

The interaction of coronary microembolization and ischemic preconditioning: A third window of cardioprotection through TNF-alpha

Philipp Heusch¹, Andreas Skyschally¹, Kirsten Leineweber¹, Michael Haude², Raimund Erbel³, Gerd Heusch¹

¹Institute of Pathophysiology, Center of Internal Medicine, University of Essen Medical School, Essen, Germany

²Lukaskrankenhaus Neuss, Germany

³Department of Cardiology, Center of Internal Medicine, University of Essen Medical School, Essen, Germany

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Corresponding author:

Gerd Heusch Prof. Dr. med. Dr. h.c.
Direktor, Institut für
Pathophysiologie
Zentrum für Innere Medizin
Universitätsklinikum Essen
Hufelandstr. 55
45122 Essen, Germany
Phone: +49 (201) 723 4480
Fax: +49 (201) 723 4481
E-mail: gerd.heusch@uk-essen.de

Abstract

With an unstable atherosclerotic coronary plaque, episodes of myocardial ischemia associated with release of debris and microembolization and subsequent restoration of more or less sufficient coronary blood flow can precede ultimate plaque rupture, complete coronary occlusion and impending acute myocardial infarction. Such scenario involves both coronary microembolization with its established detrimental consequences for the dependent myocardium and repeated, transient episodes of myocardial ischemia/reperfusion which can induce protection by ischemic preconditioning. The current review aims to study the interaction between these adverse and beneficial effects. Experimental coronary microembolization in anesthetized pigs does not induce nor prevent acute preconditioning against infarction. However, six hours after coronary microembolization a third window of protection exists, which results from the upregulation of TNF-alpha. Apparently, TNF-alpha exerts bidirectional effects, i.e. induces contractile dysfunction but protects from infarction.

Key words: infarct size, coronary microembolization, ischemic preconditioning, adenosine, TNF-alpha.

Coronary microembolization in patients

The rupture of an atherosclerotic plaque in an epicardial coronary artery with subsequent occlusive coronary thrombosis has been established as the decisive event in the pathogenesis of acute myocardial infarction [1, 2]. Milder forms of plaque rupture with subsequent embolization of atherosclerotic and thrombotic debris into the coronary microcirculation have also been recognized before [2-5], but the clinical frequency and importance of coronary microembolization in the clinical setting have only recently been appreciated [6-8].

Microemboli were identified in post-mortem autopsy of patients who had died from sudden cardiac death but without overt myocardial infarction. These patients had microinfarcts in the perfusion territories of atherosclerotic coronary arteries. Qualitatively, the microemboli were characterized by platelet aggregates, hyalin and atherosclerotic plaque material, including cholesterol

crystals. The microemboli were associated with microinfarcts and an inflammatory reaction of the myocardium (for detailed review see [8]).

The transient elevation of enzymatic markers such as creatine kinase (CK), CK-MB isoenzyme and more sensitively troponin [9] is now regarded as a characteristic sign of microinfarction in patients with unstable angina and may just reflect the consequences of coronary microembolization. The elevation of troponin is also associated with worse prognosis in patients with unstable angina [10, 11]. Such considerations have found their way into the consensus document on the definition of myocardial infarction by the European Society of Cardiology and the American College of Cardiology [12] which particularly acknowledges *microemboli from the atherosclerotic lesion that has been disrupted during angioplasty or from the particulate thrombus at the site of the culprit lesion*.

Not only in spontaneous plaque rupture, but also during coronary interventions, microembolization is in fact induced, again leading to myocardial microinfarcts, as reflected by elevated CK and troponin [13-16] and electrocardiographic (ECG) alterations [14]. New imaging techniques have facilitated the detection of epicardial coronary plaque rupture, when ulcerations have developed, and thus the potential source of coronary microembolization. Intravascular ultrasound provided evidence that indeed the reduction in epicardial atherosclerotic plaque volume in patients undergoing a percutaneous coronary intervention for acute myocardial infarction [17] or unstable angina [18] induces microembolization and contributes to inadequate reperfusion, as assessed by TIMI frame count [17], and CK-MB release [18]. Microinfarcts were also visualized by contrast-enhanced MRI technique in patients who had mild elevations of CK-MB after percutaneous coronary intervention [19, 20].

Vasoconstrictor and inflammatory mediators

Apart from the particulate debris leading to distal mechanical microvascular obstruction and infarctlets, plaque rupture also results in the release of vasomotor, thrombogenic, and proinflammatory soluble substances [7, 21, 22]. Such substances possibly contribute to impaired microvascular perfusion (the *slow or no reflow* phenomenon) [23]. We have recently studied this aspect of plaque rupture in patients who underwent stent implantation into a significant stenosis of a saphenous vein aortocoronary bypass graft; these grafts are known to be very susceptible to plaque rupture [24]. A distal balloon protection device was used to trap and aspirate particulate debris and soluble substances released from the atherosclerotic lesion during stent implantation. Since human coronary arteries and rat mesenteric arteries are

characterized by a comparable receptor arrangement for thromboxane A₂, norepinephrine, and serotonin, the vascular action of the aspirate was analyzed in isolated rat mesenteric arteries with intact and mechanically denuded endothelium using a Mulvany myograph bath chamber. Using this rat mesenteric vasomotor bioassay, we identified serotonin and thromboxane A₂ as potent endothelium-dependent vasoconstrictors in the aspirate, the action of which could be inhibited by a combined blockade of serotonin 5-HT_{2A/2C} and 5-HT_{1A/1B} receptors and of the thromboxane A₂ TP-receptor [25]. Serotonin was measured via HPLC and thromboxane A₂ indirectly via measuring its metabolite thromboxane B₂ via ELISA. Serotonin (in nmol/l: 582±81 before vs. 2540±366 after stenting, p<0.05 vs. before; n=10) and thromboxane A₂ (in pg/ml: 10±2 before vs. 30±5 after stenting, p<0.05 vs. before; n=10) were released into the aspirate in relation to the angiographic severity of the stenosis and to plaque volume, as assessed by intravascular ultrasound.

Inflammatory responses are also seen in those clinical scenarios where coronary microembolization is likely to occur. Nuclear factor-kappa-B is activated in patients with unstable angina [26], and serum C-reactive protein is increased in patients who died from an acute coronary syndrome [27]. Also the cytokine interleukin-6 was higher up to 48 h in patients with unstable angina who experienced a major adverse cardiac event [28]. These markers of inflammation were always assumed to originate from the rupturing atherosclerotic plaque, but they might be derived from microcirculatory inflammation in response to myocardial microinfarction equally well [29].

We have very recently also found an increased TNF-alpha release from stented saphenous vein bypass grafts which was not only correlated to the amount of plaque extrusion but importantly also to restenosis 5 months later [30]. The importance of inflammation secondary to coronary microembolization is also supported by a recent study, in which preprocedural treatment with HMG-CoA reductase inhibitors in patients undergoing stenting of a de novo stenosis resulted in a reduced incidence of periprocedural myocardial injury, as assessed by analysis of CK and troponin T, and better event-free survival [31]. This beneficial effect is most likely attributable to anti-inflammatory properties of HMG-CoA reductase inhibitors, such as favourably altered plaque composition and plaque stabilization, but possibly also to attenuated myocardial inflammation [32].

An experimental model of coronary microembolization

Perfusion-contraction mismatch

We have developed an experimental model of coronary microembolization using intracoronary

infusion of microspheres (diameter of 42 μm) in anesthetized dogs and pigs. The stepwise intracoronary infusion of microspheres up to a final dose of 3,000 spheres per ml/min baseline coronary blood flow causes a typical response with an immediate decrease in coronary blood flow upon infusion of the embolizing particles, followed by a more prolonged reactive increase in coronary blood flow. Concomitantly, regional myocardial function is stepwise reduced and does not fully recover [33]. During the hours following acute coronary microembolization, regional myocardial function is progressively further decreased. Interestingly, this myocardial dysfunction is not associated with a decrease in regional myocardial blood flow. Quite different from the consequences of a severe stenosis of an epicardial coronary artery, where myocardial function and myocardial blood flow are proportionally reduced in a typical perfusion-contraction matching pattern [34], the progressive myocardial dysfunction following coronary microembolization is characterized by a profound perfusion-contraction mismatch [35]. Patchy microinfarcts, which affect about 5% of the respective myocardium are observed in the microembolized myocardium [36]. Less than 0.1% of the cardiomyocytes are apoptotic, and apoptotic cells are always localized with the microinfarcts [36].

Microinfarcts and inflammation

The microinfarcts are characterized by leukocyte infiltration, including monocytes/macrophages. Such leukocyte infiltration cannot be attributed to chemoattractant properties of the embolizing microspheres per se [35]. The only small amount of infarcted myocardium and the almost unchanged myocardial blood flow after coronary microembolization raised the notion that the observed inflammatory response is responsible for the profound regional contractile dysfunction following coronary microembolization. Indeed, supporting this idea, the profound contractile dysfunction recovers spontaneously back to baseline over about one week. Moreover, unspecific inhibition of inflammation by methylprednisolone abolishes the progressive contractile dysfunction, even when given as a single bolus and even when given 30 min after coronary microembolization [37].

TNF-alpha, contractile protein oxidation and progressive contractile dysfunction

More specifically, we identified TNF-alpha to play a causal role in contractile dysfunction following coronary microembolization, supporting and extending prior findings by Arras et al. who reported enhanced TNF-alpha expression and leukocyte infiltration after coronary microembolization in pigs [38]. Importantly, the increased TNF-alpha expression is an autocrine/paracrine response of cardiomyocytes

surrounding the microinfarcts, possibly mediated by the local shear stress between contracting and non-contracting, infarcted myocardium. The increased tissue TNF-alpha concentration is causal for contractile dysfunction, since the intracoronary infusion of exogenous TNF-alpha in the absence of microembolization induces a similar progressive dysfunction and conversely, pre-treatment with TNF-alpha antibodies prevents myocardial dysfunction following coronary microembolization [36].

To further clarify the signal transduction cascade of TNF-alpha-induced dysfunction we have studied the role of nitric oxide and sphingosine, both known elements of the signal transduction cascade of TNF-alpha in ischemia-reperfusion injury and chronic heart failure [39, 40]. With coronary microembolization, both TNF-alpha and sphingosine contents in the myocardium are increased. Pre-treatment with the nitric oxide-synthase inhibitor NG-nitro-L-arginine-methylester attenuated the progressive myocardial contractile dysfunction and prevented increases in TNF-alpha and sphingosine contents [41]. Surprisingly, nitric oxide appears to be located upstream of TNF-alpha in the signal transduction of inflammatory dysfunction. Pre-treatment with N-oleoylethanolamine (NOE), which blocks the enzyme ceramidase and thus the catalytic conversion of ceramide to sphingosine [42, 43], also abolished the progressive contractile dysfunction following coronary microembolization, but the myocardial tissue concentration of TNF-alpha remained increased [41]. These results suggest that the microembolization-induced progressive contractile dysfunction is signalled through a cascade with nitric oxide located upstream of TNF-alpha and sphingosine located downstream of TNF-alpha (Figure 1) [41]. As a potential target in excitation - contraction coupling we addressed the oxidative modification of contractile proteins as a potential mechanistic link between the inflammatory signal transduction and the contractile impairment following coronary microembolization. We looked at tropomyosin as a marker protein since it contains only a single cysteine residue which can be oxidized and then forms disulfide bonds. Increased formation of disulfide crosslinks in tropomyosin were observed in pig and dog hearts 6-8 hours after coronary microembolization, a time point when there was pronounced microembolization-induced myocardial dysfunction. The extent of oxidative tropomyosin modification correlated inversely with contractile function, and TNF-alpha content was increased in parallel with tropomyosin oxidation [44]. The reversible tropomyosin oxidation is most likely caused by reactive oxygen species. In various cell types TNF-alpha synthesis depends on reactive oxygen species formation [45, 46], but on the other hand TNF-alpha

also promotes reactive oxygen species formation [47, 48]. Such bidirectional link amplifies the inflammatory response by exacerbating the oxidative stress. The notion that oxidative myofibrillar protein modification is responsible for the contractile dysfunction following coronary microembolization was further supported by experiments in which ascorbic acid was given as an antioxidant. Pretreatment with ascorbic acid prevented the microembolization-induced myocardial dysfunction, the increase in myocardial TNF- α , and the oxidation of tropomyosin [44]. The oxidation of tropomyosin might represent an end-effector of the transduction pathway triggered by microembolization that links the inflammatory response to the failure of contraction. Interestingly and supporting a causal role for tropomyosin oxidation, increased disulfide bridges are no longer observed one week after coronary microembolization when contractile function has fully recovered [44].

Ischemic preconditioning

Ischemic preconditioning is the experimental gold standard paradigm of cardioprotection since its first report by Murray et al. 20 years ago [49]. There are excellent reviews on ischemic preconditioning and its signal transduction (Figure 2) which will not be recapitulated here [50-52]. Briefly, protection by ischemic preconditioning resulting from transient episodes of myocardial ischemia/reperfusion is apparent within minutes and lasts for 2-3 hours; a second phase of delayed preconditioning re-appears 12-24 hours later and lasts for 3-4 days.

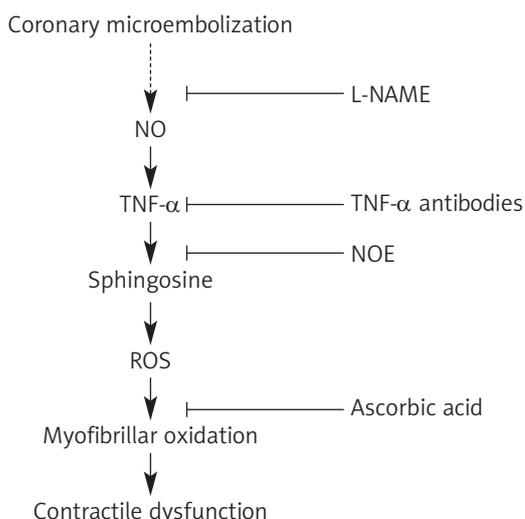


Figure 1. Schematic diagram of cellular mechanisms inducing contractile dysfunction in response to coronary microembolization. L-NAME: L-nitroarginine methylester, NO: nitric oxide, NOE: n-oleylethanolamine, ROS: reactive oxygen species

Ischemic preconditioning in humans: the evidence

The existence and significance of ischemic preconditioning in man are less clear [53, 54], largely because the most rigorous endpoint, i.e. infarct size, is not easily available for controlled, prospective studies in man, for obvious ethical reasons. Prospective clinical studies are therefore available with short episodes of fully reversible myocardial ischemia, such as induced by percutaneous transluminal coronary angioplasty (PTCA) [55-58] or by brief surgical ischemic cardiac arrest [59, 60]. Certain parts of the experimentally established signal transduction of ischemic preconditioning have been verified using PTCA, such as preconditioning by adenosine [61, 62] or bradykinin [63] and the prevention of ischemic preconditioning by the KATP blocker glibenclamide [56] or the opioid antagonist naloxone [64]. Surrogate endpoints in such studies include ST-segment shifts in the surface or intracoronary ECG, metabolic markers such as lactate and ATP, or release of CK and troponin. Whereas new troponin assays appear to provide reliable clinical markers of myocardial injury [65], the association of alterations in energy and substrate metabolism with infarct size reduction even in the experiment is still elusive. Importantly, in the animal experiment attenuation of ischemic ST-segment elevation appears to be an unreliable marker of ischemic preconditioning, reflecting activation of sarcolemmal KATP channels whereas ischemic preconditioning's protection is induced through activation of mitochondrial KATP channels [66]. Apart from the potentially unreliable endpoints, these clinical studies using PTCA are confounded by the potential of collateral recruitment that may attenuate ischemia and its consequences, independently of any preconditioning. Clearly, the exclusion of angiographically visible collaterals [67, 68] is not sufficient to exclude significant collateral recruitment, and more rigorous approaches such as the pressure-derived collateral flow index [57, 69] are required. This concern does not apply to the global ischemia during surgical cardiac arrest. All the existing clinical studies using PTCA or ischemic cardiac arrest address the early phase of ischemic preconditioning only.

Support for the existence of ischemic preconditioning in man is also derived from retrospective analyses of patients undergoing thrombolysis who had pre-infarction angina. Patients with pre-infarction angina appear to have reduced infarct size, as estimated by reduced CK release and less Q waves on their ECG [70, 71], better functional recovery [72] and better prognosis [70]. These earlier retrospective studies were confirmed in an ancillary study to the TIMI-9B trial in a prospective study design, though only in terms of improved prognosis [73]. However, patients with pre-infarction angina

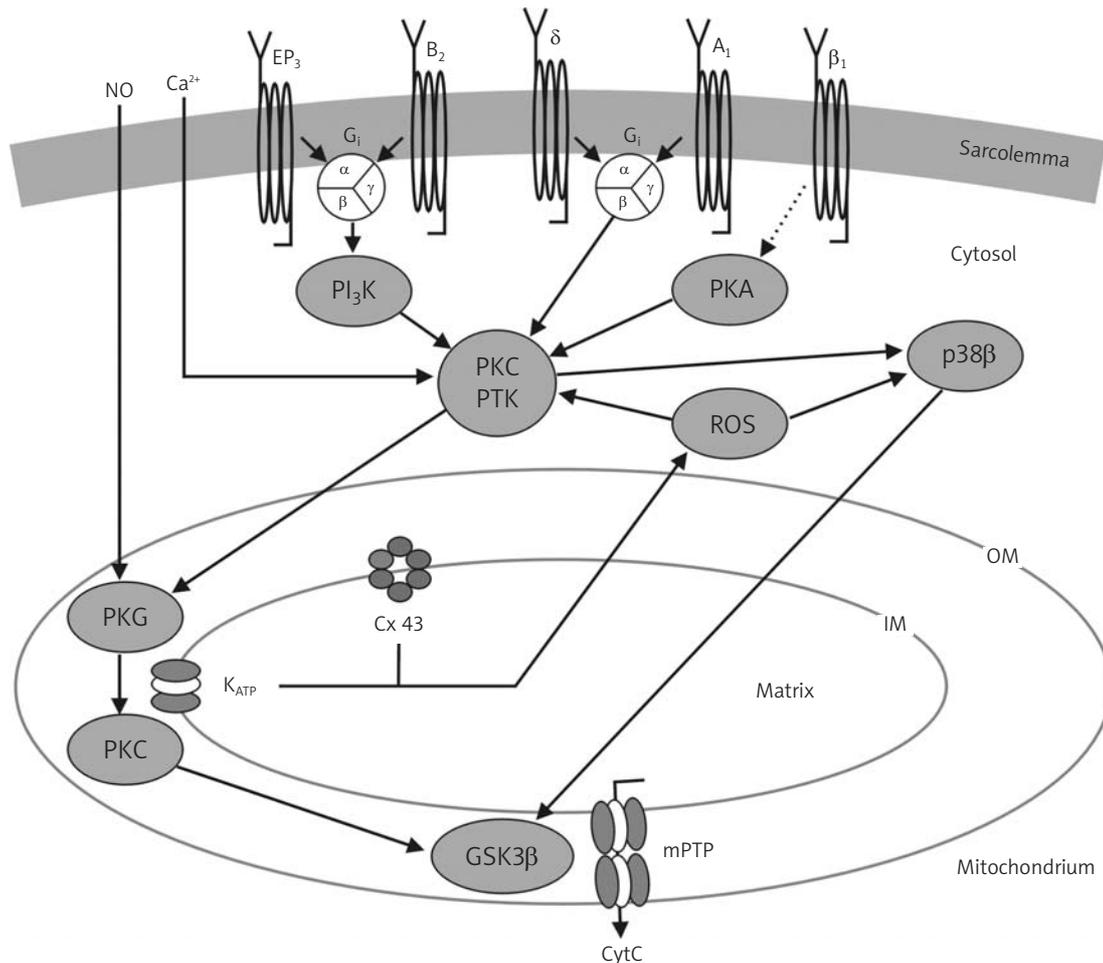


Figure 2. Schematic diagram of intracellular signal transduction of ischemic preconditioning. A₁ – adenosine A₁ receptor, ATP – adenosine triphosphate, B₂ – bradykinin B₂ receptor, β₁ – beta₁-adrenoceptor, Cx43 – connexin 43, CytC – cytochrome C, δ – δ opioid receptor, EP₃ – prostaglandin E receptor 3, Gi – inhibitory G-protein, GSK 3β – glycogen synthase kinase 3β isoform, IM – inner mitochondrial membrane, K_{ATP} – ATP-dependent potassium channel, mPTP – mitochondrial permeability transition pore, NO – nitric oxide, OM – outer mitochondrial membrane, p38β – p38 mitogen activated kinase β isoform, PI₃K – inositol triphosphate kinase, PKA – protein kinase A, PKC – protein kinase C, PKG – protein kinase G, PTK – protein tyrosine kinase, ROS – reactive oxygen species

have also more rapid thrombolysis and possibly therefore smaller infarcts and better prognosis [74]. The time frame of occurrence of angina before myocardial infarction in those studies varied widely, and the protection observed can therefore not be attributed to either early or delayed preconditioning.

Experimental studies on the interaction of coronary microembolization and ischemic preconditioning

In consideration of the above, we have conducted a series of experiments in our established porcine model of coronary microembolization (see above).

Adenosine, coronary microembolization and acute ischemic preconditioning

Common to both, ischemic preconditioning and coronary microembolization, is the involvement of

adenosine. Adenosine is an established trigger of ischemic preconditioning [51] and the hyperemic response after coronary microembolization is caused by adenosine, released from ischemic areas in the microembolized myocardium [75]. Thus, the surrounding non-embolized tissue may be protected against infarction from subsequent sustained ischemia/reperfusion. Infarct size after 90 min ischemia and 2 h reperfusion was determined by triphenyl tetrazolium chloride staining and served as the major endpoint of our studies. Under conditions of some residual blood flow during ischemia, as in the present study, it is a major determinant of final infarct size and must be taken into account [76]. Therefore, we analyzed not only infarct size but also the relationship between infarct size and subendocardial blood flow during sustained ischemia as a more specific endpoint of cardio-

myocyte protection. Coronary microembolization failed to protect the myocardium from infarction after 90 min sustained ischemia and 2 h reperfusion (Figure 3A) [77]. This lack of protection could be attributed to the lack of increase of the interstitial adenosine concentration (measured by HPLC from microdialysis samples) with coronary microembolization (Figure 3B) [77], since a transient increase in the interstitial adenosine concentration prior to the sustained ischemia is mandatory to establish protection by ischemic preconditioning [78]. The superimposition of infarction induced by coronary microembolization per se [36] even increased the final infarct size after sustained ischemia in microembolized myocardium [77].

In fact, coronary microembolization and ischemic preconditioning could interfere such that microembolized myocardium may even lose its ability to be classically preconditioned by a brief period of ischemia/reperfusion. A potential loss of protection by ischemic preconditioning could result from a critical loss of adenosine through enhanced washout with coronary blood [75, 77, 79] and lymph flow [80] subsequent to coronary microembolization. However, in our model ischemic preconditioning was still operative, even when induced upon acute coronary microembolization. Thirty minutes after coronary microembolization a preconditioning stimulus of 10 min ischemia followed by 15 min of reperfusion was still sufficient to induce the mandatory transient increase in the interstitial adenosine concentration and to reduce the final infarct size caused by subsequent sustained 90 min ischemia and 2 h reperfusion (Figures 4A and 4B) [81]; yet the infarct size was larger than with ischemic preconditioning of

naïve myocardium, due to the aggregate infarct size caused by microembolization per se.

TNF-alpha, coronary microembolization and delayed ischemic preconditioning

We now wondered whether the increased TNF-alpha expression might not only mediate progressive contractile dysfunction but also induce delayed protection against infarction. In fact, in isolated rat hearts pretreatment with TNF-alpha reduces the infarct size after ischemia/reperfusion [82]. This protective effect was confirmed in mouse [83] and rabbit models [84]. Moreover, TNF-alpha is also involved in the endogenous protection by ischemic preconditioning. In TNF-alpha knockout mice, acute [83] and delayed ischemic preconditioning [85] are abrogated, and TNF-alpha-antibodies inhibit delayed ischemic preconditioning in rats [86].

The myocardium was indeed protected against infarction at 6 h after coronary microembolization. Infarct size was reduced almost by 50% (Figure 5) [87], although coronary microembolization per se is expected to increase infarct size by about 5% [77]. The observed protection was a direct effect of TNF-alpha per se and not an indirect protection secondary to reduced contractile function before ischemia/reperfusion, since thiopental induced almost identical dysfunction but no reduction in infarct size. The pretreatment with the neutralizing TNF-alpha antibodies prior to coronary microembolization attenuated not only the progressive myocardial

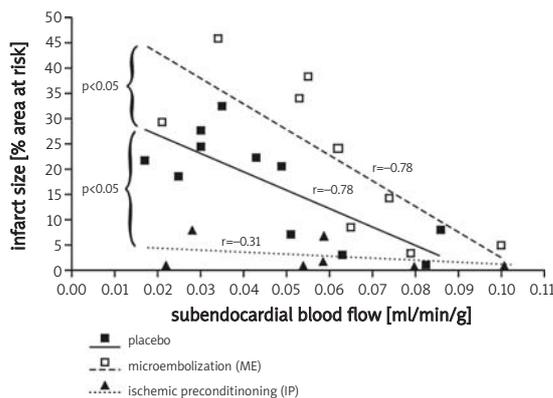


Figure 3A. In placebos, there is an inverse relationship between residual blood flow during ischemia and the infarct size resulting from 90 min ischemia and 2 h reperfusion. With microembolization, the regression line was shifted up towards higher infarct size at a given subendocardial blood flow. With ischemic preconditioning by one cycle of 10 min ischemia and 15 min reperfusion, infarct size at a given subendocardial blood flow was reduced. Reproduced with permission from [77]

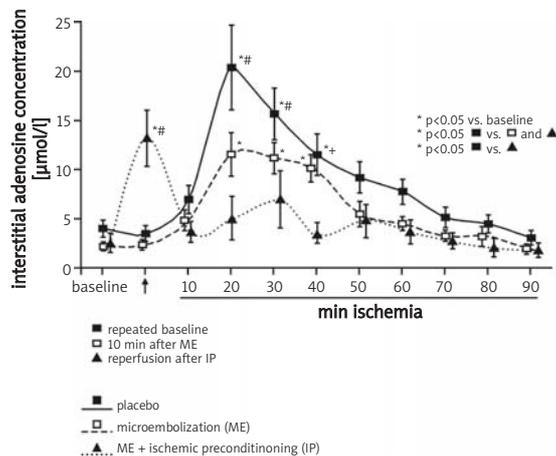


Figure 3B. In placebos, the interstitial adenosine concentration increased after the onset of the sustained ischemia. With ischemic preconditioning, the interstitial adenosine increased six-fold. The interstitial adenosine concentration remained unchanged with coronary microembolization, but increased after the onset of the sustained ischemia. With ischemic preconditioning and coronary microembolization the maximal increases in the interstitial adenosine concentration during the sustained ischemia were attenuated. Reproduced with permission from [77]

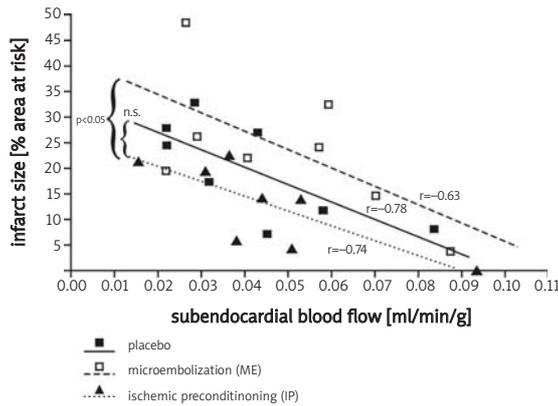


Figure 4A. The linear relationship between infarct size and subendocardial blood flow in placebos was not different from that with coronary microembolization (ME) and ME followed by ischemic preconditioning (IP). When comparing the relationships between infarct size and subendocardial blood flow in myocardium with ME (ME vs. ME plus IP) ischemic preconditioning shifted the regression line significantly downwards, reflecting smaller infarct size at a given subendocardial blood flow. Reproduced with permission from [81]

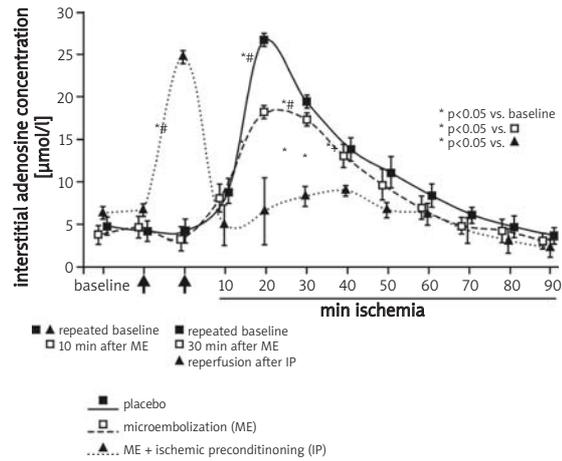


Figure 4B. In placebo controls the interstitial adenosine concentration increased after the onset of the sustained ischemia. The interstitial adenosine concentration remained unchanged with coronary microembolization, but increased after the onset of the sustained ischemia. The interstitial adenosine remained unchanged after coronary microembolization and increased 3.7-fold during reperfusion following ischemic preconditioning. With coronary microembolization alone and coronary microembolization plus ischemic preconditioning before the sustained ischemia, the maximal increases in the interstitial adenosine concentration during the sustained ischemia were attenuated. Reproduced with permission from [81]

dysfunction, as already observed in dogs [36], but also abolished the infarct size reduction by coronary microembolization, establishing that increased TNF-alpha is causal both for contractile dysfunction and protection against infarction.

Apart from the causal role of TNF-alpha in progressive myocardial dysfunction and delayed protection against infarction following coronary microembolization, there are further similarities in the signal transduction cascade. Myocardial protection by exogenous TNF-alpha depends on a sphingolipid signaling pathway. The ceramidase inhibitor NOE abolishes TNF-alpha preconditioning, and ceramide or sphingosine 1-phosphate mimic TNF-alpha protection [82]. Similarly, for the microembolization-induced progressive myocardial dysfunction we have identified a signal transduction cascade with nitric oxide upstream and sphingosine downstream of TNF-alpha [41].

Conclusions and perspective

There is solid evidence that coronary microembolization occurs in patients, both spontaneously and during percutaneous coronary interventions. The deleterious consequences of coronary microembolization, such as microinfarcts, inflammation, contractile dysfunction, arrhythmias and impairment of coronary reserve, are seen clinically and are reproduced in an experimental model with intracoronary infusion of microspheres.

There is also good, though mostly indirect evidence for ischemic preconditioning in patients, notably in patients with preinfarction angina and in patients

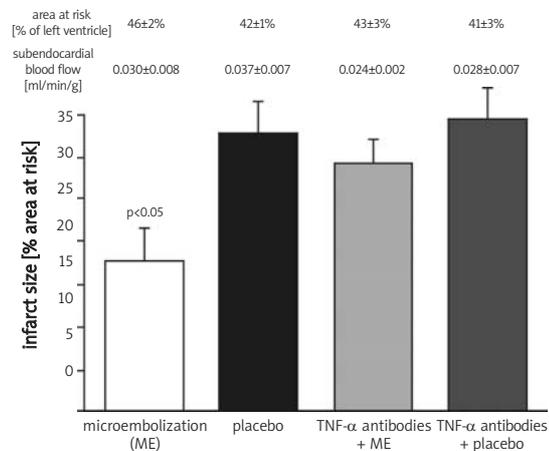


Figure 5. Area at risk (as % of the left ventricle) and subendocardial blood flow at 5 min of ischemia were comparable between groups. Infarct size was reduced by coronary microembolization compared to placebo. The protective effect of coronary microembolization was abolished by pretreatment with TNF-alpha antibodies. The TNF-alpha antibodies per se had no influence on infarct size. Reproduced with permission from [87]

undergoing repeated transient coronary occlusion during percutaneous coronary interventions.

Unstable angina preceding an acute impending myocardial infarction most likely entails both coronary microembolization and ischemic precon-

ditioning. In the experimental model, there is obviously neither the induction nor the prevention of acute ischemic preconditioning by coronary microembolization. In contrast, coronary microembolization induces profound delayed ischemic preconditioning through upregulation of TNF- α . Obviously, in this realistic model of preinfarction angina, a third window of protection exists which is separate from, located in between and mechanically distinct from the established classical first and second windows of protection by ischemic preconditioning.

The bidirectional role of TNF- α which causes contractile dysfunction on the one hand and delayed protection against infarction on the other hand might contribute to explain the controversial results of anti-TNF- α therapy in patients with heart failure.

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