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Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells

(calcium/calmodulin/tetrahydrobiopterin/cGMP/nitric oxide)

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ABSTRACT **The particulate enzyme responsible for the synthesis of endothelium-derived relaxing factor has been purified from cultured and native (noncultured) bovine aortic endothelial cells. Purification of the solubilized particulate enzyme preparation by affinity chromatography on adenosine 2′,5′-bisphosphate coupled to Sepharose followed by Superose 6 gel filtration chromatography resulted in a single protein band after denaturing polyacrylamide gel electrophoresis that corresponded to ≈135 kDa. The enzyme activity in the various fractions was assayed by its stimulatory effect on soluble guanylyl cyclase of rat fetal lung fibroblasts (RFL-6 cells), by the formation of L-citrulline from L-arginine, by measuring nitrite/nitrate formation, and by bioassay on endothelium-denuded vascular strips. Endothelium-derived relaxing factor synthase was purified 3419-fold from the crude particulate fraction of cultured bovine aortic endothelial cells with a 12% recovery (RFL-6 assay). Purified endothelium-derived relaxing factor synthase required L-arginine, NADPH, Ca^{2+}, calmodulin, and 5,6,7,8-tetrahydrobiopterin for full activity.**

Endothelial cells synthesize endothelium-derived relaxing factor (EDRF) from L-arginine (1, 2). The pharmacological and biochemical properties of EDRF are mimicked by nitric oxide (NO) (3) or NO-containing compounds (4). These agents activate soluble guanylyl cyclase (5, 6) thereby increasing cGMP and causing relaxation of vascular smooth muscle (7–9), inhibition of platelet aggregation (10), and other effects (9, 11). EDRF/NO synthase has been purified and characterized from brain (12–14), polymorphonuclear neutrophils (15), and endotoxin/cytokine-induced macrophages (16, 17). The endothelial, brain, and neutrophil EDRF/NO synthases are constitutive enzymes whereas the macrophage activity is expressed only after induction with endotoxin and/or a cytokine. The constitutive enzyme from brain is soluble and Ca^{2+}/calmodulin-regulated whereas the enzyme from neutrophils has been described as soluble and Ca^{2+}-dependent, but not calmodulin-dependent. Analysis with denaturing gel electrophoresis revealed a single band corresponding to 155 kDa and 150 kDa for the brain (12) and neutrophil (15) enzymes, respectively. The endotoxin/cytokine-induced enzyme from macrophages was reported to be soluble with no Ca^{2+}/calmodulin dependency, a denaturing molecular mass of 150 kDa in peritoneal macrophages (17) or 125–135 kDa in the RAW 264.7 macrophage cell line (16). In endothelial cells, a particulate Ca^{2+}/calmodulin-regulated enzyme accounts for >95% of the total EDRF synthase activity (18, 19). We now report the purification to homogeneity (denaturing molecular mass of 135 kDa) of this constitutive particulate EDRF synthase and characterize the enzyme as NADPH- and (6*R*)-5,6,7,8-tetrahydrobiopterin (BH_4)-dependent. Some of these observations have been reported in abstract form (20, 21).

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METHODS AND MATERIALS

Purification of EDRF Synthase from Cultured Bovine Aortic Endothelial (BAE) Cells. BAE cells (passage 6) (22) were cultured to confluence in roller bottles (850 cm^2), then scraped, and washed in phosphate-buffered saline (PBS). Cells were suspended in ice-cold 50 mM Tris·HCl (pH 7.4) containing 0.1 mM EDTA, 0.1 mM EGTA, and 0.1% 2-mercaptoethanol (buffer 1) as described (18) and homogenized with the addition of 1 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, and 1 μM pepstatin A. The crude homogenate was then centrifuged at 100,000 × *g* for 60 min. The soluble fraction was removed, 10% (vol/vol) glycerol was added, and the mixture was stored at −70°C. The particulate fraction was resuspended in buffer 2 (buffer 1 containing 10% glycerol) containing protease inhibitors and 1 M KCl for 5 min and then centrifuged at 100,000 × *g* for 30 min. The KCl-washed particulate fraction was then solubilized with the detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, Sigma; 20 mM) in buffer 2 containing protease inhibitors for 20 min at 4°C with gentle rotation of the sample and centrifuged at 100,000 × *g* for 30 min. The CHAPS extract was loaded onto a 150- to 200-μl column of preswollen adenosine 2′,5′-bisphosphate-coupled Sepharose (2′,5′-ADP-Sepharose, Pharmacia) and recirculated at least four times. The column was then washed with 1 ml of buffer 2 containing 10 mM CHAPS and 0.5 M NaCl and then with 1 ml of buffer 3 (buffer 2 containing 10 mM CHAPS). EDRF synthase activity was eluted with two 500-μl vol of buffer 3 containing 10 mM NADPH (Boehringer Mannheim). The NADPH eluate from the 2′,5′-ADP-Sepharose column was applied to a FPLC Superose 6 or 12 gel permeation column (Pharmacia) equilibrated with buffer 4 (buffer 4 = 10 mM Tris·HCl, pH 7.4/1 mM EDTA/0.1% 2-mercaptoethanol/100 mM NaCl/10% glycerol/10 mM CHAPS). EDRF synthase was eluted anomalously near the total included volume (coeluting with NADPH) due to nonspecific interactions with the column material. Superose 6 chromatography yielded a pure enzyme preparation that was partially BH_4-dependent. When Superose 12 was used for purification, the enzyme preparation was not completely pure but was substantially

Abbreviations: BAE cell, bovine aortic endothelial cell; EDRF, endothelium-derived relaxing factor; BH_4, (6*R*)-5,6,7,8-tetrahydrobiopterin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; NO, nitric oxide; SOD, superoxide dismutase; NMA, N^G-methyl-L-arginine; NNA, N^G-nitro-L-arginine.

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more BH_4-dependent. No differences were noted in the yield of purified particulate EDRF synthase from passages 6–15.

The purification of soluble EDRF synthase from cultured BAE cells (<5% of total activity) was attempted utilizing the same purification scheme as the particulate enzyme, although the buffers did not include 10 mM CHAPS. Several preparations were pooled, protease inhibitors were added, and the mixture was loaded onto a 400-μl column of 2′,5′-ADP-Sepharose and circulated overnight. The column was then washed with 3 ml of buffer 2 containing 0.5 M NaCl and then with 3 ml of buffer 2 alone. The soluble enzymatic activity was eluted with six 500-μl vol of buffer 2 containing 10 mM NADPH. A sample of the NADPH eluate was applied to a FPLC Superose 6 gel permeation column equilibrated with buffer 4 excluding 10 mM CHAPS. The soluble EDRF synthase activity also eluted near the total included volume of the column at the same retention volume as purified particulate EDRF synthase.

Protein was determined according to Bradford (23) with bovine serum albumin as a standard. The purity of enzyme preparations was assessed with 7.5% polyacrylamide gels as described by Laemmli (24) and visualized with silver staining. Purified enzyme preparations were stored at −70°C in the presence of 10% glycerol and exogenous bovine serum albumin to stabilize the activity.

Purification of EDRF Synthase from Native BAE Cells. Native bovine endothelial cells were obtained from 50 to 60 fresh bovine aortas transported from the slaughterhouse in ice-cold PBS. The aortas were cleared of connective tissue, cut longitudinally, and mounted with the luminal surface uppermost. The endothelial surface was then washed with ice-cold PBS, and the endothelial cells were removed by scraping and collected into ice-cold PBS. The cells were washed twice in PBS before being resuspended in buffer 1 and homogenized. Homogenization, solubilization, and purification followed the same protocol as for the cultured BAE cells.

Assays for EDRF/NO Activity, L-Citrulline Formation, and Nitrite/Nitrate Formation. Samples of the enzyme preparation and column fractions were bioassayed for the formation of EDRF/NO using the stimulation of cGMP formation in RFL-6 cells in the presence of 100 μM L-arginine, 100 μM NADPH, 2 mM Ca^{2+}, 30 nM calmodulin (Sigma), 3 μM BH_4 (J. B. Schircks, Jura, Switzerland), and superoxide dismutase (SOD, Sigma; 20 units/ml) as described (25, 26). Briefly, RFL-6 cells were cultured in six-well plates (1×10^6 cells per well), washed with PBS, and preincubated for 20 min in 1 ml of Locke's solution (154 mM NaCl/5.6 mM KCl/2 mM $CaCl_2$/1.0 mM $MgCl_2$/3.6 mM $NaHCO_3$/5.6 mM glucose/10.0 mM Hepes, pH 7.4) containing 0.3 mM 3-isobutyl-1-methylxanthine and SOD (20 units/ml). Samples of enzyme preparations and column fractions were added in small volumes to the RFL-6 cells in the presence of substrate and cofactors and were incubated at 37°C for 3 min. The reaction was stopped by aspirating the medium, adding 1 ml of ice-cold 20 mM sodium acetate (pH 4.0) to the RFL-6 cells, and rapidly freezing the samples in liquid nitrogen. The cGMP content was determined in each sample by radioimmunoassay as described (25, 26). The inhibitory effect of the guanidino-substituted L-arginine derivatives, N^G-methyl-L-arginine (NMA) and N^G-nitro-L-arginine (NNA), was determined in the presence of 3 μM, 10 μM, and 30 μM L-arginine. Experiments with defined Ca^{2+} concentrations were performed as described (18) in the presence of 1 mM EGTA with various amounts of $CaCl_2$ added, and free Ca^{2+} concentrations were calculated (27).

The conversion of L-arginine to L-citrulline was assayed as reported (12, 13) with minor modifications. Briefly, samples of purified enzyme (120 ng) were incubated for 3 min at 25°C in a solution of 10 μM L-[2,3-^{3}H]arginine (55 Ci/mmol; 1 Ci = 37 GBq), 1 mM NADPH, 100 nM calmodulin, 2 mM $CaCl_2$, and 10 μM BH_4 in a final volume of 100 μl. The reaction was stopped by adding 500 μl of stop buffer (2 mM EGTA/2 mM EDTA/20 mM Hepes, pH 5.5). The total volume was then applied to a 1-ml Dowex AG 50WX-8 column (Na^+ form, Bio-Rad) that had been preequilibrated with the stop buffer. L-[2,3-^{3}H]Citrulline was eluted twice with 0.5 ml of distilled water and radioactivity was determined by liquid scintillation counting. L-[2,3-^{3}H]Arginine (DuPont/NEN) was purified prior to use on Dowex AG 1-X8 (CH_3COO^- form, Bio-Rad), eluted with distilled water, acidified, concentrated on a Speed-Vac (Savant), and stored at −20°C.

The nitrite/nitrate assay was performed with purified enzyme in the presence of 50 μM NADPH, 2 mM $CaCl_2$, 100 nM calmodulin, and 3 μM BH_4 for 30 min at room temperature. Nitrate was reduced to nitrite during a 5-min incubation with nitrate reductase (Boehringer Mannheim; 0.1 unit/ml) in the presence of 5 μM FAD, and the remaining NADPH was oxidized with lactate dehydrogenase (Boehringer Mannheim; 10 units/ml) in the presence of 10 mM sodium pyruvate. Nitrite was detected according to the modified Griess reaction (28) by adding 1 mM sulfanilamide and 0.1 M HCl (5 min at 4°C) and quantified spectrophotometrically from the absorption before (A_1) and after (A_2) the addition of 1 mM naphthylethylenediamine ($A_2 - A_1 = 0.038$ for 1 μM NO_2^-) at room temperature.

The amount of detergent present in each assay described above was the amount associated with the crude or purified enzyme preparation sampled. No effect was found in each assay with these concentrations of detergent.

Vascular Relaxation in Response to EDRF Generated by Purified EDRF Synthase from Cultured BAE Cells. Rabbit aortic strips were denuded of endothelium and mounted in small plastic organ baths containing 1 ml of warmed (37°C) and gassed (95% O_2/5% CO_2) Krebs solution as described (19). Briefly, the strips were equilibrated under a tension of 2 g for 60–90 min, with washing every 15–20 min. The strips were then contracted with 10–30 nM norepinephrine to increase tension by a further 1 g. The absence of endothelium was confirmed by the addition of 1 μM acetylcholine to the bath and, if any relaxation occurred, the tissue was discarded. The Krebs solution was then changed, the tissue was contracted again with norepinephrine, and the relaxation to 1 μM 3-morpholinosydnonimine was measured. Any tissues that displayed <80% relaxation were discarded. After replacing the Krebs solution again, strips were contracted with 30 nM norepinephrine in the presence of L-arginine, NADPH, Ca^{2+}, calmodulin, and BH_4. Crude and purified particulate fractions of cultured BAE cells were then added to the baths in the presence or absence of SOD and the resultant relaxation was recorded on a Grass polygraph. The substrate/cofactor mixture had no effect on the strips in the absence of enzyme preparation.

RESULTS AND DISCUSSION

EDRF synthase in endothelial cells is predominately associated with the membrane fraction (18, 19) and is a distinct isoform of EDRF/NO-forming enzymes (12–17, 29, 30). Particulate EDRF synthase activity was solubilized from cultured and native BAE cells and purified to homogeneity in two chromatographic steps, specifically 2′,5′-ADP-Sepharose affinity and FPLC Superose 6 gel permeation chromatography. EDRF synthase was purified to a specific activity of 667 nmol of cGMP per mg of protein per 3 min (for cultured BAE cells) or 440 nmol of cGMP per mg of protein per 3 min (for native BAE cells) utilizing the stimulation of soluble guanylyl cyclase in RFL-6 cells as a bioassay (12, 18, 25, 26). This represents a 3419-fold purification from the crude particulate fraction of cultured BAE cells with a 12%

Table 1. Purification of particulate EDRF synthase from cultured BAE cells

Fraction	Protein, μg	EDRF synthase activity: Total activity, nmol of cGMP per 3 min	EDRF synthase activity: Specific activity, nmol of cGMP per mg per 3 min	Yield, %	Purification factor
Crude particulate	270,000	52.6	0.19	100	1
Particulate, KCl-washed	165,000	59.4	0.36	113	1.8
Particulate, KCl-washed and CHAPS-solubilized	88,800	154.5	1.74	294	8.9
2′,5′-ADP-Sepharose (NADPH eluate)	140	18.1	129.1	34.4	662
Superose 6 eluate	9.6	6.4	666.7	12.2	3419

Data are representative of three preparations. This preparation was performed from 80 roller bottles of cultured BAE cells. Similar data were obtained with preparations from native BAE cells.

recovery as shown in Table 1. As illustrated in Table 1, CHAPS solubilization of the enzyme preparation enhances the specific activity >4-fold. By utilizing L-[^{3}H]citrulline formation as an assay, the specific activity was 15.3 nmol of citrulline per mg of protein per min with the purified BAE preparation. Purified EDRF synthase also formed nitrite and nitrate from L-arginine at the rate of 97 nmol per mg of protein per 30 min. Denaturing polyacrylamide gel electrophoresis analysis revealed that purified EDRF synthase from native and cultured BAE cells has a denatured molecular mass of 135 kDa (Fig. 1). EDRF synthases purified from cultured and native BAE cells appear to be identical from their behavior during purification, characterization, and molecular sizing by SDS/PAGE. About 5% of the total EDRF synthase enzymatic activity was found in the soluble fraction of cultured BAE cells. The small amounts of the soluble enzyme that we could obtain were also submitted to the same chromatographic steps as the solubilized particulate enzyme. The soluble endothelial enzymatic activity eluted at the same retention volume as the particulate endothelial activity but was distinctly different from soluble brain NO synthase on the gel filtration column (data not shown). SDS/PAGE analysis of the active Superose 6 column fractions of the soluble enzyme identified a band with a similar molecular mass as the particulate enzyme; however, some low molecular mass contaminants were also present (data not shown).

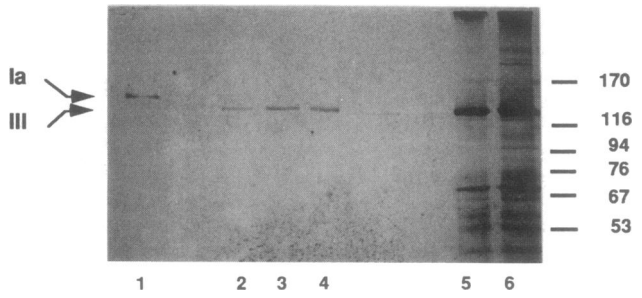

FIG. 1. SDS/PAGE of purified EDRF synthase from cultured and native BAE cells. Molecular mass standards are as follows: α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), phosphorylase *b* (94 kDa), transferrin (76 kDa), bovine serum albumin (67 kDa), and glutamic dehydrogenase (53 kDa). Lanes: 1, 200 ng of pure soluble NO synthase from rat brain (type Ia); 2, 100 ng of pure particulate EDRF synthase from native BAE cells (type III); 3, 500 ng of pure particulate EDRF synthase from cultured BAE cells (type III); 4, 500 ng of pure particulate EDRF synthase from cultured BAE cells (type III); 5, 2 μg of the NADPH eluate of 2′,5′-ADP-Sepharose from native BAE cells; 6, 3 μg of the NADPH eluate of 2′,5′-ADP-Sepharose from cultured BAE cells. A molecular mass vs. R_f logarithmic plot of the gel standards revealed that purified EDRF synthase (type III) from cultured and native BAE cells has a molecular mass of 135 kDa; whereas soluble NO synthase from rat brain (type Ia) has a molecular mass of 155 kDa.

Also, on DEAE anion-exchange chromatography, the solubilized particulate and soluble endothelial enzymes showed similar elution profiles (data not shown).

The partially purified enzyme activity in the NADPH eluate from the 2′,5′-ADP-Sepharose column was totally dependent on Ca^{2+}, but only partially dependent on exogenous calmodulin (18). However, purified EDRF synthase is completely Ca^{2+}/calmodulin-dependent with EC_{50} values for Ca^{2+} and calmodulin of 0.3 μM and 3.5 nM, respectively, as

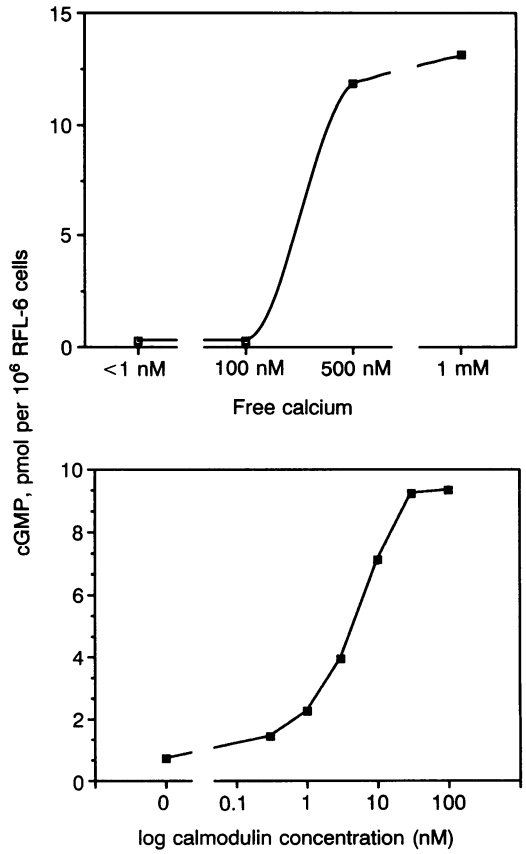

FIG. 2. Ca^{2+} and calmodulin dependency of purified particulate EDRF synthase from cultured BAE cells. Purified particulate EDRF synthase from cultured BAE cells was bioassayed for the formation of EDRF/NO by using the stimulation of cGMP accumulation in RFL-6 cells. Both assays were performed in the presence of 100 μM L-arginine, 100 μM NADPH, 3 μM BH_4, and SOD (20 units/ml). When Ca^{2+} was varied 30 nM calmodulin was present and when calmodulin was varied the Ca^{2+} concentration was maintained at 2 mM. (*Upper*) Ca^{2+} titration curve demonstrating the strict dependency of purified particulate EDRF synthase on calcium. (*Lower*) Calmodulin titration curve demonstrating that purified particulate EDRF synthase is dependent on exogenous calmodulin for activity.

Table 2. Characterization of purified particulate EDRF synthase from cultured BAE cells

Parameter	Value
Denatured molecular mass, kDa	135
V_{max}, nmol of citrulline per mg per min	15.3
K_m (L-arginine), μM	2.9
K_i (NNA), μM	0.16
K_i (NMA), μM	0.94
EC_{50} (BH_4), μM	0.1
EC_{50} (calmodulin), nM	3.5
EC_{50} (Ca^{2+}), μM	0.3
EC_{50} (Mg^{2+})	ND

Data were generated by utilizing the stimulation of cGMP formation in RFL-6 cells, with the exception of the V_{max} calculation, which was based on the conversion of L-arginine to L-citrulline. Data are representative of three determinations. ND, no dependence.

shown in Fig. 2. Presumably, the crude and partially purified enzymes have calmodulin present in the preparation or associated with the enzyme. As reported for the NO synthases isolated from the soluble fraction of either porcine brain (14) or endotoxin/cytokine-induced macrophages (29), we found that EDRF synthase in endothelial cells is BH_4-dependent with an EC_{50} value of 0.1 μM. NNA and NMA have been described as competitive inhibitors of EDRF and NO synthesis in various cell types (1, 31, 32). We found NNA and NMA to be competitive inhibitors, also. NNA was ≈6 times more potent than NMA as an inhibitor of EDRF synthase from cultured BAE cells with a K_i value of 0.16 μM for NNA and a K_i value of 0.94 μM for NMA (Table 2). The apparent K_m for L-arginine and the V_{max} of particulate EDRF synthase are reported in Table 2. The properties of the purified enzyme from cultured and native BAE cells were very similar.

To confirm that the purified enzyme can produce biologically active EDRF, endothelium-denuded rabbit aortic strips in organ baths were exposed to purified EDRF synthase. Fig. 3 illustrates that purified EDRF synthase in the presence of L-arginine, NADPH, Ca^{2+}, calmodulin, and BH_4 relaxed preconstricted rabbit aortic strips in a protein-dependent manner (0.15–1.5 μg/ml) only in the presence of SOD (Fig. 3). Crude particulate fractions from cultured BAE cells (washed once with 1 M KCl) also relaxed rabbit aortic strips in a protein-dependent manner. In the presence of 100 μM L-arginine, 100 μM NADPH, and SOD (20 units/ml), 10 μg/ml produced a relaxation of 8.8 ± 1.6% of norepinephrine (30 nM)-induced tone, 20 μg/ml produced 27.2 ± 2.6% relaxation, 40 μg/ml produced 38.6 ± 3.6% relaxation, and 60 μg/ml produced 47.2 ± 3.5% relaxation (n = 8). Crude soluble fractions from cultured BAE cells were significantly less active in relaxing rabbit aortic strips than the crude particulate fraction. In the presence of 100 μM L-arginine, 100 μM NADPH, and SOD (20 units/ml), 10 μg/ml produced

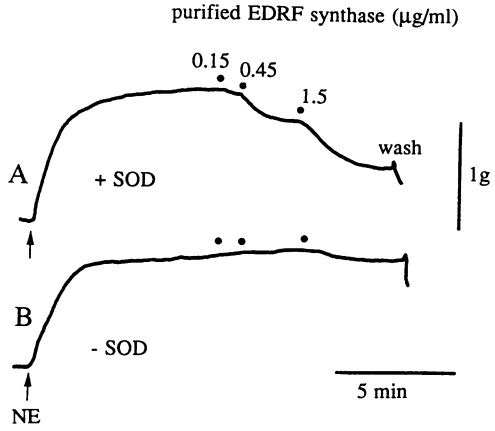

FIG. 3. Vascular relaxation in response to EDRF generated by purified EDRF synthase from cultured BAE cells. The rabbit aortic strips denuded of endothelium were contracted with norepinephrine (NE; 30 nM). Traces: A, in the presence of 100 μM L-arginine, 100 μM NADPH, 2 mM Ca^{2+}, 30 nM calmodulin, 10 μM BH_4, and SOD (20 units/ml), pure EDRF synthase (0.15–1.5 μg/ml) produced relaxations of the tissue; B, omission of the SOD from the reaction mixture virtually abolished the relaxation responses.

3.9 ± 2.3% relaxation of norepinephrine (30 nM)-induced tone, 20 μg/ml produced 9.0 ± 0.5% relaxation, 40 μg/ml produced 14 ± 3.4% relaxation, and 60 μg/ml produced 14 ± 3.4% relaxation (n = 3). Omission of L-arginine and NADPH or SOD reduced those relaxations by >90%. The mixture of substrate and cofactors in the absence of crude or purified enzyme preparations had no effect on the vascular tone of the aortic strips.

Thus far, several isoforms of NO synthase have been described and four isoforms have been purified. These isoforms and their properties are summarized in Table 3. The type I and II isoforms are soluble whereas the type III and IV isoforms are particulate. The type I and III isoforms are constitutive and Ca^{2+}-dependent whereas the type II and IV forms are inducible with endotoxin and/or cytokines and are Ca^{2+}-independent. In the cytosolic fractions of brain and endothelial cells, the Ca^{2+}-dependent enzymes are regulated by calmodulin (types Ia and Ib, respectively). In neutrophils, a constitutive enzyme has been described that is Ca^{2+}-dependent but not dependent on calmodulin (15). We tentatively classify this enzyme as type Ic (Table 3). All isoforms studied to date are NADPH-dependent and BH_4-sensitive. Antibodies and ongoing molecular cloning studies should provide more definitive data regarding the similarities, differences, and primary structures of these and perhaps other isoforms of this enzyme and their distribution in various cells and tissues.

Table 3. Isoforms of NO synthase

Type	Cofactor(s)	Regulator(s)	Molecular mass of denatured protein (SDS/PAGE) kDa	Ref.	Location: Tissue or cell	Ref(s).
Ia (soluble)	NADPH, BH_4, FAD/FMN	Ca^{2+}/calmodulin	155	12	Brain, cerebellum	12–14
Ib (soluble)	NADPH	Ca^{2+}/calmodulin	?	—	Endothelial cells	18, 30
Ic (soluble)	NADPH, BH_4, FAD	Ca^{2+} (not calmodulin)	150	15	Neutrophils	15
II (soluble)	NADPH, BH_4, FAD/FMN	Ca^{2+}-independent, induced by endotoxin/cytokines	150	17	Peritoneal macrophages	16, 17, 29, 33
			125–135	16	RAW264.7 macrophages	
III (particulate)	NADPH, BH_4, FAD/FMN*	Ca^{2+}/calmodulin	135	This paper	Endothelial cells	17, this paper
IV (particulate)	NADPH	Ca^{2+}-independent induced by endotoxin/cytokines	?	—	RAW264.7 macrophages	*

*Unpublished data.

In conclusion, the purification of EDRF synthase from cultured and native BAE cells has been accomplished in two rapid chromatographic steps. The purified enzymes from cultured and native BAE cells appear to be identical from chromatographic and kinetic properties and mobility on SDS/PAGE with a molecular mass of 135 kDa. This purified enzyme was also shown to synthesize a biologically active product capable of relaxing vascular smooth muscle, which is consistent with the hypothesis that a particulate isoform produces EDRF in BAE cells and is responsible for the phenomenon of endothelium-dependent smooth muscle relaxation described by Furchgott and Zawadzki (7).

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