Evidence supporting the Nucleotide Axis Hypothesis: ATP release and metabolism by coronary endothelium

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Buxton, Iain L. O., Robert A. Kaiser, Brian C. Oxhorn, and Dennis J. Cheek. Evidence supporting the Nucleotide Axis Hypothesis: ATP release and metabolism by coronary endothelium. Am J Physiol Heart Circ Physiol 281: H1657–H1666, 2001.—The Nucleotide Axis Hypothesis, defined and supported herein, proposes that ATP stimulates the release of vasoactive mediators from endothelium, including ATP itself. Here, we show rapid endothelium-dependent, agonist-stimulated ATP elaboration in coronary vessels of guinea pigs. Measurement of extracellular ATP metabolism in intact vessels results in the time- and substrate-dependent formation of ATP in the coronary perfusate in amounts greater than can be accounted for by release from endothelium alone. ATP formation by endothelial cells is saturable ($K_M = 38.5 \mu M$, where $K_M$ is substrate concentration at which rate is half-maximal) and trypsin-sensitive, membranes from [γ-$^{32}$P]ATP-labeled cells support ADP-dependent transphosphorylation by a 20-kDa protein, Western blots reveal the presence of a nucleoside diphosphate kinase (NDPK) of ~20 kDa in endothelial membranes, and analysis of NDPK antibody binding by flow cytometry is consistent with the presence of an ecto-NDPK on cardiac endothelial cells. Sequencing of the endothelial cell ecto-NDPK reveals a predicted amino acid sequence with 85% identity to human Nm23-H1 and consistent with a protein whose properties may confer membrane association as well as sites of regulation of activity. Our data underscore the potential importance of a nucleotide axis in cardiac blood vessels.

release of vasoactive factors; ectoenzyme; ecto-Nm23

THE NOTION THAT ATP is, despite its ubiquitous role in fueling cellular processes, strictly an intracellular molecule is invalid. It is now well established that both nucleosides and nucleotides, released as local hormones and neurotransmitters (35), act as extracellular signaling molecules in many parts of the body (16, 26). Extracellularly directed receptors for ATP exist as both ion channels (P2x) and G protein-coupled receptors (P2Y) and are ubiquitously expressed in all mammalian tissues examined to date (26). Indeed, the extracellular role of nucleotides in the regulation of multicellular communication is evident as early in the passage of evolution as the Protista (29). In the ischemic heart, adenosine released from myocytes augments blood flow (3), and ATP may play a role in the regulation of cardiac blood flow in nonpathological states (8). We (37) and others (4) have shown that endothelial cells release ATP in response to numerous stimuli, such as shear stress and vasoactive agonists, including ATP itself. Furthermore, ATP acting at the endothelial cell P2Y1 receptor, stimulates the release of the endothelium-dependent vasodilators nitric oxide (NO) and prostacyclin I2 (PGI2) (26). We have hypothesized that the release of ATP by coronary endothelium and its subsequent actions at ATP receptors on nearby endothelial cells and downstream from the site of release where it acts as the ATP metabolites (ADP and adenosine) constitute fundamental elements of an intravascular axis that serves to regulate vascular tone (37).

The Nucleotide Axis Hypothesis proposes that in response to discrete stimuli, such as the action of vasoactive agonists on endothelium or acute changes in shear stress, ATP is released from endothelium and acts as an autocrine and paracrine hormone able to do the following. First, it may cause receptor-mediated release of vasoactive factors such as NO, PGI2, and ATP from endothelium. Second, it may act in the blood vessel lumen at P2Y1 and P2Y2 receptors on nearby endothelium downstream from the site of release to amplify the ATP and vasoactive factor release response. Third, it may act on platelets in the bloodstream adjacent to endothelium as an antagonist at the thrombocyte P2r receptor to block platelet aggregation. Fourth, once dephosphorylated to ADP by ecto-ADPase of endothelium, it may act again at P2Y1 receptors and be (in part) converted back to ATP by the endothelial ectonucleoside diphosphate kinase (NDPK) to act again, thus propagating the actions of ATP in time and space within the blood vessel. Fifth, it may eventually break down to adenosine downstream from the site of release/conversion in the coronary artery, resulting in continued inhibition of platelet aggregation, vasodilation of resistance vessels, and growth maintenance/promotion of endothelium.

Whereas the documented release of ATP by endothelial cells in tissue culture is suggestive of a physiological role for ATP release in vivo, it is possible that such release is the result of the alteration or activation of endothelium during cell isolation or is a result of the

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conditions of cell culture. Thus we have employed both primary cultures of endothelial cells as well as a modified perfused heart model to determine whether ATP release occurs in intact coronary arteries of guinea pigs and to investigate the likely fate of purine nucleotides in the coronary circulation. Here we demonstrate a role for nucleotides and the peptide bradykinin in the moment-to-moment regulation of ATP release by endothelium and offer evidence for the extracellular production of ATP from ADP by endothelium in a heretofore unappreciated manner.

METHODS

Coronary artery perfusion. Hearts, removed from CO2-asphyxiated female guinea pigs (300–350 g) under an institutionally approved protocol, were cannulated via the aorta and perfused with 37°C heart perfusion buffer (HPM) at pH 7.2 (37). In Ca2+-free HPM buffer, the right and left epicardial coronary vessels could be cut 10 mm from their origin and cannulated with polyethylene tubing for collection of the perfusate. The preparation was then perfused at 4 ml/min with buffer as described above containing CaCl2 at 1.8 mM. In some experiments, the right coronary artery was perfused with distilled H2O for 3 min to remove a functional endothelium (9) and was verified microscopically (17). Experiments performed in this way were unaffected by treatments altering flow because the perfusion was controlled at a constant rate and only the epicardial portion of the vessel was employed.

Preparation of endothelial cells. Guinea pig hearts (6 females/prep) were perfused with Ca2+-free HPM containing 1 mg/ml collagenase (Worthington, type II) and purified as previously described (37). Endothelial cells liberated in this manner were grown to confluence on polymer (F-5147, Sigma)-coated plastic for 5 to 7 days. Cells were subcultured and employed as first (P-1) through third passage. In flow cytometry studies, freshly isolated cells were first labeled with a mouse anti-human monoclonal antibody (IgG1) directed against the endothelial marker CD31 (clone EN4) and positive cells retrieved by fluorescence-activated cell sorting (10). CD31+ cells were grown as above and employed for detection of Nm23-H1 as described. In studies not described here, RT-PCR studies of RNA from cells as early as the fifth passage were found to lose expression of P2 receptors.

Measurement of ATP by chemiluminescence. The presence of ATP in the coronary perfusate or cell culture incubation media was determined using luciferin-luciferase chemiluminescence (37). Effects of all treatments on the assay were always carefully controlled, such as the use of ADP at concentrations employed in our experiments, which produces a small signal in the assay.

Measurement of purines by HPLC. Metabolism of [2,8-3H]ADP sodium salt, and [8,5-3H] GDP sodium salt by cells and coronary vessels was determined by injecting filtered control or experimental buffer solutions (400 μl) over a 100-mm Whatman Partisil SAX (5 μm) HPLC column. Purines were separated by a linear gradient of NH4HPO4 (pH 3.8) from 0 to 2 M. Radioactivity was measured continuously using an INUS β-RAM pumping flow scintillation cocktail at a ratio of 3:1 resulting in an efficiency for tritium of 36%. The identity of peaks for adenine and guanine nucleoside and nucleotides were determined by separation of bona fide standards.

Western blots. Endothelial cells were removed from the growth surface with PBS (pH 7.2) containing 1 mmol/l EGTA without trypsin. The employment of trypsin to remove cells markedly reduced the density of bands that stained with antibodies for NDKP in immunoblots. Cell pellets were homogenized on ice in Kontes 1-ml glass–glass homogenizers in PBS containing leupeptin (1 μmol/l). Membrane and cytosolic fractions were prepared by a slow-speed initial centrifugation of the cell homogenate (×1 K, 10 min), followed by centrifugation of the resulting supernatant at ×88 K for 45 min. Samples for PAGE were boiled in SDS using standard protocols. Proteins separated by SDS-PAGE (15%) were blotted to nitrocellulose and probed with a rabbit polyclonal antibody (19) directed against rat liver NDKP or a monoclonal antibody directed against Nm23-H1.

Endothelial NDKP-phosphotransferase activity. Endothelial cell membranes were incubated with 100 μmol/l ATP (γ-32P[ATP, 100 μCi] + the NDKP substrate ATP (30 μmol/l) added as a phosphate acceptor. Reactions carried out in endothelial cell membranes at 21°C for 30 min in the absence of a phosphoryl donor were separated on 15% SDS PAGE gels exposed to Kodak X-OMAT AR film in cassettes with X-rayifying screens held at 4 °C for 48–72 h. Film was developed (Konica QX-70 film processor) and quantified by densitometry.

NDKP-A surface labeling by flow cytometry. Endothelial cells were incubated with mouse monoclonal antibody (IgG2a) directed against human Nm23-H1 antigen (Santa Cruz Biotechnology; Santa Cruz, CA). A secondary rabbit antibody-FITC conjugate directed against mouse IgG2a was employed to detect binding of the primary antibody. Controls were employed for nonimmune mouse IgG2a/secondary antibody binding. Cells (10,000) were analyzed by flow cytometry using a Beckman-Coulter EPICS Elite ESP instrument, and flow experiments were repeated five times with endothelial cells from different cell isolations.

RNA isolation and RT-PCR. Total RNA was isolated from 50 mg of freshly dissected guinea pig ventricle or 1 × 106 plated cardiac endothelial cells using the TRIzol method (Life Technologies; Grand Island, NY). Isolated RNA was DNase I-treated before cDNA synthesis and reverse transcribed using Superscript II. Quality of cDNA was tested for genomic contamination by performing β-actin control RT-PCRs and checking for the presence of the genomic (700 bp) or cDNA (500 bp) product. RT-PCR was performed for 40 cycles at 94°C 25 s, 58°C 25 s, and 72°C 60 s in an Eppendorf Mastercyber Gradient (Eppendorf Scientific; Westbury, NY) with primers of our own design obtained from Integrated DNA Technologies (Coralville, IA). Oligonucleotide primers for NDKP were designed for PCR based on conserved sequences of the human (GenBank X73066), mouse (M65037), and rat (D13374) NDKP genes as follows: forward GAGCGTACCTTCATTGC(G/C)ATC, reverse GCACACTCATTAAGTGG. Products were analyzed on 2% agarose gels, stained with 2 μg/ml ethidium bromide and imaged. Reverse transcribed cDNA was synthesized according to the SMART-RACE protocol (Clontech; Palo Alto, CA) using 3 μg of β-actin verified total RNA. The 3′ reaction was performed using the RACE primer and the forward primer given above. The 5′ reaction was performed using the RACE primer and the reverse primer CATGTTCTCCTGCAGTTG. RACE PCRs were performed using the following variation of touchdown PCR: 5 cycles 94°C 30 s/72°C 3 min, 5 cycles 94°C 30 s/70°C 30 s/72°C 3 min, and 28 cycles 94°C 30 s/68°C 30 s/72°C 3 min. Potential bands were isolated from a 1.0% agarose gel (QIAgen Gel extraction method, Valencia, CA) and sequenced using an ABI/prism sequencer.

Materials. All drugs and chemicals were obtained from Sigma (St. Louis, MO) unless otherwise noted. Collagenase was obtained from Worthington, and collagenase from Innovation Biochemicals (Carlsbad, CA). ATP and ADP were purchased from ICN (Costa Mesa, CA). NAD+ and NADH were from Sigma, and CHAPS from Calbiochem (La Jolla, CA). ATP-regenerating system was from Bio-Rad (Hercules, CA). Eppendorf Mastercylcer Gradient (Eppendorf Scientific; Hamburg, Germany) and Eppendorf pipet Tip Rack (Eppendorf Scientific; Westbury, NY) were used to perform all PCR reactions. DNA and RNA ladder, reverse transcriptase, and all restriction enzymes were obtained from Promega (Madison, WI). Superkine Protein A-Sepharose beads were purchased from Pharmacia (Uppsala, Sweden), and Sepharose 6B was obtained from Amersham (Arlington Heights, IL). Anti-β-actin verified total RNA. The 3′ reaction was performed using the RACE primer and the forward primer given above. The 5′ reaction was performed using the RACE primer and the reverse primer CATGTTCTCCTGCAGTTG. RACE PCRs were performed using the following variation of touchdown PCR: 5 cycles 94°C 30 s/72°C 3 min, 5 cycles 94°C 30 s/70°C 30 s/72°C 3 min, and 28 cycles 94°C 30 s/68°C 30 s/72°C 3 min. Potential bands were isolated from a 1.0% agarose gel (QIAgen Gel extraction method, Valencia, CA) and sequenced using an ABI/prism sequencer.
type-II was obtained from Worthington Biochemical (Lake-wood, NJ). Radioactive nucleotides were obtained from NEN Life Science Products, now Perkin-Elmer Life Sciences (Bos-ton, MA). Polyclonal antibody to rat liver NDPK was the generous gift of N. Kimura, Tokyo Metropolitan Institute of Gerontology (Tokyo, Japan). Monoclonal antibody to Nm23-H1 employed in immunoblots was obtained from Seikagaku America (Rockville, MD). DNAse I was obtained from Promega, Madison, WI and Superscript II from Gibco (Rockville, MD).

RESULTS

In perfused coronary artery of the guinea pig, introduction of BK results in the immediate release of ATP that can be measured in the perfusate (Fig. 1A). The time course of release to BK (1 nM) in the continued presence of the agonist (flow, 4 ml/min) peaks within the first minute; desensitizes, thereafter, consistent with action of BK at the BK2 receptor on endothelium; and is blocked by the addition of the BK receptor antagonist HOE-140. Release of ATP by coronary artery was endothelium-dependent, because removal of endothelium in the right, but not the left, coronary artery by prior perfusion with distilled water eliminated the response to BK (Fig. 1B). Addition of 2-methylthio-ATP or ADP, both agonists at the endothelial P2Y1 receptor (1), led to the immediate appearance of ATP in the perfusate (Fig. 1A). However, unlike the action of BK, stimulation of the endothelial P2Y1 receptor led to the sustained appearance of ATP in the perfusate despite the fact that ATP receptor responses are known to desensitize rapidly (36). Because ATP is known to stimulate its own release from endothelial cells in culture (37), we reasoned that such a phenomenon could explain the continued presence of ATP in the coronary perfusate. However, studies in cultured cells suggested an additional possibility.

When ADP was added in increasing concentrations to cultured guinea pig endothelial cells, ATP elaboration measured at concentrations above 10 μmol/l was significantly greater than that seen with BK at any dose (Fig. 2A, inset) and represented as much as 10% (at 100 μM ADP) of total cellular ATP (Fig. 2B). Such a large amount of ATP from cells could not be explained by release alone. Indeed, all of the ATP in the cell, defined by treatment with perchloric acid, totaled 18.8 ± 2.45 nmol/million cells, whereas total “releasable” ATP defined by treatment with ionomycin (1 μM) for 2 min (Fig. 2B), measured 12.34 ± 2.33 nmol/million cells. To test our hypothesis that ATP could be generated extracellularly by endothelium, we introduced [3H]ADP (1 μCi, 100 μM) into the coronary artery perfusate in the presence of a phosphoryl donor (GTP, 1 mM). The perfusate was collected and subjected to HPLC anion-exchange separation with radioactive flow detection. In the presence of GTP, there was a rapid formation of [3H]ATP at the expense of [3H]ADP along with the expected formation of [3H]AMP and [3H]adenosine (Fig. 3A). Although it is not strictly possible to account for all adenylyl purine in these experiments where adenosine uptake into endothelium is not prevented, it is clear that within this 1-min exposure to endothelium, the disappearance of [3H]ADP can be approximately accounted for by the [3H] products of metabolism seen on the chromatogram. Control perfusions through endothelium-de-
monolayers exposed to ADP in the presence of GTP (1 mM) yielded the rapid appearance ATP in the buffer bathing the cells in a fashion (15) suggesting the presence of an ectoenzyme (Fig. 4A). The substrate concentration at which the maximal rate of reaction \((V_{max})\) is half-maximal \((K_m)\) obtained in these experiments \((K_m = 38.5 \mu mol/l)\) was consistent with the action of an NDPK as was blockade by addition of UDP at high (5 mM) concentration, the absence of an effect of the adenylate kinase inhibitor diadenosine pentaphosphate (Fig. 3D), and the ability to see the same activity (albeit with a much decreased \(V_{max}\)) at 4°C (data not shown). Consistent with an NDPK is its promiscuity with respect to nucleotide substrate and phosphoryl donor (23). Endothelial cells were able to convert \(^{3}H\)GDP to \(^{3}H\)GTP in the presence of ATP (Fig. 3C) and could also utilize IDP or CDP as substrate (data not shown). The ability of the nonneutralizing anti-NDPK antibody we employed to activate the enzyme (Fig. 3D) was also seen when the antibody was employed with the purified rat liver NDPK (Sigma) used as a control.

We employed mild trypsin treatment of the cultures (Fig. 4B) and obtained results demonstrating an 85% reduction in ATP generated within 1 min. The effect of trypsin was reversible after a 45-min recovery period in growth medium. The presence of an ecto-NDPK was confirmed in Western blots, employing polyclonal antibodies directed against rat liver NDPK (19), which showed the presence of a 20.5-kDa membrane-associated NDPK (Fig. 5A). This same nonneutralizing antibody, when added to activity assays, stimulated the enzyme twofold (Fig. 3D). A monoclonal antibody directed against the human Nm23-H1 gene product, an NDPK isofrom expressed by numerous transformed cells (14, 31, 32) and proposed as a metastasis associated gene (27), further identified the endothelial NDPK as the guinea pig homologue of human Nm23-H1 (Fig. 5B).

Partially purified endothelial cell membranes were incubated in the presence of \(\gamma^{32}P\)ATP, washed, incubated with the NDPK acceptor (ADP), and separated by SDS PAGE followed by autoradiography. Autoradiograms demonstrated decreasing concentrations of \(\gamma^{32}P\)phosphate associated with NDPK over time (Fig. 6) dependent on the presence of the acceptor, ADP.

While the expression of NDPK as an ectoenzyme is strongly suggested by the ability to measure enzyme activity in a coronary vessel or in a cell monolayers, additional evidence for the surface expression was obtained using flow cytometry of intact cells. Incubation of cells with a fluorescent antibody directed at human NDPK (Nm23-H1 specific) and analysis of fluorescence by flow cytometry revealed that the entire population of cells acquired a fluorescence signal consistent with expression of an ecto-NDPK (Fig. 7).

RT-PCR was employed to sequence a guinea pig NDPK based on the notion that the activity we measure is homologous to Nm23-H1. The following open reading frame was obtained from the sequences of the NDPK RACE products (Genbank accession number AY017306): ATGGCCAGCAGTGACCAGCAGCTTTCA- TTGCCATCAAGCGATGGTGTCCAGCGGGAGC-
This sequence is up to 86% identical to the published mouse, human, and rat sequences and translates into a 154 amino acid protein that is up to 91% identical and over 94% homologous to these other NDPK sequences (Fig. 8). This sequence contains one more codon than the comparable sequences corresponding to a glycine at position 121. This sequence contains key conserved residues believed to be associated with phosphorylation (serines-44, -120, -123, -126), protein:protein interaction (phenylalanine-59), and catalysis of the phosphate transfer (histidine-118) (20). Serines-123 and -126 correspond to -122 and -125 in all other published NDPKs that lack glycine-121. The guinea pig NDPK contains one of the three amino acids associated with DNA binding (arginine-34); however, it lacks the other two (asparagine-69 and lysine-135) of the human Nm23-H2 (25).

**DISCUSSION**

Our results demonstrate that critical elements of our proposed nucleotide axis hypothesis can be established in coronary arteries in situ and support the notion that the elaboration of ATP through release and extracellular formation by endothelium could serve to regulate the production of endothelium-dependent vasodilators and prevent thrombosis. Indeed, when agonist is delivered to the perfused coronary, the effluent is seen to contain significant amounts of ATP as soon as a measurement can be taken. This result is consistent with the notion that the regulated release of ATP could be...
convert ADP to ATP (623...)

followed by washing (posttrypsin) reduced the ability of the cells to experiment repeated (recovery). Data are means returned to growth conditions for 45 min, washed, and the reacquired the ability to generate ATP from added ADP after cells case with the P2y1 receptor agonist ADP. Our efforts to nists such as BK does not suffer ambiguity as was the real blood vessel. receptor-mediated endothelial cell phenomenon in a damaged, supports the notion that ATP release is a blocked and is not seen, if endothelium is intentionally arterial blood (5). The fact that such release can be measurement of significant amounts of ATP seen in section of platelet aggregation and in agreement with the involvement in acute alterations in blood flow and prevention of platelet aggregation and in agreement with the measurement of significant amounts of ATP seen in arterial blood (5). The fact that such release can be blocked and is not seen, if endothelium is intentionally damaged, supports the notion that ATP release is a receptor-mediated endothelial cell phenomenon in a real blood vessel.

Studying ATP release from endothelium with agonists such as BK does not suffer ambiguity as was the case with the P2y1 receptor agonist ADP. Our efforts to

Fig. 4. Endothelial cells express an ecto-NDPK. A: intact endothelial cell monolayers were exposed to increasing concentrations of ADP in the presence of GTP (1 mmol/l) and ATP in the Krebs incubation buffer was determined at 2 min using the luciferin-luciferase assay. Data were normalized to cell number and computer fit to obtain $K_M$ and $V_{max}$ using the equation: $\frac{v}{V_{max}} = \frac{[S]}{K_s + [S]}$ where $v$, rate observed; $V_{max}$, maximal rate of reaction; $[S]$, substrate concentration; $K_s$, affinity of enzyme for substrate; $K_m$, substrate concentration at which rate is one-half maximal. Data are the means ± SE, $n = 10$. B: NDPK activity was assessed in similar cultures in the absence of ADP and GTP (basal) or in the presence of both substrate and donor (ADP 10 μmol/l + GTP 1 mmol/l) for 1 min. Treatment of the same cultures with trypsin (0.3% wt/vol in Ca²⁺-free Krebs buffer) for 90 s followed by washing (posttrypsin) reduced the ability of the cells to convert ADP to ATP (623 ± 35 = 132 ± 16 pmol/10⁶ cells). Cells reacquired the ability to generate ATP from added ADP after cells were returned to growth conditions for 45 min, washed, and the experiment repeated (recovery). Data are means ± SE, $n = 6$.

Fig. 5. Western blot demonstration of NDPK protein in cardiac endothelial cell membranes. Samples for PAGE were boiled in SDS using standard protocols. A: proteins separated by SDS PAGE (15%) were blotted to nitrocellulose and probed with a polyclonal antibody directed to rat liver to NDPK (gift of N. Kimura (19)). The blot was developed using a goat anti-rabbit antibody conjugated to alkaline phosphatase and revealed a 20.5-kDa membrane associated NDPK. Lane 1, molecular weight marker (lysozyme); lane 2, bovine NDPK (Sigma N-2635, 2 μg); lane 3, 4 μg of endothelial cell homogenate; lane 4, 2 μg of endothelial cell cytoplasm; lane 5, 3 μg of endothelial cell membrane fraction; lane 6, 4 μg of K562 leukemia cell homoge-nate (positive control). B: a monoclonal antibody (Seikagaku America) directed against human Mn23-H1 protein was employed to detect cardiac endothelial cell NDPK after incubation with rabbit anti-mouse antibody conjugated to horseradish peroxidase and detected using chemiluminescence. Lane 1, 1 μg of bovine NDPK; lane 2, 4 μg of endothelial cell homogenate; lane 3, 4 μg of endothelial cell homogenate from cells pretreated with trypsin (0.5% w/v × 5 min); lane 4, 4 μg of endothelial cell cytoplasm; lane 5, 4 μg of endothelial cell membrane; lane 6, 4 μg of K562 leukemia cell homogenate control. Blots in (A) and (B) were scanned to text and are representative of 7–10 experiments.

Fig. 6. Autoradiographic analysis of ADP-ATP phosphotransferase activity in cardiac endothelial cell membranes. A: endothelial cell membranes were incubated with [γ-32P]ATP (100 μCi; 100 μM) in the presence (lanes 3, 5, and 7) and absence (lanes 2, 4, and 6) of the NDPK substrate ADP (30 μM) as a phosphate acceptor. Enzymatic reactions carried out at 21°C for 10 min (lanes 2, 3), 20 min (lanes 4, 5), or 30 min (lanes 6, 7) were separated on 15% SDS gels and developed using a radiographic imager. Lanes 1 and 8 are purified bovine NDPK under identical conditions in the absence of acceptor for 1 min (lane 1) to 34 min (lane 8) as an assay control. Radiogram is representative of six experiments yielding similar results. B: densitometric analysis. Means ± SE, $n = 4$ experiments.
measure the effect of ADP stimulation led to the unmistakable conclusion that ADP was not only acting at a purinergic receptor to stimulate ATP release from cells but was, in addition, supporting ADP phosphorylation extracellularly. The amounts of ATP that could be generated by addition of ADP to endothelial cell monolayers was enormous in contrast to the amounts of ATP that could be measured by maximal stimulation with nonnucleotide agonists and was more than could be reasonably explained by release alone (2,800 pmoles vs. 100 pmol/10⁶ cells; data of Fig. 2). If ATP elaborated after the addition of 100 μM ADP (Fig. 2A) were the result of P2y₁ receptor stimulation alone, it would constitute ~20% of the ATP available after membrane compartments were accessed by ionomycin treatment (~10% total cellular ATP). Such a possibility was not intellectually pleasing and, furthermore, does not fit with the fact that coronary endothelium remains intact after ADP perfusions as evidenced by the tissue's ability to release ATP to subsequent introduction of BK (data not shown). The alternate possibility that ADP added in this fashion enters the cell, is phosphorylated, and returns to the extracellular space without obvious damage to the cell is also remote in the extreme. In addition, the ability to measure NDPK activity in cell cultures at 4°C suggests that under these conditions, well below the phase transition temperature of a mammalian cell membrane, nucleotides could not possibly enter and exit cells freely. We hypothesized, therefore, that ATP formation after ADP perfusion of the coronary vessel was due not to regulated release alone, but included in large measure, extracellular enzymatic formation of ATP.

Although substituted nucleotides such as 8-chloro-cAMP have been employed as NDPK inhibitors (2), there is no known potent and selective nonnucleotide inhibitor of NDPK described to date that offers utility...
in our studies. The enzyme’s ability to phosphorylate either adenyl or guanyl purine dinucleotides as well as pyrimidine dinucleotide, together with its inhibition by high levels (in mM) of pyrimidine dinucleotide substrate or EGTA, are well known (14, 30). Whereas its presence as an endothelial ectoenzyme was not known, such a possibility was apparent in perfused hearts (21), not without precedence in transformed cells (18, 19), and could be directly tested in cultured cardiac endothelial cells.

The effect of limited proteolysis to remove the ability of cells to support ADP to ATP conversion, together with the return of the activity in the same cells after trypsin removal and a period of control incubation, supports the notion that the NDPK activity we measure is assembled as an ectoenzyme and that the cells are capable of rapid recovery of activity under these conditions.

The identity of the NDPK activity of endothelium as Nm23-G1, the guinea pig homologue of Nm23-H1, is evident from immunoblots and is consistent with the ability of purified endothelial cell membranes to display a phosphoprotein that operates as a phosphoryl donor capable of transferring phosphate to ADP. The fact that this ping-pong transphosphorylase is an ectoenzyme is supported by our finding that intact cells can be immunostained with Nm23-H1-specific antibody, analyzed by flow cytometry, and found to express the antigen on the surface of the cell.

The proposed sequence of guinea pig ecto-NDPK contains three consensus protein kinase C sites at serine-4, threonine-86, and threonine-103. These sites are conserved in all of the NDPKs aligned (Fig. 8). Sequence analysis reveals that there are no transmembrane regions in NDPK, yet we find NDPK to be associated with the membrane. There are no typical glycosylation sites, but there are two conserved N-myristoylation sites shared by all of the NDPKs at glycines-15 and -102, as well as an additional myristoylation site in Nm23-G1 created at glycine-119 due to the unique presence of glycine-121. This is absent in the human, mouse, and rat proteins. This site is near the catalytic histidine-118 and interrupts the potentially phosphorylated serines at -120, -123, and -126. The role of this extra glycine is unclear, and particularly interesting due to its location in such an active region of the enzyme. The GDP binding pocket and active site are conserved with histidine-118, leucine-48, and serine-123 (24, 25).

Current dogma does not predict ADP-kinase activity of endothelium but rather predicts the rapid metabolism of adenyl purine nucleotides to adenosine (15). The notion that endothelium could generate ATP extracellularly was intriguing because it would be consistent with maintaining the actions of ATP in time and space within the blood vessel and would allow the purine nucleotide signal, originally generated by acute changes in shear or the actions of rapidly degraded peptide agonists such as BK to be carried forward well beyond the site of initial endothelial response. Furthermore, the removal of the aggregatory agonist ADP by rephosphorylation to platelet antagonist (ATP) would serve to decrease the chances of platelet activation (5). Such possibilities need not be rejected on the basis of perceived dilution of the nucleotide. ATP, released from or generated by endothelium, might reasonably be expected to act as ATP (or a metabolite) immediately adjacent to the site of release in vivo (34). On the basis of our data where ATP is measured in the bulk perfusate, ATP reaches concentrations as high as 0.1 μM in the coronary vessel. Whereas the subsequent actions of released ATP may be diffusion limited, this limitation is a special case for endothelium where the laminar flow fluid layer above endothelial cells is in motion continuously and not in immediate equilibrium with the majority of liquid flowing in the vessel (11). Although there is no objective method to determine the concentration of released ATP at the microenvironment next to the endothelial surface, if restricted there for a time, the resulting nucleotide concentration (10 to 100 times or more that determined for the bulk phase) would be vasoactive in guinea pigs (7) and may lead to formation of ADP that would achieve a local concentration sufficient to support the activity of NDPK.

Nucleotide axis hypothesis may offer a context in which to consider the elegant work of those who have established our understanding of the critical importance of NO in the regulation of vascular tone. It appears that the list of those agonists and perturbations that stimulate endothelial cells to liberate NO (6, 12, 13, 28, 33, 37) also stimulates ATP release. Our hypothesis suggests that ATP plays a central role in the signaling and responsiveness of endothelium in vivo and serves to amplify and carry forward in time and space the effects of other vasoactive agonists or shear stress beyond their initial sites of action in the blood vessel. ATP release by endothelium stimulates its own release, thus amplifying local vasodilation. Once metabolized to ADP, the nucleotide can act again at an endothelial cell P2Y1 receptor to again amplify the response (downstream) and, to the extent that ADP is rephosphorylated to ATP, can act yet again as ATP and then ADP, etc., furthering the axis cascade. The movement of the nucleotide in the bloodstream, together with the estimated fraction of ADP that can be rephosphorylated to ATP in the vessel (12%; data of Fig. 4B), mitigates this seemingly positive feedback system. Moreover, the presence of ATP elaboration by endothelium will serve to limit platelet activation both because ATP receptor activation leads to NO release and because ATP [and its ultimate vascular end product adenosine (22)], like NO, is directly antithrombogenic. Once metabolized to adenosine, the purine acts again to dilate terminal arterioles. This concept offers the pleasing feature of biological economy by employing a single purine molecule acting multiple times at disparate purinergic receptor sites in different locations within the vessel to achieve the local regulation of blood flow. Finally, the avid uptake of adenosine by capillary endothelium, where it can be reutilized, offers the ultimate in biological economy.

In studies presented herein, we have employed an extracellular phosphoryl donor in the form of either...
GTP or ATP to favor the formation of the corresponding purine triphosphate by the action of guinea pig ecto-Nm23 on a nucleoside diphosphate. Performing the NDPK assay in this manner permits an unmistakable result. However, this approach also raises questions as to the origin in vivo of the phosphoryl donor that fuels the ecto-Nm23 reaction. Does the enzyme require that the phosphoryl donor be present extracellularly as is the case with the substrate, or is the ectoenzyme phosphorylated in the membrane of the cell at the expense of intracellular nucleoside triphosphate? One is tempted to suggest that the binding of substrate and donor occur extracellularly because this is the way we assay the activity present in intact cells and on intact endothelium. Such a possibility would fit with the fact that nucleotides such as GTP and UTP as well as ATP and ADP are made abundant to the lumen in vivo from activated platelets. However, because we can label the guinea pig Nm23 enzyme in intact cells incubated with [32P]orthophosphoric acid we favor the notion that, in the normal coronary artery, the enzyme is phosphorylated at the expense of an intracellular phosphoryl donor. This notion also fits with data presented herein, showing that the enzyme is stably phosphorylated. Thus the enzyme is not only promiscuous with respect to substrate and donor but also with respect to the intracellular versus extracellular source of the donor, which would mean that the endothelium could support moment-to-moment formation of ATP in both normal and pathological states as our hypothesis proposes.

The framework we offer for considering the nucleotide axis of blood vessels may be a useful extension of our understanding of the role of purines in protecting myocardium during ischemia. Whereas these effects of purine are abuminal, they serve to increase regional blood flow in the heart. The intravascular actions of purines also regulate blood flow. To the extent that endothelial cells release ATP and that ATP is eventually hydrolyzed to adenosine, the nucleotide axis hypothesis can be viewed in context with the adenosine hypothesis as aspects of the broad role for extracellular purines in cardiovascular function.

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