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P^1,P^4-diadenosine 5′ tetraphosphate induces nitric oxide release from bovine aortic endothelial cells

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Abstract Since the infusion of P^1,P^4-diadenosine 5′ tetraphosphate (Ap4A) into animal models induces vasodilation [1,2], the present study was performed to determine whether Ap4A induces the release of nitric oxide (NO) from endothelial cells. Ap4A induced NO release was 4.2-fold greater than the amount of NO released under basal condition. Ap4A induced NO release was inhibited by N^G-nitro-L-arginine (L-NNA) and this inhibition was reversed by L-Arg. In addition, EGTA inhibits Ap4A induced NO release. These data are consistent with Ap4A inducing the release of NO from endothelial cells through the activation of endothelial nitric oxide synthase.

Key words: Nitric oxide; Receptor; Signal transduction; Bovine aortic endothelial cell

1. Introduction

Adenine dinucleotides are attracting considerable interest because of increasing evidence that they act as extracellular modulators. Adenine dinucleotides consists of α,ω-adenine dinucleotides which consist of two adenosine moieties linked via their 5′ positions by a chain of 2 to 6 phosphates (Ap_xA, where x represents the number of phosphates in the connecting chain). The α,ω-adenine dinucleotides are present in a diverse number of biological tissues including platelets, hepatocytes, adrenal medullary chromaffin granules and the central nervous system [3–7].

P^1,P^4-diadenosine 5′ tetraphosphate (Ap_4A) is one of the most abundant and best characterized of the adenine dinucleotides. Ap_4A, Ap_3A, Ap_5A, Ap_6A and ATP are co-stored in dense secretory granules of platelets, and upon platelet aggregation, are released into the extracellular milieu following stress [4,5,8,9]. In chromaffin cells, Ap_4A, Ap_5A and Ap_6A are co-stored with AMP, ADP, ATP and catecholamines [10]. It has been estimated that following localized release from platelets or chromaffin cells Ap_4A could reach physiologically significant concentrations. Furthermore, compared with ATP, Ap_4A has a relatively long half-life in whole blood [11]. Thus, Ap_4A is well suited for a role as an extracellular modulator.

Our laboratory has demonstrated the presence of membrane receptors for Ap_4A in brain, liver, kidney, cardiac, spleen and adipose tissue [12]. The presence of the receptor in a diversity of tissue types and localization of the Ap_4A receptor on the cell surface [13] supports the notion that circulating Ap_4A is a modulator of cellular functions. Indeed extracellular Ap_4A has been shown to modulate blood vessel tone [1,2,14], inhibit platelet aggregation [9], prime respiratory burst [15], regulate apoptosis in neutrophils [15], induce catecholamine release from chromaffin cells [16] and activate glycogen phosphorylase in hepatocytes [17].

The notion that circulating Ap_4A may be involved as an extracellular modulator led us to focus our efforts on defining a physiological role for Ap_4A. As a step in this direction we have demonstrated that the infusion of Ap_4A into the precava of swine triggers a dose dependent decrease in the mean arterial blood pressure without affecting the heart rate or right ventricular pressure [1]. Similar activity was noted when Ap_4A was infused into the femoral vein of dogs [2]. Other investigators have demonstrated that the infusion of Ap_4A through isolated rabbit hearts also induces vasodilation [18]. In this communication we present evidence that demonstrates Ap_4A induces bovine aortic endothelial cells (BAEC) to release nitric oxide (NO) and that Ap_4A induces the release of 4.2-fold more NO over basal levels. Since NO is the major physiological regulator of basal blood vessel tone [19,20] these data are consistent with Ap_4A exerting its vasodilation affect by inducing endothelial cells to release NO.

2. Materials and methods

2.1. Materials

BAEC were kindly supplied by Dr. Robert Auerbach of the University of Wisconsin. Minimal essential medium (MEM), penicillin and streptomycin were purchased from GIBCO. Heat inactivated fetal bovine calf serum (FSB) was purchased from HyClone. Nitrate standard solution was purchased from EM Science. N^G-nitro-L-arginine (L-NNA) was purchased from Calbiochem. N^G-methyl-L-arginine (L-NMA) was purchased from ICN Biochemicals. Arginine, superoxide dismutase derived from bovine liver and all nucleotides were purchased from Sigma. All other reagents were of analytical reagent grade or better.

2.2. Cell culture

2×10^5 BAEC were seeded in 35 mm gelatin-coated petri dishes and grown in medium, pH 7.4, consisting of MEM, 10% (v/v) FBS, 0.044 mol/l of $NaHCO_3$, penicillin (100 units/ml) and streptomycin (100 μg/ml). Cell cultures were maintained at 37°C in a humidified atmosphere of 95% air-5% Co_2. All experiments were performed with passage 6 through 10 cells.

2.3. Calibration of the electrode

NO was measured using the ISO-NO Mark II Isolated Nitric Oxide Meter using and ISO-NOP Sensor (World Precision Instruments, Inc., Sarasota, FL). The principle of measurement and the electrode design have been previously described [21,22]. Calibration of the electrode was performed by titration as described [23]. The titration method always detected NO standards with good linearity ($r = 0.99$ to 1.0) but the sensitivity showed variations between 0.85 and 3.23 nM NO/pA. Therefore calibration of the NO electrode was performed on each day.

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2.4. NO measurements

BAEC grown 35 mm gelatin-coated petri dishes were washed three times with Krebs-Henseleit buffer (0.01 mol/l HEPES (pH 7.4), 0.12 mol/l NaCl, 0.0046 mol/l KCl, 0.0015 mol/l $CaCl_2$, 0.0005 mol/l $MgCl_2$, 0.0015 mol/l NaH_2PO_4, 0.007 mol/l Na_2HPO_4, 0.01 mol/l glucose), resuspended in 2.0 ml of Krebs-Henseleit buffer and incubated for 60 min at 37°C in a humidified atmosphere of 95% air-5% CO_2. The cells were then washed three times with Krebs-Henseleit buffer (pH 7.1) prior to the addition of 2.0 ml of Krebs-Henseleit buffer (pH 7.1) containing 10 U/ml of superoxide dismutase. For experiments the sensor probe was inserted vertically into the petri dish containing BAEC so that the tip of the probe was about 1 mm into the solution. The dishes were not capped and all measurements were performed at room temperature. The data were collected and analyzed using the DUO 18 2 Channel, 18-Bit Data Recording System (World Precision Instruments, Inc., Sarasota, FL). Data were collected for 60 min (5 samples/s). The total nmoles of NO were determined after 60 min of incubation. Signal to background ratio was 475 (±50).

2.5. Determination of cell number

After each experiment the total number of cells in the petri dishes were determined by washing the dishes with 1 ml of phosphate buffered saline pH 7.4 (PBS) followed by incubating the cells with 1 ml of trypsin (1.25 μg/ml) and 1 mM EDTA at room temperature until the cells were released. Trypsin was inactivated by diluting the cells into 2 ml of trypsin inhibitor (1 mg/ml). Cell densities were determined by counting in a hemocytometer. Each sample was counted eight times.

2.6. Measurement of nucleotide degradation by BAEC

BAEC grown 35 mm gelatin-coated petri dishes were washed three times with Krebs-Henseleit buffer incubated at room temperature with either 6 μM Ap_4A (1×10^6 cpms/pmole) or 6 μM ATP (1×10^6 cpms/pmole) in a final volume of 0.1 ml of Krebs-Henseleit buffer. After incubation 2 μl aliquots, along with standards (Ap_4A, ATP, ADP, AMP and [^{32}P]-inorganic phosphate) were spotted on DC-Plastikfolien PEI-Cellulose F plates (TLC plates). The plates were developed using 1 M $LiCl_2$ as described [24]. After development, the plates were dried and autoradiography was carried out at room temperature for 6 h using Kodak X-OMATAR X-ray film and Cronex Lighting Plus intensifying screens.

Densitometer tracings of autoradiographs were obtained using a Pharamcia LKB UltraScan XL Enhanced Laser Densitometer connected to an IBM 486 Personal Computer with LKB GelScan XL (version 2.1) software.

3. Results

3.1. Effect of confluence on Ap_4A induced NO release

To determine the effect of Ap_4A on BAEC we measured the amount of NO released at different days of cell growth in response to Ap_4A (Fig. 1). Confluence was determined by visual inspection of the cells and deemed present when more than 95% of the cells were in contact with adjacent cells [25]. Under the culture conditions used in this communication the cells reached confluence 5 days after subculturing. Even after reaching confluence the cell number continued to increase while the cells decreased in size and became tightly packed before plateauing at day 8 (Fig. 1A). Both basal and Ap_4A stimulated release of NO was at least 3-fold greater in cells in early growth phase (days 3–6) as compared to cells at plateau (days 8–10). Cell viability of day 3–8 cells was greater than 95% as shown by trypan blue exclusion (data not shown). Furthermore day 3–8 cells released less than 5% of lactate dehydrogenase found in total lysates (data not shown). These data are consistent with other investigators who have demonstrated that the expression of endothelial cell NO synthase (eNOS) is increased in proliferating as compared to quiescent cells [25]. All subsequent experiments were performed using day 4 to 6 cells.

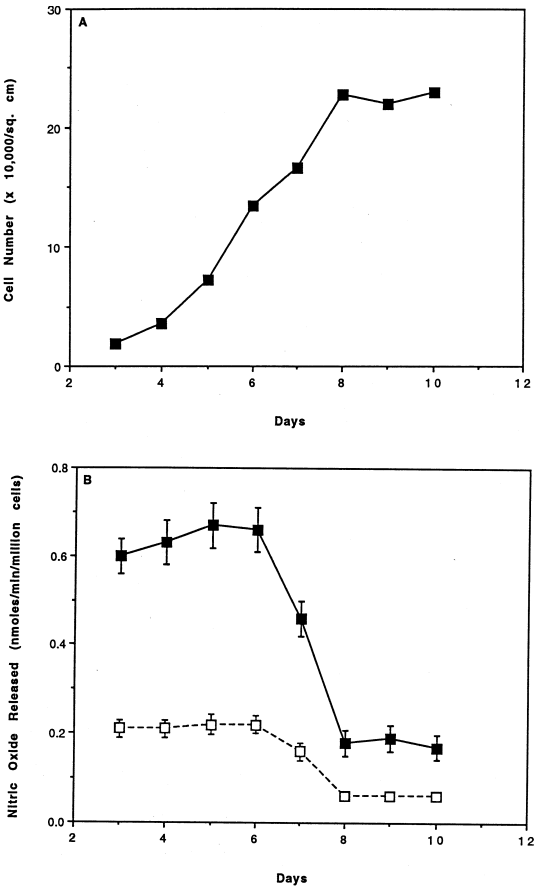

Fig. 1. Effect of BAEC cell growth on the release of NO after induction with Ap_4A. A: 2×10^5 BAEC were seeded in 35 mm gelatin-coated petri dishes and grown as described in Section 2. At the days indicated the cells were released from the dishes by trypsin digestion and counted in a hemocytometer as described in Section 2. B: 2×10^5 BAEC were seeded in 35 mm gelatin-coated petri dishes, grown and prepared as described in Section 2. At the indicated days the BAEC were washed and prepared as described in Section 2 prior to adding 1 μM Ap_4A. NO released was recorded and analyzed as described in Section 2. Ap_4A induced release of NO (■); basal release of NO (□). Data are averages of four different experiments. Error bars are shown as standard deviations.

Table 1
Effect of adenylated dinucleotides on NO release from BAEC

Adenylated dinucleotide cells	nmoles/min/10^6
Ap_4A	0.83 ± 0.044
Ap_2A	0.66 ± 0.035
ATP	0.39 ± 0.027
Basal	0.20 ± 0.021
Ap_6A	0.25 ± 0.025
Ap_5A	0.23 ± 0.026
Ap_3A	0.17 ± 0.024

2×10^5 BAEC were seeded in 35 mm gelatin-coated petri dishes, grown and prepared as described in Section 2 prior to adding 6 μM to the indicated dinucleotide. NO release was recorded and analyzed as described in Section 2. Data are averages of four different experiments and reported as standard deviations.

3.2. Effect of adenine dinucleotides on NO release

The ability of ATP and various adenylated dinucleotides to induce the release of NO from BAEC was compared (Fig. 2 and Table 1). Basal, Ap_4A, Ap_2A, Ap_3A, Ap_5A, Ap_6A and

ATP stimulated release of NO from BAEC increased with incubation time (Fig. 2). After 60 min of incubation, basal release was about 13 nmoles/10^6 cells; this release of NO increased 3.8-, 3.0- and 1.8-fold in response to 6 μM Ap_4A, Ap_2A and ATP, respectively (Fig. 2A and Table 1). On the other hand Ap_3A, Ap_5A and Ap_6A did not induce NO release above basal levels (Fig. 2B and Table 1). These data are consistent with Ap_4A, Ap_2A and ATP stimulating the release of NO from BAEC.

3.3. Nucleotide hydrolysis by BAEC

To ensure that Ap_4A is not hydrolyzed by BAEC during NO assays, 6 μM [^{32}P]-Ap_4A was incubated for various times at room temperature with BAEC suspended in 0.1 ml of Krebs-Henseleit buffer (pH 7.1). Aliquots were subjected to thin layer chromatography [24] followed by autoradiography. After 60 min of incubation 90% of the radiolabeled Ap_4A co-migrates with nonradiolabeled Ap_4A while ATP was rapidly degraded (Fig. 3). We estimated that 50% of the radiolabeled ATP was degraded between 3 to 4 min and after 60 min there was essentially no radiolabeled material co-migrating with ATP. These data are consistent with Ap_4A inducing the release of NO from BAEC. Due to the rapid hydrolysis of ATP we can not determine whether the release of NO in the presence of 6 μM ATP is due to ATP or its degradative products.

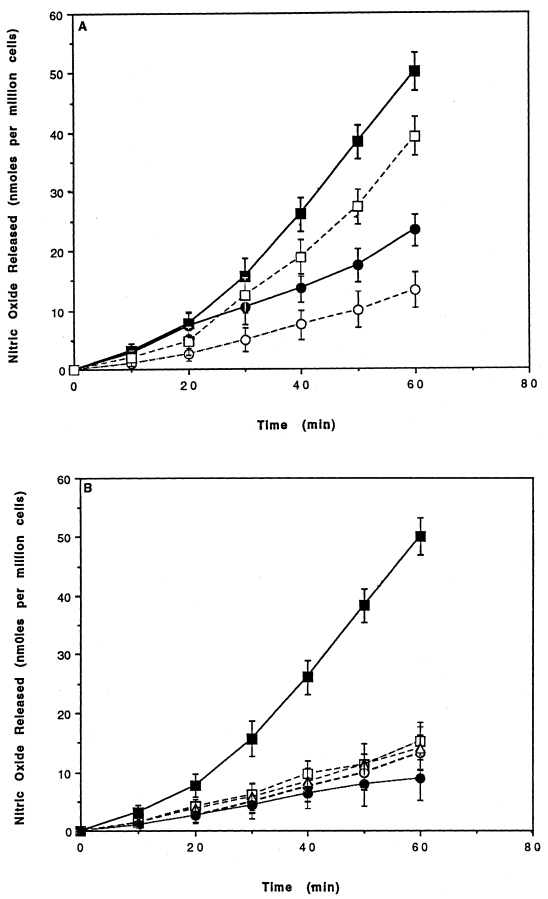

Fig. 2. Time course of adenine nucleotides induced NO release from BAEC. 2×10^5 BAEC were seeded in 35 mm gelatin-coated petri dishes, grown and prepared as described in Section 2. A: Six μM Ap_4A (■), 6 μM Ap_2A (□), 6 μM ATP (●) and no nucleotide (○) were incubated with cells for the various times indicated and the NO released was recorded and analyzed as described in Section 2. B: Six μM Ap_4A (■), 6 μM Ap_6A (□), 6 μM Ap_5A (△), 6 μM Ap_3A (●) and no nucleotide (○) were incubated with cells for the various times indicated and the NO released was recorded and analyzed as described in Section 2. Data are averages of four different experiments. Error bars are shown as standard deviations.

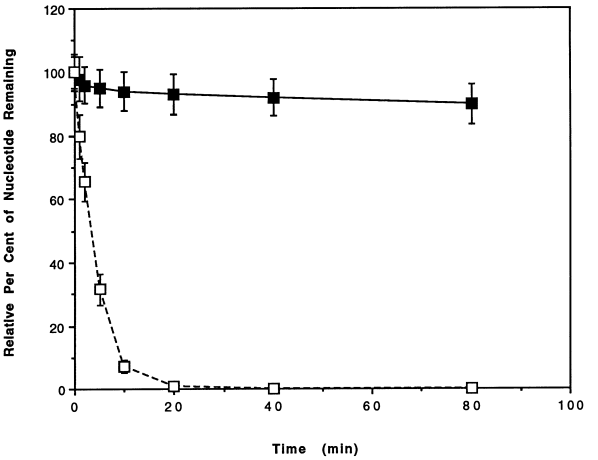

Fig. 3. Time course of [^{32}P]-Ap_4A and [α-^{32}P]-ATP hydrolysis by BAEC. 2×10^5 BAEC were seeded in 35 mm gelatin-coated petri dishes, grown and prepared as described in Section 2. These cells were incubated at room temperature with 6 μM Ap_4A (1×10^6 cpms/pmole) (■) or with 6 μM ATP (1×10^6 cpms/pmole) (□) in a final volume of 0.1 ml of Krebs-Henseleit buffer for the times indicated. After the incubations 2 μl aliquots, along with standards, were spotted on TLC plates. The nucleotides were separated as described in Section 2. The relative percentage was calculated from densitometer tracings of the autoradiographs as described in Section 2. The results are expressed as averages of three different experiments. Error bars are shown as standard deviations.

3.4. Effects of arginine antagonists on Ap_4A induced release of NO

Since L-Arg is the biosynthetic precursor of eNOS [26] and N^G-methyl-L-arginine (L-NMA) and N^G-nitro-L-arginine (L-NNA) are competitive inhibitors of eNOS [27] the effect of these analogs on Ap_4A induced NO release was determined. As shown in Fig. 4A both of these analogs inhibit the release of NO in a dose dependent fashion. We estimate that 50% of NO release is inhibited at 10 and 22 μM by L-NMA and L-NNA, respectively. To determine the ability of L-Arg to overcome the inhibitory effect of L-NNA, variable amounts of L-Arg and a fixed amount of L-NNA were incubated with BAEC for 60 min at 37°C prior to inducing NO synthesis with Ap_4A. L-Arg, as expected, effectively reverses the L-NNA inhibition of NO release (Fig. 4B). In addition, L-NNA inhibited basal release of NO and L-Arg overcame this inhibition (data not shown).

Table 2
The role of Ca^{2+} on the release of NO from BAEC

Sample	nmoles/min/10^6 cells
Basal control	0.22 ± 0.020
Basal with no Ca^{2+} plus 10 mm EGTA	0.01 ± 0.001
Ap_4A control	0.87 ± 0.043
Ap_4A with no Ca^{2+} plus 10 mM EGTA	0.03 ± 0.002

2×10^5 BAEC were seeded in 35 mm gelatin-coated petri dishes, grown and prepared as described in Section 2. In the Ap_4A experiments the concentration of Ap_4A used was 6 μM. NO release was recorded and analyzed as described in Section 2. Data are averages of four different experiments and reported as standard deviations.

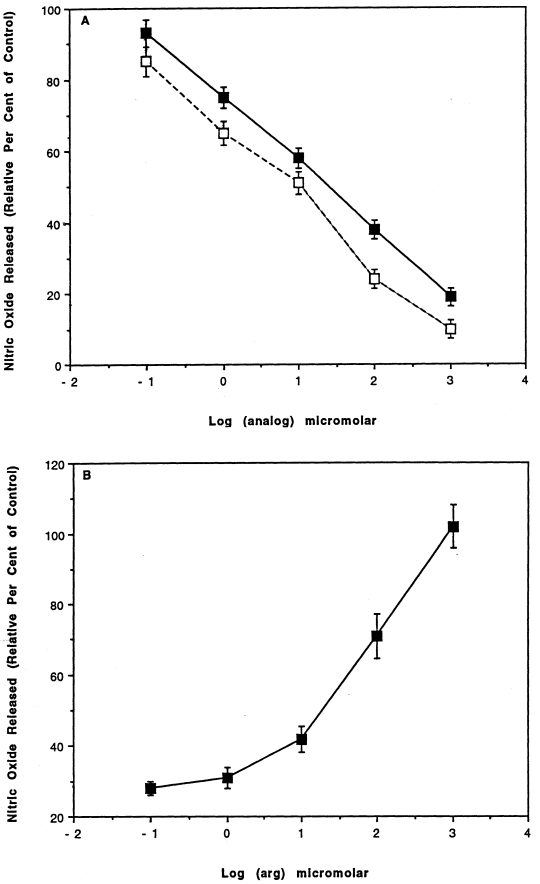

Fig. 4. Effect of L-Arg analogs on Ap_4A induced release of NO from BAEC. A: 2×10^5 BAEC were seeded in 35 mm gelatin-coated petri dishes, grown and prepared as described in Section 2. Cells were incubated for 60 min at 37°C with varying concentrations of L-NMA (□) or L-NNA (■) prior to adding 6 μM Ap_4A. NO release was recorded and analyzed as described in Section 2. B: 2×10^5 BAEC were seeded in 35 mm gelatin-coated petri dishes, grown and prepared as described in Section 2. Cells were incubated for 60 min at 37°C with 100 μM L-NNA and varying concentrations of L-Arg prior to adding 6 μM Ap_4A. NO release was recorded and analyzed as described in Section 2. Data are averages of four different experiments. Error bars are shown as standard deviations.

3.5. The role of Ca^{2+} on the release of NO from BAEC

Since eNOS has a strong requirement for calmodulin and Ca^{2+} [28], the role of Ca^{2+} Ap_4A induced release of NO was examined. The addition of 10 mM EGTA to Ca^{2+} free Krebs-Henseleit buffer inhibits both basal and Ap_4A stimulated release of NO (Table 2). Ap_4A induced release of NO in Ca^{2+} free buffer is 2.9% of the control while basal release is 4.5% of the control. These data are consistent with Ap_4A inducing NO release through the eNOS pathway.

4. Discussion

Endothelial cells are believed to mediate the vasodilatory actions of a variety of substances including adenine mononucleotides. The biological effects of adenine mononucleotides, but not the adenine dinucleotides, on endothelial cells have been well characterized. The major effect of adenine nucleotides on the endothelium is the stimulation of the synthesis and release of prostacyclin and NO [28–35]. Work presented in this communication demonstrates for the first time that Ap_4A and Ap_2A induce the release of NO from endothelial cells.

Even though eNOS was initially termed a 'constitutive' enzyme, we determined the amount of basal and Ap_4A induced NO release at different days of cell growth. Both basal and Ap_4A induced release of NO was at least 3-fold greater in growing compared to growth-arrested cells (Fig. 1). These data are consistent with other investigators who have demonstrated that basal and Ca^{2+} ionophore A-23187 induced release of NO was greater in growing BAEC [25]. Furthermore Western and Northern blot analyses revealed 3- and 6-fold increases in NO synthase protein and mRNA, respectively, in growing compared with growth-arrested cells [25].

Of the various adenylated dinucleotides tested only Ap_4A and Ap_2A induce the release of NO from BAEC (Fig. 2 and Table 1). These data are consistent with data from the infusion of Ap_4A and Ap_2A into animal models which demonstrated that these two adenylated dinucleotides induce vasodilation [1,2,14,18]. While Ap_4A is a naturally occurring adenylated dinucleotide, Ap_2A has never been found in living systems. Ap_3A did not induce BAEC to release NO above basal levels (Fig. 2B and Table 1), however other investigators have reported that the infusion of Ap_4A and Ap_3A into segments of rabbit mesenteric arteries causes vasodilation [36]. These investigators also presented evidence which suggested that Ap_4A induces an endothelium dependent dilation while Ap_3A elicits a dilator response through a direct effect on vascular smooth muscle [36]. Neither Ap_5A nor Ap_6A induce the release of NO above basal levels (Fig. 2B and Table 1). Other investigators have demonstrated that Ap_5A and Ap_6A induce vasoconstriction [7,14].

Under our experimental conditions Ap_4A induces the release of about 53% more NO than ATP (Fig. 2A and Table 1). Since endothelial cells contain ecto-enzymes that hydrolyze ATP and Ap_4A [37], it was necessary to determine under our experimental conditions whether the effect of these two nucleotides on NO release was due in part to their degradative products. ATP hydrolysis by BAEC was significantly greater than Ap_4A hydrolysis (Fig. 3). In fact, after 60 min essentially all of the ATP was hydrolyzed while only 10% of Ap_4A was hydrolyzed. Other investigators have also demonstrated that Ap_4A survives significantly longer on the endothelium than ATP [37]. Furthermore, the half-life of Ap_4A in whole blood is also longer than the half-life of ATP [11]. Thus, compared to ATP, Ap_4A appears to be well suited for a role as an extracellular modulator of NO release from the endothelium.

Previous studies have shown that eNOS generates NO from the terminal guanidino nitrogen of L-Arg yielding citrulline as a by-product [26,27]. We demonstrate that Ap_4A induced release of NO is inhibited by two competitive inhibitors of eNOS, L-NMA and L-NNA. In addition, L-Arg reversed this inhibition. Biochemical characterization of NOS has revealed a strong requirement for calmodulin and Ca^{2+} [38]. We have demonstrated that the removal of Ca^{2+} by 10 mM EGTA inhibits greater than 97% of the Ap_4A induced NO release (Table 2). These data are consistent with Ap_4A inducing eNOS to synthesize NO. At the present time we do not know whether Ap_4A induces Ca^{2+} mobilization in BAEC through the internal sink or externally through Ca^{2+} influx through receptor operated Ca^{2+} channels or through Ca^{2+} transport mechanisms. Studies are in progress directed at de-

termining the mechanism of Ap_4A induced Ca^{2+} mobilization in endothelial cells.

Our studies along with others on the infusion of Ap_4A into animal models or isolated arteries demonstrate that Ap_4A is a vasodilator. Ap_4A has a longer stability in vivo and induces the release of a greater amount of NO from endothelial cells than ATP. These data clearly support the notion that Ap_4A is a physiologically important vasoregulator.

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