Carnosine Facilitates Nitric Oxide Production in Endothelial F-2 Cells

Satoru TAKAHASHI,*^a Yukiko NAKASHIMA,^a and Ken-ichi TODA^b

^aFirst Department of Biochemistry, School of Pharmaceutical Sciences, Kyushu University of Health and Welfare; 1714–1 Yoshino, Nobeoka, Miyazaki 882–0072, Japan; and ^bDepartment of Dermatology, The Tazuke Kofukai Medical Institute, Kitano Hospital; 2–4–20 Ohgimachi, Kita-ku, Osaka 530–8480, Japan.

Received June 8, 2009; accepted September 3, 2009; published online September 4, 2009

We examined the effect of carnosine (β -alanyl-histidine) on nitric oxide (NO) production and endothelial NO synthase (eNOS) activation in endothelial F-2 cells. Carnosine enhanced NO production in a dose-dependent manner, and the stimulatory effect of carnosine was observed at concentrations exceeding 5 mM. The carnosine-stimulated NO production was inhibited by N^G -nitro-L-arginine methyl ester, but not by N^G -nitro-D-arginine methyl ester. In contrast, β -alanine, histidine (carnosine components) and anserine (N -methyl carnosine) failed to increase NO production. Carnosine had no effect on NO production for the initial 5 min, but thereafter resulted in a gradual increase in NO production up to 15 min. Carnosine did not induce phosphorylation of eNOS at Ser1177. The carnosine-induced increase in NO production was observed even when extracellular Ca^{2+} was depleted by ethylene glycol bis(2-aminoethyl ether)- N,N,N',N' -tetraacetic acid however, the effect was abolished upon depletion of intracellular Ca^{2+} by BAPTA. After F-2 cells were incubated with carnosine for 4 min, intracellular Ca^{2+} concentration gradually increased. The carnosine-induced increase in intracellular Ca^{2+} concentration occurred even in the absence of extracellular Ca^{2+} . These results indicate that carnosine facilitates NO production in endothelial F-2 cells. It is also suggested that eNOS is activated by Ca^{2+} , which might be released from intracellular Ca^{2+} stores in response to carnosine.

Key words carnosine; endothelial cell; nitric oxide; endothelial nitric oxide synthase; intracellular Ca^{2+}

Carnosine is a dipeptide composed of β -alanine and histidine. It is synthesized endogenously,¹⁾ and it is also ingested as a part of diets comprising meat, fish and chicken and is absorbed intact from the gastrointestinal tract.^{2,3)} Carnosine is found in millimolar concentrations in various tissues, and is especially abundant in the heart, muscles and brain.^{4,5)} The exact physiological role of carnosine remains unclear, but various effects of carnosine have been reported. Further, carnosine is expected to have therapeutic potential.⁶⁾ However, the effect of carnosine on endothelial function has not been investigated, although the endothelium is exposed to the dipeptide after ingestion. In addition, the effect of carnosine on vascular tone has been a matter of debate. Ririe *et al.*⁷⁾ showed that carnosine causes relaxation of the isolated rat aorta, whereas O'Dowd *et al.*⁸⁾ reported that the dipeptide induces sustained constriction of the isolated rabbit saphenous vein.

Endothelial cells constitutively produce nitric oxide (NO), which plays an important role in regulating vascular tone, platelet aggregation and endothelial viability.^{9,10)} Endothelial NO synthase (eNOS) is responsible for the physiological production of NO in endothelial cells.

We therefore examined whether carnosine affects NO production and eNOS activation in cultured endothelial cells.

MATERIALS AND METHODS

Materials Unless otherwise indicated, all the reagents used in this study and their sources are as follows: L-carnosine, L-anserine nitrate, β -alanine, L-histidine, N^G -nitro-L-arginine methyl ester (L-NAME), N^G -nitro-D-arginine methyl ester (D-NAME) and bradykinin (Sigma-Aldrich Co., St. Louis, MO, U.S.A.); protease inhibitor mixture set III and 1,2-bis(2-aminophenoxy)ethane- N,N,N',N' -tetraacetic acid (BAPTA-AM) (Calbiochem, La Jolla, CA, U.S.A.); anti-

eNOS antibody (BD Transduction Laboratories, Lexington, KY, U.S.A.); anti-peNOS antibody (phospho-Ser1177), secondary antibody linked to peroxidase and Phototope-HRP Western blot detection system (Cell Signaling, Beverly, MA, U.S.A.); Immobilon P membrane (Millipore, Bedford, MA, U.S.A.); Calcium kit-Fluo 3 (Dojin, Kumamoto, Japan); cell culture media and supplies (Invitrogen, Carlsbad, CA, U.S.A.). All other chemicals were of reagent grade.

Cell Culture F-2 cells, murine vascular endothelial cell line,¹¹⁾ were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin in the final concentration at 37 °C in 5% CO₂ and 95% air.

Determination of NO Production F-2 cells were seeded in 12-well plates and were allowed to grow to confluence. The cells were starved over night in serum-free medium containing 1 mg/ml bovine serum albumin, and then conditioned in phosphate-buffered saline (PBS) containing 10 mM HEPES–NaOH (pH 7.4), and 1 mg/ml bovine serum albumin for 1 h before the experiments. The cells were pretreated with vehicle or 5 mM L-NAME for 10 min and then stimulated with 1 μ M bradykinin, 10 mM carnosine, 10 mM anserine, 10 mM β -alanine or 10 mM histidine, unless otherwise indicated. At appropriate times, the medium was sampled. NO was determined as nitrite by colorimetry after the Griess reaction.^{12,13)} The released NO into the medium was completely oxidized to nitrite when left standing over night at 4 °C. In a preliminary study, the amount of nitrite was confirmed to be stable under this condition. The amount of nitrite was measured using a NOx analyzer (ENO-200; EICOM, Kyoto, Japan).

The depletion of extracellular or intracellular Ca^{2+} was achieved by incubating the cells in Ca^{2+} -free PBS containing 10 mM HEPES–NaOH (pH 7.4), 1 mM EGTA and 1 mg/ml bovine serum albumin with or without 50 μ M BAPTA-AM

* To whom correspondence should be addressed. e-mail: satorutaka@phoenix.ac.jp

for 30 min. Thereafter, the medium was replaced with Ca^{2+} -free PBS containing 10 mM HEPES–NaOH (pH 7.4), 0.1 mM EGTA, 1 mg/ml bovine serum albumin.

Immunoblotting of eNOS eNOS was detected as described previously.¹⁴ F-2 cells were seeded in 35-mm dishes and were allowed to grow to confluence. After incubation with 1 μM bradykinin or 10 mM carnosine for 10 min, the F-2 cells were lysed in 20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 100 mM NaCl, 10 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM β -glycerophosphate, 1% Triton X-100, 0.5% sodium deoxycholate and the protease inhibitor mixture. The lysates were placed on ice for 10 min and were then centrifuged at 10000 rpm for 10 min at 4 °C. The supernatants were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then blotted onto Immobilon P membranes. The blots were incubated over night with the primary antibody at 4 °C and then probed with the secondary antibody linked to peroxidase. Immunoreactive proteins were visualized using an enhanced chemiluminescence kit.

Determination of Intracellular Ca^{2+} Concentration Intracellular Ca^{2+} concentration was measured in the cells loaded with Fluo 3.¹⁵ F-2 cells were incubated with 5 mg/ml Fluo 3-AM for 90 min. The cells were washed with PBS and then held for 15 min. Relative fluorescence intensity was monitored at an excitation and emission wavelength of 485 nm and 535 nm, respectively.

Statistical Analysis Data are presented as means $\pm$ S.E. Statistical differences in the dose–response study were evaluated by applying Dunnett's multiple comparison test. Student's *t*-test was used for comparing two groups. A *p* value of <0.05 was regarded as significant.

RESULTS

Carnosine-Induced Increase in NO Production in Endothelial Cells Endothelial F-2 cells produced NO under the control condition without any stimulus (Fig. 1A). When F-2 cells were incubated with carnosine for 10 min, carnosine enhanced NO production in a dose-dependent manner. The stimulatory effect of carnosine was observed at concentrations above 5 mM, and reached a plateau level at 10 mM. Carnosine at 10 mM significantly stimulated NO production, although the effect of carnosine was lower than that of 1 μM bradykinin (Fig. 1B). Pretreatment with 5 mM L-NAME potently reduced both basal, carnosine- and bradykinin-stimulated NO production. In contrast, 5 mM D-NAME did not affect basal or stimulated NO production. These results indicate that the observed NO production is mediated by eNOS.

We examined whether β -alanine, histidine (carnosine components) and anserine (N^1 -methyl-carnosine) also stimulate NO production in F-2 cells (Fig. 2). However, none of the compounds at 10 mM had any effect on NO production for 10 min. Further, the combination of 10 mM β -alanine and 10 mM histidine failed to increase NO production.

Carnosine at 10 mM had no effect on NO production up to 5 min, and thereafter gradually increased NO production in a time-dependent manner (Fig. 3). The carnosine-stimulated NO production for 10 and 15 min was 171.1 and 201.3%, respectively, and was significantly higher than the control. However, the effect of carnosine disappeared between 15–30 min, and the NO accumulation rate at this stage was al-

Fig. 1. Effect of Carnosine on NO Production in Endothelial Cells

(A) F-2 cells were incubated with vehicle (control) or 0.1–20 mM carnosine for 10 min. The released NO into the medium was determined. (B) After F-2 cells were pretreated with vehicle, 5 mM L-NAME or 5 mM D-NAME, the cells were stimulated with vehicle (control), 10 mM carnosine or 1 μM bradykinin for 10 min. The released NO into the medium was determined. The data are mean $\pm$ S.E. for five experiments. * Significantly different from the control.

Fig. 2. Effect of Carnosine-Related Compounds on NO Production in Endothelial Cells

F-2 cells were incubated with vehicle (control) or the indicated compound at 10 mM for 15 min. The released NO into the medium was determined. The data are mean $\pm$ S.E. for five experiments. * Significantly different from the control.

most the same as that of the control. In contrast, bradykinin at 1 μM rapidly and markedly enhanced NO production, as determined from the high rate of NO accumulation 0–2 min after the start of the reaction as compared with the later stage. NO production stimulated by bradykinin was 390.6% for 2 min and 528.9% for 10 min, compared with the control.

Mechanism Underlying the Carnosine-Induced Increase in NO Production eNOS activity primarily depends on intracellular Ca^{2+} levels and is further regulated by protein kinases such as Akt.^{14,16–18} It was examined whether the increased NO production caused by carnosine is mediated by the phosphorylation of eNOS at Ser-1177 (Fig. 4). When F-2 cells were incubated with 10 mM carnosine for 10 min, eNOS phosphorylation was not promoted. In contrast, 1 μM bradykinin clearly facilitated the phosphorylation of eNOS.

Fig. 3. Time Course of Carnosine-Induced Increase in NO Production in Endothelial Cells

F-2 cells were incubated with vehicle (control), 10 mM carnosine or 1 μ M bradykinin for the indicated times. The released NO into the medium was determined. The data are mean $\pm$ S.E. for five experiments. * Significantly different from the control.

Fig. 4. Effect of Carnosine on Phosphorylation of eNOS in Endothelial Cells

F-2 cells were incubated with vehicle (control), 1 μ M bradykinin or 10 mM carnosine for 10 min. The phosphorylation of eNOS at Ser1177 was examined by immunoblotting. The data are representative of three experiments. Lane 1, control; lane 2, bradykinin; and lane 3, carnosine.

We next examined the Ca^{2+} requirement for the stimulatory effect of carnosine (Fig. 5). The depletion of extracellular and intracellular Ca^{2+} was achieved by treating the cells with EGTA and BAPTA-AM in Ca^{2+} -free PBS, respectively. Up to 10 min, the basal NO production in the presence of EGTA or BAPTA-AM was reduced to approximately 60% of that observed under normal Ca^{2+} levels. NO production continued to remain significantly increased in response to 10 mM carnosine or 1 μ M bradykinin even though extracellular Ca^{2+} was depleted. In contrast, the depletion of intracellular Ca^{2+} abolished the effect of carnosine. Carnosine-induced NO production was slightly enhanced, and the increase was only 19.6% greater than that of the control. In addition, the effect of bradykinin on NO production was potently inhibited by BAPTA-AM.

When intracellular Ca^{2+} concentration was measured in Fluo 3-loaded F-2 cells, the Ca^{2+} level was increased by 10 mM carnosine (Fig. 6). Carnosine did not affect intracellular Ca^{2+} concentration up to 4 min, and thereafter increased the Ca^{2+} concentration up to 10 min. Similarly, the carnosine-induced increase in intracellular Ca^{2+} concentration occurred in the absence of extracellular Ca^{2+} . However, 2 mM carnosine had no effect on intracellular Ca^{2+} concentration (data not shown).

Fig. 5. Ca^{2+} -Dependency of Carnosine-Induced Increase in NO Production in Endothelial Cells

F-2 cells were pretreated with or without 50 μ M BAPTA-AM in the presence of 1 mM EGTA, and then stimulated with vehicle (control), 10 mM carnosine or 1 μ M bradykinin (BK) for 10 min. The released NO into the medium was determined. The data are mean $\pm$ S.E. for five experiments. * Significantly different from the control.

Fig. 6. Effect of Carnosine on Intracellular Ca^{2+} Concentration in Endothelial Cells

Fluo 3-loaded F-2 cells were preincubated in the absence (A) or presence (B) of 5 mM EGTA for 5 min, and then stimulated with vehicle or 10 mM carnosine. Intracellular Ca^{2+} concentration was continuously measured. Arrows indicate the addition of vehicle or carnosine. The data are mean $\pm$ S.E. for six experiments.

DISCUSSION

The present studies demonstrate that carnosine facilitates NO production in endothelial F-2 cells. The effect of carnosine was observed only at high concentrations of more than 5 mM. Under normal physiological conditions, it is unlikely that endogenous carnosine regulates endothelial NO production, because the plasma levels of carnosine are low.³⁾ However, dietary carnosine might induce an increase in NO pro-

duction in blood vessels such as the mesenteric and portal veins. The plasma concentration of carnosine is dramatically elevated after ingestion.⁷⁾ The maximal potency of carnosine to increase NO production was less than 50% of that of 1 μ M bradykinin, and its stimulatory effect was observed for about 10 min. These results suggest that dietary carnosine-induced NO production may locally dilate blood vessels, but may not reduce systemic blood pressure. Ririe *et al.*⁷⁾ reported that carnosine reduced blood pressure in rats, however, their finding can not be applied to endogenous or dietary carnosine because the decrease was observed in response to a high dose (33.3 mg/kg) of carnosine administered intravenously. Carnosine causes relaxation in rat aorta⁷⁾ and constriction of the rabbit saphenous vein,⁸⁾ implying that the effect of carnosine on endothelial cells may differ between arteries and veins.

The stimulatory effect on NO production in endothelial cells is specific for carnosine. It is reported that the antioxidant ability of carnosine might contribute to its cellular actions.^{4,6)} In addition to carnosine, related compounds such as anserine also exert antioxidative actions.¹⁹⁾ In our studies, neither of the constituent amino acids of carnosine (β -alanine and/or histidine) nor anserine had an effect on NO production, indicating that the carnosine-induced increase in NO production might be independent of antioxidant action.

It is established that receptor ligands for endothelial cells, such as bradykinin and vascular endothelial growth factor, rapidly increase intracellular Ca^{2+} levels and cause Akt-mediated phosphorylation of eNOS, resulting in the potentiation of NO production.^{14,16–18)} In contrast, carnosine-induced increases in intracellular Ca^{2+} levels and NO production were observed after a lag period of 4 min and 5 min, respectively, and the dipeptide did not induce eNOS phosphorylation. Carnosine concentrations that exceed 5 mM resulted in NO production, and these levels were remarkably higher than the effective concentrations of the receptor ligands. Thus, the actions of carnosine quite differ from those of the receptor ligands. Receptors for carnosine have not been found in any cell types. Further investigation is needed for identifying the target protein of carnosine.

Carnosine increased both NO production and intracellular Ca^{2+} concentration in the absence of extracellular Ca^{2+} . The increased NO production in response to carnosine followed intracellular Ca^{2+} mobilization, but was abolished by the depletion of intracellular Ca^{2+} . These results indicate that carnosine mobilizes Ca^{2+} from intracellular Ca^{2+} stores, leading to Ca^{2+} -dependent eNOS activation. Interestingly, Zaloga *et al.*²⁰⁾ reported carnosine-induced intracellular Ca^{2+} mobilization. Carnosine induces Ca^{2+} release from intracellular stores, thereby increasing intracellular Ca^{2+} levels in the

isolated rat hearts and skinned myocardial fibers.

In conclusion, the present studies demonstrate that carnosine increases NO production in endothelial F-2 cells. The mechanisms of carnosine action on eNOS activation have not been fully clarified, but they may include carnosine-induced Ca^{2+} mobilization from intracellular stores. The finding of our studies might contribute to the research on the therapeutic applications of carnosine.

Acknowledgments We wish to thank Dr. T. Uchiyama (Kyushu University of Health and Welfare) and Dr. A. Sugiyama (Iwate Medical University) for their assistance.

REFERENCES

- 1) Bakardjiev A., Bauer K., *Biochemistry* (Moscow), **65**, 779–782 (2000).
- 2) Ferraris R. P., Diamond J., Kwan W. W., *Am. J. Physiol.*, **255**, G143–G150 (1988).
- 3) Gardner M. L., Illingworth K. M., Kelleher J., Wood D., *J. Physiol.*, **439**, 411–422 (1991).
- 4) Zaloga G. P., Roberts P. R., Nelson T. F., *New Horiz.*, **4**, 26–35 (1996).
- 5) Crush K. G., *Comp. Biochem. Physiol.* **34**, 3–30 (1970).
- 6) Quinn P. J., Boldyrev A. A., Formazuyk V. E., *Mol. Aspects Med.*, **13**, 379–444 (1992).
- 7) Ririe D. G., Roberts P. R., Shouse M. N., Zaloga G. P., *Nutrition*, **16**, 168–172 (2000).
- 8) O'Dowd A., O'Dowd J. J., Miller D. J., *J. Physiol.*, **495**, 535–543 (1996).
- 9) Ignarro L. J., Cirino G., Casini A., Napoli C., *J. Cardiovasc. Pharmacol.*, **34**, 879–886 (1999).
- 10) Hayashi T., Yano K., Matsui-Hirai H., Yokoo H., Hattori Y., Iguchi A., *Pharmacol. Ther.*, **120**, 333–339 (2008).
- 11) Toda K., Tsujioka K., Maruguchi Y., Ishii K., Miyachi Y., Kuribayashi K., Imamura S., *Cancer Res.*, **50**, 5526–5530 (1990).
- 12) Wang Y., Shin W. S., Kawaguchi H., Inukai M., Kato M., Sakamoto A., Uehara Y., Miyamoto M., Shimamoto N., Korenaga R., Ando J., Toyooka T., *J. Biol. Chem.*, **271**, 5647–5655 (1996).
- 13) Takahashi S., Uchiyama T., Toda K., *Biol. Pharm. Bull.*, **32**, 1840–1843 (2009).
- 14) Takahashi S., Mendelsohn M. E., *J. Biol. Chem.*, **278**, 30821–30827 (2003).
- 15) Kao J. P. Y., Harootyan A. T., Tsien R. Y., *J. Biol. Chem.*, **264**, 8179–8184 (1989).
- 16) Fulton D., Gratton P. J., McCabe T. J., Fontana J., Fujio Y., Walsh K., Franke T. F., Papopetropoulos A., Sessa W. C., *Nature* (London), **399**, 597–601 (1999).
- 17) Dimmeler S., Fleming I., Fisslthaler B., Hermann C., Busse R., Zeiher A. M., *Nature* (London), **399**, 601–605 (1999).
- 18) Gratton P. J., Fontana J., O'Connor D. S., Garcia-Cardena G., McCabe T. J., Sessa W. C., *J. Biol. Chem.*, **275**, 22268–22272 (2000).
- 19) Kohen R., Yamamoto Y., Cundy K. C., Ames B. N., *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 3175–3179 (1988).
- 20) Zaloga G. P., Roberts P. R., Black K. W., Lin M., Zapata-Sudo G., Sudo R. T., Nelson T. E., *Am. J. Physiol.*, **272**, H462–H468 (1997).