Immunogold Localization of Adenosine 5'-Monophosphate-Specific Cytosolic 5'-Nucleotidase in Dog Heart

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Adenosine has a major regulatory function in the heart and many tissues. Our previous work showed that a cytosolic (not a membrane, as previously hypothesized) 5'-nucleotidase from dog heart has the kinetic properties consistent with it being the enzyme responsible for adenosine formation from adenosine 5'-monophosphate (AMP) in response to hypoxia or ischemia. In the present study, we evaluated the spatial distribution of AMP-specific cytosolic 5'-nucleotidase in dog heart using electron microscopic immunogold localization. Polyclonal antibodies raised against purified cytosolic 5'-nucleotidase recognized the 43-kd subunit of the enzyme on Western blots of both purified enzyme and the soluble fraction of dog heart homogenates but did not react with proteins extracted from the membrane fraction. Purified cytosolic 5'-nucleotidase and 5'-nucleotidase activity present in the soluble fraction of heart homogenates were inhibited by anti-cytosolic 5'-nucleotidase, but the membrane fraction was not. The monospecific antibodies against the cytosolic 5'-nucleotidase were used for electron microscopic immunogold localization of cytosolic 5'-nucleotidase in dog heart tissue sections. Cytosolic 5'-nucleotidase was found in the cytoplasm of red blood cells, cardiac myocytes, and endothelium; the plasma membrane and interstitium were devoid of gold label. These results are the first to document the presence of cytosolic 5'-nucleotidase in specific cell types in the heart and demonstrate the potential for these cell types to produce adenosine via cytosolic 5'-nucleotidase. (Hypertension 1993;21:906-910)

Key Words • adenosine • adenine nucleotides • endothelium • enzymes

Adenosine is a potent vasodilator, and its role as a local mediator of blood flow regulation has been an area of intensive investigation for 30 years.1 Ohnishi and coworkers2-3 have demonstrated that adenosine attenuates the activation and expression of the renin-angiotensin system in renin-dependent renovascular hypertension. These findings suggest that adenosine may also function as a protective factor by mitigating some of the hypertension-producing effects of angiotensin II.3

The conceptual framework for the adenosine hypothesis is that an imbalance between oxygen supply and demand leads to hydrolysis of ATP and an increase in the intracellular concentration of AMP, which is dephosphorylated by the enzyme 5'-nucleotidase to adenosine. Adenosine released into the interstitial space then binds to adenosine receptors on the cell membranes of its various effector sites to elicit responses that serve to restore the tissue oxygen supply-to-demand ratio back toward normal. The primary source of endogenous adenosine, in terms of the cell types as well as the location of the 5'-nucleotidase producing adenosine, remains to be established.4

In the heart, the conventional view is that adenosine originates in the myocytes,5 but more recent studies have demonstrated that coronary endothelial cells6,7 are also capable of producing adenosine. With respect to the biochemical pathway mediating adenosine formation, an early and still widespread belief is that during myocardial ischemia and hypoxia, cardiac adenosine is formed exclusively from the dephosphorylation of AMP by an ecto-5'-nucleotidase localized on the plasma membrane.5,8 There is now considerable evidence, however, for the involvement of an intracellular 5'-nucleotidase in the rapid increase in adenosine production that occurs under ischemic or hypoxic conditions.9-14

We recently reported the purification of an AMP-specific 5'-nucleotidase from dog heart; this enzyme has regulatory properties that are consonant with its playing a major role in cardiac adenosine production.12-14 The purpose of this study was to evaluate the distribution and localization of this cytosolic 5'-nucleotidase in cardiac tissue using polyclonal antisera against the purified enzyme. Histochemical techniques to localize the ecto-5'-nucleotidase in heart tissue have been used previously in formulating and testing aspects of the adenosine hypothesis,5,15 but the distribution of cytosolic 5'-nucleotidase has not been reported.

Methods

Polyclonal Antisera

AMP-specific cytosolic 5'-nucleotidase was purified to homogeneity, as previously described.12,13 Twenty-five mi-
crogams of the purified enzyme in 0.5 mL of buffer was mixed in an equal volume of Freund's complete adjuvant and injected subcutaneously in the scapular region of five New Zealand White rabbits (Mohican Valley Rabbity, Loudonville, Ohio). The animals were boosted three times at 3-week intervals by repeating the injections with 25 μg of purified enzyme mixed with Freund's incomplete adjuvant. Ten days after the last injection, approximately 20 mL of blood was collected from an ear vein of both immunized and nonimmunized (control, n=2) rabbits using aseptic technique. After clot contraction, sera was removed by centrifugation. All animal procedures were approved and conducted in accordance with institutional guidelines.

**Preparation of Heart Homogenate Fractions**

Dog ventricular tissue was homogenized in ice-cold glycerol buffer (25% vol/vol glycerol), 0.1 mmol/L dithiothreitol, 1 mmol/L ethylenediaminetetraacetic acid, 0.2 mmol/L α-toluene sulfonyl fluoride, and 50 mmol/L N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid buffer, pH 7.0, and centrifuged at 150,000g for 1 hour to obtain soluble and membrane pellet fractions. Each fraction was dialyzed against glycerol buffer for 6 hours and stored at -70°C for later use.

**Antisera Specificity**

The specificity of anti-cytosolic 5'-nucleotidase was evaluated by immunodetection on Western blots of purified enzyme and the soluble and membrane fractions of ventricular homogenates. An alkaline phosphatase immunoblot assay kit (Bio-Rad Laboratories, Hercules, Calif.) was used with the anti-cytosolic 5'-nucleotidase antibody as the primary antibody. Briefly, after proteins from sodium dodecyl sulfate–polyacrylamide gels were transferred to nitrocellulose, the remaining protein binding sites on the nitrocellulose membrane were blocked with 5% gelatin. Strips of nitrocellulose membrane were then incubated with anti-cytosolic 5'-nucleotidase or the control sera at dilutions of 1:50, 1:250, 1:1,000, and 1:5,000 for 1 hour. The nitrocellulose strips were then washed and incubated for 1 hour with goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase. After washing, the strips were placed in alkaline phosphatase color development reagent until visible bands were developed.

**Enzyme Inhibition Studies**

Purified enzyme and samples of the soluble and membrane fractions of dog heart homogenates were incubated with various dilutions of immunized and nonimmunized sera for 30 minutes at 37°C before addition of a substrate buffer assay solution. The substrate buffer solution used to assay AMP-hydrolyzing cytosolic 5'-nucleotidase activity contained 50 mmol/L 3-N-morpholinopropanesulfonic acid buffer, 3 mmol/L AMP, and 5 mmol/L MgCl₂ at pH 7.4. β-Glycerophosphate (20 mmol/L) was added to the mixtures containing homogenate fractions to inhibit nonspecific phosphatase activity. 5'-Nucleotidase activity was determined by the release of inorganic phosphate, according to the spectrophotometric method of Sanui.<sup>16</sup>

**Immunogold Electron Microscopy**

Samples of dog heart ventricle were cut into 1-mm³ pieces and fixed in 1% glutaraldehyde in 0.2 mol/L sodium cacodylate buffer for 1 hour. After washing in 0.2 mol/L sodium cacodylate for 5 minutes, the tissue was incubated for 2 hours in 0.5 mol/L NH₄Cl in 0.2 mol/L sodium cacodylate buffer. The tissues were washed again in sodium cacodylate and transferred through a series of graded alcohols (30%, 50%, 70%, 90%, and 95%) for 10 minutes each and then in 100% alcohol for 10-minute washes. The tissues were then transferred into acetone for 10 minutes, followed by infiltration with acetone/Spurr's resin (1:1) for 4 to 8 hours. After infiltration, the tissues were embedded in 100% resin and polymerized at 70°C overnight. Sections 90-nm thin were picked up on nickel grids (Electron Microscopy Sciences, Fort Washington, Pa.). The grids were placed in 0.1 mol/L phosphate-buffered saline (PBS), pH 7.2, then blocked with 10% fish gelatin and 0.1% Triton X-100 in 0.1 mol/L PBS for 15 minutes. Sections were incubated in primary antisera (1:100) that was diluted with 1% fish gelatin and 0.01% Triton X-100 in PBS. Control sections were incubated in normal rabbit serum at the same dilutions as the primary antisera. Sections were washed six times in PBS and incubated in gold-conjugated anti-rabbit im-

![Figure 1](image.png)
Results

The polyclonal antibody against cytosolic 5'-nucleotidase recognized the single protein band of 43 kd molecular weight on the Western blot of purified cytosolic 5'-nucleotidase (Figure 1B). Anti-cytosolic 5'-nucleotidase also recognized the 43-kd protein on the Western blot of the soluble fraction of dog ventricular homogenate (Figure 1A, lanes 6–9). The antibody did not react with any proteins extracted from the membrane fraction (data not shown). There was no cross-reactivity with other proteins present in the soluble fraction, except for a minor band present with the 1:50 dilution. Control sera under similar conditions did not stain any proteins (Figure 1A, lanes 2–5).

The effect of anti-cytosolic 5'-nucleotidase on the activity of purified dog heart cytosolic 5'-nucleotidase is demonstrated in Figure 2. The addition of anti-cytosolic 5'-nucleotidase sera to the reaction mixture resulted in a progressive decrease in enzyme activity. Figure 3 shows that cytosolic 5'-nucleotidase activity present in the soluble fraction of dog ventricle was inhibited by the antibody, whereas the 5'-nucleotidase activity present in the membrane fraction did not change in the presence of this antibody.

Representative micrographs of the electron microscopic immunogold localization of cytosolic 5'-nucleotidase in dog heart tissue are shown in Figures 4 and 5. The majority of gold labeling was intracellular, with little or none associated with the plasma membrane and interstitium. The majority of cytosolic 5'-nucleotidase was localized in red blood cells. Other predominant sites of gold labeling were the cardiac myocytes and endothelial cells.

Discussion

The cellular and subcellular location of 5'-nucleotidase can be most reliably documented by immunocytochemical techniques using either monoclonal or monospecific polyclonal antibodies. Although other laboratories have isolated AMP-specific cytosolic 5'-nucleotidase from heart,19,20 no previous studies have evaluated the localization of cytosolic 5'-nucleotidase in this tissue. In the present study, the polyclonal antibody raised against purified AMP-specific cytosolic 5'-nucleotidase recognized both the native and denatured cytosolic 5'-nucleotidase from dog heart. The recognition of a single protein band on the Western blot of total dog heart soluble fraction and the absence of any reaction to

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**Figure 2.** Line graph shows inhibition of enzyme activity by anti-cytosolic 5'-nucleotidase. Purified dog heart AMP-specific cytosolic 5'-nucleotidase (0.5 μg) was mixed with increasing amounts of anti-cytosolic 5'-nucleotidase sera and incubated at 37°C for 30 minutes before assay for 5'-nucleotidase activity. Values are means of five experiments.

**Figure 3.** Bar graph shows effect of anti-cytosolic 5'-nucleotidase sera on AMPase activity of the supernatant and membrane pellet fractions of dog heart homogenates. Fifty microliters of soluble or membrane fraction was incubated with 10 μL of anti-cytosolic 5'-nucleotidase sera for 2 hours at room temperature, and 5'-nucleotidase activity was assayed. Values are means of five experiments.

**Figure 4.** Panel A: Electron photomicrograph of immunogold labeling of dog ventricular tissue with anti-cytosolic 5'-nucleotidase. Gold particles were localized within red blood cells (RBC), cardiac myocytes, and endothelial cells (Endo). Gold labeling was absent in the interstitial fluid (ISF) and plasma membrane of the cardiac cell. Mito, mitochondria. Panel B: Control section incubated with sera from nonimmunized rabbits.
membrane-extracted proteins demonstrate the affinity and monospecificity of the antisera to cytosolic 5'-nucleotidase. Inhibition of the activity of purified enzyme and the 5'-nucleotidase activity present in the soluble fraction of heart homogenates also demonstrate the affinity of the antisera for cytosolic 5'-nucleotidase. In addition, the inability of the antisera to inhibit 5'-nucleotidase activity present in the membrane fraction indicates that the antisera does not react with the membrane-bound 5'-nucleotidase.

Electron microscopic immunogold localization using the monospecific antibody to cytosolic 5'-nucleotidase demonstrated a significant amount of cytosolic 5'-nucleotidase in red blood cells. This finding is consistent with a previous report by Bontemps et al, who provided physiological data that argue for the operation of a cytosolic 5'-nucleotidase in erythrocytes. These investigators showed that treating red blood cells with the nucleoside transport inhibitor R-51469 caused adenosine to accumulate intracellularly. These data suggest that adenosine can be formed by an intracellular pathway in red blood cells, as confirmed by our results.

In the present study, the cardiac myocytes were also identified as a major site for the localization of cytosolic 5'-nucleotidase. This finding is consistent with the hypothesis that myocytes are the source of adenosine production under hypoxic or ischemic conditions. In 1973, Rubio et al concluded from their histochemical studies of the hearts of rats, guinea pigs, and dogs that adenosine is produced predominantly by an enzyme located on the outer surface of the plasma membrane. Our present results using immunogold labeling provide direct evidence for an intracellular localization of 5'-nucleotidase in myocytes. The technique employed by Rubio et al to use lead nitrate for the histochemical staining. We have found that lead nitrate is a potent inhibitor of cytosolic 5'-nucleotidase purified from dog heart (unpublished observations); this may explain the failure of Rubio et al to detect 5'-nucleotidase in the cytoplasm.

Our results also demonstrate that cytosolic 5'-nucleotidase is found in the endothelium. Previous studies have demonstrated the capability of endothelial cells to produce adenosine, but the enzymatic pathway involved was not identified. Our finding that cytosolic 5'-nucleotidase is present in the endothelium indicates that this enzyme may be involved in adenosine production in these cells. A limitation of our methodology, however, is that it does not permit evaluation of the relative contributions of the myocytes versus the endothelial cells to total cardiac cytosolic 5'-nucleotidase activity.

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