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CO-PURIFICATION OF 130 KD NITRIC OXIDE SYNTHASE AND A 22 KD LINK PROTEIN FROM HUMAN NEUTROPHILS

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The synthesis of nitric oxide (NO) from L-arginine has been demonstrated in several cell types. Both constitutive and inducible forms of NO synthase have been described in different cells. We purified the constitutive form of NO synthase enzyme in human neutrophils using a two-column procedure. Crude 100,000g supernatant of human neutrophils was passed through a 2'-5'-ADP-agarose column followed by a DEAE-Bio-Gel A anion exchange column. NO synthase enzyme migrated as a single band (MW=130,000) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Its activity was dependent upon nicotinamide adenine dinucleotide phosphate (NADPH) and (6R)-tetrahydro-L-biopterin (BH_4). In addition, flavin adenine dinucleotide (FAD) was also found to be essential for its maximal activity. A second NADPH, FAD-dependent component (MW=22kD) was also found consistently on the SDS-PAGE gel. These observations suggest co-regulation between NO synthase enzyme and this NADPH, FAD-dependent component, which may be associated with the superoxide radical generating system.

Endothelium-derived vascular smooth muscle relaxing factor has been shown to be identical to NO (1). NO is generated by a variety of cells including endothelial cells, murine macrophages and murine and human neutrophils (2-5). During the action of NO synthase on substrate L-arginine, a guanidino nitrogen of L-arginine is oxidized to form NO and L-citrulline. Co-factors, NADPH and BH_4, have been identified to be necessary for the enzyme reaction leading to NO synthesis in these cells (4, 6). In addition, FAD is also a key component of this reaction (7). Different types of NO synthase enzymes, some with and others without Ca^{++}-dependence have been described in various tissues (8). The precise role of NO generated by neutrophils whether cytotoxic (9) or cytoprotective (10) is not clear. This in itself has generated intense interest in NO because of its role as a second messenger in cell-mediated inflammation and immunity (11). The process of NO synthesis identified in the murine macrophages and neutrophils involves the formation and transport of L-arginine following stimulation with bacterial endotoxin and/or cytokines (12). NO formed via the action of the enzyme NO synthase is rapidly broken down by free oxygen species, particularly the superoxide radicals (10, 13). A number of disease states, such as inflammation and reperfusion of tissues following temporary arterial obstruction, are characterized by release of large amounts of free oxygen radicals. The release of free radicals

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decreases NO activity, leading to diminished vasodilation (14). However, NO itself may inhibit neutrophil superoxide anion production via a direct action on the NADPH oxidase protein (10, 15).

In this report, we describe identification of NO synthase enzyme in human neutrophils. We also show that the NO synthase enzyme co-migrates with a second protein of MW≈22kD which may have a bearing on the feedback regulation of NO synthase activity.

MATERIALS AND METHODS

2,3,4,5- ^{3}H-L-arginine (77 Ci/mmol; 1Ci=37GBq; 1.0 mCi/ml) was obtained from Amersham, Arlington Heights, IL. All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, except L-NMMA, which was a gift from Dr. S. Moncada, Wellcome Research Laboratories, UK. Other supplies were obtained as specified.

Preparation of Neutrophils and Neutrophil Extract

Heparinized peripheral venous blood from healthy individuals was layered over Mono-Poly Resolving Medium (Flow Laboratories, McLean, VA), and centrifuged at 500g for 30 min at 24°C. Red blood cells in the neutrophil-rich layer were lysed by hypotonic lysis. Neutrophils were then removed and washed in Hanks Buffered Salt Solution (HBSS, pH 7.4) without Ca^{++} and Mg^{++} (16). Neutrophil suspension ($1x10^8$ cells/ml of Tris HCl buffer) contained more than 98% neutrophils, and their viability as determined by Typan blue exclusion was more than 95%. This neutrophil suspension was used to prepare cell extract for NO synthase assay.

For preparation of neutrophil extract, neutrophils were suspended in 50 mM Tris-HCl buffer, pH 7.4, and 1 mM dithiothreitol (DTT) at a density of $1x10^8$ cells/ml. The following protease inhibitors were added: phenylmethylsulfonyl fluoride (0.1 mg/ml), trypsin inhibitor (0.01 mg/ml), leupeptin (0.01 mg/ml), antipain (0.01 mg/ml), chymostatin (0.01 mg/ml), and pepstatin (0.01 mg/ml). Cells were then disrupted by sonication for 30 seconds (Microson Ultrasonic Cell Disruptor, Heat Systems-Ultrasonics, Inc., Farmingdale, NY) and centrifuged at 100,000g (Beckman Ultracentrifuge, Fullerton, CA) for 60 min at 4° C. An aliquot of the cytosol was saved at -70° C. The pellet was re-suspended in bovine serum albumin (BSA, 0.1 mg/ml) and glycerol (0.1%) and was mixed with the cytosol for purification.

Purification of NO Synthase Enzyme

The extract was applied to a column of 2′,5′-ADP-agarose (1x1 cm, Bio-Rad) equilibrated with 50 mM Tris-HCl at pH 7.4 containing 1 mM DTT. The column was washed with 5 ml of 50 mM Tris-HCl, 1 mM DTT, and 500 mM NaCl and 10 ml of 40 mM Tris HCl with 1 mM DTT, successively. The enzyme was eluted with 2 ml of 50 mM Tris-HCl, 1 mM DTT, and 1 mM NADPH and applied to an anion exchange column, DEAE-Bio-Gel A (0.5x1 cm, Bio-Rad). After washing the column with 50 mM Tris-HCl and 1 mM DTT, enzyme was eluted with 50 mM Tris-HCl, DTT, and 80 mM NaCl, followed by 50 mM Tris-HCl, 1mM DTT, and 120 mM NaCl (4).

Determination of NO Synthase Activity

NO synthase activity was measured by monitoring the conversion of ^{3}H-L-arginine to ^{3}H-L-citrulline as described previously (2). The method used was as follows: 30 µl of cytosol or purified enzyme and 25 µl of ^{3}H-L-arginine (average count 340,000 dpm/min) were added to 200 µl of buffer containing 25 mM Hepes (pH 7.4), 1.5 mM NADPH, 2 mM EDTA, 1.25 mM $CaCl_2$, 1 mM DTT, 2.5 µM FAD and 0.1 µM (6R)-BH_4. After incubation for 5 min at 37° C, the reaction was terminated with 2 ml of cold buffer (20 mM Hepes, 2 mM EDTA and 5 mM N-arginine, pH 5.5). The aliquots were applied to Dowex AG50WX-8 (Na+ form) columns (Bio Rad, Richmond, CA) and eluted with 6 ml of distilled water. ^{3}H-L-citrulline in the eluent was quantified by liquid scintillation spectroscopy. The identity of ^{3}H-L-citrulline in the eluent has been confirmed previously by thin layer chromatography (2).

Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophesis (PAGE)

SDS-PAGE was performed using a Bio-Rad Mini-Protean II dual slab cell, a discontinuous buffer system (17) and a 7.5% separating gel. Equivalent amounts of crude supernatant and purified eluent were electrophoresed on an acrylamide linear gradient SDS gel. Gels were silver-stained using the silver staining kit (Bio-Rad).

Concentration and Determination of Protein

Protein in the purified fraction was concentrated by ultrafiltering the sample through an anisotronic membrane (Amicon, Beverly, MA). Two ml of solution was added to the concentrator's reservoir and centrifuged. Samples were centrifuged according to the type of membrane used (Centricon 10 and 100 kD MW). After centrifugation, the concentrate was stored at -70° C. Protein concentrations were determined according to Bradford (17).

Superoxide Radical Generation

Superoxide radicals generated by crude neutrophil extract and purified protein were determined by measuring the superoxide dismutase (SOD) inhibitable reduction of ferricytochrome c to ferrocytochrome c as described previously (16). The stimulus used was phorbol 12-myristate 13-acetate (PMA, 100 ng/ml). All observations were made in triplicate and the data averaged.

RESULTS

The protein concentrations and NO synthase activity in crude cytosol (supernatant) and the purified protein fraction from DEAE-column are shown in Table I. The specific activity of NO synthase was greater in the purified fraction than in the cytosol (greater L-citrulline formation, Table II). A representative SDS-PAGE showing purification of NO synthase in the neutrophil cytosol and the purified protein fraction is presented in Fig 1. DEAE eluent (column 3) exhibited two distinct bands, one with MW approximately 130 kD and another with MW approximately 22 kD. The 130 kD fraction demonstrated NO synthase activity (approximately 30-40% conversion of ^{3}H-L-arginine to ^{3}H-L-citrulline). The purified enzyme fraction eluting from the ADP affinity column also showed both 130 kD protein and another NADPH co-factor protein of 22 kD protein. These results from two different purification methods showed consistent migration of the NO synthase protein as well as another NADPH-dependent protein. The migration of the ≈130 kD band was similar to that found by Hevel et al (3) on purification of murine macrophage NO synthase on SDS-PAGE.

Conversion of L-arginine to L-citrulline in human neutrophil cytosol was found to be dependent on the availability of NADPH, FAD, and BH_4, as in the murine neutrophils (6). There was no NO synthase activity without NADPH, and a higher NO synthase activity in the presence of BH_4 and FAD (data not shown). Therefore, in all experiments shown in Table II, NADPH, FAD and BH_4 were added to the buffer prior to the addition of ^{3}H-L-arginine. The conversion of

Table I. *Purification of NO Synthase*

Purification step	Protein Concentration	Specific Activity NO Synthase		Yield
	(μg/ml)	(dpm/min)[1]	(dpm/min/mg)[2]	%
100,000xg supernatant	16,000	316,070	47,411	100
2',5'ADP-Agarose Gel	U.D.[3]	—	—	75
DEAE-Bio-Gel A	1,200	69,064	138,128	12.5

[1] A unit is defined as that total amount of ^{3}H-L-citrulline formed.
[2] Calculated based on eluate collected from Dowex column.
[3] UD: undetermined.

Table II. *Conversion of ^{3}H-l-arginine to ^{3}H-l-citrulline in Human Neutrophil Cytosol and Purified Protein: Effect of Ca^{++} NOS inhibitors*

		^{3}H-L-Citrulline formation %
Neutrophil Cytosol	without Ca^{++}	12.0±0.3
	with Ca^{++}	12.3±0.3
	L-NMMA + Ca^{++}	2.8±0.3
	with N-Arg + Ca^{++}	2.0±0.7
Purified Protein	without Ca^{++}	20.7±0.7
	with Ca^{++}	21.2±1.2
	with L-NMMA + Ca^{++}	1.2±0.3
	with N-Arg + Ca^{++}	1.2±0.2

The data based on 3 experiments, each in triplicate, in mean±SD.
Abbreviations: L-NMMA: N^G-monomethyl-L-arginine, N-Arg: N^w-nitro-L-arginine.

^{3}H-L-arginine to ^{3}H-L-citrulline in human neutrophil cytosol fractions was approximately 12%. In the purified protein fraction, the ^{3}H-L-citrulline formation was approximately 21% ($P<0.01$ vs cytosol). Similar observations were made in Ca^{++}-free buffer (1mM EGTA present) and Ca^{++}-rich buffer (EGTA absent). These data indicate failure of Ca^{++} to induce NO synthase activity. In addition, the conversion of ^{3}H-L-arginine to ^{3}H-L-citrulline was inhibited by two different NO synthase inhibitors, L-NMMA and by N-arginine (Table II).

Data on NO synthase activity (^{3}H-L-citrulline formation) and superoxide generation in the crude cysotol and the purified protein fractions are shown on Table III. In both crude unstimulated cytosol and purified protein, NO synthase activity was approximately three times the value of the superoxide radicals generated. Following stimulation with PMA, NO synthase activity decreased

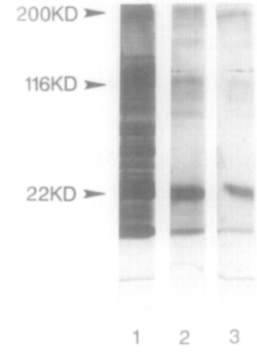

Fig. 1. SDS-PAGE of Human Neutrophil NO Synthase. Shown is a 7.5% gel that was silver-stained. Same amount of protein (45 ng) was used in lanes 2 and 3. Lane 1: Molecular weight markers of bovine carbonic anhydrase (31 kD), soybean trypsin inhibitor (21 kD), hen egg white lysozyme (14 kD) ovalbumin (45 kD), bovine serum albumin (66 kD), phosphorylase b (97 kD), β-galactosidase (116 kD), and mysosin (200 kD). Lane 2: Crude Cytosol. Lane 3: Bio-gel A eluate containing the purified protein. Both lanes 2 and 3 show a ~130 kD MW protein, which is most likely NO synthase. In addition, there is presence of a ~170 kD band which may be a part of dimer of 130 kD protein as described by Hevel et al (3). The electrophoresis of cytosol (lane 2) shows fewer than expected bands between 22 kD and 116 kD markers, probably because of the small concentration of protein used.

Table III. *NO Synthase Activity and Superoxide Generation in Cytosol and Purified Protein*

	^{3}H-l-Citrulline Formation (%)	Superoxide Generation (nmol/10^6/10min)
Unstimulated Cytosol	13.4±0.3	3.0±0.2
PMA-Stimulated Cytosol	4.6±0.3	12.3±0.7
Unstimulated Purified Protein	22.2±0.2	6.7±1.1
Stimulated Purified Protein	1.6±1.2	19.8±1.0

NO synthase activity was measured as conversion of ^{3}H-l-arginine to ^{3}H-l-citrulline. Superoxide generation was measured as conversion of ferricytochrome to ferrocytochrome. Data from experiments, each in triplicate, in mean±SD.

by about 65% and superoxide generation increased by about 100% in both crude cytosol and the purified protein. The eluent collected from the DEAE-Bio-Gel column was subjected to Centricon-10 and 100 kD microconcentrators and the ≥100 kD and <100 kD fraction used for determination of NO synthase activity and superoxide radical generation. In the <100 kD protein fraction, NO synthase activity was greatly reduced or completely lost (Table IV). In the ≥100 kD fraction, there was evidence for NO synthase activity and superoxide radical generation, but the latter was less than in the purified protein fraction (Table III).

DISCUSSION

This study demonstrates that NO synthase activity in human neutrophils is Ca^{++}-independent, but requires the presence of NADPH, FAD and BH_4 for maximal activity, characteristics similar to those of NO synthase activity in murine neutrophils (6). The activity of NO synthase was inhibited by two different analogs of L-arginine, L-NMMA and N-arginine (Table II) (18,19). We did not find stimulation of NO synthase activity in neutrophil cytosol with calcium ionophore A23187 (data not shown). These features of NO synthase activity suggest that the enzyme exists in human neutrophils primarily in a constitutive form. A comment regarding the methodology for conversion of ^{3}H-L-arginine to ^{3}H-L-citrulline needs to be made. Recent studies (20) suggest that L-citrulline is converted into L-arginine in rat macrophages which may limit the usefulness of this methodology. However, Klein et al (21) showed only insignificant conversion of L-citrulline to L-arginine. Even in studies by Wu and Brosman (20), the conversion of L-citrulline was only 300 pmol/h per 10^6 cells.

On SDS-PAGE, NO synthase migrated as a prominent band with MW of 130 kD, this MW is similar to NO synthase in rat neutrophils and macrophages (3, 4, 6). The NO synthase on SDS-PAGE was accompanied by one or two occasional faint bands with molecular weights slightly less

Table IV. *Superoxide Radical Generation in NO synthase activity in Amicon filter-concentrated Protein Fractions*

	Superoxide Radical Generation (nmol/10^6/10min)	^{3}H-l-citrulline Formation (%)
≥100 kD MW Fraction	0.82±0.12	2.2±0.9
<100 kD MW Fraction	0.93±0.10	0

Data from 3 separate experiments in triplicate, in mean±SD.

than 130 kD and these may represent degradation products of NO synthase. In addition, there was another band which appeared above the ≈130kD level which was eluted from the DEAE column. This band may be a subunit of the NO synthase protein as described earlier (3). The precise function of this band *per se* or its effect on the activity of 130 kD protein is not known. A novel feature of this report is the consistent finding of another protein fraction with a MW of 22 kD, which was observed on the SDS-PAGE of cytosol and purified protein fractoin (Fig. 1). The affinity resin that recognized NADP/NADPH identified the 22 kD protein . This band (22 kD) had the same binding affinity to the ADP/DEAE columns as the 130 kD band. In addition, we believe that this 22 kD fraction responds to PMA (Table III). The purification and delineation of the function of 130 kD and 22kD fractions was made possible by the use of simple, sensitive and specific assays for monitoring NO synthase activity (2) and superoxide radical generation (16).

A number of studies have demonstrated that superoxide radicals cause breakdown of NO (10,13). Recent reports suggest downregulation of superoxide radical generation by exogenous NO (15). These reports suggest an intimate relationship between NO synthesis and superoxide radical generation. Human neutrophil NO synthase and superoxide radical generation could be linked by an FAD-dependent protein. Fluorescence studies utilizing pure NO synthase proteins have indeed demonstrated the presence of both FAD and FMN in 1:1 ratios (3). Bredt et al (22) have shown that the NO synthase in rat cerebellum tissue contains binding sites for both FAD and FMN based on cDNA sequence and homology to cytochrome P-450 reductase.

The relationship between superoxide radical generation and NO synthase activity was dramatic. With PMA stimulation, superoxide radical generation increased 2-4 fold while NO synthase activity declined 50-70%. This relationship was observed in both cytosol and purified protein fractions (Table III).

Our consistent observations of two distinct protein fractions by two different purification procedures suggest that these two protein fractions of MW ≈ 130 kD and ≈22 kD may have an interdependence. This became quiet evident from measurements of superoxide radicals and NO synthase activity in each fraction, >100 kD and <100 kD. Whereas the first fraction demonstrated some NO synthase activity with minimal superoxide radical generation, the second fraction show some superoxide radical generation without any NO synthase activity (Table IV).

These data clearly suggest co-migration and co-dependence with some feedback inhibitory activity of two protein fractions in human neutrophils. We suggest that the 130 kD protein fraction represents NO synthase and the 22 kD protein fraction is a part of NADPH and FAD-dependent superoxide radical generating system.

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