Impaired Endothelium-Dependent Responsiveness in Porcine Coronary Arteries with Chronic Regenerated Endothelium

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관상동맥 재생 내피세포의 내피의존성 반응

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패치의 관상동맥에서 혈관내피를 제거한 후 재생된 내피세포는 백일해 독소감수성 G단백질 (pertussis toxin-sensitive G protein)과 관련된 반응들을 선택적으로 방해하여, 혈관경련의 주 원인으로 작용하는 것으로 알려져 있다. 본 실험에서는 내피세포 제거 후 4주제에 여러가지 약물에 대한 내피세포 이완반응의 장애를 in vivo와 in vitro에서 관찰하여, 재생 상태의 내 피세포가 일으키는 기능장애 기전을 밝히고자 다음과 같이 실험이다. 8~10주된 성숙한 돼지 (20~25Kg, Yorkshire)를 대상으로 대조군(n=7)과, 관상동맥의 외전허행지 내피세포를 풍선 도자를 이용하여 혈관내피세포를 제거한 군(n=11) 등 두 집단으로 분류하여 혈관조영술의 분석, 기관장기 내(organ chamber) 실험 및 관상동맥 조직의 형태학적 연구를 수행하였다. 4주제에 관상동맥내로 serotonin을 주입하여 혈관의 반응을 관찰하였는데 재생된 내피세포 군에서는 통계적으로 유의한 혈관수축(33.4±4.2%)을 일으켰으나 대조군에서는 의의있는 수축반응은 보이지 않았다. 관상동맥내로 serotonin을 주입한 결과 내피가 제거된 집단에서는 11마리중 9마리가(89%) 심전도상 심각한 허혈성 변화를 일으켰으나 대조군에서는 변화가 없었다. Organ chamber 실험상, 재생된 내피에서 serotonin, 혈소판, norepinephrine 및 endothelin-1에 대한 향진된 내피의존성 수축반응을 보였다. 패치 관상동맥의 재생된 내피에서 endothelin-1에 대한 수축반응의 증가는 cyclooxygenase에 의한 내피세포에서 유래하는 수축인자의 방출과 관련된 것이라 여겨진다. 다큐다 재생된 내피의 이완반응은 혈소판, serotonin 및 UK 14304에 대해서는 이완기능이 손상되었으나 ADP와 SIN-1에 의해서는 영향받지 않았다. 이상의
Introduction

The turnover of endothelial cells is accelerated in response to continuous injury by factors such as hyperlipidemia\textsuperscript{1)}, hypertension\textsuperscript{2)}, and mechanical stress\textsuperscript{3)}, and often the atherosclerotic process is initiated during endothelial regeneration\textsuperscript{4)}. Atherosclerosis impairs endothelium-dependent relaxations mainly because the resulting reduced production (synthesis or release) of the endothelium-derived relaxing factor\textsuperscript{5-10)}. This endothelial dysfunction appears to occur at an early stage in atherosclerotic process and may contribute to the diminution of the protective role of the endothelium. However, the cellular mechanisms for the endothelial dysfunction in atherosclerosis remain to be elucidated. In porcine coronary arteries, the regenerated endothelium similar to those in humans can be induced by balloon endothelial denudation\textsuperscript{9-11)}. Pertussis toxin, an inhibitor of certain G protein, inhibits the relaxations to serotonin and UK14304, which is a selective α\textsubscript{2}-adrenergic receptor agonist, but not relaxations to ADP, bradykinin, or the calcium ionophore A23187\textsuperscript{12)}, indicating that G protein mediated signal transduction pathway may be involved in the production of endothelium derived relaxing factor to some but not to all vasodilators. Earlier studies have demonstrated that regenerated endothelium following balloon denudation in porcine coronary arteries have selectively impaired pertussis toxin-sensitive G protein coupled responses\textsuperscript{9,10)}. Consequently, endothelium-dependent relaxations to serotonin and aggregating platelets are severely impaired at the site of endothelial regeneration, predisposing to coronary vasospasm\textsuperscript{11)}, which may be an important pathophysiologic role in a wide spectrum of ischemic heart disease\textsuperscript{13-19)}. Moreover, the migratory activity of endothelial cells and smooth muscle cells may play an important role in the development of neointimal hyperplasia following arterial injury. The possible role of the thickened intima as a functional barrier remains to be examined. The purpose of the present study was to examine the impaired endothelium dependent relaxations to various agonists in vitro and in vivo, observed 4 weeks following endothelial denudation and to elucidate the cellular mechanisms underlying the endothelial dysfunction in the regenerated state.

Methods

1. Materials

Eighteen Yorkshire pigs, 8 – 10 weeks of age (male, 20 – 25Kg), were used. They were randomly assigned to one of 2 groups: (1) controls (n=7), (2) pigs undergoing balloon endothelial denudation of the left anterior descending coronary artery (n=11). All animals were fed regular chow and the daily food intake was limited to an amount equal to 3% of the body weight to prevent excessive weight gain. The animals were housed individually in temperature-controlled animal quarters after the balloon denudation. The organ chamber experiments were performed after 4 weeks of feeding in both groups.

2. Coronary Endothelial Denudation

The animals were anesthetized with Telazol (a mixture of tiletamine hydrochloride and arylaminocycloalkane, and zolazepam hydrochloride, 100mg/animal, intramuscularly) and atropine (0.4 mg, intramuscularly) followed by inhalation of halothane (2L/min). Using aseptic surgical tech-
nique, the left carotid artery was dissected free and a 7F guiding catheter (hockey stick or multi-purpose) was introduced into the left coronary ostium under the fluoroscopic guidance. Before denudation, heparin 100 μg/kg and lidocaine HCL 20mg were administered via the arterial sheath. During the procedure, the arterial blood pressure and electrocardiogram (ECG, lead II, avL) were monitored continuously. A 2.5 or 3mm sized balloon catheter (USCI, over-the-guide wire system) was advanced through the guiding catheter into the left anterior descending coronary artery. The balloon was then gently rubbed against proximal 3−4cm of the arterial endothelium. Successful denudation of the coronary endothelium was confirmed by ischemic ECG changes (0.1mV of ST segment depression or elevation) and/or constrictive luminal diameter changes with intracoronary serotonin (10ug/kg) injection\textsuperscript{11,20,21}.

3. Quantitative Coronary Angiography

Coronary angiography was performed in 2 groups. The changes in the coronary diameter after intracoronary injection of serotonin 10μg/kg (Sigma Chemical) was examined before, immediately after and 4 weeks after endothelial denudation in denuded group and control and 4 weeks later in controls. Left coronary angiograms were obtained before the denudation and the solution of serotonin was infused gradually into the left main coronary artery over 1 minute. Repeat left coronary angiograms were obtained 2 minutes after completion of the injection or when ischemic ECG changes (≥0.1 mV ST segment elevation or depression) were noted. To quantitate the epicardial coronary artery response to intracoronary serotonin injection, angiograms were analyzed in a blind manner for coronary dimensions along the proximal 1cm (just before the first diagonal branch) and distal 1cm (end point of denudation) of the denuded left anterior descending artery using a computer-based quantitative angiographic system\textsuperscript{11,21-24}. The changes in coronary diameter were expressed as percent changes from the control angiogram. Since intracoronary injection of serotonin usually caused diffuse responses in epicardial coronary arteries\textsuperscript{21}, the responses of previously denuded arteries were reported as mean percent diameter changes of different site of coronary artery.

4. Organ Chamber Experiments

At 4 weeks, the pigs were anesthetized with Telazol (100mg i.m) and sodium pentobarbital (12.5 mg/kg i.v). After autologous blood (250−300ml) was collected from the apex of the heart for platelet preparation, the hearts were removed. Both left coronary arteries (left anterior descending coronary artery, LAD and left circumflex coronary artery, LCX) were dissected and immersed in cold modified Krebs-Ringer bicarbonate solution consisting of (mM) NaCl 118.3, KCl 4.7, CaCl\textsubscript{2} 2.5, MgSO\textsubscript{4} 2.5, MgSO\textsubscript{4} 1.2, KH\textsubscript{2}PO\textsubscript{4} 1.2, NaHCO\textsubscript{3} 25, glucose 11.1, and calcium disodium edetate 0.026 at pH 7.4 (control solution) and cleaned of connective tissue. They were then cut into rings (3−4mm length). The proximal 3−4cm portions of the LAD were used for the organ chamber experiments and same anatomic portions of the LCX were used as controls. In some rings, the endothelium was removed mechanically by inserting the tips of a watchmaker’s forceps into the lumen and gently rolling the preparation back and forth over a paper tissue wetted with cold control solution. The rings were mounted horizontally in organ chambers filled with 25ml of control solution (37°C, pH 7.4), gassed with 95% O\textsubscript{2}, 5% CO\textsubscript{2}, and stretched to the optimal point of their length active tension relation as determined by the contractile response to 60mM KCl at progressive levels of stretch. The tissues were allowed to equilibrate for 60 minutes before beginning the experiments\textsuperscript{11}. 

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5. Protocol

After equilibration, the following responses were determined (full concentration-response curves in cumulative fashion): (a) in quiescent rings with and without endothelium: contractions to 5-hydroxytryptamine, aggregating platelets, norepinephrine and endothelin-1; (b) in rings with and without endothelium, contracted with prostaglandin-F2α (PGF2α, 2 × 10^{-6} M): relaxations to 5-HT and aggregating platelets (in the presence of 10^{-6} M ketanserin for 40 minutes incubation before inducing a contraction with PGF2α to prevent direct activation of the vascular smooth muscle), UK14304, adenosine diphosphate (ADP) and SIN-1. The two rings of the LAD and the two control rings of the LCX with and without endothelium (4 rings) were assigned to the one experimental set. The studies were examined in the following orders:

Set 1 (contraction): serotonin, platelets.
Set 2 (contraction): norepinephrine, endothelin-1.
Set 3 (relaxation): serotonin, platelets.
Set 4 (relaxation): UK14304, ADP, SIN-1.

When determining contractions in quiescent rings (Set 1 and Set 2), each response was followed by 60 mM KCl to obtain the maximal contractions of vascular smooth muscle. When determining relaxation properties (Set 3 and Set 4), all rings were treated with indomethacin (10^{-5} M for 40 minutes) to prevent the formation of endogenous prostaglandin.

6. Platelet Preparation

Blood (250–300 ml) for autologous platelet preparation was collected from the apex of the heart and mixed with a citrate anticoagulant. The citrated blood was centrifuged for 40 minutes at 1,000 rpm and the platelet-rich plasma was pipetted off. An equal volume of citrate anticoagulant solution was added to the platelet-rich plasma and the mixture was centrifuged for 15 minutes at 1,600 rpm. The supernatant was discarded and the remaining platelet pellet was resuspended in a small volume (< 10 ml) of the citrate anticoagulant mixture. A platelet count of this suspension was obtained using the Coulter counter (Coulter Electronics). The volume of the suspension was adjusted so that when added to the organ chamber, the final platelet concentration in the bath was 75,000/μl.

7. Drugs

The following drugs were used: ADP, bradykinin, endothelin-1, 5-HT creatinine sulfate (serotonin), indomethacin (all from Sigma Chemical Company, St. Louis, Mo.), ketanserin tartrate (Janssen Pharmaceutica, Beerse, Belgium), norepinephrine, potassium chloride, dl-propranolol hydrochloride, prostaglandin F2α (Sigma Chemical Company, St. Louis, Mo.), UK14304 (Research Biomedicals Inc., Natick, MA), SIN-1 (Laboratories Hoechst, Paris). All drugs were prepared with distilled water on the day of the study, except indomethacin, ketanserin tartrate and propranolol. UK14304 was dissolved in dimethylsulfoxide (1%). The concentrations of the drugs are expressed as final molar (M) concentration in the bath solution.

8. Morphometric Study

The LAD rings with preserved endothelium in all four study sets used in the organ chamber experiments were prepared for morphological analysis. Each ring was fixed in 10% formaldehyde in phosphate buffer (fixation solution, Sigma), and all tissue was processed using standard paraffin histological technique. Sections were cut at 3 to 4 microns. Three different paraffin sections were collected from each paraffin block to avoid possible anatomic differences in the thickness of the intima and media, a 50 micron interval separated each of the five sections. Sections were stained with modified Masson’s Trichrome/Verhoeff elastica stain. In two cases, additional sections were taken for routine Hematoxylin and Eosin.
staining. Morphometric analysis was performed with a computer-assisted image analyzer (Optimas: Bioscan Inc.) to evaluate the cross-sectional area of the intima and media. The index of intimal thickening was defined as the ratio of intimal to medial cross-sectional area. The cross-sectional area of intima is defined as that area in a normal vessel bounded by the internal elastic lamina less the luminal area. The area of media is defined as that area in a normal vessel bounded by the external elastic lamina less the sum of the luminal area and intimal area. The areas are reported in \( \text{mm}^2 \).

9. Statistical Analysis

Results are expressed as mean± SEM. Unless otherwise specified, refers to the number of animals. In rings contracted with prostaglandin \( \text{F}_{2\alpha} \), responses are expressed as percent changes from the contracted levels. In quiescent rings, responses are expressed as a percent of the maximal contraction to KCl 60 mM. For the relaxations, the effective concentration of agonists causing 25 or 50% inhibition (IC\(_{25}\) or IC\(_{50}\)) of the contractions to PGF\(_{2\alpha}\) was calculated for each concentration-response curve, and the means of these values are presented as the negative logarithm of the molar concentration. For the contractions evoked by KCL, the effective concentration producing 50 or 70% of maximal response (ED\(_{50}\) to ED\(_{70}\)) was calculated. Statistical evaluation of the data was performed with the Student’s t test or either paired or unpaired observations. When more than two mean values were compared, an analysis variance (ANOVA) was used. If a significant value was found, the Scheffe’s test for multiple comparisons was used to identify difference among groups. Values were considered to be statistically different when \( p < 0.05 \).

Results

The averaged body weight of the 2 groups increased significantly from 22± 0.6 to 26± 0.8 kg (\( n = 18 \)) after 4 weeks. There were no significant differences between the 2 groups.

1. Hemodynamic and Electrocardiographic changes to intracoronary serotonin injection

Before balloon endothelial denudation, the blood pressure and heart rate did not change with intracoronary injection of serotonin (122± 6/85± 10 mm Hg and 116± 9 beats/min control, and 123± 8/80± 6 mm Hg and 112± 7 beats/min after serotonin injection, respectively) and no ECG changes were noted (\( n = 12 \)) in denuded group. Immediately after endothelial denudation, intracoronary injection of serotonin caused various ischemic ECG changes (\( n = 12 \), ST segment depression in 2 (17%), deep T wave inversion in 2, and non-specific T waves changes, including peaked T or flat T wave in 8). Intracoronary serotonin injection did not affect blood pressure and heart rate (122± 19/92± 20 mm Hg and 122± 12 beats/min control, and 126± 15/86± 13 mm Hg and 116± 6 beats/min after serotonin injection, \( n = 11 \)). One of 12 in denuded pigs died due to intractable ventricular fibrillation immediately after denudation. After 4 weeks of maintenance, significant resting ECG abnormalities (ST depression in 2, T inversion in 2 and QS pattern in 1) were noted in 5 (45%) of 11, but none in controls (\( n = 7 \)). Intracoronary serotonin injection provoked significant ischemic ECG changes in 9 of 11 (82%) (ST segment elevation in 1 and depression in 7 (73%), and T inversion in 1) in denuded group, but none in controls. The blood pressure did not change 2 minutes after intracoronary serotonin injection in both groups (116± 7/78± 6 mm Hg control, and 118± 7/85± 6 mm Hg after serotonin injection). The heart rate significantly increased (110± 10 beats/min before and 13± 7 beats/min after serotonin) in denuded group only.

2. Quantitative Coronary Angiography

Before balloon endothelial denudation, the
Table 1. Cross-sectional area of the intima, the media and the ratio of intima/media in left anterior descending coronary arteries

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Denuded Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intima (mm²)</td>
<td>0.12±0.03</td>
<td>0.33±0.06*</td>
</tr>
<tr>
<td>Media (mm²)</td>
<td>0.75±0.08</td>
<td>1.04±0.08*</td>
</tr>
<tr>
<td>Ratio of intima/media</td>
<td>0.14±0.03</td>
<td>0.32±0.05*</td>
</tr>
</tbody>
</table>

Data were obtained from 24 rings with endothelium in each group and expressed as mean±SEM. *p<0.05 vs controls.

0.05 vs before and immediately after denudation and minimal vasoconstriction (2.8±.4.8%, p = not significant vs 4 weeks earlier) (Fig. 1) in controls. The degree of constriction after intracoronary serotonin injection was slightly more prominent in the mid to distal segments of the left anterior descending coronary artery (21±2.6% in proximal, 37.5±4.2% in mid-portion and 41±3.2% in distal). However, there were no statistical differences.

3. Morphometric Study

No macroscopically visible region of myocardial infarction was present in any of the 18 hearts studied. At 4 weeks, no intimal and medial thickenings were noted in controls, while in previously mean diameters of left anterior descending coronary artery were comparable in 2 groups (2.8±0.1 and 2.7±0.2mm in proximal, 2.4±0.1 and 2.3±0.2 mm in mid-portion and 2.0±0.2 and 1.9±0.2mm in distal part of coronary artery in controls and denuded group respectively). Intracoronary injection of serotonin (10ug/Kg) caused comparable vasodilation in the left anterior descending artery in controls (3.0±4.8%) and denuded group (7.6±5.2%). The diameter of the denuded arteries significantly decreased (7.4±3.6%) immediately after endothelial denudation. Subsequent intracoronary injection of serotonin caused mild vasoconstriction (5.9±2.7%, p<0.05 vs before denudation). After 4 weeks of maintenance, the mean diameters of the left anterior descending coronary arteries did not change compared to those before endothelial denudation in both groups. Intracoronary injection of serotonin caused significant vasoconstriction in denuded group (33.4±4.2%, p<

Fig. 1. Responses of left anterior descending coronary artery to intracoronary injection of serotonin (10 ug/Kg) before, immediately after and 4 weeks after endothelial balloon denudation in both groups. The responses are expressed as percent change in coronary artery diameter from that in control condition.

* p<0.05 compared with change before denudation in the denuded group.

**p<0.05 compared with changes before and immediately after denudation.

Fig. 2. Cross sectional microscopic findings of the left anterior descending coronary artery of the previously balloon denuded pig [Hematoxylin and Eosin stain(A), and modified Masson’s Trichrome/Verhoeff elastica stain(B)]. Four weeks after deendothelialization, crescentic and eccentric intimal thickening occurred.
Table 2. Optimal basal tension and developed tension evoked by prostaglandin F2α 2 x 10^{-6} M and KCL 60mM

<table>
<thead>
<tr>
<th>Tension (g)</th>
<th>Controls</th>
<th>Denuded Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimal basal tension</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAD with</td>
<td>7.4 ± 0.4 (28)</td>
<td>7.3 ± 0.6 (24)</td>
</tr>
<tr>
<td>LAD without</td>
<td>6.9 ± 0.5 (28)</td>
<td>6.5 ± 0.4 (24)</td>
</tr>
<tr>
<td>LCX with</td>
<td>6.4 ± 0.4 (28)</td>
<td>6.6 ± 0.4 (24)</td>
</tr>
<tr>
<td>LCX without</td>
<td>6.0 ± 0.5 (28)</td>
<td>6.0 ± 0.5 (24)</td>
</tr>
<tr>
<td>Development tension by prostaglandin F2α</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAD with</td>
<td>5.7 ± 0.2 (14)</td>
<td>5.5 ± 0.3 (12)</td>
</tr>
<tr>
<td>LAD without</td>
<td>5.6 ± 0.6 (18)</td>
<td>5.0 ± 0.4 (12)</td>
</tr>
<tr>
<td>LCX with</td>
<td>5.4 ± 0.3 (18)</td>
<td>5.4 ± 0.3 (12)</td>
</tr>
<tr>
<td>LCX without</td>
<td>5.8 ± 0.7 (18)</td>
<td>5.2 ± 0.5 (12)</td>
</tr>
<tr>
<td>Development tension by KCL 60mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAD with</td>
<td>12.1 ± 0.3 (28)</td>
<td>10.3 ± 0.2 (24)</td>
</tr>
<tr>
<td>LAD without</td>
<td>10.3 ± 0.6 (28)</td>
<td>9.3 ± 0.4 (24)</td>
</tr>
<tr>
<td>LCX with</td>
<td>11.2 ± 0.3 (28)</td>
<td>10.6 ± 0.6 (24)</td>
</tr>
<tr>
<td>LCX without</td>
<td>9.6 ± 0.4 (28)</td>
<td>9.5 ± 0.3 (24)</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. Numbers in parenthesis are the numbers of rings tested in each experiment. LAD: left anterior descending coronary artery, LCX: left circumflex coronary artery, with: with endothelium, without: without endothelium.

denuded arteries, the cross-sectional area of the intima and media significantly increased (Table 1, Fig. 2). The intimal thickenings were focal, crescent-shaped, and eccentric in almost all sections.

4. Organ Chamber Experiments

There were no statistically significant differences in the optimal basal tension, contractions evoked by prostaglandin F2α (2 x 10^{-6}), and maximal contractions to KCL 60mM between the left anterior descending and left circumflex coronary arteries in both groups (Table 2).

1) Contractions

Aggregating platelets. In quiescent rings of left anterior descending and left circumflex coronary arteries, aggregating platelets (6,250–75,000/ml) comparable concentration-dependent contractions, which were significantly smaller in preparations with endothelium than in those without endothelium in controls. In the denuded group, platelets caused significantly greater contractions in the left anterior descending coronary artery compared with the control left circumflex coronary artery: the ED_{50} was significantly reduced. Rings

Table 3. Contractions of porcine coronary arteries

<table>
<thead>
<tr>
<th>Platelets (×10^3/ml)</th>
<th>Controls</th>
<th>Denuded</th>
</tr>
</thead>
<tbody>
<tr>
<td>with (ED_{50})</td>
<td>36.6 ± 7.0</td>
<td>17.1 ± 2.2*</td>
</tr>
<tr>
<td>without</td>
<td>10.8 ± 1.9</td>
<td>13.4 ± 1.8</td>
</tr>
<tr>
<td>Serotonin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>with (ED_{50})</td>
<td>5.63 ± 0.18</td>
<td>6.20 ± 0.11*</td>
</tr>
<tr>
<td>without</td>
<td>6.97 ± 0.07</td>
<td>6.52 ± 0.17</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>with (ED_{10})</td>
<td>5.52 ± 0.21</td>
<td>6.63 ± 0.15*</td>
</tr>
<tr>
<td>without</td>
<td>5.45 ± 0.14</td>
<td>5.71 ± 0.17</td>
</tr>
<tr>
<td>Endothelin-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>with (ED_{90})</td>
<td>7.71 ± 0.05</td>
<td>8.02 ± 0.07</td>
</tr>
<tr>
<td>without</td>
<td>8.02 ± 0.06</td>
<td>7.82 ± 0.14</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. Number of experiments are 7 in controls and 6 in the denuded group. ED_{50}, effective concentration producing 50% of the maximal response to KCL 60mM. Maximal contraction, maximal contraction to KCL 60mM. with, with endothelium: without, without endothelium. *p<0.05 compared with controls.
without endothelium showed comparable contractions (Table 3, Fig. 3).

**Serotonin.** In quiescent rings of both left anterior descending and left circumflex coronary artery, serotonin \(10^{-9} - 10^{-5}\text{M}\) caused comparable concentration-dependent contractions, which were significantly smaller in preparations with endothelium than in those without endothelium in controls. In the denuded group, serotonin caused significantly larger contractions in rings with previously denuded endothelium (LAD) than in those of control (LCX) : the ED\(_{50}\) was significantly reduced, while rings without endothelium showed comparable contractions (Table 3, Fig. 4).

**Norepinephrine.** In quiescent rings of both left anterior descending and left circumflex coronary artery, norepinephrine \(10^{-9} - 10^{-4}\text{M}\) caused comparable concentration-dependent contractions, which were significantly smaller in preparations with endothelium than in those without endothelium in controls. In the denuded group, norepinephrine caused significantly larger contractions in rings with previously denuded endothelium (LAD) compared with controls (LCX) : the ED\(_{10}\) was significantly reduced. Rings without endothelium showed comparable contractions (Table 3, Fig. 5).

**Endothelin-1.** In quiescent rings of both left anterior descending and left circumflex coronary artery, endothelin-1 \(10^{-10} - 10^{-7}\text{M}\) caused comparable concentration-dependent contractions, which were significantly smaller in rings with than in those without endothelium in controls. In the denuded group, endothelin-1 caused significantly
Fig. 4. Cumulative concentration-response curve to serotonin in quiescent rings in both groups. The contractions are expressed as percent of the maximal contractions to KCL 60mM. Four rings from control left circumflex and previously denuded left anterior descending coronary arteries with and without endothelium were studied in parallel.

larger contractions in rings with previously denuded left anterior descending coronary arteries compared with rings of control left circumflex arteries: the ED₅₀ was reduced. Rings without endothelium showed comparable contraction (Table 3, Fig. 6).

2) Relaxations

Aggregating platelets. In rings with endothelium of both left anterior descending and left circumflex coronary arteries contracted with prostaglandin F₂α, aggregating platelets (6,250–75,000/ul) caused comparable endothelium-dependent relaxations in controls. In the denuded group, the endothelium-dependent relaxations in rings with regenerated endothelium were significantly reduced compared with the control rings: the IC₂₅ was significantly increased (Table 4, Fig. 7A).

Serotonin. In rings of both left anterior descending and left circumflex coronary arteries contracted with prostaglandin F₂α, serotonin (10⁻⁹–10⁻⁵ M) caused comparable, endothelium-dependent, concentration-dependent relaxations in controls. In the denuded group, the amplitude of these relaxations were significantly reduced: the IC₂₅ for serotonin was significantly augmented in rings of previously denuded left anterior descending coronary arteries (Table 4, Fig. 7B).

UK14304. In rings of both left anterior descending and left circumflex artery contracted with prostaglandin F₂α, UK14304 (10⁻⁹–10⁻⁵ M) caused comparable, endothelium-dependent, concentration-dependent relaxations in controls. The amplitude of these relaxations were significantly reduced in rings of previously denuded left anterior descending coronary arteries (group 2): the IC₂₅ was significantly augmented (Table 4, Fig. 7C).
Adenosine diphosphate. Adenosine diphosphate (ADP, $10^{-9} - 10^{-4}$M) caused comparable concentration-dependent relaxations in rings with endothelium of left anterior descending and left circumflex coronary arteries contracted with prostaglandin $F_{2a}$, in both groups. There were no statistical differences in the degree of relaxations (Fig. 7D).

SIN-1. In rings of both left anterior descending and left circumflex coronary arteries contracted with prostaglandin $F_{2a}$, SIN-1 ($10^{-9} - 10^{-5}$M) caused comparable concentration-dependent relaxations, which was significantly augmented in rings without endothelium in both groups studied. There were no statistical differences in the degree of relaxations (Fig. 8).

Discussion

1. In Vivo Demonstration

Immediately after endothelial denudation, intracoronary injection of serotonin caused objective ischemic ECG changes in only 17% of studied cases. However, at 4 weeks after denudation, 45% of endothelial denuded cases had resting ECG abnormalities and 73% of them showed typical ischemic ECG changes with intracoronary serotonin injection. Thus, ECG changes after intracoronary serotonin injection may be an indirect index to evaluate the regenerated endothelium 4 weeks after balloon denudation, but not immedia-
Fig. 6. Cumulative concentration-response curve to endothelin-1 in quiescent rings in both groups. The contractions are expressed as percent of the maximal contractions to KCL 60mM. Four rings from control left circumflex and previously denuded left anterior descending coronary arteries with and without endothelium were studied in parallel.

Table 4. Relaxations of porcine coronary arteries

<table>
<thead>
<tr>
<th></th>
<th>IC_{25-50} (−log M) Controls</th>
<th>IC_{25-50} (−log M) Denuded</th>
<th>Maximal contraction (%) Controls</th>
<th>Maximal contraction (%) Denuded</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets (×10^5 / ul)</td>
<td>3.5 ± 0.7</td>
<td>21.8 ± 7.7 *</td>
<td>97 ± 4</td>
<td>58 ± 9 *</td>
</tr>
<tr>
<td>with (IC_{25})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serotonin</td>
<td>7.90 ± 0.11</td>
<td>7.33 ± 0.14 *</td>
<td>95 ± 4</td>
<td>48 ± 3 *</td>
</tr>
<tr>
<td>with (IC_{25})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UK14304</td>
<td>7.99 ± 0.09</td>
<td>6.79 ± 0.21 *</td>
<td>93 ± 4</td>
<td>44 ± 4 *</td>
</tr>
<tr>
<td>with (IC_{25})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td>6.29 ± 0.15</td>
<td>6.46 ± 0.24</td>
<td>106 ± 2</td>
<td>107 ± 5</td>
</tr>
<tr>
<td>with (IC_{50})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SIN-1</td>
<td>7.71 ± 0.14</td>
<td>7.44 ± 0.13</td>
<td>108 ± 3</td>
<td>113 ± 3</td>
</tr>
<tr>
<td>with (IC_{50})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.98 ± 0.14</td>
<td>7.87 ± 0.10</td>
<td>116 ± 2</td>
<td>124 ± 3</td>
</tr>
<tr>
<td>without</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as mean± SEM. Number of experiments are 7 in controls and 6 in the denuded group. IC_{50}, effective concentration causing 50% inhibition of the contractions to prostaglandin F_{2α}(2×10^{-6}M); Maximal relaxation(%), maximal relaxation in percent on the contraction induced by prostaglandin F_{2α}(2×10^{-6}M). with, with endothelium; without, without endothelium. *p<0.05 compared with controls.
Fig. 7. Cumulative concentration-response curves to platelets(A), serotonin(B), UK14304(C), and adenosine diphosphate(D) in rings with endothelium from the left anterior descending and left circumflex coronary artery, during contraction to prostaglandin F2α(2×10^(-6)M). The relaxations are expressed as percent decreases in tension from the contraction evoked by prostaglandin F2α. Data shown as mean±SEM. All rings were treated with indomethacin(10^(-5)M).

tely after denudation.

Serotonin possesses both direct activating and endothelium-dependent inhibitory effects on vascular smooth muscle. In porcine coronary arteries, the direct action is mediated by 5-HT2 receptors on the vascular smooth muscle, and the endothelium-dependent response is mediated by 5-HT1 receptors on the endothelium.\textsuperscript{12,25-27} Therefore, intracoronary injection of serotonin in native porcine coronary arteries caused mild vasodilation. Immediately after endothelial denudation, the diameter of coronary arteries decreased significa-
ntly. Moreover, intracoronary serotonin injection caused moderate diffuse vasoconstriction associated with ischemic ECG changes in arteries with chronic regenerated endothelium. This enhanced responsiveness can be explained by the loss of the inhibitory effect of the endothelium and the resultant expression of direct activation of the smooth muscle by serotonin. In the chronic regenerated endothelium, the concomitant production of endothelium-derived contracting factor in response to the monoamine may be a contributing factor.

2. Chronic Regenerated Endothelium

Chronic regenerated endothelium following balloon endothelial denudation in porcine coronary arteries selectively lose the pertussis toxin-sensitive G\(_{i}\)-protein coupled responses to 5-HT\(_1\) serotoninergic and \(\alpha_2\)-adrenergic activation. In the present study, the endothelium-dependent relaxations to platelets, serotonin and UK14304 were impaired in the endothelium denuded group, but not to ADP and SIN-1. These findings are consistent with earlier observations\(^4,10,19\). In the case of aggregating platelets, the endothelium-dependent responses are due to several platelets released products and their interactions\(^28\). Thus, platelet induced relaxations are mediated by both serotonin and adenine nucleotide. However, the impaired endothelium-dependent relaxations to platelets may depend solely on the serotonergic component as the purinergic response was well maintained in the chronic regenerated endothelium. In the regenerated endothelium of the porcine coronary artery, a depressed release of endothelium derived relaxing factor(s) by serotonin may coexist with the augmented liberation of endothelium-derived contracting factor(s)\(^11,24,29\). In the present study, the augmented endothelium-dependent contractions were noted not only in the responses to serotonin, but also to platelets, norepinephrine and endothelin-1. The augmented, endothelium-dependent contractions to aggregating platelets may also be related to serotonin activa-
tion. In the case of norepinephrine, the endothe-
lium-dependent relaxation to norepinephrine is
mediated by $\alpha$-adrenergic receptors, which is kno-
wed to be impaired in regenerated endothelium.
Thus, the augmented contractions to norepineph-
rine in rings with regenerated endothelium could
be related to the depressed release of endothe-
lium-derived relaxing factor(s). However, it re-
mains to be determined whether regenerated en-
dotheial cells release a certain endothelium-deri-
ved contracting factor(s) in response to norepine-
phrine. High concentrations of endothelin-1 cau-
sed the release of cyclooxygenase dependent en-
dotheilum-derived contracting factor(s) from the
spontaneously hypertensive rat aorta30, and in
the regenerated endothelium of the porcine coro-
nary artery (author's unpublished observation).
These findings suggest that a loss of the inhibitory
function of endothelium and the concomitant re-
lease of endothelium-derived contracting factor
(s) may contribute to the augmented endothe-
lum-dependent contraction to endothelin-1 in the
regenerated endothelium of the porcine coronary
artery. Therefore, it is reasonable to assume that
in the chronic regenerated endothelium, a depres-
sed release of endothelium-derived relaxing factor
(s) (pertussis toxin-sensitive G protein coupled
responses) may coexist with the augmented libe-
ratio for endothelium-derived contracting factor
(s) to serotonin and endothelin-1. This could pre-
dispose the blood vessel to vasospasm or a further
progression of the atherosclerotic process.

The model of chronic regenerated endothelium
in the porcine coronary artery is based on the
"reaction-to-injury hypothesis", which involves
platelet adherence and degranulation, smooth
muscle cell proliferation, and ultimately, the de-
velopment of intimal hyperplasia. This hypothesis
emphasizes the relationship between activation
of aggregating platelets and subsequent cell
growth and vessel repair31. Numerous mitogens as
well as angiotensin II may play a role in the mig-
ration and proliferation of smooth muscle cells31-
34. 5-hydroxytryptamine, which is released from
aggregating platelets, appear to stimulate the mi-
gration and proliferation of smooth muscle cells,
and suppress those of the endothelial cells35-38.
In the present study, the cross-sectional area of
the intima and the media, and the ratio of in-
tima/media increased significantly in the regen-
rated endothelium. The possible role of the thic-
kened intima as a functional barrier remains to
be examined.

3. Pathophysiological Implications

Under the pathologic condition, such as hyper-
lipidemia1 and hypertension2, endothelial turn
over rate is markedly accelerated in response to
continuous endothelial injury by these factors.
Under the these condition, regenerated endothe-
ial cell seem to play an important role in media-
ting the interactions between blood component
and vascular smooth muscle. Indeed, impaired
endothelium-dependent relaxations have been re-
ported in hypercholesterolemia39-41, hypertension
42-45, and atherosclerosis39,46-48. In the present
study, in the chronic regenerated endothelium,
the endothelium-dependent and pertussis toxin-
sensitive G protein coupled relaxations impaired
selectively, which are consistent with earlier ob-
servations9,10,11. Moreover, the augmented endothe-
lum-dependent contractions were noted not only
in the response to serotonin, but also to platelets,
norepinephrine and endothelin-1. Hence, the pre-
sent findings suggest that a loss of the inhibitory
function of endothelium and the concomitant re-
lease of endothelium-derived contracting factor
(s) may contribute to the pathogenesis of vasos-
spasm associated with endothelial damage, and
of myocardial ischemia not only in patients with
variant angina but also in those with stable and
unstable forms of angina. In the view point of
"reaction-to-injury hypothesis", this endothelial
dysfunction may also help to explain the pathoge-
nosis of late occurrence of restenosis after successful percutaneous transluminal coronary angioplasty.

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