Monitoring of Pulmonary Endothelial Enzyme Function: An Animal Model for a Simplified Clinically Applicable Procedure

Hannu J. Toivonen, M.D., Ph.D.,* Nevine Makari, M.D.,† John D. Catravas, Ph.D.‡

The authors present a simple and clinically applicable method for the serial monitoring of pulmonary microvascular enzyme function in vivo. This method requires the intravenous injection of trace amounts of a radialedinated substrate and the collection of a single arterial blood sample. Simultaneous measurement of pulmonary blood flow, e.g., by dye- or thermo-dilution and the determination of blood hematocrit are also needed for the calculations. This method was compared to the triple blood sample indicator dilution method in normal anesthetized rabbits. Both methods gave identical results for the metabolism of the synthetic, hemodynamically inactive tripeptide, \( ^6 \text{H}-\text{benzoyl-Phe-Ala-Pro} \) (\( ^6 \text{H}-\text{BPAP} \), by pulmonary microvascular endothelial angiotensin converting enzyme. The parameters measured were 1) substrate utilization, expressed linearly and logarithmically, and 2) the apparent first order reaction constant. The new method was also used for the simultaneous measurement of single pass, transpulmonary metabolism of \( ^6 \text{H}-\text{BPAP} \) by angiotensin converting enzyme and of \( ^5 \) adenosine monophosphate by \( ^5 \)-nucleotidase in rabbits in vivo. The authors propose that similar enzyme kinetic measurements could be used in clinical studies to test their usefulness as an aid in the early diagnosis of incipient pulmonary endothelial dysfunction, e.g., adult respiratory distress syndrome. (Key words: Enzymes; angiotensin converting enzyme; \( ^5 \)-nucleotidase. Lung: ARDS, lung endothelium; lung injury.)

PULMONARY ENDOTHELIUM. cell damage is a dominant feature of many types of lung injury that cause severe pulmonary dysfunction and which are often associated with high mortality. Factors damaging pulmonary microvascular endothelial function include hyperoxia,1-4 endotoxins,5 cigarette smoke and other inhaled chemicals,6-9 drugs,10-13 toxic chemicals,14 cardiopulmonary bypass,15 and severe trauma and various disease states16-19 that cause the adult respiratory distress syndrome (ARDS).

In animal experiments, pulmonary endothelial dysfunction can often be detected in very early phases of injury when, e.g., impairment of gas exchange is still absent.3,14 This has supported the suggestion that the monitoring of endothelial enzyme activity could be used as an early diagnostic test of incipient pulmonary dysfunction to allow therapeutic interventions before the lungs are irreversibly damaged.15 One of the most studied endothelial enzymes is angiotensin converting enzyme (ACE). ACE is an ectoenzyme which uniformly lines the pulmonary microvascular lumen, and catalyzes the synthesis of angiotensin II from angiotensin I and the degradation of bradykinin to inactive products.16

The interpretation of measurements of pulmonary enzyme function in vivo is complicated by the inherent difficulties in separating changes in the enzyme molecule from other factors that affect enzyme-catalyzed reactions, e.g., plasma flow rate in the capillaries (determinant of the reaction time), microvascular plasma volume and flow geometry (possibly affecting the enzyme concentration, in terms of moles of enzyme per unit of plasma volume, i.e., reaction volume), and level of capillary recruitment (affecting the amount of available enzyme). Changes in per cent transpulmonary metabolism of injected tracer substrates may not, by themselves, be reliable in predicting incipient lung damage. Animal experiments suggest that additional useful information may be obtained from the simultaneous measurement of apparent enzyme kinetics and substrate utilization in the pulmonary circulation, which could be used to correct for certain of the aforementioned confounding hemodynamic influences.17-20 In the laboratory, these techniques require the collection of serial, accurately timed blood samples of exactly known volume, by means of a special fraction collector. This kind of equipment is not commonly available in hospitals; its use is not only cumbersome in clinical situations, but also contains several potential risk factors. In this paper, we

* Postdoctoral fellow, Department of Pharmacology and Toxicology. Present address: Resident in Anesthesiology, Turku University Central Hospital, Turku, Finland.
† Graduate Student, Department of Pharmacology and Toxicology.
‡ Associate Professor of Pharmacology and Toxicology. Department of Pharmacology and Toxicology; Established Investigator of the American Heart Association.

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Address reprint requests to Dr. Catravas: Department of Pharmacology and Toxicology, Medical College of Georgia, Augusta, Georgia 30912.

ABBREVIATIONS

\( ^6 \text{H}-\text{BPAP} \) = \( ^6 \text{H}-\text{benzoyl-Phe-Ala-Pro} \)
ARDS = Adult Respiratory Distress Syndrome
CG = Cardiogreen, indocyanine green
AMP = \( ^5 \) adenosine monophosphate
\% M = Per cent metabolism
ACE = Angiotensin converting enzyme
NCT = \( ^5 \)-nucleotidase
\( k_c \) = Constant of product formation from [ES]
[ES] = Concentration of the substrate-bound enzyme
FIG. 1. Diagram of the multiple sample and single sample methods used in determining endothelial enzyme function, in vitro. A bolus containing radio labelled substrates and dye was injected into the central venous circulation, and blood samples were withdrawn from the arterial side with a peristaltic pump either into a fraction collector (A) or into a single disposable syringe (B). Blood flow was measured via a densitometer. The rest of the equipment is for the anesthesia and monitoring of vital signs. c = carotid artery catheter; j = jugular vein catheter; t = pressure transducer; d = densitometer cuvette and densitometer; p = peristaltic withdrawal pump; r = recorder; AP = airway pressure tracing; SAP = systemic arterial pressure tracing; ICG = indocyanine green (Cardiogreen) tracing; v = ventilator.

present a relatively safer, easier method that allows the measurement of pulmonary endothelial enzyme kinetics in vivo, and can be easily extrapolated to the clinic. The results obtained with this method were compared to results obtained with classical multiple sample indicator dilution measurements under similar conditions in anesthetized rabbits.

Materials and Methods

New Zealand white male rabbits (2.8–3.6 kg) were anesthetized with 6–8 ml, iv, of a solution containing 2.2 M urethane and 0.24 M allobarbital, and were artificially ventilated with oxygen enriched (35–40%) room air. Previous experience has shown that this procedure provides adequate anesthesia for approximately 6 h. Polyethylene catheters were advanced to the levels of the right atrium and ascending aorta via the right jugular vein and left carotid artery, respectively. All drugs were given iv. Subsequently, the animals were heparinized (1000 IU/kg) and paralyzed with 1 mg of pancuronium bromide (Pavulon, Organon, Inc, NJ), followed by 0.5 mg additional doses when needed during the day. All procedures described in this study have been approved by the Committee of Animal Use in Research and Education of the Medical College of Georgia. Airway pressure and systemic arterial pressure were monitored continuously on a Gould 2400 recorder (Gould Instruments, Columbus, OH) via Statham P231D pressure transducers (Statham Instruments, Hato Rey, PR). After the operation, the animals were allowed to equilibrate for approximately 30 min, during which time the ventilation was adjusted to give normal arterial blood gas values. Subsequently, the single-pass transpulmonary metabolism of the synthetic tripeptide, 3H-benzoyl-Phe-Ala-Pro (3H-BPAP) by microvascular endothelial angiotensin converting enzyme (ACE) was measured in vivo in one group of 11 rabbits. Two measurements were performed in each animal by each of the two methods in randomized order as follows.

METHOD A: MULTIPLE ARTERIAL BLOOD SAMPLE COLLECTION

3H-BPAP metabolism was measured in vivo according to the conventional indicator dilution principle under first-order reaction conditions, as described earlier. Briefly, fresh saline solution (0.9 ml) containing 0.37 mg (0.5 μmol) indocyanine green (Cardiogreen, CG, Hynson, Wescott & Dunning, Baltimore, MD) and trace amounts (1.5 μCi) of 3H-BPAP (20 Ci/ml) was injected as a bolus into the jugular venous cannula and flushed into the circulation with 1 ml saline. Simultaneously, blood was withdrawn from the arterial catheter with a peristaltic pump (rate: 28 ml/min, Coleman Instruments, Chicago, IL) into a Gilson® ESCargot fraction collector (Gilson Instruments, Lexington, MA) equipped with 13 × 100-mm borosilicate tubes advancing at the rate of one tube per 0.7 s (0.33 ml blood/tube). The arterial withdrawal line was in series with a Waters densitometer cuvette, and the appearance of Cardiogreen in the arterial blood was monitored via a Waters DC 410 Densitometer (Waters Instruments, Inc, Rochester, MN) on the recorder (fig. 1). The dye dilution curve was used to calculate pulmonary blood flow. Each collection tube contained 2 ml of 0.4 mg/ml captopril in normal saline (approximately 1 mM) to prevent the further breakdown of 3H-BPAP by plasma.
angiotensin converting enzyme. Immediately after each blood sample collection, an additional 1-ml blood sample was withdrawn (total blood withdrawal: 6 ml) from the carotid artery for pH/blood gas and hematocrit determinations.

The samples were gently mixed and centrifuged for 10 min at 1900 g (Centra® 7R; International Equipment Corporation, Needham Heights, MA) to separate the blood cells. Two 0.5-ml aliquots of the supernatant were transferred into 7-ml polyethylene scintillation vials (Fisher Scientific, Norcross, GA). The first 0.5 ml aliquot was used to measure total $^3$H activity in the presence of 6 ml Hydrofluor (National Diagnostics, Somerville, NJ) in a LS 7500 Beckman Liquid Scintillation Spectrometer (Beckman Instruments, Irvine, CA). The second 0.5-ml aliquot was mixed with 2.5 ml of 0.12 N hydrochloric acid, and the radioactivity in it was counted in the presence of 3 ml Ventrex cocktail #2 (Ventrex Laboratories, Portland, ME). With this method, 62% of the metabolite ($^3$H-benzoyl-Phe) and 7% of the parent (unmetabolized substrate, $^3$H-BPAP) is transferred to the counting phase. A 24-h equilibration period was allowed before counting.

**METHOD B: SINGLE ARTERIAL BLOOD SAMPLE COLLECTION**

The saline solution containing $^3$H-BPAP and Cardiogreen was prepared and injected into the jugular catheter as in Method A; blood was similarly withdrawn and passed through the densitometer cuvette (fig. 1). However, instead of collecting 15–20 fractionated arterial blood samples, one blood sample was collected into a disposable 10-ml syringe containing 4.5 ml of 3.7 mM captopril in ice-cold saline. Collection proceeded until 4.5 ml of blood was withdrawn, a volume empirically arrived at to contain the peak of the recorded Cardiogreen curve, which was also used for blood flow measurements. The blood sample was centrifuged as above, duplicate 0.5-ml aliquots were transferred into 20-ml polyethylene scintillation vials, and total radioactivity was estimated in the presence of 15 ml of Hydrofluor®. Additional duplicate 0.5-ml samples were mixed with 6.5 ml of 0.12 N HCl each, and the radioactivity in each was counted in the presence of 7 ml of Ventrex #2 cocktail after 24 h equilibration, as described above. Larger scintillation vials were necessary with this method, as the higher concentration of plasma in the samples tended to decrease the counting efficiency of tritium in small scintillation vials below acceptable levels. In large vials, 8% of $^3$H-BPAP and 59% of the metabolite ($^3$H-Benzoyl-Phe) extracted into the counting phase in 24 h. To calculate parent and product recovery, two similar blood samples were withdrawn from each animal (blood: collection fluid 1:1) before isotope injections. A small aliquot of the injectate ($^3$H-BPAP) was added to one of these samples, and the other sample received the radioactive metabolite ($^3$H-Benzoyl-Phe) previously prepared in the laboratory. These samples were then processed as above, together with the experimental arterial blood samples collected from the same animal.

**REPRODUCIBILITY OF THE MEASUREMENT**

To test the variability of these measurements, we repeated the single blood sample collection experiments five times in a second group of three rabbits. In addition to Cardiogreen and $^3$H-BPAP, the radioactive injectate also contained 0.5 μCi $^{14}$C-adenosine monophosphate (AMP) and 0.15 mg diprydamole. This triple indicator system allows the simultaneous monitoring of two distinct microvascular enzyme functions; it is, the hydrolysis of $^3$H-BPAP by angiotensin converting enzyme and the dephosphorylation of $^{14}$C-AMP by 5'-nucleotidase (NCT), an enzyme also located on the luminal surface of pulmonary microvascular endothelium. Diprydamole was included in the injectate to prevent the cellular uptake of formed adenosine. To prevent AMP metabolism and adenosine uptake and metabolism by red cells in the sample tubes, the collection fluid also contained adenosine 7.5 mM, AMP 1 mM, and diprydamole 0.2 mM. Samples were analyzed as above, and the total radioactivity (dpm) of $^{14}$C and $^3$H were estimated in the same aliquot (corrected for counter-efficiency and $^3$H/$^{14}$C cross-spill). In addition, two 1-ml aliquots of the supernatant were mixed with 1 ml of 1.2 M ZnSO$_4$ solution, and the AMP was then precipitated by adding 2 ml of 0.6 M Ba(OH)$_2$ solution. The precipitate was separated by centrifugation, and the radioactivity in 1-ml aliquot (containing $^{14}$C-adenosine) was counted in 20-ml scintillation vials together with 15 ml of hydrofluor. $^{14}$C-adenosine recovery was close to 100% as tested by adding $^{14}$C-adenosine together with $^3$H-Benzoyl-Phe in the test blood collected before the experiments. The spill of $^{14}$C-AMP to the supernatant was below 14%.

The following protocol was used: after the animals had equilibrated from surgery, the experiment was repeated three times at 30-min intervals, followed by two additional measurements at 1-h intervals. Thirty seconds before the third measurement, the animals received 10 nmol/kg captopril to inhibit angiotensin converting enzyme. This allowed us to test the reproducibility of our measurements, as well as test the ability of the method to detect selective enzyme dysfunction, since captopril would not affect $^{14}$C-AMP breakdown by 5'-nucleotidase. The last measurement was per-
formed 2 h after the captopril injection, to provide sufficient time for pulmonary angiotensin converting enzyme to recover from the inhibitory effects of the drug, as has been shown under similar conditions, in vivo.20

**Mathematical Calculations**

The theory and calculations of parameters for in vivo enzyme kinetic measurements under first order conditions have been published in detail earlier.19–21 Briefly, we employed three different parameters to describe microvascular enzyme function. First, substrate utilization is expressed as the per cent of the injected substrate metabolized during one passage through the lungs, i.e., %M. Secondly, substrate utilization is presented as \( (\frac{A_{\text{max}}}{K_m})/100 \text{ ml blood flow} \), a derived parameter (=\((\text{hematocrit} \times \ln (1/(1-M)))\)) that we consider to be a more sensitive expression of changes in pulmonary microvascular enzyme function because it reflects the true logarithmic nature between substrate utilization and enzyme function, and also corrects for possible fluctuations in hematocrit between the measurements.§ Altered hematocrit results in altered plasma volume. Although large vessel and capillary hematocrit may not be the same, we have used carotid vessel hematocrit values to correct for changes in capillary plasma volume (and, therefore, reaction volumes). The contribution of the hematocrit correction varies with different protocols; thus, in group 1, where only two measurements per animal were performed, hematocrit did not change (table 1). In group 3, however, performing five consecutive determinations progressively decreased hematocrit (see Results), and the above corrections were of obvious significance. The third term, \( \frac{A_{\text{max}}}{K_m} (=\text{Plasma Flow} \times \ln (1/(1-M))) \) is the modified first order rate constant reflecting both the properties of the reacting enzyme and the total amount of the enzyme present. If the enzyme itself is not altered, changes in \( \frac{A_{\text{max}}}{K_m} \) reflect changes in perfused microvascular surface area.20

**Statistics**

Data were analyzed with the aid of a Zenith-148 microcomputer equipped with the appropriate statistical software (Statpack, version 3.1, Northwest Analytical, Inc., Portland, OR). Statistical comparisons utilized student’s \( t \) test for paired samples or one-way ANOVA for repeated measures followed by Dunnett’s \( t \) test, as appropriate.22

| Table 1. Physiological Data during the Measurement of Pulmonary Endothelial Enzyme Function (Means ± SE; \( N = 11 \)) |
|-------------------------------------------------|-------------------------------------------------|
| Multiple Blood Sample Collection Method | Single Blood Sample Collection Method |
| Pulmonary blood flow (mL/min) | 368 ± 26 | 357 ± 24 |
| Pulmonary plasma flow (mL/min) | 255 ± 17 | 229 ± 16 |
| Mean aortic blood pressure (mmHg) | 83 ± 4 | 81 ± 4 |
| Peak airway pressure (mmHg) | 9 ± 1 | 9 ± 1 |
| Ventilation frequency (inspirations/min) | 25 ± 2 | 24 ± 2 |
| Hematocrit | 36 ± 1 | 36 ± 1 |
| Arterial pH | 7.45 ± 0.02 | 7.46 ± 0.02 |
| Arterial \( F_{\text{EO}_{2}} \) (mmHg) | 36 ± 1 | 36 ± 1 |
| Arterial \( F_{\text{O}_{2}} \) (mmHg) | 273 ± 28 | 278 ± 24 |

Results

Averages of 11 measurements of pulmonary endothelial ACE function by the multiple arterial blood sample method and 11 additional measurements by the single arterial blood sample method in 11 animals are presented in table 2. There is an obvious statistical and biological similarity in the values of per cent metabolism (87% vs. 88%), \( (\frac{A_{\text{max}}}{K_m})/100 \text{ ml blood flow} \) (134 vs. 140), and \( \frac{A_{\text{max}}}{K_m} \) (508 vs. 510 ml/min) between the two methods. The average values of various monitored parameters, as shown in table 1, indicate that, with either method, measurements of enzyme function were performed under similar physiological conditions.

The reproducibility and sensitivity of the single blood sample method is presented in figures 2 and 3. In these experiments, five sequential determinations of endothelial ACE and NCT function were performed in three rabbits. Exactly 30 s before the third determination, each animal was given 10 mmol/kg captopril, an ACE inhibitor with no effects on NCT and at a dose previously reported to partially inhibit ACE.17 Per cent metabolism of \( ^{3} \text{H-BPAP} \) by ACE or \( (\frac{A_{\text{max}}}{K_m})/100 \text{ ml blood flow} \)

| Table 2. \( ^{3} \text{H-BPAP} \) Interaction with Pulmonary Angiotensin Converting Enzyme, In Vivo (Means ± 1 SE; \( N = 11 \)) |
|-------------------------------------------------|-------------------------------------------------|
| Multiple Blood Sample Collection Method | Single Blood Sample Collection Method |
| Percent metabolism of \( ^{3} \text{H-BPAP} \) (% M) | 87 ± 2 | 88 ± 1 |
| \( (\frac{A_{\text{max}}}{K_m})/100 \text{ ml blood flow} \) | 134 ± 8 | 140 ± 8 |
| \( A_{\text{max}}/K_m \) (mmHg) | 508 ± 59 | 510 ± 56 |

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§ By definition, \( A_{\text{max}} = k_w \times \text{enzyme mass} \), and, since \( V_{\text{max}} = k_w \times \text{enzyme concentration} \), \( A_{\text{max}} \) is also equal to \( V_{\text{max}} \times \text{microvascular plasma volume} \).
blood flow of ACE for BPAP were virtually identical in determinations one and two (Fig. 2). Thirty seconds after captopril, however (determination #3), both parameters decreased precipitously, but quickly recovered as, in determinations 4 and 5, they had returned to levels indistinguishable from those in determinations 1 and 2 (Fig. 2). Similar changes were observed with \( \frac{A_{\text{max}}}{K_m} \) of ACE for BPAP (Fig. 3).

On the other hand, the simultaneous measurement of pulmonary endothelial NCT function revealed steady reproducible determinations of % M, \( \frac{A_{\text{max}}}{K_m} \)/100 ml blood flow (Fig. 2), and \( A_{\text{max}}/K_m \) (Fig. 3), throughout the five determinations, including the one in the presence of captopril. In those studies, % M of 5′AMP by NCT varied slightly between 75–77%, \( \frac{(A_{\text{max}}/K_m)}{100} \) ml blood flow ranged from 80–90 and \( A_{\text{max}}/K_m \) varied from 300 to 308 ml/min.

As in the first part of the study, pulmonary plasma and blood flow (Fig. 2) remained constant throughout the five determinations; however, hematocrit decreased because of the blood loss, catheter flush, and fluid replacement, the consecutive values being 39 ± 1, 37 ± 1, 34 ± 2, 29 ± 2, and 25 ± 2. Other hemodynamic and respiratory variables remained constant in these experiments, and they were not significantly different from the values presented for the first part of the study in Table 1. This, viewed together with the unchanged \( A_{\text{max}}/K_m \) values of NCT for AMP, suggest that the captopril-induced decrease in the \( A_{\text{max}}/K_m \) of ACE for BPAP during the third determination was due to the increase in apparent \( K_m \), as the unchanged \( A_{\text{max}}/K_m \) values for 5′-NCT indicate that the perfused surface area (\( A_{\text{max}} \) corresponds to the reacting enzyme mass and, thus, surface area) has remained unchanged.

**Discussion**

Studies in animal models of pulmonary damage have clearly shown that pulmonary disposition of circulating amines or peptides is selectively disturbed during early phases of lung damage, even when morphological or clinical signs of pulmonary dysfunction are absent. Healthy human lungs also metabolize circulating substances, and there is evidence of altered endothelial function in humans during various conditions, including cardiopulmonary bypass and pulmonary hypertension, non-cardiogenic pulmonary edema, and ARDS. In spite of promising animal experiments, in vivo kinetic analyses of endothelial enzyme function has not been used extensively in clinical studies.

There have been two approaches to the experimental evaluation of pulmonary endothelial enzyme function, in vivo. They involve measurements of substrate (endogenous or synthetic) utilization during a single transpulmonary passage under either 1) first order or 2) mixed order reaction conditions. Thus far, the majority of the studies have been performed under first order conditions (i.e., when the concentrations achieved by the substrate in the pulmonary circulation are much lower than its suspected \( K_m \) for the enzyme). Studies under mixed order conditions, such as those originally reported by Linehan and Dawson, require high (pharmacological) concentrations of the substrate, and multiple arterial blood samples; for both of these reasons, their potential clinical applicability may be limited. Until recently, first order type studies involved measurement of per cent
metabolism of "trace" amounts of (purely radiolabelled) substrate during a single transpulmonary passage. In 1983, Ryan proposed that additional valuable information could be obtained from these data; we subsequently reported first order kinetic constants for angiotensin converting enzyme and 5'-nucleotidase, utilizing multiple arterial sample methods.20

A detailed description of the kinetic parameters available from first order studies in vivo was recently presented.21 Briefly, in addition to % M (per cent metabolism), we also calculate: \( \frac{A_{\text{max}}}{K_m} \) and \( \frac{(A_{\text{max}}/K_m)/100 \text{ ml blood flow}}{\text{The term } K_m \text{ reflects the affinity of the substrate for the enzyme.}} \)

\( A_{\text{max}} \) (defined as \( k_c \times \text{enzyme mass and having units of mass/time, e.g., } \mu \text{mole/min} \)) is closely related to the classical \( V_{\text{max}} \). Since \( V_{\text{max}} = k_c \times \text{enzyme concentration and the microvascular plasma volume can be considered as the reaction volume, } V_{\text{max}} = k_c \times \text{enzyme mass/microvascular plasma volume or } V_{\text{max}} = A_{\text{max}}/\text{microvascular plasma volume (units: nmol/ml/min).} \)

Whereas \( V_{\text{max}} \) is proportional to total enzyme concentration, \( A_{\text{max}} \) is proportional to total enzyme mass and is independent of pulmonary plasma volume. There is an important biological difference between \( A_{\text{max}} \) and \( V_{\text{max}} \). If, for example, microvascular derecruitment is accompanied by proportional loss in lung blood, endothelial enzyme concentration (and, hence, \( V_{\text{max}} \)) will not change, whereas endothelial enzyme mass (and, hence, \( A_{\text{max}} \)) will decrease. Thus, the ratio \( A_{\text{max}}/K_m \) depends both on enzyme "function" (i.e., affinity of substrate for the enzyme) and perfused endothelial surface area. In the absence of injury, \( A_{\text{max}}/K_m \) is a direct reflection of surface area only. It should be noted that the above discussion assumes uniform enzyme distribution on, and shape of, the pulmonary microvessels. The effects of heterogeneous enzyme concentration or capillary volume on the calculation of kinetic parameters remains to be tested. The terms \( (A_{\text{max}}/K_m)/100 \text{ ml blood flow} \) and % M can be viewed as a reflection of enzyme function within a capillary unit, and largely independent of total enzyme mass. We have reported, for example, that, in healthy rabbits, derecruitment induced by high airway pressures reduces \( A_{\text{max}}/K_m \) without affecting % M or \( (A_{\text{max}}/K_m)/100 \text{ ml blood flow}.21 \)

Conceptually, % M and \( (A_{\text{max}}/K_m)/100 \text{ ml blood flow} \) are very similar; however, the latter term additionally corrects for possible alterations in hematocrit, and more accurately reflects the logarithmic relationship which exists between enzyme activity and substrate utilization under first order conditions. This can be seen in figure 2, where \(^3\text{H}-\text{BPAP utilization after captopril decreased by 70\% (to about 30\% of the control value) when expressed as } (A_{\text{max}}/K_m)/100 \text{ ml blood flow, but only by 40\% (to about 60\% of control value) when expressed as percent metabolism.}}

The evaluation of these data becomes more complicated during endothelial injury as changes in the intrinsic enzyme properties (i.e., \( K_m \)) or diffuse disappearance of the enzyme from capillary surfaces will also alter \( A_{\text{max}}/K_m \) values. The present experiments were performed on healthy animals; in two recent studies, however, we have utilized Method A to detect pulmonary endothelial enzyme dysfunction in animals injured with either phorbol myristate acetate (PMA35) or radiation to the chest.34 From these studies have been reanalyzed after first taking the sum of the dpm from all 15–20 tubes in each determination, and then calculating the various parameters (% M, \( (A_{\text{max}}/K_m)/100 \text{ ml Blood Flow, } A_{\text{max}}/K_m \)), thus simulating conditions of Method B. A summary of some of these findings is presented in table 3. In the first model, PMA was administered iv (20 \( \mu \text{g/kg} \)) into conscious rabbits equipped with carotid and jugular catheters. Five consecutive determinations of ACE function were performed (one before

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**Fig. 5.** Modified first order rate constant \( (A_{\text{max}}/K_m; \text{ upper panel}) \), pulmonary blood and plasma flow (lower panel) from the same experiments as in figure 2.
and four after PMA) as described for Method A in this study. In the second model of injury, two groups of rabbits equipped with carotid and jugular catheters were briefly anesthetized with xylazine/ketamine and one group served as control (CTRL), whereas the other was subjected to 3000 rad to the chest area (from the clavicle to the xyphoid process; EXP group). Four consecutive determinations of enzyme activity were performed as with Method A, one 24 h before irradiation, and three more over the next 48 h. In both models, injury significantly decreased % M and (Amax/Km)/100 ml blood flow without significantly affecting the same parameters in the sham-treated group (radiation study). It is of particular interest that electron microscopic examination of lung tissue revealed normal structure. These data strongly suggest that our proposed measurements are sensitive to change during the early phase in two models of endothelial damage.

In some cases, better information may be gained by simultaneously monitoring two enzymes. We demonstrated this principle by repeatedly measuring both angiotensin converting enzyme and 5'-nucleotidase function (figs. 2, 3). Inhibition of angiotensin converting enzyme by captopril (which could be viewed as a simulation of selective injury) caused a decrease in Amax/Km for angiotensin converting enzyme (i.e., enzyme dysfunction), whereas the value for 5'-nucleotidase remains unchanged (i.e., no changes in perfused endothelial surface area). Several studies have shown that, during lung damage, some endothelial functions are disturbed early in the process, whereas others are rather resistant to toxic substances. It is possible that, when a sensitive marker is used together with a rather insensitive marker, the latter could be used to estimate changes in microvascular surface area among repeated measures, while the “sensitive” probe would give information about enzymatic dysfunction that one hopes would predict incipient clinical pulmonary dysfunction. It is also possible that the ideal combination of probes for the detection of lung microvascular injury might be different for different damage processes. In this respect, both animal experiments and human confirmation are needed to find out the best possible combination of the enzyme monitors suitable for monitoring of endothelial damage.

The single blood sample method is a rather easy and safe procedure to perform even in clinical situations. The radioactive mixture can be injected in any antecubital vein, not necessarily a central vessel, since the first microvascular bed, where substrate metabolism can occur following any iv injection, is the pulmonary microvasculature. In this work, we have used a peristaltic pump to collect the blood into a disposable syringe in order to maintain the withdrawal system similar for comparison with the multiple blood sample method. In practice, the blood sample can be collected with a normal withdrawal syringe pump from a cannula located in any artery (e.g., radial artery). The amount of blood collected from the artery should contain sufficient amount of radioactivity for analysis (1000 dpm/ml), and the collection should be stopped before recirculation of the injected substrate occurs. In the present

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<th>Table 3. 1H-BPAP-ACE and 14C-5—AMP-NCT Interaction in Two Models of Lung Microvascular Injury, In Vivo</th>
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<tr>
<td><strong>Flurbiprofen Myristate Acetate</strong></td>
</tr>
<tr>
<td>% M</td>
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<td>(Amax/Km)/100 ml blood flow</td>
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<tr>
<th><strong>1H-BPAP-ACE</strong></th>
<th><strong>14C-5—AMP-NCT</strong></th>
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<td><strong>C</strong></td>
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<td>CTRL</td>
<td>83 ± 2</td>
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<tr>
<td>EXP</td>
<td>84 ± 2</td>
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<tr>
<td>(Amax/Km)/100 ml blood flow</td>
<td>105 ± 4</td>
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<tr>
<td>CTRL</td>
<td>122 ± 7</td>
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<tr>
<td>EXP</td>
<td>601 ± 85</td>
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<tr>
<td>Amax/Km (ml/min)</td>
<td>546 ± 101</td>
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</table>

* Means ± SE of seven conscious rabbits. C = control measurements, approximately 30 min before 20 μg/kg PMA, iv. Subsequent measurements were taken at indicated times after PMA (from ref 33).
† Means ± SE of five control rabbits (CTRL) or seven experimental rabbits administered 3000 rad radiation to the chest (EXP). C = control measurements, approximately 24 h before radiation. Subsequent measurements were taken at indicated times after irradiation (from ref 34).
§ P < 0.05 from control value (C) within the same group.
© P < 0.05 from time-corresponding value in the CTRL group.
work, we collected 4.5 ml of blood but, if necessary, the measurements can be done from a smaller sample taken at lower blood withdrawal rates. We used the output of the Cardiogreen® arterial concentration curve on the recorder to signal the appearance of the first-pass radioactive peak into the collection syringe; the collection is then stopped as soon as the Cardiogreen peak has disappeared. The size of the blood sample can be reduced, if the densitometer cuvette is omitted and pulmonary blood flow is measured otherwise during the experiment. In the clinic, this can be done, for example, either with a Cardiogreen® densitometer ear probe, or by using the thermodilution principle. An implicit assumption with this method is that substrate utilization remains constant throughout the Cardiogreen® curve. It is possible that certain types of injury may cause flow heterogeneities which would invalidate this assumption. It is encouraging that we have not encountered such a case in any of our animal models of injury yet.

The blood sample is collected into a syringe that contains saline and selected enzyme inhibitors. This is necessary, as the blood is also able to metabolize the same substrates, although at a much slower rate than the lungs, which is inconsequential during the substrate's transit through the lung in vivo. From our experience, the volume of the inhibitor solution in the syringe must be at least equal to the volume of collected blood, as, otherwise, mixing of blood with the inhibitor solution in the syringe tends to be inadequate.

In this paper, we have used a simple analytical method for the separation of 3H-BPAP from its metabolites. The extraction with this method requires 24 h to equilibrate, and this waiting period is a disadvantage if the measurements are aimed towards the early diagnosis of pulmonary damage. This analysis, however, can be reduced to approximately 2 h, as presented earlier.

In the present experiments, we used dipyriramole in the injectate to prevent the cellular uptake of formed adenine in 5'-nucleotidase measurements. In human experiments, this may prove unnecessary, since erythrocytes appear to account for much of the uptake of adenine in humans, and adenine taken up by red cells can be recovered for the analysis by lysing the red cells in the collected blood sample.

Certain limitations must be kept in mind when analyzing results from the single sample method. When blood is collected as a single sample, it is assumed that the metabolism of the substrate is relatively constant throughout the collection period. This is true only at first-order reaction conditions. We have tested the method for two enzymes that reside on the luminal membrane of the endothelial cell (i.e., ectoenzymes) and where there is no cellular uptake of the substrates. The measurement of carrier or intracellular enzyme functions with this method is complicated by the back diffusion of the substrate from inside the cells. In spite of this, it may still be possible to collect clinically relevant data of transporter enzymes with this kind of approach, as employed by Morel et al., for measurements of propranolol and serotonin removal by lungs of ARDS patients. The authors were able to correlate pulmonary serotonin extraction with the severity of ARDS in their patients. Pulmonary metabolism of BPAP has not been tested in man, but, given the longer substrate transit time expected in human pulmonary capillaries, BPAP would probably be totally metabolized in less than one passage through normal human lungs. In this respect, other synthetic tripeptide substrates of ACE, e.g., Benzyl-Ala-Gly-Pro, may prove more useful.

In summary, we present here a rather simple method for obtaining kinetic data of pulmonary microvascular endothelial enzyme function. It requires injection of the substrate solution into the venous circulation and collection of one arterial blood sample. In addition, hematocrit and pulmonary blood flow must be measured, e.g., by dye- or thermo-dilution. The results obtained compare favorably to results obtained with the more complicated multiple arterial blood sample indicator dilution assay, as tested in anesthetized, healthy rabbits. We believe that similar measurements can be performed in human studies when looking for early warning indicators of incipient lung damage.

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