Expression of metallopeptidases and kinin receptors in swine oropharyngeal tissues: effects of ACE inhibition and inflammation.

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List of non-standard abbreviations:
AE: angioedema; ACE: angiotensin I-converting enzyme; ACEi: angiotensin I-converting enzyme inhibitor; AUC: area under curve; des-Arg⁹-BK: des-arginine⁹-bradykinin; mAPP: membrane-bound aminopeptidase P; LPS: lipopolysaccharide; GAPDH: glyceraldehydes 3-phosphate dehydrogenase; T0: time point before experimental protocol; TS: time point at sacrifice; vWF: von Willebrand factor.
Abstract

Angiotensin I-converting enzyme inhibitors (ACEi) cause both chronic and acute side effects, including rare but potentially life-threatening angioedema (AE). The main hypothesis to be tested in this study was that metallopeptidases and kinin receptors are present in oropharyngeal tissues and that their expression is modulated by ACEi and inflammation. Novel real-time polymerase chain reaction analysis was developed and allowed the relative quantification of tissue’s gene expression for neprilysin, membrane-bound aminopeptidase P (mAPP) and both B₁ and B₂ kinin receptor subtypes in tongue, parotid gland and laryngeal tissue (areas especially involved in the gravest clinical forms of AE) and in kidney in a porcine model (single injection or 7-day ACEi oral treatments applied, or lipopolysaccharide (LPS) injected as a positive inflammatory control). The results provide evidence of the expression and activities of kininases in oropharyngeal tissues in the swine. ACEi treatment modulated the expression of neutral endopeptidase and aminopeptidase P mRNA, but the corresponding enzyme activities and that of angiotensin I-converting enzyme (ACE) were generally stable in tissues. The 7-day ACEi treatment upregulated both kinin receptor’s mRNAs in the oropharynx, and the B₁ receptor mRNA in the lingual vascular endothelium (immunohistochemistry). The inhibition of ACE in plasma is responsible for an accumulation of bradykinin and des-arginine⁹-bradykinin (des-Arg⁹-BK) generated during activation of the contact system with glass beads. The expression of critical components of the kallikrein-kinin system in the oropharyngeal tissues supports the role of kinins in ACEi-induced AE.
Introduction

Despite their clinical effectiveness in various cardiovascular and metabolic diseases, angiotensin I-converting enzyme inhibitors (ACEi) cause chronic and acute side effects (Blais et al., 2000), notably potentially life-threatening angioedema (AE). Although the incidence of AE is considered low (0.1-0.5% of patients under ACEi therapy), the fact that 35 to 40 million patients worldwide receive this medication means these drugs could account for several hundred deaths per year due to laryngeal edema (Cugno et al., 2003). Moreover, two recent studies have shown AE incidence higher than previously appreciated, up to 0.68% of enalapril-treated hypertensive patients (Packer et al., 2002; Kostis et al., 2004). Also, a threefold incidence increase was reported in African-American patients, and it was nominally higher in smokers (Coats, 2002).

AE affects notably the extremities, abdominal cavity, face, larynx, and tongue. However, a laryngeal localization is at high risk. Although the clinical symptoms of AE have been attributed to the vasoactive peptide bradykinin, no definitive experimental evidence supports an obligatory role for this agent.

The nonapeptide bradykinin is the prototype of kinins, a family of powerful bioactive autacoids, involved in a series of physiological and pathological cardiovascular responses, mainly vasodilatation, increased capillary permeability and pain processes (Leeb-Lundberg et al., 2005). Bradykinin and kallidin (Lys-bradykinin) exert their pharmacological activities by binding to their constitutively expressed kinin B2 receptor before being metabolized by multiple peptidases (Leeb-Lundberg et al., 2005). The identity of the metallopeptidases involved in bradykinin metabolism in vitro and their relative importance vary according to the biological medium considered. In various cell types and tissues such as kidney, endothelial cells and cardiomyocytes, neutral endopeptidase
24.11 (NEP, nepriyisin) plays an important role in the degradation of bradykinin (Raut et al., 1999). In human plasma, bradykinin is metabolized mostly by three metallopeptidases (Decarie et al., 1996; Cyr et al., 2001). Angiotensin I-converting enzyme (ACE) constitutes the main degradation pathway. Carboxypeptidase N transforms bradykinin and kallidin into their active metabolite, des-arginine⁹-bradykinin (des-Arg⁹-BK) and Lys-des-Arg⁹-BK (des-Arg¹⁰-kallidin), respectively (a minor metabolic pathway in plasma unless ACE is inhibited). We have also shown that aminopeptidase P (X-prolyl aminopeptidase, APP) plays an important role in the metabolism of kinins in plasma, mostly for des-Arg⁹-BK (Cyr et al., 2001). Although the carboxytruncated metabolites of bradykinin and kallidin are largely inactive under normal conditions, they are the agonists of the strongly regulated B₁ receptors, of which receptor synthesis is increased in experimental models of inflammation under the control of cytokines, MAP kinases and specific transcription factors (Leeb-Lundberg et al., 2005).

We have previously reported a decreased degradation of endogenous des-Arg⁹-BK in the plasma of hypertensive patients who, while treated with an ACEi, experienced an AE (Molinaro et al., 2002). This anomalous breakdown was linked to a decreased aminopeptidase P plasma activity, supporting a pathogenic mechanism relying on kinin catabolism (Adam et al., 2002; Molinaro et al., 2002). Human aminopeptidase P exists in both cytosolic and membrane-bound forms, the latter being most likely responsible for plasma activity (Molinaro et al., 2005). Moreover, a recent report describes a single nucleotide polymorphism within the membrane-bound aminopeptidase P (mAPP) gene XPNPEP2, linked to low plasma aminopeptidase P activity (Duan et al., 2005). Although plasma aminopeptidase P deficiency states could predispose to AE in some ACEi-treated patients, little is known about the physiological roles of this metallopeptidase in tissues. These observations based on plasma can hardly be extrapolated to a localized AE affecting the oropharyngeal tissues.
The first aim of this paper was to quantify metallopeptidases and kinin receptor mRNAs in oropharyngeal tissues in swine using novel real-time PCR analysis. As a second step, we have defined the effect of inflammation and of acute and chronic ACE inhibition on the expression of these biochemical entities that respectively limit and mediate the pharmacological activity of kinins during an episode of AE. As a sequel to our preceding \textit{in vitro} investigations, we have defined the effect of these \textit{in vivo} treatments on the metabolism of endogenous kinins in plasma.
Materials and Methods

Reagents. The ACEi enalaprilat (Vasotec I.V., 1.25 mg/mL) and enalapril (Vasotec) were from Merck Frosst Canada (Kirkland, QC, Canada); RNAlater® was from Ambion (Austin, TX); bradykinin and des-Arg⁹-BK were acquired from Peninsula Laboratories (Belmont, CA); LPS extracted from *Escherichia coli* serotype O111:B4, L-arginine and o-phthalaldehyde were from Sigma-Aldrich (Oakville, ON, Canada); TRIzol reagent was purchased from Invitrogen (Burlington, ON, Canada). The internally quenched fluorescent substrates (Abz)RGL(EDDnp) ((o-aminobenzoic acid)-Arg-Gly-Leu-(ethylenediamine 2,4-dinitrophenyl)) for neprilysin (Medeiros et al., 1997), K(Dnp)PPGK(Abz) (Lys-(2,4-dinitrophenyl)-Pro-Pro-Gly-Lys-(o-aminobenzoic acid)) for mAPP (Molinaro et al., 2005) and (Abz)YRK(Dnp)P ((o-aminobenzoic acid)-Tyr-Arg-Lys-(2,4-dinitrophenyl)-Pro) for ACE (Araujo et al., 2000) were a gift from Pr A Carmona (Dept. of Biophysics, Escola Paulista de Medicina, UNIFESP, São Paulo, Brazil). PCR primers were synthesized by Biocorp Inc. (Montreal, QC, Canada).

Animals. Twenty four healthy male crossbred barrows (Yorkshire × Landrace), approximately two months old and weighing 16.1 ± 1.6 kg were purchased from a specific pathogen free farm (faculté de Médecine Vétérinaire, Université de Montréal) and were kept in an isolated room. Within the room, the animals were housed in separate pens. Feed and water were provided *ad libitum* throughout the experiment.

Experimental protocol. The experimental protocol was approved by the Faculty of Veterinary Medicine ethics committee (Université de Montréal) in accordance to the Canadian Council on Animal Care Guidelines. The 24 animals were randomly allocated in four different treatment groups: (i) the 1-time ACEi treatment group (enalaprilat 100 µg/kg IV injection once); (ii) the 7-
day ACEi treatment group (enalapril maleate tablet 20 mg PO) 12 h apart for seven consecutive
days; (iii) the LPS group (5 µg/kg dissolved in 1 mL of NaCl 0.9% IV once); and (iv) untreated
animals (control group). At the end of the experiments, all animals were euthanized with an IV
lethal dose of sodium pentobarbital (540 mg/mL). The 1-time ACEi group was euthanized 4 h post-
enalaprilat IV injection, the 7-day ACEi group on the morning of the eighth day, and the LPS
animals were sacrificed 6 h post-LPS IV injection. Sacrifice immediately followed the last blood
samplings. Two control pigs were sacrificed with each group of treated animals.

Venous blood samples were collected in the 1-time ACEi group before, 45 min and 4 h post-ACEi
injection; in the 7-day ACEi group on the first day (prior to the first oral dose of ACEi), on the
third and eighth day of treatment; and in the LPS group prior to and 6 h after LPS injection. Control
group samples were taken at all time points on 6 animals. Blood samples were collected into
sodium citrate and EDTA-treated tubes (Vacutainer Becton Dickinson, Franklin Lakes, NJ). The
citrated blood samples were immediately centrifuged (2400 × g for 10 min) and the plasma from
each animal was aliquoted in polypropylene tube and immediately stored at -80°C until further
analysis.

Tissues from the oropharyngeal zone (parotid gland, tongue and laryngeal tissue), and a piece of
kidney, were quickly excised upon euthanasia, washed in cold saline solution and flash frozen in
liquid nitrogen and/or conserved in RNAlater® for protein and RNA extraction.

**Assessment of the systemic inflammatory response.** C-reactive protein, an indicator of a
systemic inflammatory response, was quantified in plasma with a commercial, solid-phase,
sandwich immunoassay kit (PHASE RANGE Porcine CRP Assay Kit; Tridelta, Maynooth,
Co. Kildare, Ireland) according to the manufacturer’s instructions. Total and differential leukocytes counts were routinely obtained with a Coulter counter and these systemic parameters were complemented by rectal temperature assessment in LPS-treated and control animals.

**Biochemical investigations in plasma.** The activity of ACE, aminopeptidase P and carboxypeptidase N was measured using the methods described previously. ACE activity was determined by Buhlmann ACE radioenzymatic assay (Angiotensin I-Converting Enzyme $^3$H-REA; ALPCO, Windham, NH) according to the manufacturer’s instructions. The activity of aminopeptidase P and carboxypeptidase N was assessed by fluorimetric assay as described (Cyr et al., 2001).

The metabolism of the endogenous kinins, bradykinin and its active metabolite des-Arg$^9$-BK, was studied through *in vitro* plasma contact system activation as described extensively elsewhere (Cyr et al., 2001; Molinaro et al., 2002), using two specific competitive chemiluminescent enzyme immunoassays, as previously described (Decarie et al., 1994; Raymond et al., 1995).

**Total RNA isolation from tissues.** Total RNA was isolated using the TRIzol reagent and RNAqueous®-4PCR kit (Ambion) according to the manufacturer’s instructions with modification. Briefly, ~50 mg of each tissue was first homogenized in the TRIzol reagent, and chloroform was added. After centrifugation, the aqueous phase was mixed with the lysis/binding solution and the RNAqueous®-4PCR protocol was subsequently followed. All RNA samples were finally DNase-treated to remove traces of genomic DNA, quantified using RiboGreen fluorescent nucleic acid stain (RNA quantification kit; Molecular Probes, Eugene, OR) and stored at -80°C until use.
Reverse transcription and Real-time Polymerase Chain Reaction (real-time PCR). The 0.5 µg amount of total RNA was transcribed into cDNA using 50 U Moloney-murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA) and 5 µM oligo(dT)₁₆ primer. Quantification of the RNAs was performed by real-time PCR using the LightCycler 2.0 apparatus (Roche Diagnostics, Mannheim, Germany). Two microliters of cDNA were brought to a final volume of 20 µL containing 2 mM MgCl₂, 2 µL LightCycler-FastStart DNA SYBRGreen I Mix (Roche Diagnostics), and 0.5 or 0.7 µM primers (Table 1) in water. After initial activation of the DNA polymerase at 95°C for 10 min, the amplification conditions were as follows: 47 cycles consisting of denaturation at 95°C for 15 s, annealing for 12 s at 62°C (glyceraldehydes 3-phosphate dehydrogenase (GAPDH), B₁ receptor, B₂ receptor, neprilysin) or 15 s at 60°C (mAPP), and extension at 72°C. The extension times were calculated from the amplicon size (base pairs/25). Fluorescence data were acquired at the end of each extension phase. After amplification, a melting curve analysis from 65 to 98°C with a heating rate of 0.1°C/s with a continuous fluorescence acquisition was made. Standard curves were created from specific PCR products. The corresponding real-time PCR efficiency \( E \) of one cycle in the exponential phase was calculated from the slopes calculated by the LightCycler software according to the equation:

\[
E = 10^{[-1/slope]} \quad (\text{Pfaffl, 2001}).
\]

Quantification was done by using a mathematical model presented below to determine the relative quantification of a target gene in comparison to a reference gene. The relative quantification of a target transcript is based on the PCR efficiency of the individual transcripts and crossing point (Cp) deviation (calculated by the second derivative maximum method) of a control and an unknown
sample, normalized by a reference transcript. This relative expression ratio can be calculated by this equation:

\[
\text{Normalized ratio} = \frac{E_T^{C_p(T)}}{E_R^{C_p(R)}} \times \frac{E_R^{C_p(R)}}{E_T^{C_p(T)}}
\]

\(E_R\) is the real-time PCR efficiency of a reference gene transcript; \(E_T\) is the real-time PCR efficiency of target gene transcript; \(C_pR\) is the deviation of reference gene transcript, \(C_pT = C_p\) deviation of the target gene transcript, \(S\) is the unknown sample and \(C\) is the calibrator. The expression was normalized against the expression of GAPDH and calculated for each animal for a given tissue and target gene.

**Measurement of ACE, mAPP and neprilysin enzymatic activities in tissues.** Tissues from the oropharyngeal zone, namely from the parotid gland, tongue and laryngeal tissue, and a piece of kidney, weighing ~500 mg were homogenized in Tris buffer (50 mM Tris, 100 mM NaCl, pH 7.4, and 1× Complete Protease inhibitor solution (Protease inhibitor cocktail tablets; Roche Diagnostics, Mannheim, Germany) with a Polytron homogenizer (Brinkmann Instruments, Rexdale, ON, Canada), and sonicated for 10 min. A solution of 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid (CHAPS) (Sigma) was added to each homogenate to final concentration of 8 mM, incubated on ice for 2 h, centrifuged (1750 x g, 4°C) and the supernatant containing total membrane proteins was collected (Raut et al., 1999). Final protein concentrations were determined using the bicinchoninic acid method (Pierce Biotechnology, Rockford, IL).

Metallopeptidase (ACE, mAPP and neprilysin) activities were measured in tissue extracts using quenched fluorescent substrates. Reactions were run in duplicate in 96-well flat bottom plates
(Costar UV plate 3635; Corning Inc., Corning, NY) in a final volume of 150 µL. Ten mg of membrane protein extracts in a 50 mM Tris·Cl buffer pH 7.4, 100 mM NaCl, 10 µM ZnCl₂ for ACE and neprilysin or 100 mM Hepes buffer, pH 7.4 for mAPP were incubated with 10 µM final concentration of (Abz)YRK(Dnp)P for ACE, (Abz)RGL(EDDnp) for neprilysin or K(Dnp)PPGK(Abz) for mAPP. Plates were preincubated at 37°C for 15 min before substrate addition. Fluorescence (excitation wavelength of 340 nm, emission wavelength of 420 nm) was assessed kinetically in a FL600 microplate fluorescence plate reader (BioTek, Winooski, VT). Fluorescent units were converted into pmol of hydrolyzed substrate based on a standard fluorescence curve.

**Immunohistochemistry.** Lingual tissue samples from all pigs were obtained at necropsy and fixed using 10% buffered formalin phosphate for histological examination. Paraffin-embedded tissue sections (5 µm thick) were prepared, submitted to an antigen-retrieving procedure (Marceau et al., 1999) and immunostained at 37°C for 1.5 h with one of the following antibodies: monoclonal anti-α-actin (Sigma-Aldrich, clone 1A4, dilution 1:100), monoclonal anti-human-B₂ receptor (Blaukat et al., 2003), polyclonal anti-von Willebrand factor (vWF; Dako, Mississauga ON, Canada; dilution 1:100), or a mixture of polyclonal antibodies raised against 6 distinct peptides from the B₁ receptor human sequence (Mazenot et al., 2001). The antibody staining was revealed using horseradish peroxidase-coupled goat anti-mouse IgG (Sigma-Aldrich, dilution 1:100) for primary monoclonal antibodies or monoclonal anti-rabbit IgG (Sigma-Aldrich, dilution 1:200) for polyclonal antibodies (30 min reactions, 37°C). The secondary antibodies were allowed to react for 5-15 min at 25°C with the Immunopure Metal Enhanced diaminobenzidine substrate (Pierce). Endogenous peroxidase was initially inhibited in tissue sections using 3% H₂O₂ (5 min).
Statistical Analysis. The systemic inflammatory response and the enzymatic activities in plasma are analyzed using a two-way analysis of variance with repeated measures on one of the factors. The factors are the groups with 4 levels (1-time ACEi treatment, 7-day ACEi treatment, LPS and control) and the time with 2 levels (before experimental protocol (T0) and at sacrifice (TS)), the latter being the repeated factor. In case of interaction, separate analyses of variance with the factor group are performed at each time. Significant differences with the control groups were further assessed using Dunnett’s test ($p < 0.05$).

For the normalized ratio of the examined target gene/GAPDH generated by the real-time PCR analysis, a logarithmic transformation was used to bring the data closer to normality. The statistical significance was established using the mean values of the log normalized ratio calculated for each animal for a given tissue. The biochemical investigations in tissues are analyzed separately for each tissue. For the log normalized ratio and the biochemical parameters, a one-way analysis of variance with a factor group with 4 levels (1-time ACEi treatment, 7-day ACEi treatment, LPS and control) was used followed by Dunnett’s test when a significant difference was observed ($p < 0.05$).
Results

Assessment of the systemic inflammatory response. Figure 1A represents the systemic inflammatory status of each group of animals, assessed by plasma C-reactive protein concentrations, immediately before experimental protocol and prior to sacrifice. There is a significant interaction between the factors time and group for C-reactive protein analysis (F (3,20) = 11.19, $p < 0.001$). No difference was observed between the groups at T0 (F (3,21) = 1.37, $p = 0.279$) but, at TS, a significant difference was shown (F (3,21) = 9.147, $p < 0.001$). The concentration of this acute phase protein was significantly higher for the LPS group when compared to the control group ($p = 0.001$). The 1-time or 7-day treatment with ACEi does not significantly modify the plasma concentrations of this acute phase protein marker.

Figure 1B shows the number of circulating leukocytes for the 4 groups before treatment and prior to sacrifice. As for C-reactive protein, a significant interaction was observed between the factors time and group (F (3,19) = 8.545, $p = 0.001$). No significant difference was noted at T0 (F (3,21) = 1.764, $p = 0.185$) between treatments but, at TS, a significant difference was obtained between the groups (F (3,23) = 19.402, $p < 0.001$): the LPS injection produced a significantly decreased total leukocyte count at TS compared to the control animals ($p < 0.001$).

Finally, the rectal temperatures measured in the LPS and control groups showed that the temperature peaked at 4 h and returned to baseline by the TS (6 h) (data not shown).

Biochemical analysis in plasma. Enzymatic activities measured in blood plasma for ACE, aminopeptidase P and carboxypeptidase N are presented in Table 2. For ACE, the four groups progressed differently and a significant interaction was observed between the factors time and
group \( (F(3,20) = 43.417, p < 0.001) \). At T0, a difference was obtained between the groups \( (F(3,20) = 4.77, p = 0.011) \); the 1-time ACEi treatment group was significantly different from the control group \( (p = 0.025); \) an unexplained variation observed before drug dosing). At TS, a significant difference was observed \( (F(3,20) = 105.81, p < 0.001) \); as expected, the 1-time and 7-day ACEi treatments lead to a significantly lower ACE activity \( (p < 0.001) \) when compared to LPS and control groups. Both ACEi and LPS treatments do not affect significantly plasma activities of aminopeptidase P at TS. Indeed, no interaction between the factors time and group and no time effect were noted \( ((F(3,20) = 0.452, p = 0.719) \) and \( (F(1,20) = 0.286, p = 0.599), \) respectively) (data not shown). For carboxypeptidase N, there is a significant interaction between the factors time and group \( (F(3,20) = 5.088, p = 0.009) \). No group effect at each time was observed but a time effect was significant only for LPS group, where carboxypeptidase N activity was \( 67 \pm 10 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mL}^{-1} \) at T0, and increased to \( 86 \pm 6 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mL}^{-1} \) at euthanasia \( (p < 0.001) \).

**Endogenous bradykinin and des-Arg\(^9\)-BK metabolism.** Figure 2 illustrates the calculated area under the curve (AUC), representing the accumulation and subsequent catabolism of immunoreactive bradykinin and des-Arg\(^9\)-BK measured during the *in vitro* activation of the contact system using glass beads in 1 mL of plasma sampled from each animal at TS. For bradykinin, a significant interaction was observed between the factors time and group \( (F(3,20) = 8.440, p = 0.001) \). No significant difference was noted for data at T0 \( (F(3,20) = 1.205, p = 0.333) \) but at TS, the difference was significant \( (F(3,20) = 60.94, p < 0.001) \) and obtained for the 1-time and 7-day ACEi treatment groups when compared to the control group \( (p < 0.001, \) respectively). For des-Arg\(^9\)-BK, the same outcome was observed \( (F(3,20) = 7.069, p = 0.002) \). No difference was noted at T0 \( (F(3,20) = 1.745, p = 0.190) \) but one occurred at euthanasia \( (F(3,20) = 11.475, p < 0.001) \); in fact, the 1-time ACEi \( (p = 0.006) \) and 7-day ACEi \( (p < 0.001) \) treatment groups were statistically
higher from the control group. For the LPS group, the AUC was not statistically different from the control group, for neither bradykinin nor des-Arg⁹-BK. There were no differences amongst groups from samples collected at every other time (data not shown).

**Real-time Polymerase Chain Reaction (real-time PCR) applied to tissues.** Specificity of PCR products was confirmed on the agarose gel illustrated in Figure 3A, and resulted in single bands, each one with the predicted length. The melting curves analyses are an exact and fast method to check PCR specificity and are displayed as first negative derivative of the fluorescence versus the temperature. Typical LightCycler melting curve analysis from the amplification of the targets and reference transcripts resulted in product specific melting temperatures, as illustrated in Figure 3B.

Table 3 shows the log of normalized ratios for metallopeptidases in oropharyngeal tissues and kidney and Figure 4 and Table 4, the same information for the kinin receptors. For mAPP, the 7-day ACEi treatment significantly increased the expression of the mRNA corresponding to this enzyme in the tongue ($p < 0.001$) and laryngeal tissue ($p = 0.027$). The expression of neprilysin mRNA was significantly increased in kidney, tongue and parotid for the 7-day ACEi treatment group ($p = 0.038$, $p = 0.007$ and $p = 0.026$; respectively) but a significant decrease in its expression was observed for the 1-time ACEi and LPS treatments in kidney ($p < 0.001$). The expression of the B₁ receptor mRNA was significantly higher in kidney and oropharyngeal tissues only for the 7-day treatment with ACEi (kidney, tongue and parotid: $p < 0.001$, laryngeal tissue: $p = 0.002$; Figure 4). Neither acute ACEi treatment, nor single LPS injection significantly affected the expression of the mRNA coding for this receptor in the kidney, tongue, parotid gland or laryngeal tissue at the tested time points. The 1-time ACEi treatment and LPS treatment led to a significantly lower expression of the B₂ receptor mRNA in the kidney ($p = 0.002$, $p = 0.008$; respectively; Table 4). In contrast, in
the tongue ($p = 0.002$) and laryngeal tissue ($p = 0.007$), B$_2$ receptor mRNA expression was significantly increased by a 7-day treatment with ACEi.

**Metallopeptidase activities in tissues.** Metallopeptidase activities measured in tissue extracts from control animals using quenched fluorescent substrates are given in Table 5. Neither acute nor 7-day ACEi treatments, nor the single LPS injection significantly affected ACE activities in the kidney, tongue, parotid gland or laryngeal tissue when compared to the control group (data not shown). However, a significant treatment effect was noted for the neprilysin activity in the kidney and for the mAPP activity in parotid gland ($F(3,20) = 19.567$, $p < 0.001$) and ($F(3,20) = 3.440$, $p = 0.036$), respectively) when compared to the control group. Indeed, the 1-time and 7-day ACEi treatment significantly increased neprilysin activity in the kidney, $20461 \pm 841$ ($p < 0.001$), and mAPP activity in the parotid gland, $294 \pm 151$ ($p = 0.027$), respectively.

**Immunohistochemistry applied to lingual tissue.** The tongue, examined below the epithelial surface, contains connective tissue and striated muscle along with blood vessels of various sizes (Figure 5, hematoxylin and eosin stain). The blood vessels are further identified by immunoreactivity for the endothelial cell marker vWF (one-cell thick lines) and by the smooth muscle cell marker $\alpha$-actin (thicker labelling, in arteriole and venule walls). Unexpectedly, tissues from all animals treated for 7 days with the ACEi exhibit only faint vWF vascular staining (Figure 5). The staining for the B$_2$ receptor is present in endothelial cells of identifiable blood vessels in all groups, but the comparatively weaker B$_1$ receptor signal is only found following treatment with LPS or 7-day ACEi.
Discussion

Angioedema (AE) is a local inflammatory reaction, yet most if not all previous biochemical investigations on AE are based on plasma analysis (Nussberger et al., 1998). Here we have set out to analyze in more detail the pathogenesis of ACEi-induced AE. To this end, we used an animal model, i.e., the pig, which is large enough to allow repeated blood sampling and harvesting of oropharyngeal tissues to support multiple analyses.

Clinical and experimental lines of evidence plead for a multifactorial mechanism of ACEi-associated AE. Angioedema could be the result of a coincidence of distinct pharmacological, metabolic and pathophysiological factors. Previously, we have shown that AE patients have an anomaly of the metabolism of des-Arg⁹-BK due to a defect of plasma aminopeptidase P activity, evidenced only in presence of ACEi (Adam et al., 2002; Molinaro et al., 2002). We have shown recently that this low aminopeptidase P depends largely on genetic factors (Duan et al., 2005). As the incidence of AE is higher in hypertensive smokers treated with an ACEi (Coats, 2002), we analyzed the effect of inflammation (a pathophysiological factor) on the tissue expression of metallopeptidases and on the plasma metabolism of kinins. Inflammation was mimicked by a sublethal dose of LPS. We chose a dose substantially less than that typically used in rats and mice because, like human and nonhuman primates, pigs are extremely sensitive to LPS (Warren et al., 1997). As AE episodes have been reported as early as on the first day (Wood et al., 1987) or later during ACEi treatment (pharmacological factor) (Hedner et al., 1992; Shionoiri et al., 1996), two groups of pigs were treated, acutely or chronically. The dosages of a single injection of 100 µg/kg enalaprilat IV and the *per oral* application of 20 mg of enalapril twice daily were chosen in preliminary studies, as they both allowed consistent inhibition of plasma ACE activity, as extensive as in our *in vitro* studies (Cyr et al., 1999; Molinaro et al., 2002).
Methods previously developed in our laboratory in order to define the metabolism of endogenous kinins (Decarie et al., 1994; Raymond et al., 1995; Cyr et al., 2001) were applied to the plasma of the experimental animals. A significant in vitro accumulation of kinins (as assessed by the AUC, Figure 2) has been monitored in the one-time and 7-day ACEi treatment groups when compared to controls. As aminopeptidase P activities in plasma were similar in both ACEi treated groups, the observed increased bradykinin and des-Arg^9^-BK concentrations are exclusively a consequence of ACE blockade and not of a non-specific inhibition of the activity of aminopeptidase P by ACEi. These observations are important as Hooper and colleagues (1992) reported a non-specific inhibition of purified pig aminopeptidase P by some ACEi (Hooper et al., 1992).

We provide evidence for the expression of kininases and kinin receptors in the oropharyngeal tissues of swine. For this purpose, we developed the relative quantification of our target gene transcripts XPNPEP2 (membrane aminopeptidase P), MME (neprilysin), BDKRB1 (B_1 receptor) and BDKRB2 (B_2 receptor) in comparison to the reference GAPDH gene transcript. After an analytical validation, a mathematical approach for data analysis was presented to calculate the relative expression of normalized logarithmic ratios.

We show that gene expression of the metallopeptidases neprilysin and mAPP in the oropharyngeal tissues is lower that measured in the kidney when expressed as a ratio to GAPDH (Table 3 and data not shown) and the enzyme activity measurements fully support this observation. The kidney is a valuable positive expression control that contains all components of the kallikrein-kinin system. A 7-day treatment with an ACEi increased mAPP mRNA expression in the tongue and laryngeal tissue, and also increased neprilysin mRNA expression in kidney, tongue and parotid gland tissues;
paradoxically, neprilysin expression was decreased after a single administration of enalaprilat, as well as in LPS treated animals, and this only in the kidney. As in the control animals, the corresponding enzyme activities were rather stable, with occasional changes that did not match the mRNA variation. Although these observations suggest that other factors regulate the activity of the peptidases, a high variation of the low activities did not reach statistical significance even when the activities are higher or lower than these measured in the control animals (Table 5 and data not shown). The mechanisms by which ACEi leads to changes in metallopeptidase mRNA expression in the present experimental model remain to be elucidated, although other laboratories have reported that ACEi increase ACE mRNA and activity in cell systems and in some tissues (Chai et al., 1992; King and Oparil, 1992). We were unable to assess the effect of ACEi on ACE expression itself, because obtaining valid and specific primers for the porcine gene was not possible and attempts to synthesize primers based on homologous mammalian sequences remained unsuccessful. To circumvent these problems, the quantification of enzyme activities in tissue homogenates, using highly sensitive and specific internally quenched fluorescent substrates for ACE was done, as well as for neprilysin and mAPP (Molinaro et al., 2005). In fact, the enzyme activities in oropharyngeal tissues could not be measured using traditional end point methods (Blais et al., 1999). The effects of ACEi treatments on tissue ACE activities contrast with those observed in plasma; no significant difference was seen in ACE catalytic activity in any tissue, for any treatment. These results parallel others in this laboratory for rat and human heart and they could be explained by a washout of the ACEi during the tissue homogenization for membrane preparation (Kinoshita et al., 1993; Blais et al., 1997; Blais et al., 2000a).

Local tissue inflammation in ACEi-treated pigs took the form of a strong upregulation of the mRNA levels for the kinin B₁ receptor in all oropharyngeal and renal tissues following the 7-day
drug treatment only. This contrasts with B₂ receptor regulation, modulated in a tissue specific manner by 7-day ACEi treatment (mRNA upregulated in the tongue and laryngeal tissue), and in an apparently inconsistent manner with other tissues or treatments. B₁ and B₂ receptor genes are well documented to be differently regulated in vivo (Leeb-Lundberg et al., 2005). B₁ receptor mRNA was expected to be low in healthy tissues, but upregulated during inflammatory conditions. In our hands, LPS administration at a mid-range level that does not overtly stress the animals (Warren et al., 1997) did not substantially alter kinin receptor mRNA expression in any tested tissues despite a marked systemic inflammatory reaction (Naess et al., 1989). The observed increased plasma carboxypeptidase N in the LPS group supports the idea that the synthesis of this protein is increased by inflammation and can be considered to be an acute-phase reactant protein, as previously observed in patients with inflammatory arthritis (Chercuitte et al., 1987). The lack of B₁ receptor mRNA upregulation in this experimental group could be explained by the low dose used and short duration of the endogenous cytokine action (as monitored by the febrile reaction). However, the lingual immunohistochemistry study suggests that some endothelial B₁ receptor protein was still present 6 h post-LPS after a hypothetic rise and fall of the corresponding mRNA; this induction reproduces published functional results for this species (Siebeck et al., 1996). ACEi-induced B₁ receptor expression has been observed in chronically treated rats (Marin-Castano et al., 2002), although 48 h treatment with enalaprilat did not induce B₁ receptor expression in the healthy rabbit (mRNA in several organs, function) despite an effective ACE blockade that should theoretically potentiate endogenous kinins (Marceau et al., 1999). A species- and physiological state-dependent toxic reaction to ACEi could explain the upregulation of B₁ receptor without overt systemic inflammatory reaction (as C-reactive protein remained low and leukocyte counts normal in ACEi treated pigs). Vasopressin or epinephrine administration to human subjects depletes endothelial vWF in biopsies from the oral mucosa (Takeuchi et al., 1988). Such depletion was observed in the
lingual vasculature in the 7-day ACEi group and may result from a slow onset hemodynamic adaptation that includes the secretion of these hormones and a form of endothelial stimulation that favours B1 receptor expression.

In conclusion, we provide here a sensitive real-time PCR analyses that permit following gene expression of metallopeptidases and kinin receptors in the oropharyngeal tissues in the pig. We have detected subtle changes following ACEi treatment. The low activity of kininases, the presence of specific receptors, mainly B1 receptor, the modulatory effect of inflammation and ACEi support a plausible mechanism whereby kinins could initiate a vasogenic inflammatory process (AE) via the sustained stimulation of either or both kinin receptor subtypes. These results obtained using ACEi could be the basis of future investigations on the pathophysiology of AE associated with more «modern» cardiovascular drugs, e.g., AT1 receptor antagonists and vasopeptidase inhibitors. Moreover, encouraging results in the clinical trial of the B2 receptor antagonist, Icatibant, support that kinin receptor blockade is of therapeutic interest in hereditary AE (Rosenkrans et al., 2004). It remains to be seen whether B1 receptor blockade could be more effective in this and other forms of AE.

Acknowledgments

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References


Footnotes

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Figure Legends

Figure 1. Systemic inflammatory response. Systemic inflammatory response represented by dot-plots. A. C-reactive protein (CRP) concentrations in pigs for each group before treatment of ACEi and endotoxin infusions in pigs (T0, open circles) and prior to sacrifice (TS, closed circles). * $p = 0.001$. B. Leukocytes counts before treatment of ACEi and LPS (T0, open circles) and prior to sacrifice (TS, closed circles). ** $p < 0.001$. The line represents the median.

Figure 2. Area under curve (AUC) of immunoreactive bradykinin and des-Arg$^9$-BK. AUC of immunoreactive bradykinin and des-Arg$^9$-BK as a function of time are illustrated in graph for each experimental group (7-day ACEi, 1-time ACEi, LPS and control) and values measured in each treatment group were compared with the control group values. Box plots display summary statistics for the distribution. The line in the box represents the median. * $p = 0.006$; ** $p < 0.001$. Insets represent the kinetic profiles of the formation and degradation of bradykinin (BK) (A) and des-Arg$^9$-BK (B) for mean 7-day (closed circles) and 1-time (open circles) treatment with ACEi and LPS (dashed line) group prior to sacrifice (TS) after activation of the contact system with glass beads in plasma. Dotted lines are values for control group.

Figure 3. Amplification specificity by real-time PCR. Specificity of PCR products for each primer was visualized in 2% agarose gel and resulted in a single band with the predicted length (A). In addition, a PCR product dissociation curve (melting temperature analysis) was performed which resulted in single product specific melting temperature (B).
Figure 4. Log of the normalized ratio $B_1$ receptor mRNA/GAPDH mRNA in oropharyngeal tissues and kidneys. Values are averaged ratios ± SD ($n = 6$). * $p < 0.002$; ** $p < 0.001$ vs controls.

Figure 5. Immunohistochemistry in porcine lingual tissue. Representative sections from each experimental group are shown (original magnification 100 $\times$). The presence of blood vessels is shown in hematoxylin and eosin (H&E)-stained sections. noAb refers to background staining of secondary peroxidase-labeled anti-rabbit IgG antibodies in the absence of primary antibodies (other sections treated with the anti-mouse IgG antibodies exhibited a similar background). Arrowheads point at endothelium-like structures labelled with the anti-kinin receptor antibodies in relatively large blood vessels.
Table 1. Oligonucleotides for real-time PCR.

Sequences derived from GenBank accession numbers AF017079 (GAPDH), AF540788 (B₁ receptor (B₁R)), AB051422 (B₂ receptor (B₂R)), U55039 (membrane-bound aminopeptidase P (mAPP), and NM_000902 (neprilysin (NEP)).

| mRNA Targets | Nucleotide Sequences (5’→3’)
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH-GAPDH</td>
<td>F834: ACT TCG GCA TCG TGG AAG GAC T&lt;br&gt;</td>
</tr>
<tr>
<td></td>
<td>B₁R-BDKRB1 gene&lt;br&gt;</td>
</tr>
<tr>
<td></td>
<td>B₂R-BDKRB2 gene&lt;br&gt;</td>
</tr>
<tr>
<td>mAPP-XPNPEP2</td>
<td>F1716: AAC AAC AGA CAT CAC CCG AA&lt;br&gt;</td>
</tr>
<tr>
<td>NEP-MME gene</td>
<td>F1246: CAG CGC AAC CTACAA GGA GTC CAG AAA&lt;br&gt;</td>
</tr>
</tbody>
</table>

a F and R indicate forward and reverse primers, respectively; numbers indicate the sequence position.

b Final concentration of forward (F) and reverse (R) primers.
Table 2. Enzymatic activity of ACE, aminopeptidase P (APP) and carboxypeptidase N (CPN) (nmol·min⁻¹·mL⁻¹) in plasma before treatment (T0) and prior to sacrifice (TS).

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Control</th>
<th>1-time ACEi</th>
<th>7-day ACEi</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>T0</td>
<td>214 ± 29</td>
<td>151 ± 57 †</td>
<td>221 ± 32</td>
</tr>
<tr>
<td></td>
<td>TS</td>
<td>222 ± 38</td>
<td>47 ± 7 †</td>
<td>33 ± 14 †</td>
</tr>
<tr>
<td>APP</td>
<td>T0</td>
<td>56 ± 5</td>
<td>52 ± 16</td>
<td>48 ± 6</td>
</tr>
<tr>
<td>CPN</td>
<td>T0</td>
<td>90 ± 22</td>
<td>76 ± 29</td>
<td>71 ± 10</td>
</tr>
</tbody>
</table>

Values are means ± SD (n=6)

* p = 0.025 vs. control at T0; † p < 0.001 vs. control at TS.
Table 3. Log of normalized ratios for metallopeptidases in oropharyngeal and kidney tissues.

The relative gene expressions vs. controls of membrane-bound aminopeptidase P (mAPP) and neprilysin (NEP) are normalized to GAPDH. Average ratios (n=6) are expressed with standard deviation.

<table>
<thead>
<tr>
<th>mAPP - Normalized Ratio (log)</th>
<th>Groups</th>
<th>Average</th>
<th>p Value vs. Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>tongue</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>-3.89 ± 0.14</td>
<td>0.567</td>
</tr>
<tr>
<td></td>
<td>7-day ACEi</td>
<td>-1.37 ± 0.62</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>1-time ACEi</td>
<td>-3.25 ± 0.41</td>
<td>0.883</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>-3.48 ± 0.85</td>
<td></td>
</tr>
<tr>
<td></td>
<td>laryngeal tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>-1.28 ± 0.20</td>
<td>0.998</td>
</tr>
<tr>
<td></td>
<td>7-day ACEi</td>
<td>0.06 ± 0.19</td>
<td>0.027</td>
</tr>
<tr>
<td></td>
<td>1-time ACEi</td>
<td>-1.19 ± 0.51</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>-1.22 ± 1.09</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NEP - Normalized Ratio (log)</th>
<th>Groups</th>
<th>Average</th>
<th>p Value vs. Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>kidney</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>1.4 ± 0.15</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>7-day ACEi</td>
<td>2.3 ± 0.25</td>
<td>0.038</td>
</tr>
<tr>
<td></td>
<td>1-time ACEi</td>
<td>1.4 ± 0.05</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>2.0 ± 0.28</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tongue</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>0.71 ± 0.18</td>
<td>0.295</td>
</tr>
<tr>
<td></td>
<td>7-day ACEi</td>
<td>1.81 ± 0.51</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>1-time ACEi</td>
<td>0.96 ± 0.19</td>
<td>0.955</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>1.04 ± 0.25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>parotid</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>1.48 ± 0.64</td>
<td>0.780</td>
</tr>
<tr>
<td></td>
<td>7-day ACEi</td>
<td>3.47 ± 0.37</td>
<td>0.026</td>
</tr>
<tr>
<td></td>
<td>1-time ACEi</td>
<td>1.88 ± 1.16</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>1.87 ± 0.64</td>
<td></td>
</tr>
</tbody>
</table>

Values are log means ± SD. Only tissues with significant differences are shown (p < 0.05).
Table 4. Log of normalized ratios for kinin B₂ receptor in oropharyngeal and kidney tissues.

The relative gene expressions vs. controls of the kinin B₂ receptor (B₂R) are normalized to GAPDH. Average ratios (n=6) are expressed with standard deviation.

<table>
<thead>
<tr>
<th>B₂R - Normalized Ratio (log)</th>
<th>Groups</th>
<th>Average</th>
<th>p Value vs. Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kidney</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>-3.72 ± 0.22</td>
<td>0.008</td>
<td></td>
</tr>
<tr>
<td>7-day ACEi</td>
<td>-1.28 ± 0.91</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>1-time ACEi</td>
<td>-4.10 ± 0.42</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>-1.23 ± 2.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tongue</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>-3.84 ± 0.28</td>
<td>0.607</td>
<td></td>
</tr>
<tr>
<td>7-day ACEi</td>
<td>1.10 ± 0.69</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>1-time ACEi</td>
<td>-3.00 ± 0.92</td>
<td>0.991</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>-2.76 ± 2.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Laryngeal tissue</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>-1.68 ± 0.72</td>
<td>0.942</td>
<td></td>
</tr>
<tr>
<td>7-day ACEi</td>
<td>0.64 ± 1.58</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>1-time ACEi</td>
<td>-2.28 ± 0.38</td>
<td>0.965</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>-2.01 ± 1.15</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are log means ± SD. Only tissues with significant differences are shown (p < 0.05).
Table 5. Metallopeptidases activities in tissues from control animals.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>ACE</th>
<th>mAPP</th>
<th>NEP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>8162 ± 2139</td>
<td>2780 ± 399</td>
<td>14740 ± 3242</td>
</tr>
<tr>
<td>Tongue</td>
<td>1058 ± 331</td>
<td>224 ± 55</td>
<td>501 ± 118</td>
</tr>
<tr>
<td>Parotid</td>
<td>768 ± 255</td>
<td>136 ± 59</td>
<td>123 ± 54</td>
</tr>
<tr>
<td>Laryngeal Tissue</td>
<td>1412 ± 258</td>
<td>225 ± 42</td>
<td>198 ± 59</td>
</tr>
</tbody>
</table>

Values are means ± SD (n=5-6)
Figure 1.

A

CRP (µg/mL)

control 1-time ACEi 7-days ACEi LPS

B

Leukocytes (10^9/L)

control 1-time ACEi 7-days ACEi LPS
Figure 2.

A

![Graph A showing AUC BK (pg/mL•15 min, X 10^3) for 7-days ACEi, 1-time ACEi, LPS, and control.]

B

![Graph B showing AUC des-Arg^9-BK (pg/mL•30 min, X 10^3) for 7-days ACEi, 1-time ACEi, LPS, and control.]

* indicates statistical significance vs. control, ** indicates statistical significance vs. ACEi.
Figure 3.

A

B

Fluorescence -d(F1)/dT

GAPDH
mAPP
NEP
B1R
B2R
control (-)

Temperature (°C)

65 70 75 80 85 90 95 100
Figure 4.

A bar graph showing the average log normalized ratio for different tissues: Laryngeal tissue, Parotid gland, Tongue, and Kidney. The graph compares the control, 1-time, and 7-day LPS treatments. The y-axis represents the tissues, and the x-axis represents the average log normalized ratio ranging from -5.00 to 3.00.
Figure 5.

<table>
<thead>
<tr>
<th></th>
<th>H&amp;E</th>
<th>noAb</th>
<th>vWF</th>
<th>α-actin</th>
<th>B₁R</th>
<th>B₂R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>LPS</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-time ACEi</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-day ACEi</td>
<td></td>
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</tbody>
</table>