and Davoodi J.** Apaf-1: Regulation and function in cell death. *Biochimie* 135: 111-125, 2017.

55. **Sharma AC.** Sepsis-induced myocardial dysfunction. *Shock* 28: 265-269, 2007.

56. **Sharma AC, Motew SJ, Farias S, Alden KJ, Bosmann HB, Law WR, and Ferguson JL.** Sepsis alters myocardial and plasma concentrations of endothelin and nitric oxide in rats. *J Mol Cell Cardiol* 29: 1469-1477, 1997.

57. **Sharma AC, Sam AD, 2nd, Lee LY, Hales DB, Law WR, Ferguson JL, and Bosmann HB.** Effect of NG-nitro-L-arginine methyl ester on testicular blood flow and serum steroid hormones during sepsis. *Shock* 9: 416-421, 1998.

58. **Shioiri T, Muroi M, Hatao F, Nishida M, Ogawa T, Mimura Y, Seto Y, Kaminishi M, and Tanamoto K.** Caspase-3 is activated and rapidly released from human umbilical vein endothelial cells in response to lipopolysaccharide. *Biochim Biophys Acta* 1792: 1011-1018, 2009.

59. **Shvetsova AN, Mennerich D, Keratar JM, Hiltunen JK, and Kietzmann T.** Non-electron transfer chain mitochondrial defects differently regulate HIF-1 α degradation and transcription. *Redox Biol* 12: 1052-1061, 2017.

60. **Smith KA, Waypa GB, and Schumacker PT.** Redox signaling during hypoxia in mammalian cells. *Redox Biol* 13: 228-234, 2017.

61. **Snyder CM, Shroff EH, Liu J, and Chandel NS.** Nitric oxide induces cell death by regulating anti-apoptotic BCL-2 family members. *PLoS One* 4: e7059, 2009.
62. **Soufli I, Toumi R, Rafa H, and Touil-Boukoffa C.** Overview of cytokines and nitric oxide involvement in immuno-pathogenesis of inflammatory bowel diseases. *World J Gastrointest Pharmacol Ther* 7: 353-360, 2016.
63. **Suzuki Y, Hasegawa H, Tsuji T, Tsuruda K, Sasaki D, Ishihara K, Nagai K, Yanagihara K, Yamada Y, and Kamihira S.** Relationships of diverse apoptotic death process patterns to mitochondrial membrane potential ($\Delta\psi(m)$) evaluated by three-parameter flow cytometric analysis. *Cytotechnology* 65: 59-70, 2013.
64. **Um HD.** Bcl-2 family proteins as regulators of cancer cell invasion and metastasis: a review focusing on mitochondrial respiration and reactive oxygen species. *Oncotarget* 7: 5193-5203, 2016.
65. **Wang C, and Youle RJ.** The role of mitochondria in apoptosis*. *Annu Rev Genet* 43: 95-118, 2009.
66. **Wang Y, Wang Y, Yang D, Yu X, Li H, Lv X, Lu D, and Wang H.** beta(1)-adrenoceptor stimulation promotes LPS-induced cardiomyocyte apoptosis through activating PKA and enhancing CaMKII and IkappaBalpha phosphorylation. *Crit Care* 19: 76, 2015.
67. **Ward PA.** Sepsis, apoptosis and complement. *Biochem Pharmacol* 76: 1383-1388, 2008.

68. **Wheeler DS.** Death to sepsis: targeting apoptosis pathways in sepsis. *Crit Care* 13: 1010, 2009.
69. **Williams LJ, Nye BG, and Wende AR.** Diabetes-Related Cardiac Dysfunction. *Endocrinol Metab (Seoul)* 32: 171-179, 2017.
70. **Yang X, Cohen MV, and Downey JM.** Mechanism of cardioprotection by early ischemic preconditioning. *Cardiovasc Drugs Ther* 24: 225-234, 2010.
71. **Zhang M, Wang S, Cheng Z, Xiong Z, Lv J, Yang Z, Li T, Jiang S, Gu J, Sun D, and Fan Y.** Polydatin ameliorates diabetic cardiomyopathy via Sirt3 activation. *Biochem Biophys Res Commun* 493: 1280-1287, 2017.
72. **Zhang M, Zheng J, Nussinov R, and Ma B.** Release of Cytochrome C from Bax Pores at the Mitochondrial Membrane. *Sci Rep* 7: 2635, 2017.
73. **Zhong H, Xiao M, Zarkovic K, Zhu M, Sa R, Lu J, Tao Y, Chen Q, Xia L, Cheng S, Waeg G, Zarkovic N, and Yin H.** Mitochondrial control of apoptosis through modulation of cardiolipin oxidation in hepatocellular carcinoma: A novel link between oxidative stress and cancer. *Free Radic Biol Med* 102: 67-76, 2017.
74. **Ziello JE, Jovin IS, and Huang Y.** Hypoxia-Inducible Factor (HIF)-1 regulatory pathway and its potential for therapeutic intervention in malignancy and ischemia. *Yale J Biol Med* 80: 51-60, 2007.