How to study the effects of platelet aggregation and thrombosis on coronary vasomotion and their clinical relevance

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Following arterial injury, platelets may activate and adhere to the damaged vessel wall, release vasoactive products, and produce vasoconstriction or even vasospasm. In the last few years the hypothesis of intracoronary thrombosis, triggered by plaque ulceration or fissuration, has gained wide acceptance as one of the key events in the pathophysiology of acute coronary syndromes. Following arterial injury, platelets readily adhere to the subendothelium and release a variety of chemical mediators which, apart from recruiting additional platelets from the circulation, are also powerful vasoactive substances. Platelet-induced coronary vasoconstriction may therefore contribute to the occurrence of myocardial ischemia in patients with acute coronary syndromes. Several studies have also focused on some components of the vessel wall and indicate that endothelial dysfunction in atherosclerosis plays a key role in altered vascular responses. We and others have suggested that augmented constrictor responses of atherosclerotic coronary arteries to platelet-derived substances, such as serotonin and thromboxane A_2, perhaps combined with an impaired release of endothelium-derived relaxing factor, may contribute to vasoconstricting responses to aggregating platelets. The purpose of this article is to summarize recent development in knowledge relating to alteration in coronary tone associated with intracoronary platelet activation.

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Introduction

During the past decade, a large number of studies have contributed considerably to our understanding in the pathophysiology of coronary atherosclerotic disease. It is now well recognized that in some patients coronary atherosclerotic lesions progress very slowly by a complex stepwise process which is favored by the presence of risk factors for atherosclerotic disease. On the other hand, some other patients suddenly develop symptoms of acute coronary artery disease syndromes, including unstable angina, acute myocardial infarction, and sudden cardiac death. To this respect, several clinical, histopathological and experimental studies have emphasized the importance of the interaction between circulating platelets and the arterial wall and activation of the coagulation cascade as primary factors leading to absolute reductions in coronary blood flow due to intracoronary thrombus formation, dynamic vasoconstriction, or both[1]. These new insights into mechanisms responsible for the sudden development of acute coronary syndromes are essential for the development of new therapeutic strategies aimed at preventing acute myocardial infarction and sudden cardiac death.

Platelets, coagulation factors, and vascular tone

The mechanisms of platelet-induced alterations in the coronary tone cannot be properly discussed apart from the pathophysiology of vascular endothelium. In fact, endothelial cells synthesize prostacyclin, a potent antiaggregatory substance with vasodilator properties. They also activate (e.g., angiotensin I) or inactivate (e.g., bradykinin, serotonin) a number of vasoactive substances present in the blood. Thus, the vascular endothelium is far from being a passive diffusion barrier, but it can actively alter the amounts of vasoactive substances reaching the deeper layers of the blood vessel wall. In addition, endothelial cells can actively modulate the responsiveness of the vascular smooth muscle of the tunica media through the production of a

labile factor, named endothelium-derived relaxing factor (EDRF), as first demonstrated by Furchgott and Zawadzki[2].

Contractile responses to aggregating platelets. Cohen et al.[3] observed that isolated rings of canine coronary artery contract if autologous platelets aggregate in the organ chamber in which the rings are suspended. Such contractions were markedly enhanced if the endothelium was removed (Fig. 1). If the rings are first contracted with prostaglandin $F_{2\alpha}$, a definite relaxation to aggregating platelets is observed in rings with endothelium, but only further contraction is observed in denuded rings. Thus, aggregating platelets release substances that trigger potent endothelium-dependent inhibitory responses of the smooth muscle in the media. Of the other canine blood vessels studied (femoral artery, femoral vein, and saphenous vein[4], pulmonary artery[5], saphenous artery[6]), only the coronary artery displays a sustained or consistent relaxation response to platelets aggregating in the organ chamber.

Mediators of the endothelium-dependent responses to platelets. *Serotonin.* In mammals, about 90% of the serotonin present in the body is stored in the gastrointestinal tract, mainly in enterochromaffin cells. Of the remaining serotonin, most is present in cells of the central nervous system and circulating platelets. As a consequence, as long as platelets do not aggregate, peripheral arterial blood contains little or no free serotonin. However, whenever platelet aggregation is initiated, and dense granules release their content, serotonin is released in the blood and can exert its pathophysiological effects through activation of specific membrane receptors.

Serotonin has complex and sometimes opposite effects on blood vessels, its net results being dependent on the species, route of administration, and the experimental conditions under which the amine is released or administered. With few exceptions, if isolated vascular smooth muscle is exposed to increasing concentrations of serotonin, contraction ensues, through direct activation of serotonergic receptors on the cell membrane of the vascular smooth muscle. The receptors involved in serotonin-induced contractions usually belong to the 5-HT_2 subtype. Hence, 5-HT_2 serotonergic blockers are potent antagonists of the vasoconstrictor responses to serotonin[7]. Similarly to exogenously-added serotonin, when strips or rings of blood vessels are exposed to aggregating platelets *in vitro*, they contract vigorously. Serotonin must play a major role in the platelet-evoked contraction, because 5-HT_2 serotonergic antagonists nearly abolish it[8].

In vivo, the vascular effects of serotonin are more complex. At the arteriolar level and in a number of large conduit vessels, serotonin causes the relaxation of vascular smooth muscle, and thus dilation. This vasodilation is mediated by activation of 5-HT_1 serotonergic receptors located on endothelial cells, and is, therefore, dependent on the presence of an intact endothelium. Several experimental studies have underlined the important role of serotonin in the regulation of coronary tone and flow. For instance, Brum et al.[9] and Lamping et al.[10] reported that in anesthetized dogs, serotonin caused dose-dependent constriction of coronary arteries with injured endothelium. In contrast, Chu and Cobb[11] showed that in conscious dogs without endothelial damage, serotonin caused a dose-related biphasic response characterized by an initial increase in coronary artery diameter followed by a delayed vasoconstriction. Furthermore, there is general agreement that serotonin exerts marked vasodilator effects on coronary arteriolar vessels in different experimental preparations[12-14]. Hence, the vascular responses to serotonin will depend on the integrity of the endothelial lining. If the latter is intact, it will form a diffusion barrier preventing the monoamine from permeating into the media; in addition, the endothelial cells take up the monoamine and degrade it enzymatically or store it. The serotonin reaching 5-HT_1 serotonergic receptors

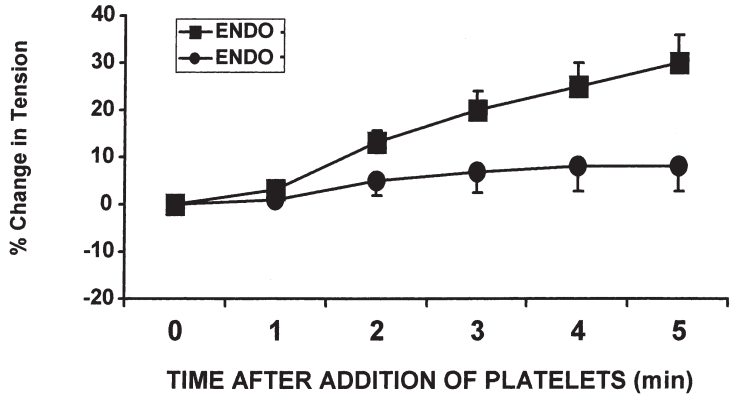

Figure 1. *Effects of aggregating platelets on vascular tone* in vitro. *Isolated human platelets were placed in an organ chamber containing isolated canine coronary arterial rings. Platelet aggregation was started by addition of collagen and coronary tension continuously recorded. A few minutes after addition of platelets to the organ chamber, a significant increase in developed tension was observed, indicating active vasoconstriction. This vasoconstriction was significantly increased if the endothelium was mechanically removed (ENDO-) from the rings. From Cohen et al.[3], modified.*

on the endothelial cells will trigger the release of EDRF. This not only will cause relaxation of the underlying vascular smooth muscle, but also will help to prevent platelet adhesion and curtail platelet aggregation. This response to serotonin (as well as the simultaneous release of EDRF evoked by platelet-derived ADP or by any thrombin formed as a result of the aggregation) contributes to the overall protective role of the endothelium in preventing unwanted coagulation in blood vessels with a normal intima. However, if the endothelial lining is interrupted by a trauma, then the platelet-derived serotonin can reach the vascular smooth muscle and cause it to contract; this reaction is not reduced by the release of EDRF, which permits the expression of the vascular phase of hemostasis.

In the Folts' experimental model of coronary artery stenosis and endothelial injury previously described, Golino et al.[15] have continuously measured coronary artery diameters by means of ultrasonic crystals implanted immediately distal to the stenosis and 1-2 cm below. In that study, a marked coronary vasoconstriction occurred at the site of the stenosis, i.e., where the endothelium had been previously damaged and where platelets and platelet-derived substances accumulate maximally in the arterial wall (Fig. 2). On the contrary, the vasoconstriction observed at the site more distal to the stenosis, i.e., where special attention was paid to maintain the endothelium intact, was much less severe and not different from that observed during a mechanical external occlusion of the coronary artery[15]. It is of note that the marked vasoconstriction observed at the site of the stenosis could be completely prevented by administration of LY53857, a selective 5-HT_2 receptor antagonist, or SQ29548, a selective thromboxane A_2 (TxA_2) receptor antagonist (see also below)[15], but not by nitroglycerin or diltiazem[16], thus indicating a prominent role of serotonin in causing this platelet-related coronary vasoconstriction.

Thromboxane A_2. TxA_2 represents the major metabolite of arachidonic acid in platelets and is produced and readily released in large amounts by activated and aggregating platelets. TxA_2 serves as an autacoid substance, as it further stimulates platelet activation, leading to recruitment of additional platelets from the circulation, but it is also a powerful vasoactive agent, as it causes contraction of coronary smooth muscle[17]. TxA_2 directly exerts its vasoconstricting effects on smooth muscle cells, as to date there are no reports of endothelium-dependent relaxation induced by TxA_2. However, the coronary response to TxA_2 is markedly reduced if an intact endothelial layer is present. As mentioned before, dogs with coronary endothelial damage and stenosis show a marked vasoconstriction that could be abolished by TxA_2 receptor antagonists[15]. The degree of this vasoconstriction, however, was significantly reduced if the endothelium was kept intact[15]. In addition, the TxA_2 synthase inhibitor, dazoxiben, reduces the ischemic response to atrial pacing in subjects with coronary artery disease[18], suggesting that TxA_2 may contribute to constriction of coronary vessels even in the absence of overt spasm.

Adenine nucleotides. ADP and ATP are contained in large quantities in the dense granules of platelets and are released during aggregation. Both compounds cause similar endothelium-dependent relaxation in a number of isolated arteries, including the canine coronary

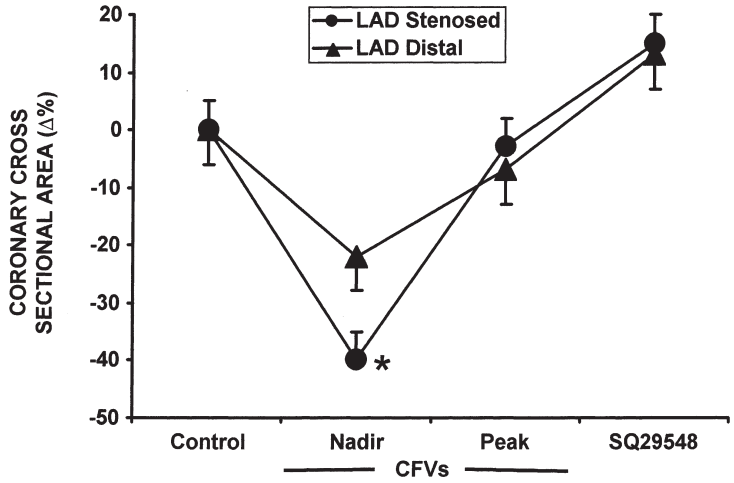

Figure 2. *Effects of aggregating platelets on coronary tone* in vivo. *Intracoronary platelet aggregation was initiated by damaging canine coronary arteries and by placing a plastic constrictor around the damaged segment (Folts' model of thrombus formation, see text for details). Coronary diameter was measured continuously during the experiment via two pairs of miniaturized ultrasonic crystals placed immediately downstream the stenosis (where the endothelium had been removed, LAD stenosed) and 1-2 cm more distal (where care had been taken to protect the endothelium, LAD distal). An intense vasoconstriction was observed during intracoronary platelet aggregation (Nadir) which was significantly more intense in the coronary segment where the endothelium had been previously removed. Administration of SQ29548, a selective thromboxane A_2 receptor antagonist, completely abolished the observed vasoconstriction, indicating that this chemical mediator is involved in the observed phenomenon. Similar data were obtained with LY53857, a selective serotonin S_2 receptor antagonist (data not shown). CFVs = cyclic flow variations; LAD = left anterior descending coronary artery. From Golino et al.[15], modified.*

artery[19-21]. The endothelium-dependent relaxation in response to platelets is almost abolished by apyrase (an enzyme that hydrolyzes ATP and ADP to AMP and inhibits the endothelium-dependent relaxations induced by adenine nucleotides). If endothelial receptors are saturated with ADP, the platelets cause contraction only, even though acetylcholine is still capable of causing relaxation[21]. Thus, the endothelium-dependent relaxation of isolated coronary arteries in response to aggregating platelets is mainly due to the release of ADP and ATP.

Platelet-activating factor. Platelet-activating factor (PAF) is a phospholipid released by leukocytes, mast cells, and platelets. *In vitro*, it is a potent aggregating agent at concentrations as low as 10^{-12} M in some species. Recently, it has been shown *in vivo* that PAF is both an early and a late mediator of intracoronary platelet aggregation at sites of endothelial injury and arterial stenosis. In a number of animals, including the rat in which it does not activate platelets, PAF causes profound hypotension[22]. Both the platelet-stimulating and hypotensive effects of PAF are antagonized by compound CV-3988, a PAF receptor antagonist. In the isolated aorta of the rat, PAF causes relaxation that is abolished by removal of the endothelium[23]. In canine coronary and femoral arteries, relaxation induced by PAF is observed only at high concentrations. This relaxation is endothelium-dependent, but it persists in the presence of CV-3988. It seems unlikely, therefore, that endothelium-dependent relaxation is due to the interaction of PAF with a receptor similar to those mediating platelet activation and hypotension. At the high concentrations required to observe it, a direct membrane effect of these lipids analogous to that observed with arachidonic and oleic acid[24] is a more likely explanation. The synthesis of prostacyclin is not involved, since incubation of the blood vessels with the inhibitor of cyclooxygenase, indomethacin, does not block the response[24].

Thrombin. Upon platelet activation and aggregation, the coagulation cascade usually activates concomitantly, mainly because stimuli that cause activation of platelets and those that activate the coagulation cascade are often simultaneously present. *In vivo*, thrombin formation represents the final step of activation of the extrinsic pathway, triggered by endothelial disruption and tissue factor exposure to flowing blood[25]. Apart from those on fibrinogen and other coagulation factors, thrombin exerts many effects, some of which mediated through stimulation of specific cellular receptors[26]. Thrombin receptors are present on a variety of cells in and around the vascular space, including endothelial cells, platelets, vascular smooth muscle cells, and fibroblasts. The consequences of thrombin receptor activation vary among cell types, but since the structure of the only known receptor appears to be identical in all cells studied, these differences presumably partly reflect differences in the effectors that are downstream from the receptor (G protein). In endothelial cells, thrombin stimulation is associated with activation of protein kinase C, increase in cAMP levels and stimulation of nitric oxide synthase[27]; conversely, in smooth muscle cells thrombin results in activation of phospholipases C A_2, and D, MAP kinase, and a transient increase in cytosolic Ca^{++} concentration[28]. Thus, in isolated vessels, the effects of thrombin are strictly dependent upon the presence of an intact endothelium: relaxation in case the endothelium is present or vasoconstriction if the endothelium is absent or malfunctioning[29]. *In vivo*, the vascular effects of thrombin are much more complex and are mediated, at least in part, indirectly through the stimulating effects on platelets which, in turn, release other vasoactive substances (see above).

Role of platelets in altering coronary tone in humans

That activated platelets may play an important role in affecting coronary tone in humans is suggested not only by the above-mentioned experimental studies, but also by the observation that patients with complex coronary artery lesions release platelet-derived substances, such as TxA_2 and serotonin, in significant amounts in the coronary circulation[30,31]. Furthermore, Rubanyi et al.[31] have also demonstrated that plasma obtained from the coronary sinus of patients with unstable angina possesses vasoconstricting activity in isolated vessels *in vitro* and that this vasoconstriction could be prevented by selective 5-HT_2 receptor antagonists. Hence, TxA_2 and serotonin may be released in the coronary circulation of patients with selected coronary artery disease syndromes in amounts sufficient to affect vascular tone of isolated vessels *in vitro*. If one takes into consideration that atherosclerosis, which almost invariably is found in patients with coronary artery disease, is associated with a marked alteration of endothelial function[32], it could be speculated that platelet-derived substances might result in profound vasoconstricting effects in such patients.

To determine whether some platelet-derived mediators, such as serotonin, may also result in vasomotor changes of the human coronary circulation *in vivo*, a study was undertaken by Golino et al.[33] aimed at determining the effects of exogenously-administered serotonin on coronary diameter and flow in patients with angiographically normal coronary arteries and patients with coronary atherosclerotic lesions. In patients with normal coronary arteries, serotonin caused a dose-dependent increase in coronary blood flow, as well as an increase in the diameter of the coronary artery where the amine was infused. These vasodilating effects were mediated through activation of non-5-HT_2 receptors, since they were not blunted but, instead, potentiated after administration of ketanserin, a selective 5-HT_2 re-

ceptor antagonist (Fig. 3). In contrast, in patients with coronary atherosclerotic lesions, intracoronary infusion of serotonin caused a dose-dependent vasoconstriction, evidenced by both a decrease in coronary artery diameter and flow. Interestingly, the vasoconstricting effects of serotonin in patients with coronary atherosclerosis were completely blocked after administration of ketanserin, thus suggesting a role of activation of 5-HT_2 subtype receptors[33]. The same findings were confirmed and extended to patients with Prinzmetal's angina by another group of investigators[34]. It should be pointed out that, although all patients were pre-treated with aspirin to reduce the risk of intracoronary platelet activation, part of the vasoactive effects of intracoronary serotonin was the consequence of a direct platelet activation by the amine with ensuing release of other vasoactive substances, such as TxA_2. Thus, these data are compatible with the hypothesis that activated platelets may importantly affect coronary diameter and flow in humans, resulting in vasodilating or vasoconstricting effects depending on the presence of a functional endothelium.

To determine whether serotonin, under different pathophysiological conditions, may be released in quantities sufficient to affect coronary tone in humans, we have measured the release of this amine in the coronary sinus of patients undergoing coronary angioplasty and correlated this release with changes in coronary diameter of the dilated vessel. A marked release of serotonin was observed in the coronary sinus of these patients following balloon inflation. This release of serotonin was accompanied by a pronounced vasoconstriction of the coronary artery distal to the dilated site that peaked at 15 min after dilation[35]. Interestingly, this vasoconstriction was blunted by ketanserin[35], thus indicating a definitive role for serotonin and activation of 5-HT_2 receptors in mediating vasoconstriction after angioplasty.

Clinical implications

It is a giant leap from studies in organ chambers with artificial solution and containing healthy animal tissue to a diseased coronary artery on a pumping human heart, so that inferences about human pathophysiology must necessarily be tentative. Nonetheless, it is appealing to propose that the body has evolved a particularly effective defense against thrombosis in a critical vascular bed with little collateral blood supply, namely the coronary circulation. Thus, one can imagine that if, for any reason, platelets began to aggregate in a normal coronary artery with an intact endothelium, the response of the smooth muscle of the blood vessel to substances released from platelets would be relaxation. Such an endothelium-dependent dilation triggered by platelet products (and possibly by thrombin) would tend to flush away the beginning aggregate before it could occlude the vessel. On the other hand, if the endothelium is absent, damaged, or for some reason fails to function properly, the response of the vessel to platelet products and thrombin would be contraction, as observed in patients with coronary artery lesions. Such contraction would further reduce the luminal area and increase the obstruction to blood flow[1].

Several lines of evidence indicate that platelet-derived mediators may be important in modulating coronary artery tone in humans, especially in pathophysiological states characterized by significant intracoronary platelet activation. In particular, patients with atherosclerotic coronary arteries have a paradoxical coronary vasoconstriction in response to serotonin, probably because the endothelium is unable to synthesize and/or release adequate amounts of EDRF, thus leaving unopposed the vasoconstricting effects of serotonin. In addition, a significant platelet activation may occur during coronary angioplasty with a consequent release of large amounts of serotonin in the coronary circulation. This

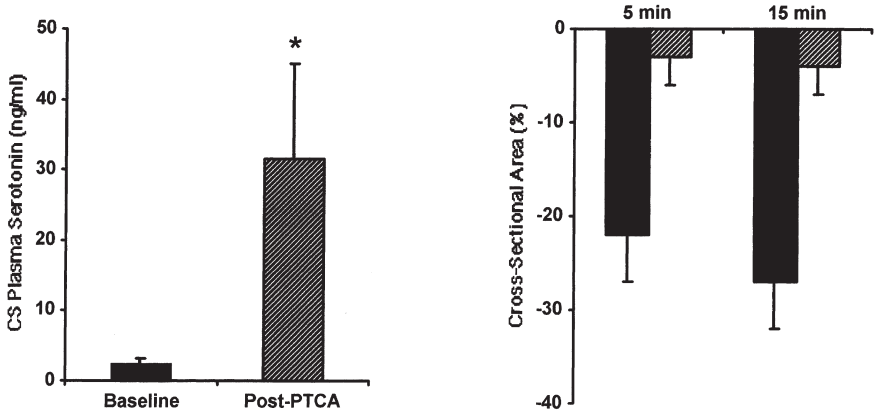

Figure 3. *Intracoronary platelet activation in patients undergoing coronary angioplasty (PTCA). Blood samples, for the measurement of plasma free serotonin levels (as an index of platelet activation), were obtained from the coronary sinus (CS) of patients undergoing PTCA at baseline and immediately after dilation of the stenosis. Free plasma serotonin concentration, indicating intracoronary platelet activation, increased markedly in the blood obtained from the CS* (left panel). *Quantitative coronary angiography revealed that a significant vasoconstriction occurred in the dilated coronary artery which was blunted by administration of ketanserin, a selective serotonin S_2 receptor antagonist* (dashed bars, right panel).

serotonin may lead to a vasoconstriction of the dilated vessel and may, if pronounced, contribute to the occurrence of early complications after a successful dilation. Finally, the observation that platelet-derived substances are released in the coronary vascular bed of patients with unstable angina strongly points toward a role of serotonin in the pathophysiology of this syndrome. Additional studies are needed to test the protective effects of several receptor antagonists in the treatment and prevention of these disorders.

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