NEW METHOD OF DETECTING NITRIC OXIDE PRODUCTION

Kazuya KIKUCHI,[a] Hiroshi HAYAKAWA,[b] Tetsuo NAGANO,[a] Yasunobu HIRATA,[b] Tsuneaki SUGIMOTO[b] and Masaaki HIROBE*,[a]
Faculty of Pharmaceutical Sciences[a] and Second Department of Internal Medicine,[b] Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan

Nitric oxide (NO) reacts with H_2O_2 to produce a rise in potent luminol-dependent chemiluminescence under neutral pH conditions. We applied this reaction to the continuous detection of NO released from the rat isolated perfused kidney with simultaneous pressure changes. Acetylcholine increased NO release dose-dependently with the pressure reduction. An NO synthesis inhibitor, N^G-monomethyl-L-arginine, abolished the release. This inhibition was reversed by the addition of L-arginine.

KEYWORDS nitric oxide; luminol; chemiluminescence; perfused rat kidney

It is well established that nitric oxide (NO) plays an important role as a signal transmitter not only in the vascular endothelium[1] but also in central[2] and peripheral neurons[3] and in phagocytic cells.[4] Several NO detection systems have been reported: chemiluminescence assay of reaction with ozone in the gaseous phase,[1] colinometric assay of the nitrite azo-coupling reaction,[5] HbO_2 oxidation[6] and GC-MS detection assay.[7] However, due to the limitations of sensitivity and/or NO detection in the gaseous phase, no system has been develpoed for the on-line and continuous assay of NO release. Furthermore, in physiological solutions NO is easily oxidized by O_2 to NO_2^- and NO_3^-, and its half life is very short (<6 seconds).[8] Generally, chemiluminescence assay is considered to be the most useful method for the highly sensitive and real time assay of unstable radicals at lower concentrations in physiological solutions. We found that the chemiluminescent reaction of NO with the luminol-H_2O_2 system was applicable to the detection of NO release.

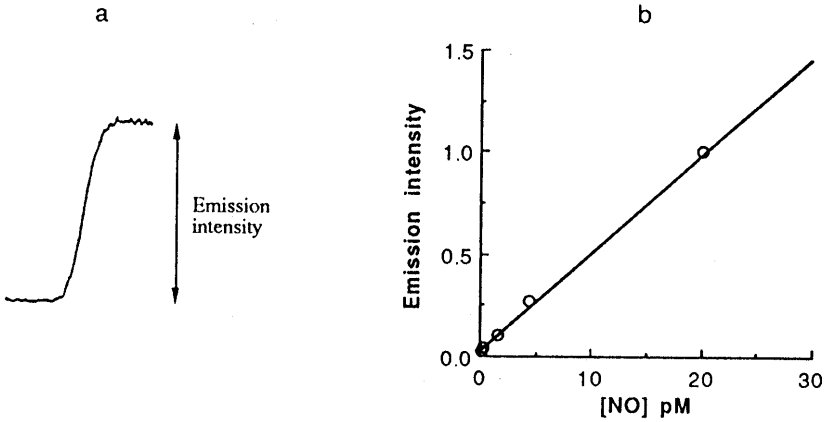

Fig. 1. Emission Profile (a) and NO Standard Curve (b)

Luminol reacts with H_2O_2,[9] but the emission intensity is very weak under neutral pH condition in the Krebs-Henselite buffer. When NO is present in the luminol-H_2O_2 system, significant emission can be monitored (Fig. 1a). Signal intensity, calibrated by NO standards, was linear between 100 fM and 1 nM (Fig. 1b). The standard NO solution was prepared according to the literature[10] and was quantified by the

HbO_2 method.[6] NO solution was set at a flow rate of 2 ml/min and was mixed with 0.5 ml/min chemiluminescence probe continuously. The mixer was connected with a chemiluminescence detector (825-CL, JASCO Corporation). NO derivatives like nitrite and nitrate did not exhibit chemiluminescence. Thio-nitroso compounds such as S-nitrosyl glutathione or S-nitrosyl cysteine exhibited emission 10^4 times less than NO. Compounds which are biologically active on the endothelium or endothelium-derived compounds: ACh, N^G-monomethyl-L-arginine(L-NMMA), L-Arg, PGI_2, endothelin-1, vasopressin, epinephrine, norepinephrine, adenosine, atrial natriuretic peptide or angiotensin II, exhibited no emission.

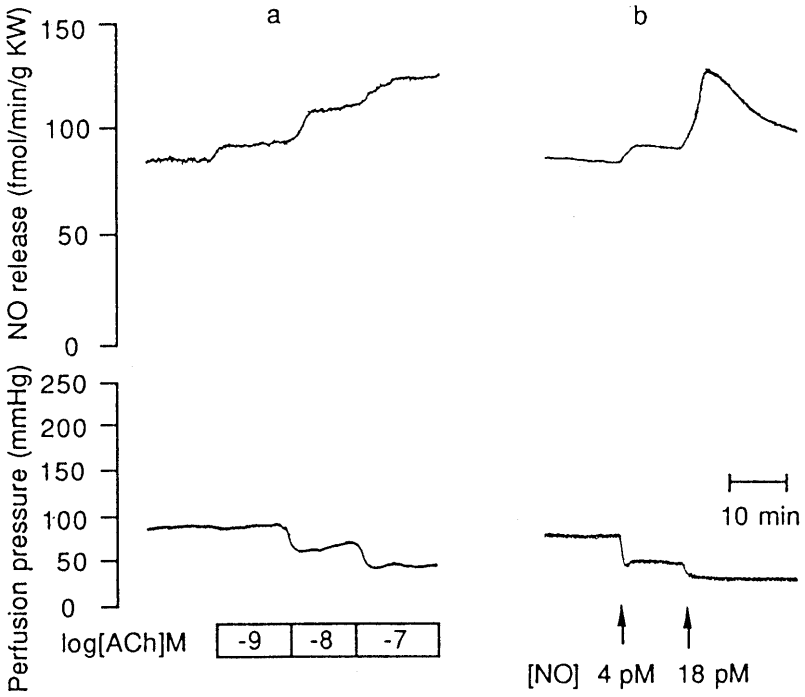

Fig. 2. Simultaneous Detection of the Renal Perfusion Pressure and NO

Kidneys from male Wistar rats were isolated and perfused as described previously.[11] The renal vein was also cannulated. The rat kidney was directly connected to the detector with a venous cannula. A rat perfused kidney was adopted as the source of NO because of its rich vascularity. Furthermore, its resistance vessel perfusion made simultaneous observation of NO release and renal perfusion pressure possible. The perfusate consisted of a Krebs-Henselite buffer and 10^{-6} M phenylephrine, oxygenated with 95% O_2-5% CO_2 at 37°C. The perfusion flow was set at 5ml/min. For chemiluminescence assay the perfusate was set at a rate of 2 ml/min, and the rest overflowed through the three-way needle. The renal effluent was mixed with 0.5 ml/min chemiluminescent probe (luminol 18 μM, desferrioxamine 150 μM, H_2O_2 10 mM, potassium carbonate 2 mM). The renal perfusion pressure and chemiluminescence were monitored simultaneously. Renal perfusion pressure and chemiluminescence became steady about 40 min after starting the perfusion. Basal NO release amounts were estimated to be 85±9 [SE] fmol/min/g KW (39 pM in the perfusate, n=5) from the chemiluminescence intensity (Fig. 2a). During renal vasodilation by 10^{-8} M ACh, NO release was quickly increased to 109±5 fmol/min/g KW ($p<0.05$, Fig. 2a). When 10^{-7} M ACh was then given, pressure fell by 49% and the released NO was further increased to 121±7 fmol/min/g KW ($p<0.05$). Thus, ACh increased NO release with a decrease in vascular resistance in a dose-dependent manner. Although changes in each NO release level and pressure occurred precisely on time, a lag time of 15 seconds was observed between changes in chemiluminescence and

pressure. When 10^{-4} M L-NMMA was added to 10^{-7} M ACh, with a rapid elevation of the perfusion pressure, NO release was thoroughly inhibited down to a value of 2%, and then gradually reversed to 58% of the level prior to L-NMMA administration. The perfusion pressure also showed a biphasic response with the same timing as NO changes. The addition of 10^{-3} M L-Arg then caused the chemiluminescence increase coincident with the pressure fall. Also, for addition of L-NMMA to the basal condition without ACh, dose-dependent inhibition of NO release was observed with the pressure elevation (data not shown). The infusion of SOD caused no significant change in either perfusion pressure or chemiluminescence. To exclude the possibility that changes in renal perfusion pressure *per se* affect the chemiluminescence, an endothelium-independent vasorelaxant, papaverine, was administered. Infusion of 10^{-5}M and 10^{-6}M papaverine reduced the pressure but did not influence the chemiluminescence (data not shown). These data also indicate that the compound which influence the chemiluminescence is endothelium-derived NO.

A standard NO solution (1 μM) in saline (0.9% NaCl) or vehicle was infused directly into the renal artery with an infusion pump through a double lumen needle at a rate of 1 ml/h. At this rate, the infusion of saline vehicle did not influence pressure or chemiluminescence. Fig. 2b demonstrates that two doses of NO solution (4 pM and 18 pM) also increased the chemiluminescence and decreased the pressure in a dose-dependent manner. To further confirm that changes in chemiluminescence and perfusion pressure are related to endothelium-derived NO, we examined the effects of the suppression of endothelial function. Treatment with 9 mM CHAPS for 30 seconds diminished the responses of NO to ACh. However, the exogenous NO infusion (4 pM) still increased the chemiluminescence and decreased the pressure. These observations in this system fully explain the involvement of changes in NO release in the regulation of vascular tone. In conclusion, a highly sensitive detection system for NO was developed using an aqua-chemiluminescence method. Application of this system to organ perfusion may enable us to promote the understanding of the pathophysiological role of NO.

Further studies on the chemiluminescence mechanism by the reaction of the luminol-H_2O_2 system with NO are now in progress.

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