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Localization and Changes of Diamine Oxidase During Cardiopulmonary Bypass in Rabbits[1]

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Background. **We previously observed increased serum diamine oxidase activity during clinical cardiopulmonary bypass, indicating small intestinal mucosal ischemia followed by bacterial translocation.**

Materials and methods. **In seven female rabbits undergoing cardiopulmonary bypass for 1 h, we analyzed the localization of diamine oxidase immunohistochemically, and measured its activity in serum and abdominal organs before and after cardiopulmonary bypass (CPB).**

Results. **Preoperatively, diamine oxidase activity and immunoreactivity were high in the small intestine, localized to villus tips. Serum activity increased significantly after CPB, whereas small intestinal diamine oxidase decreased with mucosal injury.**

Conclusions. **In this model serum diamine oxidase activity appeared to reflect CPB induced intestinal mucosal injury.**

Key Words: **small intestinal mucosal injury; diamine oxidase; bacterial translocation; cardiopulmonary bypass; rabbit.**

INTRODUCTION

Although cardiopulmonary bypass (CPB) is essential for some procedures in cardiovascular surgery, it causes peripheral hypoperfusion because of nonpulsatile flow, low blood pressure, hemodilution, and other nonphysiologic conditions. Furthermore, bacterial translocation (BT) is thought to occur in the gut during CPB [1–3]. In a more extreme but related condition, the gastrointestinal tract in multiple organ failure has been called an "undrained abscess" [4]. Study of intestinal barrier function during CPB is important, but is difficult clinically because of lack of access to the intestinal mucosa. Diamine oxidase (DAO) is localized mainly to the small intestinal mucosa, particularly near the tips of villi [5–7]. Serum DAO activity has been reported to be elevated in intestinal ischemia [8, 9]. During clinical CPB, we demonstrated simultaneous increases in serum DAO activity and peptidoglycan concentration, suggesting small intestinal mucosal ischemia and BT [10, 11]. Yet, serum and intestinal DAO activity have not been measured together during CPB, and occurrence of intestinal mucosal injury during CPB has not been proven. We studied DAO in serum and small intestinal mucosa together with small intestinal mucosal injury in a rabbit model of CPB.

MATERIALS AND METHODS

Animals

Seven female New Zealand white rabbits (3.8 ± 0.2 kg) were used (Charles River Japan, Yokohama). Animals were not fasted before surgical procedures. The study was approved by the Ehime University of School of Medicine Research Committee according to the Guidelines for Animal Experiments of Ehime University (Institute of Laboratory Animals, Ehime University School of Medicine).

DAO Activity

DAO activity was measured using histamine as a substrate. Briefly, incubation was performed at 37°C in 100 mM potassium phosphate buffer (pH 6.8), containing 10 μM histamine, plus the enzyme sample for assay. The reaction was terminated by adding 60% perchloric acid. Decrease in histamine produced by DAO per unit time was determined using high-performance liquid

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chromatography-fluorometry [12]. One unit of enzyme activity was defined as deamination of 1 mol of histamine/min under the standard assay condition.

Protein Determination

Protein concentration was determined by the method of Lowry *et al.* [13] with bovine serum albumin as a standard. Absorbance was measured at 595 nm.

Purification of DAO from Rabbit Small Intestine

All purification steps [14] were carried out at 4°C.

Step 1: Crude Extract Preparation

Rabbit small intestinal tissue (116 g) was homogenized with a Polytron homogenizer in five volumes of 100 mM potassium phosphate buffer (pH 6.8). A crude extract obtained by ultracentrifugation of the homogenate at 10,000 × *g* for 30 min was dialyzed three times against the same buffer.

Step 2: Ammonium Sulfate Fractionation

The dialyzed crude extract was saturated with 30% ammonium sulfate for 2 h, and then centrifuged at 10,000 × *g* for 15 min. The supernatant was brought to 60% ammonium sulfate saturation, allowed to stand for 2 h, and then centrifuged at 10,000 × *g* for 15 min. The precipitate, which contained DAO activity, was dissolved in 5 ml of 100 mM potassium phosphate buffer (pH 6.8), and then dialyzed three times against the same buffer.

Step 3: DEAE-Cellulose

Dialysate was applied to a DE-52 column (100 g) equilibrated with 50 mM potassium phosphate buffer (pH 6.8). The enzyme was eluted with a linear gradient of potassium phosphate buffer (50 to 300 mM) in the equilibration buffer. DAO-containing fractions were pooled, concentrated with YM-30, and then dialyzed three times against 100 mM potassium phosphate buffer (pH 6.8).

Step 4: Hydroxyapatite Column Chromatography

The dialyzed enzyme was applied to a BIO-GEL HTP column (100 ml) equilibrated with 100 mM potassium phosphate buffer (pH 6.8). The enzyme was eluted with a linear gradient of ammonium sulfate (0 to 1.0 M) in the equilibration buffer, and then dialyzed three times against 100 mM potassium phosphate buffer (pH 6.8).

Step 5: Concanavalin (Con) A-Sepharose Column Chromatography

The enzyme was kept overnight in 1 ml of a suspension of Con A-sepharose equilibrated with 50 mM potassium phosphate buffer (pH 6.8), containing 30 mM NaCl. The gel was packed into a column and then washed with equilibration buffer. The enzyme was eluted with the equilibration buffer containing 0.2 M α-methylglucoside.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed by the method of Laemmli [15] using a 7.5% PAG. The gel was then stained with a silver stain kit (Daiichi Pure Chemicals, Tokyo).

Anti-DAO Antiserum Preparation

An emulsion of purified DAO (30 μg in 0.2 ml) in an equal volume of Freund's complete adjuvant (Wako Pure Chemicals, Osaka) was injected into two female BALB/c mice. An emulsion of the enzyme (30 μg) with Freund's incomplete adjuvant (Wako Pure Chemicals) was injected as the first, second, and third booster at 2, 4, and 6 weeks, respectively, after the first injection. Anti-DAO antiserum was collected 2 days after the fourth booster.

Immunohistochemical Stain for Rabbit Intestinal DAO

Small and large intestine were sampled in the preoperative period, and fixed in 4% paraformaldehyde and 2% glutaraldehyde for 4 h at 4°C. Samples then, were washed with phosphate-buffered saline (PBS) containing 20% sucrose, and were embedded in optimal cutting temperature (OCT) compound (Tissue-Tek 4583, Sakura Finetec Japan, Tokyo), and then stored in a deep freezer at −80°C. The embedded tissue was sectioned at a thickness of 6 μm with a cryostat and affixed a glass slide. Sections were incubated in 5% normal goat serum for 30 min at room temperature to block nonspecific binding. After washing with PBS, primary antibody (mouse anti-rabbit DAO antiserum; dilution, 1:200) was allowed to react with sections overnight at room temperature. Normal mouse serum diluted at 1:200 was substituted as a negative control. Sections then were placed in 0.3% H_2O_2/methanol for 3 min to block endogenous peroxidase (POD). Next, a 1:1000 dilution of secondary antibody (peroxidase-conjugated goat anti-mouse IgG, Chemicon International, Temecula, CA) was allowed to react with sections for 3 h at room temperature. Diaminobenzidine (DAB) staining then was performed with DAKO Liquid DAB and Substrate-Chromogen System (DAKO, Carpenteria, CA) for 5 min. Sections were mounted and coversliped with Softmount (Wako Pure Chemicals) after 10 min of nuclear counter staining with methyl green and stepwise dehydration.

Cardiopulmonary Bypass [16, 17]

Each animal was anesthetized with ketamine (50 mg/kg) and pentobarbital (25 mg/kg) and then intubated with a 3-mm intratracheal tube via a tracheostomy. A mechanical ventilator was used (SN-480-5, Shinano, Tokyo). For arterial blood pressure monitoring a catheter was inserted in the right carotid artery. The electrocardiogram (ECG) and rectal temperature (RT) also were monitored.

Ringer's lactate solution was infused as maintenance fluid via the ear vein. After median sternotomy, Gore-Tex 6-0 cardiovascular suture was used to form a single purse-string layer in abdominal aorta. With total clamping of the abdominal aorta, an incision suitable for arterial cannulation was made within the purse-string. An aortic cannula (8 Fr) was inserted in a retrograde direction, and a purse-string tourniquet was secured. A 16-Fr venous cannula was inserted in the right atrium via the auricular appendage. Heparin was injected before cannulation (1000 unit/kg).

The cardiopulmonary circuit (Mera, Tokyo, Japan) consisted of a roller pump (Shiley; Stockert, Munich, Germany) and a membrane oxygenator (Mera). The main priming fluid of the circuit was Hespander (150 ml) and whole rabbit blood (120 ml). In addition, 10 ml of 15% mannitol, 40 ml of 8% sodium bicarbonate, 1 g of cefazolin, 7 ml of 2% calcium chloride, 3 ml of Poloxamer-188 (Exocorpol; Kobayashi Pharmaceutical Industry, Tokyo), and 500 units of heparin were injected into the circuit. After CPB was initiated, the flow rate was increased to 90 ml/kg/min [17, 18] and maintained for 1 h. Methylprednisolone sodium succinate (Solu-Medolol, Pfizer, Tokyo; 5 mg/kg) was injected before CPB initiation to inhibit biocompatibility. Rectal temperature was kept at 32°C to decrease tissue oxygen consumption. To eliminate residual pulsatile flow, a 16-G needle was inserted into the left ventricular apex, draining blood to the reservoir; ascending aorta was totally clamped without inducing cardiac arrest.

Sampling for DAO

Jejunum (20 cm distal to the pylorus), ileum (20 cm proximal to the ileocecal valve), ascending colon (20 cm distal to the valve), liver