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*Short Communication*

# Vasodilating dipeptide Trp-His can prevent atherosclerosis in apo E-deficient mice

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Most of the investigations for an alternative medicinal treatment on atherosclerosis have been focused on natural or dietary compounds including phytochemicals. So far, few studies regarding anti-atherosclerotic small peptides except for tetrapeptide of Lys-Arg-Glu-Ser have been reported. The present study was, thus, to investigate whether dipeptide Trp-His, which is one of vasodilating small peptides, could reduce atherosclerotic lesions in apo E-deficient mice fed a high-fat diet. The animal study involved a 9-week-successive administration of Trp-His at a dose of 0, 10 or 100 mg/kg per d. After 9-week administration, *en face* analyses provided the first direct evidence that the atherosclerotic lesion area was significantly reduced by 27 and 38 % for Trp-His dosed at 10 and 100 mg/kg per d, respectively, compared with the control group. Administration of Trp-His did not affect growth parameters such as body weight and feeding efficiency ($P > 0{\cdot}1$). Total serum cholesterol and HDL-cholesterol as well as lipid profiles in the liver did not differ between the tested groups. Taken together, the anti-atherosclerotic effect of dipeptide Trp-His should be addressed into physiological functions of bioactive peptides, in which the dipeptide may elicit the power by alternative mechanism(s), not by the regulation of lipid metabolism.

**Dipeptides: Atherosclerosis: Vasodilation: Hypertension**

Clinical evidence in human studies provides useful information that small peptides attribute preventive properties with regard to hypertension disease; in particular, the intake of anti-hypertensive foods containing Val-Tyr[1] or Ile-Pro-Pro[2], which have been accepted as a food for specific health use product in Japan, was proven to be benefit for improving blood pressure in mild hypertensive subjects. These beneficial properties are thought to be due to the suppression of renin–angiotensin system, since anti-hypertensive peptides showed a power to inhibit *in vitro* angiotensin I-converting enzyme (ACE) that is a key player to produce potent pressor peptide, angiotensin II. However, some conflicting results such as weak ACE inhibitory activity at micro molar level of $IC_{50}$ value[3], no significant decrease in plasma ACE activity and no increase in plasma renin activity in human study[1] allowed us to exclude the predominant involvement of ACE inhibition of anti-hypertensive peptides in lowering blood pressure and to include alternative mechanism(s) underlying the regulation of blood pressure.

In a series of our studies regarding underlying mechanism(s) of anti-hypertensive peptides, we have reported useful findings of dipeptides on (1) a favourable aortic ACE inhibition in transgenic mice bearing both human renin and angiotensinogen genes[4], (2) a significant anti-proliferative action in angiotensin II- or $Ca^{2+}$ channel agonist-stimulated vascular smooth muscle cells[5], and (3) an endothelium-independent relaxation effect in KCl-induced constrictive rat aorta rings[6,7]. A possible involvement of anti-hypertensive peptides in the regulation of vessel functions has also been reported by some researchers, who demonstrated accumulation of Ile-Pro-Pro into abdominal aorta[8] and stimulation of soluble guanylyl cyclase/cyclic GMP vasodilation pathway by Met-Tyr via haem oxygenase-1 activation in endothelial cells[9]. These *in vitro* and *ex vivo* observations raise the speculation that the intake of vasoactive small peptides could reduce vascular dysfunctions including atherosclerosis.

The aim of the present study was, thus, to demonstrate the *in vivo* anti-atherosclerotic effect of dipeptide Trp-His in apo E-deficient (ApoE −/−) mice. The selection of Trp-His in the present study was based on the finding that it evoked the most potent vasodilation activity in a 50 mM KCl-contracted Sprague–Dawley rat thoracic aorta ring among sixty-seven

**Abbreviations:** ACE, angiotensin I-converting enzyme; MCP, monocyte chemoattractant protein.

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synthetic dipeptides in an endothelium-independent manner[7]. The anti-atherosclerotic effect of peptides has been reported for the first time by Navab *et al.* [10], who demonstrated tetrapeptide of Lys-Arg-Glu-Ser reduced the atherosclerosis in ApoE − /− through the reduction in LPL hydroperoxides. However, no evidence was reported that the smaller dipeptides alone could attenuate the development of atherosclerotic onsets, except for a report on prevention of atherosclerosis by a mixture of diverse peptides of soya protein isolate[11].

## Experimental methods

### *Materials*

Trp-His was synthesised using an Fmoc solid-phase synthesis method according to the manufacturer's instructions (Kokusan Chemicals, Osaka, Japan), and the sequence was confirmed on a PPSQ-21 amino acid sequencer (Shimadzu Co., Ltd, Kyoto, Japan). All other chemicals were of analytical reagent grade and used without further purification.

### *Animal experiment*

Apo E-deficient (ApoE − /− ) mice purchased from Jackson Laboratory (Bar Harbor, ME, USA) in 1994 were used[11]. Mice were bred and maintained at the Laboratory of Animal Experiments in Kyushu University School of Medicine (Fukuoka, Japan). Male ApoE − /− mice (8–18 weeks old) were divided into three groups, and were fed the following diets for 9 weeks. Diets were based on the AIN-76 formulation as described previously[11]. The diet contained (g/kg) sucrose 449·0, casein 200·0, maize starch 150·0, olive oil 100·0, cellulose 50·0, mineral mixture (AIN-76) 35·0, vitamin mixture (AIN-76) 10·0, DL-methionine 3·0, choline bitartrate 2·0 and cholesterol 1·0.

A 9-week-successive administration of Trp-His sample was performed daily in each mouse, in which the dosage of 10 or 100 mg/kg per d dissolved in 1 ml deionised water was injected by intubation with nutritional catheter. Control mice were administered with the same volume of deionised water. The animals were individually housed at 22°C with a 12 h light–dark cycle (lights on, 08.00–20.00) and given free access to the diet and deionised water throughout the experimental period. The age before experiment was not significantly different between three groups (control; 15·0 (SEM 0·8), 10 mg/kg per d; 14·3 (SEM 1·2), 100 mg/kg per d; 14·6 (SEM 1·3)).

The mice were deprived of food for 6 h before sacrificing. The mice were put off and sacrificed by collecting blood from heart. Livers and white adipose tissues of retroperitoneal, mesenteric, subcutaneous areas and epididymal area were immediately removed from the carcasses, frozen in liquid $N_2$ and stored. Aorta and heart were immersed in formalin. These experiments were carried out under the guidelines for Animal Experiments in the Faculty of Agriculture and the Graduate Course, Kyushu University, Fukuoka, Japan, and the Law (No. 105) and Notification (No. 6) of the Government of Japan.

### *Measurement of serum and liver lipids*

Serum HDL-cholesterol was fractionated from obtained serum according to the method of Finley *et al.* [12]. Serum TAG ((TAG E test from Wako Pure Chemicals, Osaka, Japan), total-cholesterol and HDL-cholesterol concentrations were measured using enzyme assay kits (for both assays, total-cholesterol Kainos from Kainos, Tokyo, Japan). Serum monocyte chemoattractant protein (MCP)-1 concentration was measured using an ELISA kit (Mouse MCP-1 ELISA kit from Invitrogen, Carlsbad, CA, USA). Liver lipids extracted by $CHCl_3$–$CH_3OH$ mixture were chemically determined as previously described[12].

### *Aortic and* en face *analyses*

Formalin-fixed heart was used for determining a cross-sectional lesion area. Hearts containing aortic roots were subjected to our reported quantitative atherosclerosis assay[11] with a slight modification of the method described by Paigen *et al.* [13]. Briefly, paraffin-embedded cut along plane between heart and aortic valve was stained with elastic Van Gieson and haematoxylin. Formalin-fixed aorta was determined using an *en face* preparation according to the reported method[13] with a slight modification[14].

### *Statistical analyses*

Results are expressed as the mean and standard error of the mean. Statistical significance was estimated using a one-way ANOVA followed by Tukey–Kramer's multiple comparison *post hoc* tests. Value of $P<0{\cdot}05$ was considered to be statistically significant. All analyses were performed with Stat View J 5.0 software (SAS Institute, Cary, NC, USA).

## Results

### *Body weight and feeding efficiency*

After 9-week administration, body weight, organ weight, food intake and feeding efficiency did not differ between the three groups (Table 1). Though data were not shown, body fat content (control: 8·9 % (SEM 1·2), *n* 8; 10 mg/kg Trp-His: 7·1 % (SEM 0·7), *n* 7; 100 mg/kg Trp-His: 7·5 % (SEM 0·6), *n* 8) as well as fat contents of mesentery, testicles and hypodermis also did not differ between groups.

### *Blood lipids and monocyte chemoattractant protein-1*

In the blood analysis, no significant differences were found in total cholesterol, HDL-cholesterol and MCP-1 concentrations among the three groups (Table 1). Relatively high serum cholesterol (> 10 000 mg/l) may be due to a high cholesterol diet for the mice. Lipid profiles in the liver were not affected by Trp-His intake.

### *Aortic sinus and* en face *analyses*

After 9-week administration, *en face* analyses of aortic tree showed that the atherosclerotic lesion was less in both Trp-His groups than in control group (Fig. 1(a)). As shown in Fig. 1(b), the lesion areas for both Trp-His groups

**Table 1.** Growth parameters, serum and liver lipid levels and monocyte chemoattractant protein (MCP)-1 concentrations (Mean values with their standard errors)

| | Trp-His groups | | | | | |
|---|---|---|---|---|---|---|
| | Control | | 10 mg/kg | | 100 mg/kg | |
| | Mean (*n* 8) | SEM | Mean (*n* 7) | SEM | Mean (*n* 8) | SEM |
| Initial body weight (g) | 28·2 | 0·8 | 26·6 | 1·3 | 28·4 | 0·8 |
| Final body weight (g) | 34·4 | 2·1 | 30·2 | 0·8 | 33·1 | 1·0 |
| Body weight gain (g) | 6·2 | 1·7 | 3·6 | 1·0 | 4·7 | 0·8 |
| Food intake (g/d) | 4·0 | 0·2 | 4·1 | 0·2 | 4·1 | 0·1 |
| Food efficiency (mg weight gain/g food intake) | 23·9 | 5·9 | 14·9 | 4·4 | 18·3 | 3·4 |
| Liver weight (g) | 1·76 | 0·29 | 1·30 | 0·14 | 1·67 | 0·10 |
| Relative liver weight (g/100 g body weight) | 4·97 | 0·52 | 4·27 | 0·39 | 5·10 | 0·38 |
| Heart weight (g) | 0·21 | 0·01 | 0·23 | 0·01 | 0·23 | 0·01 |
| Relative heart weight (g/100 g body weight) | 0·63 | 0·04 | 0·75 | 0·05 | 0·69 | 0·02 |
| Serum (mg/l) | | | | | | |
| Total cholesterol | 12 380 | 1410 | 11 260 | 1320 | 13 280 | 1920 |
| HDL-cholesterol | 271 | 70 | 306 | 62 | 305 | 40 |
| TAG | 3370 | 490 | 3610 | 550 | 3820 | 930 |
| MCP-1 (pg/ml) | 47·4 | 1·4 | 47·9 | 2·7 | 46·4 | 3·7 |
| Liver (mg/g liver) | | | | | | |
| Cholesterol | | | | | | |
| Total | 12·5 | 0·55 | 13·6 | 0·55 | 12·9 | 0·42 |
| Non-esterified | 4·85 | 0·40 | 4·56 | 0·56 | 3·09 | 0·23 |
| Esterified | 7·60 | 0·66 | 9·05 | 0·84 | 9·86 | 0·45 |
| TAG | 104 | 21 | 94 | 29 | 89 | 7 |

(10 mg/kg per d: 9·5 % (SEM 1·3), *n* 7; 100 mg/kg per d: 8·1 % (SEM 1·6), *n* 8, $P<0·05$) were significantly reduced, compared with control group (13·0 % (SEM 1·0), *n* 8), indicating that dipeptide Trp-His inhibited the development of atherosclerosis lesions. Between both Trp-His groups, no significant dose-dependent reduction in the area was observed, suggesting that a Trp-His diet at a dose of 10 mg/kg per d may be enough to elicit the effect (Fig. 1(b)). In contrast, the atherosclerosis lesion in the aortic sinus (Fig. 1(c)) and the lesion area (Fig. 1(d)) did not differ between the three groups ($P>0·05$).

## Discussion

So far, many studies have been performed to prevent atherosclerosis lesions by natural compounds from the viewpoint of developing alternative medicinal foods. Beneficial anti-atherosclerotic compounds have been reported to be dietary fibres such as pectin and apple fibre showing LDL-cholesterol-lowering effect[15] or plasma uric acid-lowering effect[16], antioxidant polyphenols such as blueberry flavonoids[17] and red wine polyphenols[18] showing matrix metalloproteinase inhibitory activity and PUFA such as EPA[19] showing anti-inflammatory effect. In addition to these phytochemicals-induced anti-atherosclerotic effects, a recent report by Navab *et al.* [10] have revealed that small tetrapeptides of Lys-Arg-Glu-Ser also had a power to prevent an atherosclerotic onset in ApoE −/− mice. The tetrapeptide-induced anti-atherosclerotic effect, thus, led us to examine the atherosclerotic effect of smaller dipeptides, since some dipeptides showed *in vivo* bioactivity such as anti-hypertension[1].

In the present study, we explored whether the long-term administration of dipeptide possessing *ex vivo* vasodilation activity could ameliorate the development of atherosclerotic lesions in ApoE −/− mice. As a result of 9-week administration of vasodilating dipeptide Trp-His[7] to the mice, we found the first direct evidence that the administration of Trp-His reduced the atherosclerotic lesion area in aortic tree and was efficient for preventing atherosclerosis diseases without any affection on growth parameters and lipid profiles. Regarding the functionality of dietary proteins as a possible source of bioactive peptides, we have already reported an apparent anti-atherosclerotic action of soya protein isolate in ApoE −/− mice, in which the lesion size was reduced in mice fed a soya diet[11]. A recent study by Nagarajan *et al.* [20] has clearly showed evidence that the anti-atherosclerotic effect of soya protein diet was caused not only by isoflavones including genistein and daidzein, but also by any peptides produced by gastrointestinal digestions. Interestingly, both soya protein studies did not show any change in serum lipid profiles, being matched with the present finding in the dipeptide study. By contrast, Cho *et al.* [21] recently reported that a soyabean 'hydrolysate' prepared by bacterial proteases had a power to upregulate LDL receptor transcription at the liver so as to reduce blood cholesterol level. So far, Ile-Ile-Ala-Glu-Lys[22] and His-Ile-Arg-Leu[23] were reported as hypocholesterolaemic peptides showing the novel effect in animal studies through the inhibition of intestinal cholesterol absorption and the activation of dopamine $D_2$ receptor, respectively. These conflicting findings strongly suggest an extensive role of peptides that the underlying anti-atherosclerotic mechanisms would be determined by peptide structure or sequence. The result that the anti-atherosclerotic action of Lys-Arg-Glu-Ser was caused by reducing serum LDL hydroperoxides and activating HDL-associated enzyme paraoxonase[10], different from the present result of Trp-His with no change in lipid profiles, also supported the distinct mechanism(s) by peptide sequence.

A possible explanation of the anti-atherosclerotic effect of Trp-His without change in blood lipids may be due to

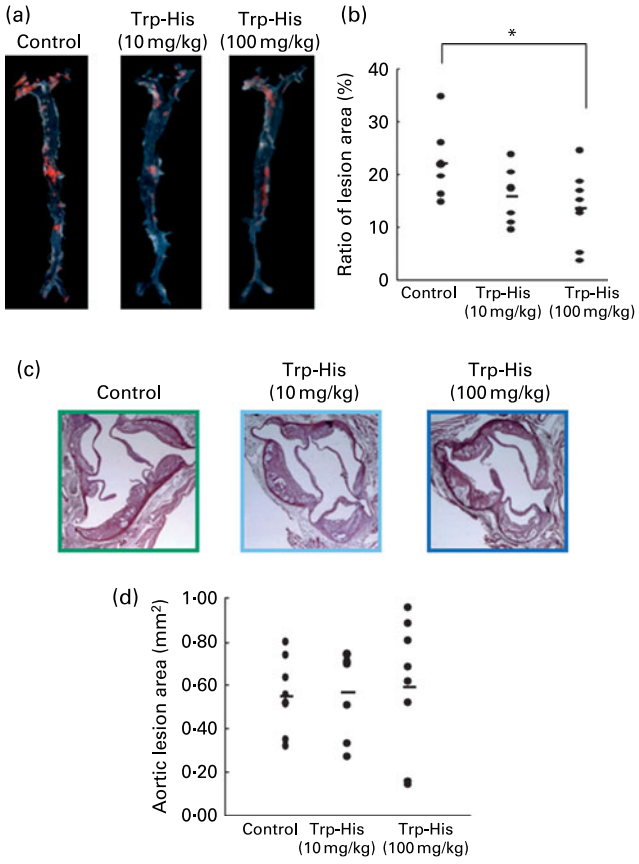


**Fig. 1.** Measurements of atherosclerotic area in aortic tree and aortic sinus of male ApoE − /− mice. (a) Male ApoE − /− mice were daily administered Trp-His (10 mg/kg per d, *n* 7 or 100 mg/kg per d, *n* 8) or not (control group, *n* 8) for 9 weeks. Atherosclerotic plaques in the aorta tree were visualised by en face Sudan IV staining. (b) The extent of straining positive areas was measured and expressed as percentage. (c) Atherosclerotic plaques in the aorta sinus were visualised by Van Gieson and haematoxylin. (d) The extent of straining positive areas was measured and expressed as percentage. A total of five slides per mouse were analysed. Values are means with the standard errors depicted by vertical bars. Mean values were significantly different from those of control group: $^*P<0{\cdot}05$.

a direct regulation of vessel functions, like soya protein suppressing an expression of inflammatory cytokines[20], Met-Tyr stimulating an induction of haem oxygenase-1[9] that produces CO gas for protecting intimal thickening[24] or oligopeptides showing vasorelaxation via bradykinin receptor stimulation[25] in endothelial events. Our recent report concerning endothelial-independent vasodilation action of Trp-His[7] may also provide alternative mechanism(s) that the proliferation or migration of vascular smooth muscle cells could be inhibited through a suppression of extracellular $Ca^{2+}$ influx like Val-Tyr[5]. In the present study, the anti-atherosclerotic effect induced by Trp-His was specific for the development at the aorta tree, while no preventive effect was observed at the aortic sinus. Provided that the effect was induced by a restrictive binding of Trp-His to the voltage-gated L-type $Ca^{2+}$ channel as clarified in a previous paper[7], no preventive effect at the aortic sinus might result from different subtype of $Ca^{2+}$ channel as T-type associated with hypertrophy[26], but the specific role of Trp-His still remains unclear. Alternatively, an explanation that higher serum cholesterol levels (> 10 000 mg/l) for all groups in the present protocol may diminish the anti-atherosclerotic effect against developed atherosclerosis onsets at the aortic sinus cannot be ruled out[27]. The involvement of suppression of peripheral sympathetic nervous system[28] would be excluded for a role of Trp-His, because of no change in growth parameters and feeding efficiency among the tested groups. In the present study, we observed no change in serum MCP-1 level in the groups, which is a biomarker of monocyte-related inflammation in response to developing atherosclerosis[29,30], as Nakano *et al.*[31] have demonstrated that a Ca channel blocker azelnidipine showing anti-atherosclerotic effect in cynomolgus monkeys affected less serum MCP-1 level. However, the suppression of local MCP-1 expression or platelet-derived growth factor, like azelnidipine, should be also taken into consideration for underlying anti-atherosclerotic mechanism of Trp-His. Collectively, further studies must be needed to clarify the Trp-His-induced anti-atherosclerotic mechanism(s) and are now in progress regarding intact absorption of Trp-His, anti-atherosclerotic effect of the constituent amino acids and expression of atherosclerosis-related mRNAs or proteins in another set of ApoE − /− mouse experiments. Additionally, it may be also of benefit to examine whether Trp-His ingestion to ApoE + /+ mice possesses the preventive potential of vascular dysfunctions, since in the anti-hypertension study of dipeptide, Val-Tyr, in borderline hypertensives, the ingestion did not affect the blood pressure of normotensives[1].

In conclusion, we provided the first direct evidence that dipeptide Trp-His possessing vasodilation activity has an ability to inhibit the development of atherosclerosis onsets in ApoE − /− mice at a dose of 10 mg/kg per d not by the regulation of lipid metabolism, but by alternative mechanism(s). The effect of Trp-His should be addressed into potential physiological functions of small peptides as a candidate for preventing atherosclerosis onsets.

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