Development of Polyethylene Glycol-Conjugated Poly-S-Nitrosated Serum Albumin, a Novel S-Nitrosothiol for Prolonged Delivery of Nitric Oxide in the Blood Circulation in Vivo

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Received April 5, 2005; accepted May 17, 2005

ABSTRACT

S-Nitrosothiols are an interesting class of nitric oxide (NO) donors used for the treatment of circulation disorders. In this study, we developed a novel macromolecular NO donor in which 10 NO molecules were covalently bound to polyethylene glycol (PEG)-conjugated bovine serum albumin (BSA) through S-nitrosothiol linkages (PEG-poly SNO-BSA). Intermolecular disulfide linkages possibly formed during the introduction of thiol groups to BSA were prevented in PEG-poly SNO-BSA. Electron spin resonance study indicated that PEG-poly SNO-BSA, the carrier part of S-N-succinimidyl S-acetylthioacetate (SATA)-BSA, the carrier part of PEG-poly SNO-BSA, was 1.7 times greater than that of 111In-SNO-BSA after intravenous injection in mice. After intravenous injection in rats at an equivalent NO dose (3 μmol of NO per kilogram), the duration of reduction in the blood pressure was 2.3 to 3.7 times longer in PEG-poly SNO-BSA than in classic S-nitrosothiols such as S-nitroso-N-acetyl penicillamine, S-nitrosothiol, and NO-BSA. The release half-life of NO from PEG-poly SNO-BSA was 11 to 108 times longer than those of the classic S-nitrosothiols examined, and this slow release rate of NO would explain the sustained reduction in the blood pressure after intravenous injection of PEG-poly SNO-BSA in rats. No cross-tolerance between PEG-poly SNO-BSA and nitroglycerin was also observed. These findings indicate that the novel S-nitrosothiol PEG-poly SNO-BSA is a promising compound that exhibits unique characteristics of sustained release of NO in the blood circulation in vivo, which would be beneficial for the treatment of circulation disorders.

Nitric oxide (NO) is a molecule that dynamically modulates the physiological functions of the cardiovascular system. They include vascular smooth muscle relaxation (Moncada et al., 1986; Ignarro et al., 1988), inhibition of platelet aggregation (Furlong et al., 1987), and immune regulation (Stuehr et al., 1989). Because NO elicits protective and beneficial actions in various disease states, NO delivery is expected to achieve therapeutic effects on essential hypertension, stroke, mean arterial pressure.

So far, compounds that can release NO have widely been used as therapeutic agents because of the limited utility of NO gas itself and its short half-life in vivo. Organic nitrates such as nitroglycerin, which have been used for many years to treat patients with ischemic heart diseases, are thought to act by generating NO upon administration. However, they induce tolerance, a state where the response to the treatment is diminished during long-term administration to patients (Glasser, 1999; Ignarro et al., 2002b). Sodium nitroprusside, another NO donor that is in clinical use, can induce cyanide toxicity (Ignarro et al., 2002b). The diazeniumdialates (NONOates) are potentially useful compounds that spontaneously generate NO (Ignarro et al., 2002b). However, they can be converted to N-nitroso compounds, which are potential carcinogens (Ragdale et al., 1965).
S-Nitrosothiols have several advantages over these other NO donors. They represent circulating endogenous reservoirs of NO and do not induce oxidant stress or vascular tolerance (Bauer and Fung, 1991; Jaworski et al., 2001). S-Nitrosothiols have been shown to improve recovery from dysfunctional conditions of organs suffering ischemic reperfusion injury in models of ischemic reperfusion injury (Gourine et al., 2002). Furthermore, S-nitrosothiols, such as S-nitrosoglutathione (GSNO), have also been administered to humans in small clinical trials (Leopold and Loscalzo, 2000). These properties of S-nitrosothiols make them attractive NO donors for the treatment of circulation disorders.

Because of its very short half-life and cytotoxic effect at high concentrations (Hibbs et al., 1988; Beckman and Crow, 1993; Laval and Wink, 1994), the tissue distribution of NO needs to be controlled to obtain its therapeutic benefits. However, little attention has been paid to the tissue distribution of NO donors. Of the various strategies available, conjugation of S-nitrosothiol to macromolecules seems to be a good approach to deliver NO to a target site, because the tissue distribution of macromolecules can be controlled by various techniques of chemical modification (Takakura and Hashida, 1996). In a recent study, we reported that a macromolecular S-nitrosothiol, S-nitrosated bovine serum albumin (NO-BSA), is a promising compound for the delivery of NO in vivo (Katsumi et al., 2004). However, we have also encountered several problems with NO-BSA; the number of molecules bound to BSA (0.25–0.28) is limited because only one free cysteine in BSA is available for conjugation, and the half-life of NO release in vivo is as short as that of other S-nitrosothiols, such as S-nitroso-N-acetyl penicillamine (SNAP) and GSNO. Some groups have attempted to synthesize poly SNO-BSA (Marks et al., 1995; Ewing et al., 1997), in which several S-nitrosothiols were conjugated to BSA after reduction of the disulfide linkages. However, poly SNO-BSA derivatives synthesized in this manner were easily aggregated because of the formation of intermolecular disulfide linkages during synthesis. The release rate of NO from S-nitrosothiols has been reported to be accelerated by heat and low molecular weight thiols, such as glutathione, and a trace amount of copper (Singh et al., 1996). Accordingly, controlled release of NO from S-nitrosothiols in vivo has hardly been achieved so far. Previous studies have shown that the thermal stability of S-nitrosothiols is increased in polyethylene glycol (PEG) solution, because the cage effect of PEG increased the stability of S-NO bonding (Shishido and de Oliveira, 2000). Therefore, we hypothesized that the conjugation of PEG to BSA controls the release rate of NO from S-nitrosothiol on BSA, increases the plasma retention of the conjugate in vivo, and prevents the intermolecular disulfide linkages induced by chemical introduction of thiol groups to BSA during synthesis.

The aim of this study is to develop a novel macromolecular S-nitrosothiol for prolonged delivery of NO in the blood circulation. To this end, we designed a new macromolecular NO donor in which 10 molecules of NO are covalently bound to polyethylene glycol-conjugated bovine serum albumin through S-nitrosothiol linkages (PEG-poly SNO-BSA). Then we examined the release rate of NO in a buffer solution and the tissue distribution after intravenous injection in mice. Finally, the vasodilating effects of PEG-poly SNO-BSA were evaluated in rats after its intravenous injection.

Materials and Methods

Animals. Male ddY mice (25–27 g) and male Sprague-Dawley rats (240–260 g) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Animals were maintained under conventional housing conditions. All animal experiments were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocols for animal experiments were approved by the Animal Experimentation Committee of the Graduate School of Pharmaceutical Sciences of Kyoto University.

Chemicals. BSA, sodium nitrite, sulfanilamide, and N-(1-naphthyl)ethylenediamine dihydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). NO-BSA was prepared by nitrosation of BSA with sodium nitrite as reported previously (Katsumi et al., 2004). Methoxypolyethylene glycol Nsuccinimidyl succinate (activated PEG; average molecular mass, 5200 Da) was supplied by NOP Corporation (Tokyo, Japan). GSNO, SNAP, N-(dithiothreitol)sarcosine disodium salt dihydrate (DTCS), diethylenetriaminepentaacetic acid (DTPA) anhydride, and EDTA were purchased from Djinga Laboratory (Kumamoto, Japan). 111Indium chloride ([111InCl3] was supplied by Nihon Medi-Physics (Takarazuka, Japan). N-Succinimidyl S-acetylthioacetate (SATA) was purchased from Pierce Chemicals (Rockford, IL). Nitroglycerin (Mirisrol, 0.5 mg/ml) was purchased from Nihon Kayaku Co. (Tokyo, Japan). DAF-FM was purchased from Daiichi Pure Chemicals Co. (Tokyo, Japan). All other chemicals were obtained commercially as reagent-grade products.

Synthesis of PEG-Poly SNO-BSA. PEG-poly SNO-BSA was synthesized as described below.

Conjugation of PEG with BSA. PEG-BSA conjugate was synthesized by reacting activated PEG with BSA in 50 mM borate buffer (pH 9.2) for 24 h at 4°C in the dark (Abuchowski et al., 1977). The reaction mixture was then washed and concentrated by ultrafiltration against distilled water.

Conjugation of SATA with PEG-BSA. PEG-BSA (15 mg as BSA) was dissolved in 3 ml of 50 mM sodium phosphate buffer containing 1 mM EDTA (pH 7.4), and then 10 mg of SATA dissolved in 40 μl of dimethyl sulfoxide was added (Duncan et al., 1983). The mixture was stirred for 90 min at room temperature, and PEG-poly SATA-BSA was purified by gel filtration at 4°C using a Sephadex G-25 column (Amersham Biosciences Inc., Piscataway, NJ) to remove unreacted SATA. The fractions containing the product were collected and concentrated by ultrafiltration at 4°C.

Deacetylation of PEG-poly SATA-BSA and measurement of thiol content. The acetyl group protecting the thiol group of SATA was removed with hydroxylamine as reported previously (Katsumi et al., 2004). The number of free amino groups was determined by Ellman’s reagent (Ellman, 1959).

Conjugation of NO. PEG-poly thiol-BSA (5 mg) and a 20-fold molar excess of sodium nitrite were dissolved in 1 ml of 0.1 M HCl. The mixture was stirred for 40 min at 37°C, and the reaction was terminated by neutralizing the solution at pH 7.5 by the addition of 0.1 M NaOH and 0.5 M Tris buffer.

Number of NO Adducts on PEG-Poly SNO-BSA. The number of NO adducts on PEG-poly SNO-BSA was determined by Saville assay (Saville, 1958), which has been described in a previous paper (Katsumi et al., 2004). The number of free amino groups was determined with trinitrobenzene sulfonic acid using glycine as a standard (Habeb, 1966).
Molecular Weight and Purity of PEG-Poly SNO-BSA. To estimate the apparent molecular weight and confirm the purity of PEG-poly SNO-BSA, samples were evaluated by nonreducing SDS-PAGE using a standard curve prepared with a set of marker proteins (full range rainbow marker; Amersham Biosciences Inc.) at 4°C.

Tissue Distribution Experiment. For the tissue distribution experiments, BSA and PEG-poly SATA-BSA, the carrier part of PEG-poly SNO-BSA, were radiolabeled with $^{111}$In using the bifunctional chelating agent DTPA anhydride according to the method of Hamamoto et al. (1982), which has been described in a previous paper (Katsumi et al., 2004). Each $^{111}$In-labeled compound was injected into the tail vein of mice at a dose of 1 mg of protein per kilogram. At appropriate times after injection, blood was collected from the vena cava under ether anesthesia, and the mice were then killed. Heparin sulfate was used as an anticoagulant. Plasma was obtained from the blood by centrifugation. The liver, kidneys, spleen, heart, and lungs were removed, rinsed with saline, and weighed; urine was also collected. The radioactivity in each sample was obtained from the blood by centrifugation. The liver, kidneys, spleen, heart, and lungs were removed, rinsed with saline, and weighed; urine was also collected. The radioactivity in each sample was counted using a well-type NaI scintillation counter (ARC-500; Aloka, Tokyo, Japan).

Calculation of Pharmacokinetic Parameter. The $^{111}$In radioactivity concentrations in plasma were normalized with respect to the percentage of the dose per milliliter and analyzed using the nonlinear least-squares program MULTI (Yamaoka et al., 1981). Although the plasma disappearance of the $^{111}$In-labeled proteins examined showed biexponential profiles, potential metabolites derived from $^{111}$In-labeled protein may partly contribute to the radioactivity in the plasma in later time periods. Therefore, we calculated the area under the concentration-time curve (AUC) based on a one-compartment model using sampling data except for the ones at the last time point.

In vitro NO Release from NO Donors. In vitro NO release from NO donors was assessed using the method described by Miles et al. (1996) with slight modifications. DAF-FM, a fluorescence probe of NO oxidant, was dissolved in 0.1 M phosphate buffer solution containing 1% bovine serum to give a concentration of 7 μM. NO donors were then added to the solution to give an equivalent NO concentration (160 μM) at 37°C in the dark. The excitation wavelength was 485 nm, and the emission was monitored at 535 nm. The fluorescence was continuously recorded with 1-min intervals in Wallac 1420 Multilabel Counter (PerkinElmer Life and Analytical Sciences, Boston, MA).

Calculation of Half-Life of NO Release from NO Donors in Vitro. The remaining NO donor concentration was estimated by subtracting the concentration of NO oxidized produced from the initial value. The half-life of NO release from NO donors was calculated using the least-squares regression from a semilogarithmic plot of the remaining NO donor concentration versus time.

Electron Spin Resonance Spectrometry. NO radical release from PEG-poly SNO-BSA in mice was confirmed by electron spin resonance (ESR) spectrometry. The mice received a saline solution of Fe$^{II}$-DTCS complex (200 mM, 4 ml/kg) by intraperitoneal injection followed by intravenous injection of PEG-poly SNO-BSA (1.5 μmol NO per kilogram) after a 30-min interval. Thirty seconds after the injection of PEG-poly SNO-BSA, blood was collected from the vena cava under ether anesthesia, and the mice were then killed. The blood was transferred to a flat quartz cuvette and placed in the cavity of a JM-FE3 ESR spectrometer (JEOL, Tokyo, Japan), and then the signal of the NO-Fe$^{II}$-DTCS spin adduct formed was measured immediately. ESR settings were as follows: magnetic field, 3295 G; microwave power, 5 mW; modulation frequency, 100 KHz; modulation amplitude, 3.2 G; response time, 0.1 s; amplitude, 10 × 100; and sweep width, 2 min.

Determination of Plasma Concentration of Nitrite Derived from NO Donors in Mice. Each NO donor was injected into the tail vein of mice at an equivalent NO dose (1.5 μmol of NO per kilogram). At appropriate times after injection, blood was collected from the vena cava under ether anesthesia, and the mice were then killed. Plasma was obtained from the blood by centrifugation. The plasma obtained was then subjected to ultrafiltration to remove proteins. The ultrafiltrate was immediately frozen in liquid nitrogen and stored at −30°C until assayed for the total nitrite ($\text{NO}_2^-$) level, which was measured by an automated NO detector-HPLC system (ENO-20; Eicom, Kyoto, Japan). This automated NO detector-HPLC system showed that the peak area of nitrite correlated well with nitrite concentration over a wide range (30 nM-10 μM, r$^2 = 1.000$) as reported previously (Yamada and Nabeshima, 1997), indicating that the measurement of nitrite within this range can be precisely performed. The lower limit of the detection was 30 nM in a 3-μl aliquot (coefficient of variation, <20%). The AUC of nitrites was calculated by a trapezoidal method using the observed values up to 60 min.

Measurement of Blood Pressure. Under pentobarbital anesthesia, the right femoral artery of rats was cannulated with polyethylene tubing, and the blood pressure was continuously recorded using a PowerLab/4sp (Bioresearch, Nagoya, Japan), with the animals being conscious. Each NO donor was administered to the rats by intravenous injection.

Vasodilating Effect of PEG-Poly SNO-BSA in the Nitroglycerin-Induced Tolerance Model. Nitroglycerin tolerance was induced by continuous 2 to 3 ml/h infusion of 0.5 mg/ml nitroglycerin solution over a 24-h period via a polyethylene cannula connected to the jugular vein using a syringe pump (Minamiyama et al., 2001) (Micro Feeder; Furue Science Co., Tokyo, Japan). At the end of the infusion, additional nitroglycerin (0.1 mg/kg) or PEG-poly SNO-BSA (27 mg of protein per kilogram) was given by an intravenous bolus injection, and the blood pressure was recorded as described above.

Results

Synthesis of PEG-Poly SNO-BSA. PEG-poly SNO-BSA had 10.3 molecules of NO conjugated via S-nitrosothiol linkages. The average molecular mass was estimated to be 250 kDa by the number of PEG on the molecule (Table 1). To evaluate the purity of the product, we performed nonreducing SDS-PAGE (Fig. 1). Without PEG, oligomers over a broad molecular mass were detected. On the other hand, there was only one band for PEG-poly SNO-BSA, indicating that the formation of the intermolecular disulfide linkages during the synthesis was prevented by the presence of PEG.

Distribution of $^{111}$In-BSA and $^{111}$In-PEG-Poly SATA-BSA after Intravenous Injection in Mice. Figure 2 shows the time courses of the concentration in plasma (top panels) and accumulation in the liver and kidney (bottom panels) of $^{111}$In radioactivity after intravenous injection of $^{111}$In-BSA and $^{111}$In-PEG-poly SATA-BSA in mice at a dose of 1 mg of protein per kilogram. As reported previously, $^{111}$In-BSA slowly disappeared from the blood circulation. The plasma retention of $^{111}$In-PEG-poly SATA-BSA, the carrier part of PEG-poly SNO-BSA, was much longer than that of $^{111}$In-BSA. $^{111}$In-PEG-poly-SATA-BSA underwent little initial uptake by any organ after intravenous injection but then gradually accumulated in the liver over time. Except for the liver

<p>| TABLE 1 |
|------------------|--------------|--------------|------------------|</p>
<table>
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<tr>
<th>SNO/BSA</th>
<th>Total NO/BSA</th>
<th>PEG/BSA</th>
<th>Molecular Mass</th>
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<td>NO-BSA</td>
<td>0.2</td>
<td>0.8</td>
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<tr>
<td>PEG-poly SNO-BSA</td>
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<td>10.3</td>
<td>36.6</td>
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<tr>
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Physicochemical characteristics of PEG-poly SNO-BSA

The molecular weights of compounds were estimated by nonreducing SDS-PAGE. The total NO adducts and S-NO adducts on BSA were estimated by the Saville assay. The number of PEG on the molecules was estimated by the trinitrobenzenesulfonate method.
and kidney, no significant radioactivity was recovered in any of the tissues examined after administration of $^{111}$In-BSA or $^{111}$In-PEG-poly-SATA-BSA (data not shown).

**Calculation of Pharmacokinetic Parameters.** To quantitatively compare the distribution profiles of $^{111}$In-BSA and $^{111}$In-PEG-poly SATA-BSA, the total body clearance ($\text{CL}_{\text{total}}$) and volume of distribution ($V_d$), as well as the area under the concentration-time curve, were calculated based on the distribution data (Table 2). The AUC of $^{111}$In-PEG-poly SATA-BSA was 1.7 times greater than that of $^{111}$In-BSA. The $\text{CL}_{\text{total}}$ and $V_d$ of $^{111}$In-PEG-poly SATA-BSA were much smaller than those of BSA, suggesting that the distribution of PEG-poly SNO-BSA from the blood circulation to tissues is limited by the presence of PEG.

**In Vitro NO Release from NO Donors.** Figure 3 shows the concentration of the remaining NO donors (total NO concentration, 160 $\mu$M) incubated in 0.1 M phosphate buffer solution containing 1% bovine serum at 37°C in the dark. The half-life of PEG-poly SNO-BSA was 11 to 108 times longer than that of classic S-nitrosothiols, such as SNAP, GSNO, or NO-BSA (Table 3).

**NO Radical Release from PEG-Poly SNO-BSA in Mice.** The water-soluble iron-DTCS complex reacted with NO to give an $[\text{Fe}^{II}(\text{DTCS})_2(\text{NO})]^2-$ (NO-iron-DTCS) complex with a three-line ESR signal ($g = 2.040; a_N = 1.27$ mT) near 700 MHz and X-band frequencies at room temperature. Because the stable and water-soluble NO-iron-DTCS complex produces an intense ESR signal at room temperature, the iron-DTCS complex can be used as a spin-trapping reagent for in vivo NO assay (Yoshimura et al., 1996; Yasui et al., 2004). A three-line ESR signal ($g = 2.040; a_N = 1.27$ mT) from mouse blood was observed 30 s after intravenous injection of PEG-poly SNO-BSA in mice (Fig. 4, Aa). No ESR signal was detected with saline (Fig. 4, Ab) and PEG-BSA-treated mouse blood (data not shown). These data indicate that PEG-poly SNO-BSA does release the NO radical in the blood circulation.

**Determination of Plasma Concentration of Nitrite Derived from NO Donors in Mice.** Figure 4B shows the plasma concentration of nitrite after intravenous injection of PEG-poly SNO-BSA or NO-BSA in mice. NO-BSA rapidly increased the plasma nitrite level at the early time point, whereas PEG-poly SNO-BSA gradually increased it. Furthermore, the nitrite in plasma was detected for a longer period of time after injection of PEG-poly SNO-BSA than after injection of NO-BSA. The AUC of nitrite from PEG-poly SNO-BSA calculated for 1 h after injection (124 $\mu$mol min/l) was greater than that from NO-BSA (94 $\mu$mol min/l). These results indicate that PEG-poly SNO-BSA gradually release NO in vivo.

**Vasodilating Effects after Intravenous Injection of NO Donors in Rats.** Figure 5 shows the mean arterial blood pressure (MAP) of rats after intravenous injection of SNAP, GSNO, and NO-BSA and PEG-poly SNO-BSA at a dose of 3 $\mu$mol of NO per kilogram. PEG-poly SNO-BSA showed a sustained reduction in the MAP, whereas SNAP, GSNO, and
NO-BSA resulted in a very short reduction. BSA and PEG-BSA had no significant effects on the blood pressure (data not shown). Figure 6 shows the dose dependence of the reduction of S-nitrosothiols. All of these parameters changed depending on the species and dose of S-nitrosothiols. Although the maximum effect of PEG-poly SNO-BSA on the reduction in the MAP was much smaller than that of SNAP and GSNO, the recovery time of the reduced blood pressure was much slower with PEG-poly SNO-BSA than SNAP and GSNO. All of the parameters of NO-BSA were the smallest of all of the S-nitrosothiols examined.

**Discussion**

Although there are many reports of possible therapeutic benefits of S-nitrosothiols in a variety of clinical situations, the therapeutic potential of many existing S-nitrosothiols is limited by their rapid NO release and poor distribution to the target site (Leopold and Loscalzo, 2000; Richardson and Benjamin, 2002). Therefore, the release rate of NO from S-nitrosothiols and its tissue distribution need to be well controlled to develop more effective NO delivery systems. Some groups have already attempted to synthesize poly SNO-BSA (Marks et al., 1995; Ewing et al., 1997), but intermolecular disulfide linkages were formed during synthesis. These observations suggest that poly SNO-BSA is easily aggregated because of the presence of highly reactive free thiol groups during synthesis. Those aggregates would not be suitable for in vivo application, because the physicochemical properties of BSA are markedly changed so that its tissue distribution cannot be controlled. To avoid aggregation, we first introduced PEG to BSA and succeeded in preventing the formation of intermolecular disulfide linkages during synthesis. Thus, PEG-poly SNO-BSA was successfully synthesized in a highly pure form.

PEG-poly SATA-BSA, the carrier part of PEG-poly SNO-BSA, exhibited a prolonged retention in the plasma after intravenous injection into mice, which was comparable with the results of another protein conjugate with PEG (Fujita et al., 1994). The plasma retention of PEG-poly SATA-BSA was much longer than that of PEG-poly BSA. Therefore, PEG-poly SNO-BSA can circulate in the blood for a long time, and it may have some advantages as far as the treatment of circulation disorders is concerned. Although 111In-PEG-poly SATA-BSA showed a sustained reduction in the MAP, whereas SNAP, GSNO, and NO-BSA induced a very short reduction. We successfully determined the NO radicals from PEG-poly SNO-BSA in vivo using an NO-trapping technique combined with ESR that has been applied to detect NO radicals in biological systems (Yoshimura et al., 1996; Yasui et al., 2004), although it is difficult to determine NO radicals in vivo because of their short half-life in vivo. This is the first direct demonstration that S-nitrosothiol releases NO radicals in the blood circulation. The ESR results and no significant changes in MAP following the administration of PEG-BSA strongly support the hypothesis that the released NO results in a reduction of blood pressure in rats. The nitrite in plasma from PEG-poly SNO-BSA after intravenous injection was detected for a longer period of time than that from NO-BSA and SNAP (data not shown). The measured release rates of NO in vitro from these compounds were in good agreement with the order of their duration of detectable nitrite in mice. This steady increase in NO levels without a peak release rate of NO would explain the sustained reduction in MAP after intravenous injection of PEG-poly SNO-BSA.
in rats. Although the release rate of NO from NO-BSA was much greater than that in PEG-poly SNO-BSA, NO-BSA showed a much weaker reduction in the blood pressure than PEG-poly SNO-BSA. This is probably due to the fact that NO-BSA contains not only S-nitrosothiol but also N-nitrosated tryptophan residues that have different effects on the blood pressure than S-nitrosothiols (Zhang et al., 1996). The pharmacological duration of NO-BSA and PEG-poly SNO-BSA in the MAP was much shorter than their duration of detectable nitrite in mice, probably due to the fact that blood pressure is also regulated by the nervous system regulating the cardiovascular system. Micromolar nitrite in plasma was detected at least 1 h after intravenous injection of PEG-poly SNO-BSA, indicating that PEG-poly SNO-BSA can release NO after recovery of the blood pressure. This prolonged release rate of NO from poly SNO-BSA in vitro was as fast as that from SNAP (unpublished data). These results indicate that conjugation of PEG can prolong the release rate of NO from S-nitrosothiol. Thus, the controlled release of NO was successfully achieved by the incorporation of PEG. The release of NO from S-nitrosothiols has been reported to be accelerated by heat and low molecular weight thiols, such as glutathione, and a trace amount of copper ion (Singh et al., 1996). However, it has been reported that the thermal decomposition of S-nitrosothiol is markedly inhibited in PEG solution because the cage effect of PEG increased the stability of S-NO bonding (Shishido and de Oliveira, 2000). Moreover, it has been reported that a flexible hydrophilic shell composed of PEG and its bound water would cover antigenic determinants and render the albumin inert to immune processes (Abuchowski et al., 1977). Such evidence indicates that the S-nitrosothiol surrounded by PEG is stabilized and prevented from decomposing by trace amounts of thiols and copper ion.

A major drawback of nitroglycerin is the induction of tolerance following repeated use. To our knowledge, this is the first report showing that macromolecular S-nitrosothiols show no cross-tolerance to the vasodilating effects, although it is well known that low molecular S-nitrosothiols, such as SNAP, do not induce tolerance (Bauer and Fung, 1991). The results showing no cross-tolerance suggest that PEG-poly SNO-BSA retains properties similar to those of classic low molecular S-nitrosothiols, in spite of the incorporation of S-nitrosothiols into the macromolecule. Furthermore, it has advantages over tolerance-producing nitrates that are currently in clinical use. Although the mechanism of nitroglycerin-tolerance remains to be elucidated, it has been reported that the vasodilating effect produced by nitroglycerin could be attributed to the formation and action of short-acting intermediate S-nitrosothiols at or near the vascular smooth muscle cell membrane, and depletion of endogenous low molecular thiols could cause nitroglycerin tolerance (Glasser, 1999; Fung, 2004). Because low molecular S-nitrosothiols, such as SNAP, can pass through the vascular endothelium, it is thought that low molecular S-nitrosothiols do not induce tolerance. In our preliminary experiments, the release of NO
from PEG-poly SNO-BSA in vivo was accelerated by the presence of thiols, such as reduced l-cysteine, as detected by ESR spectrometric measurement of nitrite and Dr. H. Yasui (Kyoto Pharmaceutical University, Kyoto, Japan) for helpful advice on the measurement of nitric oxide.

In conclusion, we have successfully developed a novel macromolecular S-nitrosothiol, PEG-poly SNO-BSA, that can release pharmacologically active NO at a sustained rate in vivo. PEG-poly SNO-BSA showed a relatively long circulation in plasma and a prolonged reduction in blood pressure. We also demonstrated an absence of cross-tolerance between PEG-poly SNO-BSA and nitroglycerin. These findings indicate that the novel S-nitrosothiol PEG-poly SNO-BSA is a promising compound with unique characteristics of sustained release of NO in vivo, which would be beneficial for the treatment of various circulation disorders.

Acknowledgments
We thank Dr. H. Nishino (Eicom, Kyoto, Japan) for providing the measurement of nitrite and Dr. H. Yasui (Kyoto Pharmaceutical University, Kyoto, Japan) for supporting the ESR spectrometric measurements. We also thank Dr. T. Kume and F. Osakada (Kyoto University) for helpful advice on the measurement of nitric oxide.

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