Bedside assessment of pulmonary endothelial function

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Abstract - The pulmonary endothelium (PE) is a highly active metabolic organ, possessing numerous physiological and pharmacological properties necessary for the maintenance of homeostasis in both pulmonary and systemic circulations. PE metabolic function in health and disease may be assessed by means of several methods. Among these methods, estimating pulmonary endothelial angiotensin converting enzyme activity by indicator dilution-type techniques, allows direct measurements of enzyme kinetic indices, and provides a means of estimating and quantifying pulmonary endothelial function at the bedside. We provide detailed information on these techniques and discuss related studies in the normal and the diseased human lung.

Keywords - Pulmonary metabolism, pulmonary endothelium, angiotensin converting enzyme, indicator-dilution technique

Introduction
Endothelium is the intimal lining of all blood vessels, composed of a single continuous layer of squamous epithelial cells, called endothelial cells (EC). Until the 1970s vascular endothelium was mainly credited for being part of a semi-permeable barrier that separates blood from the surrounding tissues and, in the lungs, blood from air. At that time however, methods of EC isolation and culturing were developed, resulting in a burst of endothelial cell culture studies [1]. These investigations along with pulmonary metabolism studies performed in isolated perfused lung preparations, or intact animals in vivo, have since provided extensive information on pulmonary endothelial physiology and pathobiology. As a result, it is now well recognized that the vascular endothelium is a metabolically dynamic organ exhibiting different phenotypes (depending on the organ and vascular bed), which possesses numerous pharmacological, physiological and immunological functions [1,2]. In the human lung, endothelial cells occupy a surface area of approximately 130 m² [1]. The location of the lungs along with the tremendous surface area of the pulmonary capillary endothelium make the latter a strategically positioned metabolic filter; through this filter the entire blood volume passes, getting enriched with and cleaned from several bioactive compounds, before it enters into the systemic circulation [3,4]. Thus, pulmonary endothelial functional and structural integrities are essential for adequate pulmonary and systemic cardiovascular homeostasis. Disruption of this integrity will lead and/or further contribute to acute and chronic lung vascular pathologies [3-5].

Pulmonary endothelial metabolic properties
Major pulmonary endothelial functions include, i. synthesis and release of vasoactive compounds carrying endocrine and paracrine properties, such as angiotensin II (AT II), prostacyclin, thromboxane, nitric oxide (NO), and endothelins; ii. clearance of vasoactive compounds such as endothelin-1 (ET-1); iii. expression of enzymes such as angiotensin converting enzyme, endothelin converting enzyme, several nucleotidases, NO synthase and lipoprotein lipase; iv. expression of receptors and signal transduction molecules; v. removal and biotransformation of drugs; vi. regulation of coagulation and thrombolysis; vii. participation in immune reactions; viii. binding of immune complexes; ix. interaction with bacteria (phagocytosis) and blood components such as leukocytes and platelets; x. participation in local vasoregulation; xi. expression of adhesion molecules; xii. production of cytokines, chemokines, and growth factors; and xiii. endothelial barrier regulation. For a detailed analysis of these most important endothelial functions, the reader is referred to [3].

Most of the above-mentioned functions are constitutive while others, such as the expression of adhesion molecules and/or receptors to circulating immune complexes, are induced upon endothelial exposure to various noxious and pro-inflammatory stimuli. Under health, the major pulmonary endothelial properties are related to a) the promotion of anti-aggregation and haemofluidity, b) barrier function enforcement, and c) the synthesis, metabolism or clearance of vasoactive compounds. Substances that promote vasoconstriction (such as ET-1 and AT II) usually carry additional pro-inflammatory and proliferative properties, while vasodilators (such as prostacyclin and NO) usually carry anti-inflammatory and anti-proliferative properties [3].

Many of these metabolic functions require the presence of ectoenzymes located on the luminal EC surface, with their
catalytic sites exposed to the bloodstream. Due to their location, they can interact with blood-borne substrates or inhibitors without the time and energy expense required for interactions with cytosolic enzymes. One such ectoenzyme is the angiotensin converting enzyme (ACE) [1,2].

Angiotensin converting enzyme is a monomeric zinc dipeptidyl carboxypeptidase that hydrolyzes the conversion of the decapeptide angiotensin (AT) I to the octapeptide AT II, as well as the degradation of the nonapeptide bradykinin [6]. AT II is a potent vasoconstrictor; it acts on vascular smooth muscle cells, interacts with the nervous system and causes volume expansion and fluid retention. At cellular level, AT II promotes migration, proliferation and hypertrophy. Bradykinin promotes vasodilation mainly through production of NO, arachidonic acid products and endothelium-derived hyperpolarizing factor. ACE appears thus to regulate the balance between the vasodilatory properties of bradykinin and the vasoconstrictive properties of AT II, promoting vascular tone and acting as a regulator between the rennin-angiotensin and the kalikrein-kinin systems. ACE is widely distributed as both a membrane-bound ectoenzyme and as a soluble one [7]. ACE molecules are uniformly distributed throughout the endothelial plasma membrane, including the membrane caveolae [8]. Due to the tremendous capillary endothelial surface and high enzyme concentrations [2], the pulmonary microvascular bed appears to be the major site of ACE activity in the body [7].

Additional pulmonary endothelial ectoenzymes are expressed in different species [9], including: 5'-nucleotidase (NCT) responsible for the dephosphorylation of extracellular adenosine-5'-monophosphate (5'-AMP) to adenosine, a molecule with vasodilatory and anti-thrombogenic properties; various nucleotidas; aminopeptidase P, which contributes to the breakdown of bradykinin; lipoprotein lipase, which hydrolyzes triglycerides to fatty acids [9]. The most important endothelin converting enzyme (ECE) is expressed in several isoforms, and produces endothelin-1 (ET)-1, ET-2 and ET-3 from their precursor big endothelin [3]. ET-1 is the most potent vasoconstrictor circulating in the body, also carrying pro-inflammatory and proliferative properties. The lung vascular bed is an important site for both the production and clearance of ET-1 via the endothelial ET B receptor [10]. In the human, there is under normal conditions a dynamic balance between ET-1 clearance and release in the pulmonary circulation [11].

Assessment of pulmonary endothelial metabolic function

Quantitative estimation of the pulmonary endothelial metabolic function can be achieved in vivo, ex vivo or in situ, by means of two methods. The first is by estimating the transpulmonary gradient of a substrate or an inhibitor of an enzyme, or of a substance that is cleared by the pulmonary circulation. This is achieved by measuring the inflow and outflow concentrations of the compound under investigation in mixed venous and aortic blood. The second is by means of radioisotopic indicator-dilution type techniques. Both methods provide information on the metabolism or extraction from the pulmonary circulation of a certain compound. However the former method although easier in its application provides limited information [1]. In contrast, the more sophisticated but arduous indicator-dilution technique can differentiate between endothelial dysfunction at the capillary level and loss of functional capillary surface area; it additionally provides information on lung transit time, volume of distribution of a compound, as well as measurements of blood flow [1,3,7,12].

The transpulmonary gradient technique, although “crude”, has been in use in both animal and human investigations, mostly focusing on chronic lung pathologies. In this respect, Stewart et al. [13] estimated arterial and venous plasma concentrations of ET-1 in patients suffering from pulmonary hypertension (PH) of diverse aetiologies and showed that such patients exhibit higher venous ET-1 concentrations than normal volunteers; patients suffering from primary pulmonary hypertension appeared to have increased pulmonary production of ET-1, implying a pathogenic role of the latter in the increased pulmonary vascular resistance observed in this disease [13]. The aforementioned study was the first in the literature to provide evidence that ET-1 pathobiology is present in PH, opening the way for a tremendous amount of related research that led, among others, to the development of ET-1 receptor antagonist-based specific treatments [14].

Similar techniques have been also applied in the critically-ill. Patients suffering from acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) exhibited abnormal pulmonary ET-1 metabolism, expressed by an early decrease of the net balance between pulmonary ET-1 clearance and release, a phenomenon that was reversed in patients who subsequently recovered [15]. More recently, AT II formation and ET-1 clearance were assessed in ARDS patients when in supine or prone position. Positioning changes were followed by acute oxygenation improvement; however the estimated short-term pulmonary ET-1 net clearance and AT II net formation were not affected [16].

Indicator-dilution techniques for pulmonary metabolism assessment

i) Studies on the activity of endothelial carrier proteins

Initially, most research groups studied the removal of several biologically active compounds from the pulmonary circulation. These are carrier-mediated processes that require energy and a metabolically healthy pulmonary endothelium. Such compounds included the biogenic amines norepinephrine and serotonin, prostaglandin E 2, and propranolol [1]. Data interpretation of such studies has been complex, since it should take into consideration parameters such as related endothelial transport properties, possible release of metabolites into the bloodstream, binding to blood elements, as well as correct for changes in blood flow [1,17].

Several such indicator-dilution type studies were performed in humans, focusing on acute pulmonary injury. Dargent et al. [18] performed simultaneous measurements of serotonin and propranolol pulmonary extractions, before and after extracorporeal circulation applied during coronary bypass operations.
surgery. Serotonin extraction exhibited no changes, but propranolol extraction decreased post surgery while it increased in patients who received positive airway pressure (CPAP). Thus propranolol extraction appeared to be a more sensitive index of lung functional changes in this patient setting. In a similar respect, prostaglandin E1 and serotonin pulmonary removal were measured in patients undergoing cardiopulmonary bypass surgery and in patients suffering from ARDS [19]. No changes were seen in the former group, while the latter group exhibited decreases in both compound extractions that were attributed to ARDS-related endothelial injury. An additional study confirmed that serotonin pulmonary extraction decreases in patients with ARDS and correlates with the severity and the evolution of the syndrome [20]. However, such studies failed to predict which patients at risk for ARDS would subsequently develop the syndrome. At that time assessing lung serotonin metabolism was approached as a potential marker of early ARDS diagnosis in high risk patients [21]. The fact that such a validation was not established, combined with the aforementioned technical complexities, resulted in the loss of interest in this kind of study.

ii) Bedside assessment of ET-1 clearance by the pulmonary endothelium
Assessing the first passage pulmonary clearance of ET-1 by means of indicator-dilution techniques has been more recently introduced in humans. However, these studies have thus far focused on chronic lung pathologies such as PH, and have not yet provided substantial information on the critically-ill. Consequently, there will be no related analysis in this review. For detailed information on this methodology and its application the reader is referred to [11,22].

iii) Assays of endothelial ectoenzyme activity in vivo
Since the early 1980s attention has been focused on monitoring pulmonary capillary endothelial ectoenzyme activity [23]. The pulmonary capillary bed provides an ideal environment for the function of these enzymes because it offers very high enzyme concentrations due to the combination of low plasma volume and high surface area. The most common assay for measuring the activity of pulmonary endothelium-bound ectoenzymes in vivo is a modification of the indicator-dilution technique, originally introduced for cardiac output (CO) estimations. The most extensively studied ectoenzyme in animals, and the only one studied in humans, is the pulmonary endothelium-bound ACE. This technique is based on measuring the transpulmonary hydrolysis of a synthetic, radio-labelled, highly specific for ACE substrate [24], and should be viewed as a clinical pharmacological application that allows estimations of pulmonary capillary endothelium-bound (pulmonary endothelial; PE) ACE activity. For a detailed analysis of the methodology used, the reader is referred to [7,12].

iv) Enzyme activity indices
PE-ACE activity is estimated as substrate transpulmonary hydrolysis (v) and percent metabolism (%M) [12,25-27]. Both v and %M should be seen as reflections of ACE activity per capillary. Hydrolysis (v) has the advantage of being directly proportional to the three factors that determine product formation under first order reaction conditions: [E], t, and kcat/Km standing for capillary enzyme concentration, reaction time (capillary mean transit time), and the ratio of the catalytic rate constant vs the Michaelis-Menten constant respectively [12]. Percent metabolism (%M) has the advantage of being a parameter more familiar to investigators and clinicians.

Further analysis of the data obtained [28] allows estimations of the functional capillary surface area (FCSA) previously known as Amax/Km . FCSA is proportional to the enzyme mass available for reaction and kcat/Km reflecting ACE activity per vascular bed. Under normal conditions, FCSA is an index of dynamically-perfused capillary surface area (DPCSA; i.e. surface area that is perfused and metabolically active) [8,12,28,29]. Under toxic conditions FCSA is related to the existing enzyme quantity and quality (i.e. to enzyme mass that has survived injury and participates in the reaction and the probably altered enzyme kinetic constants). For a more detailed analysis of the methodology and the pharmacological equations used the reader is referred to [12].

Pulmonary endothelial ACE activity under normal conditions

i) Animal studies
In vivo assays under normal conditions have established the normal range of PE-ACE activity in several animal models, and allowed estimations of the corresponding pulmonary capillary vascular bed. The correlation between changes in Amax/Km (now termed FCSA) and DPCSA has been demonstrated in rabbits, dogs, guinea pigs and sheep in vivo, and in isolated lung preparations [7,30]. This correlation is valid only if PE-ACE kinetic constants are not affected by increases of pulmonary blood flow [12,28], which is indeed the case as proven in animal studies where co-injecting a substrate and an inhibitor at different blood flows revealed unchanged catalytic and binding ACE properties [1,31].

ii) Bedside assessment of pulmonary endothelial ACE activity under normal conditions
The PE ACE activity indicator-dilution technique was assessed at the bedside, in subjects with no lung disease [12]. They all received two rapid bolus injections of an ACE synthetic substrate through a pulmonary artery catheter: one into the vascular bed of both lungs; the other into the vascular bed of one lung (the right lung in all but one). Similar transpulmonary substrate hydrolysis and %M were observed in one and both lungs, suggesting among others homogeneous PE-ACE concentrations and capillary transit times in both human lungs [1,7]. Amax/Km (now termed FCSA) in the right lung was 54% of total Amax/Km in both lungs suggesting that, as in the animals, Amax/Km is a reliable and quantifiable index of DPCSA in humans [12].

The validity of Amax/Km as a DPCSA index in humans was additionally assessed in a pilot study performed in brain-dead
subjects with no ALI: repeated PE-ACE activity determinations were performed at different cardiac outputs in each patient. Unchanged substrate hydrolysis was observed over a wide range of CO while \( \frac{A_{\text{max}}}{K_m} \) increased linearly with flow, depicting patterns similar to those observed under normal conditions in animals [9].

**Pulmonary endothelial ACE activity under pathological conditions**

**i) Animal studies**

Pulmonary endothelial injury may begin as a subtle metabolic dysfunction and then progress to overt structural alterations and cell death. PE-ACE activity reduction appears to be among the earliest signs of lung damage, preceding changes in all commonly measured parameters such as acid-base balance, gas exchange, haemodynamic parameters, increased permeability, and morphological changes at the light and electron-microscopy level [3,7]. This is the case in the rabbit, following bleomycin and PMA administration [32,33], exposure to hyperoxia [34], and chest irradiation [35,36]. More recently, normoxaemic resuscitation of haemorrhagic shock in the same animal was associated with PE-ACE activity reduction and lung inflammation; hypoxaemic resuscitation appeared to preserve the former and attenuate the latter [37]. Pathological processes that will produce substrate hydrolysis reduction are presented in Figure 1.

Assessing PE-ACE activity in lung injury may help distinguish between abnormalities secondary to endothelial dysfunction per se and decreased pulmonary vascular surface area. If endothelial dysfunction is related to either decreased enzyme mass or kinetic constant alterations, then substrate hydrolysis would be altered (Figure 1). In such a case, FCSA which is related to both enzyme quantity and functional integrity will also be decreased. In contrast, if loss of DPCSA occurs with neither endothelial dysfunction nor changes in capillary transit times, substrate hydrolysis would not change (Figure 1), while FCSA would be decreased reflecting the loss of enzyme mass available for reaction. This is the case in alveolar pressure and acid-base imbalance that affected PE-ACE activity through changes in DPCSA [38,39].

**ii) Bedside assessment of Pulmonary Endothelial ACE activity in the diseased lung**

PE-ACE activity was assessed in 33 critically-ill, mechanically ventilated patients belonging in high-risk groups for ARDS development, and suffering from various degrees of lung injury with a lung injury score (LIS) ranging from 0 to 3.7 [40]. Both BPAP transpulmonary hydrolysis (v) and \( \frac{A_{\text{max}}}{K_m} \) decreased early during the ALI/ARDS continuum and were inversely related to LIS and the APACHE II score. Contrary to the observations made in animals and humans with normal/healthy lungs, substrate hydrolysis decreased with increasing CO, suggesting decreasing \( t_c \) at higher cardiac outputs, a phenomenon probably reflecting the vascular loss occurring from occluded or obliterated vessels (Figure 1).

Although \( \frac{A_{\text{max}}}{K_m} \) (i.e. FCSA) increased with higher CO in healthier survivors, such increases did not occur in the more severely ill; consequently higher blood volumes in the latter should have been accommodated mainly through dysfunctional capillaries, whereas no reserves of FCSA were available to be recruited. High \( \frac{A_{\text{max}}}{K_m} \) values were associated with better survival, raising the intriguing possibility that ARDS patients who maintain high FCSA might have a better outcome [40].

PE-ACE activity was also assessed in patients undergoing total knee arthroplasty, a surgical procedure often related to fat embolism syndrome after tourniquet release (TR) [41]. In most patients capillary recruitment appeared to occur post TR, probably denoting a mechanism by which the lungs are able to accommodate the burden of emboli at the time of TR, thus preventing or minimizing pulmonary hemodynamic decompensation.

PE-ACE activity was further investigated in subjects suffering from systemic sclerosis (SSc), in an effort to investigate if pulmonary endothelial dysfunction is an early feature of the pathological condition. Schematic presentation of pathological conditions that will result in reduced substrate hydrolysis by PE-ACE: (I) Under normal conditions a certain hydrolysis (v) will occur. (II) Under injury endothelial cells should be either destroyed or damaged, resulting in crude ACE mass loss and thus enzyme concentration reductions. Injury may also alter enzyme kinetic constants. (III) PEACE down-regulation, for any reason, will result in enzyme concentration reduction. (IV) Under injury, capillary plugging by debris or blood cells will stop flow within, and will consequently zero capillary mean transit time (i.e. enzyme-substrate reaction time). (V) If for any reason (such as plugging by debris, or vessel remodelling and loss like in pulmonary hypertension) no reserves of healthy capillaries exist to accommodate higher pulmonary blood volumes resulting from higher cardiac outputs, pulmonary blood flow should pass through the remaining open capillaries causing, i. capillary distension (and thus a degree of enzyme concentration reduction), and mainly ii. higher pulmonary capillary plasma flows, resulting in capillary mean transit time reductions [1,30]. E. enzyme mass available for reaction, [E]: capillary enzyme concentration, \( t_c \): reaction time (capillary mean transit time), \( k_{cat} \): catalytic rate constant, \( K_m \): Michaelis-Menten constant. Grey crosses represent blood-borne parent substrate molecules; white crosses represent product molecules.

![Figure 1](image-url)
disease [42]. SSc comprises two subsets: the diffuse (dcSSc) and the limited (lcSSc) subset. PE-ACE activity was found to be decreased at an early disease stage (absence of PH or pulmonary interstitial fibrosis) in both subsets, while the degree of reduction appeared mostly related to the SSC subset (lower in dcSSc) and the underlying pulmonary haemodynamic parameters.

PE-ACE activity was more recently assessed in patients suffering from PH from diverse aetiologies [43]. Nineteen patients with pulmonary arterial hypertension (PAH) related to connective tissue disease (PAH-CTD) and 25 patients with idiopathic PAH (IPAH) [44], who had not received at the time PAH-specific treatment, were evaluated. Patients’ enzyme activity parameters were compared with those of 23 controls with no past or present lung disease. %M and hydrolysis (i.e. indices reflecting enzyme activity per capillary) were reduced in PAH-CTD patients, as compared to both IPAH and controls; no differences were observed between IPAH and the latter. The FCsA (originally termed Amax/Km) normalized to body surface area (BSA) was significantly reduced in both IPAH and PAH-CTD patients. The decreases obtained in substrate %M and hydrolysis in the PAH-CTD subjects could be related to capillary transit time reductions (i.e. reaction time reductions; Figure 1), secondary to vascular remodelling. However such a phenomenon should also produce inverse relationships of the above-mentioned indices to CO [1], which was not the case. It thus appears that pulmonary endothelial ACE dysfunction or loss is present in PAH-CTD, raising the intriguing probability that the two PAH subgroups possess different pulmonary endothelial phenotypes. In contrast with the above, functional capillary surface area was equally reduced in both PAH subgroups, reflecting the PH-related small vessel loss and remodelling. This study also provided the first functional evidence that the reduced carbon monoxide diffusing capacity (DLCO) observed in PAH-CTD is related to the degree of FCSA loss [43].

Pulmonary endothelial metabolic function was additionally studied in patients with chronic thromboembolic pulmonary hypertension CTEPH [45]. Patients exhibited FCSA reductions, while substrate %M and hydrolysis were preserved. Taken together, these findings imply a reduction of the metabolically functional pulmonary capillary bed rather, than reduced ACE activity on the pulmonary endothelial cell. This reduction could be the result of decreased capillary recruitment because of upstream vascular plugging by organized thrombus, an abnormality that could probably be corrected after pulmonary thromboendarterectomy [45].

Conclusions
Studies of pulmonary endothelial metabolism have been providing important information on pulmonary vascular physiology and pathobiology in both animals and humans, being the only techniques that offer direct and quantifiable means of assessing pulmonary endothelial function. It should be noted that the indicator dilution techniques are arduous procedures, not easily performed in everyday clinical practice. However, information from an old animal study provides evidence that they might be replaced by the much simpler “one sample” technique [46]. Future studies should test this “less demanding” technique at the bedside, in an effort to develop clinically useful tests.

References
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