

## Research Article

# Bradykinin-Induced Shock Increase Exhaled Nitric Oxide, Complement Activation and Cytokine Production in Pigs

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## Abstract

Bradykinin is an important mediator in blood pressure regulation, ischemic precondition and capillary leakage, allergy, anaphylaxis, inflammation, and nociception, at least partly via the generation of nitric oxide (NO). Macrophages are particularly abundant in the porcine lung circulation. Upon bradykinin binding macrophages release cytokines and endothelial cells increase plasma leakage. Both cells produce NO. The complement, hemostatic, fibrinolytic and kinin plasma cascade systems crosstalk and interacts with many inflammatory systems. In the present study we investigated the effect of the shock induced by intravenously infused bradykinin on the cascade systems, cytokines, plasma leakage and exhaled NO in pigs. The metabolite of bradykinin, BK1-5, was measured in plasma by a sensitive, specific and reliable liquid chromatography-mass spectrometry method to verify exposure and *in vivo* metabolism of bradykinin. We show for the first time *in vivo* how bradykinin exposure induced shock and increased exhaled NO, activated complement and hemostasis and induced cytokine production and capillary leakage. The results broaden our understanding of how bradykinin activates endothelial cells and macrophages to induce shock and inflammation. This should encourage further studies.

## INTRODUCTION

Bradykinin is a potent nine amino acid short peptide generated from kininogen in plasma. Bradykinin is involved in blood pressure regulation, capillary leakage and ischemic preconditioning of the heart [1]. Bradykinin also mediates allergy, anaphylaxis, inflammation and nociception [2]. Bradykinin itself has an extremely short half-life (<30 s) due to breakdown by angiotensin converting enzyme (ACE) and other exopeptidases present in plasma. It is therefore difficult to detect *in vivo* [3]. The kinin system, the intrinsic pathway of coagulation, the complement system and the fibrinolytic system all cross-talk [4]. Macrophages and endothelial cells release nitric oxide (NO) and bradykinin markedly increase NO from endothelial cells [5]. Ovine and porcine lung parenchyma have large amounts

of macrophages in their circulation, and the macrophage densities are similar to that of Kupfer cells in the rat liver [6]. More than 25 years ago, bradykinin and its metabolite des Arg-bradykinin were shown to stimulate murine macrophages to release tumor necrosis factor (TNF) and interleukin-1 (IL-1) [7]. The quantitative relation between bradykinin and its effects on circulation and inflammation are largely unknown in all species. Therefore, we infused pigs with bradykinin to obtain shock and investigated the effects of bradykinin on exhaled NO, the plasma cascades and inflammation.

## MATERIALS AND METHODS

## Study design

In this porcine study we administered bradykinin in three

different ways. Animal A was given a continuing and steadily increasing infusion of bradykinin with bolus doses late in the experiment. Animal B was exposed to a high bolus dose of bradykinin early in the experiment. Animal C was pretreated with 1.5 mg captopril (ACE-inhibitor) prior to infusion of bradykinin. In all the pigs, the infusion of bradykinin was adjusted to maintain a critically low mean arterial pressure (MAP) of 40 mmHg.

## Animals

Three Norwegian Landrace pigs of either sex (23 kg  $\pm$  2) were treated in accordance with the Norwegian laboratory animal regulations and the study was approved by the Norwegian Animal Research Authority (FOTS #2843). Housekeeping, anesthesia, surgery and euthanasia were performed as previously described [8]. Ringer's acetate was given intravenously at 10 ml/kg/h.

## Monitoring and examinations

Electrocardiography, end-tidal CO<sub>2</sub>, pulse oximetry, regular interval blood gas, systemic intra-arterial blood pressure and pulmonary artery pressure were recorded as described previously [8]. Basic echocardiographic parameters were obtained by a Vivid S6 scanner (GE Vingmed, Horten, Norway) with a 1.5-3.6 MHz matrix array probe. All echocardiographic values are an average of five consecutive measurements with standard deviations.

## Nitric oxide in exhaled gas

NO was measured online by the multiple breath method with a chemiluminescence analyzer, as described elsewhere [9,10].

## Laboratory analyses

Cytokines, hemostatic markers and the terminal complement complex (TCC) were measured in blood samples at baseline and during bradykinin infusion by methods described previously [11].

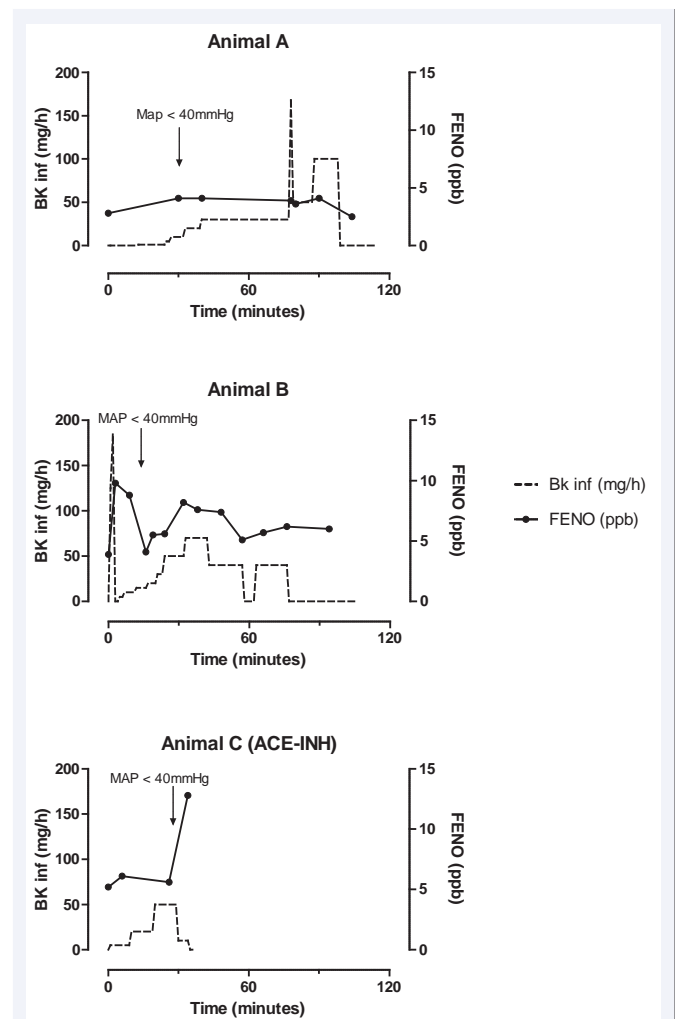
## Measurements of bradykinin and BK1-5

Measurement of the stable bradykinin metabolite BK1-5 was performed by a previously described liquid chromatography-mass spectrometry (LC-MS/MS) method [12]. Quantification of bradykinin was performed by a commercial ELISA-kit from Uscn Life Science Inc (Wuhan, China) that was validated by measuring the internal standard of bradykinin.

## RESULTS

### Bradykinin increased exhaled NO and induced shock and capillary leakage

Bradykinin dose dependently increased exhaled NO (Figure 1). Even though a large decrease in MAP was seen, mean pulmonary artery pressure (MPAP) remained constant (20-30 mmHg) throughout the infusion. Systemic vascular resistance index (SVRI) was reduced by 46% in animal A, 65% in animal B and 23% in animal C. A 37% and 30% fall in albumin was seen in animal B and C, respectively, but not in animal A. A complete echocardiographic assessment of left ventricular dimensions in animal A showed 2.4 cm (+/- 0.2 cm) end systolic parasternal long axis at baseline and 0.3 cm (+/- 0.1 cm) at peak bradykinin infusion.



**Figure 1** Effects of bradykinin infusion on exhaled NO.

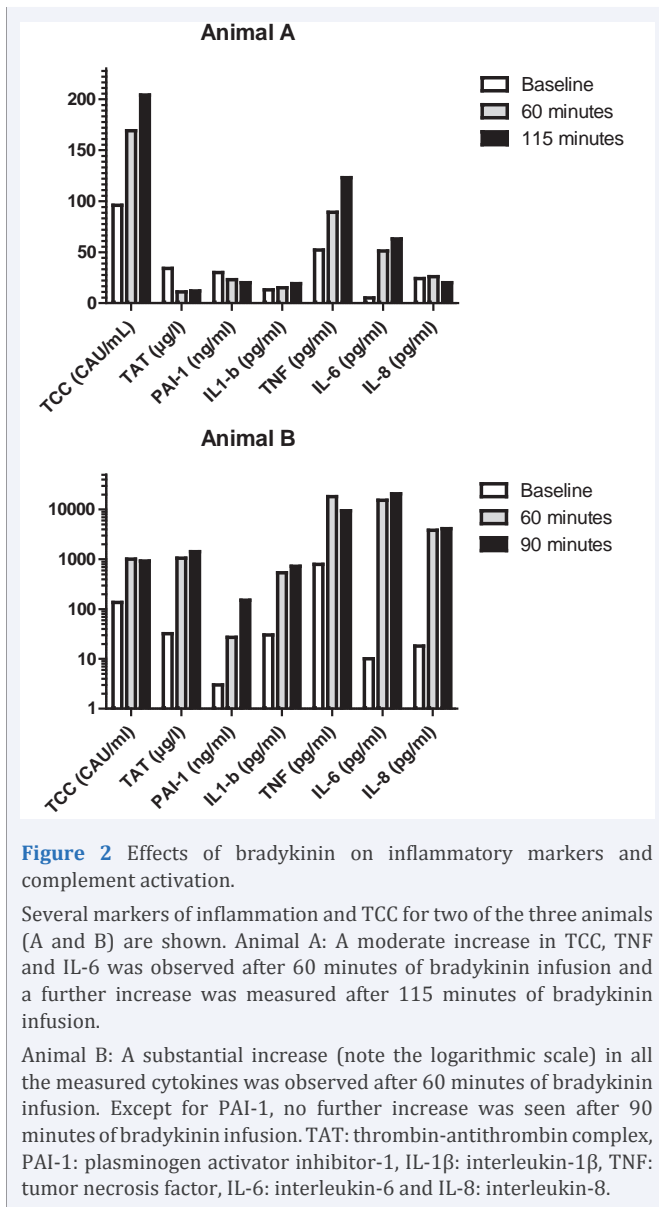
Animal A: The infusion of bradykinin was gradually increased with a corresponding increase in exhaled NO. MAP fell below 40 mmHg after 30 minutes and was maintained at a low level as the infusion rate was kept constant.

Animal B: An initial bolus dose of bradykinin was given (initial peak) which induced a substantial increase in exhaled NO. The infusion rate was quickly escalated after the initial bolus and the levels of exhaled NO closely followed the infusion rate and remained at a relatively high level throughout the experiment. In this case, MAP dropped below 40 mmHg after 15 minutes.

Animal C: Captopril was given before bradykinin and exhaled NO quickly increased to more than twice the initial concentration after bradykinin infusion was started. The animal died after only 34 minutes after a large fall in MAP. BK inf. Bradykinin infusion, FENO: fractional exhaled nitric oxide, ppb: parts per billion.

### Bradykinin activated both complement and hemostasis and induced cytokine production

Inflammatory and hemostatic markers, including TCC, thrombin-antithrombin complex (TAT), plasminogen activator inhibitor-1 (PAI-1), IL-1 $\beta$ , TNF, interleukin-6 (IL-6) and interleukin-8 (IL-8), were measured during the experiments. After bradykinin infusion, TCC, TNF and IL-6 increased markedly in Animal A, while all markers rose in Animal B (Figure 2). Animal



**Figure 2** Effects of bradykinin on inflammatory markers and complement activation.

Several markers of inflammation and TCC for two of the three animals (A and B) are shown. Animal A: A moderate increase in TCC, TNF and IL-6 was observed after 60 minutes of bradykinin infusion and a further increase was measured after 115 minutes of bradykinin infusion.

Animal B: A substantial increase (note the logarithmic scale) in all the measured cytokines was observed after 60 minutes of bradykinin infusion. Except for PAI-1, no further increase was seen after 90 minutes of bradykinin infusion. TAT: thrombin-antithrombin complex, PAI-1: plasminogen activator inhibitor-1, IL-1β: interleukin-1β, TNF: tumor necrosis factor, IL-6: interleukin-6 and IL-8: interleukin-8.

C developed an uncontrollable hypotension and died too early to deliver data on these markers.

### Massively increased BK1-5 measured by LC-MS/MS

After 60 minutes, animal A and B showed substantially elevated levels of BK1-5. The signals were about two orders of magnitude above the detection method's validated maximum (17.7 nmol/mL). Animal C, which was given an ACE-inhibitor before bradykinin, had lower (15.8 nmol/mL), but elevated levels of BK1-5 after 30 minutes of bradykinin infusion. The baseline levels were below the method's detection limit (35.4 pmol/mL) for all the animals. ELISA measurements of bradykinin were done in animal A, but failed to show the extensive exposure to bradykinin as they never reached the lowest standard of 24 pg/ml, which is consistent with its short half-life.

## DISCUSSION

In this study, effects of intravenously infused bradykinin in

the absence or presence of an ACE-inhibitor was investigated in pigs. We found an increase in exhaled NO, activation of complement and hemostasis, increased cytokine production and capillary leakage.

In all the animals, there was a clear link between the infused amount of bradykinin and a severe lowering of MAP, whereas MPAP remained unaffected. The sizeable reduction in end systolic diameter of the left ventricle during peak infusion also indicates that bradykinin reduces afterload and increases contractility. This is consistent with earlier findings on the hemodynamic effects of bradykinin infusion [13], and bradykinin's known activity as a vasodilator [14]. Our results also fit well with an early study of bradykinin infusion to humans [15], and suggest a different effect of bradykinin in the pulmonary circulation than in the systemic circulation. It is tempting to speculate that the high concentration of ACE on pulmonary endothelium protects the lungs from bradykinin induced edema. This could also explain why pulmonary edema is rare in patients with hereditary angioedema who excessively produce bradykinin [16].

By measuring the NO concentration in expiration gas, the effects of bradykinin could be indirectly monitored. A close and rapid relation between exhaled NO and rate of bradykinin infusion was seen (Figure 1). Whether bradykinin released NO directly or indirectly via preformed cytokine cell stores, remains to be investigated. However, the slow and moderate increase of cytokines we measured suggest bradykinin as dominant directly. Elevated exhaled NO after infused bradykinin is, to our knowledge, for the first time observed and documented in the present study. The increased exhaled NO was related to a quantifiable shock. Stimulation of the B2-receptor by bradykinin is known to release plasminogen-activator and produce NO and prostaglandin I<sub>2</sub> (PGI<sub>2</sub>) from the endothelial cells [14,17,18]. Upon stimulation by lipopolysaccharides macrophages per cell release ten times more NO than do endothelial cells upon exogenously added bradykinin [5]. The enzyme endothelial NO synthase (eNOS) is released within seconds upon receptor stimulation and could explain the rapid NO response [19]. A difference in exhaled NO was seen between animal A and B (Figure 1). This should be expected as animal A and B received bradykinin infusions at varying rates. The difference may suggest a desensitizing effect by prolonged and gradually increased exposure to bradykinin. A previous publication also found desensitization after internalization of the bradykinin-B2 receptor complex [20].

Both NO and PGI<sub>2</sub> relax smooth muscles in the arterial wall and systemic vascular resistance drops accordingly. The shock we observed mimicked severe sepsis, and bradykinin mediates inflammation [8]. A fall in albumin, as we observe during bradykinin infusion, is also seen during sepsis [8]. It supports the role of bradykinin as a mediator of capillary leakage in these conditions. In a recent study exogenously infused bradykinin to rats reduced the tight junction proteins claudin-5 and occludin and increased plasma leakage. The mechanism in this brain tumor model was up regulation of the eNOS and neuronal NOS and the transcriptional repressor ZO-1 associated nucleic acid binding protein ZONAB [21]. In macrophages the inducible NOS (iNOS) is the key inflammatory enzyme and produce such high levels of NO that in one study setting upon LPS administration

to mice genetically defect of iNOS, the fall in blood pressure was markedly attenuated and early death averted [22]. Consequently, the relative contribution by macrophages and endothelial cells to the capillary leakage and NO-production we observed after bradykinin infusion is difficult to ascertain. The presence of approximately 4000 bradykinin receptors per guinea pig macrophage [23], and the reciprocal activation by NO and cytokines on macrophages and endothelial cells gives the impression that both cell types contribute to the results in our study.

The inflammatory markers TCC, TNF and IL-6 were markedly increased after bradykinin infusion (Figure 2). Interestingly, in animal B (Figure 2), where a large bolus dose of bradykinin was instantly followed by a large increase in exhaled NO and a marked drop in MAP, these increases were several orders of magnitude higher than in animal A. A marked increase in coagulation, represented by TAT and PAI-1 was also present. It is possible that this massive generation was a result of the very early bradykinin bolus. It also suggests that bradykinin might be better tolerated when its infusion rate is gradually increased. This also fits well with the previously mentioned desensitizing theory from prolonged bradykinin exposure. Even more interesting is the possibility of a direct cross-talk between the kinin system on one side and the complement and coagulation pathways on the other. To our knowledge, we present here the first *in vivo* evidence that bradykinin alone activates these cascade systems directly.

The BK1-5 measurements showed a massive signal after infusion of bradykinin (possibly more than 100 000 times increase compared to baseline levels), whereas measurements of bradykinin by ELISA did not reveal any changes in concentration. This illustrates the very rapid metabolism of bradykinin [3]. The significance was further exemplified in animal C. Here the increase in BK1-5 was modest, as the conversion of bradykinin into BK1-5 is hampered by inhibition of ACE [24]. Bönner et al. found that an ACE-inhibitor potentiated a bradykinin infusion 20-fold [15]. This is in line with our observations in animal C. Although the bradykinin infusion in animal C was reduced compared to animal A and B, the presence of an ACE-inhibitor caused a massive bradykinin buildup and a rapid circulatory collapse. It also explains why the exhaled NO in animal C reached the highest value in our experiment.

## CONCLUSION

This *in vivo* study shows for the first time how infused bradykinin induced shock and markedly increased exhaled NO, activated the coagulation and complements system and induced several pro-inflammatory cytokines. The rise in these markers was related to the stable bradykinin metabolite BK1-5 with a sensitive LC-MS/MS method. These results suggest that bradykinin directly activates macrophages and endothelial cells and contributes to cytokine response, plasma leakage and increased exhaled NO. Our study expands the understanding of bradykinin's many roles *in vivo*.

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