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Author(s)	Lee, MYK; Sørensen, GL; Holmskov, U; Vanhoutte, PM
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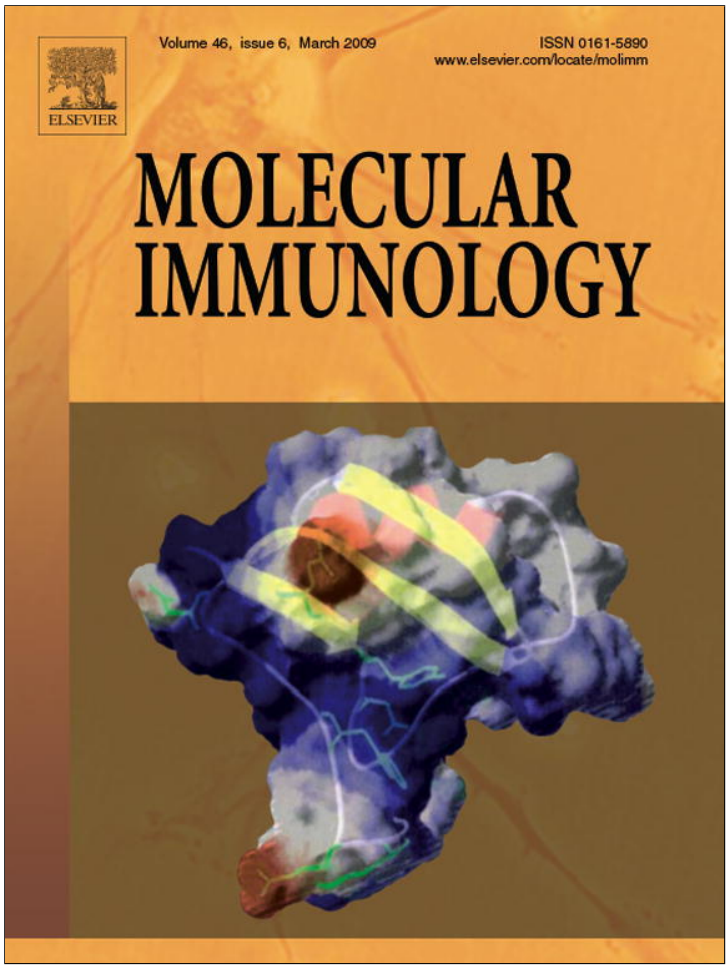

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The presence and activity of SP-D in porcine coronary endothelial cells depend on Akt/PI_3K, Erk and nitric oxide and decrease after multiple passaging

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 anti-thrombotic 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

(67.5 mM, pH 6.8); β-mercaptoethanol (100 mM), and sodium dodecyl sulfate (SDS; 2%, w/v). After extensive washing, the filters were incubated in blocking buffer and subsequently with the primary antibody and developed (Fisslthaler et al., 2003).

2.5. Data analysis

Data are expressed as means ± S.E.M. The Western blot image intensity was calculated by the Multi-Analysis computerized program (Multi-Analyst version 1.1; Bio-Rad Laboratories, Inc.; Hercules, CA, USA). Densitometric analysis was normalized with the respective house-keeping gene, GAPDH, or the protein, β-actin. Statistical analysis of the data was performed using Student's *t*-test for paired or unpaired observations. *P*-values equal to or less than 0.05 were considered to indicate statistically significant differences.

2.6. Drugs and reagents

Recombinant human TNF-α was obtained from Invitrogen (Carlsbad, CA, USA). Antibody against porcine SP-D was a kind gift from Peter Heegaard (Danish Veterinary Institute, Copenhagen, Denmark). The antibody against Erk 1/2 (1:1000), captopril and the Erk/MEK inhibitor PD 98059 were purchased from Calbiochem (Behring, La Jolla, CA, USA). Antibodies against protein kinase B or Akt 1/2 (1:1000) and phospho-Erk 1/2 (1:1000) were from Cell Signaling (Beverly, MA, USA). Antibodies against beta-actin (1:2000), insulin, L-NAME and wortmannin were obtained from Sigma (St. Louis, MO, USA). Diethylenetetraamine NONOate was purchased from Cayman (Ann Arbor, MI, USA). The antibody against endothelial nitric oxide synthase (eNOS; 1:2500) was from Transduction Laboratories (Lexington, KY, USA).

3. Results

3.1. Localization of SP-D in porcine endothelial cells

In the lung homogenates, immunoblotting using the monoclonal antibody against porcine SP-D detected a band with a molecular weight of 50 kDa as previously reported (van Eijk et al., 2002). In freshly collected coronary endothelial cells (Fig. 1) two bands at 37–38 kDa and a single band at 50 kDa were detected by the monoclonal antibody. In addition, immunoreactive proteins with estimated molecular masses lower than the mass of the intact protein appeared in the blots at 27–28 kDa and at 19 kDa with varying intensity. The 37–38 kDa variants were the predominant forms at #1 (Fig. 1) and 4 (data not shown). The 50 kDa variants were detected only in freshly collected cells but not in cultured cells. Therefore, only the intensity of the 37–38 kDa variants was analyzed before and after treatments. Cross-reactivity between the monoclonal anti-SP-D antibody and components in the cell culture medium was tested using a four times concentrate or FBS. No cross-reactivity was observed (data not shown).

3.2. Basal and TNF-α induced levels of endothelial SP-D expression is reduced with successive passaging

The endothelial mRNA expression of SP-D was reduced significantly (by 50%) at #4 compared to #1 (Fig. 2a). Incubation with TNF-α (10 ng/ml) for 2 h significantly augmented (by 200%) the expression of SP-D mRNA at #1. At #4, TNF-α no longer caused a significant increase in the expression of SP-D using the same treatment conditions (Fig. 2b).

The expression level of SP-D protein was significantly reduced (by 40%) at #4 compared to #1 (Fig. 3a). TNF-α augmented the

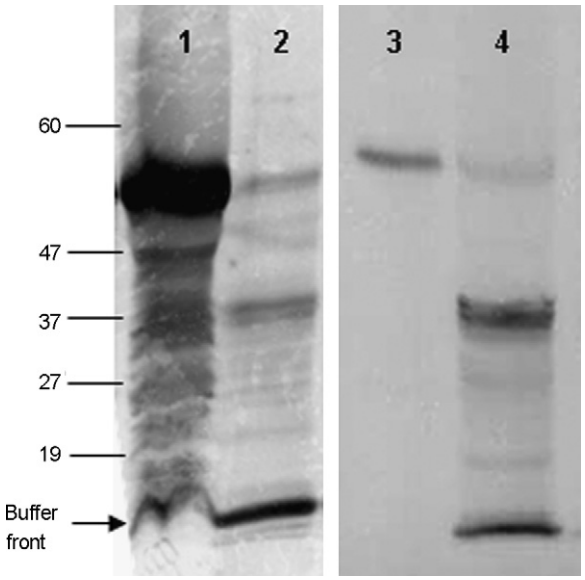

Fig. 1. Basal detection by Western blotting of SP-D in porcine lung lysates, freshly collected porcine coronary endothelial cells before and after primary cell culture (#1). Lanes 1 and 3, lung lysates; lane 2, freshly collected coronary endothelial cells without culture; lane 4, cultured coronary endothelial cells at #1.

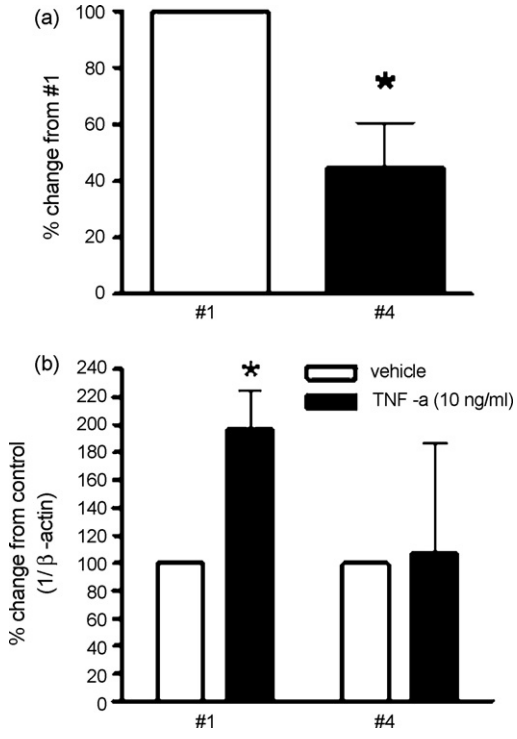

Fig. 2. Basal and stimulated SP-D gene expression in cultured porcine coronary endothelial cells at #1 and #4. Total RNA from cultured endothelial cells with or without treatment was harvested for the detection of SP-D carbohydrate-recognition domain (CRD) gene by real time-PCR. Genetic sequence encoding for the CRD region of porcine SP-D was selected to give a PCR product of 257 bp. (a) Basal genetic expression of SP-D CRD at passages (#) 1 and 4. Data are expressed as % change and shown as mean ± S.E.M. of the basal level at #1. (b) Stimulated level of SP-D CRD gene by incubation with tumor necrosis factor α (TNF-α; 10 ng/ml) for 2 h in endothelial cells at passages (#) 1 and 4. Data are expressed as % changes ± S.E.M. from the corresponding vehicle control of the same passage. The asterisk indicates a statistically significant ($P < 0.05$) difference from the corresponding control ($n = 3–5$).

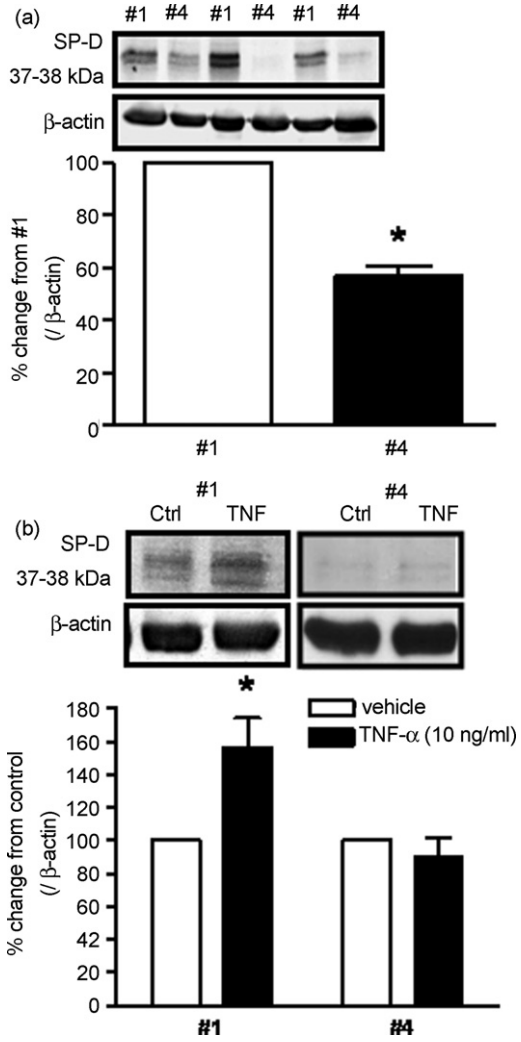

Fig. 3. Basal and stimulated SP-D protein presence in cultured porcine endothelial cells at #1 and #4. Total protein from cultured endothelial cells with or without treatment was harvested, lysed, and processed for SP-D protein detection using immunoblotting analysis. A monoclonal antibody against porcine SP-D was used to probe the SP-D monomer at approximately 37–38 kDa. The membrane were stripped and reprobed with antibody against β-actin for normalization of sample loading. The representative SP-D protein expression is shown as determined by densitometric analysis. (a) Upper: the basal SP-D protein at #1 and #4. Lower: data expressed as % change and shown as mean ± S.E.M. from the basal level at #1. (b) Upper: stimulated level of SP-D protein after incubation with TNF-α (10 ng/ml) for 2 h under serum-free condition in endothelial cells at #1 and #4. Ctrl, vehicle; TNF, TNF-α-treated. Lower: data expressed as % change and shown as mean ± S.E.M. of the corresponding vehicle control from the same passage. The asterisk indicates a statistically significant ($P < 0.05$) difference from corresponding control ($n = 5–7$).

expression level of SP-D protein significantly at #1 (by 170%), but not at #4 (Fig. 3b).

3.3. Porcine endothelial SP-D expression is dependent on endothelial nitric oxide synthase, Akt 1/2 and Erk 1/2

Porcine coronary endothelial cells were treated with various types of metabolic inhibitors under serum-free medium and time-dependent responses were obtained at 2-h and 4-h intervals. The SP-D protein expression was diminished significantly by incubation with the nitric oxide synthase inhibitor, L-NAME (100 μM) (55% reduction, $P < 0.05$), the phosphatidylinositol-3 kinase/Akt inhibitor, wortmannin (20 nM) (75% reduction, $P < 0.01$), and the Erk/MEK inhibitor, PD 98059 (50 μM) (60%, $P < 0.05$) at the 4-h interval (Fig. 4a). The reduction in SP-D expression at #4 was reversed partially by incubation for 4 h with either the exogenous NO donor, DETA NONOate (100 μM) or the PI_3K/Akt activator insulin (1 nM) (Fig. 4b).

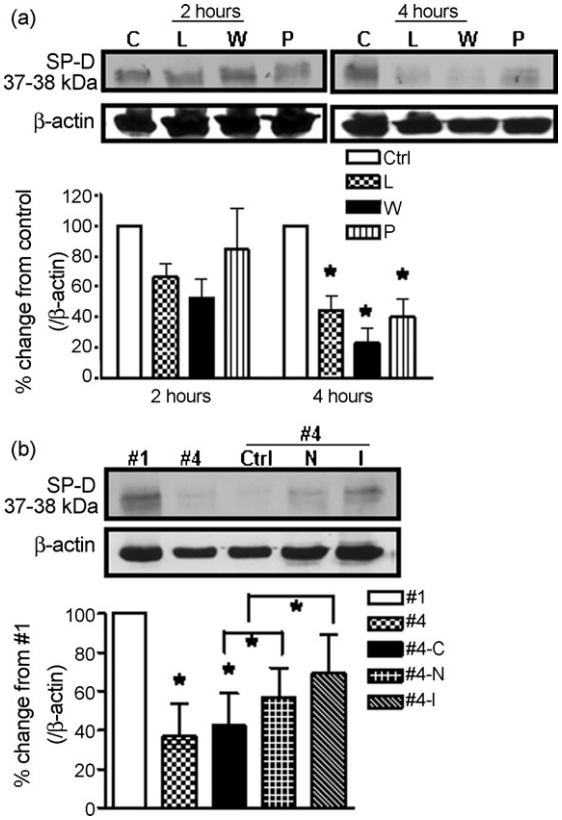

Fig. 4. Effects of inhibitors of nitric oxide synthase, PI_3K/Akt 1/2 and Erk 1/2 on the basal expression of SP-D protein at #1 and effects of a nitric oxide donor and an activator of PI_3K/Akt on SP-D at #4. (a) Upper: endothelial cells at #1 were treated with L-NAME (100 μM), wortmannin (20 nM) and PD 98059 (50 (μM) under serum-free conditions at 2-h and 4-h interval. C, vehicle; L, L-NAME; W, wortmannin; P, PD 98059. Lower: data expressed as % change and shown as mean ± S.E.M. of the corresponding vehicle control from the same passage ($n = 3$). (b) Upper: endothelial cells were sub-cultured until #4 and DETA NONOate (100 μM) and insulin (1 nM) were supplemented under serum-free conditions for 4 h. C, vehicle; N, DETA NONOate; I, insulin. Lower: data expressed as % change and shown as mean ± S.E.M. of the corresponding vehicle control or basal level at #1 ($n = 3$). No significant difference was found between basal and vehicle-treated SP-D level at #4. The asterisk indicates a statistically significant ($P < 0.05$) difference from corresponding control.

Western blotting also revealed a significantly reduced basal expression of eNOS protein at #4 compared to #1 (Lee et al., submitted). By contrast, the basal protein levels of both Akt 1/2 and Erk 1/2 were increased significantly at #4 (Fig. 5a and b). Treatment with TNF-α for 2 h of cells at #1 significantly reduced the protein level of eNOS but significantly increased that of Akt 1/2 and p-Erk 1/2 (Fig. 6a–c), compared to the corresponding vehicle controls.

4. Discussion

SP-D is a large and complex protein which exists as structural variants with different proportions of trimers, dodecamers, and high multimers (Crouch et al., 1993) probably due to the individual polymorphic variation (Leth-Larsen et al., 2005). Different oligomeric forms of SP-D result in different functions. Thus, SP-D of high molecular weight binds preferentially to intact viruses and bacteria, whereas the low MW SP-D interacts with lipopolysaccharide (Leth-Larsen et al., 2005; Hartshorn et al., 2007) and this may lead to differential contribution to inflammation of the dif-

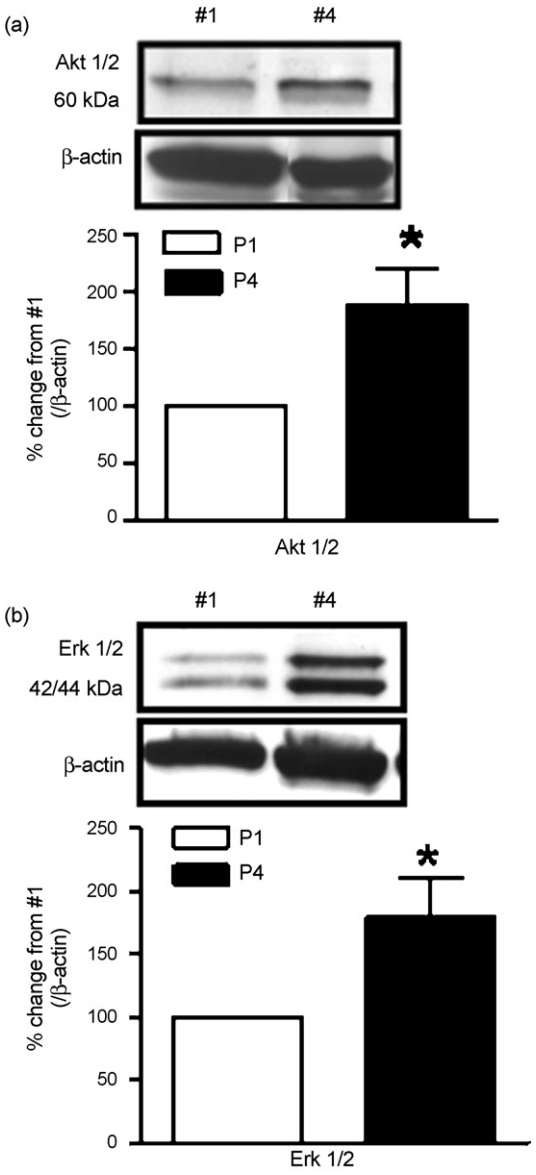

Fig. 5. Basal detection of (a) Akt 1/2 and (b) Erk 1/2 proteins in cultured porcine coronary endothelial cells at #1 and #4. Upper: representative Western blots. Lower: data of 5–7 independent experiments expressed as % change between #1 and #4 and shown as means ± S.E.M. of the corresponding basal level at #1. The asterisk indicates a statistically significant ($P < 0.05$) difference from corresponding control.

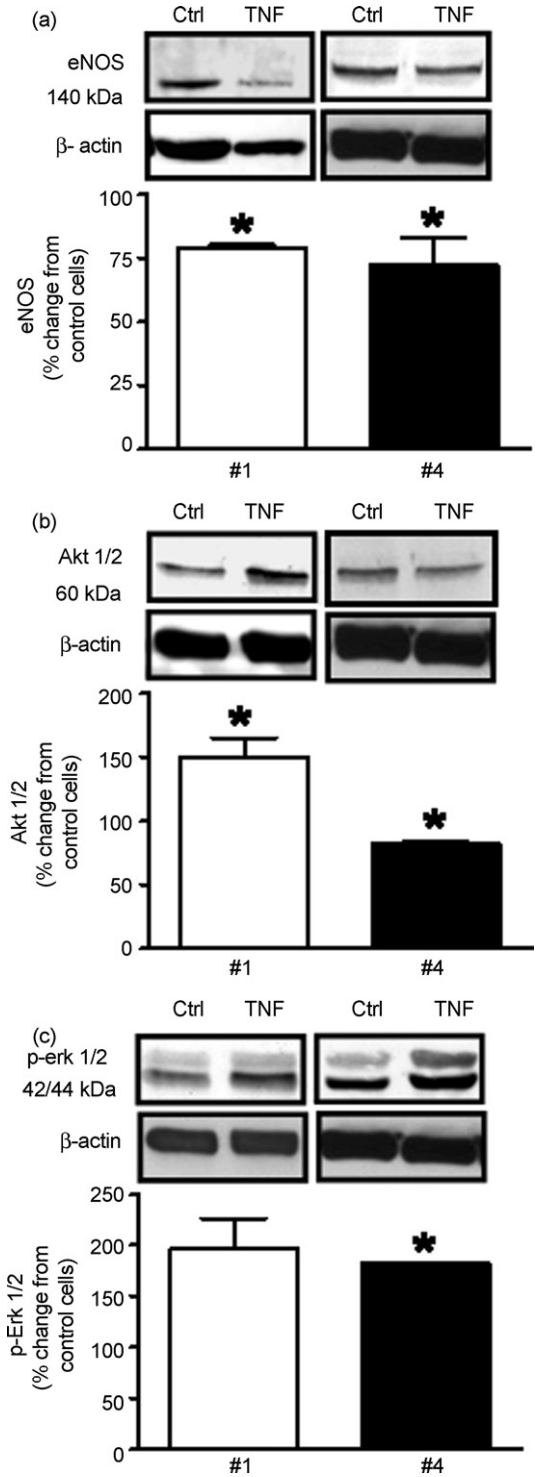

Fig. 6. Detection of (a) eNOS, (b) Akt 1/2 and (c) p-Erk 1/2 proteins in cultured porcine coronary endothelial cells at #1 after stimulation with tumor necrosis factor α (TNF-α; 10 ng/ml) for 2 h under serum-free condition. Upper: representative Western blots; Ctrl, vehicle; TNF, TNF-α-treated. Lower: data expressed as % change and shown as means ± S.E.M. of the corresponding vehicle control at #1. The asterisk indicates a statistically significant ($P < 0.05$) difference from corresponding control ($n = 3–6$).

ferent molecular SP-D forms. However, SP-D may also act in a dual manner, to enhance or suppress inflammatory mediator production depending on its binding orientation with the effectors (Gardai et al., 2003) as well as the inflammatory status of the host (Janssen et al., 2008). The present study demonstrates the presence of three isoforms (37–38 kDa and 50 kDa) in endothelial cells. SP-D has likewise been detected in human alveolar lavage (43 kDa) with a higher molecular weight than the 37 kDa variant present in human endothelial cells (Sorensen et al., 2005). The 50 kDa form of SP-D in the porcine coronary endothelial cells was consistent with the native SP-D being detected in lung of the same species (van Eijk et al., 2002). However, in primary culture of porcine coronary endothelial cells, the 50 kDa, 27–28 kDa and probably the 19 kDa breakdown products were detected at a minimal level while the 37–38 kDa form was dominant. This 37–38 kDa bands could be due to proteolytic degradation or alternative glycosylation or a representative splice variant form of the intact SP-D polypeptide found in freshly harvested cells. Due to the low detection level of the native 50 kDa SP-D in cultured endothelial cells, the further studies focused on the 37–38 kDa protein, since it was the dominant SDS-PAGE product in the present experimental setting. Both the mRNA

expression and the 37–38 kDa SP-D protein expression decreased from #1 to #4. To further understand the possible mechanism leading to the basal expression of SP-D in endothelial cells at #1, the effects of various blockers, known to cause changes in endothelial responses compatible with dysfunction, were examined on the expression level of the protein. Since this level was reduced when the cells were treated with L-NAME [inhibitor of eNOS; Grumbach et al., 2005], wortmannin [inhibitor of PI_3K/Akt; Bernier et al., 2000] and PD 98059 [inhibitor of Erk 1/2; Bernier et al., 2000], nitric oxide, phosphotidylinositol-3 kinase (PI_3K/Akt) and Erk must contribute to maintain the basal presence of SP-D protein in endothelial cells. Supplementation with exogenous NO [by DETA NONOate; Braam et al., 2004] or treatment with insulin [which activates PI_3K/Akt; Fisslthaler et al., 2003] at #4 significantly counteracted the effects of *in vitro* passaging on the expression of the protein. Thus, the present observations reveal the importance of nitric oxide in permitting the basal expression of SP-D in native/young endothelial cells, a role that diminishes with multiple passaging. To date, it is still not clear whether or not SP-D can be taken up by endothelial cells from the systemic circulation especially since nitric oxide can serve as a facilitator to initiate endocytosis in these cells (Lowenstein, 2007). Since no protein presence of SP-D could be detected in the culture medium and FBS, the possibility of its presence by endocytosis in endothelial cells under culture conditions can be eliminated, at least in the present study.

The development of atherosclerosis involves a complex process of inflammatory and atherothrombotic events which result in part from endothelial dysfunction and involves an inflammatory response (Ross, 1999; Koh et al., 2005 Libby, 2008). Obesity is one of the factors that may lead to endothelial dysfunction (Meyers and Gokce (2007)). Low systemic levels or even lack of SP-D are associated with obesity in mice and humans, respectively (Sorensen et al., 2006; Zhao et al., 2007). However, the relation between SP-D levels, obesity and endothelial dysfunction has not been investigated. In human vascular smooth muscle cells, SP-D may play an anti-inflammatory role. Thus, overexpression of SP-D in these cells enhanced uptake and clearance of pathogens like *C. pneumoniae* while the production of IL-8, a proinflammatory mediator, was attenuated (Snyder et al., 2008). The level of SP-D augmented in response to TNF-α and lipopolysaccharide, suggesting a protective immunomodulating role in inflammatory situations (Snyder et al., 2008). By contrast to the observations in human vascular smooth muscle, data obtained in genetically modified mice suggests that SP-D may play a pro-atherogenic role. Indeed, atherosclerotic lesions are reduced in homozygous $SPD^{-/-}$ mice fed an atherogenic diet (Sorensen et al., 2005). Lower serum TNF-α and higher HDL-C levels were measured in the $SPD^{-/-}$ compared to wild type mice on the same diet, suggesting a modified lipid metabolism, reduced stimulation of inflammatory processes, and a pro-atherogenic role for SP-D (Sorensen et al., 2005) in that particular mouse model. The differential effects of SP-D on inflammatory responses observed in the mouse (Sorensen et al., 2005) and in human tissues (Snyder et al., 2008) may reflect species differences and effects specific for the chosen model system. The present data obtained in porcine endothelial cells are in line with those reported in human vascular smooth muscle rather than with the findings in genetically modified mice.

One of the important physiological roles of the endothelium is to release nitric oxide as a major factor to control the tone of the underlying vascular smooth muscle and to prevent events leading to formation of the atherosclerosic plaque (Moncada et al., 1991; Joannides et al., 1995; Vanhoutte, 1997). Endothelial cells undergo aging both *in vivo* and *in vitro* (Rubin, 1997). Such aged cells are viable and metabolically active but display altered gene and protein expressions, resulting in phenotypic changes (Fournet-Bourguignon et al., 2000; Brandes et al., 2005). In endothelial cells at #4, the eNOS protein presence is reduced (Vanhoutte et al., 2002), indicating a decreased capability to produce nitric oxide, and thus endothelial dysfunction. Akt 1/2 and Erk 1/2 are involved in insulin-related and extracellularly regulated signaling pathways, respectively, to modulate longevity (Secchiero et al., 2003; Qiao et al., 2004) and promote proliferation. They also activate eNOS to release nitric oxide (Jin et al., 2005; Joy et al., 2006). Nevertheless, the Akt activity increases with cellular senescence and its inhibition extends the lifespan of primary cultured endothelial cells (Miyauchi et al., 2004). The mechanism by which Akt affects cellular lifespan in primary cell cultures is complex due to its interactions with several components of the cell cycle and the regulation of cell death (Zhang et al., 2001). Nonetheless, it is still not clear why in the present study the protein presence of Akt 1/2 and Erk 1/2 are increased at #4 despite the reduced protein presence and activity of nitric oxide synthase.

TNF-α, an inflammatory cytokine, can be produced under various pathological conditions contributing to endothelial dysfunction and atherosclerosis (Krasinski et al., 2001; Sack, 2002; Makino et al., 2005; Chen and Easton, 2008). The up-regulation of SP-D in early passaged endothelial cells in response to TNF-α, observed in the present study both at the genomic transcriptional and proteomic levels may indicate that SP-D plays an immunomodulating role in host cell defense mechanisms in response to vascular stress or injury in order to reduce inflammation. The involvement of SP-D in modulating the response in association with nitric oxide may help to prevent the shift of nitrosylated components under inflammatory conditions (Atochina et al., 2004). This phenomenon is not observed in cells at #4. In line with other studies, the protein expression of endothelial nitric oxide synthase is reduced in endothelial cells at #1 (Lee et al., submitted) and #4 when treated with TNF-α, suggesting a detrimental effect on nitric oxide production by cytotoxic stimulation (Makino et al., 2005). Treatment with TNF-α for 2 h also induced a significant increase in Akt 1/2 and p-Erk 1/2 in endothelial cells at #1, promoting cell survival (Secchiero et al., 2003). The inability to up-regulate SP-D by TNF-α in cells at #4 might be explained by the reduction in eNOS protein despite activation of p-Erk 1/2 (Hoffmann et al., 2001; Fisslthaler et al., 2003; Secchiero et al., 2003). As a consequence, the progressive loss of nitric oxide (Csiszar et al., 2004) after multiple passaging permits the expression of proinflammatory mediators, a stage at which the protein presence of SP-D is reduced concomitantly.

In summary, the present data indicate that SP-D is expressed in porcine coronary artery endothelial cells in two or three isoforms. The basal expression level can be augmented in response to TNF-α. The expression of SP-D protein requires the production of nitric oxide, and is also Akt- and Erk-dependent. Thus, the lower level of SP-D protein observed in cultured endothelial cells at #4 is probably related to the reduced production of nitric oxide. These results suggest a possible anti-inflammatory role of SP-D in endothelial cells, with its role being diminished in dysfunctional cells, as obtained by multiple passaging. These findings support a favorable role of SP-D in curtailing processes in the vascular wall facilitating the occurrence of atherosclerosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2008.09.027.

Appendix B. Supplementary data

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