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STUDIES ON CYTOSOLIC GUANYLATE CYCLASE FROM HUMAN PLACENTA+

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We have purified the soluble form of guanylate cyclase from human placenta >2400-fold. The enzyme shared several characteristics with the enzyme purified from other sources including molecular mass and subunit composition, activation by divalent cations, inhibition by ATP and Michaelis constants. The enzyme, however, had a lower absorption maximum in the Soret region (417 ± 1 nm) than the enzyme from other sources and was activated only one-fifth as much by nitric oxide as the bovine lung enzyme. It appears that the heme prosthetic group in the human placental enzyme may be hexa-coordinate and in the bovine lung enzyme the heme group may be penta-coordinate.

Guanylate cyclase exists as a soluble, or cytosolic, form and as a particulate, or membrane-bound, form (1-3). The soluble form is a heme-containing enzyme; it is activated 20-200-fold by NO' and is the target of endogenously-generated NO, i.e., endothelium-derived relaxing factor, and of NO-generating agents such as SNP and nitroglycerin (4-9). The particulate enzyme has an extracellular domain and is activated by binding of atrial naturietic peptide and other signaling peptides (10); the enzyme from bovine rod outer segments is activated 2-20-fold by NO suggesting that this enzyme may also contain heme (11).

The soluble enzyme has been purified from bovine lung and rat lung, brain and liver with the bovine lung and rat liver enzymes studied most extensively (3-8,12-14). The human enzyme has been partially purified from platelets by several groups and in this partially-purified state was activated only 4-fold by NO (15,16). Because of the importance of the soluble form of guanylate cyclase as

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The abbreviations used are: nitric oxide (NO), sodium nitroprusside (SNP) and thin-layer chromatography (TLC).

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the mediator of the actions of endothelium-derived relaxing factor and nitrosovasodilators, we decided to determine the degree of activation of the highly purified human enzyme by NO. We chose placenta as our source and found that the enzyme was activated only 5-fold by NO compared to a 25-fold activation of the bovine lung enzyme. It appears that the microenvironment of the heme group in the human placental enzyme is different than in the enzyme from other sources.

MATERIALS

Human placentas were obtained within 30 min of delivery according to a protocol approved by the Institutional Review Board. The NO was >99.9% pure; prior to use it was dissolved in oxygen-free 500 mM triethanolamine, pH 7.5 to a saturating concentration of 1.91 mM and diluted as needed. DEAE-Sephacel was from Pharmacia, GTP-agarose (22-carbon spacer) was from Sigma and Matrex gel blue A was from Amicon.

METHODS

Measurement of Guanylate Cyclase Activity

Guanylate cyclase activity was measured by following [α-^{32}P]GTP conversion to [^{32}P]-cGMP; substrate and product were separated by TLC. The reaction mixture contained in a final volume of 20 µl: 25 mM triethanolamine, pH 7.4, 5 mM $MnCl_2$, 1 mM isobutylmethylxanthine, 100 µM GTP, 0.2 µCi of [α-^{32}P]GTP and 20 ng of either human placental enzyme or bovine lung enzyme (where indicated 3 mM $MgCl_2$ was substituted for the $MnCl_2$). The reaction was started by adding substrate, except in NO activation experiments where the NO solution was added to the incubation mixture lacking enzyme; in these latter experiments the reaction was started by adding enzyme which had been previously incubated for 60 min at 4°C in 0.01 mM hemin and 10 mM cysteine in extract buffer (defined below). Reactions were terminated after 10 min at 37°C by adding 5 µl of 8 M HCOOH.

Substrate and product were separated by spotting 18 µl of the reaction mixture on TLC plates pre-spotted with a marker mixture containing GTP, GDP, GMP and cGMP. The plates were developed for 6 h in a butanol/acetone/ water/acetic acid/ammonium hydroxide (35/25/22.5/15/2.5) solvent system. The nucleotides were visualized under ultraviolet light and had the following R_f values: GTP, 0.15; GDP, 0.22; GMP, 0.30; and cGMP, 0.48. The spots corresponding to each nucleotide were cut out and radioactivity was measured by liquid scintillation counting. For each sample, the radioactivity recovered in cGMP was divided by the sum of radioactivity recovered in GMP, GDP, GTP and cGMP to calculate a conversion factor. The data are expressed as nmol of GTP converted to cGMP per min per mg of protein.

Because we separated substrate and product by TLC, rather than on columns as is usually done (2-4), we were able to determine the exact amount of conversion of substrate to product in each assay tube without the problem of variable recovery from sample to sample. Moreover, this method allowed us to determine that <10% of the GTP was catabolized to GDP and GMP during the incubation period; we, therefore, did not include a GTP-regenerating system in the assay mixture.

Michaelis constants were determined by linear regression analysis of Lineweaver-Burke double reciprocal plots. The degree of guanylate cyclase activation by NO was calculated according to the formula $\varepsilon_a = \frac{V - V_o}{V_o}$ where ε_a was the degree of activation, v_o was the reaction velocity in the absence of NO and v was the reaction velocity in the presence of NO.

Purification of Human Placental Guanylate Cyclase

All steps were performed at 4°C using nitrogen-flushed solutions. Placentas were washed in 25 mM triethanolamine, pH 7.8, 20 mM 2-mercaptoethanol, 5 mM dithiothreitol, 0.5 mM phenylmethylsulfonylfluroide (extract buffer) and cut into approximately 100 g pieces which were homogenized in a Waring blender. The extract was centrifuged at 100,000 X g for 1 h and added to DEAE-Sephacel resin. After gently mixing the resin for 1 h, the resin was washed with four volumes of extract buffer and six volumes of 50 mM NaCl in extract buffer. Guanylate cyclase was eluted with one volume of 350 mM NaCl and after concentration and dialysis was applied slowly to a 10 ml GTP-agarose column; the column was washed with extract buffer until protein was undetectable by the Bradford method (17). Guanylate cyclase was eluted with 5 mM GTP; the enzyme was detected by measuring the absorbance of fractions at 417 nm which coincided with the peak of maximal activity after dialysis.

The GTP-agarose column eluate was concentrated and incubated for 30 min with 50 μM hemin, and then applied to a 25 ml Matrex gel blue column which was washed with 500 ml of extract buffer followed by two liters of 0.5 M NaCl in extract buffer. A 300 ml gradient from 0.5 M NaCl to 2 M NaCl in extract buffer was applied to the column with guanylate cyclase eluting at approximately 1.5 M NaCl. The purified enzyme was detected by measuring absorbance of fractions at 417 nm and appropriate fractions were combined, concentrated and glycerol was added to 30% (v/v). The enzyme was stored at -20°C under N_2 and was stable for approximately one month. We incubated the enzyme with hemin prior to the Matrex gel blue column because human serum albumin present in the early steps of purification binds hemin and could potentially have extracted hemin from the enzyme (18).

Purification of Bovine Lung Guanylate Cyclase

Although the purification scheme described above was optimized for the human placental enzyme and is not identical to methods described for the bovine lung enzyme (4,6,7), for purposes of comparison the bovine lung enzyme was purified by the above described method. The bovine lung enzyme was purified >2000-fold and had a specific activity of 19.5 nmol/min/mg protein; this compares to a 1400-2000-fold purification of the enzyme with a specific activity of 20 nmol/min/mg protein (7). Even though bovine serum albumin does not bind hemin (18), the bovine enzyme was treated like the human enzyme and incubated with hemin prior to the Matrex gel blue column.

Polyacrylamide Gel Electrophoresis

Samples were heated at 100°C for 5 min in 20 mM Tris, pH 7.0, 2.5% SDS and 25 mM 2-mercaptoethanol. They were applied to a 7% SDS-polyacrylamide gel which was stained with Coomassie brilliant blue R-250.

Absorbance Spectra

Absorbance spectra were determined in stoppered, gas-tight cuvettes in nitrogen-flushed solutions.

RESULTS

Purification of Human Placental Guanylate Cyclase

Soluble guanylate cyclase from human placenta was purified >2400-fold with an approximate 3% recovery. When subjected to SDS-polyacrylamide gel electrophoresis, the highly purified enzyme yielded two major protein bands corresponding to apparent molecular masses of 70 kDa and 88 kDa (Fig. 1). Thus, as has been reported for the soluble form of the enzyme from several sources (14,19,20), the soluble form of human placental guanylate cyclase appeared to be a heterodimer. There was no evidence of contamination of the soluble enzyme by either hemoglobin or particulate guanylate cyclase, since the latter has a molecular mass ranging from 120 to 180 kDa (10).

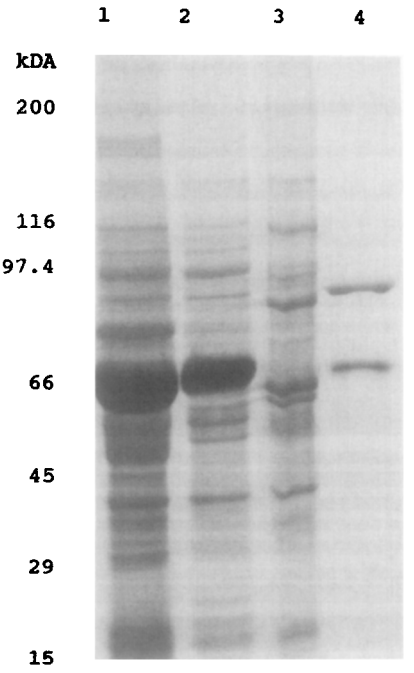

Fig. 1. SDS-gel electrophoresis of fractions from purification of human placental guanylate cyclase.
The soluble form of human placental guanylate cyclase was purified as described in Methods. Samples from each fraction were applied to a 7% SDS-polyacrylamide gel and after electrophoresis the gel was stained with Coomassie brillant blue R. In lane #1 is the 100,000 X g supernatant, in lane #2 the DEAE-Sephacel fraction, in lane #3 the GTP-agarose column fraction and in lane #4 the Matrex blue column fraction. The migration of molecular mass standards is shown in the left of the figure.

Effect of Mn^{++}, Mg^{++}, Ca^{++}, ATP and pH on Activity of Human Placental Guanylate Cyclase

Over a range from 0.5 mM to 5 mM of Mn^{++} and Mg^{++}, enzyme activity was approximately 2-fold greater with Mn^{++} than with Mg^{++}; optimal concentrations of these two metal ions were 5 mM and 3 mM, respectively. At concentrations between 80 μM and 5 mM, Ca^{++} increased enzyme activity by approximately 2-fold; this occurred when either Mn^{++} or Mg^{++} were the metal cofactor. Enzyme activity was inhibited markedly by physiological concentrations of ATP: at 0.5 mM ATP enzyme activity was inhibited approximately 80% and at 1 mM ATP enzyme activity was inhibited >95%. Maximal enzyme activity occurred at a pH of 8.5 and was approximately 2-fold greater than that observed at a pH of 7.4. These data are similar to what has been reported previously for the soluble enzyme purified from bovine lung and rat lung and liver (1-3). It should be noted that particulate guanylate cyclase is activated by physiological concentrations of ATP (2) providing further evidence that our preparation was free of the particulate

enzyme; in addition, the particulate enzyme is not activated by Ca^{++} but rather can be inhibited by this cation, possibly by a Ca^{++}-binding protein (10,11).

Determination of the Michaelis Constants of Human Placental Guanylate Cyclase

When determining the enzyme's Km values for MnGTP and MgGTP, the concentrations of Mn^{++} and Mg^{++} were kept constant at 5 mM and 3 mM, respectively, while varying the concentration of GTP from 2.5 µM to 1 mM. When Mn^{++} was the divalent cation the Km for GTP was 10.4 µM and when Mg^{++} was the divalent cation the Km for GTP was 105 µM; these Km's are similar to values reported for the enzyme purified from other sources suggesting that the GTP-binding site of the human placental enzyme is similar to that of the enzyme from other sources (1-3,7).

Absorbance Spectrum of Human Placental Guanylate Cyclase

Guanylate cyclase purified from human placenta showed an absorption maximum in the Soret region at 417 ± 1 nm (Fig. 2). This is lower than the absorption maximum of 430 ± 2 nm reported for the bovine lung enzyme (7,21,22). Adding dithionite to the human placental enzyme caused a minimal decrease in absorbance and gave rise to a shoulder at 424 nm; adding oxygen to the native enzyme by flushing the cuvettes with air had no effect on the spectrum (data not shown). These results are in contrast to the findings with the bovine lung enzyme which shows no change in absorbance maximum on adding dithionite and a marked and slow decrease in absorbance on adding oxygen (21).

Effect of NO and SNP on the Activities of Human Placental and Bovine Lung Guanylate Cyclase

In the presence of 3 mM $MgCl_2$, the activity of human placental guanylate cyclase was increased approximately 5-fold by 1.91 µM NO and 2-fold by 100 µM SNP

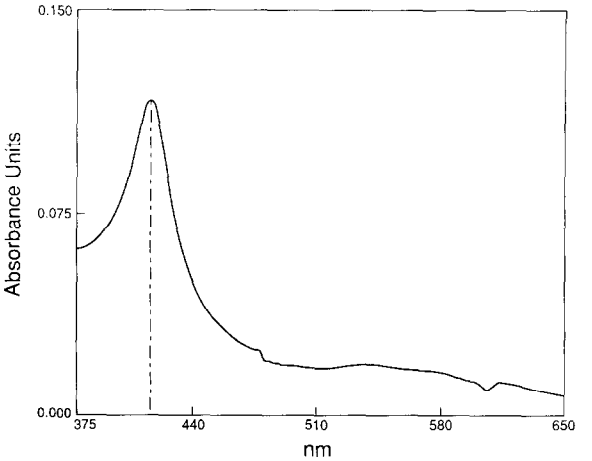

Fig. 2. Absorbance spectrum of human placental guanylate cyclase. The absorbance of human placental guanylate cyclase was measured under anaerobic conditions between 375 and 650 nm; the peak of absorbance occurred at 417 nm.

Table I. NO and SNP Activation of Soluble Guanylate Cyclase Purified From Human Placenta and Bovine Lung

Source	Enzyme Activity		
	No Additions	1.91 μM NO	100 μM SNP
	nmol/min/mg prot		
Human Placenta	25.3 ± 2.4	128 ± 1.4	53.8 ± 5.9
Bovine Lung	19.6 ± 2.1	495 ± 52	303 ± 32

Enzyme activity in the absence or presence of 1.91 μM NO and 100 μM SNP was measured as described in Methods. The values are the mean ± S.D. of four independent experiments performed in duplicate.

(Table I); less activation was seen when Mn^{++} was the divalent cation. This degree of activation by NO and SNP is considerably less than that reported for the soluble enzyme purified from bovine lung and rat liver (4-8,19). We, therefore, compared the degree of activation of human placental guanylate cyclase by NO and SNP to that of the bovine lung enzyme. We could reproduce the degree of activation by NO and SNP reported for bovine lung guanylate cyclase (4,6,7,19,23) but found that the human placental enzyme was activated only one-fifth as much by these agents as the bovine lung enzyme (Table I).

This difference between the human placental enzyme and the bovine lung enzyme was confirmed by comparing the rate constants of the two enzymes in basal and NO-activated states. In Fig. 3 the formation of cGMP is shown as a function of time; less than 3% of substrate was converted to product over the short time intervals of the experiment, even under activated conditions. The reaction catalyzed by guanylate cyclase can be expressed as follows:

$$GTP + GC \rightleftharpoons GTP\text{-}GC \xrightarrow{k} cGMP + GC$$

where GC is guanylate cyclase and k is the first order rate constant. Since:

$$\frac{d[cGMP]}{dt} = k[GTP\cdot GC] \quad \text{and} \quad \frac{d[GTP\cdot GC]}{dt} = 0, \quad \frac{d[cGMP]}{dt} = k'$$

where k' is a pseudo zero-order rate constant equal to k[GTP·GC]. The slopes in Fig. 3 yield k' = 0.013 nmol/min in the absence of NO and 0.064 nmol/min in the presence of NO for the human placental enzyme indicating a 5-fold activation of the enzyme by NO. Similar calculations for the bovine lung enzyme indicated that it was activated approximately 25-fold by NO. At high substrate concentrations most of the enzyme is bound to GTP and, therefore, [GTP·GC] = [GC] and k = k'/[GC]. The values of k for human placental guanylate cyclase were 0.96 min^{-1} and 4.73 min^{-1} in the absence and presence of NO, respectively; the corresponding values for the enzyme from bovine lung were 0.84 min^{-1} and 19.8 min^{-1}, respectively.

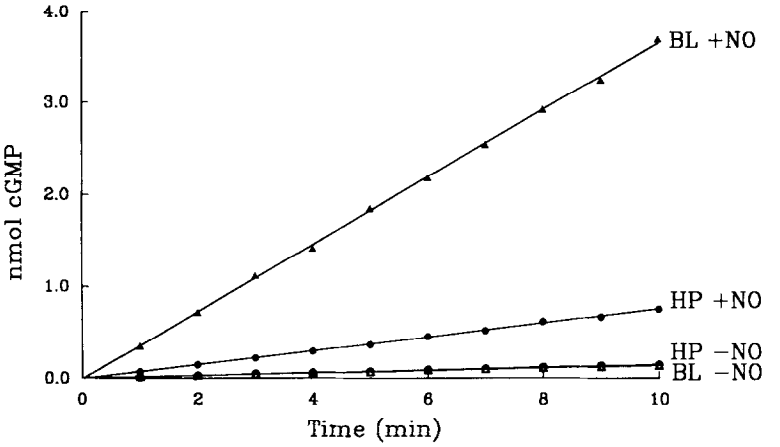

Fig. 3. Effect of NO on the activities of human placental guanylate cyclase and bovine lung guanylate cyclase.
The activities of human placental guanylate cyclase (circles) and bovine lung guanylate cyclase (triangles) were measured as described in Methods in the absence (open symbols) or presence of 1.91 μM NO (closed symbols). In separate tubes the reaction was terminated every minute over a 10 min interval. The nanmoles of GTP converted to cGMP are plotted versus time; each data point is the mean of two independent experiments performed in duplicate. BL, bovine lung guanylate cyclase; HP, human placental guanylate cyclase.

DISCUSSION

The soluble form of human placental guanylate cyclase shares several characteristics with the bovine lung enzyme since both enzymes: 1) are heterodimers with molecular masses of the two subunits being approximately 70 and 85 kDa (14,19,20); 2) are more active with Mn^{++} as the divalent cation than with Mg^{++} (6-8); 3) are modestly activated by Ca^{++} and markedly inhibited by ATP (1-3); and (4) have similar Km's for MgGTP and MnGTP (1-3,7).

The microenvironment of the heme group of the two enzymes appears to be different, however, since the Soret spectrum of human placental guanylate cyclase differed considerably from that of the bovine lung enzyme. The λ_{max} of the human placental enzyme in the absence or presence of oxygen was 417 ± 1 nm and adding dithionite caused the appearance of a shoulder at 424 nm. This is in contrast to what has been found for the bovine lung enzyme which in the absence and presence of oxygen shows a λ_{max} in the Soret region at 430 ± 2 nm and dithionite has no effect on the λ_{max} (21,22). By comparison with the data for hemoglobin (λ_{max} HbO_2 = 415 nm and λ_{max} Hb = 430 nm) and myoglobin, it would appear that the iron in the heme prosthetic group in the bovine lung enzyme is in a penta-coordinate divalent state and in the human placental enzyme the iron is in a hexa-coordinate state (24).

These observations provide a possible explanation for less NO activation of human placental guanylate cyclase than of the bovine lung enzyme: the prosthetic heme group in the human placental enzyme may be hexa-coordinate and,

therefore, not as readily available for ligation with NO as it is in the bovine lung enzyme. It should be noted that NO has a very high affinity for iron heme and can bind both to penta- and hexa-coordinate hemes (25,26). In the case of reaction with hexa-coordinate heme, the binding affinity of NO would be considerably reduced because it must replace the distal axial ligand; even after being replaced, the distal ligand could provide a steric hindrance to NO.

Although the degree of guanylate cyclase activation by NO can vary from tissue to tissue in the same animal (23), it is possible that the soluble form of guanylate cyclase from human tissues other than placenta, e.g., vascular smooth muscle cells, is also less activated by NO than the enzyme from bovine and rat sources. In this context it is interesting to note that nitroglycerin and SNP are less potent arterial vasodilators when administered to humans than when administered to rats (27).

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