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Edema in Oral Mucosa after LPS or Cytokine Exposure

ABSTRACT

Lowering of interstitial fluid pressure (P_{if}) is an important factor that explains the rapid edema formation in acute inflammation in loose connective tissues. Lipopolysaccharide (LPS) and the pro-inflammatory cytokines interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) are pathogenetic in gingivitis. To test if these substances induce lowering of P_{if} in rat oral mucosa, we measured P_{if} with a micropuncture technique. IL-1β and TNF-α caused lowering of P_{if}, whereas LPS induced an immediate increase in P_{if}, followed by lowering after 40 min. Measurements of fluid volume distribution showed a significant change in interstitial fluid volume (V_i) 1.5 hr after LPS exposure as V_i changed from 0.41 ± 0.02 to 0.51 ± 0.03 mL/g wet weight ($p < 0.05$), confirming edema. These findings show that LPS, IL-1β, and TNF-α induce lowering of P_{if} in the rat oral mucosa and contribute to edema formation in LPS-induced gingivitis.

KEY WORDS: IL-1β, TNF-α, interstitial fluid pressure, micropuncture, inflammation.

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A supplemental appendix to this article is published electronically only at http://www.dentalresearch.org.

INTRODUCTION

Edema formation resulting from increased net transcapillary filtration is one of the cardinal signs of inflammation. In the skin, trachea, and nasal mucosa, lowering of interstitial fluid pressure (P_{if}) is shown to be a very potent factor in the development of edema, leading to increased net transcapillary hydrostatic pressure and fluid flux during the initial stages of inflammation (Rodt *et al.*, 1990; Koller and Reed, 1992; Berg *et al.*, 1998). Recently, cytokines such as interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) have been found to lower P_{if} in the rat paw skin significantly when given systemically or locally (Nedrebø *et al.*, 1999), suggesting that these substances have a mechanistic role in the development of edema.

Gingivitis is characterized by redness, edematous swelling, bleeding upon probing, and an increase in gingival crevicular fluid flow. All clinical signs are due to pathological tissue alterations that occur at the level of gingival microcirculation (Attström and Egelberg, 1970; Page and Schroeder, 1976; Del Fabbro *et al.*, 2001). Bacteria and their by-products present in the gingival sulcus (Löe *et al.*, 1967; Lindhe *et al.*, 1973) induce inflammatory processes that are maintained and amplified by the *in situ* production of mediators such as IL-1β and TNF-α (Van Dyke *et al.*, 1993; Gorska *et al.*, 2003). A vascular component in the development of edema has been demonstrated in oral mucosa, since IL-1β has been shown to increase vascular permeability (Daffonchio *et al.*, 2002), but so far, the role of the interstitium in edema formation in oral mucosa has not been investigated. By lowering the P_{if}, probably due to the loss of fibroblast adhesion to collagen in the interstitial tissue (Rodt *et al.*, 1990; Koller and Reed, 1992; Berg *et al.*, 1998), the interstitium plays an active role in the development of edema formation by promoting an increase in net transcapillary fluid transport.

The question raised in this study is: Are cytokines and lipopolysaccharide (LPS) important for edema formation in gingivitis/periodontitis *via* effects on the connective tissue? We therefore investigated the effects of LPS, IL-1β, and TNF-α on P_{if} in the rat oral mucosa to test the hypothesis that these substances can induce lowering of P_{if}, thereby inducing edema. Furthermore, to quantify a potential edema formation as an effect of the lowering of P_{if}, we measured fluid distribution volumes in oral mucosa after LPS exposure.

MATERIALS & METHODS

All the procedures described in this article were performed with the approval of and in accordance with the Norwegian State Commission for Laboratory Animals.

For a more detailed description of the methods used, please see the APPENDIX.

Experimental Protocol for P_{if} Measurements

Guided by a stereomicroscope, we performed P_{if} measurements with sharpened glass micropipettes (tip diameters, 2-6 μm), filled with 0.5 M NaCl stained with Evans Blue. The micropipette was connected to a servocontrolled counterpressure system, as first described by Wiederhielm and co-workers (Wiederhielm *et al.*, 1964), and was inserted 0.5-1 mm into the oral mucosa with a Leitz-Wetzlar micromanipulator (see APPENDIX for detailed description of the method).

P_{if} was recorded in the submucosa for 10 min before any injections were made (*in vivo* measurements). From 1 to 3 min after administration of the test substances or vehicle, cardiac arrest was induced with an intravenous injection of 0.5 mL saturated potassium chloride (KCl). Cardiac arrest was induced to limit fluid filtration and edema formation, so that we could measure the full effect of lowering the P_{if} (Lund *et al.*, 1988; Koller and Reed, 1992).

Thereafter, P_{if} was repeatedly recorded for 60-90 min. Test substances or vehicle was given either systemically or locally. The animals were divided into groups as follows:

Control groups: Seven rats received 0.5 mL of 0.9% NaCl with 1% bovine serum albumin (BSA) intravenously, whereas 6 rats received 5 μL of 0.9% NaCl with 1% BSA submucosally.

TNF-α group: Nine rats received 0.5 mL of TNF-α (400 ng/mL) intravenously, whereas 6 rats received 5 μL TNF-α (100 ng/mL) submucosally.

IL-1β group: Ten rats received 0.5 mL of IL-1β (30 ng/mL) intravenously, whereas 6 rats received 5 μL IL-1β (20 ng/mL) submucosally.

LPS group: Six rats received 5 μL LPS (5 mg/mL) submucosally.

Experimental Protocol for Fluid Volume Measurements

After the 1.5-hr equilibration period of the extracellular marker ^{51}Cr-EDTA, the rats received a dose of 4 mg LPS/kg body weight (*LPS group*, n = 6) or the equivalent volume of vehicle i.v. (*control group*, n = 8). Ninety minutes after LPS or vehicle administration, the intravascular marker ^{125}I-HSA was given, and the rats were killed 5 min later (see APPENDIX for detailed description of the method).

Statistical Analysis

Data are presented as means ± SE. Statistical analysis was performed by one-way analysis of variance with repeated measures (RM-ANOVA), followed by the Bonferroni or Fisher LSD *post hoc* test for P_{if} measurements. An unpaired *t* test was performed for fluid volume measurements. $P < 0.05$ was considered statistically significant.

RESULTS

(1) P_{if} Measurement Experiments

Systemic mean arterial blood pressure averaged 118.5 ± 3.0 mm Hg. No changes in blood pressure were observed throughout the control measurements of P_{if}.

Intravenous administration

Control group: Control P_{if} averaged 2.0 ± 0.5 mmHg before cardiac arrest had been induced. An intravenous injection of 0.5 mL of saline with 1% BSA followed by circulatory arrest did not change P_{if} over the 60-minute observation period (Table).

Table. Effect of Systemic Administration of TNF-α and IL-1β on Interstitial Fluid Pressure in Rat Oral Mucosa

Substance*	n	Control	Time after Cardiac Arrest (min) 0-20	21-40	41-60
		Interstitial Fluid Pressure (mm Hg)			
1% BSA	7	2 ± 0.5	1.1 ± 0.7	0.9 ± 0.4	0.8 ± 0.3
TNF-α (400 ng/mL)	9	2.7 ± 0.5	1.9 ± 0.6	0.7 ± 0.5[a]	-0.3 ± 0.6[ab]
IL-1β (30 ng/mL)	10	2.6 ± 0.5	2.2 ± 1.1	0.6 ± 0.4[ab]	0.2 ± 0.7[ab]

* All substances were administered as 0.5 mL i.v. Saturated KCl was given 3 min after injections of substances, to induce circulatory arrest. TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β. Values are means ± SE; n = number of rats. RM-ANOVA.
[a] $p < 0.05$ compared with own control.
[b] $p < 0.05$ compared with 1% BSA at same time interval.

TNF-α group: Intravenous administration of TNF-α lowered P_{if} significantly at the 21-40- and 41-60-minute periods compared with its own control ($p < 0.001$) (Table). In the 41-60-minute period, a significant drop was observed when compared with the control group at the same time interval ($p < 0.05$) (Table).

IL-1β group: When IL-1β was given intravenously, P_{if} was lowered significantly, from 2.6 ± 0.5 to 0.6 ± 0.4 mm Hg at 21-40 min and to 0.2 ± 0.7 mm Hg at 41-60 min, compared with its own control (Table). In the same time periods, P_{if} was statistically lowered ($p < 0.05$) when compared with the control group (Table).

Submucosal administration

Control group: Control P_{if} averaged 0.2 ± 0.3 mm Hg before cardiac arrest had been induced for rats receiving saline with 1% BSA. Local injection of vehicle followed by circulatory arrest after 2-3 min did not change P_{if} over the 90-minute observation period (Fig. 1).

TNF-α group: Submucosal injection of TNF-α significantly lowered P_{if} to -0.8 ± 0.5 mm Hg at 41-60 min and to -0.6 ± 0.5 mm Hg at 61-90 min, compared with its own control (Fig. 1A). Surprisingly, control values (*in vivo* measurements) in this group (0.8 ± 0.1 mm Hg) were significantly higher than the respective values of the control group (0.2 ± 0.3 mm Hg) (Fig. 1A).

IL-1β group: Local injection of IL-1β lowered P_{if} from 1.4 ± 0.4 mm Hg to -0.4 ± 0.6 mm Hg at 41-60 min, and the effect lasted until the end of the experimental period (-0.4 ± 0.4 mm Hg at 61-90 min), compared with its own control (Fig. 1B).

LPS group: Control P_{if} averaged 0.1 ± 0.4 mm Hg in the animals that received LPS. Submucosal injection of LPS raised P_{if} to 1.0 ± 0.2 mm Hg at 0-20 min (Fig. 1C). This rise in P_{if} occurred immediately after the administration of LPS, it was consistent in the group (although not statistically significant), and it could potentially cause an underestimation of the lowering of P_{if} if compared with its own control. We therefore performed, in this particular group, all the pairwise multiple comparison procedures (Fisher LSD method). LPS significantly lowered P_{if} to -1.3 ± 0.6 mm Hg at 41-60 min and

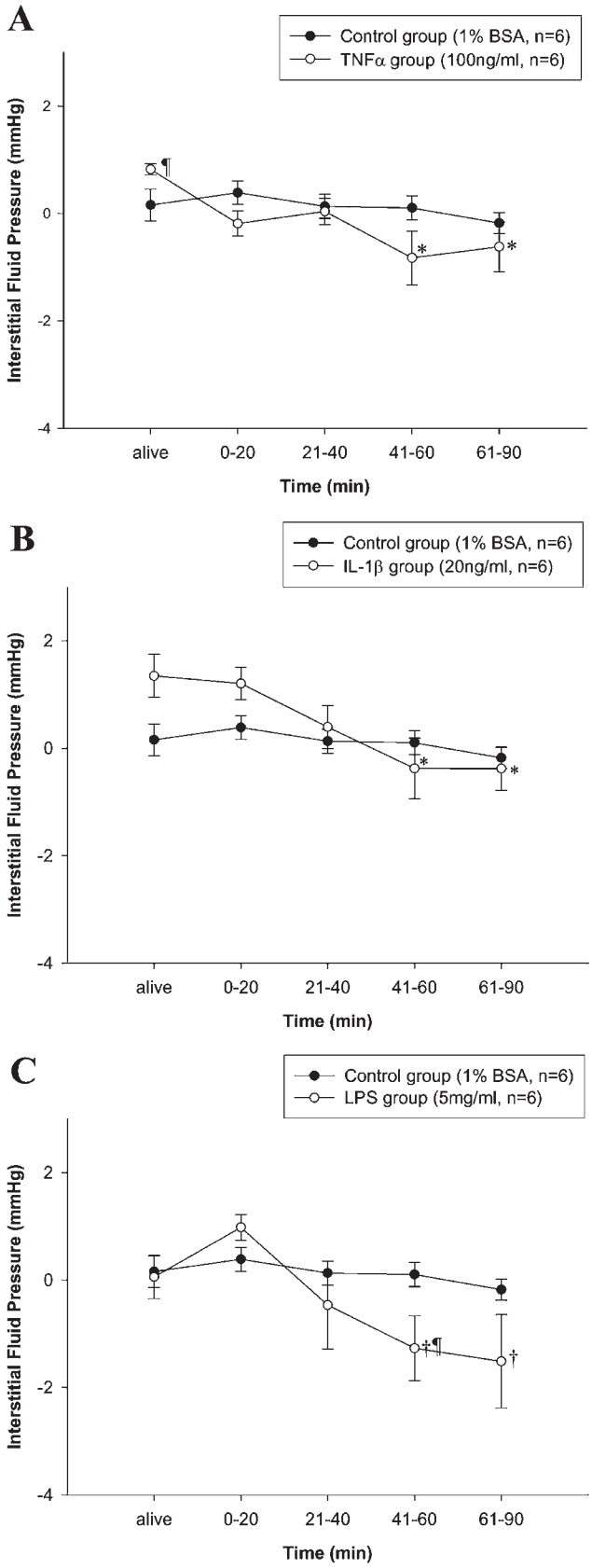

Figure 1. Effects of submucosal injections of albumin, TNF-α, IL-1β, and LPS on interstitial fluid pressure. Values are mean ± SE. n = 6 in every group. RM-ANOVA. *$p < 0.05$ compared with own control. † $p < 0.05$ compared with 0-20 min in the same group. ¶ $p < 0.05$ compared with 1% BSA at same time interval.

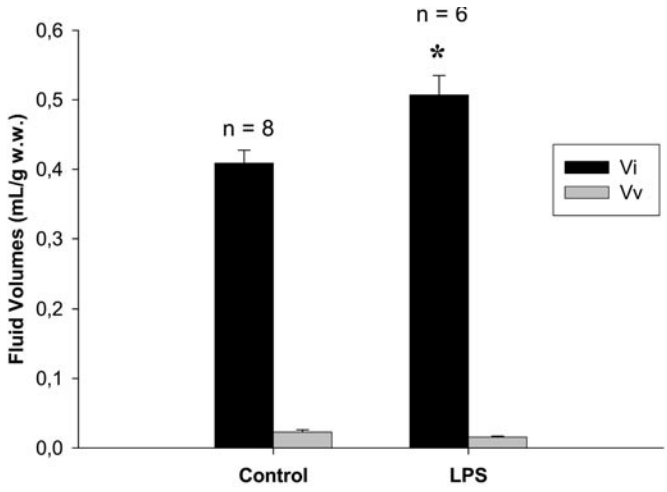

Figure 2. Distribution of fluid volumes in rat oral mucosa under normal conditions and 1.5 hr after LPS exposure. V_i, interstitial fluid volume; V_v, intravascular fluid volume. Values are mean ± SE, n = 8 controls (0.9% NaCl with 0.1% BSA) and n = 6 LPS-treated rats (4 mg/kg b.w.). Unpaired *t* test, *$p < 0.05$ compared with control group.

to -1.5 ± 0.9 mm Hg at 61-90 min, compared with the initial rise in P_{if} at 0-20 min (Fig. 1C). The drop in P_{if} at 41-60 min was also significant when compared with the control group (Fig. 1C).

(2) Fluid Volume Measurements

Skin: We measured fluid distribution volumes in skin to determine normal distribution of the isotopes in the extracellular and intravascular compartments. Total extracellular fluid volume (V_x) averaged 0.44 ± 0.01 mL/g wet weight (w.w.) and vascular volume (V_v) averaged 0.005 ± 0.0004 mL/g w.w., in agreement with previous data (Gyenge *et al.*, 2003). No differences were observed in skin fluid volumes 1.5 hr after LPS exposure.

Oral mucosa (Fig. 2): Control V_x averaged 0.43 ± 0.02 mL/g w.w. and V_v averaged 0.02 ± 0.003 mL/g w.w., resulting in a V_i = 0.41 ± 0.02 mL/g w.w. After LPS administration, V_i was significantly increased to 0.51 ± 0.03 mL/g w.w., $p < 0.05$, whereas V_v remained unchanged. The increase in V_i (~ 24.4%) shows edema formation in oral mucosa.

DISCUSSION

This study was designed to investigate the role of P_{if} in edema formation after LPS-, IL-1β-, and TNF-α-induced inflammation in the oral mucosa. Our results showed that LPS, TNF-α, and IL-1β induce lowering of P_{if} and therefore contribute to edema formation in inflammation.

The rate and direction of transcapillary fluid exchange are related to local differences in hydrostatic and colloid osmotic pressures between blood vessels and the interstitium (Starling, 1896). Edema will result when the net capillary filtration exceeds lymph drainage. A high driving pressure across the capillary is required to explain the rapid tissue swelling in acute inflammation. In skin, P_{if} falls as low as -150 mm Hg after burn injury (Lund *et al.*, 1988), providing the filtration pressure required for quick edema formation by creating a 'suction' on the fluid in the capillaries. This observation demonstrates that the biophysical properties of a tissue can be altered in a few

minutes *in vivo*, and that connective tissues can play an active role in fluid exchange (Berg *et al.*, 2001). Thus, the observed drop in P_{if} measured in the present study after LPS, IL-1β, and TNF-α exposure shows a mechanistic role of the connective tissue that may lead to the formation of inflammatory edema in the oral mucosa.

Cardiac arrest was induced 1-3 min after the administration of the inflammatory substances, to limit further filtration and thereby focus on the role of the extracellular matrix (Lund *et al.*, 1988). Circulatory arrest did not change P_{if} for up to 90 min in the controls. When LPS was administered locally at the gingiva, an immediate rise in P_{if} occurred. Although the rise was not statistically significant, it was consistent in all rats. Such a rise in P_{if} was not observed with either TNF-α or IL-1β. This observation indicates that LPS has an immediate effect in the mucosa, since interstitial fluid volume and subsequently P_{if} had started to rise before cardiac arrest was introduced, followed by lowering of P_{if} at 41-60 and 61-90 min when compared with the 0-20-minute period. The effect of LPS cannot be solely explained by the release of TNF-α and/or IL-1β, and must be attributed to mechanisms other than the release of these cytokines. In contrast, many cytokine-positive cells exist in the area of our measurements as a result of continuously invading irritants from the gingival sulcus (Miyauchi *et al.*, 2001; Bletsa *et al.*, 2004). It seems logical that LPS might have stimulated the release of cytokines from these cells, partly explaining the drop in P_{if} observed after 40 min. To our knowledge, fluid distribution volumes in oral mucosa have not been reported previously. In this study, we measured fluid distribution under normal conditions and after LPS exposure to investigate if the inflammatory mediators induced edema formation. In the controls, the interstitial and vascular fluid volumes represented 41% and 2% of tissue w.w., respectively, whereas LPS exposure resulted in a V_i of 51% of tissue w.w., showing significant tissue fluid accumulation in the inflammatory condition.

The roles of LPS and pro-inflammatory cytokines in the development of edema have been explored previously: LPS induced accumulation of pulmonary neutrophils and lung edema (Tate and Repine, 1983; Uchiba *et al.,* 1995). The administration of FR167653, a potent suppressor of IL-1β and TNF-α production, decreased serum IL-1β and TNF-α concentrations, which were associated with decreased lung injury and edema in rats (Yoshinari *et al.*, 2001). However, there are few data regarding the mechanism(s) behind such a relationship. Our results are in accordance with the findings observed in skin (Nedrebø *et al.*, 1999) after the application of cytokines. Both systemic and local administration of IL-1β and TNF-α induced significant lowering of P_{if}, with systemic administration inducing the drop in P_{if} earlier (21-40 min) than local administration (41-60 min). The cytokine concentration injected locally in this study was in accordance with cytokine levels found in mouse and human gingival tissue extracts from a periodontitis model and patients with periodontal disease, respectively (Stashenko *et al.*, 1991; Nishida *et al.*, 2001).

The β_1-integrin receptors have been assigned a mechanistic role in transcapillary fluid flux and edema formation in connective tissues (Reed *et al.*, 1992). These receptors are responsible for keeping the connective tissue under tensile forces. If the interaction between connective tissue cells and the extracellular matrix proteins is lost, the tissue is allowed to expand, resulting in lowering of P_{if}. *In vivo* blockade of β_1-integrin adhesion receptors in rat skin causes local edema concomitant with increased negativity of P_{if} (Reed *et al.*, 1992). The fibroblast-populated collagen gels represent an *in vitro* model of the phenomenon described above, and the integrin receptors mediate collagen gel contraction, whereas both IL-1β and TNF-α inhibit it (Tingstrom *et al.*, 1992; Zhu *et al.*, 2001). It therefore seems reasonable to speculate that cytokines induce lowering of P_{if} *via* perturbation of the β_1-integrin receptors in the oral mucosa.

An unexpected finding in this study was the variability in P_{if} in live animals. Tissue pressure in the oral mucosa is normally above ambient pressure (Johannessen *et al.*, 1987). The continuous formation of gingival fluid is presumably due to the fact that P_{if} in gingiva is higher than atmospheric pressure (Fjærtoft *et al.*, 1992; Del Fabbro *et al.*, 2001). We measured control P_{if} in all the rats in every group before any test substance was introduced, and the pressures varied between 0.2 ± 0.3 mm Hg (control group receiving 1% BSA locally) and 2.7 ± 0.5 mm Hg (group receiving TNF-α systemically). Although P_{if} in the rat oral mucosa has been very dependent on region (Johannessen *et al.*, 1987), such variation cannot explain the initial low P_{if} observed in some groups in this study. All P_{if} measurements were made by one operator at the exact location, and, therefore, it is more likely that differences are due to variability in animal stocks. Such discrepancies in P_{if} in control animals have been previously reported (Gjerde *et al.*, 2004) and explained by genetic or environmental factors. Despite the variations in *in vivo* P_{if}, the effects of the investigated agents were clear: No changes in P_{if} were observed in the control groups during the experimental period, whereas lowering of P_{if} occurred with the administration of cytokines and LPS. It is likely that a more stable control P_{if} would result in more statistical differences between control and experimental groups for the same time period.

To summarize, the present study demonstrates that LPS, IL-1β, and TNF-α induce lowering of P_{if} in rat oral mucosa, illustrating the important role of oral connective tissue in the development of inflammatory edema in gingiva.

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APPENDIX

MATERIALS & METHODS

Animal Preparation

Sixty-four female Wistar Møller rats (204-220 g) (Taconic M&B, Ry, Denmark) were used. Animals were fed a standard pellet diet and tap water *ad libitum*. All animals were anesthetized with pentobarbital sodium (Mebumal 50 mg/mL; Svaneapoteket, Bergen, Norway), 50 mg/kg body weight, given intraperitoneally. The rats were tracheotomized and placed on a heating pad thermostatically controlled to maintain rectal temperature at 37.5-38.5°C. The right femoral artery and vein were cannulated with PE-50 catheters for systemic arterial blood pressure (P_A) recordings and intravenous administration of substances, respectively. When P_{if} was measured (n = 50), the head was immobilized and fixed to the operating table by a stereotaxic frame, with the rat lying on its right side. The lips were pulled gently with thread to increase accessibility to the oral mucosa of the left maxillary region adjacent to the incisor, without causing any distortion to the tissue.

Test Agents and Administration

TNF-α and IL-1β (R&D systems, Minneapolis, MN, USA) were dissolved in 0.9% NaCl containing 1% bovine serum albumin (BSA, Fraction V; Sigma-Aldrich Chemie, Schnelldorf, Germany) at final concentrations of 400 ng/mL and 100 ng/mL for TNF-α and 30 ng/mL and 20 ng/mL for IL-1β, respectively. Lipopolysaccharide from *Escherichia coli* 0127:B8 (Sigma) was dissolved in 0.9% NaCl containing 1% BSA, to a final concentration of 5 mg/mL. For the fluid distribution experiments, the same type of LPS was dissolved in 0.9% NaCl containing 0.1% BSA, to a final concentration of 2.5 mg/mL.

Intravenous administration of test substances (TNF-α, IL-1β) or vehicle (0.9% NaCl with BSA) was done through the cannulated femoral vein in a volume of 0.5 mL. The submucosal injections of the test substances (TNF-α, IL-1β, LPS) or vehicle were performed with a 10-μL syringe (Hamilton, Bonaduz, Switzerland) in a volume of 5 μL of the test solution stained with Evans blue for visualization. A blue circle with diameter 1.5-2 mm appeared at the injection site, and P_{if} measurements were performed on the outer edge of this circle.

P_{if} Measurements

Pipettes were made from glass capillaries (1.00 mm OD x 0.58 mm ID) (Harvard Apparatus LTD, Kent, UK). They were pulled on a micropipette puller (P-87, Sutter Instruments Co., Novato, CA, USA) and sharpened to achieve a tip diameter of 2-7 μm (MB3/T-PSU5 microbeveller, World Precision Instruments LTD, Aston, UK). The pipettes were filled with 0.5 M NaCl solution stained with Evans blue and inserted into the tissue with a micromanipulator (Leica, Heerbrugg, Switzerland), as previously described (Johannessen *et al.*, 1987). Punctures of the oral mucosa were performed 1-2 mm distal-buccally of the maxillary left incisor at a depth of 0.2-0.6 mm below the surface under visual guidance *via* a stereomicroscope (MZ16, Leica, Wetzlar, Germany). Epi-illumination was provided by a two-armed fiber-optic lamp (CLS 150X, Leica). All micropuncture measurements were performed at heart level. P_{if} and P_A were monitored with a pressure transducer (model 1280C; Hewlett-Packard Medical Electronics Division, Waltham, MA, USA) connected to an amplifier and recorder (model 8188 2201 06; Gould Instrument Systems Inc., Valley View, OH, USA). The transducers were calibrated before each experiment.

We measured zero pressure before and after each recording by placing the micropipette in a drop of 0.9% NaCl on the mucosa or in a cup containing 0.9% NaCl at the level of the puncture site. A measurement was accepted when the following criteria were met:

(1) There was no visible distortion of the mucosa during measurement.

(2) Feedback gain could be altered without changing the recorded pressure.

(3) After criterion 2 was met, fluid communication between pipette and tissue was verified by the application of suction to the servo-controlled pump. When fluid could be moved into the pipette, this was visualized as increased electrical resistance in the pipette, due to lower tonicity of the fluid entering the pipette.

(4) Zero measurements before and after the P_{if} registration were unchanged.

Fluid Volume Measurements

Fourteen rats were used for measurements of fluid distribution volumes in oral mucosa.

Total extracellular fluid volume (V_x) and intravascular volume (V_v) were measured with the isotopes ^{51}Cr-labeled EDTA (^{51}Cr-EDTA) and ^{125}I-labeled Human Serum Albumin (^{125}I-HSA), respectively. After anesthesia and placement of catheters, both kidney pedicles were ligated *via* flank incisions, and 60-70 μCi of

^{51}Cr-EDTA was injected i.v. After a 90-minute equilibration period, 3-4 μCi of ^{125}I-HSA was given i.v. and allowed to circulate for 5 min. A blood sample of 0.5-0.7 mL was obtained from the arterial catheter, and the rat was killed with 0.5 mL KCl i.v. The rat was transferred to an infant incubator kept at 20-24°C and 100% relative humidity. A small area at the back of the rat was shaved, and a 2x2 cm piece of skin was cut with scissors and placed in a pre-weighed airtight tube. Oral mucosa was excised and transferred to the pre-weighed airtight tube, to avoid evaporation of fluid from the tissue. All tubes were re-weighed to obtain w.w. of the tissues. The blood samples were centrifuged at 11,000 rpm for 10 min. Known volumes of plasma were removed and used for further analysis. Samples were counted in a LKB γ-counter (Wallac 1282; Compugamma, Turku, Finland) with window settings of 15-75 keV for ^{125}I and 290-350 keV for ^{51}Cr. We counted standards in every experiment to obtain spillover corrections, and corrected counts for background and spillover.

Fluid volumes were calculated as the plasma equivalent distribution volumes of the tracers, assuming that the labeled EDTA will distribute in the extracellular fluid phase, and the labeled HSA will distribute only in plasma. Intravascular plasma volume (V_v) in a tissue sample was calculated as the 5-minute distribution volume of ^{125}I-HSA:

$$V_v\ (mL/g) = \frac{\text{Counts } ^{125}\text{I-HSA/g tissue}}{\text{Counts } ^{125}\text{I-HSA/mL terminal plasma}} \qquad (1)$$

Since ^{125}I-HSA was circulated in the animal for only 5 min, extravasation was negligible. Tissue extracellular fluid volume (V_x) was calculated as the 90-minute distribution volume of ^{51}Cr-EDTA:

$$V_x\ (mL/g) = \frac{\text{Counts } ^{51}\text{Cr-EDTA/g tissue}}{\text{Counts } ^{51}\text{Cr-EDTA/mL terminal plasma}} \qquad (2)$$

The tissue interstitial fluid volume (V_i) was calculated as the difference between extracellular fluid and plasma volume: $V_i(mL/g) = V_x - V_v$.