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The presence and activity of SP-D in porcine coronary endothelial cells depend on Akt/PI_3K, Erk and nitric oxide and decrease after multiple passaging

Mary Y.K. Lee[a], Grith L. Sørensen[b], Uffe Holmskov[b], Paul M. Vanhoutte[a,*]

[a] Department of Pharmacology, Li Ka Shing Faculty of Medicine, The University of Hong Kong, L2-48, Laboratory Block, 21 Sassoon Road, Pokfulam, Hong Kong
[b] Medical Biotechnology Center, Institute of Medical Biology, University of Southern Denmark, Winsløwparken 25.3, DK-5000 Odense, Denmark

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ABSTRACT

Surfactant protein D (SP-D) mediates clearance of microorganisms and modulates inflammation in response to cytotoxic stimulation. It is present in various epithelia, but also in vascular smooth muscle and endothelial cells. Experiments were designed to determine whether or not SP-D is present in porcine coronary arterial endothelial cells and if so, to investigate the molecular mechanisms underlying this presence. The expression of SP-D, NO synthase, Akt 1/2 and Erk 1/2 proteins was determined in cultures at passages 1 (#1) and 4 (#4). SP-D in primary cells existed in three isoforms (37–38 kDa and 50 kDa). The 37–38 kDa SP-D forms were the dominant isoforms in the porcine endothelium and were prominent at #1 but partially lost at #4. Tumor necrosis factor-α (TNF-α) significantly augmented the level of SP-D expression at #1 but not at #4. The basal level of 37–38 kDa SP-D isoforms at #1 was reduced by L-NAME, wortmannin and PD 98059. The low basal expression at #4 could be increased by DETA NONOate (donor of NO) or insulin (activator of PI_3K/Akt). The presence of nitric oxide synthase was reduced while that of Akt 1/2 and Erk 1/2 was increased at #4. In cells both at passages 1 and 4, TNF-α downregulated NO synthase and up-regulated p-Erk 1/2 protein. The present findings demonstrate the presence of SP-D in endothelial cells which is NO-, PI_3K/Akt- and Erk-dependent. They suggest a protective role of SP-D in these cells.

1. Introduction

Surfactant protein D (SP-D) is a collectin involved in the innate immune defense of epithelial surfaces including the lung (Motwani et al., 1995; Madsen et al., 2000). Pulmonary SP-D is produced by alveolar type II and Clara cells and secreted to the alveolar surface. The carbohydrate recognition domain of SP-D binds specifically to the surface of pathogenic microorganisms to initiate their elimination by aggregation, opsonization for phagocytosis or direct lysis of the microbial cell membrane (Holmskov et al., 2003; Kingma and Whitsett, 2006). In the mouse, the SP-D knockout phenotype is characterized by alveolar infiltrations with leucocytes and foam cells, increased levels of proinflammatory cytokines and metalloproteinases and the spontaneous development of emphysema. The production of SP-D increases during development, is under hormonal influences, and is up-regulated in pulmonary inflammatory diseases (Holmskov et al., 2003; Sorensen et al., 2007). Moreover, low systemic levels of SP-D are correlated to obesity in humans (Sorensen et al., 2006; Zhao et al., 2007).

SP-D is further localized to mucosal surfaces in non-pulmonary tissues like the gastrointestinal tracts and genital system (Bourbon and Chailley-Heu, 2001; Leth-Larsen et al., 2004). SP-D is also present in endothelial and vascular smooth muscle cells of humans and mice (Sorensen et al., 2005; Snyder et al., 2008). In human vascular smooth muscle cells, it exerts an anti-inflammatory action (Snyder et al., 2008). The role of SP-D in endothelial cells is unknown.

Nitric oxide produced by the endothelial cells helps to control vascular tone and maintain an anti-inflammatory and antithrombotic surface within the vasculature (Moncada et al., 1991; Flavahan, 1992; Vanhoutte, 2003). The turnover and resulting regeneration of endothelial cells *in vivo* can be accelerated by mechanical disruption (Shimokawa et al., 1987; Lee et al., 2007). Cultures derived from regenerated endothelium are dysfunctional with reduced NO synthesis, enhanced apoptosis (Lüscher and Noll, 1995; Tschudi et al., 1996; Asai et al., 2000; Vanhoutte et al., 2002; Lee et al., 2007), and genomic changes compatible with increased oxidative stress and reduced anti-coagulant properties (Lee et al., 2007). This dysfunction probably favors the development of atherosclerosis (Hoffmann et al., 2001; Brandes et al., 2005; Lee et al., 2007). The endothelial dysfunction of regeneration can be mimicked by multiple passaging *in vitro* as regards reduced

* Corresponding author. Tel.: +852 2819 9258; fax: +852 2817 0859.
E-mail address: vanhoutt@hkucc.hku.hk (P.M. Vanhoutte).

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nitric oxide bioavailability in terms of cyclic GMP production and increased oxidative stress (Rubin, 1997; Antropova et al., 2000; Shi et al., 2004). Akt and Erk are involved in the activation of eNOS and augmented the release of nitric oxide by various stimuli including shear stress, insulin and estrogens (Jin et al., 2005; Joy et al., 2006), resulting in the release of nitric oxide. They also promote endothelial survival and proliferation after inflammatory stimulation (Zhang et al., 2001; Secchiero et al., 2003). When the cells were activated by inflammatory cytokines, in particular tumor necrosis factor-α (TNF-α), both Akt and Erk are activated and mediate the signaling leading to stress-induced apoptosis (Secchiero et al., 2003; Chen and Easton, 2008).

The present experiments were designed to test the hypothesis that endothelial dysregulation would result in reduced SP-D expression. Endothelial dysregulation was induced by culture after multiple passaging and quantified by diminishing and inhibiting NO-synthase, and measuring Erk and Akt kinase activity. The experiments were performed on endothelial cells from the porcine coronary artery since the coronary circulation in that species closely resembles that of the human.

2. Materials and methods

2.1. Cell isolation and primary culture

The present study was approved by the Committee on the Use of Live Animals in Teaching & Research of The University of Hong Kong. Female pigs (weight 25–30 kg) were anaesthetized and euthanized by exsanguination. The heart was removed and placed in fetal bovine serum (FBS)-containing culture medium. The aorta and coronary arteries were excised, cleaned of surrounding fat and connective tissue, and opened longitudinally. Endothelial cells were harvested by gentle scraping of the luminal surface using a scalpel blade (Lee et al., 2007). They were either studied without further culture (both aortic and coronary endothelial cells) or seeded (coronary endothelial cells only) on collagen type I-coated culture plates for mRNA and protein analysis (Lee et al., 2007). The cultures were maintained in Eagle's minimal essential medium (Gibco-BRL; Grand Island, NY, USA) containing 10% FBS, and penicillin–streptomycin (100 unit/ml) and kept at 37 °C, 95% humidity, 5% CO_2. The medium was changed every 48 h until the cells reached confluence (passage 0). Cells were detached for passaging with trypsin–EDTA and were passaged on a weekly basis at a ratio of 1:3 to produce cells at passage 1 (#1).

Cultured cells were passaged (split at 1:3 ratio; 100 mm^2 plate) further weekly until cells at passage 4 (#4) were obtained. After reaching 70–80% confluence (which takes 2–3 days after subculture), cells were changed into FBS-free medium for 30 min prior to drug treatment. Cells at #1 and #4 were exposed to TNF-α (10 ng/ml) for 2 h. For inhibition experiments, cells at #1 were treated with either N-nitro-L-arginine methyl ester (L-NAME; 100 μM), wortmannin (20 nM) or PD 98059 (50 μM) for both 2 h and 4 h. For stimulation experiments, diethylenetetraamine NONOate (DETA NONOate; 100 μM) and insulin (1 nM) were administered for 4 h in cells at #4. The presence of SP-D was detected by Western blotting after the various drug treatments.

2.2. Isolation of porcine lung homogenates for immunoblotting

Lung tissue was homogenized in buffer (10 mM Tris–HCl/100 mM NaCl, 0.25 M sucrose, 2 mM EDTA; pH 7.4) containing a cocktail of protease inhibitors (10 μM leupeptin; 10 μM pepstatin A and 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 200 × *g* at 4 °C for 10 min to remove cellular debris. The supernatant was centrifuged at 208,000 × *g* at 4 °C for 16 h. The pellet was resuspended in Tris-buffered saline (TBS) for immunoblotting. The protein content was quantified using the Bradford Assay.

2.3. RNA preparation and real time-PCR

The total RNA (~5 μg to 10 μg) from cells at #1 and #4, both under conditions basal and after TNF-α-stimulation was extracted with TRIZOL according to the manufacturer's instructions. One microgram of total RNA was added to the reverse transcription mixture [(20 μl; first-strand buffer, 10 mM DTT, 0.5 mM DNTPs, 10 ng/μl Oligo(dT) (Gibco-BRL; Grand Island, NY, USA), 1 unit/μl Rnasin;, 1 unit/μl moloney murine leukemia virus reverse transcriptase (M-MLV RT; Gibco-BRL, USA)] for 10 min at room temperature followed by 37 °C for 60 min. The product was denatured by placing at 94 °C for 7 min to produce the first-strand cDNA. One microliter of the total reverse transcription product was added to PCR reaction mixture (20 μl) containing 10 μl 2× SYBR® Green PCR master mix (Applied Biosystems, UK) and the primers (sense and anti-sense; 1 μM) for the polymerase chain reaction (PCR). Real-time PCR technique was used to determine the mRNA expression. The PCR products were amplified using a primer pair of sequence specific oligonucleotides for porcine SP-D carbohydrate recognition domain (CRD): 5′-CGG AGG GCA ATT TCA CCT AC-3′ and 5′-TGG CCA GCA GAA GGT CAC-3′ (257 bp) (van Eijk et al., 2000) and GAPDH, 5'-AATGACCCCTTCATTGACCTCC-3' and 5'-GCTTCCCATTCTCAGCCTTGAC-3' (100 bp).

Unknown samples and gene-specific PCR products for the standard (porcine SP-D CRD at different dilutions) were amplified using a 7900HT Fast Real-Time PCR System (Applied Biosystems; Foster City, CA, USA). The cycling conditions to amplify PCR products of SP-D CRD and GAPDH when using real time PCR were 50 °C for 2 min; 95 °C for 10 min; 95 °C for 15 s, 60 °C for 1 min; and 95 °C for 15 s. Each sample was studied in duplicates. Results were normalized to the copy numbers of GAPDH gene products in the samples. The PCR products were visualized on 1.2% (w/v) agarose gels using ethidium bromide to ensure that the PCR products produce a single band at the expected size.

2.4. Western blotting

After treatment with various blockers or activators, cells were washed twice with cold phosphate buffer solution and then lysed in ice-cold lysis buffer (20 mM Tris–HCl, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate) containing a cocktail of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 100 ng/ml trypsin inhibitor, 20 μg/ml leupeptin and 1 μM pepstatin). The protein concentration was determined using the Bradford Assay. Thirty to 40 μg of total protein were separated on a polyacrylamide gel (10%) and blotted on nitrocellulose membranes (200 mA, 1.5 h). The blot was incubated for 1 h in TBS containing 5% fat-free milk. Membranes were incubated with antibodies at 4 °C overnight. This was followed by incubation of the HRP-labeled secondary antibody (Amersham; Freiburg, Germany) prior to image detection by enhanced chemiluminescence using a commercially available kit (Amersham). For SP-D, the membranes were incubated with 10 μg/ml mAb 1.7 anti-porcine SP-D (Soerensen et al., 2005) overnight and then with alkaline phosphate-coupled goat anti-mouse immunoglobulin (DAKOCytomation; Glostrup, Copenhagen, Denmark) for 2 h. The SP-D band was developed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) tablets (Roche, Penzberg, Germany). For reprobing with antibodies, the nitrocellulose membranes were incubated at 50 °C for 30 min in a buffer containing Tris/HCl