

Altered cardiac bradykinin metabolism in experimental diabetes caused by the variations of angiotensin converting enzyme and other peptidases

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Article type: Full paper submitted for the Proceedings of Peptide Receptors – Kinin 2009 Joint Symposium

Sources of support in the form of grants: This study was supported by a grant from Bristol-Myers Squibb (to A.A.) and the Canadian Institutes of Health Research (grant to F.M. and A.A.).

Abstract

The peptidases angiotensin converting enzyme (ACE) and neutral endopeptidase 24.11 (NEP) mediate most of the kinin catabolism in normal cardiac tissue and are the molecular targets of inhibitory drugs that favorably influence diabetic complications. We studied the variations of those kininases in the myocardium of rats in experimental diabetes. ACE and NEP activities were significantly decreased in heart membranes 4-8 weeks post-streptozotocin (STZ) injection. However, insulin-dependent diabetes did not modify significantly bradykinin (BK) half-life ($t_{1/2}$) while the effect of both ACE (enalaprilat) and ACE and NEP (omapatrilat) inhibitors on BK degradation progressively decreased, which may be explained by the upregulation of other unidentified metallopeptidase(s). In vivo insulin treatment restored the activities of both ACE and NEP. ACE and NEP activities were significantly higher in hearts of young Zucker rats than in those of Sprague-Dawley rats. BK $t_{1/2}$ and the effects of peptidase inhibitors on $t_{1/2}$ varied accordingly. It is concluded that kininase activities are subjected to large and opposite variations in rat cardiac tissue in type I and II diabetes models. A number of tissue or molecular factors may determine these variations, such as remodeling of cardiac tissue, ectoenzyme shedding to the extracellular fluid and the pathologic regulation of peptidase gene expression.

Keywords: bradykinin metabolism, diabetes, angiotensin converting enzyme, neutral endopeptidase

1. Introduction

Bradykinin (BK) is a nonapeptide released from high molecular weight kininogen by nonspecific and specific kininogenases. It exerts its vasodilatory effect mainly by stimulation of B₂ receptors (Leeb-Lundberg et al., 2005). However, the pharmacological actions of BK are short-lived due to intense metabolism (Erdös and Skidgel, 1997). At the level of the heart, we have shown previously that 2 metallopeptidases, angiotensin-converting enzyme (ACE) and neutral endopeptidase 24.11 (NEP) respectively, play important roles in BK degradation (Blais et al., 1997; Dumoulin et al., 1998; Raut et al., 1999). Owing to its kinetic characteristics, ACE is a particularly effective kininase (Brunning et al., 1983; Erdös and Skidgel, 1997). Acute myocardial infarction and chronic left ventricular hypertrophy modify the relative participation of these 2 zinc peptidases in the inactivation of BK. These observations demonstrate that pathophysiological conditions may influence cardiac metallopeptidase activities, leading to metabolic modifications that are physiologically relevant. In addition to ACE and NEP, aminopeptidase P has been reported by some authors to inactivate BK in the endothelium (Ryan et al., 1994; Prechel et al., 1995), and kininase I, a generic name for several arginine carboxypeptidases, transforms native kinins into their des-Arg⁹ fragments which exert their pharmacological actions via B₁ receptors. However, based on our experimental approaches, des-Arg⁹-BK formation is only a minor metabolic pathway (Blais et al., 1997; Dumoulin et al., 1998).

ACE and NEP degrade BK, other vasoactive peptides and (in the case of ACE) activates angiotensin. A new class of drugs, the vasopeptidase inhibitors (VPI), suppresses ACE and NEP simultaneously; an agent from this class, omapatrilat, exhibits a similar nanomolar inhibitory constant (K_i) for the 2 enzymes (Trippodo et al., 1998) and is a useful in dissecting kinin

metabolism. ACE inhibitors (ACEi) are in clinical use and have been proven to reduce mortality and morbidity in diabetic patients (Tatti et al., 1998; Gazis et al., 1998; HOPE investigators, 2000). There is accumulating experimental evidence suggesting that the increase in BK concentration resulting from ACE inhibition may play a major role in the beneficial effects of these drugs, particularly as it relates to diabetes. For example, Uehara et al. (1994) demonstrated that BK infusion in vivo mimicked the impact of ACEi on insulin action and glucose uptake. These effects were inhibited by a B₂ receptor antagonist.

The present study has 2 objectives: firstly, to define the influence of experimental type I and type II diabetes, and also the effect of insulin treatment, on the activities of ACE and NEP in the rat heart, a representative tissue where ACE blockade may exert kinin-mediated protective effects. Finally, the participation of both metallopeptidases in BK degradation was quantitatively examined in heart membrane extracts from the same animals.

2. Methods

2.1. *Drugs, peptides and reagents*

BK was purchased from Peninsula Laboratories (Belmont, CA). Enalaprilat, an ACEi, was obtained under the commercial solution form Vasotec IV (Merck & Co.). Omapatrilat, the VPI which inhibits both ACE and NEP, was kindly provided by Bristol-Myers Squibb (Princeton, NJ). Streptozotocin (STZ) in citrate buffer, pH 4.5, for the induction of diabetes was obtained from the pharmacy of the Royal Victoria Hospital (Montréal, QC). Alzet 2ML2 osmotic minipumps were from Alza Corporation (Palo Alto, CA), and insulin from Eli Lilly (Humulin R ADNr source, Indianapolis, IN). Hypnorm (fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml) was obtained from Janssen Pharmaceuticals (Belgium), and sodium pentobarbital (Somnotol) from M.T.C. Pharmaceuticals (Mississauga, ON). Glycosuria was measured with Diastix urinary strips (Bayer, Etobicoke, ON), and glycemia by the glucose oxydase method (Lifescan Canada, Burnaby, BC). Heparin and bovine serum albumin, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) were purchased from Sigma-Aldrich (Mississauga, ON). Alkaline phosphatase-labeled anti-digoxigenin Fab fragments, *o*-phenanthroline, *p*-chloromercuriphenyl sulfonate (PCMS), and phenylmethylsulfonyl fluoride (PMSF) were from Boehringer Mannheim (Laval, QC). The bicinchoninic acid protein assay was from Pierce (Rockford, IL). Other reagents and chemicals of analytic grade were from Fisher Scientific (Montréal, QC).

2.2. *Type I diabetes model*

All research protocols conformed to the guidelines of the Canadian Council on Animal Care and were approved by the Committee for Animal Research of the Université de Montréal. Diabetes

was induced by a single dose of STZ (55 mg/kg) via caudal vein injection in male anesthetized (Hypnorm, 0.4 ml/kg, i.m.) Sprague-Dawley rats (125-150g, Charles River Canada, St-Constant, QC, Canada). The animals were housed at constant room temperature (21-23°C) and humidity for the duration of the experiments. They had free access to normal rat chow and tap water. The evolution of diabetes was monitored each day for the first week and then every week for a period of 8 weeks by measurement of glycosuria and glycemia. Animals presenting glycemia over 17 mmol/l 12 days after STZ administration were considered diabetic (Tschöpe et al., 1996). Age-matched Sprague-Dawley rats receiving no treatment were used as controls. These animals were housed and fed under identical conditions and at the same time as the treated groups.

In some diabetic rats, Alzet miniosmotic pumps containing regular insulin were implanted intraperitoneally in 8 weeks post-STZ injection anesthetized rats (sodium pentobarbital). Insulin was administered at a constant rate (3 U/day) for 7 days. Weight, glycemia and glycosuria were monitored as described above.

2.3. Type II diabetes model

Zucker fa/fa and lean rats (Charles River Canada, St-Constant, QC, Canada) served as type II diabetic animals and their respective controls (Bray, 1977).

2.4. Organ sampling and membrane preparation

Three groups of rats with induced type I diabetes were sacrificed 2, 4 or 8 weeks post-STZ injection along with their age-matched controls. Moreover, another group of 8 weeks STZ-injected rats was sacrificed 7 days post-insulin treatment. Zucker lean and fa/fa rats were sacrificed at 8 weeks of age.

Briefly, all animals were anesthetized with sodium pentobarbital (65 mg/kg, i.p.) before the thorax was opened. Heparin (1000 U/kg) was injected into the jugular vein before they were exsanguinated by transection of the carotid artery. The heart was excised after rapid perfusion in situ with Krebs-Heisenleit buffer through a cannula inserted in the aortic stump. All hearts were frozen at -80°C until cardiac membrane preparation. Membranes were extracted from cardiac tissues by a method used previously in our laboratory for normal, infarcted and hypertrophied rat and human hearts (Blais et al., 1997; Raut et al., 1999). Briefly, each heart was weighed and then cut in small pieces of 3 to 4 mm. Heart tissues were homogenized in a 50 mM Tris-HCl buffer, pH 7.4, at 4°C (10 ml/g of tissue) with a Polyron homogenizer (Brinkman Instruments, Rexdale, ON) at setting 8 for 15 seconds. The total homogenate was then centrifuged at 40,000 × g for 20 minutes at 4°C and the cytosolic supernatant was discarded. The pellet containing heart membranes was suspended in 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, at 4°C with a Wheaton-Potter-Elvehjem tissue grinder (Fisher Scientific, Pittsburgh, PA) driven by a T-line motorized stirrer (Talboys Engineering, Emerson, NJ) rotating at setting 8 for 60 seconds. The protein concentration of the cardiac membrane suspensions was determined by the bicinchoninic acid method, before biochemical investigation.

2.5. Measurement of insulin in rat serum

Serum insulin was evaluated by competitive radioimmunoassay (ImmuChem Insulin Assay, ICN Pharmaceuticals Inc., CA) using a rabbit anti-human insulin antibody that presents 90% cross-reactivity with rat insulin.

2.6. Measurement of membrane ACE and NEP activities in myocardium

Heart membrane suspensions (1 ml) were incubated with CHAPS at a final concentration of 8 mM for 2 hours and then centrifuged ($40000 \times g$ for 15 min at 4°C) for the solubilization of proteins (Costerousse et al., 1994). Protein concentration of the solubilized membranes was assessed by the bicinchoninic acid method. The Buhlmann ACE radioenzymatic test (ALPCO, Vindham, NH) was employed according to the manufacturer's instructions to quantify ACE activity in these protein solutions.

NEP activity was measured using the tritiated substrate (tyrosyl-[3,5- ^3H])(D-Ala₂-Leu-enkephalin (50 Ci/mmol) (Research Product International Inc., Mount Prospect, IL), as described previously by Le Moual et al. (1991). Incubations were conducted in 50 mM MES, pH 6.5, at 37°C .

Incubation time for both the ACE and NEP assays was prolonged to 4 hours. Each sample was quantified in duplicate. ACE and NEP activities were expressed respectively as pmol and fmol of hydrolyzed substrate /min/mg of proteins.

2.7. Assessment of BK metabolism by rat heart membranes

The metabolic profile of BK was measured at 37°C by incubating, in 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, synthetic BK (final concentration of 471 nM) in the presence of resuspended heart membranes (final protein concentration of 2.5 mg/ml). After various incubation periods (2 to 20 minutes), the reaction was stopped by adding cold anhydrous ethanol (final concentration of 80% vol/vol). The samples were then centrifuged for 15 minutes at 4°C and $2000 \times g$ to allow the complete precipitation of proteins. The clear supernatant containing BK was collected and evaporated to dryness in a Speed Vac Concentrator (Savant, Farmingdale, NY). The residues were stored at -80°C until quantification of residual BK was performed. The effects of enzyme inhibitors on BK metabolism were determined in parallel experiments. For that

purpose, enalaprilat (final concentration of 130 nM) and omapatrilat (final concentration of 50 nM) were preincubated for 15 min with the membrane suspensions before the addition of exogenous BK.

Finally, to characterize the nature of other peptidases different from ACE or NEP responsible for BK metabolism, membranes were first incubated in the presence of omapatrilat and then with the following enzyme inhibitors: *o*-phenanthroline (metallopeptidase inhibitor), *p*-chloromercuricphenyl sulfonic acid (PCMS, cysteine protease inhibitor) and phenylmethanesulphonylfluoride (PMSF, serine protease inhibitor) at a final 10^{-4} M concentration.

Evaporation residues were resuspended in 500 μ l of 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl and 0.05% Tween-20. Immunoreactive BK was quantified with a highly specific and sensitive non-isotopic immunoassay developed in our laboratory (Blais et al., 1997; Décarie et al., 1994). This competitive enzyme immunoassay method uses highly specific polyclonal rabbit immunoglobulins raised against the carboxy-terminal end of BK, digoxigenin-labeled BK as a tracer and alkaline phosphatase-labeled anti-digoxigenin Fab fragments with the substrate *p*-nitrophenyl phosphate to detect and quantify the immune complexes. Each sample was measured in triplicate. Typical calibration curves were characterized by half-maximal saturation values of 0.78 pmol/ml.

2.8. Kinetic parameter analysis

The BK hydrolysis rate constant (*k*) was evaluated using the first-order equation

$$[\text{BK}] = [\text{BK}]_0 \times e^{-kt},$$

where [BK] is the BK concentration at a given time and [BK]₀ is [BK] at time (t) = 0. BK half-life ($t_{1/2}$) was represented as $t_{1/2} = \ln(2)/k$ (Moore et al., 1981). The different $t_{1/2}$ values were expressed for 1 mg of protein.

2.9. Statistical analysis

All data were expressed as means \pm standard error of the mean (s.e.m.) for n values. Paired Student's t-test was used to analyse the effect of enalaprilat and omapatrilat on BK $t_{1/2}$ within the same group of rat hearts. One-way ANOVA was assessed the statistical significance between several groups of rats. All pair-wise comparisons were made with Scheffé's method of post hoc testing. A difference was accepted as significant at $P < 0.05$ (Wallenstein et al., 1980).

3. Results

3.1. Type I experimental diabetes: Animal characteristics

Overt diabetes developed in 49 STZ-treated rats as confirmed by glycemia and glucosuria values observed during the first week post-STZ injection. The characteristics of these animals at different times of the experiment are summarized in Table 1. Administration of insulin 8 weeks post-STZ injection led to the disappearance of glycosuria as early as 1 day after miniosmotic pump implantation.

3.2. Time-course of NEP and ACE activities during the development of insulin-dependent diabetes and insulin treatment

The mean heart membrane ACE activity of control animals was 9.00 ± 0.76 pmol/min/mg of protein (n = 5) and was not influenced by age. It was not statistically different at 2 weeks post-STZ injection (7.72 ± 0.47 pmol/min/mg of protein, n = 6) but was decreased significantly at 4 (3.00 ± 0.52 pmol/min/mg of protein, n = 5, $P < 0.05$) and 8 (5.29 ± 0.21 pmol/min/mg of protein, n = 7, $P < 0.05$) weeks after diabetes induction (Figure 1a). Treatment with insulin led to normalization of ACE activities with values similar to those of control animals (7.86 ± 0.34 pmol/min/mg of protein, n = 5).

Diabetes also modified cardiac NEP activity. Although NEP activity was not different from that of the controls at 2 weeks post-STZ injection (2.06 ± 0.15 fmol/min/mg of protein, n = 5 and 1.62 ± 0.13 fmol/min/mg of protein, n = 5, respectively), a significant decrease was observed at 4 (1.00 ± 0.13 fmol/min/mg of protein, n = 5, $P < 0.05$) and 8 (0.80 ± 0.07 fmol/min/mg of protein,

n = 5, $P < 0.05$) weeks post-injection (Figure 1b). Like ACE, NEP activity was restored to control values after 7 days of insulin treatment (1.88 ± 0.14 fmol/min/mg of protein, n = 5).

3.3. Effect of insulin-dependent diabetes and insulin treatment on BK metabolism

Control Sprague-Dawley groups age-matched with 2 weeks (n = 4), 4 weeks (n = 4) and 8 weeks (n = 7) post-STZ-injected rats were regrouped into a single group (n = 15) after statistical evaluation revealed that all BK $t_{1/2}$ values were not significantly different.

BK $t_{1/2}$ increased significantly 2 weeks post-STZ injection when compared to control rats (202 ± 8 sec, n = 6 vs. 152 ± 7 sec, n = 15, $P < 0.05$) but was similar to control values at 4 (155 ± 7 sec, n = 7) and 8 weeks post-STZ injection (149 ± 8 sec, n = 7) (Figure 2a).

Two weeks post-diabetes induction, preincubation of membrane preparations with enalaprilat significantly increased the BK $t_{1/2}$ (262 ± 11 sec vs. 202 ± 8 sec without inhibitor, n = 6, $P < 0.05$). At that time, the potentiating effect of omapatrilat (455 ± 33 sec; n = 6, $P < 0.05$) was more pronounced than that of enalaprilat. In addition, the $t_{1/2}$ of BK incubated with enalaprilat or omapatrilat at 2 weeks post-diabetes induction was statistically higher ($P < 0.05$) when compared to the $t_{1/2}$ of the control group (205 ± 5 sec; and 314 ± 13 sec; respectively; n = 15). Nevertheless, their relative potentiating effects (enalaprilat $30 \pm 8\%$ and omapatrilat $128 \pm 25\%$) were not higher than in the control group ($38 \pm 8\%$ and $112 \pm 13\%$, respectively) because of the concomitant elevation of $t_{1/2}$ without the inhibitor.

At 4 weeks post-diabetes induction, the potentiating action of enalaprilat was modest and could not be detected after 8 weeks. Disappearance of the effect of omapatrilat could only be observed 8 weeks post-STZ injection (Figure 2a).

Treatment of rats with insulin 8 weeks post-STZ injection had no significant influence on BK degradation in the absence of peptidase inhibitor (Figure 2b). In fact, BK $t_{1/2}$ 7 days after insulin treatment was not different (153 ± 8 sec, $n = 8$) from that measured in diabetic rats before insulin treatment (149 ± 8 sec, $n = 7$). In the insulin-treated group of animals also, preincubation with both ACEi and VPi increased BK $t_{1/2}$ (236 ± 20 sec, $n = 8$ and $292 \text{ sec} \pm 16$, $n = 8$, respectively) in a similar way as in the control group. In other words, the potentiating effects of both inhibitors that disappeared 8 weeks post-diabetes induction were restored to their initial values by insulin treatment (Figure 2b).

The preincubation of cardiac membranes from 8 weeks post-STZ-injected rats (pools of membranes from 6 hearts) in the presence of omapatrilat plus different enzyme inhibitors allowed evaluation of the contribution of other peptidase families to BK metabolism once ACE and NEP were inhibited. Only *o*-phenanthroline (a metallopeptidase inhibitor) totally prevented the degradation of BK (Figure 3). PMSF (serine protease inhibitor) and PCMS (cysteine protease inhibitor) were found to partially inhibit the BK metabolism in membranes of control rats. These results excluded the participation of serine and cysteine proteinases in the BK metabolism by the diabetic rat heart and showed that besides ACE, and NEP, only metallopeptidases are involved in the BK metabolism in this preparation.

3.4. Type II experimental diabetes: NEP and ACE activities

ACE activity was significantly higher in Zucker lean rats (55.5 ± 4.1 pmol/min/mg of protein, n = 6, $P < 0.05$) when compared to the heart values (9.00 ± 0.76 pmol/min/mg of protein, n = 5) of Sprague-Dawley rats but this value was statistically lower than in the Zucker fa/fa group (75.5 ± 3.1 pmol/min/mg of protein, n = 6, $P < 0.05$) (Figure 4a).

NEP activity exhibited similarly higher values in both groups of Zucker rats (lean: 2.59 ± 0.20 fmol/min/mg of protein, n = 5, $P < 0.05$; fa/fa: 2.89 ± 0.30 fmol/min/mg of protein, n = 5, $P < 0.05$) when compared with Sprague-Dawley rats (2.06 ± 0.15 fmol/min/mg of protein, n = 5). No difference could, however, be found between lean and fa/fa animals (Figure 4b).

3.5. BK metabolism in cardiac membranes from Zucker rats

Similar BK $t_{1/2}$ degradation was recorded for both lean (58 ± 5 sec, n = 7) and fa/fa (41 ± 2 sec, n = 8) rats. These values were, however, significantly lower than those of Sprague-Dawley rats (152 ± 7 sec, n = 15, $P < 0.05$) (Figure 5). Although BK $t_{1/2}$ in the presence of enalaprilat (lean: 147 ± 9 sec; n = 7, and fa/fa: 140 ± 6 sec; n = 8) and omapatrilat (lean: 264 ± 14 sec; n = 7, and fa/fa: 257 ± 10 sec; n = 8) was statistically similar in both Zucker groups, the relative potentiating effect of the inhibitors was significantly higher in fa/fa rats (enalaprilat $240 \pm 14\%$ and omapatrilat $532 \pm 42\%$ $P < 0.05$) compared to lean rats (enalaprilat $163 \pm 24\%$ and omapatrilat $370 \pm 40\%$). In addition, the potentiating actions of both inhibitors were higher than in Sprague-Dawley rats ($38 \pm 8\%$ for enalaprilat and $112 \pm 13\%$ for omapatrilat, $P < 0.05$).

4. Discussion

In this paper, we demonstrate that experimental diabetes influences the activities of 2 metallopeptidases (ACE and NEP) in the rat heart. In STZ-induced type I diabetes, a model in which insulin levels are reduced, the activities of both ACE and NEP are decreased; yet, BK metabolism was unchanged due to increased activity of other as yet unidentified metallopeptidase(s), a conclusion supported by the complete inhibition provided by *o*-phenanthroline treatment under these conditions. However, when the metabolism of BK was normalized following insulin treatment, as shown by glucose blood level and the end of glycosuria, the activities of both ACE and NEP were back to normal and BK $t_{1/2}$ remained unchanged, indicating the disappearance of the unidentified metallopeptidase(s) upregulated in this model of diabetes (operationally defined as the *o*-phenanthroline-sensitive, but omapatrilat-resistant component of BK degradation). MMP expression is well known to be controlled by the inflammatory cytokines, notably in endothelial cells (Mackay et al., 1992) and the present data suggests that their potential kininase activity has been overlooked.

The genetically obese *fa/fa* rat is a model used to study the pathogenesis of obesity and the possible evolution of this syndrome to diabetes. These animals are characterized by marked insulin resistance. Initially, they are both normoglycemic and hyperinsulinemic but, with time, they develop type II diabetes which becomes evident after 12 weeks (Bray, 1977; Liu et al., 2002). In Zucker *fa/fa* rats and their lean controls, BK $t_{1/2}$ at 8 weeks of age was significantly lower than in control Sprague-Dawley animals. This shorter $t_{1/2}$ is in agreement with the higher participation of ACE and NEP in BK metabolism. Consistent with a cause and effect relationship, when both enzymes are inhibited, the relative potentiating effect on BK $t_{1/2}$ is higher than that

measured in the Sprague-Dawley control group. At 8 weeks of age, fa/fa rats exhibited hyperinsulinemia but were nearly euglycemic, as previously reported (Bray, 1977). At 8 weeks, ACE and NEP activities of Zucker obese and lean rats were higher than in Sprague-Dawley rats. On the other hand, ACE activity in obese rats was significantly higher than in lean animals whereas NEP activity was similar in both groups.

The regulation of heart ACE and NEP in experimental diabetes is a novel finding of interest. ACE and NEP decreasing activities after STZ treatment are not inversely correlated to glycemia, because the increasing glycemia in 8 week-old fa/fa Zucker rats is rather associated with very high ACE and NEP. Other than genetic strain difference, the opposite effects of the two diabetes models on ACE and NEP expression could be explained by a number of cellular/molecular adaptations that regulate peptidase presence in rat heart membranes. Firstly, diabetes-induced remodeling of cardiac tissue may modulate the relative abundance of cell types, e.g. decrease the endothelial cells density where ACE resides. Secondly, ectopeptidases like ACE and NEP could theoretically be regulated by cleavage and shedding from the cell surface (Ramchandran et al., 1996), and there is evidence for an increased plasma ACE concentration in some diabetic patient subgroups and some animal diabetes model (Scherthaner et al., 1984; van Dijk et al., 2001). Finally, the pathology may alter the expression of the ACE and NEP genes. The regulation of at least the ACE gene product that has previously been studied in cultured endothelial cells (Villard et al., 1998; Saijonmaa et al., 2001a; 2001b). Some changes may be determined by the inflammatory phenotype of insulin-deprived animals: untreated diabetes is associated with high endogenous production of inflammatory cytokines like TNF- α , which increases early (as soon as 5 days) and intensely (88-fold at 5 weeks) in the rat myocardium following STZ treatment

(Drimal et al. 2008). TNF- α decreases ACE expression in endothelium (both mRNA and protein; Papapetropoulos et al., 1996; Saijonmaa et al., 2001a).

Fatty Zucker rats exhibit in their vascular tissue an increased concentration of the endogenous protein kinase C (PKC) stimulant diacylglycerol and an increased activity of several PKC isoforms (Naruse et al., 2006). If the PKC activity alteration is anterior to the insulin resistance in these animals, it could also determine in vivo the previously described PKC-mediated endothelial upregulation of ACE (Villard et al., 1998; Saijonmaa et al., 2001b). It is also of interest that the serum of obese Zucker rats contain more VEGF than that of lean controls (Lamarre et al., 2007), because VEGF is coupled to PKC to upregulate ACE in human umbilical vein endothelial cells (Saijonmaa et al., 2001b). The Zucker rats were studied up to the age of 8 weeks in the present study, but overt hyperglycemia and diabetic complication arise later. It is not excluded that ACE and NEP decrease at a later age owing to the particular inflammatory alterations that are common to type I and II diabetes, such as those induced by advanced glycation end products that are determined by persistent hyperglycemia (Wautier et al., 1994). In a previous study, tissue TNF- α concentration was not increased in 6-week old fat Zucker rats, but became elevated at 15 weeks (Liu et al., 2002). In a different model of type II diabetes, the db/db mice, heart ACE was normal but renal ACE was decreased at 8 weeks of age, raising the possibility of complex time-dependent transitions of peptidase expression (Ye et al., 2004). Little is known on the regulation of the peripheral NEP gene expression, but it was largely parallel to that of ACE in the diabetes models that we have studied.

Some of the observations reported in the present paper were unexpected and remain preliminary. Future aims in the study of kininase expression in diabetes models include the *in vivo* study of the regulation of peptidase gene expression at the levels of mRNA and protein as a function of the organ/tissue and of the time course of the pathology, the identification of a postulated but unknown BK-destroying metalloenzyme(s) that compensates for the loss of ACE in NEP in the type I diabetes model, the definitive localization of cell types that express ACE, NEP and the unknown peptidase(s) in healthy or diabetic hearts, and the elucidation of a role for TNF- α and/or other inflammatory cytokines in the downregulation of ACE during insulin-dependent diabetes. Untreated type I diabetes is a rapidly fatal wasting disease that is nowadays less clinically relevant because insulin-dependent diabetic patients are not left untreated without insulin. Thus, the decreased abundance of ACE and NEP expression seen in streptozotocin-treated rats may be associated with a serious clinical state rarely seen in patients now, because insulin treatment reversed these alterations in rats. The metabolic syndrome and aging may rather be statistically associated with the prediabetic upregulation of kininases in the heart, as observed in young Zucker fat rats, with detrimental effects such as increased angiotensin II formation and BK breakdown. The precise biochemical mechanisms for ACE upregulation in Zucker rats remain to be elucidated, with the PKC-mediated activation in vascular tissue being a prime candidate, as discussed above.

Our findings have special significance because BK is thought to mediate at least part of the beneficial cardiovascular (Linz et al., 1995) and metabolic actions of ACEi (Carvalho et al., 1997; Henriksen et al., 1995; Uehara et al., 1994). As the heart represents a major site of the complications of diabetes and a target for the cardiovascular effects of ACEi and the new VPI, disease-driven alterations of ACE and NEP and the rise of a novel kininase component in

diabetes models is of considerable interest. More molecular work is needed to better characterize the mechanism of metallopeptidase variation in each model.

Source of funding and Disclosure

This study was supported by a grant from Bristol-Myers Squibb (to A.A.) and the Canadian Institutes of Health Research (grant to F.M. and A.A.). The authors state no other conflict of interest.

Acknowledgements

We thank Mr. Charles Blais, Jr. and Mr. Jean-François Bouchard for their useful scientific advice, and Ms. Nicole Gervais for her excellent technical assistance.

References

Blais Jr, C., Drapeau, G., Raymond, P., Lamontagne, D., Gervais, N., Venneman, I., Adam, A., 1997. Contribution of angiotensin-converting enzyme to the cardiac metabolism of bradykinin: an interspecies study. *Am. J. Physiol. Heart Circ. Physiol.* 273, H2263-H2271.

Bray, G.A., 1977. The Zucker fatty rat: a review. *Fed. Proc.* 36, 148-153.

Brunning, P., Holmquist, B., Riordan, J.F., 1983. Substrate specificity and kinetic characteristics of angiotensin converting enzyme. *Biochemistry* 22, 103-110.

Carvalho, C.R.O., Thirone, A.P.C., Gontijo, J.A.R., Velloso, L.A., Saad, M.J.A., 1997. Effect of captopril, losartan, and bradykinin on early steps of insulin action. *Diabetes* 46, 1950-1957.

Costerousse, O., Allegrini, J., Huang, H., Bounhiko, J., Alhenc-Gelas, F., 1994. Regulation of ACE gene expression and plasma levels during rat postnatal development. *Am. J. Physiol. Endocrinol. Metab.* 267, E745-E753.

Décarie, A., Drapeau, G., Closset, J., Couture, R., Adam, A., 1994. Development of digoxigenin-labeled peptide: application to chemiluminoenzyme immunoassay of bradykinin in inflamed tissues. *Peptides* 15, 511-518.

Drimal, J., Knezl, V., Navarova, J., Nedelceva, J., Paulovicova, E., Sotnikova, R., Snirc, V.,
Drimal, D., 2008. Role of inflammatory cytokines and chemoattractants in the rat model of
streptozotocin-induced diabetic heart failure. *Endocr. Regul.* 42, 129-135.

Dumoulin, M.J., Adam, A., Blais Jr, C., Lamontagne, D., 1988. Metabolism of bradykinin by the
rat coronary bed. *Cardiovasc. Res.* 38, 229-236.

Erdös, E.G., Skidgel, R.A., 1997. Metabolism of bradykinin by peptidases in health and disease.
In: Farmer, S.G. (Ed.). *The Kinin System*. Academic Press, San Diego, CA , pp. 111-141.

Gazis, A., Page, S.R., Cockcroft, J.R., 1998. ACE inhibitors, diabetes and cardiovascular disease.
Diabetologia 41, 595-597.

Henriksen, E. J., Jacob, S., 1995. Effects of captopril on glucose transport activity in skeletal
muscle of obese Zucker rats. *Metabolism* 44, 267- 272.

HOPE investigators, 2000. Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on
cardiovascular events in high-risk patients. *N. Engl. J. Med.* 342, 145-153.

Lamarre, N.S., Ruggieri, M.R., Braveman, A.S., Gerstein, M.I., Mydlo, J.H., 2007. Effect of
obese and lean Zucker rat sera on human and rat prostate cancer cells: implications in obesity-
related prostate tumor biology. *Urology* 69, 191-195.

Le Moual, H., Devault, A., Roques, B.P., Crine, P., Boileau, G., 1991. Identification of glutamic acid 646 as a zinc-coordinating residue in endopeptidase 24.11. *J. Biol. Chem.* 266, 15670-15674.

Leeb-Lundberg, L.M., Marceau, F., Müller-Esterl, W., Pettibone, D.J., Zuraw, B.L., 2005. International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. *Pharmacol. Rev.* 57, 27-77.

Linz, W., Wiemer, G., Gohlke, P., Unger, T., Schölkens, B.A., 1995. Contribution of kinins to the cardiovascular actions of angiotensin-converting enzyme inhibitors. *Pharmacol. Rev.* 47, 25-49.

Liu, R.H., Mizuta, M., Kurose, T., Matsukura, S., 2002. Early events involved in the development of insulin resistance in Zucker fatty rats. *Int. J. Obesity* 26, 318-326.

Mackay, A.R., Ballin, M., Pelina, M.D., Farina, A.R., Nason, A.M., Hartzler, J.L., Thorgeirsson, U.P., 1992. Effect of phorbol ester and cytokines on matrix metalloproteinase and tissue inhibitor of metalloproteinase expression in tumor and normal cell lines. *Invasion Metastasis* 12, 168-184.

Moore, J.W., Pearson, R.G., 1981. Complex reactions. In: Moore, J.W., Pearson, R.G. (Eds.), *Kinetics and Mechanism* (3rd ed.), Wiley, New York, pp.284-296.

Naruse, K., Rask-Madsen, C., Takahara, N., Ha, S.W., Suzuma, K., Way, K.J., Jacobs, J.R., Clermont, A.C., Ueki, K., Ohshiro, Y., Zhang, J., Goldfine, A.B., King, G.L., 2006. Activation of

vascular protein kinase C-beta inhibits Akt-dependent endothelial nitric oxide synthase function in obesity-associated insulin resistance. *Diabetes* 55, 691-698.

Papapetropoulos, A., Antonov, A., Virmani, R., Kolodgie, F.D., Munn, D.H., Marczin, N., Ryan, J.W., Gerrity, R.G., Catravas, J.D., 1996. Monocyte- and cytokine-induced downregulation of angiotensin-converting enzyme in cultured human and porcine endothelial cells. *Circ. Res.* 79, 512-523.

Prechel, M.M., Orawski, A.T., Maggiora, L.L., Simmons, W.H., 1995. Effect of a new aminopeptidase P inhibitor, apstatin, on bradykinin degradation in the rat lung. *J. Pharmacol. Exp. Ther.* 275, 1136-1142.

Ramchandran, R., Kasturi, S., Douglas, J.G., Sen, I., 1996. Metalloprotease-mediated cleavage secretion of pulmonary ACE by vascular endothelial and kidney epithelial cells. *Am. J. Physiol. Heart Circ. Physiol.* 271, H744-H751.

Raut, R., Rouleau, J.L., Blais Jr, C., Gosselin, H., Molinaro, G., Sirois, G.M., Lepage, Y., Crine, P., Adam, A., 1999. Bradykinin metabolism in the postinfarcted rat heart: role of ACE and neutral endopeptidase 24.11. *Am. J. Physiol. Heart Circ. Physiol.* 276, H1769-H1779.

Ryan, J.W., Berryer, P., Chung, A.Y.K., Sheffy, D.H., 1994. Characterization of rat pulmonary vascular aminopeptidase P in vivo: role in the inactivation of bradykinin. *J. Pharmacol. Exp. Ther.* 269, 941-947.

Saijonmaa, O., Nyman, T., Fyhrquist, F., 2001a. Downregulation of angiotensin-converting enzyme by tumor necrosis factor- α and interleukin-1 β in cultured human endothelial cells. *J. Vasc. Res.* 38, 370-378.

Saijonmaa, O., Nyman, T., Kosonen, R., Fyhrquist, F., 2001b. Upregulation of angiotensin-converting enzyme by vascular endothelial growth factor. *Am. J. Physiol. Heart Circ. Physiol.* 280, H885-H891.

Schernthaner, G., Schwarzer, C., Kuzmits, R., Müller, M.M., Klemen, U., Freyler, H., 1984. Increased angiotensin-converting enzyme activities in diabetes mellitus: analysis of diabetes type, state of metabolic control and occurrence of diabetic vascular disease. *J. Clin. Pathol.* 37, 307-312.

Tatti, P., Pahor, M., Byington, P.R., Di Mauro, P., Guarisco, R., Strollo, G., Strollo, F., 1998. Outcome results of the Fosinopril versus Amlodipine Cardiovascular Events Randomized Trial (FACET) in patients with hypertension and NIDDM. *Diabetes Care* 21, 597 -603.

Trippodo, N.C., Robl, J.A., Asaad, M.M., Fox, M., Panchal, B.C., Schaeffer, T.R., 1998. Effects of omapatrilat in low, normal, and high renin experimental hypertension *Am. J. Hypertens.* 11, 363-372.

Tschöpe, C., Gavrilluk, V., Reinecke, A., Seidl, U., Riester, U., Hilgenfeldt, U., Ritz, E., Unger, T., 1996. Bradykinin excretion is increased in severely hyperglycemic streptozotocin-diabetic rats. *Immunopharmacology* 33, 344-348.

Uehara, M., Kishikawa, H., Isami, S., Kisanuki, K., Ohkubo, Y., Miyamura, N., Miyata, T., Yano, T., Shichiri, M., 1994. Effect on insulin sensitivity of angiotensin converting enzyme inhibitors with or without a sulphhydryl group: bradykinin may improve insulin resistance in dogs and humans. *Diabetologia* 37, 300-307.

van Dijk, D.J., Boner, G., Giler, S., Erman, A., 2001. Increased serum angiotensin-converting enzyme activity and plasma angiotensin II levels during pregnancy and postpartum in the diabetic rat. *J. Renin Angiot. Aldo. Syst.* 2, 193-198.

Villard, E., Alonso, A., Agrapart, M., Challah, M., Soubrier, F., 1998. Induction of angiotensin I-converting enzyme transcription by a protein kinase C-dependent mechanism in human endothelial cells. *J. Biol. Chem.* 273, 25191-25197.

Wallenstein, S., Zucker, C.L., Fleis, J.L., 1990. Some statistical methods useful in circulation research. *Circ. Res.* 47, 1-9.

Wautier, J.L., Wautier, M.P., Schmidt, A.M., Anderson, G.M., Hori, O., Zoukourian, C., Capron, L., Chappey, O., Yan, S.D., Brett, J., Guillausseau, P.J., Stern, D., 1994. Advanced glycation end products (AGEs) on the surface of diabetic erythrocytes bind to the vessel wall via a specific receptor inducing oxidant stress in the vasculature: a link between surface-associated AGEs and diabetic complications. *Proc. Natl. Acad. Sci. U.S.A.* 91, 7742-7746.

Ye, M., Wysocki, J., Naaz, P., Salabat, M.R., LaPointe, M.S., Battle, D., 2004. Increased ACE2 and decreased ACE protein in renal tubules from diabetic mice: a renoprotective combination? Hypertension 43, 1120-1125.

Figure legends

Figure 1

Time course of changes in ACE (a) (pmol/min/mg of protein) and NEP (b) (fmol/min/mg of protein) activities in type I experimental model of diabetes in Sprague-Dawley control rats (CTL), STZ-treated rats 2, 4 and 8 weeks post-injection, and STZ-treated rats 8 weeks post-injection that received insulin for 7 days (+ INS-1 week). Values are means \pm s.e.m. for 4 to 6 rats per group. * $P < 0.05$ versus CTL, † $P < 0.05$ for the indicated pair.

Figure 2

(a) Time course of changes in the half-life of exogenous BK incubated with heart membranes from Sprague-Dawley control rats (CTL) and STZ-treated rats 2, 4 and 8 weeks post-injection in the absence enzymatic inhibitor (open columns) or in the presence of enalaprilat (cross-hatched columns) or omapatrilat (closed columns). (b) Influence of insulin treatment on half-life of exogenous BK incubated with heart membranes from STZ-treated rats 8 weeks post-injection that received insulin for 7 days (+ INS-1 week). Values are means \pm s.e.m. for 15 rats (control Sprague-Dawley) and 6 to 8 rats for other groups. * $P < 0.05$ versus without inhibitor. † $P < 0.05$ versus enalaprilat.

Figure 3

Time course of the degradation of exogenous BK when incubated with pools of heart membranes from (a) Sprague-Dawley control rats and (b) STZ-treated rats 8 weeks post-injection in the absence of enzymatic inhibitor or in the presence of omapatrilat alone (Oma) or omapatrilat plus 1 of the following enzymatic class inhibitors: PMSF, PCMS, or *o*-phenanthroline (*o*-PNT).

Figure 4

Time course of changes in ACE (a) and NEP (b) activities in type II experimental diabetes in Sprague-Dawley rats (S-D) and Zucker (Z) lean and fa/fa rats. Values are means \pm s.e.m. for 5 to 6 rats per group. * $P < 0.05$ versus S-D. † $P < 0.05$.

Figure 5

Influence of rat strain and type II diabetes on the half-life of exogenous BK incubated with heart membranes from Sprague-Dawley rats (S-D) and Zucker (Z) lean and fa/fa rats and as influenced by drugs (absence enzymatic inhibitor, open columns; presence of enalaprilat, cross-hatched columns, or omapatrilat, closed columns). Values are means \pm s.e.m. for 15 rats (S-D group) and 7 to 8 rats for other groups. * $P < 0.05$ versus without inhibitor. † $P < 0.05$ versus enalaprilat.

Table 1. PHYSIOLOGICAL CHARACTERISTICS OF RATS USED IN THIS STUDY

Type I diabetes

Groups	Weight (g) (initial)	Weight (g) (sacrifice)	Glycosuria (mM)	Insulin (μ IU/ml) (sacrifice)	Glycemia (mM) (sacrifice)
Controls 2 weeks	292 \pm 2	361 \pm 10	negative	173 \pm 3	7.4 \pm 0.5
STZ 2 weeks	301 \pm 5	320 \pm 2	> 111	69 \pm 7	21.8 \pm 2.0
Controls 4 weeks	296 \pm 4	424 \pm 10	negative	-	6.2 \pm 0.2
STZ 4 weeks	293 \pm 3	342 \pm 16	> 111	106 \pm 9	21.7 \pm 1.5
Controls 8 weeks	295 \pm 5	483 \pm 12	negative	-	7.0 \pm 0.5
STZ 8 weeks	265 \pm 4	304 \pm 6	> 111	33 \pm 5	24.9 \pm 1.0
STZ 8 weeks + Insulin 7 days	252 \pm 3	312 \pm 12	negative	48 \pm 3	5.9 \pm 0.8

Type II diabetes

Groups	Weight (g) (initial)	Weight (g) (sacrifice)	Glycosuria (mM)	Insulin (μ IU/ml)	Glycemia (mM) (sacrifice)
Zucker lean	119 \pm 3	219 \pm 5	negative	123 \pm 2	7.6 \pm 1.2
Zucker fa/fa	168 \pm 12	312 \pm 12	negative	272 \pm 1	10.0 \pm 1.5

Figure 1
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Fig. 1

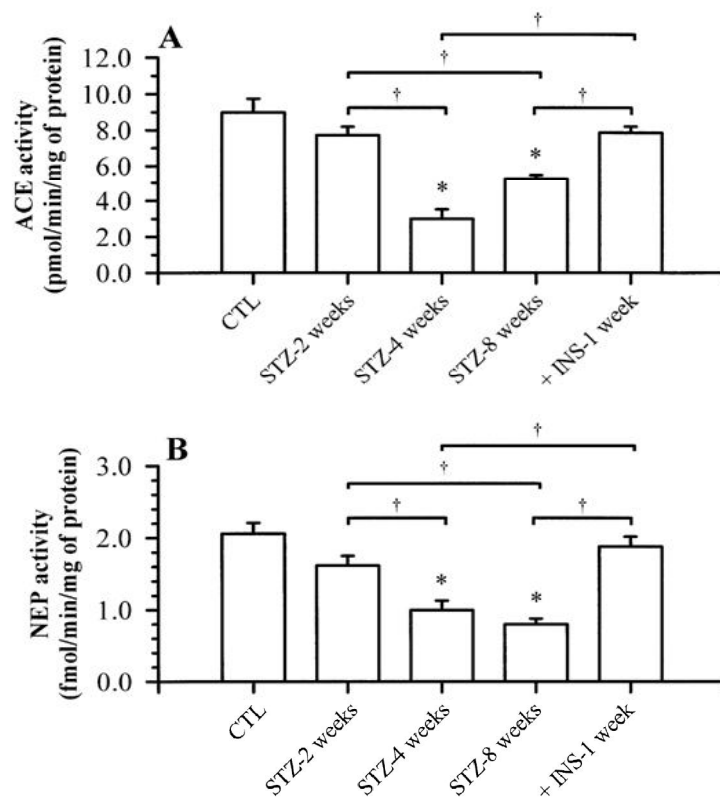


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Fig. 2

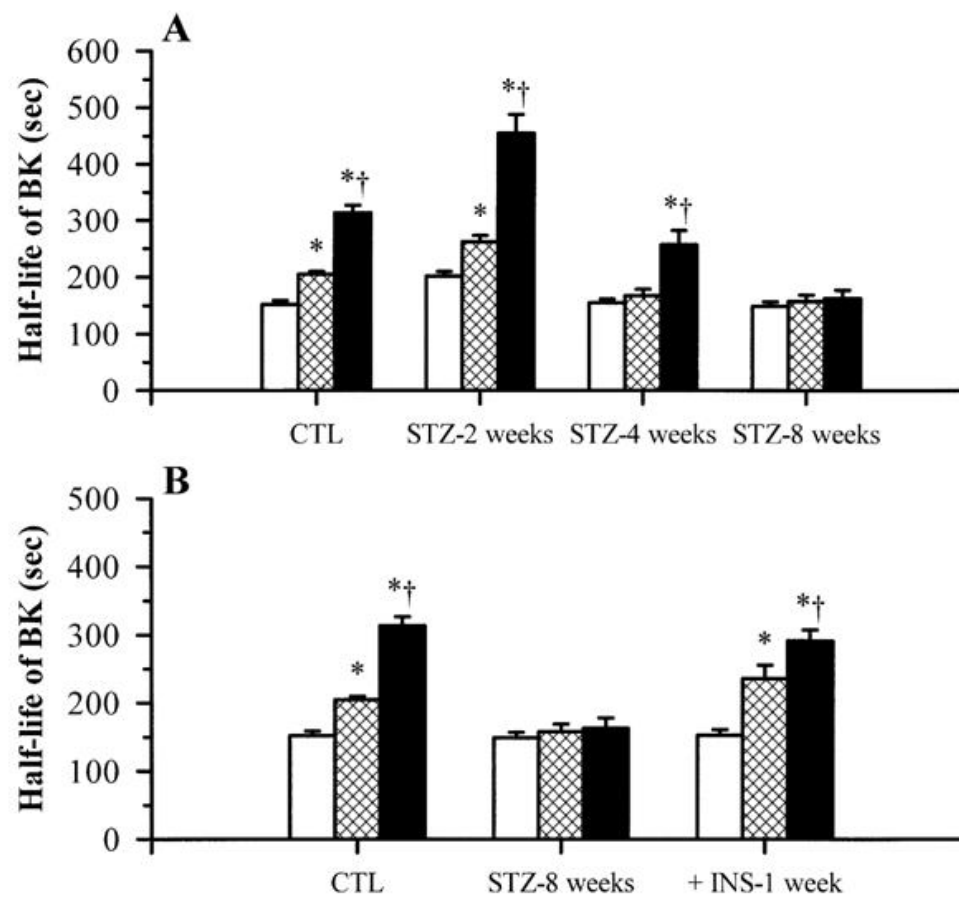


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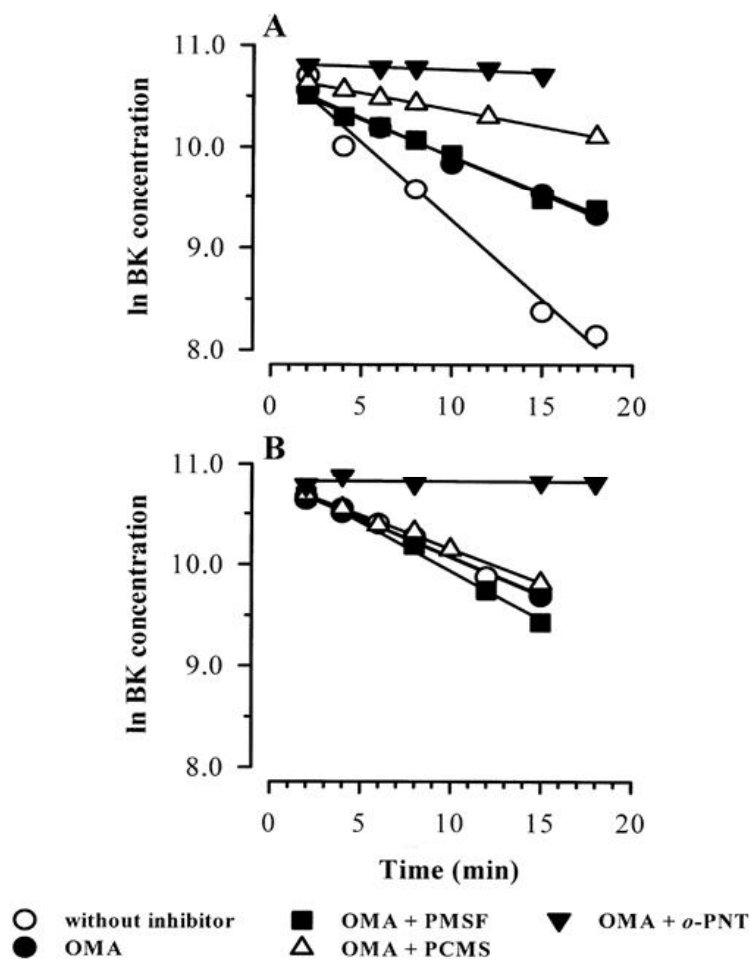


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Fig. 4

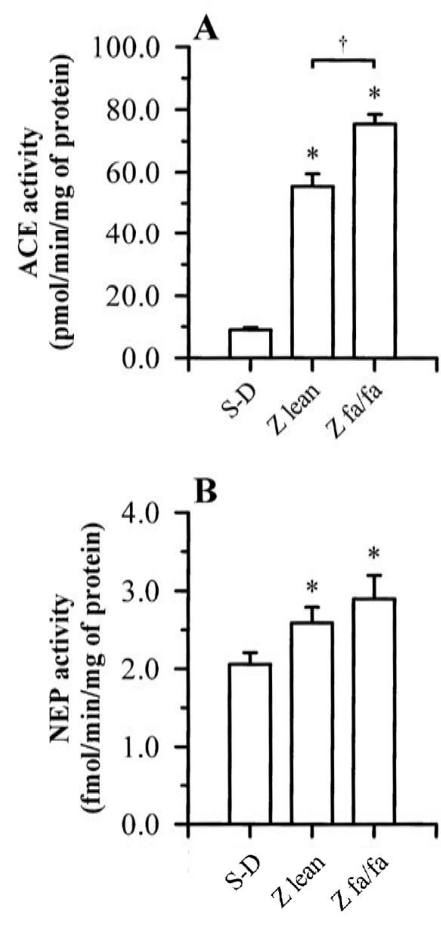


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Fig. 5

