**Prostaglandin E₂–Mediated Relaxation of the Ductus Arteriosus**

Effects of Gestational Age on G Protein-Coupled Receptor Expression, Signaling, and Vasomotor Control

Nahid Waleh, PhD*; Hiroki Kajino, MD*; Anne Marilise Marrache, PhD; David Ginzinger, PhD; Christine Roman, BS; Steven R. Seidner, MD; Timothy J.M. Moss, PhD; Jean-Claude Fouron, MD; Alejandro Vazquez-Tello, PhD; Sylvain Chemtob, MD, PhD; Ronald I. Clyman, MD

**Background**—In the preterm newborn, a patent ductus arteriosus is in large part a result of the increased sensitivity of the immature ductus to prostaglandin E₂ (PGE₂). PGE₂ acts through 3 G protein–coupled receptors (EP₂, EP₃, and EP₄) that activate both adenyl cyclase and Kₐᵥ₅ channels. We explored these pathways to identify the mechanisms responsible for the increased sensitivity of the immature ductus to PGE₂.

**Methods and Results**—We measured EP receptor content (mRNA and protein), receptor binding, cAMP production, and isometric tension in rings of ductus taken from immature (65% gestation) and mature (95% gestation) sheep and baboon fetuses. Ductus relaxation and cAMP generation were augmented in response to selective EP receptor agonists in the immature ductus. 8-Br-cAMP, a stable cAMP analogue, produced greater relaxation in the immature ductus. In the presence of a selective protein kinase A inhibitor, Rp-8-CPT cAMPS, the developmental differences in sensitivity to PGE₂ could no longer be demonstrated. EP₂, EP₃, and EP₄ receptor densities were higher in immature ductus, despite similar receptor mRNA and protein contents at the 2 gestational ages. In contrast, forskolin and NaF, direct activators of adeny cyclase and Gₛ, respectively, elicited comparable increases in cAMP in both age groups. Kₐᵥ₅ channel inhibition also had similar effects on PGE₂-induced relaxation in both age groups.

**Conclusions**—Two mechanisms explain the increased sensitivity of the immature ductus to PGE₂: (1) increased cAMP production because of increased binding of PGE₂ to the individual EP receptors and (2) increased potency of cAMP on protein kinase A–regulated pathways.

---

**Key Words:** muscle, smooth; receptors; signaling; vasomotor control; PGE₂; EP receptors; signal transduction; vasodilation; vessels
Fetal baboons (Papio sp) were delivered by cesarean section at either 175 days (n=8) or 125 days (n=8) gestation (full term is 185 days) and euthanized with pentobarbital sodium. Their ductus arteriosus was removed for polymerase chain reaction (PCR) studies. These procedures were approved by the Committees on Animal Research at the University of California, San Francisco, at the Department of Agriculture, Western Australia, and at the Southwest Foundation for Biomedical Research, San Antonio, Tex.

**Isometric Tension In Vitro**

The relaxing effects of prostaglandins were measured in rings of ductus arteriosus that were precontracted with oxygen and in which endogenous prostaglandin and nitric oxide production were maximally inhibited.1 Rings were suspended at 38°C in a modified Krebs solution (in mmol/L: 118 NaCl, 4.7 KCl, 2.5 CaCl2, 0.9 MgSO4, 1 KH2PO4, 11.1 glucose, 23 NaHCO3 [pH 7.4], equilibrated with 5% CO2/30% O2/65% N2) containing indomethacin 5.6×10−5 mM and IV-nitro-L-arginine methyl ester (L-NAME) 10−5 mM/L). The bath solution was changed every 20 minutes. Each of the rings was stretched to an initial length that resulted in a maximal isometric contractile response to increases in oxygen tension.3 After the rings reached a steady-state tension (≈100 to 120 minutes), potassium (K⁺)-Krebs solution (containing KCl 10−3 mM) was used to measure the maximal contraction that could be developed by the ductus (maximal contraction).

The (K⁺)-Krebs solution was washed out of the bath (prerelaxation tension), and cumulative relaxation dose-response curves were constructed for prostaglandin E2 (PGE₂), Butaprost (an EP₂ agonist), M&B 28767 (EP4 agonist), and 8-bromoadenosine 3′,5′ cAMP (8-Br-cAMP, a nonhydrolyzable cAMP mimetic). The concentrations that produce 50% of the maximal response to the drug (EC50 values) were determined from each dose-response curve. In some experiments, a cumulative dose-response curve was performed to a relaxing agent after the tissue had equilibrated with either an EP₄ receptor antagonist (AH23848B), a selective inhibitor of KₐtP channels (glibenclamide), or a selective inhibitor of protein kinase A, 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphorothioate, Rp isomer (Rp-8-CPT-cAMPS).3 Sodium nitroprusside (10−4 mmol/L) was added to each ring at the end of the experiment to determine its minimal tension.3 The difference in tensions between the prerelaxation tension and the tension after sodium nitroprusside was considered the net tension (Immature = 273±139 g/cm²; Mature = 324±207 g/cm²). Tensions are expressed as a percentage of the net tension. After the experiment, the tissues were removed from the baths and blotted dry, and their wet weights were determined.

**Supplies**

AH23848B was generously provided by Dr Simon Lister (Glaxo-Wellcome); M&B 28767 by Dr Jean Hough (Rhone-Poulenc Rorer). PGE₂, butaprost (Cayman Chemical), and Rp-8-CPT-cAMPS (Biolog, Life Science Institute) were purchased. All other chemicals were from Sigma Chemical Co.

**EP Receptor Binding, cAMP Generation, and Immunoblotting of EP Receptors**

Assays of EP receptor binding, cAMP generation, and EP receptor immunoblotting have been published in detail previously.1,4 [3H]PGE₂ binding and displacement studies with 16,16-dimethyl-PGE₂ (a nonselective EP agonist), butaprost, M&B 28767, and AH23848B were performed on ductus membranes; total and individual EP receptor densities (Bₐmax) were determined.3,4 For cAMP generation, ductus homogenates were incubated for 10 minutes (in mmol/L: 1 ATP, 7.5 MgCl₂, 15 creatine phosphate, 0.5 EGTA, 0.5 IBMX, 1 dithiothreitol, 1 benzamidine, and 0.1 PMSF, and 185 U/mL creatine phosphokinase, 200 μg/mL aspirin, and 100 μg/mL soybean trypsin inhibitor), and cAMP was measured by radioimmunoassay.3,5 Immunoblots of EP, and EP, were performed on ductus arteriosus membranes using a monoclonal antibody against EP, (3E6, from Dr J. Castracane, Exalpha) and a polyclonal anti-EP₄ antibody.3,4 No antibody is currently available for EP,D.

**EP Receptor Expression: Preparation of Total RNA, Reverse Transcription, and Quantitative PCR**

Total RNA was isolated from fetal sheep and baboon ductus arteriosus as described elsewhere.1 We used the TaqMan Universal PCR master mix of PE Applied Biosystems to quantify the expression of EP receptors. TaqMan probes were designed using the Primer Express program and labeled with fluorophores FAM (6-carboxy-fluorescein) and TAMRA (6-carboxy-tetramethyl-rhodamine) as reporter and quencher dyes, respectively. An ABI PRISM 7700 Sequence Detection system was used to determine number of PCR cycles required for product detection (the cycle threshold [CT] value). Reactions were performed in triplicate. The smaller the number of starting copies of a gene, the higher the CT value required for product detection. All reactions were repeated on at least 3 separate days. Data were analyzed using the Sequence Detector version 1.6.3 program.

In preliminary experiments, we found that the CT values of baboon and sheep malate dehydrogenase (MDH) and GAPDH genes were constant throughout gestation: baboon MDH (CT [125 days]=27.9±0.6 cycles, n=8; CT [175 days]=27.9±0.6, n=8), sheep MDH (CT [100 days]=25.4±0.3, n=7; CT [138 days]=25.4±0.4, n=7), and sheep GAPDH (CT [100 days]=24.0±0.2, n=7; CT [138 days]=23.7±0.4, n=7). Therefore, MDH and GAPDH were used as internal controls to normalize the degree of expression of the EP receptors using the relative gene expression method.

**Statistics**

Statistical analyses of unpaired and paired data were performed with the appropriate t test or Mann-Whitney test. When >1 comparison was made, the Bonferroni correction was used. Values are expressed as mean±SD. Drug doses refer to their final molar concentration in the bath. A probability value of P<0.05 was considered significant.

**Results**

We first looked at the effect of gestation on the ductus’ relaxation response to PGE₂. In the presence of 30% oxygen, indomethacin, and L-NAME, the immature fetal ductus develops a net tension (75±14% of maximal active tension) similar to that of the mature fetal ductus (78±9% of maximal active tension). The immature ductus is more sensitive to the relaxing effects of both nonselective (PGE₃) and selective (EP₂=butaprost, EP₄=M&B 28767) EP receptor stimulation (Figure 1). The EP₄ receptor antagonist AH23848B shifts the EC₅₀ of PGE₂ to the right. AH23848B tends to have a greater effect on the PGE₂ EC₅₀ in the immature (5.8±4-fold increase) than in the mature (2±1-fold increase, P<0.10) ductus (Figure 1).

To determine whether the increased sensitivity of the immature ductus to PGE₂ could be a result of differences in signaling through KₐtP channels (Figure 2) or protein kinase A (Figure 3), we used a selective KₐtP channel inhibitor, glibenclamide (10−⁷ mmol/L) and a selective protein kinase A inhibitor, Rp-8-CPT-cAMPS. Glibenclamide decreased the sensitivity of the ductus to PGE₂ at both gestational ages. However, it did not eliminate the significant difference in PGE₂ sensitivity between the 2 age groups (Figure 2).

Rp-8-CPT-cAMPS (250 μmol/L) blocked the relaxation caused by 8-Br-cAMP and decreased the sensitivity of the ductus to both nonselective and selective EP agonists (Figure
3) Rp-8-CPT cAMPs had a significantly greater effect on the PGE$_2$ EC$_{50}$ in the immature (6.1±1.5-fold increase) than in the mature (2.2±0.8-fold increase, P<0.01) ductus. In the presence of Rp-8-CPT cAMPs, the statistically significant difference in PGE$_2$ sensitivity between the 2 age groups could no longer be demonstrated. This suggests that the increased sensitivity of the immature ductus to PGE$_2$ may be due to increased signaling through the protein kinase A pathway. Therefore, we examined whether the increased sensitivity of the immature ductus to PGE$_2$ might be due to increased cAMP production after PGE$_2$ stimulation. Both the nonselective EP receptor agonist PGE$_2$ and the selective EP receptor agonists butaprost (EP$_2$) and M&B 28767 (EP$_3$) increased cAMP production in the lamb ductus arteriosus (Figure 4). To test the role of EP$_2$, we used the EP$_2$ antagonist AH23848B in the presence of PGE$_2$. AH23848B decreased PGE$_2$-induced cAMP formation (Figure 4). This suggests that some of the cAMP formation that results from PGE$_2$ stimulation is due to activation of EP$_2$ receptors.

Both the selective and nonselective EP receptor agonists produced a greater increase in cAMP production in the immature ductus than in the mature ductus. Similarly, the EP$_2$ receptor antagonist AH23848B caused a greater inhibition of cAMP production in the immature ductus (Figure 4).

We used forskolin and NaF to determine whether differences in maximal adenyl cyclase activity could account for the increased PGE$_2$-induced cAMP formation in the immature ductus. NaF maximally activates G protein–coupled adenyl cyclase; forskolin maximally activates adenyl cyclase through a mechanism that is independent of G proteins. There was no difference between the immature and mature ductus in the amount of forskolin- or NaF-induced cAMP production (Figure 5).

In addition to the increased production of cAMP after PGE$_2$-stimulation, the immature ductus is more sensitive to relaxation (Figure 1). Immature ductus=104±1.5 days gestation; Mature ductus=138±2 days gestation. A, PGE$_2$: Immature (n=8), Mature (n=9); EC$_{50}$(Immature)=1.1±0.3×10$^{-10}$ mmol/L, EC$_{50}$(Mature)=6.9±2.4×10$^{-9}$ mmol/L, P<0.05; maximum relaxation(Immature)=93±2%, maximum relaxation(Mature)=90±4%, P=NS. B, Butaprost: Immature (n=6), Mature (n=9); EC$_{50}$(Immature)=2.4±1.0×10$^{-8}$ mmol/L, EC$_{50}$(Mature)=5.7±3.0×10$^{-9}$ mmol/L, P<0.05; maximum relaxation(Immature)=65±11%, maximum relaxation(Mature)=33±10%, P<0.05. C, PGE$_2$,AH23848B: Immature (n=7); EC$_{50}$(PGE$_2$)=2.0±1.0×10$^{-10}$ mmol/L, EC$_{50}$(PGE$_2$, AH23848B)=11±9×10$^{-10}$ mmol/L, P<0.05; maximum relaxation(Immature)=90±3%, maximum relaxation(PGE$_2$)=90±3%, maximum relaxation(PGE$_2$, AH23848B)=87±9%; Mature (n=8); EC$_{50}$(PGE$_2$)=7.2±3.0×10$^{-10}$ mmol/L, EC$_{50}$(PGE$_2$, AH23848B)=14±6×10$^{-10}$ mmol/L, P<0.05; maximum relaxation(Immature)=83±6%, maximum relaxation(PGE$_2$, AH23848B)=80±8%; Mature (n=8); EC$_{50}$(Immature)=4.2±1.5×10$^{-8}$ mmol/L, EC$_{50}$(Mature)=29±11×10$^{-8}$ mmol/L, P<0.05; maximum relaxation(Immature)=74±5%, maximum relaxation(Mature)=55±10%, P<0.05. D, 8-Br-cAMP: Immature (n=6); Mature (n=8); EC$_{50}$(Immature)=5.1±1.3×10$^{-4}$ mmol/L, EC$_{50}$(Mature)=9.0±1.3×10$^{-4}$ mmol/L, P<0.05; maximum relaxation(Immature)=95±1.3%, P=NS.
the relaxing effects of cAMP itself. Both 8-Br-cAMP, the nonhydrolyzable analogue of cAMP, and forskolin have twice the potency in the immature ductus (Figure 1).

We next looked for differences in EP receptor density. Maximal specific binding of \(^{3} \text{H}\)PGE\(_{2}\) to immature ductus membranes was 2.6-fold greater than that to mature ductus membranes (Figure 6). The B\(_{max}\) for all 3 EP receptors was increased in the immature ductus. The increased receptor binding in the immature ductus was not due to increased expression of EP receptor mRNA (Figure 7) or protein.

**Figure 2.** \(K_{\text{ATP}}\) channel inhibitor glibenclamide alters vasomotor response of fetal ductus arteriosus to PGE\(_{2}\). Ductus rings were precontracted with 30% oxygen, indomethacin, and L-NAME. Dose responses to PGE\(_{2}\) were tested in presence and absence of glibenclamide(10\(^{-6}\) mmol/L), EC\(_{50}\)(glibenclamide)=3.2±0.7×10\(^{-10}\) mmol/L, P<0.05, maximum relaxation(Control)=93±2%, maximum relaxation(glibenclamide)=89±5%. Note: in presence of glibenclamide, there still was a significant difference in response of immature [EC\(_{50}\)(glibenclamide)=2.0±10\(^{-10}\) mmol/L ductus to PGE\(_{2}\) (P<0.05).

**Figure 3.** Protein kinase A inhibitor Rp-8-CPT cAMPS alters vasomotor response of fetal ductus arteriosus to prostaglandins and 8-Br-cAMP. Ductus rings were precontracted with 30% oxygen, indomethacin, and L-NAME. Dose responses to 8-Br-cAMP, butaprost (a selective EP\(_{2}\) agonist), PGE\(_{2}\), and M&B28767 (a selective EP\(_{3}\) agonist) were also tested in presence and absence of Rp-8-CPT cAMPS (2.5×10\(^{-4}\) mmol/L). Tension is expressed as a percentage of cAMP formation in basal (unstimulated) state. Effects of PGE\(_{2}\) were also tested in presence of EP\(_{4}\) antagonist AH23848B (5×10\(^{-6}\) mol/L). Basal rate of synthesis was 17±10 pmol·mg protein\(^{-1}\)·10 minutes\(^{-1}\) for immature and 21±8 pmol·mg protein\(^{-1}\)·10 minutes\(^{-1}\) for mature ductus. *P<0.05: immature vs mature; **P<0.05: PGE\(_{2}\) vs PGE\(_{2}\)+AH23848B. Note: EP\(_{4}\) antagonist AH23848B by itself did not alter cAMP production (data not shown).

**Figure 4.** Effects of PGE\(_{2}\) analogues on cAMP synthesis in immature and mature ductus arteriosus. Ductus homogenates (100 µg) were incubated with indicated agents for 10 minutes at 37°C. CAMP synthesis (after addition of stimulant) was expressed as a percentage of cAMP formation in basal (unstimulated) state. Effects of PGE\(_{2}\) were also tested in presence of EP\(_{4}\) antagonist AH23848B (5×10\(^{-6}\) mol/L). Basal rate of synthesis was 17±10 pmol·mg protein\(^{-1}\)·10 minutes\(^{-1}\) for immature and 21±8 pmol·mg protein\(^{-1}\)·10 minutes\(^{-1}\) for mature ductus. *P<0.05: immature vs mature; **P<0.05: PGE\(_{2}\) vs PGE\(_{2}\)+AH23848B. Note: EP\(_{4}\) antagonist AH23848B by itself did not alter cAMP production (data not shown).

**Figure 5.** Protein kinase A inhibitor Rp-8-CPT cAMPS alters vasomotor response of fetal ductus arteriosus to prostaglandins and 8-Br-cAMP. Ductus rings were precontracted with 30% oxygen, indomethacin, and L-NAME. Dose responses to 8-Br-cAMP, butaprost (a selective EP\(_{2}\) agonist), PGE\(_{2}\), and M&B28767 (a selective EP\(_{3}\) agonist) were also tested in presence and absence of Rp-8-CPT cAMPS (2.5×10\(^{-4}\) mmol/L). Tension is expressed as a percentage of cAMP formation in basal (unstimulated) state. Effects of PGE\(_{2}\) were also tested in presence of EP\(_{4}\) antagonist AH23848B (5×10\(^{-6}\) mol/L). Basal rate of synthesis was 17±10 pmol·mg protein\(^{-1}\)·10 minutes\(^{-1}\) for immature and 21±8 pmol·mg protein\(^{-1}\)·10 minutes\(^{-1}\) for mature ductus. *P<0.05: immature vs mature; **P<0.05: PGE\(_{2}\) vs PGE\(_{2}\)+AH23848B. Note: EP\(_{4}\) antagonist AH23848B by itself did not alter cAMP production (data not shown).
We examined the changes in mRNA expression in 2 separate species; although the relative expression of individual EP receptors appeared to differ between baboon and sheep, the immature ductus in both species had either the same amounts of or less mRNA than the mature ductus.

**Discussion**

The present studies confirm our previous findings that the immature fetal ductus arteriosus is more sensitive than the late-gestation fetal ductus to the relaxing effects of PGE2. The immature ductus is also more sensitive to stimulation of each of the individual EP receptors than is the ductus near term (Figure 1). EP receptor stimulation relaxes the fetal lamb ductus arteriosus by increasing cAMP and activating KATP channels (Figures 2 to 4). We found that the increased sensitivity of the immature ductus to EP receptor stimulation can be explained by differences in cAMP signaling (rather than by differences in KATP channel activity). Stimulation of EP receptors in the immature ductus produces a greater degree of cAMP production (Figure 4) and protein kinase A–mediated relaxation (Figure 3) than is found in the mature ductus. When protein kinase A activity is inhibited, the immature ductus loses its increased sensitivity to PGE2 and behaves like the mature ductus (Figure 3).

Our data suggest that the increased cAMP-mediated relaxation in the immature ductus is in part a result of both increased binding of PGE2 to EP receptors and increased potency of cAMP on protein kinase A–regulated pathways. There does not seem to be a difference in the amount of EP receptor mRNA or protein between the 2 gestational ages studied, nor is there a difference in the maximal activity of adenyl cyclase after forskolin or NaF stimulation.

Our findings differ somewhat from those of Smith et al. These authors did not detect EP2 receptors in the sheep ductus arteriosus. We detected the presence of EP2 receptor protein...
and mRNA in all of our ductus samples and demonstrated that the highly selective EP2 agonist butaprost relaxed the fetal lamb ductus (Figures 1, 7, and 8). The reduction in EP2 expression observed by Smith et al is in some ways consistent with the decreased binding that we observed with advancing gestation. However, in contrast with Smith et al, we found that although binding decreased with advancing gestation, EP receptor expression (mRNA and protein) was unchanged. This suggests that in the mature ductus, the availability of EP receptors for PGE2 is decreased. Changes in receptor affinity (altered G protein binding–induced conformational changes) and/or differences in receptor phosphorylation pattern (affecting ligand binding and receptor internalization) could be responsible for the decrease in Bmax with advancing gestation. Elevated PGE2 levels have been associated with EP receptor relaxes the lamb ductus by increasing cAMP (Figures 3 and 4). Although stimulation of EP3 receptors caused a small constriction of the rabbit ductus arteriosus, its major effect was relaxation consistent with our observations. In our hands, cumulative dose-response curves to 2 selective EP3 agonists (M&B 28767 [10−9 to 10−6 mmol/L] and GR63799X [10−8 to 10−5 mmol/L]) elicited relaxation even in the presence of forskolin, indomethacin, L-NAME, and K-channel inhibitors (n=3, data not shown). It is interesting to note that the protein kinase A inhibitor Rp-8-CPT cAMPS affected the efficacy of EP3-induced relaxation to a much smaller degree than the efficacy of either butaprost or 8-Br-cAMP (which are mediated exclusively through protein kinase A–dependent pathways) (Figure 3). This finding is consistent with our other observations that signaling through EP3 involves both protein kinase A–dependent and protein kinase A–independent pathways.

Figure 8. Immunoblot of EP2 and EP4 receptor proteins (arrows) in individual ductus from 4 separate immature and mature sheep fetuses. Ordinate represents arbitrary densitometry units. There were no significant differences between immature and mature ductus in either EP2 or EP4 protein expression.

Our studies do not address the possibility that decreased phosphodiesterase activity may play a role in the increased sensitivity of the immature ductus to EP receptor–mediated relaxation. Our experiments were designed to measure cAMP production; therefore, in our cAMP generation assays, ductus membranes were treated with nonselective phosphodiesterase inhibitors. In preliminary studies, we have found that phosphodiesterase inhibitors have a more potent effect on ductus relaxation in the immature fetus than in the late gestation ductus (unpublished results).

We have previously shown that stimulation of the EP2 receptor relaxes the lamb ductus by activating glibenclamide-sensitive KATP channels. In the present study, we also found that stimulation of the EP2 receptor relaxes the ductus by increasing cAMP (Figures 3 and 4). Although stimulation of EP3 receptors caused a small constriction of the rabbit ductus arteriosus, its major effect was relaxation consistent with our observations. In our hands, cumulative dose-response curves to 2 selective EP3 agonists (M&B 28767 [10−9 to 10−6 mmol/L] and GR63799X [10−8 to 10−5 mmol/L]) elicited relaxation even in the presence of forskolin, indomethacin, L-NAME, and K-channel inhibitors (n=3, data not shown). It is interesting to note that the protein kinase A inhibitor Rp-8-CPT cAMPS affected the efficacy of EP3-induced relaxation to a much smaller degree than the efficacy of either butaprost or 8-Br-cAMP (which are mediated exclusively through protein kinase A–dependent pathways) (Figure 3). This finding is consistent with our other observations that signaling through EP3 involves both protein kinase A–dependent and protein kinase A–independent pathways.

Acknowledgments

This study was supported by grants from the US Public Health Service (HL-46961 and HL-56061), the Medical Research Council of Canada, the Heart and Stroke Foundation of Quebec, the Fonds de la Recherche en Santé du Québec, and a gift from the Perinatal Associates Research Foundation. NHLBI grant HL-52636 funded the BPD Resource Center. The authors thank Drs Alan Jobe, Machiko Ikegami, and John Newnham for their invaluable help in obtaining sheep ductus and Hendrika Fernandez and Francoise Maurray for their expert technical assistance.

References


Prostaglandin E$_2$—Mediated Relaxation of the Ductus Arteriosus: Effects of Gestational Age on G Protein-Coupled Receptor Expression, Signaling, and Vasomotor Control

_Circulation_. 2004;110:2326-2332; originally published online October 11, 2004; doi: 10.1161/01.CIR.0000145159.16637.5D

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/110/16/2326

**Permissions:** Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

**Reprints:** Information about reprints can be found online at:
http://www.lww.com/reprints

**Subscriptions:** Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/