

## Protective Effects of Quinaprilat and Trandolaprilat, Active Metabolites of Quinapril and Trandolapril, on Hemolysis Induced by Lysophosphatidylcholine in Human Erythrocytes

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We examined the effects of the angiotensin converting enzyme (ACE) inhibitors captopril, enalaprilat, quinapril, and trandolapril, and their active metabolites quinaprilat and trandolaprilat, on hemolysis induced by lysophosphatidylcholine (LPC) in human erythrocytes. LPC induced hemolysis at the concentrations above the critical micelle concentration (4  $\mu$ M). Propranolol, used as a reference drug, attenuated the 50% hemolysis induced by 6  $\mu$ M LPC at concentrations ranging from 100 nM to 100  $\mu$ M. Similarly, quinaprilat (10  $\mu$ M) and trandolaprilat (10, 100  $\mu$ M) significantly attenuated the LPC-induced hemolysis, but other ACE inhibitors did not. Since propranolol possesses a membrane stabilizing action correlated with high lipophilicity, it appears that the high lipophilicity of quinaprilat or trandolaprilat is responsible for the protection from the damage induced by LPC. However, quinapril and trandolapril were not effective, although both drugs have higher lipophilicity than quinaprilat and trandolaprilat. Hence, it is suggested that the high lipophilicity alone may not contribute to the protective effects of ACE inhibitors against LPC-induced hemolysis. None of ACE inhibitors attenuated the hypotonic hemolysis (60 mM NaCl), although propranolol did. Furthermore, neither propranolol (100  $\mu$ M) nor quinaprilat (50  $\mu$ M) and trandolaprilat (50  $\mu$ M) affected LPC micelle formation, suggesting that these drugs do not directly bind to LPC. We therefore believe that the protective effects of quinaprilat and trandolaprilat on the LPC-induced hemolysis may be related physicochemically to their highly lipophilic and ACE inhibitory structures, which probably maintain erythrocyte membrane integrity by a mechanism other than ACE inhibition, prevention of LPC micelle formation or protection against osmotic imbalance.

**Key words** angiotensin converting enzyme inhibitor; lysophosphatidylcholine; hemolysis; lipophilicity

There is ample evidence indicating that angiotensin converting enzyme (ACE) inhibitors attenuate myocardial cell damage induced by acute ischemia of the heart. The cardioprotective effect of ACE inhibitors may be due to their indirect effect through hypotension and/or a decrease in contractile force. Data concerning the direct effect of ACE inhibitors on cardiac cells during acute ischemia, however, are limited.

The direct effect of ACE inhibitors on the cell can be examined using cardiomyocytes. There is a problem, however, in that cardiomyocytes have both ACE activity and angiotensin II receptors.<sup>1,2)</sup> Therefore, it is difficult to determine whether the direct effect of ACE inhibitors is due to their specific effect (ACE inhibition) or a nonspecific effect when cardiomyocytes are used. Human erythrocytes are a convenient cell type in this regard, because they lack both ACE activity and angiotensin II receptors.<sup>3)</sup> Thus, cell damage can be easily detected by hemolysis. A problem associated with using erythrocytes is the means with which to inflict ischemic or hypoxic damage because erythrocytes are resistant to hypoxia. It is therefore necessary to find a way to inflict ischemia-like damage on the erythrocyte.

Lysophosphatidylcholine (LPC) inflicts ischemia-like damage on the cell. It has been shown that LPC accumulates in the myocardium to a large extent during ischemia, and that it inflicts damage on the cell.<sup>4)</sup> Due to these findings, LPC has been assumed to be one of the substances responsible for ischemia-induced cell damage. Therefore, LPC can be used to inflict ischemia-like damage on the erythrocyte.

Several reports have discussed the effect of ACE inhibitors on the LPC-induced cell damage. Ma *et al.*<sup>5)</sup> demonstrated

that quinaprilat, an active metabolite of quinapril, attenuates damage to isolated rat cardiomyocytes induced by LPC, indicating that quinaprilat has a direct cytoprotective action against LPC-induced damage, which may contribute to the cardioprotection against ischemia-induced cell damage. Nevertheless, it is uncertain whether the cytoprotective effect of quinaprilat on cardiomyocytes is due to its specific action or not, because rat cardiomyocytes have both ACE activity and angiotensin II receptors.<sup>1,2)</sup>

The present study was designed to answer the following two questions. 1) Do ACE inhibitors other than quinaprilat also have a direct cytoprotective action against LPC-induced cell damage? 2) Is the inhibition of either ACE activity or angiotensin II receptors responsible for the cytoprotective action of ACE inhibitors against LPC-induced cell damage? Human erythrocytes were used as a model of the cell. Human erythrocytes lack  $\beta$ -adrenoreceptors,<sup>6)</sup> ACE-activity, and angiotensin II receptors. Propranolol was used as a reference drug based on established data<sup>7)</sup>; propranolol is known to attenuate LPC-induced hemolysis by a nonspecific mechanism that is different from  $\beta$ -adrenoreceptor antagonism.

### MATERIALS AND METHODS

**Materials** Quinapril hydrochloride and quinaprilat were supplied by WellFide Corporation (Osaka, Japan) that merged with Mitsubishi Pharma (Tokyo, Japan) in October 2001, and trandolapril and trandolaprilat were obtained from Roussel Uclaf (Romainville Cedex, France). Captopril, propranolol (*dl*-propranolol HCl), and LPC (palmitoyl C16:0) were pur-

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chased from Sigma Chemical Co. (St Louis, MO, U.S.A.), and enalaprilat was purchased from Merck & Co., Inc. (Rahway, NJ, U.S.A.). 8-Anilino-1-naphthalenesulphonic acid was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Other chemicals were purchased from Wako Fine Chemicals (Osaka, Japan).

LPC was dissolved in distilled water to achieve the desired concentrations and then allowed to stand at 4 °C for at least 12 h. Quinaprilat was dissolved in 0.01 N NaOH and then further diluted with distilled water so that the final concentration of NaOH would be 2–200  $\mu$ M. Trandolapril and trandolaprilat were dissolved in ethanol and then diluted with distilled water to a final ethanol concentration of 0.0002–0.02%. Other drugs were dissolved in distilled water. All drug solutions tested were prepared immediately before use. NaOH and ethanol did not produce hemolysis at the concentrations used in the present study (data not shown).

**Blood Sampling** Human blood was supplied by the Blood Center of the Asahikawa Red Cross (Asahikawa, Japan); the blood was drawn from healthy donors within several days before use. Each blood sample was washed four times with isotonic buffer (10 mM phosphate buffer containing 154 mM NaCl, pH 7.4) to remove the buffy coat and plasma to yield a 40% (V/V) erythrocyte suspension.

**Hemolysis Induced by LPC and Hypotonic Solution** Preliminary experiments were performed to determine the extent of hemolysis in response to different concentrations of LPC or NaCl. The 40% erythrocyte suspension (1 ml) was diluted with isotonic buffer solution (5 ml) and incubated for 30 min at 37 °C. The diluted erythrocyte suspension (200  $\mu$ l) was mixed with isotonic or hypotonic buffer (5 ml) containing different concentrations of LPC (2–20  $\mu$ M) or NaCl (45–75 mM). This resulting mixture (sample) contained about 0.26% erythrocytes. Each sample was incubated for 30 min at 37 °C and centrifuged at 1300 $\times$ *g* for 5 min. After centrifugation, the extent of hemolysis was determined from the absorbance of hemoglobin released into the supernatant using a Hitachi model 200-10 spectrophotometer at a wavelength of 543 nm. The maximum hemolysis obtained with distilled water was taken as 100%, and the minimum (spontaneous) hemolysis obtained in the isotonic buffer was taken as 0%. The preliminary experiments revealed that experimental conditions producing around 50% hemolysis, which were obtained by 6  $\mu$ M LPC or 60 mM NaCl (Fig. 1), were suitable for evaluation of the effects of drugs on erythrocytes. In the main study, we therefore used 6  $\mu$ M LPC and 60 mM NaCl for LPC- and NaCl-induced hemolysis, respectively.

**Effects of ACE Inhibitors and Propranolol on LPC-Induced Hemolysis** The effects of drugs (six ACE inhibitors and propranolol) on LPC-induced hemolysis were examined. First, the 40% erythrocyte suspension (200  $\mu$ l) was mixed with the isotonic buffer (1 ml) containing each of the drugs at various concentrations, and incubated for 30 min at 37 °C. Samples containing one of the drugs and LPC were then prepared to measure the extent of hemolysis. The concentrations of the drugs in the isotonic buffer solutions were 10, 100 nM, 1, 10, 100  $\mu$ M or 1 mM, and that of LPC was 6  $\mu$ M. Each sample was further incubated for 30 min at 37 °C and then centrifuged at 1300 $\times$ *g* for 5 min. The extent of hemolysis was measured as described above. Neither ACE inhibitors nor propranolol interfered with the measurement of hemolysis.

**Effects of ACE Inhibitors and Propranolol on Hypotonic Hemolysis** The effects of ACE inhibitors and propranolol on hypotonic hemolysis were examined. The measurement procedures for the extent of hemolysis were the same as those described in LPC-induced hemolysis, except that hypotonic buffer (10 mM phosphate buffer containing 60 mM NaCl, pH 7.4) was used for hemolysis.

**Determination of Micelle Formation** We examined whether the ACE inhibitors used in the present study or propranolol affected the formation of LPC micelles. The extent of micelle formation was measured by a fluorescence method using 8-anilino-1-naphthalenesulphonic acid.<sup>8)</sup> Isotonic buffer containing 8-anilino-1-naphthalenesulphonic acid (10  $\mu$ M) and LPC (2–20  $\mu$ M) was incubated in the presence of an ACE inhibitor (50  $\mu$ M) or propranolol (100  $\mu$ M) for 30 min at 37 °C. The intensity of 8-anilino-1-naphthalenesulphonic acid fluorescence was measured using excitation and emission wavelengths of 375 and 480 nm, respectively, with a Hitachi model 240-R fluorescence spectrophotometer. The intensity of 8-anilino-1-naphthalenesulphonic acid obtained in isotonic buffer containing LPC (20  $\mu$ M) was taken as 100%, while that in LPC-free isotonic buffer was taken as 0%. Neither the ACE inhibitors nor propranolol interfered with the measurement of fluorescence intensity.

**Statistical Analysis** All values are expressed as the means $\pm$ S.E.M. Statistical analysis was performed using ANOVA followed by the *post hoc* Dunnett's *t*-test for comparison of the control group with each of the drug-treated groups. A probability value of  $p < 0.05$  was considered statistically significant.

## RESULTS

**Concentration–Response Curves of LPC and NaCl for Hemolysis** Figure 1 shows the concentration–response curves for LPC and NaCl solutions; LPC produced hemolysis at concentrations above 3  $\mu$ M (Fig. 1A) and NaCl produced hemolysis at concentrations below 75 mM (Fig. 1B). Figure 1 also shows that 6  $\mu$ M LPC and 60 mM NaCl produced  $53 \pm 2.7$  and  $58 \pm 2.6$ % of the total hemolysis, respectively. We used 6  $\mu$ M LPC and 60 mM NaCl for the following experiments examining the effects of drugs on LPC- and NaCl-induced hemolysis.

**Effects of ACE Inhibitors and Propranolol on LPC-Induced Hemolysis** Propranolol significantly attenuated the LPC-induced hemolysis at concentrations ranging from 100 nM to 100  $\mu$ M ( $p < 0.01$ , Fig. 2). However, propranolol accelerated the LPC-induced hemolysis at the highest concentration (1 mM). Captopril, enalaprilat, quinapril, and trandolapril had no effect on LPC-induced hemolysis at concentrations between 100  $\mu$ M and 1 mM. In contrast to these ACE inhibitors, quinaprilat (10  $\mu$ M) and trandolaprilat (10, 100  $\mu$ M) significantly attenuated LPC-induced hemolysis ( $p < 0.01$  or  $p < 0.05$ ). The highest concentration of quinaprilat or trandolaprilat (1 mM) significantly ( $p < 0.01$ ) accelerated the LPC-induced hemolysis. The biphasic effect of quinaprilat or trandolaprilat on LPC-induced hemolysis was similar to that of propranolol.

**Effects of ACE Inhibitors and Propranolol on Hypotonic Hemolysis** Propranolol at concentrations between 10 nM and 10  $\mu$ M attenuated the hypotonic hemolysis induced

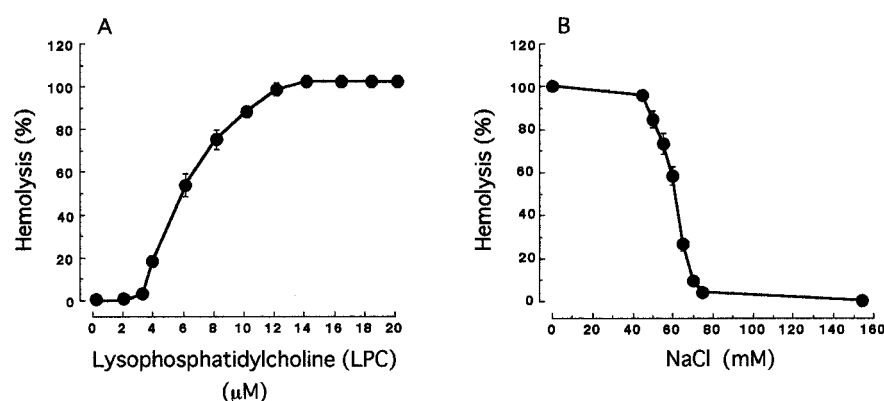


Fig. 1. Extent of Hemolysis in Response to Different Concentrations of LPC (A) or NaCl (B)

(A) Erythrocytes and LPC were incubated in isotonic buffer (10 mM phosphate buffer containing 145 mM NaCl; pH 7.4) and (B) erythrocytes were incubated in hypotonic buffer (10 mM phosphate buffer containing 45 to 75 mM NaCl; pH 7.4) for 30 min at 37 °C. Each point is the mean  $\pm$  S.E.M. of five experiments.

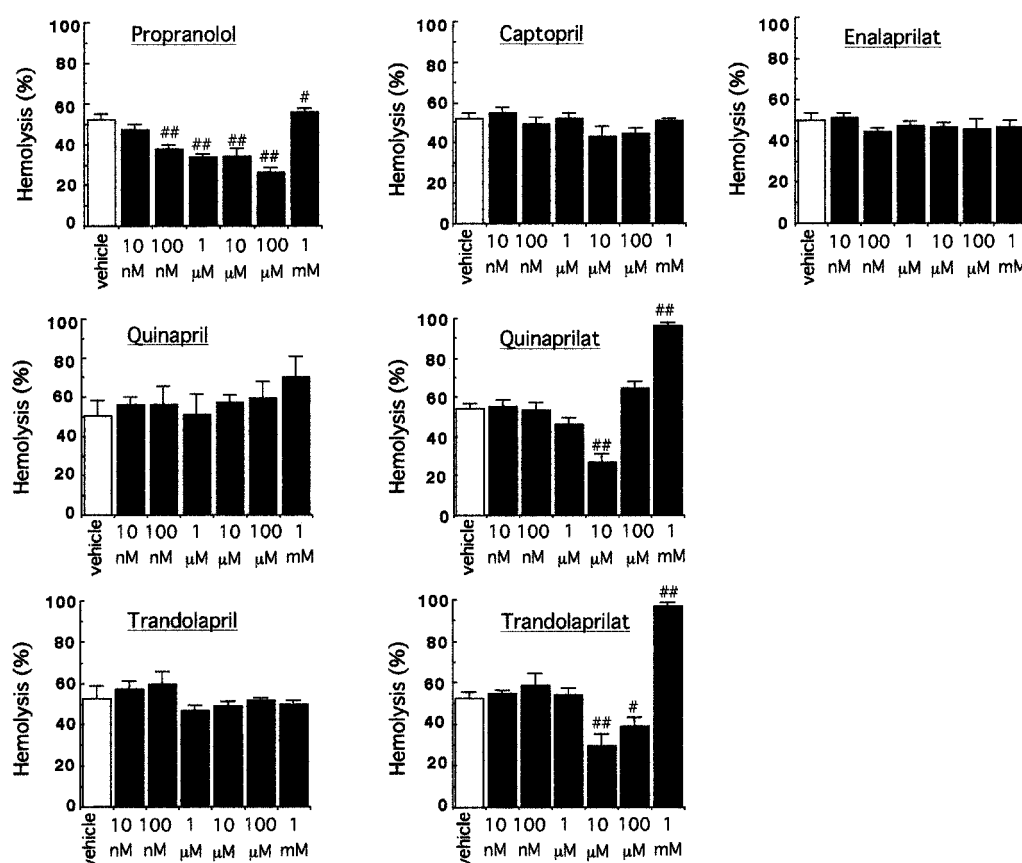


Fig. 2. Effects of Propranolol and ACE-Inhibitors on Hemolysis Induced by 6  $\mu\text{M}$  LPC

Each value is the mean  $\pm$  S.E.M. ( $n=4$  or 5). # $p<0.05$ , ## $p<0.01$ , these values are significantly different compared with the control (vehicle) group.

by 60 mM NaCl (Fig. 3). At concentrations above 100  $\mu\text{M}$ , however, propranolol did not attenuate the hypotonic hemolysis, while 1 mM propranolol significantly accelerated the hypotonic hemolysis ( $p<0.05$ ). ACE inhibitors did not attenuate the hypotonic hemolysis. Quinapril and trandolapril had no effect at lower concentrations, but did accelerate the hypotonic hemolysis at higher concentrations ( $p<0.01$ ).

**LPC Micelle Formation** The fluorescence intensity began to increase markedly when the concentration of LPC increased to 4  $\mu\text{M}$  or higher, suggesting that the critical micelle concentration for LPC was approximately 4  $\mu\text{M}$ . None

of the ACE inhibitors or propranolol modified the increase in fluorescence intensity induced by LPC, suggesting that these drugs did not affect LPC micelle formation.

## DISCUSSION

In the present study, LPC clearly caused hemolysis only at those concentrations which induced micelles, suggesting that LPC may cause hemolysis by the formation of micelles.<sup>9)</sup> Propranolol significantly attenuated the hemolysis induced by LPC at concentrations of 100 nM to 100  $\mu\text{M}$ . Similarly,

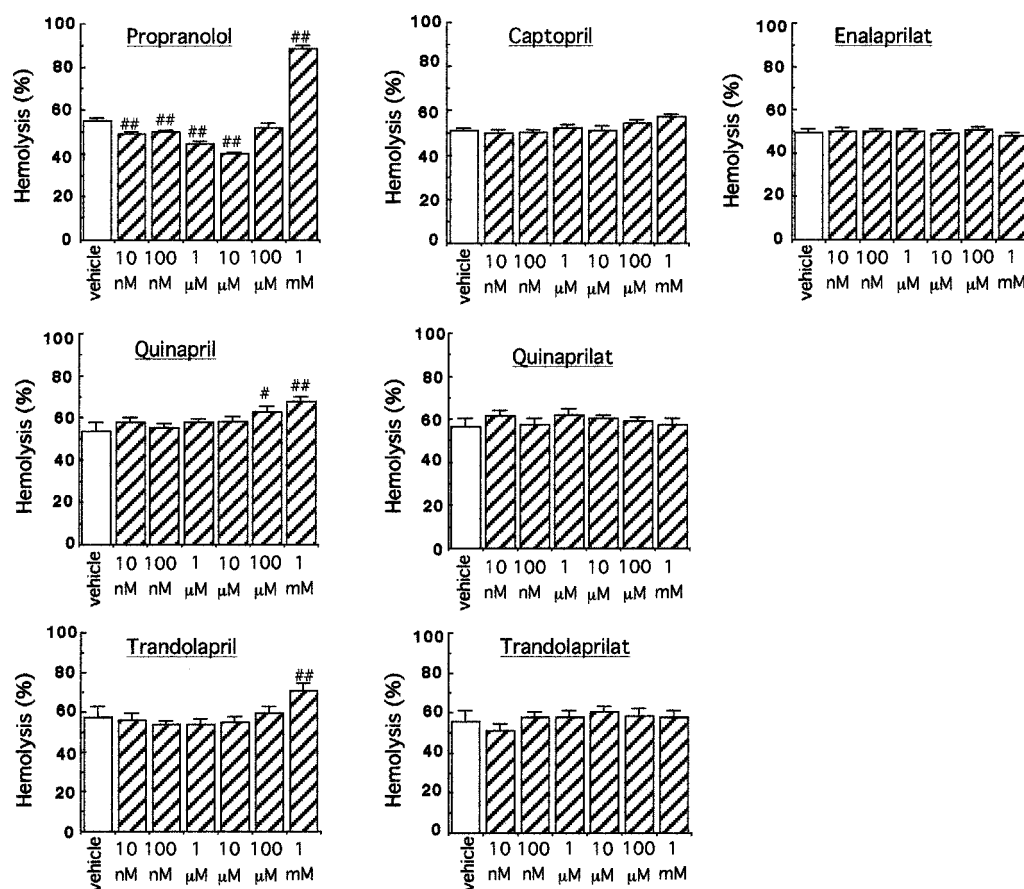


Fig. 3. Effects of Propranolol and ACE-Inhibitors on Hypotonic Hemolysis Induced by 60 mM NaCl

Each value is the mean  $\pm$  S.E.M. ( $n=4$  or 5). #  $p<0.05$ , ##  $p<0.01$ , these values are significantly different compared with the control (vehicle) group.

quinaprilat and trandolaprilat also attenuated the hemolysis. These results suggest that either quinaprilat, trandolaprilat, or propranolol has a direct cytoprotective action against LPC-induced cell damage. It has been proposed that the hemolytic capacity of LPC is determined by the chemical structure of the LPC molecule.<sup>10</sup> According to this hypothesis, quinaprilat, trandolaprilat, and propranolol might directly bind with LPC to lengthen its aliphatic chain, perhaps resulting in larger micelles and thereby reduced hemolytic activity. It should be noted, however, that the protective effects of quinaprilat, trandolaprilat, and propranolol are not the result of a change or inhibition of micelle formation, because neither quinaprilat (50  $\mu$ M), trandolaprilat (50  $\mu$ M), nor propranolol (100  $\mu$ M) affected LPC micelle formation.

Bierbaum *et al.*<sup>11</sup> found that exogenous LPC micelles liberated several cell surface proteins from erythrocyte membranes to increase sodium permeability which was inhibited by tetrodotoxin. Furthermore, the consequent hemolysis was also suppressed by tetrodotoxin or sucrose, suggesting that an osmotic imbalance induced by abnormal  $\text{Na}^+$  uptake was the primary cause of the hemolysis induced by LPC. It is, therefore, possible that drugs preventing the osmotic imbalance can inhibit or reduce the hemolytic effect of LPC. However, the present results indicate that the anti-hemolytic action of quinaprilat or trandolaprilat on hypotonic hemolysis was not observed at concentrations (10 or/and 100  $\mu$ M) that inhibit LPC-induced hemolysis. If the hemolytic action of LPC is a consequence of osmotic imbalance, quinaprilat or

trandolaprilat should attenuate the hypotonic hemolysis. There must be other mechanisms through which these two ACE inhibitors reduced LPC-induced hemolysis.

Propranolol significantly inhibited the hemolysis induced by LPC and hypotonic solution. It has been reported that propranolol protects erythrocytes from damage induced by bioactive phospholipids or hypotonic solution *via* a membrane-stabilizing action.<sup>7</sup> Membrane stabilization decreases membrane fluidity as well as permeability.<sup>12</sup> Because propranolol possesses a membrane stabilizing action correlated with a high lipophilicity,<sup>13</sup> it is possible that the high lipophilic properties of ACE inhibitors may play a role in the protection against hemolysis induced by LPC. Table 1 summarizes the effects of ACE inhibitors and propranolol in terms of their ACE inhibitory activity, lipophilicity, and anti-hemolytic action on LPC. The high lipophilicity of quinaprilat and trandolaprilat may be related to the protective effects of these drugs on hemolysis induced by LPC. However, quinapril and trandolapril were not effective, although both drugs have greater lipophilicity than quinaprilat and trandolaprilat.<sup>14</sup> Therefore, high lipophilicity alone cannot completely explain the protective effect of ACE inhibitors on hemolysis induced by LPC. Quinapril and trandolapril are metabolized into quinaprilat and trandolaprilat (diacid metabolites) in the body, hence they possess high ACE inhibitory activities. These findings suggest that ACE inhibitors require an active structure to inhibit the hemolysis induced by LPC. Because of the amphiphilic properties of LPC, LPC or LPC micelles

Table 1. ACE Inhibitory Activities, Distribution (Octanol/Water) Coefficients, and Anti-hemolytic Effects of ACE Inhibitors and Propranolol

Drugs	Inhibitory activity of ACE <sup>a)</sup> (enalaprilat=1)	Octanol/water coefficient <sup>b)</sup> (at 37 °C, pH 7.4)	Anti-hemolytic effect
<b><math>\beta</math>-Blocker</b>			
Propranolol	—	25.704	Yes
<b>ACE inhibitors</b>			
Captopril	0.07	0.001	No
Enalaprilat	1	0.617	No
Quinaprilat	14.3	6.607	Yes
Trandolaprilat	11.9	10.471	Yes
<b>ACE inhibition prodrugs</b>			
Quinapril	0.02	89.125	No
Trandolapril	0.001	81.283	No

a) The values of captopril, quinapril, and quinaprilat were cited from Fabris *et al.*,<sup>18)</sup> and those of trandolapril and trandolaprilat were from Chevillard *et al.*<sup>19)</sup> and Okunishi,<sup>17)</sup> respectively. b) Data were cited from the papers by Lüllmann *et al.*<sup>20)</sup> and Genes *et al.*<sup>14)</sup>

bind to and associate with the cell membrane.<sup>9,15)</sup> ACE inhibitors with a physicochemically lipophilic and diacidic structure such as quinaprilat and trandolaprilat might compete with LPC to penetrate into the membrane matrix, thereby preserving the membrane integrity of erythrocytes.

At the highest concentration (1 mM), the protective effects of quinaprilat, trandolaprilat and propranolol on LPC-induced hemolysis were nullified and deteriorated. It is known that propranolol accelerates hemolysis by partitioning into the inner membrane of the lipid bilayer at high concentration.<sup>16)</sup> Similarly, quinaprilat and trandolaprilat at high concentrations may accelerate LPC-induced hemolysis because these ACE inhibitors can easily enter the cell membrane.<sup>17)</sup> Further investigations are necessary to clarify the detailed mechanisms of the effects of quinaprilat and trandolaprilat on hemolysis induced by LPC.

In conclusion, quinaprilat and trandolaprilat, active metabolites of the lipophilic ACE inhibitors quinapril and trandapril, respectively, directly protected the erythrocyte membrane damaged by LPC. We believe the anti-hemolytic mechanism of the two ACE inhibitors might depend on their

lipophilic and ACE inhibitory structures.

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