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Increasing Serum Albumin in Patients with Hypoalbuminemia Does Not Inhibit Serum Angiotensin-Converting Enzyme Activity

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ABSTRACT

Background and Aim: Angiotensin - converting enzyme (ACE) plays a pivotal role in the production of angiotensin II and the inactivation of bradykinin. Recent studies have suggested that human serum albumin may possess ACE - inhibitory properties, serving as a potential endogenous ACE inhibitor that primarily affects serum ACE levels. Interestingly, the infusion of albumin in the postoperative phase of cardiac surgery has been associated with the development of hypotension.

Methods: This study aimed to assess serum ACE activity in 27 hypoalbuminemia patients admitted to the ICU before and after a protein-rich diet was administered to raise their serum albumin levels. Serum ACE activity was quantified using raas (HPLC), measuring the formation of hippuric acid, a product generated during the incubation of serum with Hip - His - Leu, a substrate, at 37°C for 30 minutes.

Results: In vitro experiments demonstrated that the addition of albumin to human sera led to a significant reduction in ACE activity compared to control groups ($P < 0.0001$). This reduction was consistent across all serum samples. Specifically, the maximum velocity (Vmax) of ACE activity significantly decreased from 14.90 U/L in the control group to 3.210 U/L in the albumin - added group ($P = 0.0262$). Notably, there was no significant change in the Michaelis constant (Km) between the control group (0.5263 mM) and the albumin group (0.2742 mM) ($P = 0.6763$), indicating a non-competitive inhibitory effect of albumin on ACE activity. Interestingly, in this study, elevating serum albumin levels in hypoalbuminemia patients following a protein - rich diet resulted in both ACE inhibition and a slight increase in activity ($P = 0.0201$). This increase correlated mildly with serum albumin levels across all samples.

Conclusion: In conclusion, contrary to in vitro findings, raising serum albumin levels in hypoalbuminemia patients did not further inhibit serum ACE activity.

INTRODUCTION

The renin - angiotensin - aldosterone system (RAAS) is a vital hormonal system that plays a pivotal role in regulating blood pressure and maintaining fluid and electrolyte balance.

The intricate cascade of events begins when juxtaglomerular kidney cells convert pro - renin to rein in response to reduced kidney blood flow.

Renin, in turn, acts on angiotensinogen, synthesized by the liver, converting it into angiotensin I. This precursor is then transformed into the biologically active angiotensin II by the action of angiotensin - converting enzyme (ACE), predominantly found in the vascular endothelial cells of the lung. Angiotensin II exerts its blood pressure-raising effects through two primary mechanisms: as a potent peptide vasoconstrictor and by stimulating the adrenal cortex to release aldosterone (1).

Pharmacological inhibition of the RAAS, using agents such as captopril, enalapril, losartan, and valsartan, has proven effective in managing hypertension, renal failure, heart failure, and complications associated with diabetes mellitus.

Notably, Ryan et al. were the first to propose the existence of endogenous ACE inhibitors (ACEIs) with a molecular mass

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of less than 10 kDa (2). Subsequent experiments have identified additional molecules with ACE inhibitory activity, including Des - Leu - Angiotensin I, N-terminal fragments of P-neuropeptides, and Acein-2 (Leu - Ile - Tyr tripeptide) (3-5). Further investigations by Lieberman and Ikemoto demonstrated the presence of endogenous ACEIs with molecular masses greater than 50 kDa and 10 kDa, respectively (6, 7). Interestingly, recent research has revealed that the 50-100 kDa ACEI corresponds to human serum albumin (HSA) (8). In their study, Fagyas et al. observed that the IC_{50} values for HSA (5.7 mg / mL) in ACE inhibition were significantly lower than the physiological HSA concentrations (35-52 mg / mL), indicating complete in vivo suppression of ACE activity by HSA.

These findings underscore the importance of tissue-bound ACE as the primary site of ACE activity in the human body, which can be inhibited by pharmacological ACE inhibitor drugs. The research has demonstrated that HSA exerts inhibitory effects on serum ACE, thereby partially inhibiting tissue - bound ACE as well (8). Interestingly, HSA exhibits a higher level of selectivity towards the C-terminal active site of serum ACE, leading to distinct impacts on the hydrolytic activity of ACE towards angiotensin I and bradykinin (8).

Interestingly, it has been suggested that a 20-fold diluted serum sample exhibits higher ACE activity compared to a 4-fold diluted sample due to the lower concentration of serum albumin in the more diluted sample (9). The addition of angiotensin I and HSA to the saphenous vein has demonstrated a significant reduction in vascular contractile function, indicating a decrease in ACE activity in the presence of HSA (8). HSA plays a critical role in transporting various advantageous molecules through the bloodstream and tissues (10).

In the present study, we aimed to evaluate ACE activity in serum samples obtained from patients with hypoalbuminemia, both before and after albumin correction through the prescription of a protein - rich diet. Rather than using direct albumin infusion, which is associated with postoperative hypotension, we employed a novel approach of assessing ACE activity in undiluted serum samples from hypoalbuminemic patients who received a protein-rich diet instead of albumin infusion. This approach allowed us to investigate if a protein-rich diet could substantially reduce ACE activity in vivo, presenting a unique aspect of our study distinct from the conventional confirmation of albumin as the primary endogenous ACE inhibitor.

MATERIAL and METHODS

Materials

Albumin 10% vial was obtained from Octapharma (Lachen, Switzerland). Hippuryl histidyl laceucine (HHL) and hippuric acid (HA) were purchased from Sigma (St. Louis, Mo. USA). Hydrochloric acid, boric acid, potassium chloride, magnesium chloride, sodium chloride, phosphoric acid, and sodium hydroxide were analytical grades from Merck. Water and methanol were HPLC grade.

Sample collection

The research was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (Ethics Code: IR.SBMU.RETECH.REC.1398.336). All participants signed written informed consent. Blood samples were obtained from 27 ICU admitted patients at Masih Daneshvari Hospital, Tehran, Iran, with hypoalbuminemia (serum albumin < 3.5 g / dL) before and after increased serum albumin by protein - rich diet (primarily casein and leucine). All patients were over the age of 18, nonsmokers, and did not use ACEIs (captopril, enalapril) or angiotensin receptor blockers (losartan, valsartan). We also collected 26 blood samples from hypoalbuminemia and 26 normoalbuminemic patients, with the same inclusion criteria, to study the effects of in vitro addition of HSA to serum samples and its effect on ACE activity. Collected serums were stored at -20°C until the ACE activity was assayed.

ACE activity measurement

Serum ACE activity was measured using the following method (11): Briefly, the HHL substrate was dissolved in a 0.25 M sodium borate buffer (pH 8.3) containing 1.7 M KCl. The assay was performed by incubating a serum sample of 10 µL with a substrate (HHL) of 40 µL at 37°C for 30 minutes, then 150 µL of 1 M Hydrochloric acid was added to stop the reaction. A Shimadzu HPLC system was used to determine the hippuric acid (HA) as the product of the ACE reaction at the wavelength of 228 nm. The HPLC analysis was performed on a C18 column (250 * 4.6 mm i.d.), particle size 5 µm. The column was eluted at a flow rate of 1 mL / min with methanol / phosphate buffer (50:50; adjusted to pH 3 with H3PO4) solvent system. The amount of enzyme catalyzing the release of 1 µ/mol of hippuric acid from substrate per minute at 37°C was considered as one unit of ACE activity per liter (U/L). The ACE - specific activity was determined based on the micromole hippuric acid (HA) produced by the ACE action on the substrate per microgram protein content of the serum sample for one minute at 37° C.

To observe in vitro effect of albumin on ACE activity, 100 microliters of HSA (normal saline (NS) for control) were added to 100 µL of each serum sample and incubated at room temperature for 10 min. The ACE activity was assayed in both HSA and NS. K_m and V_{max} were determined by the activity measurement at the 0.1, 0.5, 1, 3, 6, and 8 mM of HHL substrate concentrations.

Statistical analysis

Statistical comparisons were performed by paired and unpaired t-tests analyzed by Graphpad Prism software. Differences were found to be significant when P was < 0.05.

RESULTS

In vitro addition of albumin to the human sera showed significant inhibition of ACE activity in most of the samples (Figure 1).

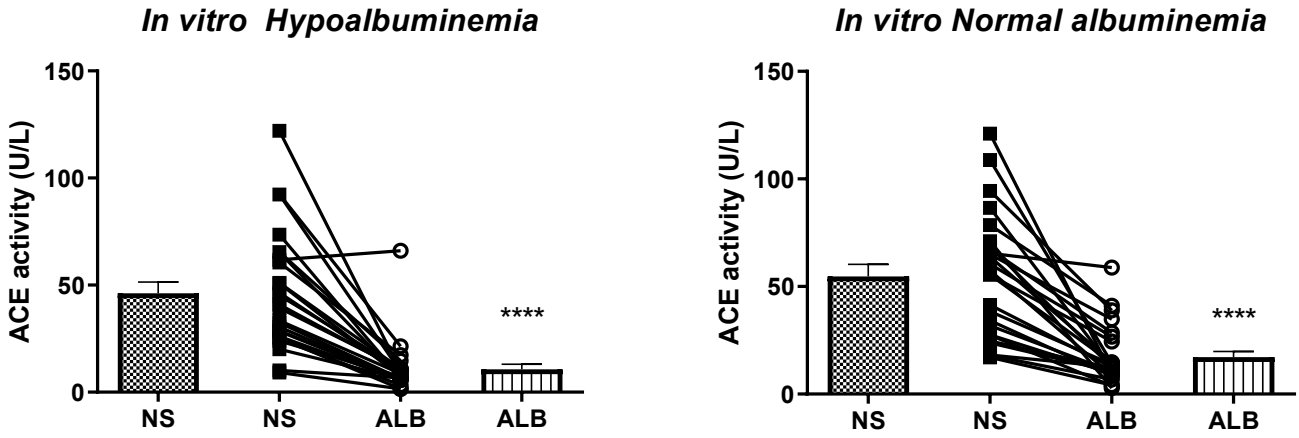

FIGURE 1. Significant decrease in ACE activity after *in vitro* albumin addition in patients with hypoalbuminemia and normal serum albumin. 100 µL of HSA or NS (normal saline) was added to 100 µL of serum and ACE activity was measured by incubation of prepared samples with HHL substrate according to the method (P-value < 0.0001).

In the HSA - added samples, ACE activity was substantially reduced relative to their controls (P < 0.0001) (Figure 1), and this was shown with each serum examined. V_{max} was significantly reduced from 14.90 U/L in the control group to 3.210 U/L in the albumin-added group (P = 0.0262). K_m was not significantly changed in the control group (0.5263 mM) compared to the albumin group (0.2742 mM) (P = 0.6763), suggesting a non-competitive inhibitory effect of albumin on ACE activity (Figure 2).

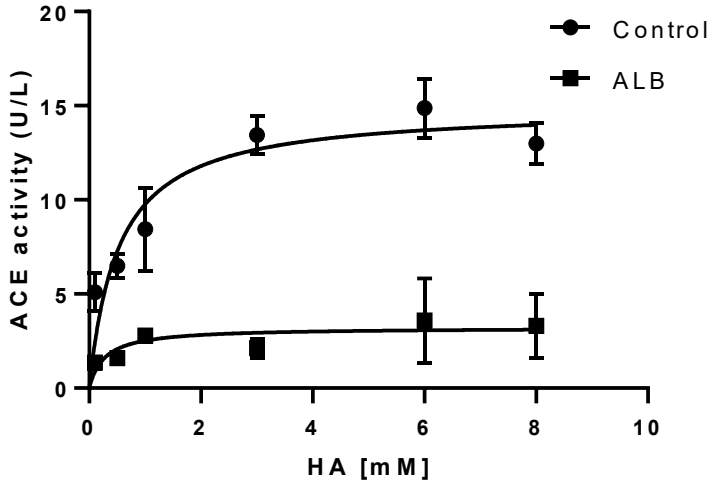

FIGURE 2. Michaelis - Menten curve in the control group and albumin treated (ALB) group showed a significant decrease in V_{max} after in vitro albumin addition and an almost unchanged K_m; which suggests the noncompetitive inhibitory effect of albumin on ACE activity.

The in vivo study showed that despite a significant increase in patients' serum albumin content followed by a high - protein diet prescription in hypoalbuminemic patients (Figure 3), ACE activity increased significantly following an increase in the HSA group ($p = 0.0201$) but the specific activity increased non-significantly ($p = 0.1951$) (Figure 4).

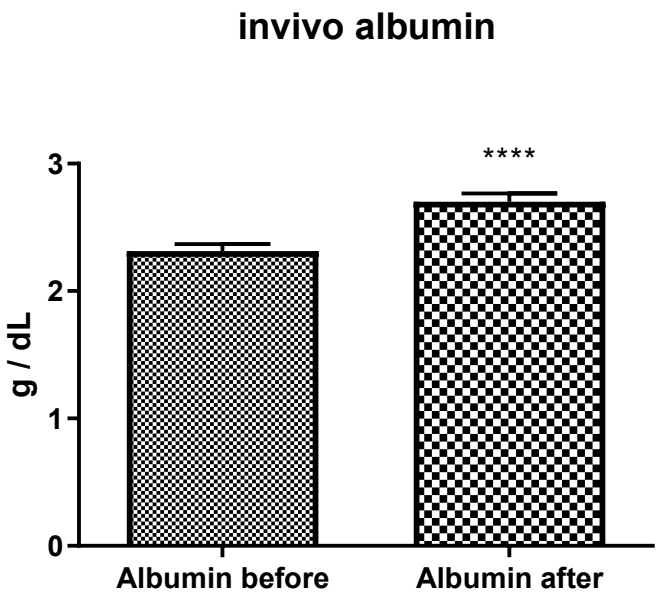

FIGURE 3. Albumin changed significantly after *in vivo* high - protein diet regimen in hypoalbuminic patients.

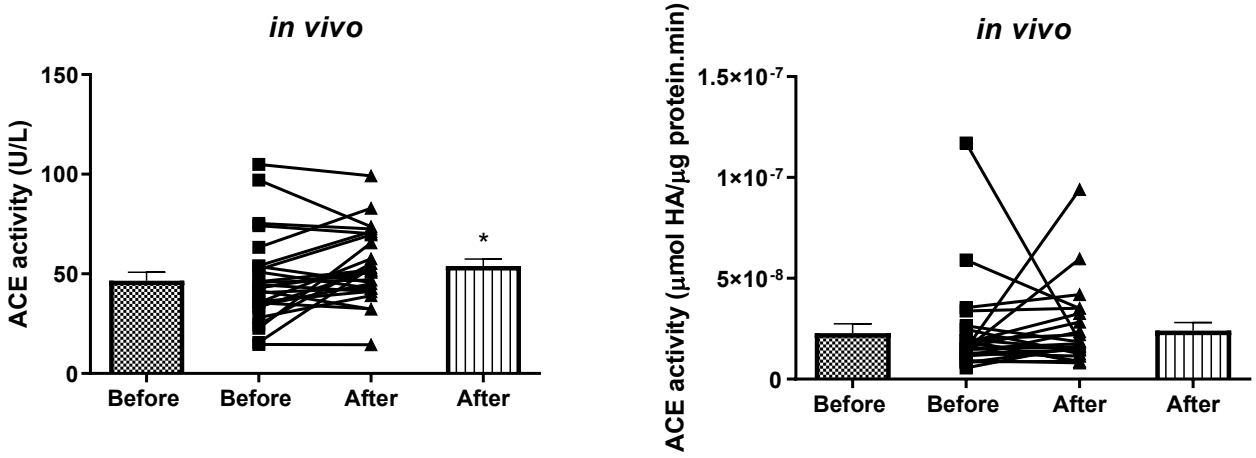

FIGURE 4. ACE activity increased significantly after an in vivo increase in HSA due to a high protein diet ($p = 0.01201$), and specific activity increased non-significantly ($p = 0.1951$).

Serum ACE activity non-significantly was higher in normal albumin patients than hypoalbuminemic patients after receiving a protein-rich diet, which significantly raised their serum albumin (serum albumin ≥ 3.5 g / dL) and there was also no decrease in serum ACE specific activity in hypoalbuminemia patients (Figure 5), which indicates that the *in vivo* results were controversy with the in vitro study. The Pearson correlation between ACE activity and serum albumin concentration showed a weak positive correlation ($r^2 = 0.185$; $p = 0.054$), implying that serum albumin increases ACE activity.

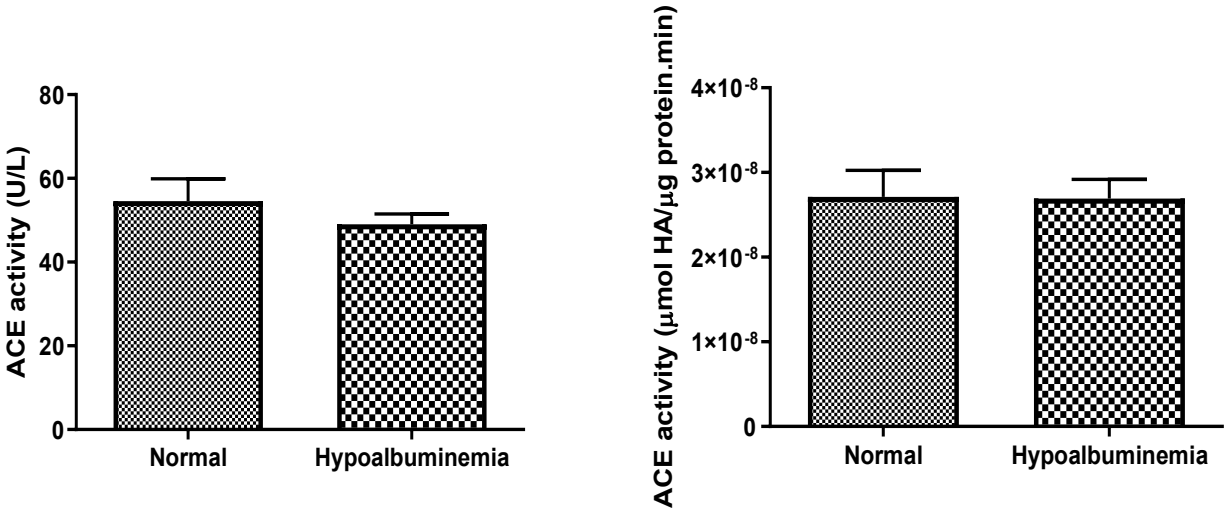

FIGURE 5. ACE - specific activity in normoalbuminic patients (≥ 3.5 g / dl) compared to hypoalbuminic patients indicates that pathological low serum albumin did not affect ACE activity.

DISCUSSION

In this study, we measured ACE activity in hypoalbuminemic patients before and after increasing albumin levels with high protein diets. Our findings showed that, despite in vitro inhibition of ACE activity by human albumin, the ACE activity not only decreased but also increased as serum albumin levels increased, and that ACE activity in normal albumin patients was slightly higher than in hypoalbuminemic patients.

ACE is one of the best - studied enzymes involved in the conversion of angiotensin I to II, and its inhibition is known to protect against hypertension, myocardial fibrosis, remodeling, and renal dysfunction.

It has been discovered that ACE serum activity is not completely associated with the concentration of ACE. A 4.1-fold increase in ACE concentration resulted in a 2.1-fold increase in the patient's serum ACE activity, which proves ACE activity is a deceptive factor in the estimation of ACE concentration (9). In several studies, genetic determinants of ACE expression have shown no correlation with ACE activity. Various polymorphisms have not been found to alter the occurrence of cardiovascular diseases in studies (12). It has also been reported that a point mutation in the ACE gene stalk region resulted in a 5-fold increased circulating ACE concentration (13). At least eight families were affected by this mutation, but ACE-related clinical disorders or hypertension did not occur (13). Another mutation followed by a 13-fold rise in serum ACE concentration also occurred without an increase in cardiovascular disease incidence (14). All of these observations suggest that substantial differences in ACE concentrations are well tolerated in vivo. This tolerance can be explained by the presence of endogenous ACE inhibitors such as serum albumin as one of the main factors (8, 15).

It has been shown that dietary factors such as bacterial digested milk casein have ACE inhibitory properties (8, 16). There are also extensive experiments to identify ACE inhibitors among medicinal plants and natural products (17-22). Numerous experiments have already demonstrated the existence of endogenous ACE inhibitors. In 1979, Ryan et al. identified molecules of < 10 KDa weight as endogenous ACE inhibitors for the first time (2). Acein 2, isolated from human serum tryptic hydrolysate, is proposed as another low molecular weight ACEI (5). Casein tryptic hydrolysate was first described as ACEI by Maruyama et al (23). Des - Leu 10- angiotensin I and N- terminal fragments of substance P are among other known endogenous ACEIs (3, 4). In addition to serum ACEI, there are tissue ACE inhibitors that were initially identified in rat hearts (7). Klauser et al. discovered the non - competitive ACE inhibitory role of albumin for the first time in 1979 (24).

This theory is also supported by a remarkable decline in ACE activity after in vitro albumin addition to 52 serum samples in our study, and a substantial decrease in V_{max} in the albumin group was also observed in vitro, although k_m remained unchanged (Figure 2), suggesting a non - competitive ACE inhibition with human serum albumin. In noncompetitive inhibition, the inhibitor binds to a site on the enzyme that is distinct from the active site. This binding alters the enzyme's shape and reduces its activity, irrespective of the concentration of the substrate.

Noncompetitive inhibition of enzymes does not have a linear relationship with the inhibitor. Typically, noncompetitive inhibitors follow an exponential or sigmoidal relationship with the degree of inhibition. At low inhibitor concentrations, the enzyme activity may be slightly reduced, but as the inhibitor concentration increases, the inhibition becomes more pronounced. However, there is no direct proportionality or linear relationship between the inhibitor concentration and the degree of inhibition in noncompetitive inhibition.

Albumin infusion in the postoperative phase of cardiac surgery has caused hypotension as described by Howard et al. (25). The new protocols, on the other hand, forbid injecting albumin into critically ill patients, and albumin depletion should be compensated by consuming protein sources. Lower concentrations of albumin are found in renal failure, hepatic failure, and malnutrition. The indications for the use of albumin administration in critically ill patients are a source of debate; research to resolve these issues are ongoing (26). In patients with hypoalbuminemia, a decrease in serum albumin levels may lead to an increase in ACE activity, potentially contributing to cardiovascular complications in these individuals. However, it is important to note that certain investigations have not reported a direct association between decreased serum albumin levels and the development of cardiovascular diseases (9). The novelty of this study lies in our investigation of this effect in vivo and within a clinical setting. Due to a treatment protocol issue in hypoalbuminemia correction, which did not allow direct albumin infusion, we administered a protein-rich diet to 24 hypoalbuminemic patients. The aim was to observe whether an increase in serum albumin would result in a further decrease in ACE activity. The IC50 for HSA on serum and tissue - bound ACE was 0.57 g/dl in Fagyas et al's study, and physiological serum albumin concentration (3.5 g/dl) is much higher (almost 6-fold) compared to albumin IC50 for ACE inhibition (8). They concluded that further albumin increases will no longer suppress ACE because it is already completely inhibited. Even simultaneous addition of captopril and albumin to angiotensin I in a saphenous vein does not change ACE activity remarkably in comparison to albumin and angiotensin I alone, which indicates that serum ACE activity is almost completely suppressed by albumin and ACEI drugs may mainly affect tissue-bound ACE (8).

In our study, albumin was significantly increased but did not repair hypoalbuminemia (serum albumin > 3.5 g / dl). This may be the result of a low increase in serum albumin, but it was also previously found that HAS levels of more than 0.3 g / dL would dramatically suppress ACE activity, but contrary to our expectations, ACE activity increased slightly rather than decreased. Our study was unique in that we compared ACE activity in serum samples from hypoalbuminemic patients before and after a protein - rich diet rather than the albumin infusion.

Our ACE activity assay method was based on the HHL substrate and the HPLC method. A quick, sensitive, and reliable technique is desirable to facilitate the identification of ACE in hypoalbuminemic patients. Numerous methods, such as spectrophotometric (27), fluorometric (28, 29), and radiochemical (30), have been reported for the measurement of ACE activity. Various substrates are also ideal for measuring ACE activity. Among them, synthetic peptide hippuryl - L - histidyl - L - leucine (HHL) is a popular ACE activity assay substrate (27), which has been developed to be detected by high-performance liquid chromatography (HPLC) to reduce the wavelength interaction with sample components and to provide reliable results (31). One of the key disadvantages of Fagyas and colleagues' work was that due to serum light absorption mainly at 340 nm, where exogenous substrate conversion is observed, ACE activity in undiluted human sera could not be directly determined and ACE activity was measured at a 4-fold dilution (8). According to Fagyas et al., after extrapolating and correcting the results of ACE activity in the 4-fold diluted serums, the normal physiological concentration of HSA in undiluted human serum samples should suppress ACE activity to 8.56 ± 2.2 U/L (9). However, in our study, the actual ACE activity in undiluted serum with normal albumin concentration was 54.55 ± 5.37 U/L. The benefit of our approach was the isolation of the pure product (HA) using HPLC, so that there was no interaction of contamination of the wavelength and therefore no dilution of the samples was required in our research.

Contrary to the assumption, our in vivo results indicate a significant positive correlation between HSA and ACE activity, as well as a slight increase in ACE activity associated with an increase in serum albumin. The increase in ACE activity after protein rich diet may be explained by the fact that the increase in total serum protein coincides with an increase in other serum proteins like ACE. To support this theory, further studies are suggested to be done to investigate ACE activity changes in serum samples of patients after in vivo albumin infusion. However, according to some studies, HSA is unlikely to be the only endogenous inhibitor of ACE, making it difficult to estimate its contribution (9). Given the complications associated with the acute injection of albumin, our study opted for the correction of patients' albumin levels through the prescription of a protein-rich diet. During this period, it is plausible that compensatory mechanisms within the body may have balanced the ACE activity levels. In contrast, our controlled in vitro experiments specifically examined the effects of adding pure albumin. Notably, the in vitro tests demonstrated an approximate 50% increase in

albumin concentration, while the patients experienced a modest 10-15% rise in albumin levels (Figure 3). Moreover, due to the non - linear relationship between inhibitor concentration and non - competitive inhibition, the observed level of enzyme inhibition in the in vitro tests may not directly translate to in vivo conditions. Therefore, despite observing the inhibition of enzymes in our in vitro experiments, the applicability of these findings to clinical settings is limited. This limitation can be attributed to compensatory mechanisms, including increased production of ACE following non - acute conditions, indirect elevation in albumin levels, and the presence of other enzymes like Chymase and Cathepsin G, which may contribute to the controversy. Chymase, predominantly found in mast cells, is a serine protease capable of directly converting Ang I to Ang II, bypassing the requirement for ACE. The production of Ang II via chymase is particularly notable in specific tissues such as the heart and blood vessels. Cathepsin G, released by neutrophils and other immune cells, is also a serine protease that can directly convert Ang I to Ang II, similar to chymase (32).

CONCLUSION

The angiotensin - converting enzyme (ACE) activity observed in 27 patients with hypoalbuminemia following a high - protein diet did not correspond to the in vitro ACE inhibitory effect of albumin.

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CONFLICT OF INTEREST

The authors have no relevant financial or non-financial interests to disclose.

ETHICS APPROVAL

The research was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (Ethics Code: IR.SBMU.RETECH.REC.1398.336), and was conducted according to the recommendations of the Declaration of Helsinki. Informed consent was obtained from all participants in the study.

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