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EFFECT OF CHRONIC ENALAPRIL TREATMENT ON ENZYMES RESPONSIBLE FOR THE CATABOLISM OF ANGIOTENSIN I AND FORMATION OF ANGIOTENSIN II

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Abstract—We have investigated the effect of chronic administration of enalapril on the carboxypeptidases responsible for the formation of angiotensin II from angiotensin I and other peptidases known to recognize angiotensin I as a substrate in the rat. These studies have shown an increase in activity in rate of formation of des-Leu-angiotensin I in both kidney S2 and P2 centrifugal fractions as well as a decrease in the rate of degradation of angiotensin I substrate. Similar increases in the formation of A(1-8) have been observed in kidney using A(1-9) as substrate. These two enzyme activities have been named carboxypeptidase K_1 and K_2, respectively to reflect their presence in rat kidney. These changes were acompanied by significant decreases in the activity of an amastatin-sensitive aminopeptidase and endopeptidase 24.11 in the kidney P2 fraction. These data suggest that chronic treatment with ACE inhibitors may differentially affect the activity of other enzymes capable of degrading angiotensin causing a substantial re-direction of angiotensin metabolism.

Angiotensin II [AII, A(1–8)] is a potent vasopressor octapeptide involved in the regulation of fluid and electrolyte balance [1]. The formation of this peptide not only occurs by the action of angiotensin converting enzyme (ACE) [EC 3.4–15.1] on the decapeptide angiotensin I [AI, A(1–10)] but also by sequential carboxypeptidase action on AI producing des-Leu-angiotensin I [A(1–9)] as an intermediate peptide [2, 3]. Carboxypeptidase-mediated formation of A(1–9) and A(1–8) can be detected in kidney centrifugal fractions by monitoring the formation of these peptides from their respective precursor substrates A(1–10) and A(1–9) [2–5]. This alternative pathway to ACE has also been implicated in being involved in the regulation of AII production by inhibition of ACE by A(1–9) [6].

Furthermore, it has been established that chronic administration of ACE inhibitors cause an increase in the activity of ACE in plasma of rats and humans and in many other tissues of the rat after withdrawal of inhibitor [7, 8]. However, the effect of ACE inhibitors on the activity of other peptidases hydrolysing vasoactive peptides has not been established. Inhibitors of ACE, such as captopril and enalapril, which are now used clinically to treat hypertension and congestive heart failure may, by suppressing the active of ACE, redirect metabolism of AI to AII through the carboxypeptidase pathway.

In order to establish whether carboxypeptidase-mediated formation of AII is affected by chronic administration of an ACE inhibitor we have investigated the effects of chronic enalapril treatment on the carboxypeptidase enzyme activities responsible for the formation of A(1–9) and AII in the rat as well as some other peptidase activities known to recognize AI.

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MATERIALS AND METHODS

Chemicals. Amastatin, 7-amino-4-methyl coumarin (AMC), fast garnet green, hippuryl-L-arginine (Hipp-Arg), iodoacetamide, β-naphthylamine, phenylmethylsulfonyl fluoride (PMSF), thiorphan (Sigma Chemical Co., St Louis, MO), angiotensin I [A(1–10)], angiotensin II [A1–8], angiotensin III [A(2–8)], des-Leu-angiotensin I [A(1–9)] (Auspep Pty Ltd, Melbourne, Australia), Z-arginyl-arginyl-methoxycoumarinamide (Z-Arg-Arg-MCA), hippuryl-histidyl-leucine (Hipp-His-Leu) Peptide Institute Inc., Osaka, Japan), 1-arginyl-β-naphthylamine (Arg-β-NA) (Cambridge Research Biochemicals Ltd, U.K.), acetonitrile (HPLC grade, Mallinckrodt Australia Pty Ltd), trifluoroacetic acid (TFA) (HPLC grade, Pierce Chemicals Co., Rockford, IL), ethylene diaminetetraacetic acid, di-sodium salt (EDTA) (Ajax Chemicals, Melbourne, Australia) were obtained from the stated suppliers. Captopril and enalapril (MK421) were generous gifts from E. R. Squibb & Sons Inc. (Melbourne, Australia) and Merck & Co. (Sydney, Australia). All other chemicals and reagents were analytical reagent grade. Distilled deionized water was used for all solutions.

Treatment of animals. Wistar-Kyoto rats (weighing 200–260 g) in groups of six were given enalapril in their drinking water for up to 4 weeks. Two separate groups (A and B) were treated for 4 weeks at a concentration of 0.2 g/L. In another group (C) rats were exposed for 14 days with a concentration of enalapril of 0.5 g/L in their drinking water. In all groups a parallel group of six rats were included who received only vehicle (water).

Enzyme preparations. Treated rats or control rats

were anaesthetized with halothane and their kidneys perfused with ice-cold heparinized saline (75 IU/mL) to remove blood. Kidneys were removed and homogenized in 20 vol. of ice-cold 50 mM Tris buffer, pH 7.0 containing 180 mM NaCl and centrifuged for 1000 *g*/min to sediment unlysed cells and tissue fragments. The resultant pellet, which was largely composed of incompletely homogenized cell debris and fibrous tissue, was discarded and the supernatant was further centrifuged at 10,000 *g*/min at 4°. The pellet (P2) was resuspended in 10 mL of ice-cold buffer. The supernatant was labelled as the S2 fraction. The S2 fraction was enriched two-fold in the activity of the lysosomal marker enzyme cathepsin B, while the activity of the cell marker enzyme ACE was enriched seven-fold in the P2 fraction (see Results). All fractions were frozen at −15° until use.

Enzyme assays. Aminopeptidase (AP) activity was measured using 200 μM Arg-β-NA in 50 mM Tris pH 7.4 and 180 mM NaCl as the substrate solution. The amount of β-NA liberated was determined by reacting it with fast garnet green and measuring the change in absorbance at 525 nm essentially as described by Hopsu *et al.* [9].

Angiotensin-converting enzyme activity was measured fluorometrically using Hipp-His-Leu as the substrate according to the method of Friedland and Silverstein [10].

Carboxypeptidase B (CB-B) activity was measured using Hipp-Arg as substrate essentially as described by Schweisfurth [11] except that the hippuric acid produced in the reaction was measured by HPLC by evaporating the ethyl acetate extract under a stream of air at 60°. The residue was reconstituted with 100 μL of HPLC mobile phase and 10-μL aliquots were injected. HPLC conditions were as follows: column, NOVAPAK-C18 (Waters Associates, Melbourne, Australia); flow rate, 0.8 mL/min; mobile phase, 15% acetonitrile in 0.1% orthophosphoric acid; detector, variable wavelength M450 (Waters Associates); wavelength was 228 nm and sensitivity was 0.10 aufs.

Cathepsin B activity was measured using Z-Arg-Arg-MCA as substrate as described by Barrett and Kirschke [12]. In these assays the total volume was 2 mL using a final concentration of substrate of 5 μM and 100 μL enzyme. The reaction proceeded for 15 min. The fluorescence formed in the reaction was compared to standards of AMC prepared in the incubation buffer.

Protein concentration was determined according to the method of Bradford using bovine serum albumin as standard [13].

HPLC enzyme assays. Enzyme activities were measured similar to that described before [2, 3]. In particular enzyme activities responsible for the production of A(1–9) and A(1–8) were obtained by incubating either A(1–10) or A(1–9) at a concentration of 100 μM with S2 and P2 centrifugal fractions of rat kidney in a 50 mM TRIS buffer, pH 7.0 containing 180 mM NaCl for 10 min at 37° in a total volume of 200 μL. Reactions were started by the addition of substrate and terminated by the addition of 100 μL 3% (v/v) TFA in acetonitrile. All incubations were preincubated for 10 min at 37° before the reaction was started with substrate. For incubations with A(1–10) in kidney S2 captopril (20 μM) was present to inhibit ACE activity and in kidney P2, captopril (20 μM) and thiorphan (10 μM) were present in the incubation mixture to inhibit ACE and enkephalinase activities. For the determination of endopeptidase 24.11 activity, thiorphan was omitted from the inhibition mix and the peak corresponding to the elution of A(8–10) was used to calculate enzyme activity. The amounts of enzyme used were adjusted to give approximately 50% digestion of substrate in 10 min. For most incubations 20 μL of enzyme was used except those with A(1–10) as substrate in kidney P2 when no inhibitors were added (except for captopril) in which case 2 μL was used. The samples were then centrifuged at 5000 *g*/min and 15 μL of the supernatant was injected into a 15 cm Spherisorb 5S ODS-2 column using an automatic sample injector. The samples were eluted isocratically with 22.5% acetonitrile in 10 mM ammonium phosphate buffer, pH 4.25 at a flow rate of 1.0 mL/min. Detection was by UV at 214 nm. The amounts of peptides present were calculated by comparing peak areas of unknown with those of authentic standards.

Enzyme activities (in nmol/min/mg protein) were calculated for the rate of digestion of substrate (A(1–10) or A(1–9) and for the rate of formation of peptide products (A(1–9) or A(1–8), respectively.

Statistical evaluation of data. Data are presented as means ± SE and were analysed by two-way analysis of variance for the effects of treatment and experiment, using the program CLR-ANOVA (Clear Lake Research Incorporated, Version 1.1) run on an Apple Macintosh personal computer. A probability (P) of less than 0.05 was taken as representing a significant difference.

RESULTS

Chromatographic conditions

The HPLC conditions chosen allowed complete separation of all known angiotensin fragments as previously reported [2–3] and included A(1–10), A(1–9), A(1–8), A(1–7), A(2–8), A(2–10), A(1–4), A(8–10). Peaks corresponding to elution of A(1–9) and A(1–8) were confirmed as such in some experiments by the collection of corresponding fractions and checking these for immunoreactivity against antisera specific for those two peptides.

Digestion studies with AI

Incubation of A(1–10) with the kidney S2 centrifugal fraction resulted in the formation of A(1–9). As there were no significant trends or differences among the three chronic preparations in terms of their ability to produce A(1–9) from A(1–10), the results of the three separate preparations were pooled. This activity was significantly increased by 36% in rats treated chronically with enalapril (Table 1), but was not associated with a significant change in the rate of digestion of substrate A(1–10). The proportion of A(1–10) converted to A(1–9) in this tissue was 42% in control animals and 56% in the treated animals ($P < 0.01$).

In the kidney P2 fraction both captopril and thi-

Table 1. Effect of chronic enalapril treatment on the degradation of A(1–10) and formation of A(1–9) in rat kidney centrifugal fractions

Group/Subcellular fraction	Rate of A(1–10) degradation*	Rate of A(1–9) formation* (Carboxypeptidase K_1 activity)
(a) Kidney S2†		
Control	28.1 ± 2.7	11.9 ± 1,1
Enalapril treated group	28.9 ± 2.2	16.2 ± 1.7§
(b) Kidney P2‡		
Control	32.5 ± 0.5	14.3 ± 0.6
Enalapril treated group	26.6 ± 2.9	18.8 ± 1.2§

* Digestion of A(1–10) in Tris/NaCl buffer with subcellular fractions obtained from control rats or rats treated chronically with enalapril. Activity in nmol/min/mg protein, mean of 17–18 tissues from three separate experiments.

† Incubated in the presence of captopril (20 μM) to inhibit ACE activity.

‡ Incubated in the presence of captopril (20 μM) and thiorphan (10 μM) to inhibit ACE and enkephalinase activities, respectively.

§ $P < 0.025$ (ANOVA) with respect to control group.

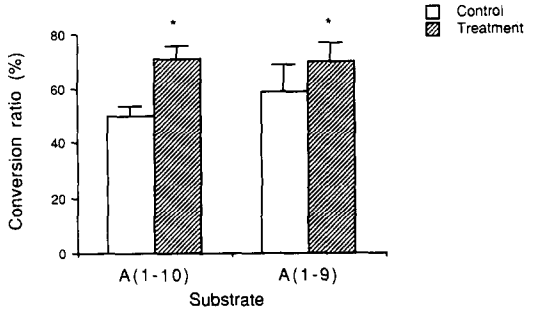

Fig. 1. Ratio of enzyme activity producing A(1–9) and A(1–8) peptides from respectively, A(1–10) and A(1–9) substrates to rate of degradation of substrate expressed as a percentage in control animals and in animals treated with enalapril chronically. Data shown is the mean of 18 tissues obtained from three separate experiments ± SE *$P < 0.05$.

ophan were added to incubations to inhibit ACE and enkephalinase (endopeptidase 24.11) activities, respectively. Under these conditions a significant increase (31% of control) in carboxypeptidase-mediated formation of A(1–9) was also observed in tissues obtained from rats treated chronically with enalapril (Table 1). In contrast to kidney S2, there was a reduction in overall A(1–10)-degrading activity in kidney P2 fractions obtained from treated animals. Although this did not reach statistical significance the proportion of A(1–10) converted enzymatically to A(1–9) changed significantly from 50 ± 3% to 70 ± 6% ($P < 0.05$, Fig. 1).

Digestion studies with A(1–9)

Incubation of A(1–9) with the kidney S2 centrifugal fraction resulted in the production of A(1–8). This carboxypeptidase-mediated production of A(1–8) was increased significantly in tissues obtained from rats treated chronically with enalapril (Table 2). In contrast there was a reduction in the rate of degradation of A(1–9), although, as with the A(1–10) studies, this did not reach statistical significance. The proportion of A(1–9) converted to A(1–8) in this tissue increased from 59 ± 10% to 70 ± 7% (Fig. 1), however this also was not statistically significant.

Incubation of A(1–9) in the kidney P2 fraction did not in any of the 18 kidney preparations taken from the 18 control rats produce A(1–8). However, in all of the animals treated chronically with enalapril (N = 18) small amounts of A(1–8) (1.3 nmol/min/mg protein) were observed (Table 2). This was associated with a significant ($P < 0.01$) reduction in the rate of degradation of the substrate A(1–9) compared with the control animals. Again there were no significant differences in the observed changes with any of the three chronic experiments.

Effect of inhibitor mixtures on the digestion of AI in kidney P2

To investigate any differential effect enalapril treatment may have had on the rate of breakdown of AI in the kidney P2 centrifugal fraction we incubated AI in the presence of a number of inhibition mixtures. These included a captopril-only mixture (mixture 1) and mixtures (mixtures 2–4) containing other inhibitors additional to captopril. These data are shown in Fig. 2.

All inhibition mixtures used showed the formation of A(1–9). The most effective mixtures in producing A(1–9) was mixture 4 containing capropril (20 μM) and thiorphan (10 μM). This also had the lowest rate of degradation of A(1–10). Mixture 3 containing captopril (20 μM), iodoacetamide (1 mM), amastatin (20 μM), EDTA (1 mM) and PMSF (50 μM) was almost as effective as mixture 4 in terms of A(1–9) formation but showed a greater rate of breakdown (almost two-fold) of substrate A(1–10) compared to that observed with mixture 4 ($P < 0.05$). Mixture 2, which contained the same inhibitors as mixture 3 with the exception of EDTA, produced a significant increase ($P < 0.05$, 3–4-fold) in the rate of breakdown of A(1–10) together with the formation of significant quantities of both A(1–7) and A(1–9) fragments. These fragments were probably associated with the action of enkephalinase on A(1–10) breaking the Pro^7-Phe^8 bond since both EDTA and

Table 2. Effect of chronic enalapril treatment on the degradation of A(1–9) and formation of A(1–8) in rat kidney centrifugal fractions

Group/subcellular fraction	Rate of A(1–9) degradation*	Rate of A(1–8) formation* (Carboxypeptidase K_2 activity)
(a) Kidney S2		
Control	20.2 ± 2.8	7.9 ± 1.0
Enalapril treated group	16.4 ± 2.00	9.7 ± 0.8
(b) Kidney P2		
Control	182 ± 21	<0.5
Enalapril treated group	142 ± 11†	1.32 ± 0.24†

* Digestion of A(1–9) in Tris/NaCl buffer with kidney subcellular fractions obtained from control rats or rats treated chronically with enalapril. Activity in nmol/min/mg protein, mean of 18 tissues from three separate experiments ± SE.

† $P < 0.01$ (ANOVA) with respect to control group.

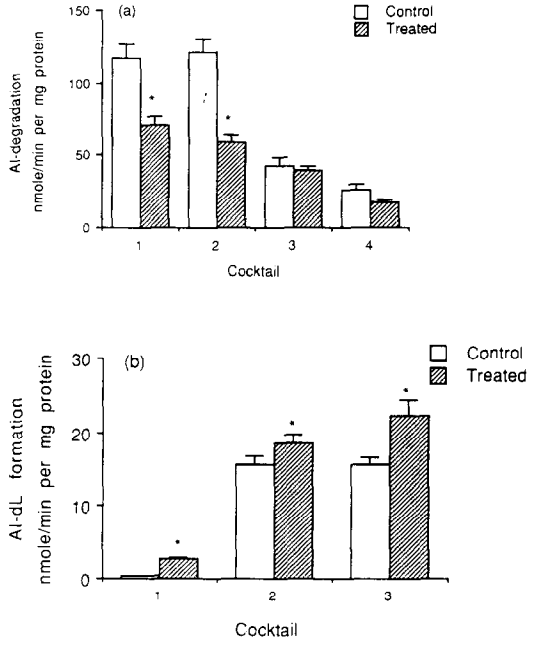

Fig. 2. (a) Rate of degradation of angiotensin I [A(1–10)] substrate and (b) rate of des-Leu angiotensin I formation [A(1–9)] in the kidney P2 centrifugal fraction obtained from control and enalapril-treated rats using various inhibitor mixture cocktails. Cocktail 1: captopril (20 μM) only. Cocktail 2: captopril (20 μM), PMSF (50 μM), iodoacetamide (1 mM), amastatin (20 μM). Cocktail 3: captopril (20 μM), PMSF (50 μM), iodoacetamide (1 mM), amastatin (20 μM) and EDTA (1 mM). Cocktail 4: captopril (20 μM) and thiorphan (10 μM). Data shown is the mean of 18 tissues obtained from three separate experiments ± SE. *$P < 0.05$.

thiorphan inhibited this activity. It was of interest that this enkephalinase activity observed using mixture 2 decreased significantly from 51 ± 5 to 25 ± 3 nmol/min/mg protein in the treated animals ($P < 0.01$) (Table 3). Incubations using mixture 1 which only contained captopril to inhibit ACE showed a further increase in the rate of degradation of A(1–10). Again these digestions with mixture 1 showed evidence of significant enkephalinase activity on A(1–10).

Miscellaneous enzyme activities

Plasma ACE activity was substantially reduced (36% of control) but not blocked completely in animals treated chronically with enalapril (Table 3).

Measurement of carboxypeptidase B (CP-B) activity in both kidney S2 and P2 fractions showed no significant difference ($P > 0.05$) in the control and treated groups (Table 3). Similarly, cathepsin B activity was unaffected by chronic treatment with enalapril (Table 3). However, there was a significant reduction (58% of control) in aminopeptidase (AP) activity in kidney P2, but not S2 fractions, following chronic treatment with enalapril. The activity in kidney P2 was almost totally inhibited (>95%) by amastatin (20 μM) whereas the activity in kidney S2 was only partially inhibited by amastatin (60%).

DISCUSSION

Peptides constitute a major class of chemical mediators in biological systems. The formation and inactivation of peptides are largely controlled by the action of peptidases. While some peptidases such as ACE may be ascribed specific functions it is not clear whether these peptidases can also affect the metabolism of other peptides [14]. For example, while ACE is known *in vitro* to hydrolyse many other peptides such as bradykinin, substance P and enkephalins it is not known what role ACE plays in the physiological regulation of these peptides. Similarly many other enzymes such as the aminopeptidases, carboxypeptidases and endopeptidases have no characterized specific function. However, changes in the amounts of these peptides by the addition of exogenous agents are likely to affect the metabolism of peptides.

Treatment of rats with enalapril chronically in our studies produced some profound and unexpected changes in enzyme activities. If A(1–10) was used as substrate significant increases in the carboxypeptidase activity producing A(1–9)) occurred in both S2 and P2 fractions following treatment with enalapril. This was associated with a

Table 3. Miscellaneous enzyme activities in control animals and in animals treated chronically with enalapril

Enzyme activity	Tissue fraction	No. of tissues assayed	Control group	Treated group	Statistical significance
ACE* nmol/min/mL	Plasma	N = 18	75 ± 3	27 ± 6	$P < 0.01$
Cathepsin B pmol/min/mg	KS2	N = 12	392 ± 97	384 ± 84	NS
	KP2	N = 12	225 ± 22	220 ± 26	NS
CP-B† nmol/min/mg	KS2	N = 6	0.69 ± 0.07	0.48 ± 0.08	NS
	KP2	N = 6	2.26 ± 0.16	2.64 ± 0.42	NS
AP‡ nmol/min/mg	KS2	N = 18	1.53 ± 0.18	1.61 ± 0.25	NS
	KP2	N = 18	122 ± 7	71 ± 9	$P < 0.01$
Endopeptidase 24.11 nmol/min/mg	KP2	N = 12	50.6 ± 5.0	24.6 ± 2.6	$P < 0.01$

* Angiotensin converting enzyme.
† Carboxypeptidase B activity.
‡ Aminopeptidase activity.
NS = $P > 0.05$.

decreased rate of degradation of substrate suggesting that the activity of other enzymes capable of degrading A(1–10) had been reduced. One of these may be aminopeptidase B (AP-B), as the activity responsible for the hydrolysis of the synthetic substrate Arg-β-NA [10], was significantly decreased by enalapril pretreatment. This is not a direct effect on the enzyme since *in vitro* enalaprilat (the active form of enalapril) has no direct effects on carboxypeptidases mediating formation of A(1–9) and A(1–8) and on AP-B activity (O. Drummer, unpublished observations).

In contrast, both cathepsin B activity and carboxypeptidase B activity were unchanged by enalapril pretreatment. Cathepsin B is a lysosomal cysteine-dependent enzyme capable of showing carboxypeptidase activity [12], while CP-B activity is often associated with kininase I activity, both of which are related to carboxypeptidase H and can be measured using the same synthetic substrate [15].

When other inhibitor mixtures were used that did not contain captopril or thiorphan in the kidney P2 digestions of A(1–10) an additional group of peaks were seen in the HPLC chromatograms. The mixtures used contained amastatin, iodoacetamide and PMSF to inhibit aminopeptidase, cysteine-dependent proteases and serine-dependent proteases, respectively. These additional peaks in the elution profile corresponded to the formation of A(1–7) and A(8–10) (Phe-His-Leu), which is consistent with the action of enkephalinase on A(1–10) [5, 16]. Moreover, this enkephalinase activity was halved by the chronic administration of enalapril to rats. Neither enalapril nor captopril are known to inhibit enkephalinase *in vitro* at the concentrations encountered in these studies [17].

The use of A(1–9) as substrate to monitor carboxypeptidase-mediated formation of AII also demonstrated an increase in this enzyme activity in kidney S2 by chronic administration of enalapril. Whilst kidney P2 does not normally show carboxypeptidase activity on this substrate [3], some activity was observed in all of the rats treated with enalapril. The rate of digestion of A(1–9) was also reduced in kidney although this effect was most noticeable in the P2 fraction.

These results demonstrate a profound change in the metabolism of A(1–10) and A(1–9) in kidney following chronic administration of enalapril to rats. It is already established that ACE activity is induced by chronic administration of ACE inhibitors in both humans and rats [7, 8]. This effect takes time to develop and appears to result from an increased production of ACE [7, 8]. Our studies now show that chronic administration of enalapril decreases the activities of amastatin-sensitive aminopeptidase and endopeptidase 24.11 in the kidney particulate fraction. Both these enzymes, like ACE, are metallopeptidases. In contrast, these same experiments demonstrate an increase in carboxypeptidase activities mediating formation of A(1–8) from A(1–10). These carboxypeptidase enzymes have been called K_1 and K_2 to reflect their ability to produce A(1–9) and A(1–8), respectively in rat kidney. Both enzymes behave like cysteine-dependent peptidases and are inhibited by para-chloromercuribenzoate (PCMB) but not by iodoacetamide, or any of the inhibitors used in these studies [3].

Our observations have potentially important implications not only for angiotensin metabolism but also for metabolism of other vasoactive peptides. For angiotensin a shift in the degree of metabolism by chronic exposure to enalapril and probably other converting enzyme inhibitors may be associated with an increased production of A(1–8) by carboxypeptidase-mediated processes. This may provide an explanation for the observed resistance of some patients to ACE inhibitors and to the inability to completely abolish plasma AII [(A(1–8)] concentrations [18, 19]. Doses of enalapril used in these studies were chosen to cause significant inhibition of plasma ACE in rats mimicking the degree of inhibition observed in humans following chronic therapy.

Our studies may also provide an explanation for the observed respiratory side-effects of ACE inhibitors in humans such as cough and bronchospasm including aggravated bronchospasm [20] which may be related to the effect enalapril has on the metabolism of other vasoactive peptides.

It will be important to assess the effects of chronic ACE adminstration on the metabolism of other vasoactive peptides. Since enzymes affected may not be present in plasma, measurement of plasma concentrations of vasoactive peptides may not be sufficient to show any real and significant changes within tissues.

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