Comparative Study of the L-arginine-NO Pathway Gene Expressions between Human and Porcine Pulmonary Artery Endothelial Cells in Response to Lipopolysaccharide

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Lipopolysaccharide (LPS) is found in the outer membrane of gram-negative bacteria and acts as an endotoxin. The endothelium functions as a barrier to gram-negative infection in the blood. We studied the effects of LPS on gene expressions related to the L-arginine-NO pathway, with a focus on the inducible form of nitric oxide synthase (iNOS) and two types of arginase (Arg-1 and Arg-2) in response to different concentrations of LPS in human pulmonary artery endothelial cells (HPAEC) and porcine pulmonary artery endothelial cells (PPAEC). We demonstrated significant differences in response to LPS between HPAEC and PPAEC. While we found that both PPAEC and HPAEC responded to LPS by up-regulation of classical inflammation genes, PPAEC were much more responsive to LPS in gene expressions, and could survive in higher concentrations of LPS. iNOS and Arg-2 were induced by LPS alone only in PPAEC. We also showed that, without iNOS induction in response to high concentrations of LPS, HPAEC faced almost total cell death, and that the addition of an NO donor, NOC-18, protected HPAEC from toxic effects of LPS.

INTRODUCTION

Gram-negative bacteremia is the 13th leading cause of death in the U.S. It affects about 400,000 people, causing roughly 100,000 deaths annually in the U.S. alone.[1] Infection by gram-negative bacteria could cause septic shock within hours, which can affect multiple parts of the body, including the heart, brain, kidney, liver, and intestines, while causing low blood pressure, rapid heart rate, fever, lightheadedness, and shortness of breath.[2-4] Bacteremia and septic shock are caused by the release of endotoxin into the blood stream.[4]

LPS is a part of the outer membrane of gram-negative bacteria that acts as an endotoxin by binding to the CD14/TLR4/MD2 receptor complex on various cells. In this study, we compared the effects of LPS on gene expression related to the L-arginine-NO pathway in the pulmonary artery endothelial cell of porcine and human origins: CAT-1, CAT-2B, Arg-1, Arg-2, and iNOS.

iNOS is an inducible isoform of nitric oxide synthase. Once induced by stimulatory signals, iNOS produces a robust amount of NO, which helps cells fight infection and plays a major role in airway tone.[5] iNOS has been reported to be up-regulated in endothelial cells by LPS.[6, 7]

Arginase is an enzyme that competes with iNOS for the same substrate, L-arginine, and converts it to urea and L-ornithine. Ornithine from arginase could be involved in the synthesis of collagen and polyamine regulation of cell proliferation in wounds and inflammation.[8-10] Arginase and NOS enzymes are expressed simultaneously under a wide variety of inflammatory conditions.[11] Arginase activity in some circumstances limits production of NO by iNOS.[12]

An increase in arginase expression has been shown to lower the effects of iNOS activation and cytotoxic response in macrophages.[13] Increases in iNOS expression could also limit arginase activity by releasing NG-hydroxy-L-arginine, an intermediate in NO synthesis and a potent inhibitor of arginase.[14] There are two types of arginase, type I and II, which are different genes and found in different chromosomes.[15] Type 1 arginase (Arg-1) is cytosolic and mostly expressed in the liver, while type 2 arginase (Arg-2) is found in mitochondria and expressed in extrahepatic tissues.[16] This experiment studied the response to LPS in Arg-1 and Arg-2 expression in addition to iNOS expression.

We also studied the expression of CAT-1 and CAT-2B, two members of the cationic amino acid transporters (CAT). CAT-1 and CAT-2B are encoded by separate genes, but perform the same task with nearly identical substrate patterns.[17, 18] CAT proteins provide a major entry for cationic amino acid in non-epithelial cells. They supply L-arginine for the synthesis of NO by NOS, and for the production of urea and L-ornithine.[19-21]

Another common response of endothelial cells to LPS is up-regulation of ICAM-1.[22] ICAM-1 is involved in both innate and adaptive immune responses to bacterial infection, facilitating interaction between antigen presenting cells and T cells and the translocation of leukocytes from blood vessels through endothelial cells to sites of inflammation.[23] We used ICAM-1 expression to confirm that our cells respond to LPS.

MATERIALS AND METHODS

Reagents

All cell culture reagents, unless specified, were purchased from Invitrogen (Carlsbad, CA). All chemicals not specified are from Sigma-Aldrich (St. Louis, MO). 1-hydroxy-2-oxo-3, 3-bis(2-aminoethyl)-1-triazene (NOC-18), an NO donor, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture and Treatment

Porcine pulmonary artery endothelial cells (PPAEC) were isolated from the main pulmonary artery of 6-7 month-old pigs using collagenase digestion, as previously reported.[24] Third to sixth passage cells in a monolayer culture were maintained in a RPMI-1640 medium containing 4% fetal bovine serum and antibiotics (10 u/mL penicillin, 100 µg/mL streptomycin, 20 µg/mL gentamicin, and 2 µg/mL Fungizone), and were used 2 or 3 days after confluence. Human pulmonary artery endothelial cells (HPAEC) were purchased from Lonza (Walkersville, MD) and cultured in EBM-2 media (Lonza) supplemented with 5% FBS, antibiotics, and growth factors (Bullet Kit, Lonza). HPAEC were used at passage 3 to 6.

Confluent HPAEC and PPAEC were treated with 0, 1, 10, and 100 µg/mL LPS (*E. coli* 011:B4, Sigma) in EBM-2 media supplemented with 1% FBS incubated at 37°C with air-5% CO_2 for 24 h. We also treated HPAEC with 100 ng/mL IFN-γ, or a cocktail consisting of 100 ng/mL IFN-γ + 10 ng/ mL TNF-α + 4 ng/mL IL-1β for 8 h to see iNOS up-regulation.

Measurement of Gene Expression by Real-time PCR

The total RNA was isolated from PPAEC and HPAEC using the RNeasy Mini kit (Qiagen, Valencia, CA) and treated with DNAse I (Ambion) according to the manufacturers' instructions. cDNA was synthesized from 0.9 µg of total mRNA using Oligo $d(T)_{20}$ primers and High Capacity RNA-to-cDNA Kit (Applied Biosystem, Foster City, CA). Relative quantification real-time PCR was done using Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) in a 7500 Real-time PCR System (Applied Biosystems). The PCR primers used are summarized in Table 1 and Table 2. All samples were run in duplicates. Melting curve analyses for amplification products indicated one specific product for each gene and no primer-dimer formation. Gene expression levels were normalized to β-actin and calculated based on the $\Delta\Delta C_T$ method.[25]

Cell Viability and Cytotoxicity Assay

To see the toxic effects of LPS on cell viabilities of PPAEC and HPAEC, we treated confluence cells in 96-multiwell clear-bottom plates for 24 h. Cell survival was measured with PrestoBlue™ Cell Viability Reagent (Invitrogen), according to the manufacturer's instructions.

Table 1. Human Real-Time PCR Primers

Gene	Access number	Primers: Sequence (5' -> 3')
β-Actin	NM_001101	GCCAACCGCGAGAAGATGA
		CATCACGATGCCAGTGGTA
Arginase I	NM_000045	GGAGACCACAGTTTGGCAAT
		CCACTTGTGGTTGTCAGTGG
Arginase II	NM_001172	AAGCTGGCTTGATGAAAAGGC
		GCGTGGATTCACTATCAGGTTGT
CAT-1	NM_003045	ATCTGCTTCATCGCCTACTT
		TAGCAGTCCATCCTCAGCGATG
CAT-2B	U76369	TTCTCTCTGCGCCTTGTCAA
		CCATCCTCCGCCATAGCATA
ICAM1	NM_000201	GCTATGCCTTGTCCTCTTG
		ATACACACACACACACACGC
iNOS	AF068236	GGTGGAAGCGGTAACAAAGG
		TGCTTGGTGGCGAAGATGA
VCAM	NM_001078	CAAATCCTTGATACTGCTCATC
		TTGACTTCTTGCTCACAGC

Table 2. Porcine Real-Time PCR Primers

Gene	Access number	Primers: Sequence (5' -> 3')
β-actin	U07786	GGACCTGACCGACTACCTCA
		GCGACGTAGCAGAGCTTCTC
β-actin	U07786	CCAGCACCATGAAGATCAAG
		ACATCTGCTGGAAGGTGGAC
Arginase I	AY039112	TGAAAGATTACGGGGACCTG
		CTTTTCCCACAGACCTTGGA
Arginase II	NM_009705	CACCCCTCACCACTTCATCT
		GAAAATCCTGGGAGTTGTGG
CAT-1	NM_001012613.1	ATGGCCTTCCTCTTTGACCT
		GGCTGGTACCGTAAGACCAA
CAT-2B	NM_001110420.1	TTCTCTCTGCGCCTTGTCAA
	(CAT-2A)	TTGAAAAGCAACCCATCCTC
		CATCCTCCGCCATAGCATA
ICAM	AF156712.1	GCCCAATTGAAGCTGAATGT
		CACCTGGGTCTGGTTCTTGT
iNOS	U59390	CCCTTCAATGGCTGGTACAT
		ATCCTTCTGCCCACTTCCTC
GAPDH	U48832	ACCCAGAAGACTGTGGATGG
		AAGCAGGGATGATGTTCTGG

RESULTS

Cytotoxic Effects of LPS in HPAEC and PPAEC

HPAEC and PPAEC were treated with different concentrations of LPS ranging from 0-100 μg/mL for 24 h. In both cell lines, treatment with 1 and 10 μg/mL LPS did not cause cell death. Treatment of PPAEC with 100 μg/mL LPS only slightly decreased the survival rate to 89±4%. In contrast, HPAEC treated with 100 μg/mL LPS showed only 13±2% survival rate (Figure 1).

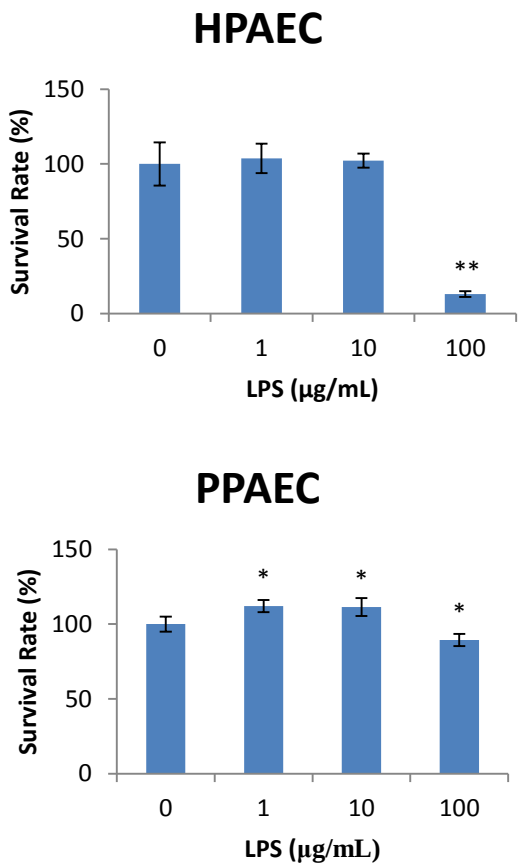

Figure 1. Survival of PPAEC treated with 0 – 100 μg/mL LPS and HPAEC treated with 0-100 μg/mL LPS without and with IFN-γ for 24 hr. Survival rate measured from the total amount of cellular nucleic acids. Treatment with 100 μg/mL LPS decreases cell survival of both PPAEC and HPAEC but more so in HPAEC where only 13% of cells survive. *P < 0.05 and **P < 0.001 compared to the untreated cells.

Effects of LPS on Gene Expressions in HPAEC

Since most of the HPAEC treated with 100 μg/mL LPS died, we tested the effect of 1 and 10 μg/mL LPS on the expression of genes involved in the arginine-NO pathway. We also used ICAM-1 as a marker of cellular response to LPS. HPAEC treated with LPS for 24 h showed increased mRNA expression of ICAM-1 with no significant change in the expression of the genes involved in the arginine-NO pathway, except CAT-2B. We could not detect iNOS and Arg-1 mRNA expression in the control and the treated cells. The expression of Arg-2 and CAT-1 did not change with LPS treatments. Since we saw no changes in the expression of iNOS or Arg-2, we added IFN-γ, a known mediator of inflammation that can increase the response of endothelial cells to LPS.[26-28] Although IFN-γ by itself did not up-regulate any studied gene, the addition of IFN-γ to these LPS treatments decreased the expressions of Arg-2, CAT-1, and CAT-2B mRNAs when compared with the corresponding treatments without IFN-γ. In contrast, IFN-γ up-regulated ICAM when added to cells treated with LPS but not by itself (Figure 2). Still, we found no iNOS mRNA.

Induction of iNOS Expression by a Cocktail

We tried to find the right treatment to induce iNOS expression in HPAEC. Published studies of iNOS showed that the gene can be induced in human endothelial cells with the right combination of cytokines, and that the maximum of iNOS expression can be detected after 6 to 8 h.[29] Hence, we treated HPAEC with several combinations of cytokines and LPS for a shorter period of 8 h. We found that TNF-α alone or with LPS did not induce iNOS expression and caused no change in Arg-2 or CAT-1 expression (data not shown). However, HPAEC treated with LPS and IFN-γ showed up-regulation of iNOS mRNA by 30±1.4 times compared to the non-treated cells. The highest increase of 560 times in iNOS expression was detected in cells treated with 10 μg/mL LPS and a cocktail of 10 ng/mL TNF-α, 100 ng/mL IFN-γ and 4 μg/mL IL-1β. Treatment with this cocktail also caused more up-regulation of CAT-1, CAT-2B, and ICAM-1 when compared to the treatment with LPS and IFN-γ alone. In both treatments, the expressions of Arg-2 were significantly lower than the control (Figure 3).

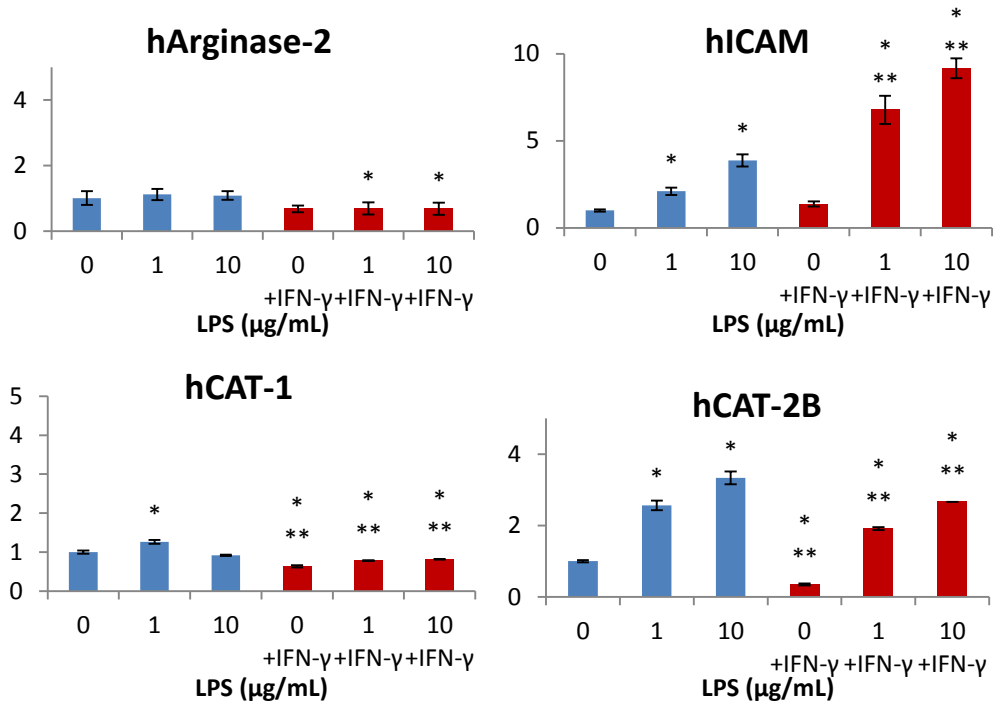

Figure 2. Response of mRNAs expression to 1-100 ug/mL LPS with and without 100 ng/mL IFN-γ in human pulmonary arterial endothelial cells (HPAEC) treated for 24 h in EBM-2 complete media supplemented with 1% FBS. Treatment with IFN-γ resulted in down-regulation of Arg-2, CAT-1, and CAT-2B mRNAs, but ICAM-1 is up-regulated with treatment with both LPS and IFN-γ. Arginase type I and inducible-nitric oxide synthase mRNA are not detectable with these treatments. The bars represent the mean of relative quantification ± S.D. *P < 0.05 compared to the untreated cells. **P < 0.05 compared to cells treated with LPS of the same concentration alone.

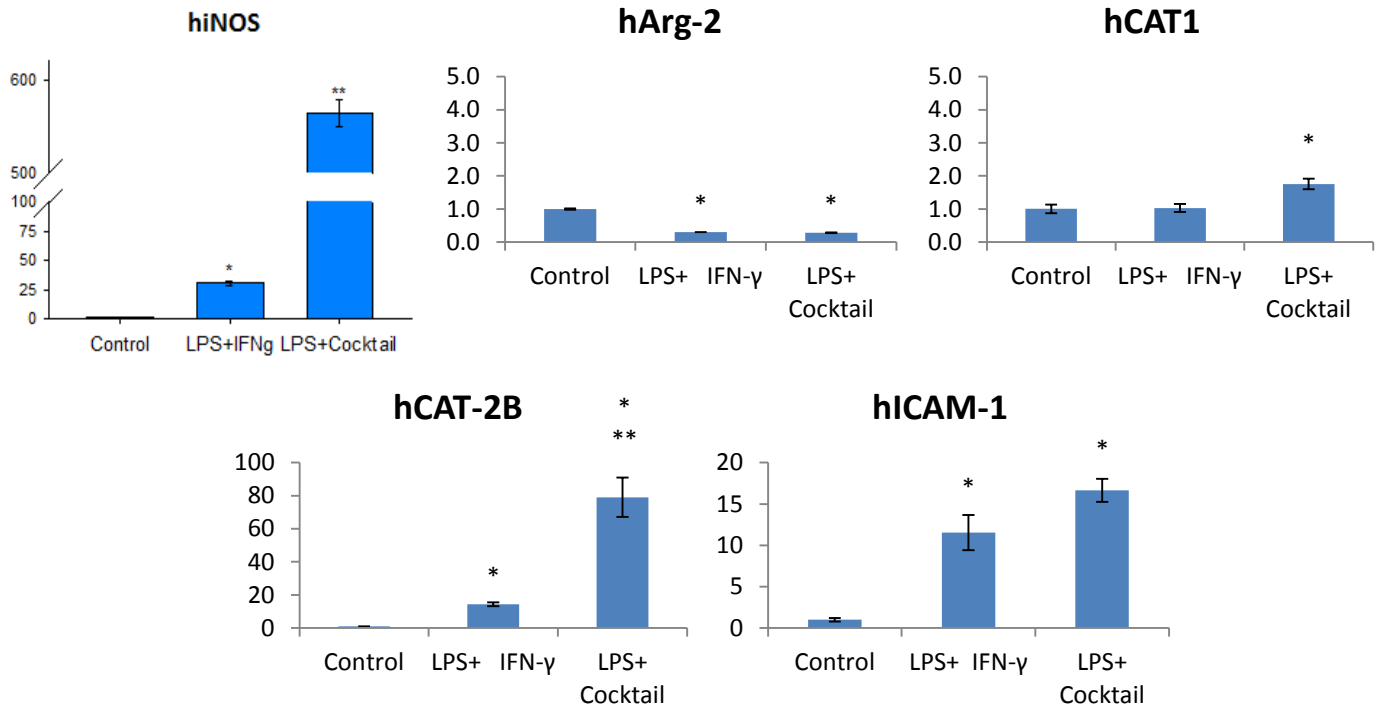

Figure 3. Induction of inflammatory-response related genes by treatment of HPAEC with 10 ug/mL LPS and 100 ng/mL IFN-γ or with 10 ug/mL LPS and cocktail (100 ng/mL IFN-γ + 10 ng/ mL TNF-α + 4 ng/mL IL-1β) for 8 h in EBM-2complete media supplemented with 1% FBS. Only Arg-2 is down-regulated by either treatment. iNOS, ICAM, CAT1, and CAT-2B are up-regulated with both. The addition of cocktail to treatment with LPS significantly increases the expression of iNOS, CAT1, and CAT-2B when compared with treatment with LPS + IFN-γ alone. No Arg-1 mRNA detected. Bars represent mean of relative quantification ± S.D. *P < 0.05 compared to the control, ** P < 0.005 compared to treatment with LPS + IFN-γ.

Effects of LPS on Gene Expressions in PPAEC

PPAEC was subject to the same LPS treatments as HPAEC. Stimulation of PPAEC with 1 μg/mL LPS for 24 h was sufficient to elicit significant changes in mRNA expression of iNOS, ICAM, Arg-1, Arg-2, CAT-1 and CAT-2B. An increase in LPS treatment concentration to 10 μg/mL further induced mRNA expression of ARG1, but it did not increase any other mRNA expression compared to the cells treated with 1 μg/mL LPS. iNOS mRNA expression was the most highly up-regulated, with an increase of more than a thousand-fold when compared with the non-treated cells. The treatment with 100 μg/mL LPS resulted in decreased mRNA expressions of all genes studied, relative to those treated with 1 or 10 μg/mL LPS, and thus could be associated with cytotoxic effect and enhanced cell death (Figure 4). Additions of human IFN-γ to LPS treatments did not show any significant difference in the expressions of the genes studied (data not shown). The lack of effects of human IFN-γ to PPAEC confirms that IFN-γ was species-specific and that human IFN-γ did not work on porcine cells.[30]

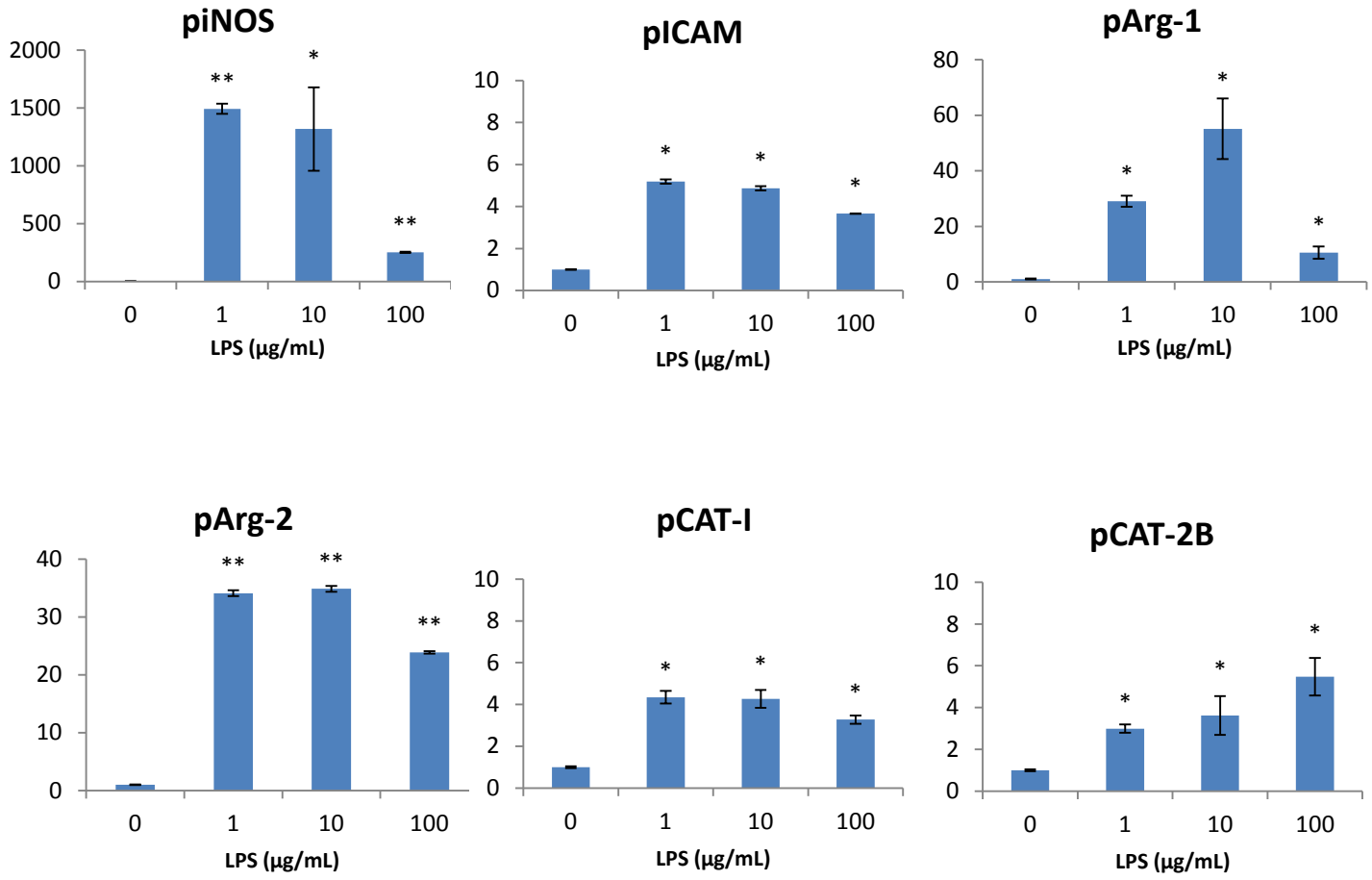

Figure 4. Up-regulation of the L-arginine-NO pathway's mRNAs expressions was induced by 1-100 ug/mL LPS in porcine pulmonary artery endothelial cells (PPAEC) treated for 24 h in EBM-2 media supplemented with 1% FBS. Bars represent mean of relative quantification ± S.D. *P < 0.05 compared to the untreated cells. **P < 0.001 compared to the untreated cells.

NO Protects HPAEC from Toxic Effects of LPS

We wanted to know whether NO was responsible for the higher survival rate in PPAEC in 100 μg/mL LPS, so we treated HPAEC with LPS and an NO donor, NOC-18, for 24 h. Treatment of HPAEC with 100 μg/mL LPS killed them, while almost 80% of the cells survived with the addition of NOC-18. Survival rates between cells treated with the two concentrations NOC- 18 were the same (Figure 5).

DISCUSSION

The treatment of cells with toxic LPS mimics gram-negative bacterial infection and stimulates inflammatory responses. We looked for differences in gene expressions

related to the L-arginine-NO pathway in response to LPS in endothelial cells from two species: human and porcine.

iNOS, the inducible form of NOS, is not constitutively expressed in most cells and has to be induced by cytokines and bacterial products.[31,5] Regulation of iNOS is complex and varies among species and cell types.[31] LPS can induce iNOS in certain cell types. For example, in mouse macrophages, LPS caused up-regulation of iNOS, Arg-1, Arg-2, and other genes.[32] LPS has also been shown to induce iNOS mRNA expression in human macrophage cells.[33] iNOS is required for macrophages to produce reactive nitrogen species (RNS), which plays a role in eliminating pathogens, including gram-negative bacteria. Unlike macrophages, endothelial cells primarily act as a barrier and a biosensor membrane between the blood stream and other tissues.[34]

We found that PPAEC responded to LPS by a robust induction of iNOS, which suggested that PPAEC were capable of producing NO in response to gram-negative infection, a role normally performed by macrophages. In HPAEC, we were unable to induce iNOS expression with the same LPS treatment given to PPAEC for 24 h. iNOS mRNA expression was undetectable in HPAEC, even when we used 100 μg/mL LPS, a much higher concentration than those used to induce iNOS in murine and porcine endothelial cells. This concentration of LPS was so toxic that the treatment killed 85% of HPAEC.

We confirmed that HPAEC responded to LPS since ICAM-1 was induced by LPS in both HPAEC anPPAEC. Inflammation increased the expression of ICAM-1, a gene which coded for an essential protein for arrest and extravasation of leukocytes to the site of inflammation.[23] In the arginine-NO pathway, CAT-1 and CAT-2B provide the substrate for NOS and arginase.[19, 35] CAT-1 has been shown to be expressed constitutively in various cell types, except in adult hepatic cells.[17, 18] On the other hand, CAT-2B was found only after treatments with LPS or cytokines. We showed that CAT-2B was up-regulated by LPS in both HPAEC and PPAEC; however, CAT-1 was only induced in PPAEC.

While LPS alone cannot induce iNOS, the addition of cytokines to LPS treatment was able to stimulate iNOS expression. We found that although IFN-γ by itself did not induce iNOS expression, the addition of IFN-γ to LPS or to IL-1β did. This result was consistent with a published study by Asano K et al.[29] The requirement of IFN-γ in the activation of iNOS in HPAEC could be due to its role in the enhancement of TLR signal transduction. IFN-γ is a cytokine that mediates immunity and inflammatory interactions, such as macrophage activation and cellular inflammation. IFN-γ has been shown to increase TLR expression, induce required transcription factors for certain TLR responsive genes, promote activation of NF-κB, and shut down the feedback inhibition of activation by TLR in human and murine macrophage.[26,36] Specifically, IFN-γ was shown to up-regulate expression of TLR-4—a main receptor for LPS—on cell surfaces in human mononuclear phagocytes.[37]

Although TNF-α has been implicated in septic shock and inflammation, treatment with TNF-α alone or TNF-α with LPS caused no change in iNOS mRNA expression in HPAEC. Thus, TNF-α was not critical in iNOS induction in HPAEC. [38,39]

Another striking difference in the up-regulation of iNOS mRNA was the fact that, unlike the up-regulation of iNOS in PPAEC which could be detected after 24 h, the up-regulation of iNOS in HPAEC produced via the combination of LPS and cytokines could only be detected after 8 h and not 24 h. This could be due to the difference in magnitude of up-regulation. These data were consistent with iNOS mRNA expression in human hepatocytes, in which the peak expression was detected after 8 h of treatment with LPS and cytokines and then decreased until becoming undetectable after 48 h.[5]

The fact that LPS alone induces ICAM-1—but not iNOS, Arg-1, or Arg-2—indicates that HPAEC themselves do not perform some of the functions of macrophages, such as the elimination of gram-negative bacteria via production of NO. Instead, HPAEC act as cells that facilitate cellular immunity by the up-regulation of adhesion molecules (ICAM), which attracts leukocytes to the site of inflammation. PPAEC could perform such a task, but not HPAEC.

LPS also showed dramatic differences in the induction of arginases in HPAEC and PPAEC. There are two types of arginase: the cytosolic Arg-1 is mostly expressed in the liver, while Arg-2 is expressed in extrahepatic tissues.[16] We found that HPAEC constitutively expressed only the Arg-2 isoform, while PPAEC expressed both Arg-1 and Arg-2. LPS up-regulated both Arg-1 and Arg-2 in PPAEC. Since ornithine, a product of arginase, could be involved in the synthesis of collagen and the regulation of polyamines, the up-regulation of these arginases suggested that PPAEC may respond better to LPS exposure in the context of wound healing. HPAEC did not up-regulate Arg-2 upon LPS exposure. In our attempt to induce iNOS, the addition of IFN-γ to LPS or cocktail actually led to down-regulation of Arg-2 in HPAEC. Ultimately, this illustrated that HPAEC responded to LPS and cytokines very differently from PPAEC.[8-10]

We also found different sensitivities between HPAEC and PPAEC to LPS. While even low concentrations of LPS were able to increase gene expression in PPAEC dramatically, they could tolerate LPS in high amounts as demonstrated by high survivability in LPS concentration that was very toxic to HPAEC. Less than 20% of HPAEC survived treatment with 100 μg/mL LPS. Could the lack of iNOS expression at high concentrations of LPS cause excessive cell death in HPAEC? Studies have shown that LPS induced apoptosis in ovine and bovine endothelial cells.[40-43] However, the apoptotic effects of LPS can be inhibited by the overexpression of iNOS via suppression

of caspase-3-like protease activity in sheep pulmonary artery endothelial cells.[44, 45] We speculated that the iNOS up-regulation also protected PPAEC from cell death at high concentrations of LPS, and, due to the lack of iNOS expression, HPAEC could not tolerate high LPS concentration. We showed that the presence of NOC-18, an external donor of NO, rescued HPAEC from LPS induced cell death.

In summary, we demonstrated that the same cell type from two species of mammals responded very differently to LPS. PPAEC were more responsive to LPS than HPAEC in regards to induction of genes in the L-arginine-NO pathway. PPAEC responded to low concentrations of LPS by induction of all of the genes studied: Arg-1, Arg-2, CAT-1, CAT-2B, and iNOS. In HPAEC, LPS could only induce CAT-2B. Due to the difference in up-regulation of iNOS, we found that PAECs could survive in a medium with the amount of LPS that was deadly to HPAEC. We showed that, with an external source of NO, HPAEC could also survive in that toxic media. The absence of NO production by iNOS in HPAEC could be the reason that most HPAEC died in concentrated LPS media. The different mechanisms in regulation of response to LPS between PPAEC and HPAEC are still to be investigated.

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