A2B adenosine receptor mediates human chorionic vasoconstriction and signals through arachidonic acid cascade

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and typifying the putative adenosine receptor subtypes that might mediate these effects in isolated human chorionic vessels and intact perfused cotyledons. We further aimed at characterizing the intracellular signaling pathways that may govern the adenosine response in a tissue-specific manner. In view of the paramount role of prostanoids in placental vasculature, we ascertained whether eicosanoids might act as the mediators of the adenosine-induced vasomotor action in human chorionic vessels.

The present vascular reactivity assays and ex vivo cotyledon perfusion protocols show that both endothelial cells and vascular smooth muscles from human chorionic vessels and cotyledons have predominantly A2B receptors, which are coupled to the arachidonic acid cascade and cause a vasoconstriction, mediated apparently by the release of a thromboxane or related prostanoid. RT-PCR studies confirmed the expression of A2B receptors in both endothelial and smooth muscles of these vessels. The finding that placental vessels contract to applications of adenosine, in contrast to most other arteries or veins in which adenosine is known to cause vasorelaxation, illustrates the concept that the vascular reactivity of adenosine varies in humans in a tissue-specific manner.

MATERIALS AND METHODS

Adenosine Receptor Ligands and Sources of These Compounds

Adenosine and related structural analogues such as 5′-(N-ethylcarboxamido)adenosine (NECA), 2-p-(carboxyethyl)phenethylamino-5′-N-ethylcarboxamido-adenosine HCl (CGS-21680), N′-cylohexyladenosine (CHA), 9-chloro-2-(2-furanyl)-5′-(phenylacetyl)aminol-1,2,4-triazolo[1,5-c]-quinazoline (MRS-1220), serotonin hydrochloride (5-HT), 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-3-indoleacetic acid (indomethacin), prostaglandin E2 and prostaglandin F2 were purchased from Sigma-Aldrich (St. Louis, MO). 1-[2-Chloro-6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-1-deoxy-N-ethylcarboxamidoadenosine HCl (CGS-21680), 5′-N′-ethylcarboxamido-adenosine (NECA), 2-p-(carboxyethyl)phenethylamino-5′-N-ethylcarboxamido-adenosine HCl (CGS-21680), N′-cylohexyladenosine (CHA), 9-chloro-2-(2-furanyl)-5′-(phenylacetyl)aminol-1,2,4-triazolo[1,5-c]-quinazoline (MRS-1220), serotonin hydrochloride (5-HT), 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-3-indoleacetic acid (indomethacin), prostaglandin E2 and prostaglandin F2 were purchased from Sigma-Aldrich (St. Louis, MO). 1-[2-Chloro-6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-1-deoxy-N-ethylcarboxamidoadenosine HCl (CGS-21680), 5′-N′-ethylcarboxamido-adenosine (NECA), 2-p-(carboxyethyl)phenethylamino-5′-N-ethylcarboxamido-adenosine HCl (CGS-21680), N′-cylohexyladenosine (CHA), 9-chloro-2-(2-furanyl)-5′-(phenylacetyl)aminol-1,2,4-triazolo[1,5-c]-quinazoline (MRS-1220), serotonin hydrochloride (5-HT), 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-3-indoleacetic acid (indomethacin), prostaglandin E2 and prostaglandin F2 were purchased from Sigma-Aldrich (St. Louis, MO). 1-[2-Chloro-6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-1-deoxy-N-ethylcarboxamidoadenosine HCl (CGS-21680), 5′-N′-ethylcarboxamido-adenosine (NECA), 2-p-(carboxyethyl)phenethylamino-5′-N-ethylcarboxamido-adenosine HCl (CGS-21680), N′-cylohexyladenosine (CHA), 9-chloro-2-(2-furanyl)-5′-(phenylacetyl)aminol-1,2,4-triazolo[1,5-c]-quinazoline (MRS-1220), serotonin hydrochloride (5-HT), 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-3-indoleacetic acid (indomethacin), prostaglandin E2 and prostaglandin F2 were purchased from Sigma-Aldrich (St. Louis, MO).

Source of Human Placentas

Over 160 full-term placentas from normal pregnancies delivered by vaginal or cesarean section were transported from the delivery room to the laboratory within <20 min after childbirth. The Committee on Ethics of the Medical Research Department approved these protocols and consent forms, and ethical regulations were strictly followed.

Dissection of Placentas to Obtain Rings from Superficial Chorionic Vessels

Immediately after placentas arrived in the laboratory, a 2- to 4-cm segment of second- and third-order superficial chorionic artery and vein vessels were routinely dissected to obtain 0.5-cm rings and mounted on a bath chamber used for vascular reactivity studies as detailed by Valdecantos et al. (54). Most of the protocols were repeated at least four times, using vessels dissected from different placentas each time. Studies on artery or vein rings were performed with intact (E\(^+\)) or manually endothelium-denuded (E\(^-\)) vessels (2, 54). Vessel rings were placed in Krebs-Ringer buffer maintained at 37°C within a double-jacketed organ bath chamber bubbled with a 95% O\(_2\)-5% CO\(_2\) mixture. Isometric muscular tension from the circular layer was recorded with a Grass force displacement transducer connected to a Grass 7 multichannel polygraph. Vessel rings were manually adjusted to an artificial tension of 1.5 g, which, as described by Valdecantos et al. (54), is an optimal value obtained from length-tension curves; this tension was maintained throughout the experiment. After an equilibration period of 60 min, with buffer washouts every 15 min, the rings were challenged with three successive applications of 70 mM KCl to evoke the maximal smooth muscle contraction, used to quantify and standardize responses within vessels. This concentration of KCl proved optimal for this caliber of vessels [see Valdecantos et al. (54) and Racchi et al. (44) for human saphenous vein biopsies]. This long-lasting protocol was routinely followed with each vessel ring preparation; it allowed a thorough washout of hormones or drugs that could have been present in the tissues due to parturition or cesarean section surgery.

Vascular Reactivity Bioassays in Intact and Endothelium-Denuded Vessels

Determination of agonist potency. The contractile potency of adenosine, the endogenous ligand for all four adenosine receptor subtypes, and agonists with preferential selectivity for each of the alleged receptor subtypes, CHA (a preferential A1 receptor ligand), CGS-21680 (a rather selective A2A receptor agonist), NECA (a nonselective A2 receptor agonist), and 2-Cl-IB-MECA (recognized as a relatively selective A2 ligand), was assessed by means of noncumulative concentration-response protocols. Varying adenosine concentrations were added directly to the tissue bath chambers in random order within two or three orders of magnitude concentration range. The adenosine applications were spaced every 45 min, a time lag required to avoid desensitization of the adenosine-induced contraction. Parallel control experiments were conducted to examine whether the adenosine vasoresponse decayed over time during the 4- to 5-h course of the experiment. In the particular case of NECA, CHA, or CGS-21680, because the second application caused a much diminished response, a single concentration was assessed per bioassay, i.e., in the concentration-response curve protocols, each data point is derived from separate vessel rings, each obtained from an individual placenta. In every case, at least three to five rings were used to quantify the contractions elicited by NECA, CHA, or CGS-21680. In addition, we also examined whether, in precontracted vessels, adenosine and analogs also elicited contractions. For this purpose, a single concentration of 19 μM adenosine, 57 μM CHA, 37 μM CGS-21680, or 1 μM NECA was also applied in 20 mM KCl-precontracted rings.

In every single bioassay protocol, rings were challenged with a 70 mM KCl standard at the beginning and the end of the experiment, a concentration previously demonstrated to cause the maximal contraction of these vessels (54). This procedure allowed the standardization of the concentration-response protocols described. Furthermore, adenosine concentration-response studies were performed in E\(^+\) and E\(^-\) vessels. Likewise, in the case of NECA, a single concentration was assayed in tissues with and without the endothelial cell layer.

Studies with adenosine receptor antagonists. Intact vessel rings were challenged repeatedly with adenosine at 19 μM, a value close to its EC\(_{50}\), which did not evidence desensitization after successive applications. Each antagonist was examined in a wide range of concentrations (6-1,500 nM); the antagonists were preapplied 10 min before the standard 19 μM adenosine challenge. The results are plots of the percentage of 70 mM KCl values of the vasomotor action of the adenosine challenge vs. the antagonist concentration. Independent protocols, performed in separate vessels, examined the potency of DPCPX, ZM-241385, or MRS-1220 to block the standard adenosine-evoked contractions. These antagonists have relative selectivity for the A1, A2A, and A3 receptor subtypes, respectively. The concentration of these antagonists that halved the adenosine-induced contraction was interpolated from the respective concentration-response curves and expressed as the mean ± SE for each receptor blocker. To
assess whether more than one adenosine receptor mediates the adenosine response, additional studies were performed applying 200 nM ZM-241385 and 200 nM DPCPX together.

**Blockade of tissue cyclooxygenase and thromboxane receptor.** Because eicosanoids often act as mediators of the vasomotor actions elicited by serotonin and other vasoconstrictors in umbilical vessels (3, 7, 13), we assessed the involvement of the arachidonic acid cascade in adenosine-induced vasoconstrictions. For this purpose, tissue cyclooxygenases were blocked with indomethacin (42), a non-selective COX-1/COX-2 inhibitor. The indomethacin concentration necessary to reduce the 19 μM adenosine-induced vasoconstriction was determined in a first set of experiments. Next, choriionic rings were incubated with 100 nM indomethacin for 40 min before the performance of a complete adenosine concentration-response protocol. As control, tissues were maintained in the bath chamber during the same 40 min but without drug treatments, except for the challenge with 19 μM adenosine. Tissue preincubation with 100 nM indomethacin was also used to assess the blockade of the vasoconstriction elicited by 1 μM NECA. Reversibility was examined by challenging the tissues several times until the original contractile response was recovered. Analogous protocols were performed in endothelium-denuded vessels.

In the next series of protocols we assessed whether the thromboxane receptor blocker GR32191 (33) antagonizes the adenosine-induced contraction. Adenosine concentration-response protocols were performed in the absence of GR32191 and then after a 5-min exposure to 10, 70, or 210 nM GR32191. Additional experiments assessed whether 140 nM GR32191 also reduced the vasomotor response elicited by 1 μM NECA in E+ and E- vessels. Separate experiments addressed the specificity of the GR32191-induced blockade of the adenosine-evoked contractions by challenging vessels with 70 mM KCl before and after a 5-min incubation with 140 nM GR32191.

**RT-PCR Amplification Studies**

Four- to five-centimeter segments of choriionic arteries or veins with and without the endothelial cell layer were placed in RNA stabilizing buffer. The corresponding primers were obtained from the tissues several times until the original contractile response was recovered. Analogous protocols were performed in endothelium-denuded vessels.

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**RT-PCR Amplification Studies**

Four- to five-centimeter segments of choriionic arteries or veins with and without the endothelial cell layer were placed in RNA stabilizing solution for RNA extraction and PCR studies. Total RNA from each tissue was extracted by the standard Chomczynski and Sacchi procedure (5). The oligonucleotide amplification primers for each human (h) adenosine receptor subtype and the length of the expected PCR products (in parentheses) were A1; sense 5'-CTCTTAGAGTGGCCTCCTC-3', antisense 5'-CGGAATTCCTACGGCCAGGA-3' (311 bp) (35); A2A; sense 5'-AGATGGAGGCGACGACCTC-3', antisense 5'-GCTAAAGGAGGCAACTGCTG-3' (427 bp) (22); A2B; sense 5'-GAGGCTGATCATCGGACCCCA-3', antisense 5'-ACACGGAGAGCACGGTAC-3' (342 bp) (22); and A3; sense 5'-ACCCCGCTGTTGAGCTGCT-3', antisense 5'-GCCAAGCTGTGTAACCTCA-3' (361 bp) (29).

The PCR reactions were performed in 25-μl final volumes, containing each primer at 0.5 μM, 2 μl of cDNA, 200 μM dNTPs, 1 U of Taq DNA polymerase, and 1× Taq DNA polymerase reaction buffer. The PCR thermal profile for hA1, hA2A, and hA2B were 3 min at 94°C and 30 cycles of 1 min at 94°C, 1 min at 60°C, and 3 min at 72°C. A final extension at 72°C for 7 min was completed. PCR conditions for hA3 were 2 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C and a last step of 10 min at 72°C. A sample without cDNA was subjected to this protocol as a negative control. The PCR products were separated by electrophoresis in agarose gel and visualized with ethidium bromide staining. The specificity of product bands was confirmed by analysis of the nucleotide sequence with an ABI 3100 Sequencing Automatic Analyzer.

Additional protocols were followed to evaluate the presence of human CD31 and human myosin alkaline light chain (MALC) isoform 6, selective endothelium and vascular smooth muscle markers, respectively. The corresponding primers were obtained from the GenBank database as detailed by Buvnic et al. (4). For this purpose, RNA extracts from choriionic vessels with or without the endothelial layer were utilized. All molecular biology reagents and buffers were obtained from GIBCO-BRL Life Technologies, Promega, and Ambion RNA.

**Perfusion of Placental Cotyledons and Assessment of Vascular Resistance**

Individual cotyledons were perfused with Krebs-Ringer buffer gassed with 95% O2-5% CO2 through plastic tubing cannulas inserted into the corresponding placental artery. After a 40-min equilibration period, 300 μM adenosine or 10 μM NECA dissolved in buffer was perfused through the arterial cannula for 1 min. Perfusion pressure was monitored continually on a Grass polygraph; the changes in perfusion pressure were recorded through a strain gauge connected to the artery. Generally, for adenosine we tested seven concentrations in four different placentas, whereas for NECA four concentrations were tested; a single agonist concentration was examined per cotyledon, and each concentration was examined in at least four separate placentas. This procedure was determined to be optimal for concentration-response determinations, because we noted in a preliminary series of experiments that a second perfusion of NECA, but not adenosine, performed 25 min later, evoked a markedly reduced contraction. In a further set of three protocols, the vasomotor action elicited by 300 μM adenosine was assessed in cotyledons precontracted with 0.3 μM 5-HT, a procedure that allowed us to discard the idea that adenosine might act as a vasorelaxant in precontracted tissues. Results are plotted as the mean changes in perfusion pressure elicited by both adenosine and NECA.

**Data Quantification and Statistical Analysis**

The median effective contractile concentration (EC50) was interpolated from each agonist concentration-response curve (at least 4 points defined a curve, and each point was repeated in individual vessel rings from at least 3–8 separate placentas) and served to establish the relative order of potency of adenosine and structurally related analogs; EC50 values are expressed as micromolar. The contraction elicited by each agonist concentration was recorded in grams of tension, normalized according to the KCl standard, allowing comparisons within multiple vessel rings, and plotted in a standard concentration-response curve format. With regard to the relaxant effect induced by 2-CI-IB-MECA, this effect was quantified by normalizing the percent relaxation of the 20 mM KCl-induced tension; the median agonist concentration required to elicit relaxation was interpolated from each concentration-response curve protocol. In the case of the receptor subtype antagonist studies, the concentration required to halve the contractile response to the standard adenosine challenge (IC50) was interpolated for each antagonist experiment (n = 2, 4).

GraphPad software (GraphPad, San Diego, CA) was used to fit concentration-response curves. Analysis of variance established the statistical significance of several treatments on the concentration-response curves. When necessary, the Student’s t-test tables (paired or nonpaired) were used; Dunnett’s tables for multiple comparisons with a single control were likewise used when appropriate. Significance was established by a P value <0.05.

**RESULTS**

**Vasomotor Action of Adenosine and Structurally Related Analogs in Intact Vessels**

Adenosine contracted isolated choriionic arteries or veins concentration-dependently, reaching slightly more than 40% of the KCl standard contracture. The adenosine EC50 was almost identical in artery and vein and averaged 37 μM (Table 1 and
Fig. 1). NECA, a highly potent but nonselective adenosine receptor agonist, was almost 100-fold more potent than adenosine and reached 60% of the KCl contracture, and CHA, a classic A1 receptor ligand, was 2-fold more potent than adenosine and maintained the maximal response; that is, they both reached 40% of the KCl standard contracture, whereas the potency of CGS-21680, a prototype A2A receptor agonist, was indistinguishable from that of CHA, but its maximal contracture did not exceed 15% (Table 1). Furthermore, adenosine and congeners also raised the tension of precontracted vessels; the magnitude of the contraction was similar to that obtained in nonprecontracted tissues (see tracings in Fig. 1), although only 2-Cl-IB-MECA, an alleged A2 receptor agonist, which per se was inactive as a vasoconstrictor, concentration-dependently relaxed KCl-precontracted chorionic vessels (Fig. 2). The potency of 2-Cl-IB-MECA to induce relaxations was 27.5 ± 7 μM (n = 4) in vein rings; a similar value was found in arteries (18.5 ± 8.5 μM n = 3). The maximal relaxation achieved 58.7 ± 7% of the KCl-induced tension (n = 4).

Successive CHA, CGS-21680, or NECA applications in the range of their EC50 values resulted in a gradual and substantial loss of vasomotor activity (Table 2). In contrast, three or more sequential adenosine applications elicited contractions of similar magnitude (Table 2).

The KCl standard challenge elicited a significantly larger contraction in chorionic vein than in artery rings [1.54 ± 013 g (n = 29) vs. 0.88 ± 0.08 g (n = 29), P < 0.001]; however, we did not observe significant differences between the KCl contracture in vessels derived from cesarean section placentas vs. vaginal birth placentas, ruling out the influence of drugs used during the surgical and/or anesthetic procedures.

**Adenosine-Induced Vasoconstriction Raises Perfusion Pressure of Isolated Cotyledons**

Consistent with the finding that NECA is much more potent than adenosine to contract isolated chorionic ring vessels, NECA was at least 100-fold more potent than adenosine to raise the perfusion pressure of human placenta cotyledons (Fig. 3). Consonant with the vascular reactivity observations in the isolated rings from the chorionic vessels, a second NECA application did not cause a rise in the perfusion pressure, in contrast to adenosine, which showed essentially no desensitization (see recordings in Fig. 3). Moreover, paralleling the observations in isolated vessel rings, adenosine caused a further rise in perfusion pressure in precontracted cotyledons (Fig. 3). Interestingly, the magnitude of the rise in perfusion elicited by 300 μM adenosine in noncontracted and precontracted tissues was the same (28.3 ± 4 mmHg; n = 4).

Additional experiments demonstrated that prostaglandin F2α elicited a concentration-dependent rise in the perfusion pressure (its estimated EC50 was 3 ± 0.43 μM), whereas the application of 5 μM prostaglandin F2α evoked a rise in the perfusion pressure of 137.5 ± 12.5 mmHg (n = 3) and that of 0.5 μM 5-HT caused a 91 ± 15 mmHg rise in perfusion pressure (n = 3). Therefore, prostaglandin F2α is at least 100-fold more potent than adenosine as a human cotyledon vasoconstrictor.

**Table 1. Potency and maximal response evoked by NECA, adenosine, and structurally related analogs to contract human superficial chorionic vessels**

<table>
<thead>
<tr>
<th></th>
<th>Chorionic Artery</th>
<th>Chorionic Vein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC50, μM</td>
<td>Emax (%)</td>
</tr>
<tr>
<td>NECA</td>
<td>0.4 ± 0.2 (5)</td>
<td>57 ± 9 (5)</td>
</tr>
<tr>
<td>Adenosine</td>
<td>37.6 ± 81 (8)</td>
<td>43 ± 4.7 (8)</td>
</tr>
<tr>
<td>CHA</td>
<td>14.0 ± 31 (5)</td>
<td>39.5 ± 6.6 (5)</td>
</tr>
<tr>
<td>CGS-21680</td>
<td>15.8 ± 2.9* (3)</td>
<td>11 ± 47 (3)</td>
</tr>
</tbody>
</table>

Values are means ± SE for no. of rings shown in parentheses. Emax(%), % of contraction compared with the 70 mM KCl standard; NECA, 5'-N-ethylcarboxamidoadenosine; CHA, N6-cyclohexyladenosine; CGS-21680, 2-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine HCl. *P < 0.05, †P < 0.01 compared with NECA in arteries or veins. ‡P < 0.05 compared with adenosine.
show that 36
Chorionic artery
Loss of vasomotor responses elicited by successive
Table 2.
onic arteries was 15
241385 to halve the adenosine contraction standard in chori-
contractions. The estimated potency of DPCPX and ZM-
tration-dependently blocked the challenge adenosine-induced
Receptor Antagonists
Top
veins with or without endothelium.
Fig. 2. Vasorelaxation evoked by 2-Cl-IB-MECA in precontracted chorionic
rings prepared from superficial human chorionic vessels
applications of adenosine analogs, but not adenosine, in
Different ChV rings obtained from separate placentas.
MRS-1220 did not significantly block the adenosine-induced
Table 2. Loss of vasomotor responses elicited by successive applications of adenosine analogs, but not adenosine, in
rings prepared from superficial human chorionic vessels
<table>
<thead>
<tr>
<th></th>
<th>First Application</th>
<th>Second Application, 40 Min Later</th>
<th>Third Application, 40 Min Later</th>
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<tbody>
<tr>
<td>Chorionic artery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19 µM adenosine (9)</td>
<td>12.4 ± 4.0</td>
<td>13.4 ± 3.9</td>
<td>12.7 ± 0.7</td>
</tr>
<tr>
<td>17 µM CHA (7)</td>
<td>21.9 ± 5.7</td>
<td>8.6 ± 2.3*</td>
<td>2.6 ± 1.1†</td>
</tr>
<tr>
<td>11 µM CGS-21680 (6)</td>
<td>14.5 ± 2.4</td>
<td>3.3 ± 1.7†</td>
<td>1.2 ± 0.7†</td>
</tr>
<tr>
<td>1 µM NECA (7)</td>
<td>36.8 ± 5.8</td>
<td>18.5 ± 9.9*</td>
<td>8.2 ± 2.9†</td>
</tr>
<tr>
<td>Chorionic vein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19 µM adenosine (13)</td>
<td>10.2 ± 2</td>
<td>11.9 ± 3.1</td>
<td>11.3 ± 2.4</td>
</tr>
<tr>
<td>17 µM CHA (7)</td>
<td>40.1 ± 5.2</td>
<td>8.9 ± 1.2†</td>
<td>2.1 ± 0.9†</td>
</tr>
<tr>
<td>11 µM CGS-21680 (6)</td>
<td>9.5 ± 2.9</td>
<td>3.9 ± 1.0*</td>
<td>1.1 ± 0.5†</td>
</tr>
<tr>
<td>1 µM NECA (6)</td>
<td>49.9 ± 12.5</td>
<td>16.8 ± 8.5*</td>
<td>6.2 ± 2.2†</td>
</tr>
</tbody>
</table>

Values are mean ± SE tension (expressed as % KCl standard) elicited by sequential applications for no. of rings shown in parentheses. *P < 0.05, †P < 0.01 compared with the first application.

Adenosine Receptor Classification Based on Use of Receptor Antagonists
DPCPX and to a much lesser extent, ZM-241385, concentration-dependently blocked the challenge adenosine-induced contractions. The estimated potency of DPCPX and ZM-241385 to halve the adenosine contraction standard in chorionic arteries was 15 ± 3.7 (n = 6) and 207 ± 42 (n = 3) nM, respectively (Fig. 4). Likewise, in veins, these values were 22 ± 3.8 (n = 6) and 633 ± 84 (n = 3) nM, respectively. MRS-1220 did not significantly block the adenosine-induced contractions in either chorionic arteries or veins [Fig. 4; linear regression r = 0.29 and 0.014 (not significant) in chorionic artery and vein, respectively]. Successive adenosine applications caused a similar vasoconstriction, as exemplified in Table 2, ruling out the idea that the antagonist-evoked reduction of the adenosine vasoconstriction is due to muscular damage or nonspecific metabolic alterations.

In addition, 200 nM ZM-241385 reduced the magnitude of the contractions induced by the 19 µM adenosine standard in artery rings by 47% [from 18.7 ± 2.2% of the KCl contracture (n = 21) to 8.7 ± 1.6% of the maximal KCl response (n = 7); P < 0.05], 200 nM DPCPX caused a 69% reduction of the response (from the control value to 5.8 ± 1.4% of the maximal KCl response; n = 12; P < 0.01). However, the coapplication of both antagonists only reduced the adenosine response by 77% (4.3 ± 0.7%; n = 4; P < 0.05), a value that is not significantly different from that attained by the separate application of each antagonist. Similar findings were also observed in chorionic vein rings (data not shown).

RT-PCR Products Identify Adenosine Receptor mRNAs
RT-PCR further identified the mRNAs coding for multiple adenosine receptors. In E+ and E− vessels, RT-PCR products identified the mRNAs coding for the human adenosine A2A-
cotyledons. Therefore, because the acetylcholine challenge was
line failed to relax precontracted perfused human placental
one-third of chorionic rings. Likewise, 10 –100
chorionic vessels; in fact, 1–10
activity assays. However, it did not relax the intact isolated
cept without endothelium (Fig. 5). This experiment was repeated with mRNA extracted from three separate placentas.

Role of Endothelium

Removal of the endothelial cell layer reduced by about
two-thirds the adenosine- and NECA-induced contractions. Endothelial cell removal caused a flattening and downward shift of the adenosine concentration-response curve in both artery and vein rings ($P < 0.01$, $n = 7$; Fig. 6). The NECA-induced contraction was likewise more than halved after en-
dothelem removal ($P < 0.05$; Fig. 6). Acetylcholine, a widely
accepted endothelium-dependent vasodilator, is used to func-
tionally evaluate the role of the endothelium in vascular reac-
tivity assays. However, it did not relax the intact isolated
chorionic vessels; in fact, 1–10 μM acetylcholine contracted
one-third of chorionic rings. Likewise, 10–100 μM acetylcholine failed to relax precontracted perfused human placental
cotyledons. Therefore, because the acetylcholine challenge was
not useful as a functional test of endothelium denudation, the
absence of CD31, revealed in the RT-PCR studies, confirmed
in this case the efficacy of the endothelium removal procedure.

Additionally, the 2-Cl-IB-MECA-induced relaxation was
independent of the endothelium (Fig. 2). Removal of the
endothelial cell layer did not significantly modify the potency
or the maximal relaxation evoked by this agonist in isolated
chorionic veins [15.4 ± 2.4 (n = 4) vs. 27.5 ± 7 μM (n = 4)].

Involvement of Arachidonic Cascade in Adenosine-
and NECA-Evoked Contractions

In view of the fact that arachidonate metabolites have a
preponderant role in vascular tone regulation, we assessed the
putative role of eicosanoids as mediators of adenosine-evoked
contractions.

Cyclooxygenase inhibition. Indomethacin concentration-de-
pendently inhibited adenosine-evoked contraction in both chro-
monic artery and vein rings (Fig. 7). Indomethacin blockade
was reduced by 30–60% after a 120-min washout period (Fig.
7). Moreover, 100 nM indomethacin obliterated and flattened
the adenosine concentration-response curve in both artery and
vein rings (Fig. 7). Likewise, 100 nM indomethacin reduced by
92 ± 4% ($P < 0.01$) the 1 μM NECA-induced contraction in
either vessel (Fig. 7, inset). Moreover, 100 nM indomethacin
also reduced by 80% the 1 μM NECA-evoked vasoconstriction.
in vessel rings denuded of the endothelial layer \((P < 0.01; n = 3)\). Furthermore, 2-Cl-IB-MECA-evoked relaxation was also blocked in a concentration-dependent manner by 0.1–1 \(\mu M\) indomethacin (see recordings in Fig. 2).

**Thromboxane receptor blockade.** The thromboxane inhibitor GR32191, which per se did not alter the basal tone of the rings, concentration-dependently reduced the vasoconstriction evoked by adenosine \((P < 0.05\) in arteries and veins), whereas 70 nM GR32191 halved the vasoconstrictions and increasing the concentration to 210 nM obliterated the adenosine concentration-response curve (Fig. 8). In separate experiments, 140 nM GR32191 reduced by 70–90\% the 1 \(\mu M\) NECA-induced vasoconstriction \((P < 0.05)\) in artery or vein choric rings. Likewise, GR32191 abolished the 19 \(\mu M\) adenosine- and 1 \(\mu M\) NECA-induced vasoconstriction in endothelium-denuded vessels (data not shown). In separate control protocols, 140 nM GR32191 reduced only by 8.7\% the 70 mM KCl-evoked contractions \((n = 4)\), evidencing antagonist selectivity. Previous studies from our laboratory (54) showed that the EC50 for prostaglandin F2alpha is 0.09 \(\pm 0.004\) \(\mu M\), a value 400-fold more potent than adenosine, with a maximal effect that doubled that elicited by the KCl standard challenge. The present results

![Figure 6](http://ajpheart.physiology.org/)

**Fig. 6.** Endothelium influences adenosine-and NECA-evoked vasoconstrictions. Top: representative polygraph tracings of the contractions evoked by 6.4 and 19 \(\mu M\) adenosine in a ChA ring with endothelium (left) and an adjacent segment ring that was endothelium denuded (right). Middle: adenosine concentration-response curves in ChA (left) and ChV (right) rings with and without endothelium. Symbols denote the mean average normalized tension; bars indicate SE. Each complete concentration-response curve protocol was repeated in 7 intact endothelium rings and in 7 rings without endothelium obtained from separate ChA and ChV, each derived from individual placentas. Bottom: 1 \(\mu M\) NECA-evoked contraction in ChA and ChV is also partially sensitive to endothelium denudation \((P < 0.05)\). Each experiment was replicated in 9 vessel rings with endothelium and 4 rings without endothelium from both ChA and ChV vessels; each preparation was derived from a separate placenta. Columns represent the mean average tension; bars indicate SE.

![Figure 7](http://ajpheart.physiology.org/)

**Fig. 7.** Indomethacin blocks adenosine-induced vasoconstriction, involving the participation of the arachidonic acid cascade. Top: tissue preincubation with indomethacin, a cyclooxygenase inhibitor, for 40 min concentration-dependently reduced the standard 19 \(\mu M\) adenosine-induced contractions in intact ChA \((n = 4, left)\) and ChV \((right)\); 100 nM indomethacin obliterated the adenosine response \((P < 0.05,\) Dunnett’s tables). The indomethacin-induced blockade is partially reversible after 120 min of drug washout (open columns) \((P < 0.05\) compared with the 100 nM indomethacin). Inset: 1 \(\mu M\) NECA-induced contraction was also significantly reduced by 100 nM indomethacin (Indo) in either ChA or ChV vessel rings \((P < 0.01; n = 3)\). Columns indicate the mean average of the normalized tension; bars indicate SE. Bottom: 100 nM indomethacin flattened the adenosine concentration-response curve, which was performed with 13 control rings and 3 separate indomethacin-treated rings. Symbols indicate the mean average normalized tension; bars indicate SE.
show that prostaglandin E2 is also a constrictor of isolated chorionic vein segments; although its EC50 is 170-fold less than prostaglandin F2α (15.5 ± 2.1 μM; n = 3 per antagonist concentration). Symbols indicate the mean average contraction normalized against the 70 mM KCl standard; bars indicate SE. Bottom: ring preincubation with 140 nM GR32191 significantly (*P < 0.05) reduced the 1 μM NECA-evoked contractions. Left: data from ChA rings. Right: data from ChV rings. These protocols were repeated 4 times in vessels obtained from separate placentas. Columns indicate mean average tension; bars indicate SE.

The present results support the hypothesis that the adenosine-induced vasoconstriction of human chorionic vessels is mediated by the activation of A2B receptors, an effect that appears to be related to the synthesis of an arachidonate metabolite that ultimately activates a thromboxane receptor in the vascular smooth muscles. Figure 9 summarizes and schematizes this proposal, indicating that adenosine contracts chorionic vessels via the activation of a dominating population of adenosine A2B receptors located in both endothelial and vascular smooth muscle cells. These receptors are coupled to the synthesis of an arachidonate metabolite, likely thromboxane A2, which might activate a thromboxane TP receptor, as the final effector of the adenosine contractile response. At present we cannot exclude the possible participation of related prostanooids. The mechanism that links A2B receptor signaling to phospholipase activity, and thereafter activation of tissue cyclooxygenase, remains to be further described. The present results highlight for the first time a role for adenosine as an extracellular signal involved in blood flow regulation in the human placental vasculature and links its transduction mechanism to an arachidonic acid cascade metabolite. Adenosine contracted isolated chorionic arteries and veins and, moreover, increased the perfusion pressure of cotyledons, a finding that does not imply, but suggests, commonalities in receptor distribution along the placental vasculature. We are aware that vasoconstrictors such as angiotensin II or norepinephrine do not have the same responses along the whole fetal-placental circulation, at least in the sheep, a common animal model used to study cardiovascular development. Almost 20 years ago, Adamson et al. (1) showed that angiotensin II increased umbilical artery resistance in the sheep, whereas no change was detected in cotyledon or venous resistances. Likewise, norepinephrine had no effect on cotyledon vascular resistance but constricted downstream vessels (1). Radiolabeling binding studies further confirmed the data of Adamson et al. (1) and established that only umbilical vessels expressed

Fig. 8. Involvement of thromboxane receptors in adenosine and NECA-induced vasoconstriction. Top: adenosine concentration-response curves were performed in ChA and ChV rings in the absence (n = 10) or the presence of 10, 70, or 210 nM (1R-[1α(Z),2β,3β,5α]-(+)-7-[5-[(1',1'″-biphenyl)-4-yl]methoxy]-3-hydroxy-2-(1-piperidinyl)cyclopentyl]-4-heptenoic acid (GR32191; n = 3 per antagonist concentration). Symbols indicate the mean average contraction normalized against the 70 mM KCl standard; bars indicate SE. Bottom: ring preincubation with 140 nM GR32191 significantly (*P < 0.05) reduced the 1 μM NECA-evoked contractions. Left: data from ChA rings. Right: data from ChV rings. These protocols were repeated 4 times in vessels obtained from separate placentas. Columns indicate mean average tension; bars indicate SE.
mediated predominantly by activation of the A2B receptor. The nosine-induced vasoconstriction in human placental vessels is out (28). Furthermore, the potency of NECA is within the finding that CGS-21680, a prototype A2A receptor agonist, was be mediated by a nonspecific interaction with the A2B receptor CHA concentrations required to contract chorionic vessels may what is described for the human A1 receptor, but closer to its antagonism, and that both antagonists act at a common site, the joint nature of the ZM-241385-induced antagonism could be related to the role of A1 receptors, we conclude that the large antagonism. Furthermore, MRS-1220, a specific A3 receptor antagonist (24), did not block the adenosine-induced contraction.

Consistent with this interpretation, RT-PCR studies identified the A3 receptor subtype mRNA in these vessels. A1 receptor mRNA was found only in intact veins, suggesting that the mRNA might be further restricted to endothelial cells. We are aware that the mRNAs for the A1 and A2A receptors were detected by this procedure; however, at present we have no indication for a functional role of these receptors in the placental vasculature, except for the mRNA coding for the A3 receptor, which might be linked to vasodilatation.

Regarding the intracellular signaling mechanisms of adenosine in human chorionic vessels, the present results might be interpreted to indicate that arachidonate metabolites are in all likelihood the mediators of the adenosine effects in the placental vasculature. Furthermore, we infer that the thromboxane TP receptor (3, 10, 33) is the final effector involved in the adenosine-induced contraction. The present results highlight the preponderant role of arachidonate metabolites, such as prostaglandin E2, prostaglandin F2α, and likely thromboxane A2, in the control of the placental vascular tone (40, 41). Furthermore, the present data demonstrate the potent and efficacious contractions mediated by prostanoids in isolated vessels as in perfused cotyledons, a finding that further supports their role as mediators of the adenosine contractions. Why the adenosine-induced contractions do not desensitize, in contrast to the rapid loss of response observed with NECA, CHA, or CGS-21680, remains an open question. However, we recognize that the lack of desensitization of the adenosine responses allowed us to study its physiology in human chorionic vessels.

An issue that has gained increasing interest within the past few years, and that may have profound implications for this research and clinically oriented pathophysiological studies, refers to the discovery that tissue hypoxia upregulates the expression of the adenosine A2B receptor while decreasing in both human umbilical vein endothelial cells and bronchial smooth muscle cells. Because the placentas used in this study were not oxygenated in the 20-min period that elapsed between child delivery and laboratory manipulation, we are aware that some degree of modulation might have occurred and may have a definitive influence on our vascular reactivity studies. The group of Biaggioni (16) demonstrated that 3 h of hypoxia sufficed to triple the mRNA for the adenosine A2B receptor in endothelial cells from the umbilical vein, while it reduced by almost 90% the mRNA coding for the A2A receptor. However, it is difficult at present to evaluate the sole influence of this regulatory mechanism in vascular reactivity.

Although the present research was not aimed at determining the origin of the local placental adenosine production, it is important to keep in mind that adenosine in blood vessels derives from platelet release, endothelial cells, nerve endings, tissue damage, and so forth. These processes may be exacerbated by a variety of stimuli, including, among others, ischemia, local hypoxia, and platelet aggregation. As to the physiologic role that adenosine may play in fetal adaptation, the present results indicate the importance of the adenosine receptors in the regulation of fetal-placental circulation in health and disease states, including eclampsia and related diseases.
It remains a challenge to examine whether there is a common intracellular signaling mechanism that mediates the A2B receptor activation in different tissues, confirming the generality of our proposal. Adenosine is known to cause a transient rise in blood pressure, a finding that appears unique to the kidney microcirculation and involves A1 receptor activation (36). Although at present we cannot distinguish whether the indirect adenosine-evoked vasorelaxant mechanism operates exclusively in human placental vessels, the vasomotor response developed by serotonin in placentas from indirect adenosine-evoked mechanism operates exclusively in human placental vessels is also indirect in nature and dependent on the synthesis of an arachidonate metabolite (7, 13), suggesting that in the human placenta, which lacks sympathetic innervation (48), the arachidonic acid cascade has a major role in mediating the vasomotor regulation discussed for adenosine. The pathophysiologic implications of the present findings for preeclampsia and related vascular diseases during pregnancy are open for future research.

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