RAPID COMMUNICATION Jpn. J. Cancer Res. 85, 331–334, April 1994

Enhanced Vascular Permeability in Solid Tumor Is Mediated by Nitric Oxide and Inhibited by Both New Nitric Oxide Scavenger and Nitric Oxide Synthase Inhibitor

Hiroshi Maeda,[1] Youichiro Noguchi, Keizo Sato and Takaaki Akaike

Department of Microbiology, Kumamoto University School of Medicine, Honjo 2-2-1, Kumamoto 860

A newly discovered nitric oxide radical scavenger, an imidazolineoxyl *N*-oxide derivative, was used to investigate the role of nitric oxide radical (·NO) in the vascular permeability enhancement of solid tumor. Sarcoma-180 solid tumor in ddY mice was used for this experiment. Electron spin resonance spectroscopy was used to quantitate the reacted and unreacted scavenger. The results showed that extensive extravasation, assessed by intravenous injection of Evans blue, could be greatly suppressed by both ·NO scavenger administered orally and ·NO synthase inhibitor administrated intraperitoneally. This indicates that ·NO is responsible for the vascular permeability in solid tumors.

Key words: Tumor vascular permeability — Nitric oxide — Nitric oxide scavenger — Bradykinin

We have recently reported that stable radical compounds, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide derivatives (PTIOs), can react selectively with nitric oxide (·NO) by radical-radical reaction (Fig. 1)[1] and nullify various pathophysiological functions of ·NO such as hypotension induced by endotoxin.[1–3] In this report we describe the inhibitory effect of PTIO against enhanced vascular permeability in solid tumors. This result indicates that ·NO is a crucial messenger molecule affecting both permeability and blood flow of tumor blood vessels. Further, bradykinin may be an inducer of ·NO synthase, and ·NO may ultimately facilitate extravasation of plasma components in solid tumors.

Tumor tissue has unique vascular characteristics such as extensive angiogenesis,[4] which usually results in hypervasculature, irregular morphology with defective architecture,[5,6] response to angiotensin II[7,8] and enhanced permeability.[9–12] This enhanced vascular permeability in solid tumor is observed with macromolecules and lipids.[9–13] Furthermore, tumor tissue exhibits much slower recovery of these substances from the tumor interstitium into the lymphatics.[9–13] These phenomena are known as the EPR (enhanced permeability and retention) effect of solid tumors.[9–12]

The enhanced vascular permeability, which facilitates tumor growth and perhaps metastasis, is now known to be mediated by a number of factors including tumor vascular permeability factor (a protein with a molecular mass of about 40,000 Da),[14] bradykinin (and also [hydroxyprolyl^3]bradykinin),[15–17] tumor necrosis factor,[18] interleukin-2,[18] and others. However, we know of no report on the effect of ·NO. Here we present evidence that the enhanced vascular permeability of tumor tissue is indeed mediated by ·NO.

In this study S-180 tumor cells passaged in ascitic form were implanted in ddY mice subcutaneously with an inoculum size of 2×10^6 cells per injection site at the back. Tumors were allowed to grow to a palpable size, usually 3–5 mm in diameter, for about 10–12 days. Then, 0.2 ml of 0.2% Evans blue in physiological saline was injected via the tail vein, followed after 6 h by tumor removal under ether anesthesia. The removed tumor was minced for the extraction of Evans blue with formamide at 60°C for 48 h, and extracted dye was quantitated spectroscopically at 620 nm as described previously.[9]

To examine the possible role of ·NO in vascular permeability enhancement in solid tumors, the ·NO scavenger PTIO (Fig. 1) was dissolved at 43 m*M* in medium chain triglyceride (C_8: 95%) obtained from NOF Corporation, Tokyo, and a 0.5 ml aliquot was administered orally to S-180 tumor-bearing mice. The plasma concentration of PTIO was then determined at different time points after oral administration by electron spin resonance spectroscopy (ESR).[1] The results in Fig. 2 show ESR spectra of PTIO in blood; a sufficient concentration was attained and remained in blood for a few hours after oral administration.

Fig. 3 shows extravasated Evans blue in solid tumors (S-180 in ddY mice) in the presence or absence of PTIO and L-NAME (L-N^{ω}-nitro-L-arginine methylester). Since a sufficient plasma level of PTIO can be maintained for about 2.5 h (Fig. 2B), it was administered every 2 h (4 times), and Evans blue was injected at 1 h after the initial administration of PTIO via the tail vein of tumor-bearing mice as described. Similarly, aqueous L-NAME in saline was administrated intraperitoneally every 2 h (4 times), a total of 4.2 mg/kg in 8 h. Tumors with diameter between

[1] To whom correspondence and reprint requests should be addressed.

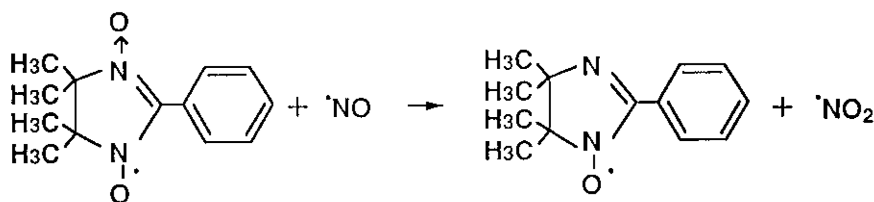
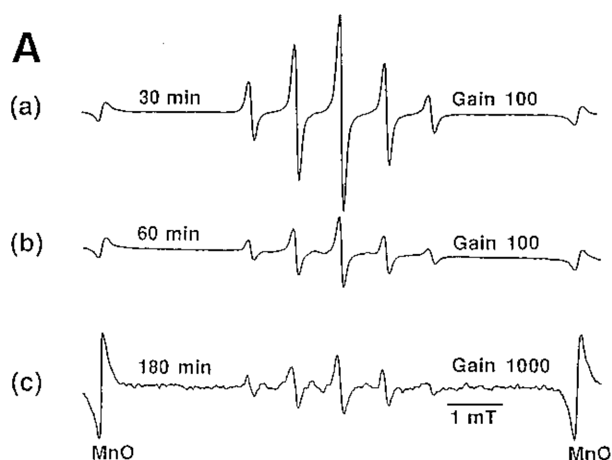


Fig. 1. Structure of PTIO, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, and reaction scheme with ·NO. The reaction products are 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl and $\cdot NO_2$.



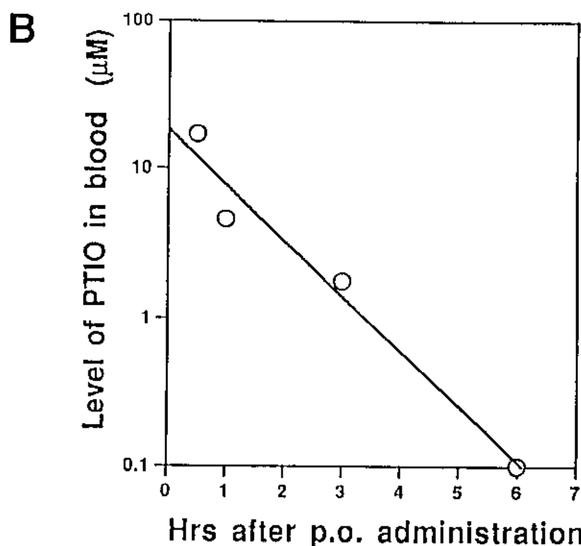


Fig. 2. Detection of PTIO in plasma by electron spin resonance spectroscopy. The ESR spectra shown in A are typical spectra, the same as that of authentic PTIO, and the concentrations of (a), (b), and (c) were quantified as 11.9 μM, 3.9 μM and 0.8 μM, respectively. PTIO (21.5 μmol in 0.5 ml) was given orally in mice and the ESR measurement for quantitation of PTIO was performed at the time points indicated. The amounts of PTIO found in the blood plasma are shown in B. (See text for details.)

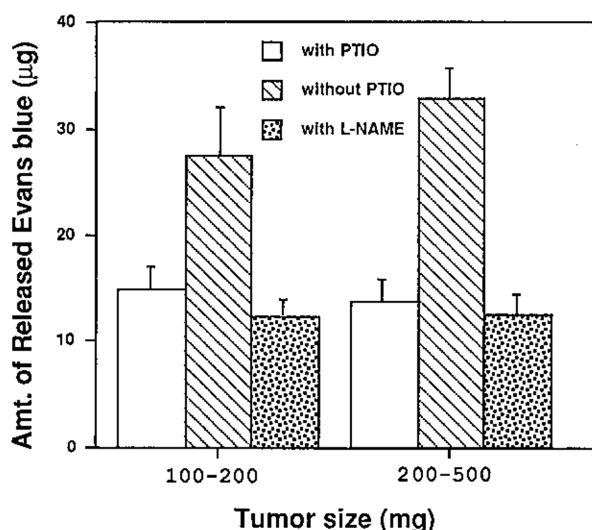


Fig. 3. Extravasation of Evans blue in S-180 tumor and inhibition by PTIO and L-NAME given via the oral and intraperitoneal routes in mice, respectively. (See text for details.) N=6 except for the group with PTIO, 200–500 mg tumor, in which N=8 and the L-NAME group, N=3. Each bar indicates SD.

3 mm and 7 mm were used in this study. A clear difference in extravasation with or without PTIO or L-NAME was seen (Fig. 3). Namely, PTIO suppressed the extravasation of Evans blue by 47 and 59% for small (100–200 mg) and large (200–500 mg) tumors, respectively. Similar extents of suppression were seen with L-NAME. Detailed analyses may be needed to clarify the dose-response relationship of PTIO, which will be described elsewhere.

We also tested the vascular permeability-enhancing effect of ·NO itself in guinea pig skin. ·NO gas was dissolved in biocompatible oil solution (similar to oily PTIO solution) at different concentrations, and 0.05 ml of the solution was injected intradermally into animals which had received Evans blue as described above. Oily PTIO was injected intraperitoneally into the guinea pigs, and the specimens of normal skin at the site of ·NO

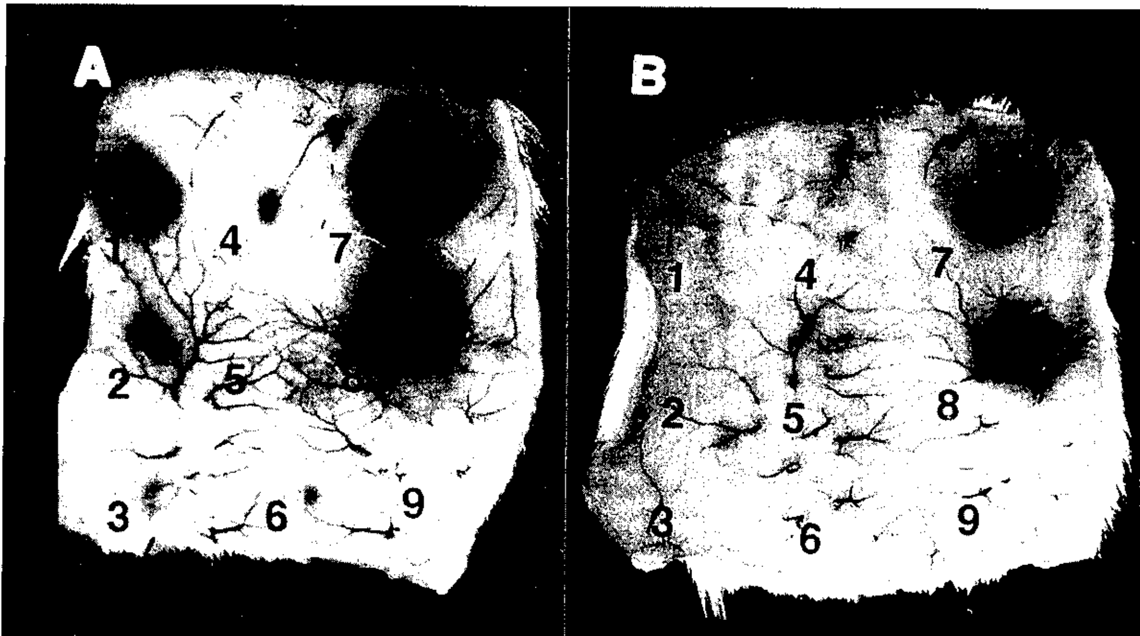


Fig. 4. Extravasation of Evans blue in guinea pig skin after intradermal injection of nitric oxide in oil, bradykinin in physiological saline and controls, and effect of the nitric oxide scavenger PTIO. (A) In the absence of PTIO. (B) In the presence of PTIO given intraperitoneally. 1, ˙NO, 0.85 μmol; 2, ˙NO, 0.44 μmol; 3, ˙NO, 0.29 μmol; 4, ˙NO, 0.14 μmol; 5, ˙NO, 0.08 μmol; 6, MCT, control vehicle; 7, bradykinin, 3 μg; 8, bradykinin, 1 μg; 9, saline control. (See text for details.)

injection were removed and quantified in the same manner as described for S-180 tumor, except that dye was allowed to permeate for 2 h. The results of this series of experiments showed that PTIO inhibited extravasation which was artificially induced with authentic ˙NO. These data support the hypothesis that the inhibitory effect of PTIO on extravasation in tumor tissue can be interpreted in terms of the generation of ˙NO by solid tumor tissues, possibly tumor vessels (Fig. 4).

We reported previously that vascular permeability enhancement is also mediated by bradykinin (and also [hydroxyprolyl^3]bradykinin),[15-17] and bradykinin may induce ˙NO production.[19-21] Myers *et al.*[21] and others[11, 20, 22] have suggested that this mechanism, i.e., stimulation of ˙NO production by bradykinin (as well as the cycloxygenase system) may be operating in solid tumor tissue and vasculature. Suppression of the inducible isoform of ˙NO synthase by dexamethasone in tumor tissue, especially in endothelium of tumor neovasculature, was reported during the preparation of this manuscript.[23] That finding is concordant with the present results if ˙NO is involved in the vascular permeability increase in tumor tissue via the following cascade; bradykinin → ˙NO synthase → ˙NO release → vascular relaxation → permeability-enhancing effect.

Our results show that (1) PTIO was delivered into blood via oral administration as judged by ESR measurement of PTIO, (2) both PTIO and L-NAME suppressed ˙NO-induced extravasation in normal guinea pig as well as S-180 tumor in mice due to this ˙NO-suppressing activity, and (3) enhanced extravascular permeation in solid tumor induced by bradykinin may be mediated by ˙NO.

A part of this work was supported by Grants-in-Aid for Science Research from the Ministry of Education, Science and Culture and the Ministry of Agriculture, Forestry and Fisheries of Japan, 1993 and 1994, to H.M.

(Received November 19, 1993/Accepted January 25, 1994)

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