A fresh look at reperfusion injury 1

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1. Introduction

In clinical therapy of evolving acute myocardial infarction, coronary reperfusion has proven to be the only way to limit infarct size, provided it occurs soon enough after coronary artery occlusion. However, there is also evidence that reperfusion is accompanied by detrimental manifestations known as ‘reperfusion injury’. Reperfusion injury refers to a causal event associated with reperfusion that had not occurred during the preceding ischemic period and can be entirely attenuated by an intervention given only at the time of reperfusion. It classically includes myocardial stunning, reperfusion arrhythmias and lethal reperfusion injury. Clearly, reperfusion arrhythmias do not represent an important problem for the clinician because their incidence is very low and they can be quite easily treated. Myocardial stunning is usually not a major clinical problem in the context of acute myocardial infarction because it disappears spontaneously and is very sensitive to inotropic agents. Myocardial stunning becomes of serious concern only if the affected portion of the myocardium is very large.

Lethal reperfusion injury could be an interesting target for the clinician who is now able to promptly revascularize acutely ischemic myocardium. However, the existence of lethal reperfusion injury has been debated for years by scientists and is still controversial [1–4]. One of the problems is that the development of necrosis cannot be accurately followed in time, both in experimental animal preparations and in man. Instead, scientists have used an indirect approach to deal with this issue; the principle is to modify the nature of reperfusion and then assess whether the extent of necrosis is reduced. Numerous studies have been performed according to this general design. Unfortunately, many of them remained inconclusive, partly for technical reasons [5]. In most, actually hundreds of studies on lethal reperfusion injury, the effects of antioxidants were investigated. These studies were based on the hypothesis that oxygen-derived free radicals produced upon reperfusion of ischemic myocardium represent the predominant cause of lethal reperfusion injury. This hypothesis was based on two well-documented facts: free radicals are indeed produced during reperfusion, and exogenously applied free radicals have negative effects on many cellular and subcellular systems. A limitation of the hypothesis was, however, the lack of a precise formulation of the mechanisms that were considered to link free radical damage with hypercontracture and cell death. Another confounding factor in this hypothesis was the confusion between the mechanism of stunning and reperfusion necrosis. In contrast to reperfusion necrosis, there is now convincing evidence that oxygen-derived free radicals play an important role in the pathogenesis of stunning, and antioxidants alleviate stunning [6]. This mixture of reasons has led many to conclude that reperfusion necrosis does not exist, whereas it only suggests that oxygen-derived free radicals are not important for lethal reperfusion injury. Oxygen-derived free radicals are not the only candidate for the cause of reperfusion injury, and other mechanisms must be looked at in order to further reduce morbidity and mortality following acute myocardial infarction. These alternative mechanisms will be discussed, and the discussion will be confined to the most severe forms of reperfusion injury terminated by cell deterioration, i.e. lethal reperfusion injury.

Lethal reperfusion injury is defined as injury caused by restoration of blood flow after an ischemic episode leading...
to death of cells that were only reversibly injured during that preceding ischemic episode. For lethal reperfusion injury to occur, ischemia has to set the stage without producing irreversible injury already itself. The definition thus comes with the corollary that ischemic alterations of cellular conditions are necessary prerequisites for lethal reperfusion injury, but not in themselves sufficient causes for cell death. Unfortunately, it is technically impossible to date to evaluate manifestation of irreversible injury in the same piece of myocardium both before and after reperfusion. It is also not feasible to distinguish from the analysis of developing damage in reperfused myocardium alone whether cell death is caused entirely by the ischemic history or by reperfusion. Even though such direct assessments are not feasible, there is one valid, albeit indirect, criterion for the presence of lethal reperfusion injury. This criterion is whether modification of the conditions of reperfusion can prevent cell death, otherwise occurring in ischemic-reperfused myocardium. This criterion appears simpler than it is to apply, since many modifications of reperfusion conditions are possible and failure to find one that reduces cell death in reperfused myocardium does not dispute the existence of reperfusion injury. As we argue below, causes of certain forms of lethal reperfusion injury have now been identified and thus some modifications of the conditions of reperfusion are indeed known to provide protection. If such a modification preventing cell death in reperfused myocardium has been identified yet another question may be raised. In experimental models, cell death is normally assessed within a few hours of reperfusion. It therefore remains possible that the tested modifications of reperfusion conditions only delay the full expression of cell death beyond the time of assessment and thus falsely suggest prevention of lethal reperfusion injury. This problem is not specific for studies on reperfusion protection, however. It applies to all research directed at reducing infarct size.

The whole topic of lethal reperfusion injury can be broken down into a logical tree of problems (Fig. 1). Reversible injury must be differentiated from lethal, irreversible injury. In this overview, we will concentrate on the development of the acute form of cell death, i.e. necrosis occurring within the first minutes to first few hours of reperfusion. Further below, we will also discuss the possibility of delayed cell death by processes of necrosis or apoptosis. In the normal clinical setting, myocardial injury due to ischemia-reperfusion may appear as a single entity. For the understanding of reperfusion injury ischemic injury must be clearly separated and the dependency of reperfusion injury on preceding ischemic changes must be clarified. Injury of ischemic-reperfused myocardium is complex, involving injury of vascular cells as well as cardiomyocytes. The problems of an inadequate redistribution of blood flow in reperfused myocardium are not here addressed. We will primarily discuss whether lethal reperfusion injury of cardiomyocytes (as opposed to other cells in myocardium) can occur during the first minutes after adequate reflow in the setting of ischemia-reperfusion, i.e. we will concentrate on immediate lethal (necrotic) reperfusion injury. This can be distinguished from delayed lethal (necrotic) reperfusion injury which, e.g., may be delivered to the cardiomyocytes by activated polymorphic neutrophils, and also from induction of apoptosis of cardiomyocytes in reperfused myocardium.

2. Three initial causes of immediate lethal reperfusion injury

We address the question whether causes for immediate lethal reperfusion injury of cardiomyocytes exist in mammalian hearts and how one can interfere with their mechanisms. Since specific strategies against immediate reperfusion injury have never been applied to human myocardium in vivo except for antioxidative treatment, it must remain open now if the lessons learned from experimental animal studies can be applied to the human case. Three potential initial causes of immediate reperfusion injury, apart from oxygen radicals, have been experimentally investigated in considerable detail, and will be briefly discussed: cause 1, re-energization; cause 2, rapid normalization of tissue pH; and cause 3, rapid normalization of tissue osmolality.

These potential initial causes are not entirely independent. As outlined below, mechanical disruption of the sarcolemma appears to be the endpoint of immediate lethal reperfusion injury. Hypercontracture of the myofibrils is probably one of the major final causes. Hypercontracture is made possible by re-energization of the ischemic cell [cause 1] in which destructive contractile forces are gener-

![Fig. 1. Conceptual differentiation of ischemia-reperfusion injury of myocardial tissue. This review is focused on lethal injury, caused by necrosis as opposed to apoptosis. The eliciting cause originates during reperfusion as opposed to ischemia. The affected cells of interest are cardiomyocytes as opposed to other constituent cells of the myocardium. The injury appears immediately upon reperfusion as opposed to delayed development.](image-url)
ated due to Ca\(^{2+}\) overload and increased cytoskeletal fragility. Ischemic acidosis can attenuate this contractile activation. Rapid normalization of tissue pH (cause 2) can act as a permissive factor for hypercontracture elicited by re-energization and can also contribute to further Ca\(^{2+}\) overload. Cell swelling is the other major final cause for immediate lethal reperfusion injury. It originates in the reperfusion situation from a too rapid normalization of extracellular osmolality (cause 3), leaving the intracellular fluid hyperosmolar.

2.1. Role of re-energization

About two decades ago, Hearse and co-workers [7] demonstrated that in the oxygen-depleted and reoxygenated myocardium, the severe myocardial injury, characterized by myofibrillar hypercontracture and sarcolemmal disruption, may develop with the onset of reoxygenation. It has been demonstrated by Ganote and co-workers [8,9] that this injury is due to the resumption of energy production upon reoxygenation. This phenomenon of severe cell injury immediately provoked by re-energization has been termed ‘oxygen paradox’. It has remained an open question for a long time whether the oxygen paradox represents genuine ‘reoxygenation injury’ or just a dramatic manifestation of injury that has already developed during the oxygen depletion period. The presence of contraction bands in infarcted myocardium is a histological indicator of oxygen paradox injury in ischemic-reperfused myocardium. Histologic analysis clearly demonstrates that when reperfusion is performed early enough to produce some myocardial salvage, infarcts are composed almost exclusively of contraction band necrosis reflecting hypercontracture of myocytes, and there is evidence indicating that this hypercontracture occurs during the first minutes of reflow. Although contraction bands can be observed in the absence of necrosis in specific circumstances (as an artifact in biopsies), in the setting of reperfusion, hypercontractured myocytes invariably present signs of necrosis, indicating that, as opposed to what happens in isolated cardiomyocytes, reperfusion-induced hypercontracture is associated with sarcolemmal disruption and cell death.

Details of the causal mechanism of the oxygen paradox have now been identified in experimental studies using isolated cardiomyocytes (see below). These studies have shown that the oxygen paradox is indeed injury: (1) brought about by the process of reoxygenation; and (2) based on a mechanism within the myocardial cell. Re-energization causes lethal cell injury by provocation of hypercontracture. The mechanism is the following: after prolonged energy depletion, cytosolic Ca\(^{2+}\) concentration is dramatically increased. Upon re-energization of the myocardial cell, made possible by resupply of oxygen to mitochondria, two processes are simultaneously activated: (1) the energy supply to cation pumps initiates recovery of the cellular cation balance; and (2) resupply of energy to the myofibrillar elements initiates contractile activation.

(1) Under conditions of energy depletion, i.e. in ischemic or hypoxic myocardium, the cytosol of the myocardial cells becomes loaded with Na\(^{+}\) and Ca\(^{2+}\). Recovery of energy production upon the resupply with oxygen and metabolic substrates rapidly reactivates two major cation pumps (Fig. 2): namely the Ca\(^{2+}\) pump (Ca\(^{2+}\)-ATPase) of the sarcoplasmic reticulum (SR) and the Na\(^{+}\) pump (Na\(^{+}\)-K\(^{+}\)-ATPase) of the sarcolemma, unless these pumps are themselves injured by the preceding ischemic conditions. Activation of the Ca\(^{2+}\) pump of the SR leads to a temporary sequestration of excess Ca\(^{2+}\) within this intracellular storage organelle [10,11]. If the capacity of this organelle is too small for the amount of Ca\(^{2+}\) accumulated in the cytosol, a cycle of continuous release and reuptake of Ca\(^{2+}\) from and into the SR is initiated. These spontaneous oscillations come to an end only if the major mechanism for Ca\(^{2+}\) extrusion from the cytosol is sufficiently activated, i.e. the Na\(^{+}\)/Ca\(^{2+}\) exchanger of the sarcolemma operating in its ‘forward mode’ [11]. The ability of this exchanger to remove Ca\(^{2+}\) from the cytosol depends on the magnitude of the transsarcolemmal Na\(^{+}\) gradient. Restoration of a sufficiently large Na\(^{+}\) gradient across the sarcolemma is therefore the prerequisite for extrusion of Ca\(^{2+}\) from reoxygenated myocardial cells. It is essential that the Na\(^{+}\) pump of the sarcolemma is rapidly activated to remove excess Na\(^{+}\) from the interior of the cell. It was shown on the cellular level that the re-energized cardiomyocyte can retain sufficient metabolic competence to rapidly reactivate the SR Ca\(^{2+}\) pump and the sarcolemmal Na\(^{+}\) pump during the early phase of reoxygenation, even if the cell had been extensively de-
pleted of its energy stores and suffered from severe Ca\(^{2+}\) and Na\(^{+}\) overload before re-energization [10–12]. It seems that cells in which these pumps have been crucially damaged during the ischemic period are in principle unable to recover and cannot be, therefore, subject to reperfusion injury in the sense of the definition given above. The intracellular accumulation of Ca\(^{2+}\) is likely to continue in such cells as a diminished Na\(^{+}\) gradient favors Ca\(^{2+}\) entry through a Na\(^{+}\)/Ca\(^{2+}\) exchanger operating in ‘reverse mode’.

(2) It is the resupply of energy to the myofibrillar elements in the presence of an increase of cytosolic Ca\(^{2+}\) concentration which may become deleterious for the re-oxygenated cell (Fig. 3). This is because during the initial phase of reoxygenation, the cytosolic Ca\(^{2+}\) is still largely elevated and myofibrillar activation therefore leads to uncontrolled, excessive force generation. This sustained force generation causes hypercontraction. A hypercontracting cardiac muscle cell becomes severely injured in its cytoskeletal structures as the deformation of cytoskeletal elements beyond the degree found under normal contractile shortening is no longer readily reversible. The resulting state of irreversible cell shortening is called ‘hypercontracture’. In tissue, hypercontraction of adjacent cells may lead to mutual cellular disruptions and necrosis. This pathomechanism of reoxygenation-induced mechanical injury can be prevented if the contractile machinery is inhibited during the first stage of energy recovery, for the time needed to re-establish a normal cellular cation control. It has been demonstrated in several studies that a direct blocker of the myofibrils, 2,3-butanedionemonoxime (BDM), can be used experimentally to inhibit the myofibrillar machinery during the early ‘vulnerable phase’ of reoxygenation [13–17]. These studies from different groups have involved different models (isolated myocytes [13], isolated rat heart [14], isolated guinea pig heart [17], in situ pig [15] and dog heart [16]), different conditions (anoxia-reoxygenation [13,14], ischemia-reperfusion [17] transient coronary occlusion [15,16]) and different end-points including ventricular function [17], hypercontracture [13], histochemically determined infarct size [15,16] or extension of myocardial necrosis as assessed by quantitative histology after 24 h of reperfusion [15]. In the study by García-Dorado et al. [15], for example, the left descending coronary artery was occluded for 45 min in an in vivo model (pig) of regional ischemia. Upon reperfusion, BDM was added to the coronary flow and remained there for the first 60 min of normoxic reperfusion. Infarct size determinations after 24 h of reperfusion then demonstrated a reduction by half in BDM-treated hearts.

It has recently been demonstrated that hypercontraction may also be elicited by a closely related mechanism [19]. In cells capable of re-establishing a normal cation control, the initial phase of Ca\(^{2+}\) recovery in the reoxygenated cell may be divided in two stages: (1) an early stage during which the cytosolic Ca\(^{2+}\) level falls due to uptake of Ca\(^{2+}\) into the SR; and (2) a second stage during which Ca\(^{2+}\) is shifted in oscillatory manner between cytosol and SR until a sufficient proportion of the Ca\(^{2+}\) level is extruded across the sarcolemma [10]. The Ca\(^{2+}\) oscillations of stage 2 can also cause hypercontraction. This is not solely explained, however, by the magnitude of the Ca\(^{2+}\) peak concentrations occurring during oscillations. It has been shown that the susceptibility of reoxygenated cardiomyocytes to develop hypercontracture at a given elevation of cytosolic Ca\(^{2+}\), is increased after a prolonged period of hypoxic energy depletion [18]. This means that hypercontraction in energy-depleted and -repleted myocardial cells may be elicited by Ca\(^{2+}\) concentrations in the cytosol which would not cause harm to a normal cell. The cause for this increased susceptibility seems to reside in an increased fragility of cytoskeletal elements which can no longer resist large contraction forces. An alternative explanation would be that the myofibrillar sensitivity to Ca\(^{2+}\) is increased in reoxygenated cardiomyocytes. This has not yet been studied directly. It seems unlikely, however, since studies on reperfused myocardium after short-lived ischemia, exhibiting stunning, have shown rather a reduction of myofibrillar Ca\(^{2+}\) sensitivity [19].

In vitro, it is possible to protect the reoxygenated myocardial cell from hypercontracture by damping the oscillatory movements between Ca\(^{2+}\) and cytosol, thus reducing the high-peak concentrations of cytosolic Ca\(^{2+}\) [18]. This can be achieved by specific blockade of Ca\(^{2+}\) uptake into or release from the SR, as with cyclopiazonic acid or ryanodine, respectively [19]. Interestingly, the volatile anesthetic halothane can also be used to inhibit SR function and thereby provide protection [20]. Halothane applied upon reoxygenation has been shown to protect isolated cardiomyocytes, hypoxia-reoxygenated hearts [21] and ischemic-reperfused myocardium in vivo [22] against hypercontracture and lethal reperfusion injury.
2.2. Role of rapid normalization of tissue pH

The cytosolic pH in cardiomyocytes in reperfused myocardium has a pronounced influence on the development of hypercontracture. After prolonged ischemia, the cytosolic pH is markedly lowered because anaerobic metabolism and the breakdown of ATP produce an excess of H⁺. This leads to an acidification of both the intracellular and the interstitial space. Upon reperfusion, the pH in the interstitial space is rapidly renormalized and a gradient is thus generated between the cytosol, containing still high H⁺ concentration, and the interstitium, where the H⁺ concentration is already re-normalized. This causes an activation of the H⁺ extruding mechanisms of the cardiomyocytes, i.e., the Na⁺/H⁺ exchanger and the Na⁺/HCO₃⁻ symporter [23,24]. This process has two consequences: (1) intracellular acidosis is rapidly reduced. Intracellular acidosis inhibits, however, the myofibrillar machinery, i.e. it exerts an effect similar to the presence of BDM during the early phase of reperfusion [25]. Rapid extrusion of excess H⁺ from the reoxygenated cell thus removes a potentially protective agent (Fig. 4); and (2) activation of the Na⁺/H⁺ exchanger causes a net influx of Na⁺ into the cytosol. Depending on the ability of the Na⁺ pump to remove this excess load of Na⁺, it may come to a secondary activation of the Na⁺/Ca²⁺ exchange mechanism, transporting Na⁺ in the outward direction and Ca²⁺ in the inward direction (‘reverse mode’). This coupled mechanism may enhance the pre-existing Ca²⁺ overload of the cells.

Rapid H⁺ removal and secondary Ca²⁺ uptake thus both favor the development of hypercontracture if ischemic-reperfused myocardial cells are allowed to restore a normal intracellular acid-base balance. It has been demonstrated in vitro that continuation of extracellular acidosis, and thereby intracellular acidosis, during the early phase of reoxygenation protects myocardial cells against the development of hypercontracture during this phase. For reperfusion of myocardium in vivo, the situation is less clear. Inhibitors of the Na⁺/H⁺ exchanger were found to protect against the development of hypercontracture and necrosis during reperfusion only when present during the previous ischemic period [26–28]. The most likely explanation for the discrepancy between the in vitro and the in vivo studies is at present that in blood-perfused hearts, intracellular acidosis cannot be maintained for a sufficiently long time after initiation of reperfusion if only the Na⁺/H⁺ exchanger is inhibited. This is because the myocardial cell possesses another route for the transsarcolemmal extrusion of acid equivalents, i.e. the Na⁺/HCO₃⁻ symporter which works in parallel to the Na⁺/H⁺ exchanger and is active in normal bicarbonate-containing fluids. Unfortunately, specific inhibitors for this mechanism are not yet available for research or therapy.

2.3. Role of rapid normalization of tissue osmolality

One of the major causes for water influx into the ischemic-reperfused myocardial cell seems to be cytosolic Na⁺ overload. The Na⁺/H⁺ exchanger plays a major role in cell volume regulation [29,30]. In ischemic myocardium, the end products of anaerobic metabolism also accumulate, thus increasing the osmotic load in the intracellular and the interstitial space [30]. If, during reperfusion, the extracellular excess of osmotically active molecules is rapidly washed out, an osmotic gradient between the intracellular and the extracellular space is generated [31]. Cellular uptake of water and, through the consecutive increase in intracellular pressure, mechanical stretch of the sarcolemma occurs with a myocardial cell whose mechanical fragility is increased during the preceding energy depletion [32–34]. As is the case for hypercontracture, swelling per se is normally not able to disrupt the sarcolemma, as demonstrated by the maintenance of sarcolemmal integrity in isolated cardiomyocytes subjected to osmotic stress in normoxic conditions. However, the mechanical stress caused by swelling may add up with other sources of stress and then result in cell deterioration (Fig. 5). In isolated cardiomyocytes, osmotic stress results in sarcolemmal disruption only if the cell develops hypercontracture and has previously been submitted to prolonged energy deprivation [35]. The combination of these factors seems to increase sarcolemmal fragility and render the cell thus more susceptible to damage by osmotic stress. The results of studies with highly hyperosmotic reperfusion indicates that attenuation of the additional mechanical stress imposed by swelling can limit myocardial necrosis during reperfusion [36–39].

The mechanism of sarcolemmal fragility secondary to energy deprivation is not understood in detail. Alterations
in the lipid composition of the cell membrane, as suggested by the protective effect of treatments preserving the turnover of phospholipids during energy deprivation [34], changes in sarcolemmal proteins [40–42] or changes in the sarcolemma–cytoskeleton anchorage [43,44] could play a role. There is also evidence that sarcolemmal fragility induced by ischemia can be aggravated during the first moments of reperfusion [33,42]. It has recently been demonstrated that enhanced susceptibility of reoxygenated myocardial cells to osmotic injury can be reduced by specific interventions during the early phase of reoxygenation [33]. Effective measures were additions of NO donors in high concentration and of an antilipid peroxidant or means increasing the cellular glutathione pool. The results suggest that mechanical fragility of the sarcolemma is increased by radical mechanisms during the early period of reoxygenation. It must be said clearly that, in this role of enhancing sarcolemmal fragility, oxygen radicals are a factor of secondary importance for reperfusion injury, when seen in relation to the whole scenario.

3. Following initial causes: spreading of necrosis

Several lines of evidence indicate that reoxygenation-indu ed hypercontracture and sarcolemmal disruption are markedly influenced by cell-to-cell interactions [45,46]. Histologic observations have shown that the areas of contraction-band necrosis induced by transient coronary occlusion followed by reperfusion are composed of hypercontracted myocytes connected to each other to form a continuum, of which the often complex geometry cannot be explained by gradients of flow or microvascular distribution [47]. Computer simulation studies indicate that some kind of cell-to-cell interaction must be taken into account to explain these histological features, and that in the absence of such interaction, hypercontracted myocytes should be scattered across the area of risk instead of forming continuous zones of necrosis. It has been suggested that this cell-to-cell interaction could be mechanical, the exchange of forces imposed by tight intercellular junctions tearing apart the sarcolemma of myocytes hypercontracting during in situ reperfusion, and damaging the sarcolemma of adjacent cells [45,46] (Fig. 6). But the interaction between adjacent myocytes leading to cell-to-cell progression of hypercontracture could be also chemical. Ca\(^{2+}\) and other second messengers may diffuse freely through gap junctions and thereby transmit the trigger for hypercontracture. Recent studies in pairs of isolated cardiomyocytes have demonstrated that hypercontracture of one cell induced by sarcolemmal disruption or microinjection of Ca\(^{2+}\) can induce hypercontracture of adjacent cells. This transmission of injury is associated with passage between adjacent cells of dye permeating gap junctions. The gap junction uncoupler heptanol prevents both dye passage and transmission of hypercontracture. The intracoronary administration of heptanol during the first minutes of reperfusion significantly reduces infarct size in the in situ pig heart submitted to transient coronary occlusion.

Fig. 5. Scheme of osmotic control in the cardiomyocyte upon reperfusion in the cardiomyocyte. Ischemia creates an intra- and extracellular increase of osmolality. Upon reperfusion the extracellular osmolality is rapidly normalized (‘small Osm’) and thus a transsarcolemmal osmotic gradient is created. This leads to water influx and cell swelling. Circumstances of ischemia and reperfusion independently augment sarcolemmal fragility. Increased sarcolemmal fragility and cell swelling together favor rupture of the sarcolemma.

Fig. 6. Scheme of spreading of necrosis. Once a first cell has developed Ca\(^{2+}\) overload this can be communicated in a gap junction-dependent way to adjacent cells creating hypercontracture also in these cells, favored by their increased susceptibility to hypercontracture. Exchange of large mechanical force between a hypercontracting cell and its neighbors through the intercalated (interc.) discs can cause their mechanical disruption. Thus, both chemical and physical cell–cell interactions can contribute to spreading of necrosis.
These results are consistent with the hypothesis that cell-to-cell transmission of hypercontracture may cause spreading of necrosis contributes to reperfusion injury.

4. The problem of delay of necrosis and initiation of apoptosis

Some interventions applied at the time of reperfusion can apparently reduce the extent of irreversible tissue injury when this is investigated early, but, in fact, may only delay the manifestation of irreversible injury (discussed in [1,5]). If this is the case, such an intervention does not provide true reperfusion protection. There are indeed some cases known where an apparent protective effect was later found to be only imitated by a time delay in development of the markers of necrotic tissue injury. This must be distinguished from injury of myocardium by causes appearing not during the early, but during the late phase of reperfusion, e.g. tissue injury by invasion of activated neutrophils.

It is another question whether reperfused myocardium may also become subject to apoptosis, i.e. programmed cell death, even if effectively protected against immediate necrotic injury. Apoptosis is a transcriptionally controlled cellular response to moderate cell injury or to the influence of various cytokines. In contrast, necrotic cell death is the consequence of severe structural cell damage and is not transcriptionally regulated. Cells which have entered the apoptotic process retain physical integrity of the plasmalemma initially even though its chemical structure may change. The point where the process becomes irreversible seems to be the activation of endonucleases severing genomic DNA at internucleosomal sites what is then taken as a characteristic feature of cell death by apoptosis. In a number of recent papers, evidence for apoptotic cell injury in ischemic-reperfused myocardium and in border zones of ischemic myocardium has been demonstrated [49–55]. This gives rise to the question if reperfusion of severely ischemic myocardium can be followed by delayed apoptotic cell death which could abolish all short-lived protective effects against the acute onset of necrosis during reperfusion. The real contribution of apoptosis to cardiomyocyte death has not yet been established. To date, the factors initiating apoptosis in ischemic-reperfused myocardium are still unclear. It is also an open question whether the apoptotic process observed in reperfused myocardium is due to triggers during the time of ischemia or during reperfusion. If the latter is the case, it seems reasonable to expect that possibilities are found to inhibit the full development of apoptotic cell death as the whole process of apoptosis is a multi-stage metabolic mechanism with many possible sites of inhibition.

5. Conclusions

After prolonged periods of energy depletion the ischemic myocardial cell can be jeopardized by specific causes within the reperfusion period (Fig. 7). These causes can be viewed as unwanted aspects of the recovery process itself limiting its efficiency. Understanding of the basic causes has opened novel perspectives for specific interference with these serious pathomechanisms. The experimental results encourage the development of therapeutic approaches to reduce infarct size by specific measures applied during the early phase of reperfusion. The principles of the protective interventions during the early stage of

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Fig. 7. Scheme of factors contributing to immediate lethal (necrotic) reperfusion injury of the cardiomyocyte. Enhancing influences are marked with a plus sign, inhibitory influences with a minus sign.
reperfusion are: (1) inhibition of contractile activation; (2) prevention of intracellular Ca\textsuperscript{2+} oscillations; (3) preservation of intracellular acidosis; (4) reduction of cell swelling; (5) prevention of spreading of necrosis. Suppression of generation of oxygen radicals may be beneficial as oxygen radicals can augment the fragility of the sarcolemma initiated already by the ischemic conditions. Enumerating these principles for early interventions is not meant to deny that measures aimed to limit necrosis or apoptosis occurring at a later stage of reperfusion can be of additional value. And, of course, it is not to deny either that interventions prior to the onset of ischemia may, when applicable, represent the most effective measures to protect the ischemic-reperfused heart.

The principles of protective interventions directed specifically at reperfusion injury should be further tested and validated in animal and human studies. In experimental animals, transient coronary occlusion and reperfusion of myocardium in situ can be rigorously controlled. Animal models with negligible native collaterals, such as pigs, are preferable to those with large, variably developed collaterals, in which the confounding effect of collateral flow must be accounted for by statistical methods. The area of necrosis is to be measured after a prolonged time of reperfusion and referred to the area at risk. A prolonged reperfusion time of 24–72 h allows a more precise histological quantitation of necrosis than shorter times and excludes the possibility that an apparent reduction of infarct size observed early after onset of reperfusion is subsequently overcome by delayed necrosis or apoptosis. Clinical studies on lethal reperfusion injury require not only a high degree of certainty about its existence and mechanisms, but the availability of safe and applicable treatments. For example, while transient contractile blockade during reperfusion with BDM fulfills the design requirements of animal studies, the toxicity of this drug has prevented so far its use in human studies. On the contrary, the protective effect of halothane against reperfusion injury could be easily tested in humans. The development of coronary angioplasty as a technique of reperfusion during acute myocardial infarction gives, to the clinicians, the opportunity to evaluate protective interventions applied just at the time of reflow. The intravenous or even intracoronary administration of the cardioprotective agent to be tested would start just at the end of the first balloon inflation. It would then be present during the first phase of reflow to mimic experimental designs used in anesthetized animal models. Measurement of infarct size in humans is subject to many limitations, but adequate enzymatic and isotopic methods have been proven able to detect differences in infarct size between groups of patients. Although there are no exact methods to quantify the mass of myocardium at risk in patients with evolving acute myocardial infarction, ECG and regional wall motion can be used as means for a clinical classification. Ultimately, the therapeutic value of protective treatments has to be established in large scale studies evaluating the improvement of posts ischemic myocardial function and mortality. The problems arising for evaluating new strategies of protection against lethal reperfusion injury in patients do not differ from those for any other new clinical strategy aiming at reduction of infarct size in the ischemic-reperfused heart.

In summary, the novel strategies for reperfusion protection here discussed have, to date, only been studied experimentally and the number of studies is relatively small. The results of these studies has conclusively demonstrated that it is possible to markedly reduce myocardial necrosis by treatments applied at the time of reperfusion, and provided precise mechanistic explanations for this beneficial effect within the frame of our current understanding of myocyte death during ischemia-reperfusion. This is in contrast to the weight of investigations testing the strategy of preventing injury due to oxygen free radicals in reperfusion injury. The failure of the latter strategy has hindered research on this important pathophysiological problem during the recent years. The knowledge on the basic causal mechanisms of reperfusion injury, reviewed in this paper, no longer justifies abstention from intensive research on new principles of reperfusion protection.

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