

Ischemic post-conditioning: an effective strategy of myocardial protection?

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There is no doubt that a prompt reperfusion is required to prevent the extension of a myocardial infarction and to allow revascularization. Paradoxically, however, reperfusion itself can lead to a worsening of the ischemic injury with an increase of cell death (Follette et al., 1981; Braunwald & Kloner, 1985; Buckberg, 1986). As a consequence the final damage of an infarction is the result of both ischemia and reperfusion (IR). In myocardial infarction cell death can be due either to necrosis or to apoptosis. While necrosis is the result of both ischemia and reperfusion, apoptosis is mainly an outcome of reperfusion (Zhao & Vinten-Johansen, 2002).

Before we consider myocardial protection, we must summarise the main aspects of reperfusion injury. This takes place mainly in the early phase of reperfusion. As soon as the occlusion of the coronary artery is removed, superoxide anion (O_2^-) is produced by vascular endothelium and neutrophils. Xantin-oxidase and NADHP-oxidase are the enzymes responsible for such a production. While the former is mainly present in endothelial cells and acts on hypoxanthine released by the previously ischemic cardiomyocytes, the latter is mainly located in neutrophils and causes the production of O_2^- when acting on the large amount of oxygen delivered by the postischemic reactive hyperemia.

Superoxide anion is responsible not only for an oxidative stress on cardiomyocytes, but also for the scavenging of nitric oxide (NO) which combines with O_2^- to produce peroxynitrite ($ONOO^-$). Apart from being an indication of a reduced availability of NO, the presence of $ONOO^-$ contributes to the oxidative stress (Kaeffer et al., 1997; Beauchamp et al., 1999).

Also Ca^{2+} overload contributes to IR injury. It initiates during ischemia as a result of the inactivation of Ca^{2+} and Na^+ pumps by the hypoxic unavailability of ATP. While the inactivity of the Ca^{2+} pump leads to Ca^{2+} overload directly, the inactivity of Na^+ pump leads to the same results by increasing the cell concentration of Na^+ , which in turn activates the Ca^{2+}/Na^+ exchanger. During reperfusion the same exchanger is activated by the high cell concentration of Na^+ brought about by the H^+/Na^+ exchanger (Hoffman et al., 2004). Also O_2^- and other reactive oxygen species favour Ca^{2+} overload (Zhao, 2004).

Two are the main effects of Ca^{2+} overload: a) an increase in cell osmolarity with swelling of cardiomyocytes, which

sometimes is so severe to induce cell membrane rupture (explosive swelling); b) an enhancement release of preapoptotic genes from mitochondria (Zhao, 2004).

The IR reduced availability of NO is responsible for an up-regulation of cellular adhesion molecules leading to the adhesion of neutrophils to the endothelial cells with possible extravasation into the interstitial compartment. Adhesion molecules are *B-integrins*, *selectins* and *immunoglobulins*. The last ones include *intercellular adhesion molecules-1* (ICAM-1), *vascular cell adhesion molecules* (VCAM) and *platelet-endothelial cell adhesion molecules-1* (PECAM-1). In the lumen of the small coronary vessels, the lack of NO allows platelet aggregation and microthrombosis.

The reduced availability of NO may also favour vasoconstriction. The combined effect of the adhesion of neutrophils to the endothelial cells with the presence of microthrombi in the small coronary vessels and vasoconstriction can impair myocardial perfusion to such an extent to produce the so called *no-reflow phenomenon* (Reffelman & Kloner, 2004), i.e. a severe blood supply reduction that follows an initial hyperemia after the reopening of an occluded vessel. The administration of NO-donors before IR limits reperfusion injury.

Ischemia and reperfusion injury can be limited by ischemic preconditioning (IP), which consists in one or more brief (a few minutes) coronary occlusions of a large coronary artery before an occlusion long enough to produce an infarction. IP reduces infarct size (Murry et al., 1986; Schott et al., 1990), limits reperfusion arrhythmias (Parrat & Vegh, 1994), prevents endothelial dysfunction of the coronary vessels (DeFily e Chilian, 1993; Gattullo et al., 1999; Pagliaro et al., 2001; 2001b; 2002) and impairs apoptosis (Nakamura, 2000). Myocardial protection by IP is at the same time direct and mediated by the prevention of reperfusion endothelial dysfunction. In fact endothelial dysfunction damages myocardium with two mechanisms: the release of reactive oxygen species and the no-reflow phenomenon. After IP there are two windows of protection: the first window begins immediately after the preconditioning manoeuvre and lasts 1-3 hours. It is followed by a 20-24 hour period without protection, after which the protection reappears (second window) to last 72 hours or longer. While the first window is mainly characterized by a reduction of the infarct size, the second window is mainly

characterized by a reduction of the postischemic stunning. Among the mechanisms responsible for the first window, we must remember the role of adenosine and NO in the opening of mitochondrial ATP-sensitive K^+ (mito K-ATP) channels, a compulsory step to myocardial protection (Pagliaro et al., 2001; Pagliaro et al., 2002). Adenosine is released by ischemic myocardium and leads to the opening of the channels via the activation of protein-kinase C (PKC), whereas NO has been proposed to open the same channels via the activation of a protein-kinase G (PKG) by cyclic GMP (cGMP). Adenosine is also known to induce the release of NO from the endothelial cells thus activating NO-cGMP pathway to the opening of mito K-ATP channels. Endothelial release of NO is also induced by the link of bradykinin (BK) to B_2 endothelial receptors. The occlusion-induced reduction of pH in the vascular wall activates a kininogenase which is responsible for the release of BK from a plasma kininogen.

The study of the signalling cascade in ischemic preconditioning allowed the set up of pharmacological preconditionings. Unfortunately, the use of any kind of preconditioning is limited by the unpredictability of the onset of a myocardial infarction. As a consequence the attention has been focused on the possibility to find out procedures that could be employed after ischemia has taken place. An important observation revealed that some protection may be achieved with a graded reperfusion after ischemia. The awareness that myocardial damage can increase during reperfusion drove Zaho et al. (2003) to investigate in the anesthetized dog whether three brief coronary occlusions right at the beginning of reperfusion after a prolonged ischemia can exert a protective effect. Starting 30 s after the beginning of reperfusion, they performed three 30 s occlusions separated from each other by a 30 s interval. The procedure was named ischemic post-conditioning (Post-C). It was seen to reduce infarct size, tissue oedema and endothelial dysfunction, which, as said above, contributes to myocardial reperfusion injury. In these experiments the protection occurred to the same extent as it were due to IP. As regards endothelial dysfunction it is noteworthy that Post-C reduced the adhesion of neutrophils to endothelium in vessels of the infarcted part of myocardium.

In our laboratory we wanted to see whether the effects of Post-C can also be obtained in the absence of neutrophils. We used isolated rat hearts perfused at constant flow with oxygenated Krebs-Henseleit solution. After stabilization the heart was submitted to 30 min of global ischemia, followed by 2 hours of reperfusion. Using this model we saw that the protection occurs to a greater extent than IP even in the absence of blood, i.e. of neutrophils.

We used two types of occlusions. In the attempt to integrate Post-C with graded perfusion, in one group of hearts the occlusions were progressively shorter in duration and separated from each other by progressively longer intervals; in the other group the duration of occlusions and intervals were kept constant. The infarct size was determined with nitro-blue tetrazolium technique and confirmed with the measurement of the release of lactic-dehydrogenase (LDH) during reperfusion. Contractile

recovery was tested from ventricular pressure after ischemia. No difference between the two groups was observed.

In isolated perfused rat hearts, we also tried to identify the signalling cascade leading to protection. Since our opinion was that NO is likely to be involved, in a further series of experiments we prevented NO release with the NO-synthase inhibitor L-N-nitroarginine (L-NNA) which was infused for 20 min starting a few seconds after the beginning of reperfusion. The prevention of NO release reduced the protection by 50%.

Since NO activates soluble guanylyl-cyclase (GC) which induces the production of cGMP, initially we blocked soluble GC with 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ). Surprisingly enough, ODQ abolished protection completely. Consistent with this finding, test ELISA revealed an increase of cGMP throughout the entire time-course of reperfusion after Post-C, an increase that did not occur in the case GC was blocked. Interestingly, after NO-synthase was blocked cGMP production took place only during the first hour of reperfusion. Thus the signalling cascade seems to activate mitoK_{ATP} channels via cGMP and PKG.

Now a question arises: why does GC blockade completely suppress protection which is only 50% reduced by NO-synthase blockade? It may be suggested that GC is activated not only by enzymatic NO (i.e. produced by NO-synthase) but also by non-enzymatically produced NO. Another possibility is the activation of CG by atrial natriuretic peptide, which in some circumstances is also produced by the ventricles. However in the literature nothing is said about an ischemia-induced production of the peptide. In another series of experiments we observed that protection may be abolished either by blockade of adenosine A_1 receptors with cyclo-pentyl-xanthine (CPX) or by the blockade of PKC with chelerythrine. These findings show that both adenosine and PKC are involved in Post-C as well as in IP.

Some experiments were performed by perfusing the hearts at constant pressure and not at constant flow. In these preparations we studied the protective effect of Post-C either after inhibition of NO-synthase or after blockade of GC. We found that with this kind of perfusion the infarct size was limited to a lesser extent than with constant flow perfusion.

Recently, in the isolated rat heart we tried to protect the heart against IR injury with the administration of an NO-donor or an antioxidant compound during the first 20 min of reperfusion. The antioxidant compound was much more effective than the NO-donor. The result is consistent with the remarkable contribution of the reactive oxygen species to reperfusion injury. When the NO-donor and the antioxidant were given together, the protection was similar to the one induced by the antioxidant alone. Moreover, the effect was more marked with regards to the recovery of developed left ventricular pressure. Since sometimes the use of either the antioxidant compound or mixture reduced the infarct size to more than 10% of the risk area, it may be argued that, at least in rat myocardium, when ischemia lasts no longer than 30 min, most of the injury occurs during reperfusion and depends mainly on oxidative stress.

In conclusion, the possibility to limit IR injury by mechanical (i.e. brief coronary occlusions at the beginning of reperfusion) or pharmacological means may have positive clinical implications in an intensive care unit if carried out immediately after a previously occluded coronary artery has been reopened.

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