Biochemical Pharmacology, Vol. 41, No. 2, pp. 273–279, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

CONTAMINATION OF ADENOSINE DEAMINASE BY SUPEROXIDE DISMUTASE

STABILIZATION OF ENDOTHELIUM-DERIVED RELAXING FACTOR

JOEL LINDEN,* M. RENEE PRATER, GAIL W. SULLIVAN,† ROGER A. JOHNS‡ and AMRAT PATEL

Departments of Internal Medicine (Cardiology), Physiology, †Infectious Diseases, and ‡Anesthesiology, University of Virginia, Health Sciences Center, Charlottesville, VA 22908, U.S.A.

(*Received* 9 *May* 1990; *accepted* 14 *August* 1990)

Abstract—We have measured cyclic GMP accumulation in co-cultures of bovine aortic endothelial cells and rat smooth muscle cells as an index of endothelium-derived relaxing factor (EDRF) production. Adenosine deaminase (EC 3.5.4.4, Sigma type VI) produced a 5- to 10-fold increase in the basal and bradykinin-stimulated cyclic GMP content of co-cultures but had no effect on smooth muscle cells alone. Cyclic GMP accumulation in response to adenosine deaminase was not blocked by adenosine deaminase inhibitors or affected by adenosine, the products of adenosine deamination (inosine and ammonia), or adenosine receptor antagonists. Since superoxide anion is known to destroy EDRF and nitric oxide (NO) (which is similar or identical to EDRF in composition), we tested for superoxide dismutase (SOD, EC 1.15.1.1) in single lots of eight commercial sources of adenosine deaminase by measuring inhibition of the superoxide-mediated reduction of cytochrome *c*. SOD activity was found in all sources of adenosine deaminase, but varied widely. One lot of Sigma type VI enzyme contained 0.08 units SOD/unit adenosine deaminase. The EC_{50} values of purified SOD (0.23 units/mL) and Sigma type VI adenosine deaminase (2.1 units/mL) needed to increase the cyclic GMP content of co-cultures differed by a similar factor, 0.11. Thus, the SOD activity in adenosine deaminase is sufficient to account for its effect on cyclic GMP accumulation. One lot of Boehringer Mannheim adenosine deaminase contained much less SOD contamination (0.006 units SOD/unit adenosine deaminase) and produced much less accumulation of cyclic GMP in co-cultures. Cyclic GMP accumulations in response to adenosine deaminase and SOD were both abolished by the NO synthetase inhibitor N^G-monomethyl-L-arginine (0.1 mM), consistent with the idea that these enzymes act by stabilizing EDRF. Adenosine deaminase and the SOD activity contaminating it were found to have similar molecular masses of 33–34 kD as assessed by gel permeation chromatography. When run under reducing conditions to dissociate homodimeric SOD into monomers, a 16.6 kD peptide which co-migrates with purified cupro-zinc SOD was visible in silver-stained sodium dodecyl sulfate–polyacrylamide gels of the Sigma type VI but not the Boehringer Mannheim adenosine deaminase. We conclude that commercial sources of adenosine deaminase are variably contaminated by SOD. Since EDRF is synthesized by many tissues, the use of adenosine deaminase contaminated with SOD may produce numerous effects not attributable to the deamination of adenosine.

In order to examine radioligand binding to adenosine receptors, or to evaluate the biochemical and physiological consequences of adenosine receptor activation, it is necessary to deplete tissues, cells, or membranes of endogenously produced adenosine [1]. This is usually done by adding adenosine deaminase (EC 3.5.4.4) which converts adenosine to inosine and ammonia. Inosine is a very weak activator of adenosine receptors. To measure the effects of adenosine receptor occupancy in the presence of adenosine deaminase, analogs of adenosine which activate adenosine receptors and are resistant to adenosine deaminase can be added.

Adenosine and the adenosine deaminase resistant adenosine analog, 5′-*N*-ethylcarboxamidoadenosine (NECA), relax vascular smooth muscle through A_2 receptors. Although an intact endothelium is not required for adenosine receptor-mediated relaxation of vascular smooth muscle, some studies indicate that the response is mediated in part by endothelial cells [2, 3]. In this report we describe an effect of adenosine deaminase to enhance the action of endothelium-derived relaxing factor (EDRF) released from co-cultures of endothelial and smooth muscle cells.

Endothelial cells produce superoxide anion [4] which destroys EDRF [5]. Consequently, the half-life of EDRF is prolonged by superoxide dismutase [5] (SOD, EC 1.15.1.1). Superoxide anion readily oxidizes nitric acid (NO) which is consistent with recent evidence which suggests that EDRF is similar or identical in composition to NO [6]. We now report that adenosine deaminase enhances cyclic GMP accumulation in co-cultures of endothelial and

* Address correspondence to: Dr. Joel Linden, University of Virginia, Box 158, Health Sciences Center, Charlottesville, VA 22908.

smooth muscle cells and that this activity can be attributed to contaminating SOD. Contaminating SOD may complicate the interpretation of many studies in which adenosine deaminase has been used to evaluate the effects of purines on vascular and other tissues.

MATERIALS AND METHODS

Cyclic nucleotide determinations in cultured cells. Co-cultures of bovine aortic endothelial cells and rat aortic smooth muscle cells were prepared as described [7, 8]. In brief, aliquots of confluent endothelial cells were resuspended in serum-free medium (M199) and added to 24-well cluster plates containing confluent smooth muscle cells. Endothelial aliquots contained $3–5 \times 10^5$ cells on 25 mg of 100 μm diameter Cytodex microcarrier beads. After a 3-h equilibration period the medium in each well was replaced with 1 mL of phosphate-buffered saline containing various enzymes and drugs, and incubated for 3 min. When bradykinin was used to stimulate EDRF production, it was added for the last 40 sec of the incubation period. Assays were terminated by aspirating off the saline and replacing it with 0.5 mL of 0.1 M HCl. ^{125}I-Labeled monosuccinyl cyclic nucleotide tyrosine methyl esters were prepared and used for the automated radioimmunoassay of cyclic nucleotides as described [9]. Cyclic nucleotides were acetylated by adding 12.25 μL of triethylamine/acetic anhydride (3.5:1, v/v) to 250 μL of the HCl extract. In some experiments cyclic AMP accumulation was measured in 24-well cluster plates containing only confluent monolayer cultures of endothelial cells. For these experiments adenosine deaminase (2 units/mL), Ro20-1724 (a phosphodiesterase inhibitor, 20 μM) and various concentrations of NECA were co-incubated for 1 min. Cyclic nucleotide data are reported as the means ± SEM of quadruplicate determinations, each assayed in duplicate. To compare cyclic GMP responses within experiments the data were analyzed by ANOVA and the Dunnett or Tukey test. Results were considered significantly different at $P < 0.05$. Each figure is representative of 2–3 similar experiments. To calculate the EC_{50} values of adenosine deaminase and SOD needed to elevate cyclic GMP accumulation in co-cultures, the data were fit to the equation:

$$\text{response} = \text{basal} + \text{maximal} \times [\text{enzyme}]/(EC_{50} + [\text{enzyme}]).$$

Gel permeation chromatography. Samples of adenosine deaminase or molecular weight standards (thryoglobulin, 670 kD; γ-globulin, 158 kD; ovalbumin, 44 kD; myoglobulin, 17 kD; and vitamin B12, 1.35 kD) were prepared in 0.1 N NaCl, 5 mM NaH_2PO_4, pH 6, and applied at a rate of 0.5 mL/min to a 7.5 × 300 mm Bio-Sil TSK-250 gel filtration column. The column eluate was monitored at 280 nm and 1-mL fractions were collected for subsequent analyses of adenosine deaminase and SOD activities. The standards were fit to the equation: $\log M_r = C_1 + C_2[(V_e - V_o)/V_b - V_o)]$ where V_e is the elution volume, V_o is the void volume, V_b is the bed volume, and C_1 and C_2 are constants. The correlation coefficient of the least squares line fitting the standard curve was 0.991.

Adenosine deaminase activity. The rate of conversion of adenosine to inosine was determined by continuously measuring $A_{260} - A_{248.5}$ following the addition of 0.05 to 1 units/mL of adenosine deaminase to 1 mL of buffer (5 mM phosphate, 150 mM NaCl, pH 6.0). In some cases the enzyme was preincubated with the adenosine deaminase inhibitors erythro-9(2-hydroxy-3-nonyl)-adenine (EHNA, 0.1 mM) or 2′-deoxycoformycin (0.1 mM) for 1 min prior to initiating the reaction by adding 0.1 mM adenosine.

Superoxide dismutase activity. SOD activity was assessed as described by Salin and McCord [10]. The enzyme inhibits superoxide-mediated reduction of cytochrome *c*. SOD was prepared in buffer containing 20 mM Na_2CO_3, pH 10.5 (to inhibit spontaneous dismutation at neutral pH), 0.1 mM EDTA (to chelate free copper which has dismutase activity) and 1 mM NaN_3 (to inhibit catalase and peroxidase activities). To 0.675 mL of this buffer was added xanthine (0.1 mL of 50 μM), cytochrome *c* (0.1 mL of 2 μM from Sigma) and SOD standards or samples. These were incubated for 5 min prior to recording baseline absorbance at 418 nm. Then 0.025 mL of xanthine oxidase (1 unit/mL from Boehringer Mannheim) was added to generate superoxide anion. The incubation was continued for 5 more minutes, and the change in cytochrome *c* absorbance was recorded. Standards consisted of seven concentrations of purified bovine erythrocyte SOD (Sigma) ranging from 0.025 to 1.6 units/mL. The standard curve was well fit to a quadratic equation.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Protein samples or molecular mass standards (Bio-Rad) were dissolved in 2% SDS with 6% (v/v) β-mercaptoethanol and applied to 12% polyacrylamide gels. Gels were stained with Coomassie blue or silver [11].

RESULTS

Cultured endothelial cells contain A_2 adenosine receptors as evidenced by the fact that they responded to 100 μM NECA with a 3.6 ± 0.2 fold increase in cyclic AMP content (mean ± SEM, N = 3 experiments; $P < 0.01$). The average basal level measured in the presence of 20 μM Ro20-1724 was 2.6 ± 0.3 pmol/well, N = 12). The EC_{50} of NECA was 0.7 ± 0.15 μM (N = 3). During studies to determine if NECA influences cyclic GMP accumulation in co-cultures, we added adenosine deaminase to convert endogenous adenosine to inosine which does not activate adenosine receptors. We were surprised to find that adenosine deaminase alone increased the cyclic GMP content of co-cultures under basal conditions, and potentiated bradykinin-stimulated cyclic GMP accumulation. Figure 1A illustrates the results of a typical experiment in which adenosine deaminase and bradykinin both significantly ($P < 0.01$) enhanced cyclic GMP levels in co-cultures, but adenosine had no significant effect. Both bradykinin and adenosine

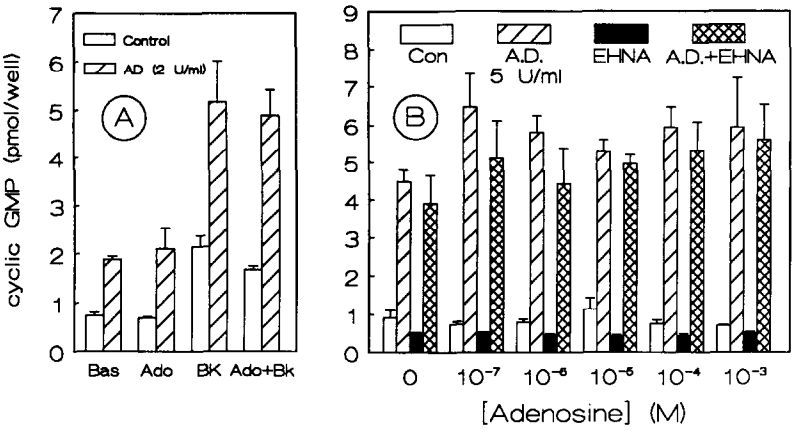

Fig. 1. Effects of adenosine deaminase and adenosine on cyclic GMP accumulation in co-cultures of endothelial and smooth muscle cells (A) Cells were treated with Sigma type VI adenosine deaminase (AD) alone, or together with 0.1 mM adenosine (Ado) and/or 1 μM bradykinin (BK). (B) cells were treated with various concentrations of adenosine, adenosine deaminase (5 units/mL), and/or EHNA (0.1 mM). Values are means ± SEM, N = 4.

deaminase elevated cyclic GMP only in co-cultures; neither endothelial cells nor smooth muscle cells treated alone responded with an increase in cyclic GMP. In the absence of added adenosine deaminase, neither NECA (100 μM) nor the potent adenosine receptor antagonist BWA-1433 [1,3-dipropyl-8-(4-acrylate)phenylxanthine, 10 μM] had any effect on cyclic GMP accumulation in co-cultures. Thus, we considered the possibility that the stimulus for cyclic GMP accumulation might involve inosine or ammonia, the products of adenosine deamination. However, as shown in Fig. 1, adding adenosine (10^{-7}–10^{-3} M) in the presence or absence of adenosine deaminase did not influence the ability of the enzyme to elevate basal or bradykinin-stimulated cyclic GMP accumulation. Moreover, the addition of inosine and ammonia, alone or together, also had no effect on cyclic GMP accumulation at concentrations between 0.01 and 1 mM (data not shown). These results indicate that the stimulatory effect of adenosine deaminase on cyclic GMP accumulation in co-cultures does not involve adenosine receptors or the products of adenosine deamination.

We next considered the possibility that some factor or activity other than adenosine deaminase is responsible for the ability of the enzyme to enhance cyclic GMP accumulation. Addition of an inhibitor or adenosine deaminase, EHNA (0.1 mM), had no significant effect on the action of the enzyme to stimulate cyclic GMP accumulation (Fig. 1B), but reduced the rate of conversion of adenosine to inosine measured spectrophotometrically by 98%. Another very potent adenosine deaminase inhibitor, 2′-deoxycoformycin, also was without effect on cyclic GMP accumulation (Fig. 2A), at a concentration (0.1 mM) that completely abolished the conversion of adenosine to inosine. EHNA and 2′-deoxy-coformycin were also found not to significantly affect the concentration dependency by which adenosine deaminase enhanced cyclic GMP accumulation (Fig. 2A). The EC_{50} of one lot of Sigma type VI enzyme was found to be 2.1 ± 0.3 units/mL in the absence or presence of these adenosine deaminase inhibitors. One lot of Boehringer Mannheim adenosine deaminase (5 units/mL) produced only 12% as much cyclic GMP accumulation as the Sigma type VI enzyme. These data suggest that cyclic GMP accumulation in co-cultures is the result of a contaminant of adenosine deaminase.

SOD has been reported to protect EDRF by preventing its destruction by superoxide anion [5]. Therefore, we reasoned that adenosine deaminase may be contaminated with SOD. The addition of purified bovine erythrocyte SOD to co-cultures elevated cyclic GMP accumulation (Fig. 2B). The EC_{50} for this response was 0.23 ± 0.02 units/mL. In co-cultures, cyclic GMP accumulation stimulated by bradykinin and other endothelium-derived vasodilators is inhibited by the NO synthetase inhibitor N^G-monomethyl-L-arginine (L-NMMA) [8]. The effects of adenosine deaminase and SOD to enhance cyclic GMP were abolished by the inclusion of 100 μM L-NMMA. Moreover, adenosine deaminase had no effect when added to a maximally effective concentration of superoxide dismutase (10 units/mL). These data suggest that adenosine deaminase and SOD elevate cyclic GMP in co-cultures through the same mechanism.

We analyzed single lots of eight commercial sources of adenosine deaminase for SOD activity. The results are summarized in Table 1. All sources of the enzyme contained detectable SOD activity, but the amount of contamination varied widely. The Sigma type VI enzyme contained 0.08 units SOD/unit adenosine deaminase. This is consistent with the observation that the EC_{50} values of Sigma type VI adenosine deaminase and purified SOD required to enhance cyclic GMP accumulation differed by a similar ratio, 0.11 (Fig. 2). The Boehringer Mannheim adenosine deaminase was relatively low in SOD activity (Table 1) which is consistent with the fact that it is a weak activator of cyclic GMP accumulation in co-cultures.

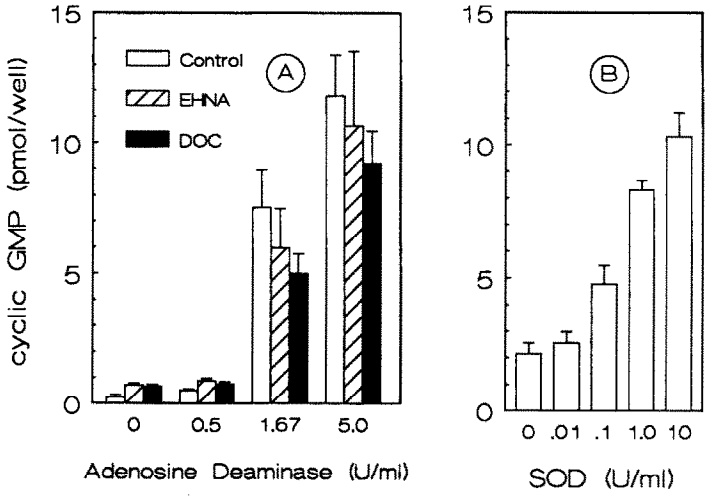

Fig. 2. Concentration–response curves for adenosine deaminase and SOD to increase cyclic GMP levels in co-cultures. (A) Cells were treated with three concentrations of Sigma type VI adenosine deaminase as indicated. Cyclic GMP was elevated ($P < 0.05$) by 1.67 and 5 units/mL. (B) Cells were treated with four concentration of SOD purified from bovine erythrocytes. Cyclic GMP was elevated by concentrations of SOD $\geq$ 0.1 units/mL ($P < 0.01$). Values are means ± SEM, N = 4.

Table 1. Quantitation of SOD activity contained in commercial sources of adenosine deaminase

Source (lot)	Tissue	SOD/AD activity ratio (units/unit)
Boehringer Mannheim (11593721-26)	Intestine	0.006 ± 0.002
Serva (01079C)	Intestine	0.006 ± 0.002
Sigma type V (34F81454)	Spleen	0.007 ± 0.002
Sigma type X (39F9581)	Spleen	0.007 ± 0.001
Worthington (J8A064Y)	Intestine	0.011 ± 0.004
Calbiochem (801022)	Intestine	0.017 ± 0.005
Sigma type VI (89F3871)	Intestine	0.080 ± 0.03
Sigma type II (89F8235)	Intestine (crude)	1.990 ± 0.59

All enzymes were derived from bovine tissues. Between 0.5 and 10 units of each source of adenosine deaminase was assayed in a volume of 1 mL. Each point is the mean ± SEM of triplicate determinations.

We attempted to resolve adenosine deaminase in two commercial sources of the enzyme from SOD contaminants by gel permeation chromatography. As shown in Fig. 3, there was one major peak of UV absorbing protein in both the Sigma type VI and the Boehringer Mannheim enzymes which was eluted between ovalbumin (44 kD) and myoglobin (17 kD), at a molecular mass that was calculated to be 33.5 kD. This molecular mass is consistent with several previous estimates of the molecular mass of bovine intestinal adenosine deaminase as ranging between 31 and 35 kD by gel filtration [reviewed in Ref. 12]. Gel filtration fractions (1 mL) were collected and analyzed for enzyme activities. For both sources of enzymes the peaks of adenosine deaminase and SOD activities were found in fraction 12 (11–12 mL) corresponding to the peak of UV absorbance (Figs. 3 and 4). These data indicate that the SOD contaminant contained in these sources of adenosine deaminase has a molecular mass on gel filtration of about 33 kD, very close to that of adenosine deaminase. Quantitation of SOD in gel filtration fractions of adenosine deaminases (Fig. 4) verified the data in Table 1 which indicates that

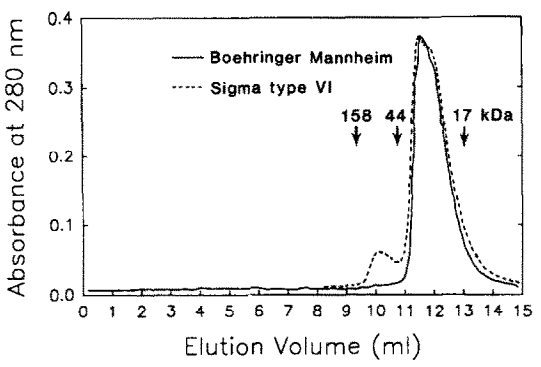

Fig. 3. High pressure gel permeation chromatography of adenosine deaminases. UV absorbance at 280 nM was monitored during chromatography as described under 'Materials and Methods'. One hundred units of each enzyme was applied to the column. The elution position of standards is indicated by arrows.

the total SOD activity contaminating adenosine deaminase was greater in the Sigma type VI than in the Boehringer Mannheim enzyme.

To gain further information about the nature of the SOD contaminating adenosine deaminase, the Sigma type VI and Boehringer Mannheim enzymes were subjected to SDS–PAGE (Fig. 5). The molecular mass of the major protein corresponding to adenosine deaminase was calculated to be 44 kD by electrophoresis and was the major polypeptide visible in Coomassie blue stained lanes (Fig. 5A). In the Sigma preparation, an additional polypeptide with a molecular mass of 16.6 kD was visible in silver stained gel lanes (Fig. 5B), and this peptide co-migrated with purified SOD monomers. Based on the intensity of silver staining in the 16.6 kD band, the Sigma type VI adenosine deaminase appears to contain close to the 0.08 units of SOD expected on the basis of the data in Table 1.

DISCUSSION

We have shown that adenosine deaminase enhances cyclic GMP accumulation in co-cultures of endothelial and smooth muscle cells. The effect was not due to adenosine deaminase activity *per se*, but rather to contaminating SOD. We measured the contamination of adenosine deaminase by SOD in single lots of the enzyme from eight different sources. We did not measure lot-to-lot variations, and hence it should be kept in mind that the amount of contamination may vary with the lot as well as with the source of the enzyme. It is known that SOD protects EDRF by preventing its degradation by superoxide anion which is released from endothelial cells [4]. SOD catalyzes the destruction of the superoxide anion radical by converting it to oxygen and H_2O_2.

The use of adenosine deaminase to remove endogenous adenosine is often essential in order to be able to discern adenosine receptor-mediated tissue responses [1]. The enzyme is also used in the study of P_2 purinergic receptor-mediated responses in order to deaminate adenosine formed from the degradation of adenine nucleotides. Thus, SOD activity contaminating adenosine deaminase could constitute a significant complicating factor in the study of the function of purinergic receptors in many systems.

Recent reports suggest that endothelial EDRF contains NO derived from the amino acid L-arginine by the enzyme NO synthetase which can be inhibited by L-NMMA [6, 13–15]. We found that L-NMMA prevented the action of SOD to elevate cyclic GMP

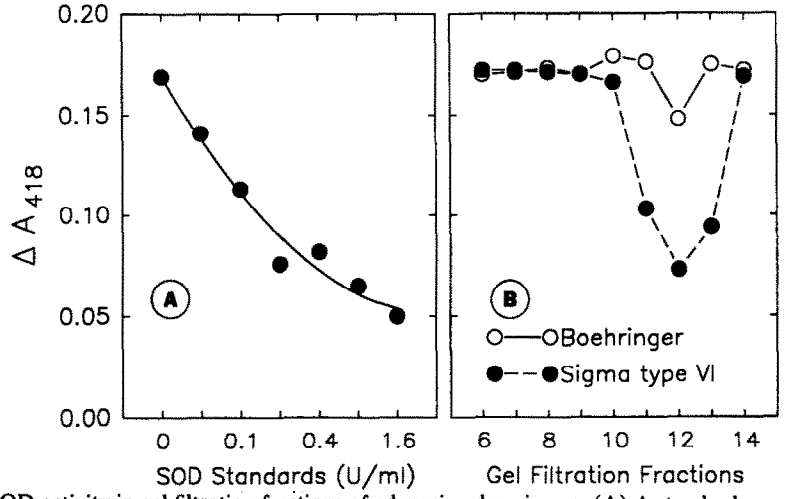

Fig. 4. SOD activity in gel filtration fractions of adenosine deaminases. (A) A standard curve for SOD activity was constructed using the purified bovine erythrocyte enzyme. Each point is the mean of closely agreeing triplicates. (B) Fractions of adenosine deaminases (100 units) eluted during gel permeation chromatography (Fig. 3) were analyzed for SOD activity. From each 1-mL fraction 100-μL aliquots were analyzed for SOD activity. The average of duplicate determinations is plotted. Peak SOD activities were found in fraction 12 corresponding to a molecular mass of 31–35 kD.

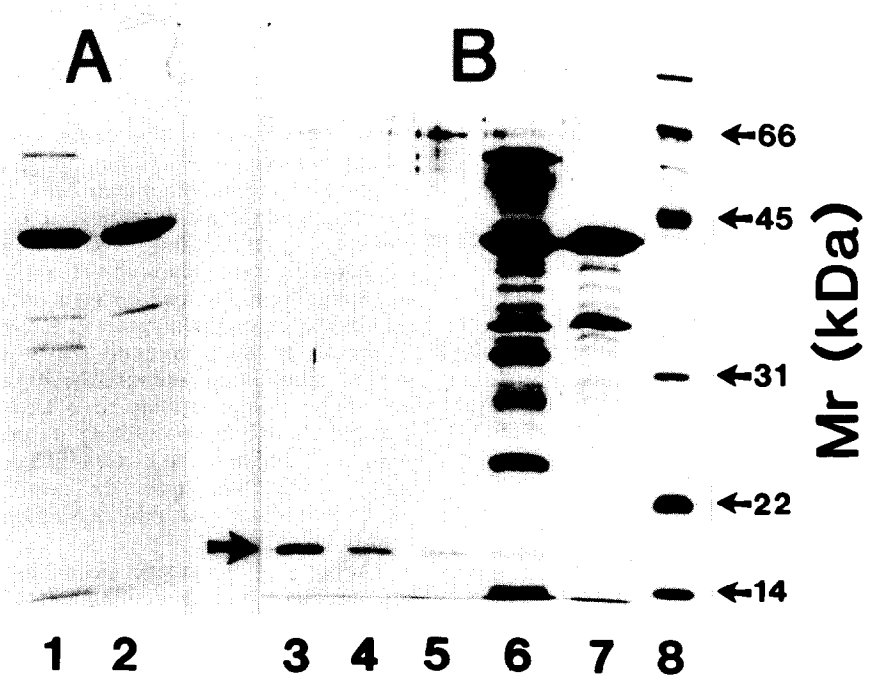

Fig. 5. SDS–PAGE of SOD and adenosine deaminases. (A) Sigma type VI (lane 1) and Boehringer Mannheim (lane 2) adenosine deaminase (1 unit, 5–6 μg protein) were subjected to electrophoresis and stained with Coomassie blue. (B) Silver-stained proteins; lanes 3–5 contained 0.2, 0.1 and 0.05 units bovine erythrocyte SOD respectively. The location of the 16.6 kD SOD monomer is indicated by an arrow between lanes 2 and 3. Lanes 6 and 7 contained 1 unit each of Sigma type VI Boehringer mannheim adenosine deaminases respectively. Lane 8 contains molecular mass standards.

in co-cultures. This is consistent with the idea that SOD acts by protecting EDRF from degradation.

The fact that cyclic GMP accumulation in co-cultures is elevated by SOD implies that EDRF and superoxide anion are both released by cultured endothelial cells. If this is also the case *in situ*, then superoxide production by endothelial cells may produce some degree of vasoconstriction due to its ability to chemically destroy EDRF. Adenosine metabolism provides one source of superoxide anion in tissues which contain xanthine oxidase since the major route of adenosine metabolism is to xanthine via inosine and hypoxanthine [16]. Superoxide anion is generated as a by-product of the conversion of xanthine to uric acid by xanthine oxidase. In this study, adding adenosine had no effect on cyclic GMP accumulation in co-cultures (Fig. 1) suggesting that xanthine oxidase is not a major source of superoxide anion production in aortic endothelial cells. This is consistent with reports which indicate that xanthine oxidase, although abundant in microvascular endothelial cells, is absent in macrovascular cells [reviewed in Ref. 16]. However, adenosine metabolism may lead to superoxide production and antagonize the dilatory action of EDRF in the microcirculation where most adenosine is converted to uric acid. Reductases which can be uncoupled by compounds which undergo redox cycling provide another source of superoxide anion in macrovascular endothelial cells such as those used in this study [4].

A number of tissues in addition to blood vessels have been found recently to produce NO and could potentially be influenced by SOD. These tissues include cerebellum, forebrain, neutrophils, Kupffer cells, hepatocytes, kidney epithelial cells, adrenal gland and probably others [reviewed in Ref. 15]). The extent to which superoxide is also produced by these tissues has yet to be carefully explored. In the cerebellum, activation of *N*-methyl-D-aspartate (NMDA) receptors is a stimulus for EDRF release [17]. It is interesting that Canonico *et al.* [18] recently reported that adenosine deaminase facilitates glutamatergic transmission in cultured cerebellar neurons. As in the present study, the action of adenosine deaminase was found not to be reversed by adding adenosine deaminase resistant adenosine analogs, or to be mimicked by adenosine receptor antagonists. Thus, the effect of adenosine deaminase on cultured cerebellar neurons may be due to contaminating SOD. This would implicate superoxide anion as well as NO as potential modulators of neurotransmitter release from cerebellar neurons.

A striking finding of this study is the universal contamination of commercial sources of adenosine deaminase by SOD. This may be attributable to difficulty of resolving these enzymes by commonly used chromatographic procedures. Two mammalian superoxide dismutases can be distinguished by differences in metal content. A blue-green Cu (II)-Zn(II) cytosolic enzyme which has been purified

from bovine erythrocytes consists of two identical 17 kD subunits joined by a disulfide bond [19]. A Mn(III) mitochondrial enzyme has been purified from liver and is a tetramer with a subunit molecular mass of 25 kD [20]. In this study, the SOD contaminant which co-migrated with adenosine deaminase during gel permeation chromatography had a molecular mass of 33 kD (Fig. 4). On reduced SDS–PAGE gels, a 16.6 kD polypeptide in Sigma type VI adenosine deaminase could be seen corresponding to reduced SOD (Fig. 5). This band was not visible in the Boehringer Mannheim enzyme which had 13 times less SOD activity. These data indicate that SOD contaminating adenosine deaminase is probably of the copper–zinc type, which in its native state is very close in molecular mass to adenosine deaminase.

Schrader *et al.* [21] have reported previously that adenosine deaminase purified from human erythrocytes by gel filtration and ion exchange chromatography is contaminated by cupro–zinc SOD. Each of several fractions of adenosine deaminase eluted from a DEAE Sephadex column by a salt gradient also contained SOD activity. Thus, the combined use of gel filtration and ion exchange chromatography may not be sufficient to completely resolve these enzymes.

Although we have identified commercial sources of adenosine deaminase which are relatively low in SOD activity, contamination may vary with lot, and it would be useful to be able to completely remove SOD from adenosine deaminase. Contaminating SOD could be destroyed by treatment with H_2O_2 which rapidly inactivates the enzyme [22]. Another approach to removing SOD is the use of an immunoadsorbant column containing anti-SOD antibodies. Such a column has been shown to completely remove SOD from erythrocyte adenosine deaminase [21].

Acknowledgements—Supported by NIH Grants HLRO1-37942 (J.L.), PO1HL19242 (J.L. and R.A.J.) and Virginia Heart Grant F880010 (A.P.). J.L. is an Established Investigator of the American Heart Association, and R.A.J. is a recipient of the Anesthesia Young Investigator/Parker B. Frances Award.

REFERENCES

1. Linden J, Adenosine deaminase for removing adenosine: How much is enough? *Trends Pharmacol Sci* **10**: 260–262, 1988.
2. Sabouni MH, Ramagopal MV and Mustafa SJ, Roles of calcium and the endothelium in the relaxations produced by 5′-*N*-ethylcarboxamidoadenosine (NECA). *Eur J Pharmacol* **166**: 311–314, 1989.
3. Rubanyi G and Vanhoutte PM, Endothelium-removal decreases relaxations of canine coronary arteries caused by α-adrenergic agonists and adenosine. *J Cardiovasc Pharmacol* **7**: 139–144, 1985.
4. Rosen GM and Freeman BA, Detection of superoxide generated by endothelial cells. *Proc Natl Acad Sci USA* **81**: 7269–7273, 1984.
5. Gryglewski RJ, Palmer RMJ and Moncada S, Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature* **320**: 454–456, 1986.
6. Palmer RMJ, Ferrige AG and Moncada S, Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **327**: 524–525, 1987.
7. Loeb AL, Owens GK and Peach MJ, Evidence for endothelium-derived relaxing factor in cultured cells. *Hypertension* **7**: 804–807, 1985.
8. Johns RA, Peach MJ, Linden J and Tichotsky A, L-N^G-Monomethyl arginine (LNMMA) inhibits endothelium-derived relaxing factor (EDRF) Stimulated cyclic GMP accumulation in co-cultures of endothelial and vascular smooth muscle cells by an action specific to the endothelial cell. *Circ Res*, in press.
9. Patel A and Linden J, Purification of ^{125}I-labeled succinyl cyclic nucleotide tyrosine methyl esters by high performance liquid chromatography. *Anal Biochem* **168**: 417–420, 1988.
10. Salin ML and McCord JM, Superoxide dismutases in polymorphonuclear leukocytes. *J Clin Invest* **54**: 1005–1009, 1974.
11. Wray W, Boulikas T, Wray VP and Hancock R, Silver staining of proteins in polyacrylamide gels. *Anal Biochem* **118**: 197–203, 1981.
12. Zielke CL and Suelter CH, Purine, purine nucleoside, and purine nucleotide aminohydrolases. In: *The Enzymes* (Ed. Boyer PD), pp. 47–78. Academic Press, New York, 1971.
13. Knowles RG, Palacios M, Palmer RMJ and Moncada S, Formation of nitric oxide from L-arginine in the central nervous system—A transduction mechanism for stimulation of the soluble guanylate cyclase. *Proc Natl Acad Sci USA* **86**: 5159–5162, 1989.
14. Marletta MA, Yoon PS, Iyengar R, Leaf CD and Wishnok JS, Macrophage oxidation of L-arginine to nitrite and nitrate: Nitric oxide is an intermediate. *Biochemistry* **27**: 8706–8711, 1988.
15. Collier J and Vallance P, Second messenger role of NO widens to nervous and immune systems. *Trends Pharmacol Sci* **10**: 427–431, 1989.
16. Gerlach E, Becker BF and Nees S, Formation of adenosine by vascular endothelium: A homeostatic and antithrombogenic mechanism? In: *Topics and Perspectives in Adenosine Research* (Eds. Gerlach E and Becker BF), pp. 309–320. Springer, Berlin, 1987.
17. Garthwaite J, Charles SL and Chess-Williams R, Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature* **336**: 385–388, 1988.
18. Canonico PL, Catania ML and Sortino MA, Adenosine deaminase facilitates glutamatergic transmission in cultured cerebellar neurons. *Soc Neurosci Abstr* **15**: 234, 1989.
19. Keele BB Jr, McCord JM and Fridovich I, Further characterization of bovine superoxide dismutase and its isolation from bovine heart. *J Biol Chem* **246**: 2875–2880, 1971.
20. Ishikawa T, Hunaiti AR, Piechot G and Wolf B, Isolation and characterization of basic superoxide dismutase consisting of Mr-25,000 subunits in rat liver. *Eur J Biochem* **170**: 317–323, 1987.
21. Schrader WP, Stacy AR and Pollara B, Purification of human erythrocyte adenosine deaminase by affinity chromatography. *J Biol Chem* **251**: 4026–4032, 1976.
22. Bray RC, Cockle SA, Fielden EM, Roberts PC, Rotilio G and Calabrese L, Reduction and inactivation of superoxide dismutase by hydrogen peroxide. *Biochem J* **139**: 43–48, 1974.