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Coronary endothelial dysfunction after ischemia and reperfusion: a new therapeutic target?

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Abstract

Although cardiac ischemia is usually characterized as a disease of the myocyte, it is clear that the vasculature, and especially endothelial cells, is also a major target of this pathology. Indeed, using a rat model of ischemia/reperfusion, we were able to detect severe endothelial dysfunction (assessed as a decreased response to acetylcholine) after acute or chronic reperfusion. Given the essential role of the endothelium in the regulation of vascular tone, as well as platelet and leukocyte function, such a severe dysfunction could lead to an increased risk of vasospasm, thrombosis and accelerated atherosclerosis. This dysfunction can be prevented by free radical scavengers and by exogenous nitric oxide. Endothelial dysfunction can also be prevented by preconditioning with brief periods of intermittent ischemia, thus extending to coronary endothelial cells the concept of endogenous protection previously described at the myocyte level. Experiments performed on cultured cells showed that the endothelial protection induced by free radical scavengers or by preconditioning was due to a lesser expression of endothelial adhesion molecules such as intercellular adhesion molecule-1, leading to a lesser adhesion of neutrophils to endothelial cells. Identification of the mechanisms of this protection may lead to the development of new strategies aimed at protecting the vasculature in ischemic heart diseases.

Key words
• Coronary endothelium
• Ischemia/reperfusion
• Preconditioning

Introduction

Vascular endothelial cells play an essential role in the control of vascular tone, but also in the regulation of smooth muscle cell growth, as well as of platelet and leukocyte function. Numerous experimental and clinical data suggest that these essential physiological functions of the endothelium are altered in various pathophysiological situations, such as hypertension, hypercholesterolemia or diabetes. Such dysfunction can be characterized by an altered capacity of the endothelium to release nitric oxide (NO), associated with an increased production of oxygen-derived free radicals such as superoxide anions.

A similar impairment in the endothelial synthesis of NO has also been detected at the level of the coronary circulation after myocardial ischemia and acute or chronic reperfusion. Given the central role of NO as a vasodilating agent, but also as an inhibitor of platelet aggregation and leukocyte adhesion,

it is likely that such a persistent impairment may have important deleterious consequences for the coronary arterial wall. Thus, coronary endothelial cells may be considered as a major therapeutic target of anti-ischemic treatments.

Endothelium and myocardial ischemia

Evidence that myocardial ischemia leads to coronary endothelial dysfunction was first obtained by Ku (1) who showed that a 90-min period of ischemia followed by 1-2-h reperfusion was associated with a decreased endothelium-dependent relaxation in response to thrombin in canine coronary arteries. These findings were rapidly extended to other endothelium-dependent vasodilators such as acetylcholine (2). The impaired endothelium-dependent responses were accompanied by marked structural injury to endothelial cells (assessed by electron microscopy). However, the response to nitroprusside, an endothelium-independent vasodilator, was intact.

Most of the experimental studies of endothelial dysfunction after ischemia/reperfusion involve *in vivo* coronary occlusion in large species (especially dogs and pigs) followed by studies of endothelium-dependent vasorelaxation in isolated coronary arteries. In a few studies, endothelial dysfunction is evaluated in isolated hearts submitted to ischemia *in vitro.* In our laboratory, we developed a rat model of myocardial infarction to assess coronary endothelial dysfunction following ischemia and reperfusion. This model consists of submitting Wistar rats to 20-min ischemia followed by 60-min reperfusion. At the end of this infarct protocol, hearts are removed and placed in physiological buffer for vascular study. Thus, coronary artery segments (1.5-2 mm long and 200-300 µm in diameter) are taken distal to the site of occlusion and mounted on small vessel wire myographs to study their reactivity in the presence of vasoactive agents. This model allows us to focus on the consequences of ischemia/reperfusion at the level of large epicardial or medium-size intramyocardial coronary arteries.

The first results obtained with the use of this model were that ischemia/reperfusion decreased significantly the endothelium-dependent relaxation of the coronary artery segments in response to acetylcholine, compared with those of sham animals (3). However, no alteration in the relaxing response to acetylcholine appeared with the arteries taken from animals subjected to ischemia alone, in accordance with previous results (4), suggesting that the endothelial dysfunction observed with the myocardial ischemia is a manifestation of reperfusion injury. Moreover, the contractile responses to serotonin, which in this rat coronary preparation is not associated with the release of endothelium-derived relaxing factors, and thus only induces direct smooth muscle contraction (5), and the relaxing responses to SIN-1, an NO donor, were not modified by ischemia alone or followed by reperfusion.

Thus, myocardial ischemia/reperfusion is associated with an altered NO-dependent relaxation of coronary arteries. Additional experiments showed that incubation of the arterial segments with the substrate of NO synthase L-arginine could not reverse this impairment, in contrast to other diseases such as hypercholesterolemia. These results suggest that the impaired response to acetylcholine is mostly the consequence of the absence of structurally intact endothelial cells and not that of a selective defect in NO synthase activity, or a specific impairment of the transduction pathway linking muscarinic receptors to NO synthase. This hypothesis is in accordance with electron microscopy studies showing structural changes in the vascular wall (6).

Endothelial dysfunction after reperfusion is not a transient phenomenon, since it persists for at least 4-6 weeks in experimental

models (6,7). However, while acute dysfunction is the consequence of structural injury to the endothelial cells, chronic changes are rather a manifestation of dysfunctional regenerated endothelium, as previously demonstrated after balloon injury (8).

Mechanisms of reperfusion-induced coronary endothelial dysfunction

The observation that the reintroduction of molecular oxygen at reperfusion is required to produce postischemic endothelial dysfunction is consistent with the view that ischemia/reperfusion injury to the endothelium may result from the generation of reactive oxygen species. This has now been clearly demonstrated by the observation that endothelial dysfunction can be attenuated or prevented by scavengers of these species. In our rat model of myocardial ischemia/reperfusion, a continuous intravenous infusion of the free radical scavenger *N*-2-mercaptopropionyl glycine (MPG) during ischemia and reperfusion resulted in the prevention of the coronary endothelium-dependent relaxation in response to acetylcholine (9). There is also evidence in species other than rats that the reperfusion-induced endothelial injury is the consequence of the production of oxygen-derived free radicals because impaired endothelium-dependent relaxation can be restored by treatment with superoxide dismutase and catalase in both large coronary arteries (4,10) and the microcirculation (11).

Several mechanisms may explain the endothelial injury induced by free radicals. Once produced, superoxide anions may directly inactivate NO (12,13). Since NO is a potent inhibitor of neutrophil activation and adhesion (14), the decreased NO production may lead to the development of an acute inflammatory response. Moreover, since free radicals produced during reperfusion also trigger the rapid adhesion of neutrophils to endothelial cells through the induction of adhesion molecules such as selectins or intercellular adhesion molecule-1 (ICAM-1) (15), the combined effects of the decreased production of NO and the increased production of free radicals will reinforce the adhesion of neutrophils to the endothelial cells, setting the stage for an amplification of the neutrophil-mediated endothelial injury.

Protection of coronary endothelial cells

Prolonged ischemia and reperfusion are responsible for a cascade of reactions leading to endothelial injury characterized by a decrease in NO production. Given the important vasodilator property of NO, such an impairment may lead to an increased coronary vasoconstriction and an increased risk of vasospasm. Moreover, endothelial dysfunction after reperfusion may favor platelet aggregation and thus increase the risk of thrombosis. Taken together, those observations indicate that the prevention of endothelial dysfunction/injury is an important therapeutic goal. However, little is known about the endothelial effects of most of the known anti-ischemic interventions.

One of the most potent anti-ischemic interventions known to date is the concept of endogenic protection of ischemic myocardium described by Murry et al. (16), and termed "preconditioning". According to these experiments, submitting the heart to short episodes of ischemia separated by intermittent reperfusion renders the heart more resistant to prolonged ischemia and markedly limits infarct size. Preconditioning also confers protection against the severe ventricular rhythm disturbances that occur during subsequent ischemia and reperfusion (17,18).

Using our rat model of myocardial ischemia/reperfusion *in vivo*, we demonstrated that such a protection can be obtained at the level of the coronary endothelial cells (3). Indeed, 3 cycles of 5-min ischemia/5-min reperfusion prevented the impaired endothe-

lium-dependent relaxing responses to acetylcholine when the artery was subjected to immediate prolonged ischemia/reperfusion (Figure 1). Moreover, we demonstrated that this was not a transient phenomenon and that the protective effect can be prolonged at least 4 weeks after reperfusion (6).

The mechanisms of the endothelial protection induced by preconditioning are still not well established. Given the central role of neutrophil adhesion in endothelial injury during reperfusion, we hypothesized that the protective effect of preconditioning is due in part to a decreased production of endothelial adhesion molecules leading to a lesser adhesion of neutrophils to endothelium. Indeed, as mentioned above, reperfusion injury to the endothelium is mediated by neutrophils which adhere via the expression of adhesion molecules such as ICAM-1 (19,20). To test this hypothesis, we subjected cultured rat aortic endothelial cells to *in vitro* anoxia and reoxygenation. In this context, we found that preconditioning abolished the increased ICAM-1 expression and reduced the adhesion of neutrophils observed with anoxia-reoxygenation alone (21). These protective effects of preconditioning can be mimicked by the free radical scavenger MPG. Moreover, incubation of endothelial cells with MPG, chelerythrin (an inhibitor of protein kinase C) or N^G-nitro-L-arginine (an inhibitor of NO synthases) during preconditioning abolished its protective effects on ICAM-1 expression and neutrophil adhesion, suggesting a role for protein kinase C, free radicals and NO as triggers of preconditioning in this model.

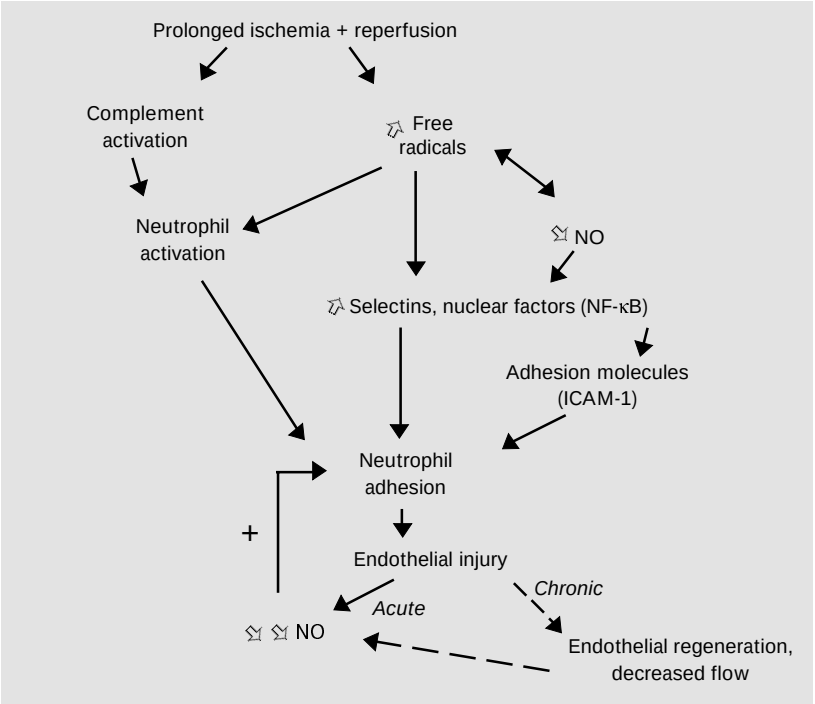

Figure 1 - Possible mechanisms of endothelial injury after ischemia/reperfusion. NO, Nitric oxide; ICAM-1, intercellular adhesion molecule-1.

Although preconditioning the heart has efficient protective effects, the protection induced by this intervention is transient, and disappears if the period of reperfusion separating preconditioning from prolonged ischemia extends over 2-3 h. This time course possibly limits its therapeutic application. However, recent evidence showed that myocardial protection reappears several hours after preconditioning. Indeed, preconditioning performed 24 h before prolonged coronary occlusion is associated with a significant limitation of infarct size in dogs (22) or in rabbits (23), and extends to other aspects of ischemia/reperfusion injury such as myocardial stunning (24). To assess whether such a "second window" of protection exists at the level of coronary endothelial cells, we subjected rats to 3 periods of intermittent left coronary artery occlusion and allowed them to recover. Twenty-four hours later, rats were re-anesthetized and subjected to the standard ischemia/reperfusion protocol described above (9). Those experiments showed that, in addition to having beneficial effects on infarct size and postischemic myocardial contractile dysfunction, delayed preconditioning also protects coronary endothelial cells against ischemia and reperfusion injury (9). Moreover we found that the beneficial effect of delayed preconditioning on endothelial protection was abolished by administration of MPG (9). These results suggest a

triggering role of free radicals in delayed preconditioning, which may act through stimulation of the expression of various protective proteins, including NO synthases and antioxidant enzymes (Figure 2). Similar results were obtained about infarct size (25), as well as myocardial stunning (26).

Again, the mechanisms of such delayed preconditioning were evaluated in cultured endothelial cells; as observed with "classic" preconditioning, we found that delayed preconditioning was associated with a decrease in neutrophil adhesion and a complete abolition of ICAM-1 expression (27). These changes in endothelium-neutrophil interactions were accompanied by an increased expression of several antioxidant enzymes and of NO synthases.

Among the different possible triggers and mediators of delayed preconditioning, NO seems to play a dominant role. Indeed, it has been demonstrated that brief ischemia was associated with a delayed increase in the coronary flow response to acetylcholine and bradykinin (28). Moreover, numerous studies have shown that NO plays a central role in the delayed protective effects of preconditioning in myocardial stunning (29) and infarction (30,31) via the inducible nitric oxide synthase (iNOS). Thus, we tested whether a similar involvement of iNOS could be observed at the level of the endothelial cells. For this purpose, we administered the selective iNOS inhibitor, *N*-(3-(aminomethyl) benzyl)acetaminide (1400 W) to preconditioned rats (32). Although 1400 W indeed blocked iNOS activity *in vivo* under those experimental conditions, it did not affect the endothelial protection induced by delayed preconditioning. Thus, in contrast to the myocytes, iNOS is not involved as a mediator of delayed preconditioning in endothelial cells. This opposite effect of iNOS inhibition may be explained in part by the distinct effect of NO on cardiomyocytes and endothelial cells. Indeed, iNOS activation by inflammatory stimuli produces high concentrations of NO (33). In cardiomyocytes, such high levels of NO probably induce a decrease in metabolic requirements and oxygen consumption, and this effect is probably responsible for the anti-ischemic role of iNOS. Contrary to cardiac myocytes, an overproduction of NO in endothelial cells may have deleterious consequences, in part by its reaction with oxygen-derived free radicals to produce highly reactive intermediates such as peroxynitrites. In this context, NO may act as a pro-inflammatory stimulus.

Our experiments rule out the hypothesis of a role of iNOS in the endothelial effects of delayed preconditioning, but do not exclude a role for NO produced by other enzymes such as endothelial NO synthase (eNOS). Indeed, endogenous stimulation of eNOS (for example by acetylcholine) may exert marked endothelial protective effects after ischemia and reperfusion (34). Moreover, our experiments on cultured cells demonstrated an increase of the nuclear factor AP-1 in the endothelial cell nucleus after preconditioning (27), and this may be correlated to the increased level of eNOS mRNA, since AP-1 is responsible for the enhanced

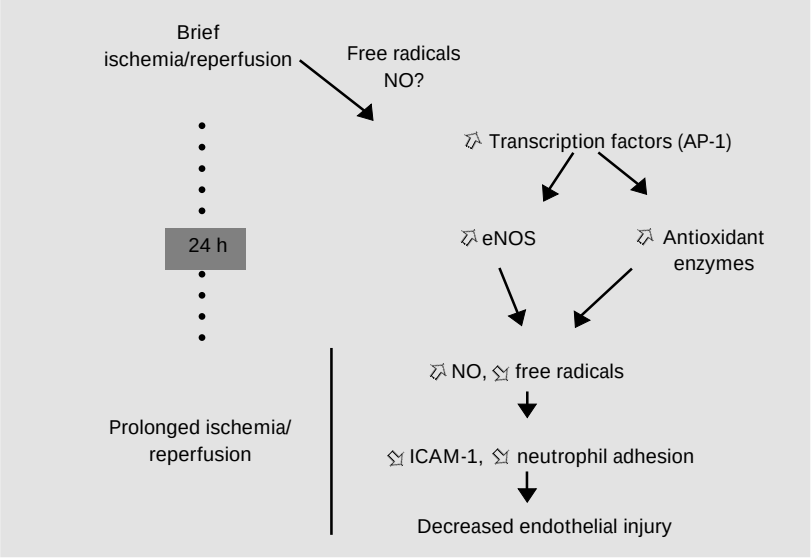

Figure 2 - Potential mechanisms of the endothelial protective effects of late preconditioning. NO, Nitric oxide; eNOS, endothelial nitric oxide synthase; ICAM-1, intercellular adhesion molecule-1.

expression and activity of eNOS (35). Whether changes in eNOS contribute to the endothelial effects of preconditioning is currently being investigated in our laboratory.

Coronary endothelial dysfunction is an important, although long overlooked, component of cardiac ischemia/reperfusion injury. Such endothelial dysfunction seems to be mainly the consequence of reperfusion-induced production of free radicals and adhesion of neutrophils. However, identification of protective pathways against reperfusion-induced endothelial injury, including preconditioning, may lead to the discovery of potent interventions aimed at limiting vascular injury after ischemia, with possible marked beneficial effects on the incidence of vasospasm, thrombosis and further atherosclerosis.

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