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Purified retinal nitric oxide synthase enhances ADP-ribosylation of rod outer segment proteins

Martin Zoche, Karl-Wilhelm Koch*

Institut für Biologische Informationsverarbeitung, Forschungszentrum Jülich, Postfach 1913, D-52425 Jülich, Germany

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Abstract Nitric oxide synthase is present in different cell layers of vertebrate retina and seems to have neuromodulatory functions in the outer retina. The enzyme, when purified from a bovine retina extract, has an apparent molecular mass of 160 kDa and resembles the neuronal constitutive NOS type I with respect to Ca^{2+}-calmodulin sensitivity, K_m value and inhibition by analogues of L-arginine. Retinal NOS is present in a preparation of rod outer segments attached to parts of the inner segments, but not in pure outer segments. We describe the enhancement of specific ADP-ribosylation of outer segment proteins by purified retinal NOS.

Key words: Retina; Nitric oxide synthase; Rod outer segment; ADP-ribosylation

1. Introduction

The inorganic gas molecule nitric oxide (NO) acts as a neuronal messenger in many cell types of the central and peripheral nervous system [1–3]. There is also evidence from recent studies that NO is involved in several different processes in the vertebrate retina, for example it influences light responses of amphibian rods [4,5], it activates a soluble guanylyl cyclase and modulates a Ca^{2+} channel and a voltage-independent conductance in inner segments of photoreceptors [6,7]. Effects of NO were also recorded on hemi-gap junction channels in horizontal cells [8], on cGMP-gated cation channels in retinal ganglion [9] and on-bipolar cells [10]. cGMP-independent effects of NO donors include the ADP-ribosylation of rod outer segment proteins [11,12] and modulation of endogenous dopamine release in the retina [13].

Presence of the NO-generating enzyme nitric oxide synthase (NOS) in the retina has been documented by immunocytochemistry and NADPH-diaphorase histochemistry [6,7,9,14–21], however, the data on cell staining are inconsistent. Most authors describe staining of subpopulations of amacrine and ganglion cells, but in some reports labelling of photoreceptor inner segments and cone outer segments was also demonstrated [6,7,17,19]. Additionally, NOS activities have been determined biochemically in crude retinal extracts [22,23] and in a photoreceptor preparation of outer segments attached to parts of the inner segment (OS-IS), where the NOS activity was almost exclusively found in the inner segment [6]. Enzymatic properties indicate a relationship between the retinal enzyme and the neuronal NOS type I from mammalian brain [24,25]. So far the retinal enzyme has not been purified or cloned and therefore knowledge of its molecular and enzymatic properties is limited. It was the aim of our study to identify and purify NOS from bovine retina in order to determine some of its key properties. Furthermore, we used isolated active retNOS in a reconstitution experiment with rod outer segment proteins to investigate changes in ADP-ribosylation of outer segment proteins.

*Corresponding author. Fax: (49) (2461) 614 216.

Abbreviations: retNOS, retinal nitric oxide synthase; ROS, rod outer segments; OS-IS, outer segments attached to parts of the inner segments; PMSF, phenylmethylsulfonylfluoride; H_4B, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; SNP, sodium nitroprusside; SIN-1, 3-morpholinosydnonimine.

2. Materials and methods

2.1. Materials

2′-5′-ADP-Sepharose 4B was obtained from Pharmacia, (6*R*)-5,6,7,8-tetrahydro-L-biopterin from ICN, [^{3}H]L-arginine and adenylate-[^{32}P]NAD from Amersham, β-NADPH from Biomol. All other reagents were obtained from Sigma unless otherwise noted. Zaprinast was a gift from Baker and May, and SIN-1 was a gift from Dr. P. Wohlfahrt (Hoechst).

2.2. Purification of retNOS

Bovine eyes were obtained from the local slaughterhouse, the retinae were directly removed and frozen at −80°C. All enzyme purification steps were performed at 4°C. 100–150 retinae were thawed and homogenized in 150 ml of buffer A (10 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, 12.5 mM 2-mercaptoethanol, 0.1 mM PMSF). The crude homogenate was centrifuged at 100,000 × *g* for 40 min. Solid ammonium sulfate (0.176 mg·ml^{-1}, 30%) was added to the supernatant to precipitate retNOS and centrifuged at 100,000 × *g* for 30 min. The pellets were washed with buffer A containing 0.176 mg·ml^{-1} $(NH_4)_2SO_4$ and then dissolved in buffer A. This fraction was centrifuged again at 100,000 × *g* for 30 min to remove unsoluble components. The dissolved ammonium sulfate precipitate (30 ml) was applied to 0.5 ml 2′-5′-ADP-Sepharose 4B (Pharmacia) equilibrated with buffer A. The solution was circulated four times through the Sepharose column. Afterwards, the column was washed with 20 ml of buffer A, 10 ml of buffer B (buffer A, 500 mM NaCl) and again with 10 ml of buffer A. The retNOS was eluted with 5 ml of buffer C (buffer A, 10 mM β-NADPH). Protein-containing fractions were pooled, and 2 mM $CaCl_2$ and 10 mM Tris-HCl, pH 7.5, were added.

The pooled fractions (6 ml) of the 2′-5′-ADP-Sepharose chromatography step were applied to 0.3 ml calmodulin-agarose (Sigma) eqilibrated with buffer D (20 mM Tris-HCl (pH 7.5), 2 mM $CaCl_2$, 12.5 mM 2-mercaptoethanol, 0.1 mM PMSF). The ADP-Sepharose eluate was passed four times through the column before the column was washed with 10 ml of buffer D, 10 ml of buffer E (buffer D, 400 mM NaCl) and again with 10 ml of buffer D. retNOS was eluted with buffer F (buffer D, 10 mM EGTA instead of 2 mM $CaCl_2$). Protein-containing fractions were pooled.

2.3. Enzyme assays

RetNOS activity was determined by the formation of [^{3}H]L-citrulline from [^{3}H]L-arginine as previously described [24,25]. Briefly, samples (50 μl) were incubated for 5 min at 37°C in the presence of 25 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$, 0.5 mM DTT, 0.1 mM β-NADPH, 5 μM H_4B, 1 μM FAD, 1 μM FMN, 0.5 μg calmodulin and 5 μM L-arginine (0.1 mCi/μmol) in a final volume of 100 μl. The reaction was stopped by adding 400 μl of 10 mM EGTA and 20 mM HEPES (pH 5.5) and

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applied to 1-ml columns of Dowex 50X8-200 (Sigma). The [^{3}H]L-citrulline was eluted twice with 0.75 ml of water and quantified by liquid scintillation spectroscopy. Free Ca^{2+} concentrations were adjusted by Ca^{2+}/EGTA buffer mixtures calculated with a calcium buffer program as previously described [26]. Functional coupling of retNOS and soluble guanylyl cyclase was determined a described [6], but Zaprinast was used as a phosphodiesterase inhibitor instead of 3-isobutyl-1-methylxanthine (IBMX). Neuronal NADPH diaphorase activity was assayed with 0.5 mM Nitro blue tetrazolium and 1 mM NADPH exactly as described [27].

2.4. [^{32}P]ADP-ribosylation of photoreceptorproteins

ADP-ribosylation was carried out following essentially the method of Pozdnyakov et al. [12]. ROS suspension (20 μg rhodopsin/assay) or ROS cytosolic fraction (8 μg protein/assay) were incubated in a final volume of 50 μl containing 50 mM Tris-HCl, pH 7.5, 5 mM DTT, 50 mM NaCl, 2 mM $MgCl_2$, 1 mM ATP, 0.1 mM GTP, 10 mM thymidine and 10 μM NAD (2.5 μCi adenylate-[^{32}P]NAD/assay). In reconstitution experiments 0.5 μg purified retNOS were added to the incubation mixture containing 1 μM FAD, 1 μM FMN, 5 μM H_4B, 100 μM L-arginine and 100 μM NADPH. After 60 min of incubation at 37°C the reaction was stopped by the addition of electrophoresis sample buffer. The samples were electrophoresed in 10% SDS-polyacrylamide gels. After Coomassie blue staining and drying, the gels were exposed to Kodak X-OMAT AR films for 72 h at −80°C.

2.5. Protein determination

Protein concentrations were determined by the method of Bradford [28] using the Bio-Rad assay solution and bovine serum albumin as a standard.

2.6. Gel-electrophoresis

SDS-PAGE was carried out with 7.5% or 10% polyacrylamide gels [29]. The gels were stained for protein using Coomassie blue dye. The high molecular mass standards (Pharmacia) included myosin (212 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), glutamate dehydrogenase (53 kDa) and the low molecular mass standard (Pharmacia) included phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carboanhydrase (30 kDa), trypsine inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

3. Results and discussion

Retinal nitric oxide synthase (retNOS) could be purified from bovine retina extract by ammonium sulfate precipitation and two subsequent chromatographic steps, first on 2′-5′-ADP-Sepharose and second on calmodulin-agarose (Table 1). Ammonium sulfate precipitation yielded a pellet that contained over 60% of NOS activity present in the retina extract and about 20% of the soluble proteins. After dissolving the ammonium sulfate precipitate retNOS was specifically bound to a 2′-5′-ADP-Sepharose. NOS activity was eluted from the column by 10 mM β-NADPH. The protein composition of this fraction is shown in Fig. 1 (lane 2). The fraction contained a distinct band at 160 kDa and several more intensely labelled bands at 36 kDa, 55 kDa, 65 kDa and a doublet at 104 and 116 kDa. Affinity chromatography on 2′-5′-ADP-Sepharose was an effective purification step for retNOS with a 1000-fold increase in specific activity. Contaminating proteins were separated from the band at 160 kDa by calmodulin-affinity chromatography. RetNOS activity was eluted by 10 mM EGTA. Eluted fractions contained a single band at 160 kDa (Fig. 1, lane 3). Occasionally we observed some minor bands below 100 kDa

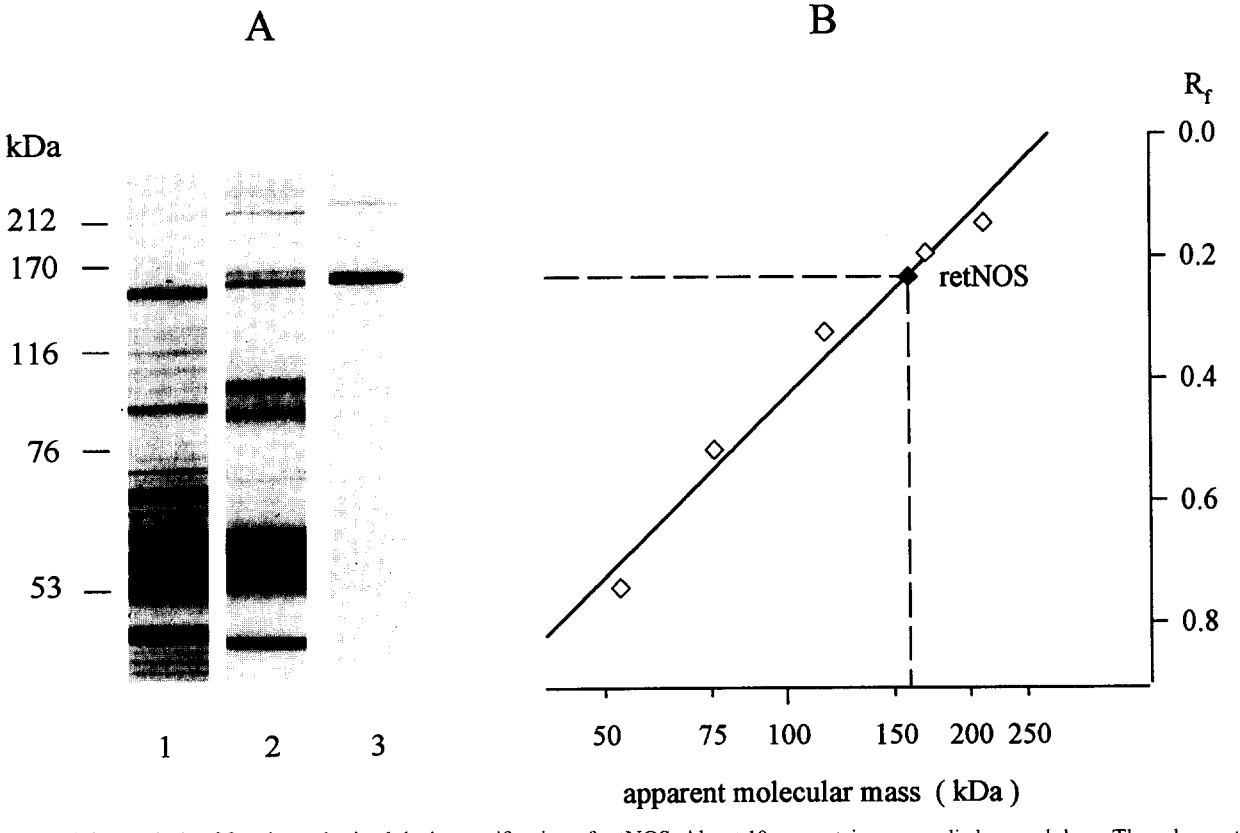

Fig. 1. SDS-PAGE analysis of fractions obtained during purification of retNOS. About 10 μg protein was applied on each lane. The gel was stained with Coomassie blue. (A) Lanes: (1) retina extract after centrifugation at 100,000 × *g*; (2) NADPH eluate of the 2′-5′-ADP-Sepharose column; (3) EGTA eluate of the calmodulin-agarose column. (B) The molecular mass of purified retNOS was determined in a R_f vs. log mass plot to be 160 kDa.

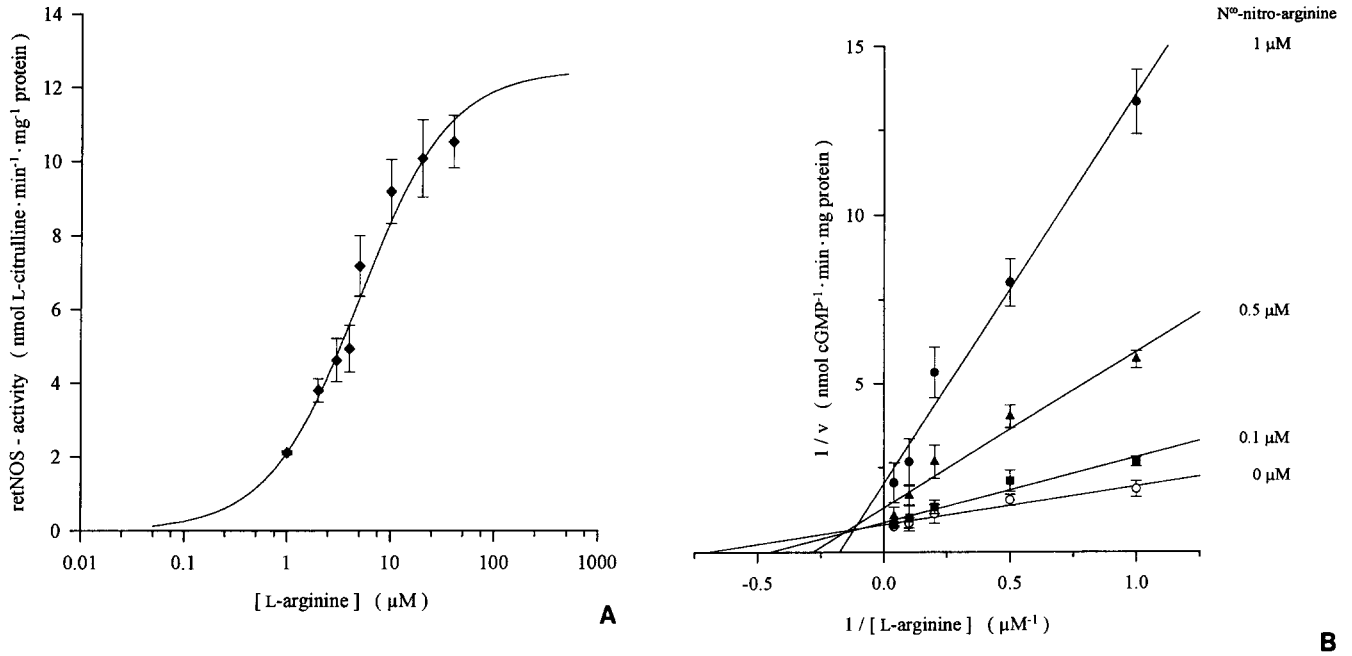

Fig. 2. Enzymatic properties of retNOS. (A) Purified retNOS (~0.5 μg) was incubated with different amounts of the substrate L-arginine. Activity was determined by measuring the formation of [^{3}H]L-citrulline. (B) Lineweaver–Burk plot showing inhibition of retNOS by N^{ω}-nitro- arginine (inhibitor concentration as indicated on the right). Activity of retNOS was monitored by the formation of cGMP catalyzed by an endogenous soluble guanylyl cyclase in rod OS-IS preparations in a coupled bioassay [6].

that could be removed mostly by ultrafiltration with Centricon-100. Purified retNOS exhibited a specific activity of 3.6 nmol L-citrulline $min^{-1} \cdot mg^{-1}$. Starting from a retina extract of 80 retinae (1.2 g protein) we obtained 0.01 mg retNOS with 0.4% recovery of total activity. The overall enrichment in purification was 1600-fold. Purified retNOS has a half-life of 12 h either at 4°C or −20°C. Activity of retNOS in a retina extract was stable over 24 h at 4°C.

The sequential use of two affinity columns, 2′-5′-ADP-Sepharose and calmodulin-agarose, closely resembled the purification scheme of the neuronal type of a NOS [24,25] and indicated a dependence of enzyme activity on NADPH and calmodulin. No retNOS activity was detected without addition of NADPH and calmodulin to the purified protein sample. Activity of retNOS was also dependent on the cofactors FAD and H_4B [6]. Omitting either FAD or H_4B in the incubation mixture yielded retNOS activities of 40% and 25%, respectively. Fractions obtained from chromatographic procedures were also assayed for neuronal NADPH-diaphorase activity [27]. All fractions that showed NOS activity in the [^{3}H]L-citrulline assay exhibited NADPH-diaphorase activity, but diaphorase activity was also detected in fractions devoid of retNOS. L-Arginine conversion by retNOS could be blocked by the diaphorase substrate Nitro blue tetrazolium.

Enrichment of retNOS activity resulted in isolation of a 160 kDa protein, which is in agreement with our previous observation that crude retinal extracts show immunoreactivity of a 160 kDa band with an anti NOS-I antibody [6]. Purified retNOS was also immunolabelled by the same antiserum (data not shown). Kinetic data for purified retNOS revealed a K_m for L-arginine of 4.8 μM and a V_{max} of 12.5 nmol $\cdot min^{-1} \cdot mg^{-1}$ (Fig. 2A). The calcium-dependent activation without competing Mg^{2+} ions but in the presence of saturating amounts of calmodulin was 0.36 μM. Enzyme activity was specifically inhibited by N^{ω}-nitro-arginine with an apparent K_I of 0.2 μM. Analysis of inhibition by N^{ω}-nitro-arginine in a Lineweaver–Burk plot (Fig. 2B) uncovered a mixed-type of inhibition. This is in agreement with the observation that only the binding of the arginine derivative to brain NOS is competitive, but that inhibition of the enzyme is more complex [30]. Inhibition by N^{ω}-nitro-arginine is a useful pharmacological tool to distinguish constitutive NOS from the inducible type as expressed in macrophages, since the latter isoform shows a much higher inhibitory constant of 212 μM [1]. Inducible NOS is expressed in bovine retinal pigmented

Table 1
Purification of NO synthase from bovine retinae

Purification step	Protein concentration ($\mu g \cdot ml^{-1}$)	Total activity ($pmol \cdot min^{-1}$)	Specific activity ($pmol \cdot min^{-1} \cdot mg^{-1}$)	Purification (-fold)
Retine extract	10,375	950	2.29	
$(NH_4)_2SO_4$ precipitation	2400	597	332	145
ADP-Sepharose elution	180	450	2488	1100
Calmodulin-agarose elution	10	4	3568	1600

The enzyme was purified as described. NOS activity of each fraction was determined by measuring the formation of L-citrulline from L-arginine.

epithelial cells and retinal Müller glial cells after treatment with interferon-γ or lipopolysaccharides [31–33].

A comparison of the neuronal NOS purified from human, rat and porcine brain [24,25,34,35] with bovine retNOS revealed in most cases similar properties (Table 2) for both enzymes, but displayed one remarkable difference. The retinal enzyme showed a 10- to 100-fold lower V_{max}, yielding a very low turnover number for the purified enzyme of 1.8 L-citrulline or NO per min. At present we cannot decide whether this low turnover number is a specific property of NOS in a highly specialized neuronal tissue (retina) or reflects a loss of enzymatic activity. There is recent evidence that NO has neurotoxic effects on retinal tissue. Phagocytosis of photoreceptor outer segments is decreased by addition of the NO donor SIN-1 to cultured RPE cells [36], and NOS inhibitors like N^{ω}-nitro-arginine can prevent photoreceptor degeneration [37]. Production of NO in the retina is probably kept low in order to save neurons from its cytotoxic potential. It will be interesting to elucidate the molecular basis of the observed differences and whether increased NO synthesis is involved in retinal degeneration.

ROS contain an ADP-ribosyltransferase activity that can be regulated by the NO donor SNP [11,12]. We observed a similar effect with the compound SIN-1 known to release NO in neutral aqueous solution. NO mainly increased the ADP-ribosylation of a 37–39 kDa protein, presumably α-transducin, as previously reported [11,12]. We also detected a stimulated labelling of a band at 110 kDa in the membrane fraction by SNP as reported in [12], but its ADP-ribosylation was not significantly altered by SIN-1 or retNOS (Fig. 3). It was previously suggested that NO generated by an endogenous NOS in ROS can have the same effect on ADP-ribosylation [12]. However, immunocytochemical, NADPH-diaphorase histochemical and biochemical studies have demonstrated the presence of retNOS in inner segments but not in ROS [6,7]. Consistent with these studies we detected no significant NO synthesis in pure ROS and consistently no change in ADP-ribosylation. However, adding purified retNOS to ROS resulted in the labelling of α-transducin that we observed after addition of SIN-1 (Fig. 3). Furthermore, using a photoreceptor preparation of rod OS-IS we spotted ADP-ribosylation to the same extent (not shown). Our data provide biochemical evidence that ADP-ribosylation of ROS proteins, particularly transducin, is regulated by NO that is synthesized from retNOS located in inner and not in outer segments. We hypothesize a feedback communication between the photoreceptor inner and outer segment transmitted by the neuronal messenger NO.

Table 2
Properties of retNOS and NOS-I

Type	retNOS	NOS-I
Source	Retina	Cerebellum
Molecular weight from SDS-PAGE (kDa)	160	150–160
L-Arginine, K_m (μM)	4.8	2–8
V_{max} ($\mu M \cdot min^{-1} \cdot mg^{-1}$)	0.0125	0.3–3.4
Nitro-L-arginine, K_I (μM)	0.2	0.05–0.9
Ca^{2+}, EC_{50} (μM)	0.36	0.2–0.4
Activity without		
Calmodulin	None	None
β-NADPH	None	None

Data of ret NOS were obtained from 3–5 sets of experiments and represent mean values. Data of NOS-I were taken from a review [1].

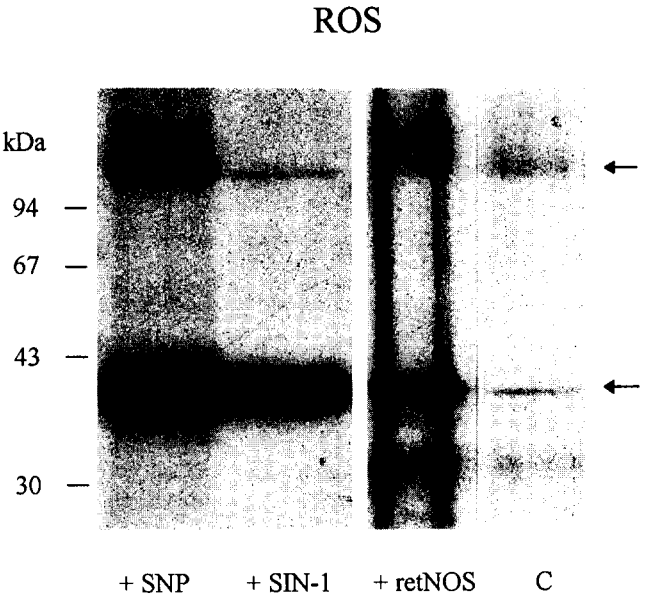

Fig. 3. Autoradiography of [^{32}P]ADP-ribosylated ROS proteins enhanced by nitric oxide. Whole ROS were incubated with either 100 μM SNP or 100 μM SIN-1 or 0.5 μg retNOS (in the presence of 100 μM L-arginine and 100 M β-NADPH. Lane C represents the control incubation without artificial or physiological NO donors.

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