Editorial

Preconditioning and limitation of stunning: one step closer to the protected protein(s)?

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Brief periods of ischaemia are known to induce a reversible deficit in myocardial function despite the maintenance of histological and metabolic integrity, a phenomenon initially described by Heyndrickx et al. in 1975, and called stunning several years after [1]. In that first study it was reported that several hours were required for complete recovery of regional contractility following a 5 min occlusion of the left anterior descending coronary artery in conscious dogs and up to 24 h were needed for complete recovery following a 15 min occlusion [1]. Therefore, myocardial stunning is considered to represent reversible injury contrasting the irreversible injury of myocardial infarction. The phenomenon has been described in every species studied so far, does occur in several clinical settings and likely plays an important role in the morbidity of patients with coronary artery disease [2]. Although the principal cause of the reversible contractile dysfunction is not fully understood, there is now consensus that loss of Ca$^{2+}$ responsiveness of the myofilaments represents a major underlying mechanism as first proposed by Kusuoka et al. in 1987 on basis of experiments with isolated perfused ferret hearts [3]. Initially some studies suggested an additional role of failing sarcoplasmic reticulum Ca$^{2+}$ pump, but this could not be substantiated [4]. There is evidence that abnormalities in myofilaments also underly stunning induced in clinically relevant large animal models. For instance, it was shown in the in situ porcine model that the potent Ca$^{2+}$ sensitizer (EMD 60263), without phosphodiesterase inhibitory properties, increased segment length shortening to a greater extent in stunned than in non-stunned myocardium [5]. These findings were consistent with the left-and upward shifts of the p[Ca$^{2+}$]/Mg$^{2+}$-ATPase curves induced by Ca$^{2+}$ sensitizer as observed with isolated myofibrils from stunned pig myocardium [6]. Moreover, another group found a decrease in the Ca$^{2+}$ sensitivity of isometric tension and the rate of cross-bridge cycling in skinned myocytes isolated from stunned pig myocardium [7].

Regarding the mechanism(s) upstream of the contractile apparatus, it has become clear that oxidant stress and Ca$^{2+}$ overload are the two initial triggers that induce myocardial stunning [8]. However, the chain of events between free radical burst, increased [Ca$^{2+}$], and development of the abnormalities in the myofilaments remains to be established.

One or several brief episode(s) of ischaemia, of insufficient duration to cause myocyte necrosis, do(es) not only induce myocardial stunning but is (are) also known to increase the heart’s tolerance to a subsequent sustained period of ischaemia, such that there is a delay of myocardial necrosis. This beneficial effect on the heart, first described by Murry et al. in 1986, is termed ischaemic preconditioning and has also been demonstrated in a wide variety of species including man [9]. The protective state afforded by the preconditioning stimulus lasts 1–2 h depending on the species and model. This is called the first window of protection in contrast to the second window of protection present after 24 h. At first, a cause and effect relationship between stunning and preconditioning was suggested because both phenomena are observed in reversibly injured myocardium. However, results described in a second report by Murry et al. [10] proved that stunning is not involved in preconditioning which was confirmed by others (see e.g. ref. [12]). For instance, whereas a 15 min coronary occlusion followed by a 5 min reperfusion period before a 40 min sustained occlusion...
significantly limited infarct size, much less salvage was realized if the reperfusion period between the brief and sustained occlusion was 120 min [10,11]. Stunning during the reperfusion interval (5–120 min) was, however, similar in magnitude.

Otherwise, a cause and effect relationship between preconditioning and limitation of stunning at first seemed more likely. Cohen et al. first showed that in addition to a delay in development of irreversible necrosis caused by sustained ischaemia, preconditioning improved the recovery of contractile function but improvement of wall motion could be entirely explained by reduction in infarct size [11]. Nevertheless, many subsequent reports kept the question alive whether improved contractile recovery following ischaemic preconditioning during the first window is solely based upon the limitation of the infarct size or also on the attenuation of stunning of salvaged myocardium [11,13–16]. As previously commented by Ovize et al. [13], the analysis of the preconditioning effect on contractile function after prolonged coronary artery occlusion in open-chest or conscious models is confounded by many factors, e.g. 1) the presence of subendocardial necrosis which influences wall motion in surrounding viable myocardium; 2) preconditioning results in stunning before the sustained ischaemia and may therefore limit or mask a beneficial effect of preconditioning on wall motion. Except for one study in favor [15], the in situ studies failed to demonstrate that early preconditioning alleviates the severity of stunning independent of reduction of infarct size [11,13,14,16]. On the other hand, Bolli et al. demonstrated that when the heart was subjected to a sequence of brief (5 min) ischaemic episodes, the first episode preconditioned against the stunning induced by the next two episodes and this protection disappears at some point between the fourth and tenth episodes [17,18]. They suggested that the protective effect on stunning in the early phase is rather weak in contrast to the powerful and long-lasting (at least 3 days) protection against stunning by brief ischaemia induced in the late phase. A major reason that the dispute on whether preconditioning limits stunning remains timely, is that many subsequent reports have extended the use of the term ischaemic preconditioning to include several endpoints in addition to the originally described limiting effect on infarct size (reviewed in [13]). For instance, it was reported that ischaemic preconditioning protects against post-ischaemic contractile dysfunction, enzyme release, increased [H+]i, and arrhythmias in the isolated perfused rat and rabbit hearts [13,19–21]. Likewise, these in vitro investigations taken on the whole are not conclusive. But in this issue of *Cardiovascular Research* Pérez et al. presents results which strongly indicate that at least in the perfused rat heart model, ischaemic preconditioning limits reversible contractile dysfunction [22]. Other studies in isolated perfused hearts, in which both myocardial necrosis (infarct size) and post-ischaemic functional recovery were measured, indicated that post-ischaemic improvement in global left ventricular function produced by preconditioning was secondary to reduced infarction size, and that preconditioning did not reduce stunning (see e.g. [23]). Thus, where we now stand is: in order to obtain definite proof of protection against stunning in vivo and reversible post-ischaemic contractile dysfunction in vitro, selective molecular markers measuring the degree of stunning of salvaged myocardium must become available.

The study of Pérez et al. [22] of this issue elegantly characterizes the principal cellular abnormality that was previously proven to cause reversible post-ischaemic contractile dysfunction in the isolated perfused rat heart by measuring Ca2+ transients and [Ca2+]/force relationships in dissected thin right ventricular trabeculae. Recovery of these isolated muscle parameters, as indicators of the degree of reversible injury, in parallel with recovery of left ventricular developed pressure was measured after 20 min reperfusion of retrogradely-perfused rat heart that had been subjected to global ischaemia for 20 min [22]. Perhaps one limitation of the chosen experimental set-up is that the possibly existing slight differences between muscle responses of right- versus left ventricle to ischaemia and reperfusion, may have become unnoticed. Nonetheless, the fact that the effect of a previous 5 min lasting preconditioning stimulus is measured too on isolated muscle parameters makes this study so noteworthy. Direct evidence is obtained for ischaemic preconditioning to prevent the development of abnormalities localized in the myofilaments that in their previous work were shown to be associated with reversible post-ischaemic contractile dysfunction [22,25]. It is also important to notice that the protection was obtained when there was no initial depression of contractile function. Therefore, the preconditioning effects on myofilament function and left ventricular developed pressure can not be ascribed to contractile dysfunction prior to the ischaemia. Another essential point is the author’s claim that 20 min of global ischaemia of the isolated perfused rat heart is a true model of stunning because it shows no signs of irreversible injury based upon intact cell–cell coupling, unaffected Ca2+ transients, absence of contracture, maintained response to inotropic stimulation, the triphenyltetrazolium and actin staining were not different from control perfused hearts (compare also ref. [24]). In view of the foregoing introduction, the findings of Pérez et al. [22] are of utmost importance because for the first time, one is directly referred to the myofilaments as the potential site to find molecular markers for stunning. The results take us one step closer to the intracellular protein(s) protected by ischaemic preconditioning. The following questions have become topical: how far are we with the identification of molecular abnormalities in the myofilaments during post-ischaemic contractile dysfunction in vitro and/or stunning in vivo? Secondly: if in general, preconditioning and limitation of stunning have a cause and effect relationship, what could
we learn from setting side by side the central steps of the currently known mechanism(s) involved in both phenomena?

Marban and coworkers [3,25] were the first to provide strong evidence for the hypothesis that post-reversible post-ischaemic contractile dysfunction is associated with reversible breakdown and replacement of damaged myocardial proteins, in particular cardiac troponin I (cTnI). Western blots revealed an additional band of 26 kDa compared with 32 kDa for the full length cTnI. This degradation could be prevented by a low Ca\(^{2+}\)/low pH reperfusion which also prevented the post-ischaemic contractile dysfunction [25]. Also calpastatin, a naturally occurring inhibitor of calpain (a Ca\(^{2+}\)-dependent protease), prevented post-ischaemic contractile dysfunction in the perfused rat heart. These results fit well with cellular Ca\(^{2+}\) overload as initial trigger in the mechanism of stunning [8]. In this respect, it is interesting to note that Pérez et al. previously showed that xanthine oxidase inhibitors, which are known to blunt the dysfunction of stunning, sensitize myocardial proteins to Ca\(^{2+}\) [26]. These data are in line with the concept that free oxygen radicals play a role in stunning as well [8]. Recently, other groups could substantiate the findings on cTnI by correlating altered thin-filament regulation with contractile dysfunction resulting from 15 min global ischaemia and more severe periods of global ischaemia (60 min) with and without 45 min of reperfusion [27,28].

The authors hypothesize on basis of the identified cTnI breakdown products and additional modifications within the Tn complex (binary covalent complexes formed by ischaemia–reperfusion-induced activation of transglutaminase (TGase)), that the balance between the formation of the stabilized covalent Tn complexes and the amount of TnI degradation (initially to cTnI\(^{2+}\)) may determine whether finally necrotic or apoptotic pathways are activated in stunned myocardium [28]. The involvement of TGase in ischaemia–reperfusion induced injury was first suggested by Gorza et al. [29] because unidentified covalent complexes containing cTnT in ischaemic–reperfused cardiomyocytes accompanied changes in cTnT immunoreactivity in cryosections (60 min ischaemia followed by 30 min of reperfusion) [28]. Van Eyk and coworkers propose on basis of the obtained data the following sequence of reactions for mildly ischaemic (15 min)–reperfusion induced TnI modification: Ca\(^{2+}\) overload, free radical burst and/or receptor-mediated activation of phospholipase C result in both proteolytic and TGase known about the mechanism(s) involved in both stunning and ischaemic preconditioning. Most reports on preconditioning have taken infarct size and not stunning as endpoint, which would complicate such an effort. Anyhow, the most favored current hypothesis for early preconditioning, first proposed by Downey coworkers [33], is that endogenous ligands such as adenosine, noradrenaline, endothelin and bradykinin initiate intracellular pathways by activation of phospholipases C and D which lead to the activation of protein kinase C isotypes (PKCs) via intracel-
lar inositol-1,4,5-trisphosphate-induced Ca\(^{2+}\) release from the sarcoplasmic reticulum and diacylglycerol formation [33,34]. Activated PKCs then phosphorylate, up to now, unknown proteins that induce protection. At present, only target proteins, e.g. the cardiac mitochondrial ATP dependent K\(^{+}\) channel and Na\(^{+}\)/H\(^{+}\)-exchanger, have been postulated, for which it is not at all clear whether they become phosphorylated by PKC in intact cardiomyocytes. However, cTnI is a well known substrate for cyclic AMP-dependent protein kinase (PKA) as well as PKC [30]. Its phosphorylation represents a major mechanism by which myofilaments are normally able to sense sympathetic and parasympathetic control of the heart. At present, the physiological role of PKA induced phosphorylation in β-adrenergic stimulation of the heart is well understood but, unfortunately, not that of PKC. In intact cardiomyocytes PKC-mediated cTnI phosphorylation takes actually place when the cells are stimulated with e.g. α\(_{1}\)-adrenergic agonists [35]. However, the effects of phosphorylation of myofibrils by PKC in vitro are not consistent, either stimulation or inhibition of the Ca\(^{2+}\) stimulated Mg\(^{2+}\)-ATPase of isolated cardiac myofibrils or reconstituted actomyosin preparations has been observed [35,36]. Returning then to the possible interactions of preconditioning and stunning mechanism(s), of crucial importance may turn out to be the in vitro studies that have shown that prior phosphorylation of cTnI by PKA reduces the rate of its degradation by calpain [37]. Furthermore, recently it was reported as an unpublished observation that treatment of experimental animals with β-adrenergic agonist provides protection against TnI degradation [28]. On basis of these recent results it might be speculated that PKC and/or PKA-mediated phosphorylation of cTnI by (partially) preventing its proteolytic breakdown, contributes to the mechanism by which ischaemic preconditioning limits stunning.

**References**


[27] Van Eyk JE, Powers F, Law W et al. Breakdown and release of myofilament proteins during ischemia and reperfusion in rat hearts: