Alteration of Endothelium-Dependent Hyperpolarizations in Porcine Coronary Arteries With Regenerated Endothelium

Catherine Thollon, Jean P. Bidouard, Christine Cambarrat, Isabelle Delescluse, Nicole Villeneuve, Paul M. Vanhoutte, Jean P. Vilaine

Abstract—The present study was designed to test the ability of regenerated endothelium to evoke endothelium-dependent hyperpolarizations. Hyperpolarizations induced by serotonin and bradykinin were compared in isolated porcine coronary arteries with native or regenerated endothelium, 4 weeks after balloon endothelial denudation. The experiments were performed in the presence of inhibitors of nitric oxide synthase (N^G-nitro-L-arginine) and cyclooxygenase (indomethacin). The transmembrane potential was measured using conventional glass microelectrodes. Smooth muscle cells from coronary arteries with regenerated endothelium were depolarized in comparison with control coronary arteries from the same hearts. Spontaneous membrane potential oscillations of small amplitude or spikes were observed in some of these arteries but never in arteries with native endothelium. In coronary arteries from control pigs, both serotonin and bradykinin induced concentration-dependent hyperpolarizations. In the presence of ketanserin, 10 μmol/L serotonin induced a transient hyperpolarization in control coronary arteries. Four weeks after balloon denudation, the response to serotonin was normal in arteries with native endothelium, but the hyperpolarization was significantly lower in coronary arteries with regenerated endothelium. In control arteries, the endothelium-dependent hyperpolarization obtained with bradykinin (30 nmol/L) was reproducible. Four weeks after balloon denudation, comparable hyperpolarizations were obtained in coronary arteries with native endothelium. By contrast, in arteries with regenerated endothelium, the hyperpolarization to bradykinin became voltage-dependent. In the most depolarized cells, the hyperpolarization to bradykinin was augmented. The changes in resting membrane potential and the alteration in endothelium-dependent hyperpolarizations observed in the coronary arteries with regenerated endothelium may contribute to the reduced response to serotonin and the unchanged relaxation to bradykinin described previously. (Circ Res. 1999;84:371-377.)

Key Words: regenerated endothelium ■ endothelium-derived hyperpolarizing factor ■ serotonin ■ bradykinin

By releasing endothelium-derived relaxing factors (EDRFs) such as nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF), the endothelium plays a key role in modulating the responses of the underlying vascular smooth muscle cells. All the major cardiovascular risk factors have been associated with endothelial dysfunction, which precedes clinically apparent vascular disease and its complications. Hypertension, diabetes mellitus, hypercholesterolemia, and atherosclerosis are associated with decreased responses to endothelium-dependent vasodilators. Experiments in animal models suggest that the occurrence of abnormal endothelium-dependent relaxations is an early event in the development of vascular disease. Because the turnover of endothelial cells is accelerated under most of these conditions, the functional alterations in the regenerated endothelium could play an important role in the genesis of coronary disease. Previous studies demonstrated that, in porcine coronary arteries with regenerated endothelium after balloon denudation, endothelium-dependent relaxations are normalized 8 days after the procedure. However, 4 weeks after denudation, the relaxations to UK 14,304 (a selective α_{1}-adrenergic agonist), serotonin and aggregating platelets were reduced, whereas those induced by ADP, bradykinin, and the calcium ionophore A23187 were maintained. Because the decrease in endothelium-dependent relaxations seems restricted to stimuli that activate endothelial Gi-2 proteins, a selective Gi protein dysfunction seems likely. This selective endothelium impairment and the resulting marked dysfunction in the NO-EDRF pathway does not result from a change in the expression of Gi proteins but rather reflects their reduced function in regenerated endothelial cells. However, because EDHF contributes to the endothelium-dependent relaxations to bradykinin and A23187, an upregulation of the release of this factor rather than a selective Gi protein dysfunction may explain the better preservation of endothelium-dependent relaxations to these agonists. The present experiments were designed to test the ability of regenerated endothelium to...
evoke hyperpolarizations of the underlying smooth muscle of the porcine coronary artery.

**Materials and Methods**

**Animals**

Sixty-three Large White pigs (male or female), 8 to 12 weeks of age, were used for the electrophysiological study. The animals were purchased from a commercial swine producer (Frenelles E.A.R.L., Boisemont, France) in accordance with the French Ministry of Agriculture. All experiments were performed in accordance with governmental and institutional guidelines for the use and care of animals.

**Coronary Endothelium Denudation**

Thirty-four pigs, 8 weeks old (mean weight 21±0.5 kg), were anesthetized with an intramuscular injection of a mixture composed of tiletamine plus zolazepam (20 mg/kg) and atropine sulfate (50 μg/kg). Additional doses of anesthetic (sodium thiopental) were given intravenously as necessary. Animals were intubated with an endotracheal tube and mechanically ventilated with a respirator. Heparin (250 IU/kg) and lysine acetylsalicylate (10 mg/kg) were administrated intravenously to prevent thrombus formation and limit inflammation, respectively. Using aseptic surgical techniques, a percutaneous transliminal coronary angioplasty (PTCA) guide catheter (Cordis, model AR 7F) was introduced via the femoral artery into the left or right coronary ostium, under fluoroscopic guidance (Radiologeectan contrast product, x-ray imager, model BV25, Philips). A PTCA dilation catheter (Cordis, balloon of 2 to 3 mm in diameter and 20 to 30 mm long) was then introduced into the chosen coronary artery through the guide catheter. The diameter of the balloon was adapted according to the size of the coronary artery. The endothelium was removed by inflating the balloon 3 times for 30 seconds. The pressure of inflation was adjusted so that the blood vessel was not overstretched (from 2 atm at the distal side to 8 atm at the proximal side). At the end of the surgical procedure, Terramycin (20 mg/kg) was given intramuscularly as an antibiotic prophylaxis. After recovery from anesthesia, the animals were housed in individual cages for 4 weeks. At the time of killing (12 weeks of age), the body weight of these animals was 32±0.7 kg. The control animals (nonoperated) were killed at 10 weeks of age (n=29, body weight 25±0.8 kg).

**Membrane Potential Recording**

After anesthesia with tiletamine plus zolazepam (20 mg/kg, IM), the heart was removed and rapidly placed in an ice-cold oxygenated Krebs-Ringer solution. Then the left anterior descending, left circumflex, and right coronary arteries were dissected-free, cleaned of adherent fat and connective tissue, and maintained in an oxygenated Krebs-Ringer solution. Then the left anterior descending, left circumflex, and right coronary arteries were dissected-free, cleaned of adherent fat and connective tissue, and maintained in an oxygenated Krebs-Ringer solution. Then the left anterior descending, left circumflex, and right coronary arteries were dissected-free, cleaned of adherent fat and connective tissue, and maintained in an oxygenated Krebs-Ringer solution. Then the left anterior descending, left circumflex, and right coronary arteries were dissected-free, cleaned of adherent fat and connective tissue, and maintained in an oxygenated Krebs-Ringer solution. 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slow depolarization on the first 20 minutes was observed, ie, before the addition of agonists causing hyperpolarization, a vascular strips were superfused with the incubation solution, oscillations in resting membrane potential in coronary arteries with regenerated endothelium. Example of recordings obtained in 3 different coronary arteries. A, Membrane potential fluctuations of small amplitude (3 to 4 mV) and frequency (7/min) during the resting period (Krebs-Ringer solution) before any pharmacological intervention. B, Recordings of membrane potential during the resting period (Krebs-Ringer solution) before any pharmacological intervention. B, Recordings of membrane potential in the presence of the NOS blocker L-NA (30 μmol/L) in 2 preparations with regenerated endothelium that were quiescent during the resting period. a, Membrane potential fluctuations of the same frequency as in panel A (7/min) but with higher amplitude (8 to 9 mV). Recordings in panels b and c were obtained in 2 cells from the same vascular preparation. In these cells, some of the slow waves with regenerated endothelium that were quiescent during the resting period. a, Membrane potential fluctuations of the same frequency as in panel A (7/min) but with higher amplitude (8 to 9 mV). Recordings in panels b and c were obtained in 2 cells from the same vascular preparation. In these cells, some of the slow waves of depolarization lead to the generation of spikes with an amplitude in the range of 10 to 25 mV.

vascular strips were superfused with the incubation solution, before the addition of agonists causing hyperpolarization, a slow depolarization on the first 20 minutes was observed, ie, approximating 5 mV under all experimental conditions and all vascular tissues. Bradykinin or serotonin was given when the resting membrane potential had stabilized (Table). After the superfusion with the solution containing pharmacological inhibitors, the resting membrane potential was still significantly different between smooth muscle cells from coronary arteries with regenerated endothelium and native endothelium (Table). Some coronary arteries with regenerated endothelium (n=7) that had stable resting membrane potential under control conditions showed fluctuations of the membrane potential during incubation with the NOS blocker L-NA. Under those conditions, the amplitude of the oscillations was higher (6.4±1.8 mV, n=4) (Figure 1B) and could even generate spikes (Figure 1c), with an averaged maximal amplitude of 18.8±5.7 (n=3). The upstroke of these action potentials attained maximal levels, ranging between −15 and −20 mV and never showed an overshoot. The frequency of spontaneous fluctuations in membrane potential during exposure to L-NA was identical as under control conditions (7.6±0.8/min).

Hyperpolarization to Serotonin

In the presence of indomethacin (10 μmol/L), L-NA (30 μmol/L), and ketanserin (1 μmol/L), serotonin induced endothelium-dependent hyperpolarization in coronary strips from control animals. The response to serotonin was concentration-dependent (Figure 2) with maximal changes in membrane potential of −3.0±1.1 mV (n=6), −7.8±1.6 mV (n=7), and −13.2±1.9 mV (n=6), at 0.1, 1, and 10 μmol/L, respectively. These hyperpolarizations were transient. Thus, at 10 μmol/L serotonin, the hyperpolarization was maximal after 25.1±1.6 seconds. The membrane potential returned to control level within 1 minute (58.6±4.1 seconds, n=7).

Changes in Resting Membrane Potential During the Incubation Period Before the Application of Serotonin or Bradykinin

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>Control Animals</th>
<th>Native</th>
<th>Regenerated</th>
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<tr>
<td></td>
<td>Control Animals</td>
<td>Native</td>
<td>Regenerated</td>
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<td></td>
<td>n=7 (6)</td>
<td>n=8</td>
<td>n=16**</td>
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<tr>
<td>L-NA + Indo + Ket</td>
<td>Pre</td>
<td>−54.1±3.15</td>
<td>−56.8±1.82</td>
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<tr>
<td></td>
<td>Post</td>
<td>−50.4±2.05</td>
<td>−51.3±1.70</td>
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<tr>
<td>Indo + Ket</td>
<td>Pre</td>
<td>.</td>
<td>−56.0±1.26</td>
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<td></td>
<td>Post</td>
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<td>−49.1±1.33</td>
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<td>Ket</td>
<td>Pre</td>
<td>.</td>
<td>−58.8±1.39</td>
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<td>−51.4±1.29</td>
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<tr>
<td>L-NA + Indo</td>
<td>Pre</td>
<td>−58.2±1.49</td>
<td>−57.1±1.15</td>
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<tr>
<td></td>
<td>Post</td>
<td>−49.4±0.75</td>
<td>−49.9±0.69</td>
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Recordings were performed before (pre) and after (post) the incubation with 10 μmol/L ketanserin alone (Ket), 10 μmol/L indomethacin and 10 μmol/L ketanserin (Indo+Ket), 30 μmol/L L-NA and 10 μmol/L indomethacin (L-NA+Indo), or 30 μmol/L L-NA, 10 μmol/L indomethacin, and 10 μmol/L ketanserin (L-NA+Indo+Ket). Data are mean±SEM for n experiments. When unspecified, n represents the number of animals per group. No statistical difference was observed between control coronary arteries from control and operated animals. Significant difference between native and regenerated endothelium. *P<.05; **P<.01.
Four weeks after balloon endothelial denudation, the hyperpolarization induced by serotonin at 10 $\mu$mol/L in the presence of indomethacin (10 $\mu$mol/L), L-NA (30 $\mu$mol/L), and ketanserin (1 $\mu$mol/L) was not significantly different from control in coronary arteries with native endothelium: $-210.6 \pm 1.6$ mV (n=8) versus $-210.8 \pm 1.2$ mV in coronary strips (n=7) from unoperated animals. In arteries with regenerated endothelium (Figure 3), the response to serotonin was significantly less than the corresponding control strips ($-5.6 \pm 1.7$ mV, Figure 5; $P<0.05$, n=8). Although the amplitude of the hyperpolarization was not influenced by the presence or absence of L-NA in control arteries, in arteries with regenerated endothelium, studied in the absence of L-NA, serotonin did not cause significant hyperpolarizations but rather evoked a small depolarization (Figure 3). Such depolarizations were not obtained in the presence of inhibitors of NOS, even in presence of spikes (Figure 3A) and also were never observed in coronary segments with native endothelium.

**Hyperpolarization to Bradykinin**

In control porcine coronary arteries, bradykinin evoked concentration-dependent hyperpolarizations (Figure 4) resistant to indomethacin (10 $\mu$mol/L) and L-NA (30 $\mu$mol/L). The threshold concentration to obtain endothelium-dependent hyperpolarization was 3 mmol/L. With higher concentrations, the amplitude of membrane potential changes increased and the time to peak of hyperpolarization decreased (not shown). The maximal value of hyperpolarization was obtained at 100 mmol/L. Because at the concentration of 30 mmol/L the amplitude of hyperpolarization was reproducible, albeit not maximal, this concentration was used for the evaluation of the endothelium-dependent hyperpolarization induced by the kinin in arteries with regenerated endothelium.

Four weeks after balloon endothelial denudation, the response to bradykinin (30 mmol/L) was not altered in coronary strips with native endothelium (Figure 5): $-20.9 \pm 0.7$ mV (n=16) versus $-21.5 \pm 0.8$ mV hyperpolarization in arteries from control animals (n=8). The temporal changes in membrane potential were not modified: the more hyperpolarized potential was reached after $\sim 1$ minute, and the hyperpolarization lasted longer than 3 minutes. In the corresponding coronary segments with regenerated endothelium, although the average change in membrane potential was not different...
(−20.1±1.6 mV, n=16), the values of membrane potential before administration of bradykinin and that reached in its presence were significantly lower (Figure 5B). When examining the individual experiments, a high variability in the response to bradykinin was noticed in preparations with regenerated endothelium, showing identical, reduced, or increased hyperpolarizations (Figure 5A). The analysis of individual pairs of results showed that although similar responses were recorded in control arteries, the changes in membrane potential for segments with regenerated endothelium were related to the value of membrane potential immediately before the administration of the kinin (Figure 6). In the more depolarized cells, bradykinin evoked a larger hyperpolarization (up to −40 mV) (Figure 5A, bottom panel, and Figure 6).

**Discussion**

There are 2 main findings in the present study: (1) Four weeks after balloon endothelial denudation, the smooth muscle cells from porcine coronary arteries with regenerated endothelium showed intrinsic alterations of the resting membrane potential. (2) The changes in the endothelium-dependent hyperpolarizations of these cells may contribute to the reduced response to serotonin and the unchanged relaxation to bradykinin described previously.13,17,18

**Resting Membrane Potential in Arteries With Regenerated Endothelium**

The present study demonstrates that the smooth muscle cells from coronary arteries with regenerated endothelium are depolarized in comparison to those from the corresponding control arteries. The depolarization of these cells imply alterations of ionic conductance(s) implicated in the control of the resting membrane potential. This could be achieved by an increase in a depolarizing current (inward calcium or nonspecific currents and/or outward chloride current), a decrease in an outward repolarizing or hyperpolarizing potassium current, or both. Such alterations...
could include changes in the number of active channels, variations in their open probability, or differences in their regulation. Obviously, alterations in ionic homeostasis could also change the driving force for ions and thus affect the ionic current. Whatever the cause of this relative depolarization, the change in resting membrane potential in these coronary arteries is relevant, because it could modify the myogenic tone of these vessels. This interpretation is confirmed by the observation of spontaneous electrical activities in some arteries with regenerated endothelium. Such membrane potential instability was not observed in the corresponding control arteries. The resting membrane potential of arterial smooth muscle cells is generally stable except in the microvasculature; in the cerebral microcirculation, spontaneous contractions have been associated with an unstable membrane potential of smooth muscle cells. The membrane potential of smooth muscle cells in rhythmically constricting arteries is also significantly less negative than that of quiescent ones. In the porcine coronary artery, cell membrane potential oscillations, spikes, and associated spontaneous contractions have been observed, under marked depolarization with tetrabutylammonium, a potassium channel blocker. The membrane potential oscillations obtained in the present study may be partly the result of the depolarized state of the smooth muscle cells. Indeed, action potentials can be generated only within a certain range of cell membrane potential (≈−40 to −20 mV). Furthermore, rhythmic spontaneous activity has been demonstrated in human coronary arteries and with higher frequency in vessels from older patients or those with cardiovascular diseases and atherosclerotic changes. Hence, the depolarization of smooth muscle cells, observed 4 weeks after balloon denudation, may be a key factor in the development of alterations in vasomotion of these coronary arteries. Previous studies have shown, in the same porcine model, a decreased response to serotonin while the relaxation to bradykinin remained normal, whereas it was blunted under depolarizing conditions imposed by both agonists.

**Endothelium-Dependent Hyperpolarization Induced by Serotonin and Bradykinin**

In the coronary arteries from control pigs, both serotonin and bradykinin induced concentration-dependent hyperpolarizations that are resistant to indomethacin and L-NA. The hyperpolarizations observed in the present study during exposure to bradykinin are in agreement with previous observations. Under the experimental conditions imposed, bradykinin given at 30 nmol/L as a single concentration induced an endothelium-dependent hyperpolarization of smooth muscle cells of ≈−20 mV. This level of hyperpolarization was slightly higher than that observed when concentrations of bradykinin were applied in a cumulative manner. The temporal aspects of the membrane hyperpolarization were also in agreement with previous reports. Therefore, the endothelium-dependent hyperpolarization induced by serotonin was transient, and it was observed at higher concentrations. A similar response to serotonin has been reported in the same preparation. In preliminary studies, it was noted that the response to serotonin is labile, in that many factors can influence the amplitude of the hyperpolarization: (1) rapidity of dissection of coronary arteries after heart excision; (2) stretch of the blood vessels during dissection; (3) a long period before the beginning of the experiments (the response was markedly reduced after 8 hours); and (4) previous exposure to serotonin even after a long washout (desensitization).

Although an EDRF has been demonstrated to be NO, the nature of EDHF is still unknown. It could be a cytochrome P450 metabolite, derived from arachidonic acid. When considering differences between hyperpolarizations induced by bradykinin and serotonin in the present study or those provoked by bradykinin and A23187 compared with that of thrombin, one cannot exclude the existence of several EDHFs. These hyperpolarizations, resistant to indomethacin and L-NA, differ by their amplitude and their kinetics. The fact that in the presence of regenerated endothelium, the transient response to serotonin was reduced while the sustained hyperpolarization induced by bradykinin could be increased would be also in favor of the existence of 2 different mechanisms. It is generally accepted that EDHF increases a potassium conductance leading to the hyperpolarization of the smooth muscle cells. Because all potassium channels implicated in the control of resting membrane potential of smooth muscle cells are voltage-dependent, the depolarization of smooth muscle cells in coronary arteries with regenerated endothelium would be expected to influence the response to EDHF. In the vascular strips with regenerated endothelium, the response to bradykinin was correlated with the value of membrane potential before the administration of the kinin. The maximal opening of potassium channels would lead the membrane to reach potential values close to the equilibrium potential for potassium. Therefore, in preparations with regenerated endothelium, the depolarization of smooth muscle cells induced an increase in the response to bradykinin, illustrating an augmentation of the EDHF pathway in the most altered cells. This result correlates well with previous experiments showing a maintained relaxation to bradykinin in coronary strips with regenerated endothelium whereas it was blunted under depolarizing conditions. The lack of impairment of the bradykinin-evoked relaxation, despite a reduction in the NO pathway, could be the result of a greater contribution of the EDHF pathway. Furthermore, although an altered relaxation to serotonin was described in the same experimental model, the present study demonstrates a reduction in the hyperpolarization induced by serotonin. The fact that relaxations to serotonin in porcine coronary arteries are almost completely inhibited by the presence of NOS inhibitors is in favor of a poor participation of the EDHF pathway to the response. This interpretation is supported by the weak hyperpolarizations obtained at high concentrations of serotonin in the present study.
balloon denudation, no compensatory effect of EDHF for the reduced response to this agonist is plausible. On the contrary, depolarizations were observed that were not blocked by the presence of a high concentration of ketanserin, excluding the implication of 5-HT$\text{R}$ receptor on the vascular smooth muscle. These depolarizations may have curtailed the endothelium-dependent hyperpolarizations. Shimokawa et al\textsuperscript{13} have previously shown an increase in the contraction to serotonin in the same model and have suggested that the regenerated endothelium releases endothelium-derived contracting factor in response to the monoamine.

The present findings are in favor of a minor implication of EDHF in the endothelium-dependent response of porcine coronary artery to serotonin, but the participation of EDHF in the relaxation response to bradykinin is more important. After balloon endothelial denudation, the endothelium-dependent hyperpolarization induced by serotonin is markedly reduced in the presence of regenerated endothelium, whereas that generated by bradykinin is maintained or even increased in coronary arteries showing an alteration of resting membrane potential. Because in coronary arteries with regenerated endothelium the EDRF-NO pathway is markedly reduced,\textsuperscript{17,18} the present results help to explain the better preservation of endothelium-dependent relaxations to bradykinin in this model.

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References
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