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Short communication

Quantitative determination of endogenous nitric oxide in the mouse skin in vivo by microdialysis

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Abstract

We have developed a subcutaneous microdialysis system for the determination of nitric oxide (NO) concentration in the skin. The skin was microdialyzed using a degassed solution containing 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide and the perfusate was reacted on-line with Griess' reagent. This method could reveal NO production following intradermal injection of bradykinin (10–100 nmol/site) in mice. The increase in cutaneous NO after bradykinin (100 nmol/site) was dose dependently suppressed by the NO synthase inhibitor, N^G-nitro-L-arginine methyl ester, and the bradykinin B_2 receptor antagonist, D-Arg-[Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin. This system may be useful for pharmacological and physiological experiments on the role of NO in the skin. © 1997 Elsevier Science B.V.

Keywords: Nitric oxide (NO); Microdialysis; Skin; Bradykinin; Inflammatory hyperalgesia; N^G-nitro-L-arginine methyl ester; Bradykinin B_2 receptor

1. Introduction

Nitric oxide (NO) is synthesized from L-arginine by nitric oxide synthase (Palmer et al., 1988). The substrate for, and inhibitors of, NO synthase and NO scavengers have been shown to modulate hyperalgesia (Nakamura et al., 1996) and inflammation (Ialenti et al., 1992) in the periphery, suggesting the involvement of NO in such phenomena. However, no reports have directly demonstrated an inflammagen-induced increase in the intracutaneous concentration of NO.

The measurement of NO in brain regions by in vivo microdialysis has been described (Balcioglu and Maher, 1993; Ohta et al., 1994). Free radical $^{\cdot}NO$ is very unstable with a half-life of 3 to 5 s (Ignarro et al., 1987) and is readily oxidized to NO_2^- and NO_3^- in the presence of water (Marletta et al., 1988). Thus, in many brain microdialysis experiments, NO_2^- (and NO_3^-), instead of $^{\cdot}NO$, was measured in collected perfusion samples after diazotization. In these experiments, however, it was unclear whether NO_2^- was derived exclusively from $^{\cdot}NO$ because the former can also be from NO_x in the atmosphere. To overcome this problem, the NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (carboxy-PTIO) was added into the perfusion medium. This compound reacts with $^{\cdot}NO$ in a stoichiometric manner to generate immediately NO_2^- and NO_3^- in a neutral perfusion medium in the microdialysis tube (Akaike et al., 1993). We now showed that a combination of subcutaneous microdialysis with a carboxy-PTIO-containing perfusion medium allows the reproducible measurement of the concentration of intracutaneous NO.

Bradykinin is a chemical mediator of inflammation and pain (Steranka et al., 1988; Dray and Perkins, 1993), vasodilation and hypotension (Regoli and Barabé, 1980), and increases vascular permeability (Barabé et al., 1979). This peptide was thought to induce inflammation, hyperalgesia and an increase of microvascular blood flow through the production of NO (Khalil and Helme, 1992; Warren and Loi, 1995; Nakamura et al., 1996). Therefore, using the present microdialysis method, we examined whether an intradermal injection of bradykinin would increase the intracutaneous concentration of NO.

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2. Materials and methods

2.1. Materials

The bradykinin B_2 receptor antagonist, D-Arg-[Hyp^3, Thi^{5,8}, D-Phe^7]bradykinin (Peptide Institute, Minoh, Japan), the NO synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME; Research Biochemicals International, Natick, MA, USA) and its inactive enantiomer, D-NAME (Research Biochemicals International), were dissolved in physiological saline. These agents were intradermally injected into the rostral back over the microdialysis tube in a volume of 0.05 ml. Carboxy-PTIO (Dojindo Laboratories, Kumamoto, Japan) and sodium nitrite (Wako, Osaka, Japan) were dissolved in 0.1 M phosphate-buffered saline (pH 7.4).

2.2. Subcutaneous microdialysis and NO measurement

Male ICR mice (5–6 weeks old) were used. The hair was clipped over the rostral part of the mouse back and a microdialysis tube (PNF-1700, Asahi Medical, Tokyo, Japan) was then introduced into the rostral skin (Fig. 1). All experimental procedures were conducted under chloral hydrate (400 mg/kg, i.p.) anesthesia. This tube was connected to a microsyringe pump (EP-60, Eicom, Kyoto, Japan) and the outlet was connected to a three-way tap through fine polyethylene tubes (EF-250, Eicom). The subcutaneous region was perfused with the perfusion medium, which was phosphate-buffered saline containing 0.1 mM carboxy-PTIO. Another microsyringe pump was connected to another opening of the three-way tap through the polyethylene tube and supplied Griess' reagent (1% sulfanilamide, 0.1% naphtylethenediamine dihydrochloride, 2.5% phosphoric acid). These solutions were degassed immediately before use and each flow rate was 10 μl/min. The perfused sample was mixed with the Griess' reagent in the three-way tap for diazotization and then collected into sealed polyethylene tubes at 5 min intervals. The azo dye formed was determined with a spectrophotometer at 540 nm (Ohta et al., 1994), using sodium nitrite as a standard.

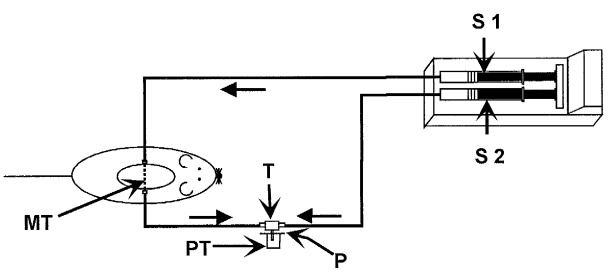

Fig. 1. Diagram of in vivo subcutaneous microdialysis for the determination of nitric oxide concentration in the skin. MT, microdialysis tube; P, parafilm; S1, syringe pump for perfusion medium containing carboxy-PTIO; S2, syringe pump for Griess' reagent; T, three-way tap; PT, polyethylene test tube.

2.3. Data processing

All data are presented as means and S.E. The statistical significance of drug effects was analyzed using one-way analysis of variance followed by Dunnett's multiple comparisons and that of the linear regression was evaluated with an analysis of variance; $P < 0.05$ was considered significant.

3. Results

3.1. Sensitivity and recovery of NO

Initial in vitro experiments were carried out to estimate the sensitivity and recovery of nitrite. To determine the sensitivity of the microdialysis system to NO_2^-, sodium nitrite solution (0.125–16 μM) was microdialyzed. The lowest detectable concentration was 0.2 μM and absorbance was approximately linear ($F(1,5) = 2445.9$, $P < 0.0001$) with the concentration of sodium nitrite in the range of 0.2 to 16 μM. To estimate the recovery of NO through the dialysis membrane, a solution containing (±)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro]-5-nitro-3-hexamide (NOR3), as NO donor, was microdialyzed. When NOR3 was dissolved in phosphate-buffered saline (0.1 M, pH 7.4) at a concentration of 100 μM, the concentration of NO gradually increased during the experimental period (1 to 4.5 h) from 8.2 to 26.3 μM, the recovery being 54.7 ± 0.6% (7 different concentrations of NO). In subsequent experiments, therefore, the extracellular concentration of NO was calculated on the basis of a 55% recovery.

3.2. Bradykinin-induced NO production in the skin

When the skin of the rostral back of the untreated mouse was microdialyzed, the basal concentration of NO was 2.1 ± 0.3 μM ($n = 21$). When bradykinin (10, 30 and 100 nmol/site) was intradermally injected near the microdialysis tube, the concentration of intracutaneous NO increased in a dose-dependent manner, with little change following saline injection (Fig. 2A). The increase in NO concentration following bradykinin (30 and 100 nmol/site) injection peaked at 5–10 min and had almost subsided by 50 min. Although the perfusion medium containing carboxy-PTIO revealed bradykinin-induced NO production in the skin, no apparent effects of bradykinin were observed when the skin was microdialyzed with the medium without carboxy-PTIO (Fig. 2B). Bradykinin itself affected neither the reaction with Griess' reagent nor the measurement of NO_2^- (data not shown).

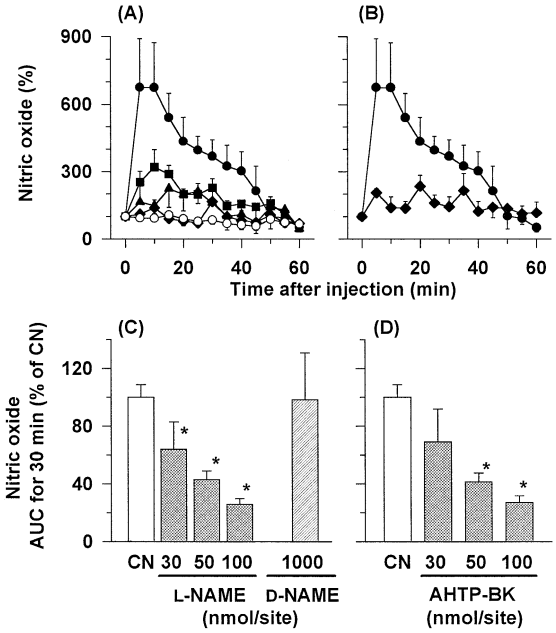

Fig. 2. Bradykinin-induced NO production in the mouse skin and its inhibition by NO synthase inhibitor and bradykinin B_2 receptor antagonist. (A) Time-course and dose–response relationship of NO production following an intradermal injection of bradykinin. The mouse was given bradykinin at doses of (▲) 10, (■) 30 and (●) 100 nmol/site, (♦) physiological saline or (○) without injection. (B) The effect of carboxy-PTIO on the detection of bradykinin (100 nmol/site)-induced NO production. The skin was microdialyzed using perfusion medium (●) with or (♦) without carboxy-PTIO. (C) Suppressive effects of NO synthase inhibitor, L-NAME, on NO production induced by bradykinin. Bradykinin (100 nmol/site) was intradermally injected alone (control, CN) or together with L-NAME or the inactive enantiomer D-NAME. (D) Suppressive effects of the bradykinin B_2 receptor antagonist, D-Arg-[Hyp^3, Thi^{5,8}, D-Phe^7]bradykinin (AHTP-BK) on NO production induced by bradykinin. Bradykinin (100 nmol/site) was intradermally injected alone (CN) or together with AHTP-BK. The ordinate indicates the extracellular concentration of NO at 5-min intervals (A and B) or the area under the curve (AUC) of the NO concentration for the initial 30 min after injection (C and D). Values are the means and S.E. for 6–7 animals. * $P < 0.05$ when compared with CN.

3.3. Effects of L-NAME and bradykinin B_2 receptor antagonist

To determine whether NO synthase activity was responsible for bradykinin-induced NO production, L-NAME was intradermally injected together with bradykinin. L-NAME at doses of 30, 50 and 100 nmol/site dose dependently ($F(3,24) = 8.81$, $P < 0.01$) suppressed the increase in NO concentration induced by bradykinin at a dose of 100 nmol/site (Fig. 2C). The inactive enantiomer, D-NAME, at a dose as high as 1000 nmol/site did not inhibit the effect of bradykinin (100 nmol/site) (Fig. 2C).

To determine whether bradykinin B_2 receptors were responsible for bradykinin-induced NO production, we examined the effect of a B_2 receptor antagonist on the bradykinin action. When D-Arg-[Hyp^3, Thi^{5,8}, D-Phe^7]bradykinin, a bradykinin B_2 receptor antagonist, at doses of 30, 50 and 100 nmol/site was intradermally injected together with bradykinin (100 nmol/site), the antagonist produced a dose-dependent suppression ($F(3,24) = 6.68$, $P < 0.01$) of bradykinin-induced NO production (Fig. 2D).

4. Discussion

The present results demonstrated that the NO concentration in the skin can be measured by intracutaneous microdialysis with carboxy-PTIO-containing perfusion medium. As PTIO reacts with $^{\cdot}NO$ to produce $^{\cdot}NO_2$, which does not require O_2 and is easily changed to NO_2^- (and NO_3^-) in the presence of water, the perfusion medium can be degassed to remove NO_x. In addition, the diazotization of NO_2^- was done on-line using degassed Griess' reagent. These procedures minimize the possibility of contamination from atmospheric NOx. Thus, the distinct features of this procedure are an in situ chemical change from $^{\cdot}NO$ to NO_2^- in the vicinity of NO production and the low possibility of contamination from atmospheric NO_x. The basal concentration of NO in the skin was determined to be about 2 μM, which was similar to the basal NO concentration in the brain (Ohta et al., 1994). Bradykinin produced a dose-dependent increase in intracutaneous NO concentration and this effect was inhibited by the NO synthase inhibitor, L-NAME, but not by D-NAME, findings strongly suggesting that the present system is useful for measuring the in situ production of NO. In contrast, a perfusion medium without carboxy-PTIO did not show the apparent effects of bradykinin, probably because of degassing of the medium.

Although it has been suggested that NO is involved in the hyperalgesia, vasodilatation and plasma extravasation induced by bradykinin (Warren and Loi, 1995; Feletou et al., 1996; Nakamura et al., 1996), there has been no direct demonstration of a bradykinin-induced increase in the concentration of NO in the skin. In the present experiments, an intradermal injection of bradykinin (10–100 nmol/site) dose dependently induced NO production in the mouse skin. The effect peaked at 5–10 min and had almost subsided by 50 min. This bradykinin action was suppressed by blocking of bradykinin B_2 receptors. On the other hand, hyperalgesia induced in the rat by an intradermal injection of bradykinin (3 nmol/site) peaks within 5 min and persist for 30 min (Nakamura et al., 1996). Assuming a significant role of bradykinin B_2 receptors in the earlier stage of inflammatory pain (Dray and Perkins, 1993; Khasar et al., 1995), these similarities of dose and time course between bradykinin-induced NO production and hyperalgesia strongly support the view that NO is responsible for bradykinin-induced hyperalgesia. Although the present results do not reveal the kind of cells that produced NO through bradykinin B_2 receptors, candidates are the endothelial cells (Sung et al., 1988; Warren and Loi, 1995), macrophages (Bockmann and Paegelow, 1995),

sensory neurons (Steranka et al., 1988) and fibroblasts (Geppetti, 1993), all of which express bradykinin B_2 receptors.

In conclusion, the present results show that intracutaneous microdialysis with perfusion medium containing carboxy-PTIO can be used to measure NO concentration in the skin. This system may be useful for pharmacological and physiological experiments on the role of NO in the skin.

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References

Akaike, T., Yoshida, M., Miyamoto, Y., Satoh, K., Kohno, M., Sasamoto, K., Miyazaki, K., Ueda, S., Maeda, H., 1993. Antagonistic action of imidazolineoxyl N-oxides against endothelium-derived relaxing factor/ ·NO through a radical reaction. Biochemistry 32, 827–832.

Balcioglu, A., Maher, T.J., 1993. Determination of kainic acid-induced release of nitric oxide using a novel hemoglobin trapping technique with microdialysis. J. Neurochem. 61, 2311–2313.

Barabé, J., Marceau, F., Theriault, B., Drouin, J.N., Regoli, D., 1979. Cardiovascular actions of kinins in the rabbit. Can. J. Physiol. Pharmacol. 57, 78–91.

Bockmann, S., Paegelow, I., 1995. Bradykinin receptors in signal transduction pathways in peritoneal guinea pigs macrophages. Eur. J. Pharmacol. 291, 159–165.

Dray, A., Perkins, M., 1993. Bradykinin and inflammatory pain. Trends Neurosci. 16, 99–104.

Feletou, M., Bonnardel, E., Canet, E., 1996. Bradykinin and changes in microvascular permeability in the hamster cheek pouch: Role of nitric oxide. Br. J. Pharmacol. 118, 1371–1376.

Geppetti, P., 1993. Sensory neuropeptide release by bradykinin: Mechanisms and pathophysiological implications. Regul. Pept. 47, 1–23.

Ialenti, A., Ianaro, A., Moncada, S., Rosa, M.D., 1992. Modulation of acute inflammation by endogenous nitric oxide. Eur. J. Pharmacol. 211, 177–182.

Ignarro, L.J., Buga, G.M., Wood, K.S., Byrns, R.E., Chaudhuri, G., 1987. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc. Natl. Acad. Sci. USA 84, 9265–9269.

Khalil, Z., Helme, R.D., 1992. The quantitative contribution of nitric oxide and sensory nerves to bradykinin-induced inflammation in rat skin microvasculature. Brain Res. 589, 102–108.

Khasar, S.G., Miao, F.J., Levine, J.D., 1995. Inflammation modulates the contribution of receptor-subtypes to bradykinin-induced hyperalgesia in the rat. Neuroscience 69, 685–690.

Marletta, M.A., Yoon, P.S., Iyengar, R., Leaf, C.D., Wishnok, J.S., 1988. Macrophage oxidation of L-arginine to nitrite and nitrate: Nitric oxide is an intermediate. Biochemistry 27, 8706–8711.

Nakamura, A., Fujita, M., Shiomi, H., 1996. Involvement of endogenous nitric oxide in the mechanism of bradykinin-induced peripheral hyperalgesia. Br. J. Pharmacol. 117, 407–412.

Ohta, K., Araki, N., Shibata, M., Hamada, J., Komatsumoto, S., Shimazu, K., Fukuuchi, Y., 1994. A novel in vivo assay system for consecutive measurement of brain nitric oxide production combined with the microdialysis technique. Neurosci. Lett. 176, 165–168.

Palmer, R.M.J., Ashton, D.S., Moncada, S., 1988. Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature 333, 664–666.

Regoli, D., Barabé, J., 1980. Pharmacology of bradykinin and related kinin. Pharmacol. Rev. 32, 1–46.

Steranka, L.R., Manning, D.C., De Haas, C.J., Ferkany, J.W., Borosky, S.A., Connor, J.R., Vavrek, R.J., Stewart, J.M., 1988. Bradykinin as a pain mediator: Receptors are localized to sensory neurons, and antagonists have analgesic actions. Proc. Natl. Acad. Sci. USA 85, 3245–3249.

Sung, C.P., Arleth, A.J., Shikano, K., Berkowitz, B.A., 1988. Characterization and function of bradykinin receptors in vascular endothelial cells. J. Pharmacol. Exp. Ther. 247, 8–13.

Warren, J.B., Loi, R.K., 1995. Captopril increases skin microvascular blood flow secondary to bradykinin, nitric oxide, and prostaglandins. FASEB J. 9, 411–418.