Mild Myocardial Stunning Affects Platelet Aggregation and Certain Hemostatic Factors in Swine

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Summary: Myocardial stunning is characterized by transient contractile dysfunction occurring subsequent to an episode of ischemia followed by reperfusion. Platelet activation and hemostatic abnormalities have been described in patients with unstable angina and acute myocardial infarction, however, their role in the pathogenesis of myocardial stunning is unknown. The purpose of this study was to determine if platelet aggregation and certain hemostatic factors change during myocardial stunning following brief coronary arterial occlusion. Nine Yorkshire swine underwent left anterior descending coronary artery occlusion for 8 minutes followed by 90 minutes of reperfusion. Blood samples were obtained at baseline, at 4 and 8 minutes of occlusion, and at 60 and 90 minutes of reperfusion. Platelet aggregability and concentrations of antithrombin III, protein C, protein S, fibronectin, endothelin 1, and the stable metabolites of thromboxane (TxB2) and prostacyclin (6-keto-PGF1α) were measured in systemic circulation. The occlusion phase was associated with a decline of endothelin 1 (−13.6%), and TxB2 (−19.6%), and elevation of antithrombin III (+40.2%) and protein C (+22.9%). Mild myocardial stunning was associated with a significant increase in platelet aggregation (+33.7%), endothelin-1 (+24.7%), 6-keto-PGF1α (+41.5%), TxB2 (+11.9%), and protein C (+42.3%) during the reperfusion phase. There were no changes in plasma fibronectin and total protein S. Thus, mild myocardial stunning following brief coronary artery occlusion is associated with substantial dynamic changes in platelet aggregability and certain hemostatic factors. These results may be relevant to understanding the mechanisms determining myocardial stunning and coronary arterial patency following reperfusion. Key Words: Myocardial stunning—Platelets—Hemostasis—Animal model.

Myocardial stunning (MS) is a transient state of contractile dysfunction occurring subsequent to an episode of ischemia followed by reperfusion (1,2). Myocardial stunning is generally considered to be a form of reperfusion injury (3). Episodes of ischemia followed by reperfusion are common in patients with coronary artery disease (4). Over the past decade, investigation of this phenomenon has yielded significant information about the potential cellular mechanisms involved. Clinically, this phenomenon may contribute to left ventricular pump failure (5). However, despite considerable efforts, a comprehensive explanation for MS is lacking. One of the theories proposes that MS results from a burst of free radicals formed within the first minute of reperfusion (6). In addition, cytosolic calcium overload, which may result from oxygen driven free radicals, has been observed during MS (1–4). Another component of reperfusion injury under active investigation is endothelial damage with alterations in hemostasis and platelets (7).

The present study was designed to investigate possible changes in platelet function and the hemostatic profile during MS. It was hypothesized that because endothelial function is altered during MS there may be resultant changes in hemostatic factors modulated by the endothelium. Platelet activation and hemostatic abnormalities have been described in unstable angina and acute myocardial infarction (AMI), however, their occurrence following very brief ischemia is unknown.

A swine model was chosen for several reasons. First, the swine anatomy is similar to the human coronary circulation with a relative absence of collateral flow (8). Second, swine hemostatic factors, especially platelets, antithrombin III (AT III), protein C, and endothelin-1 (ET-1) are identical or very similar to those of humans and are therefore relevant to thrombosis research (9). Third, the Yorkshire swine’s heart is large enough to produce a model of regional ischemia in which an uninvolved region can serve as an internal control. Finally, swine, like humans, lack significant myocardial xanthine oxidase activity and therefore express identical platelet aggregation patterns (10). The concentration of agonist...
used was based on the known optimal platelet reactivity in this particular breed of swine and absence of reversible aggregation and/or secondary disaggregation responses (11–13).

**MATERIALS AND METHODS**

This study was approved by the Institutional Animal Care and Use Committee of the University of Maryland. All procedures conformed to the guidelines established by the U.S. Department of Health and Human Services, published by the U.S. National Institute of Health (NIH publication No. 85-23, revised 1985).

**Animals**

Thirteen purebred Yorkshire female swine (36–40 kg weight) 10–12 weeks of age were housed at our institution for a minimum of 1 week prior to use. A detailed protocol for the MS experiments can be found elsewhere (14,15). Briefly, the heart was exposed and the left anterior descending coronary artery was occluded for 8 minutes followed by 90 minutes reperfusion. Brief ischemia-reperfusion resulted in significant changes of myocardial contractility that was assessed by epicardial Doppler displacement probes.

Blood was collected five times during the experiment from the femoral vein: at baseline; at 4 and 8 minutes of occlusion; at 60 and 90 minutes of reperfusion. To avoid observer bias, blood samples were coded and blinded before any measurements were made. Platelet aggregation and hemostatic factor levels were determined by an individual unaware of the experimental protocol.

**Platelet aggregation**

Blood samples were collected from an ear vein through a 19-gauge needle into a plastic syringe containing 130 mM citric acid trisodiumdihydrate (Sigma Chemical Co., St. Louis, MO, U.S.A.). Citrate and whole blood were immediately mixed in a 1:9 ratio and centrifuged at 1,200g for 2.5 minutes to obtain platelet-rich plasma (PRP) that was kept at room temperature and used within 1 hour. The platelet count was determined in each PRP sample with a Coulter Counter ZM (Coulter Co., Hialeah, FL, U.S.A.). Platelet numbers were adjusted to 5.75 x 10^8 /mL with homologous platelet-poor plasma (PPP). Platelet aggregation was induced by 5 VLM adenosine diphosphate (ADP). All supplies were obtained from the Chrono-log Corporation (Havertown, PA, U.S.A.). Aggregation studies were performed using a Chrono-Log Whole Blood Lumi-Aggregometer (model 560–Ca) and expressed as a percentage of light transmittance change from the baseline using PPP as a reference at the end of the recording time. Platelet aggregation curves were recorded for 5 minutes and analyzed according to internationally established standards.

**Endothelin-1**

Blood was collected into plastic tubes containing 7.5 mM EDTA and centrifuged immediately at 2,000g for 10 minutes at 4°C. Plasma was stored at −80°C prior to analysis. ET-1 was extracted from plasma, using Amersham Amper TM 500 mg C2 columns (Amersham International, Little Chalfont, England). First, the column was equilibrated by washing with 2 mL methanol followed by 2 mL water. Then 1 mL of plasma was acidified with 0.25 mL 2M HCL, centrifuged at 10,000g for 5 minutes, and loaded onto the column. The column was then washed twice with water, 0.1% trifluoroacetic acid and 80% HPLC grade methanol. The eluent was collected in a polypropylene tube and dried under nitrogen. Finally, the pellet was reconstituted in 250 μL assay buffer for analysis using an immunoenzymetric “sandwich” enzyme-linked immunosorbent assay (ELISA) system (Amersham International).

**Fibronectin**

The level of fibronectin was measured in EDTA-treated PPP by kinetic turbidimetry of the antigen-antibody reaction according to the principle of the fixed time method (Boehringer Mannheim, Mannheim, Germany).

**Eicosanoids**

Since eicosanoids have a short half-life under physiological conditions, the final metabolites were analyzed. Thromboxane B_2 (TxB_2), the stable breakdown product of thromboxane A_2, and 6-keto-PGF_{1a}, the stable degradation product of prostacyclin, were measured in 3.8% citrated PPP that was kept at −4°C. Prostaglandin biosynthesis in vitro was inhibited with 7.5 mM EDTA and 4 μg/mL indomethacin. Plasma samples were extracted with ethanol and stored at −80°C before final prostaglandin determination, using TiterZyme® enzyme immunoassays according to standard techniques (PerCepetive Diagnostics, Inc., Cambridge, MA, U.S.A.).

**AT III**

The level of AT III was determined in the remaining citrated PPP using a quantitative chromogenic assay (Accucolor™ Sigma Chemical, St. Louis, MO, U.S.A.).

**Protein C and protein S**

Levels of protein C and protein S were measured in citrated plasma using enzyme immunoassays (Asserachrom® Diagnostica Stago, Asnieres-sur-Seine, France).

**Statistics**

All comparisons were made using repeated measures analysis of variance (ANOVA). Tukey’s pairwise comparison procedure was performed on data sets for which significant differences were detected by ANOVA. A post hoc comparison using the Bonferroni t-test was performed to identify specific differences between baseline
values and those of ischemia reperfusion phases. The values were expressed as mean ± SEM; p < .05 was considered significant.

RESULTS

Exclusion of animals
All 13 swine under investigation survived the experiment. No animal developed ventricular fibrillation. However, four animals were excluded for the following reasons: Doppler probe instrumentation failure (2); low initial platelet count (less than 5.75 x 10^9/mL) (1), and reversible character of platelet aggregation curve in response to ADP (1). The remaining nine swine developed mild MS and were analyzed.

Laboratory data
The cumulative data on platelet aggregability, plasma levels of ET-1, fibronectin, eicosanoids, and natural antithrombotics are summarized in Table 1. We found that brief myocardial ischemia, followed by reperfusion is associated with significant systemic disturbances in platelet function and certain hemostatic factors. Platelet aggregability was decreased during the occlusion phase, however, such changes did not reach significant values (Fig. 1). The reperfusion phase of MS was characterized by a significant progressive increase in platelet aggregation. A decline in the ET-1 plasma level at the end of occlusion was observed, followed by a significant increase that remained consistent during the entire phase or reperfusion (Fig. 2). There were no differences in the plasma fibronectin profile during ischemia-reperfusion compared to the baseline (Fig. 3). In contrast, the plasma TxB2 concentrations underwent marked changes (Fig. 4). The thromboxane level decreased significantly during occlusion and then increased above the baseline value at the first hour of reperfusion with a further decline during reperfusion. Plasma prostacyclin levels remained constant during the entire occlusion and at the beginning of reperfusion followed by a significant increase during the second hour of reperfusion (Fig. 5). Antithrombin III plasma levels were significantly elevated during the early occlusion phase, without any further changes during ischemia-reperfusion (Fig. 6). Similar to fibronectin, the profile of the total protein S level was not changed during MS (Fig. 7). However, plasma protein C increased significantly during the end of occlusion reaching the highest level at the beginning of reperfusion with a further decline to the baseline level during late reperfusion (Fig. 8).

FIG. 2. Changes in the plasma level of endothelin-1. The values are expressed as mean ± SEM. *p < .05 vs. baseline.

DISCUSSION

It has been shown that the coronary endothelium may be affected by brief episodes of ischemia-reperfusion (16). Following ischemia, the endothelial release of nitric oxide is inhibited, resulting in enhanced adhesion of neutrophils and platelets to the endothelium. The immediate effect of MS on platelet function and important hemostatic factors is poorly understood. The current study suggests that brief coronary artery occlusion followed by reperfusion in the open-chested swine model is associated with significant changes in platelet aggregability and the plasma levels of certain hemostatic factors. These findings may have implications for understanding of the pathophysiological events during MS.

Platelet aggregation
Considerable data has been accumulated from animal and human studies on the association of acute myocardial infarction with platelet function. However, platelet involvement and activation of hemostasis are still poorly
TABLE 1. Effects of myocardial stunning on platelet aggregation and hemostatic profile in swine

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>4' occlusion</th>
<th>8' occlusion</th>
<th>60' reperfusion</th>
<th>90' reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet aggregation (%)</td>
<td>35.9 ± 2.2</td>
<td>33.7 ± 2.2</td>
<td>28.7 ± 1.6</td>
<td>44.1 ± 2.1*</td>
<td>48.0 ± 2.6*</td>
</tr>
<tr>
<td>Endothelin-1 (pg/mL)</td>
<td>3.92 ± 0.19</td>
<td>3.87 ± 0.24</td>
<td>3.39 ± 0.29*</td>
<td>4.92 ± 0.27*</td>
<td>4.89 ± 0.17*</td>
</tr>
<tr>
<td>Fibrinectin (µg/mL)</td>
<td>53.2 ± 1.1</td>
<td>53.0 ± 0.6</td>
<td>52.3 ± 1.7</td>
<td>51.3 ± 1.5</td>
<td>52.5 ± 1.4</td>
</tr>
<tr>
<td>Thromboxane B₂ (pg/mL)</td>
<td>509.7 ± 31.2</td>
<td>400.5 ± 36.7*</td>
<td>410.3 ± 37.9*</td>
<td>570.2 ± 42.6*</td>
<td>453.3 ± 26.3</td>
</tr>
<tr>
<td>Prostacyclin (pg/mL)</td>
<td>360.5 ± 19.6</td>
<td>364.2 ± 21.3</td>
<td>369.5 ± 16.1</td>
<td>378.5 ± 16.3</td>
<td>510.2 ± 21.1*</td>
</tr>
<tr>
<td>Antithrombin III (%)</td>
<td>98.5 ± 3.4</td>
<td>138.1 ± 3.6*</td>
<td>93.0 ± 4.7</td>
<td>86.7 ± 3.7</td>
<td>96.5 ± 2.3</td>
</tr>
<tr>
<td>Protein S (%)</td>
<td>70.3 ± 1.4</td>
<td>71.0 ± 2.0</td>
<td>71.8 ± 1.1</td>
<td>68.3 ± 1.2</td>
<td>67.8 ± 1.1</td>
</tr>
<tr>
<td>Protein C (%)</td>
<td>45.3 ± 1.8</td>
<td>45.6 ± 1.1</td>
<td>55.7 ± 1.4*</td>
<td>64.5 ± 1.4*</td>
<td>47.1 ± 1.3</td>
</tr>
</tbody>
</table>

* Significant difference when compared to baseline (p < .05).

known issues in the understanding of the pathogenesis of MS. It has been shown that platelets become activated after ischemia-reperfusion in animal models (13). An important role of platelets in the pathogenesis of MS is suggested by the alleviation of MS by platelet depletion in dogs (17). It remains uncertain whether abnormal platelet aggregability is a direct consequence of short-term coronary artery occlusion. The present data demonstrate that during brief coronary artery occlusion in swine, a trend of decreased platelet aggregability occurs and is followed by a significant increase during the reperfusion phase. Such an association does not absolutely establish a cause and effect relationship, but may provide important insight into the pathogenesis of MS, and the clinical events of reocclusion following catheter-based or pharmacologically induced reperfusion in acute coronary syndromes.

### Endothelin-1

ET-1 is the most potent naturally occurring vasoconstrictor yet discovered with an identical structure in humans and swine. Since vasoconstriction can modulate myocardial ischemia, we evaluated plasma ET-1 levels during MS. While it has been established that coronary and systemic plasma concentrations of ET-1 increase following myocardial infarction, there is lack of evidence regarding the role of ET-1 endothelial release during MS. A significant role of ET-1 in the pathogenesis of diastolic MS was reported in conscious dogs (18). Our results are concordant with a previous clinical observation that while plasma ET-1 increases dramatically during reperfusion, it does not increase during myocardial ischemia per se (19). Findings of diminished ET-1 levels during the occlusion phase of MS are also consistent with the report of no detectable ET-1 production during coronary artery thrombus formation, whereas resolved or ruptured clot, resulting in reperfusion, releases a potent stimulus for ET-1 production (20). Similarly, ET-1 has been shown to be released from the swine heart during reperfusion following brief coronary artery occlusion not leading to myocardial infarction (21) and is concordant with the results of the current study.

### Fibronectin

Although evidence concerning the role of fibronectin in hemostasis is far from conclusive, it appears that this protein enhances platelet adhesion and spreading on exposed vascular matrix components, and in excessive amounts, paradoxically inhibits platelet aggregation (22).
Plasma fibronectin levels have been shown to be significantly decreased in patients with myocardial infarction complicated by ventricular arrhythmia, left ventricular failure, or death. However, the plasma fibronectin level remains unchanged in patients following uncomplicated infarction (23). In the present study, there were no differences in plasma fibronectin during brief ischemia-reperfusion in the open-chested swine.

**Eicosanoids**

The balance between arterial wall prostacyclin production and platelet thromboxane synthesis directly influences platelet activity and hemostasis. Support for the relevance of eicosanoids during ischemia-reperfusion was found in a study demonstrating that coronary arteries produce large amounts of prostacyclin compared with low quantities of thromboxane (24). Prostacyclin and its analogs (e.g., defibrotide) were shown to reduce tissue injury occurring during myocardial ischemia (24). Increased prostacyclin production, indicated by elevated systemic 6-keto-PGF$_{1a}$ levels during the reperfusion phase of MS, may enhance endothelial protection and prevent thrombosis. The present study supports the recent observation that short-term ischemia is associated with endothelial release of prostacyclin (25). In contrast, the link between thromboxane and MS is controversial. Early studies found that thromboxane does not appear to be an important mediator of reversible ischemia-reperfusion damage (26). However, recent observations describe beneficial cardioprotective properties of thromboxane receptor blockade or thromboxane synthetase inhibition (27) on recovery after MS. In the current study we observed significant increases of plasma TxB$_2$ during

**FIG. 5.** Changes in the plasma level of prostacyclin. (See Fig. 2 for details.)

**FIG. 6.** Changes in the plasma level of antithrombin III. (See Fig. 1 for details.)

**FIG. 7.** Changes in the plasma level of protein S. (See Fig. 1 for details.)

**FIG. 8.** Changes in the plasma level of protein C. (See Fig. 1 for details.)
early reperfusion, however, the importance of these changes remains uncertain.

Natural antithrombotics

The rationale for AT III determination during ischemia-reperfusion was based on recent clinical observations linking deficiency of AT III with ischemic heart disease including unstable angina and AMI (28). Although activation of antithrombotics has been consistently shown during ischemia-reperfusion, most observations are limited to the reperfusion phase of the prolonged ischemic event (29). The consequences of MS on the plasma AT III level are not known. We found that brief ischemia-reperfusion is associated with a marked increase in AT III levels during the early occlusion phase with a further decline to the baseline values. This finding could reflect the protective release of the potent antithrombotic substance responding to enhanced thrombin generation during coronary artery occlusion. Our data is in agreement with the study showing elevation of plasma protein C and total protein S in patients with myocardial ischemia (30). However, the exact effects of MS on the status of natural antithrombotics are not fully understood. Our findings suggest that brief coronary artery occlusion followed by reperfusion induces thrombin generation that may be antagonized by augmented protein C activity.

Study limitations

The current study is descriptive and thus cannot lead to direct statements about the primary nature of events or the ultimate pathophysiological mechanisms involved in MS. We used an open-chested animal model. This design is not as physiological as a closed-chest conscious porcine model, and there are obvious concerns about potential effects of neurohumoral activation on hemostasis. The hemostatic factors studied were limited to those that previously demonstrated an experimental association with the ischemia reperfusion. It would be important to investigate hemostasis not only in the systemic circulation, but in the coronary circulation as well. Patterns of serial changes in the blood from the femoral vein could differ from those obtained from coronary blood, as demonstrated previously for platelet aggregation (19).

Conclusions

The current data demonstrate that significant serial changes in platelet aggregation and some hemostatic factors occur following very brief coronary artery occlusion followed by reperfusion. To the best of our knowledge, this is the first observation that even mild myocardial ischemia-reperfusion is associated with increased platelet aggregability, accompanied by a similar increase in thromboxane B2 production, and elevated plasma levels of ET-1. These changes may predispose to thrombosis. However, a compensatory elevation of antithrombotic factors (prostacyclin, AT III, and protein C) were also observed. The balance to these opposing forces could influence hemostasis and deserves further study.

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REFERENCES


