Necrosis and apoptosis are two forms of cell death in the myocardium that have been associated with ischemia and reperfusion. Although it has been well documented that necrosis, as a major form of myocyte cell death, rapidly leads to a destruction of a large group of cells after myocardial ischemia and reperfusion, the induction of apoptosis in myocardium, primarily triggered during reperfusion, may independently contribute to the extension of cell death (i.e. infarction) in a dynamic manner. Ischemic preconditioning (IP), an endogenous protective mechanism rapidly evoked by a brief period of ischemia, is recognized for its protection in limiting myocardial necrosis, but has also shown a profound inhibition in apoptosis. While much research has been done on the reduction of necrosis by IP, there remains no clear understanding of the time course of inhibition of apoptosis by IP after long-term reperfusion. In this review, we will show the time course of necrosis and apoptosis during reperfusion, focus on the role of apoptosis in the extension of lethal myocyte injury, and summarize the potential mechanisms involved in reducing apoptosis by IP. ‘Classic’ or ‘early’ IP has been shown to reduce apoptosis in part by inhibiting inflammatory cell activation and altering the expression of anti- and pro-apoptotic proteins as well as protein kinase C activity. It remains unknown, however, whether delayed IP participates in the attenuation of apoptosis in addition to necrosis. The demonstration of a ‘window of opportunity’ in inhibiting apoptosis by IP will provide new directions in research investigating mechanisms underlying myocyte cell death during reperfusion, and translate this information into new treatment approaches for reducing the extent of ischemia/reperfusion injury.

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Keywords: Apoptosis; Ischemia; Necrosis; Preconditioning; Reperfusion

1. Introduction

Prolonged and unresolved regional myocardial ischemia without reperfusion inescapably causes myocyte cell death. Although the early restoration of blood flow to the ischemic myocardium is necessary to salvage myocytes from eventual death, abundant evidence indicates that reperfusion after even a brief period of ischemia has additional deleterious effects on the ischemic myocardium that are not expressed during ischemia, and that can be modified by interventions given only at the onset of reperfusion [1–3]. Experimental studies have shown that myocytes undergo cell death during ischemia and reperfusion by two mechanisms: necrosis and apoptosis. Necrosis is a major form of pathological cell death that rapidly leads to the destruction of myocytes after myocardial ischemia [4]. However, we have previously found that necrosis develops over a period of time during the early phase and the late phase of reperfusion after a brief period of regional ischemia, suggesting a dynamic pathological process [5]. It has recently been reported that apoptosis, a morphologically defined form of programmed cell death, may independently contribute to irreversible myocyte injury [6–11]. Although some studies have shown that myocardial apoptosis occurs during ischemia, a growing body of evidence indicates that apoptosis is primarily expressed during reperfusion [6,7,12,13]. Furthermore, apoptotic cells from regions adjacent to necrotic tissue in myocardium and brain have been loosely associated with the extension of infarction over the course of prolonged reperfusion.
The process of necrosis and apoptosis may differ in a number of ways, and may seemingly proceed down separate paths. However, it is becoming more evident that there is overlap or cross-over (i.e. switch from apoptosis to necrosis) between these two types of cell death under some pathologically stimulated conditions that share similar mechanisms [16,17]. With this advance in knowledge, therefore, an interrelationship between necrosis and apoptosis after ischemia and reperfusion may offer new opportunities to reduce total myocardial injury after an ischemic event, with the ultimate clinical aim of reducing post-infarct morbidity and mortality.

In the last two decades, numerous investigations have been undertaken to identify therapeutic approaches to limit reperfusion-induced extension of infarct size following myocardial ischemia. Many studies have focused on targeting and exploring some pharmacological agents in an attempt to reduce experimental infarct size. In the long run, however, results have been rather unsatisfactory, and many pharmacologic agents simply delay injury, rather than permanently reduce infarct size [2,18–20]. In 1986, Murry et al. first described that ischemic preconditioning (IP), defined as a repetitive brief exposure of the myocardium to ischemia, protected the heart against infarction resulting from a subsequent longer ischemic insult [21]. The impact of this profound cardioprotection was heightened at that time by the failure of pharmacologic agents to significantly reduce infarct size. The effects of IP are manifested primarily as a reduction in infarct size [21], preservation in endothelial function [22,23] and an attenuation of the neutrophil-mediated inflammatory response in the involved myocardium [23,24]. The understanding that IP could also inhibit apoptosis has recently emerged. In this review, we will discuss recent data regarding myocyte cell death during ischemia and reperfusion, primarily focusing on the time course of ischemia/reperfusion-induced apoptosis. An understanding of possible mechanisms involved in the reduction of apoptosis by IP might be helpful to minimize myocyte loss and thereby reduce total infarct size that may consist of both necrotic and apoptotic tissue.

2. Involvement of necrosis and apoptosis in myocardial injury during ischemia and reperfusion

There is growing evidence that two general mechanisms are responsible for myocyte cell death during myocardial ischemia and reperfusion, i.e. necrosis and apoptosis. Myocytes undergoing necrosis and apoptosis show characteristic morphologically and biologically distinct features. Necrosis is, in general, a rapidly occurring form of cell death that may trigger a significant inflammatory response. Typically, changes in cells include severe cellular and organelle swelling, denaturation and coagulation of cytoplasmic proteins and breakdown of cell organelles. Ultrastructural changes are related to the lack of oxygen, depletion of ATP, loss of calcium homeostasis and defects in membrane permeability. In addition, contracture may precipitate membrane disruption and eventual cell death [25]. Apoptosis, in contrast, is an energy-dependent process in which cell death follows a genetically controlled programmed sequence of events. Its chief morphologic features include loss of cell membrane phospholipid asymmetry, condensation of chromatin, and formation of cytoplasmic blebs. In the final stage of apoptosis, cellular fragments form into membrane-bound apoptotic bodies that confine intracellular contents and prevent initiation of an inflammatory response [8–10,26]. Existence of both types of cell death simultaneously in myocardium may co-determine the final degree of lethal myocardial injury after ischemia and reperfusion [9,18,27].

2.1. Evidence showing myocyte cell death by a necrotic pathway

It has been well-documented in animal studies that myocardial necrosis rapidly develops after ischemia, progressing from subendocardial to subepicardial regions of the left ventricular free wall with increasing duration of coronary occlusion. As Reimer et al. [4] reported in a canine model of coronary occlusion, necrosis reaches its full transmural extent after 6 h of ischemia without reperfusion. In agreement with this report, we found that 72% of the area-at-risk myocardium became necrotic after 7 h of persistent ischemia in a canine model [12]. However, infarct size may be determined not only by the duration of ischemia, but also by pathological events occurring during reperfusion. Several studies have provided evidence strongly supporting the existence of reperfusion-induced necrosis in acute open-chest models after a few hours of reperfusion [28,29]. Little attention has been placed, however, on the time course of reperfusion-induced cell death over a period of days after a fixed ischemic time period. Failure to identify a progressive nature of reperfusion-induced cell death may be related to the interval between the onset of reperfusion and the time in assessing cell death. We recently quantified the extent and time course of necrosis (TTC, triphenyltetrazolium chloride vital staining) in a chronic canine model during 6–72 h of reperfusion after a fixed 1 h ischemic period. The area of necrosis extended from 27±2% after 6 h of reperfusion to a peak of 41±2% after 24 h of reperfusion, with no further increase at 48 and 72 h [5]. These results are consistent with a previous report by Rochitte et al. [30] that infarct size defined by contrast-enhanced MRI progressively developed between 2 and 48 h after the onset of reperfusion. Therefore, these data suggest that necrosis is a dynamic pathological process that continues over at least 24 h of reperfusion after a fixed duration of ischemia.
2.2. Evidence showing myocyte cell death by an apoptotic pathway

Several previous studies suggest that ischemia/reperfusion induces myocardial apoptosis within the involved myocardium [6,7,31]. However, whether apoptosis is triggered during ischemia or during reperfusion is still controversial. In an in vivo rabbit study, Gottlieb et al. [6] demonstrated that nucleosomal ‘ladders’ of DNA fragments were detected only after reperfusion, but not after a relatively short period of ischemia without reperfusion. However, Kajstura et al. [31] and Fliss and Gattinger [7] reported that apoptosis began in rat ischemic myocardium either after a prolonged period of permanent ischemia or during a much shorter period of ischemia followed by reperfusion. To further confirm that the apoptotic process is a reperfusion-triggered phenomenon, we recently analyzed the appearance of apoptosis versus necrosis in ischemic myocardium after 7 h permanent coronary occlusion or after 1 h of occlusion followed by 6 h of reperfusion in a canine model [12]. Although prolonged ischemia alone caused 72±5% infarct size, very few apoptotic cells (TUNEL staining) were detected in the necrotic area (0.2±0.1% of total normal nuclei), confirmed by an absence of DNA fragmentation. In contrast, a significantly greater number of apoptotic cells were observed in the peri-necrotic area after 1 h ischemia followed by 6 h of reperfusion (26±4% of total normal nuclei), in association with DNA laddering. The peri-necrotic area is defined as the area-at-risk myocardium immediately overlying the necrotic zone outlined by TTC staining. This sampling location avoids issues related to differentiating apoptosis from necrotic tissue. These observations are consistent with the hypothesis that the induction of apoptosis following brief ischemia is largely a reperfusion-associated event (Fig. 1). Other investigators using a canine model of global or regional ischemia and reperfusion also confirmed reperfusion-triggered or accelerated apoptosis [27,32–35]. The depletion of intracellular ATP levels during ischemia blocks the activation of the downstream pro-apoptotic genes, which prevents the typical apoptotic changes from taking place. However, reperfusion rapidly restores the intracellular ATP levels, thereby providing the energy necessary to allow the apoptotic pathway to proceed [36].

It is not clear whether apoptosis precedes or is followed by necrosis, or whether both mechanisms of cell death occur simultaneously by separate pathways. The time course of the development of apoptosis relative to necrosis was investigated in a recent study from our laboratory using a canine model of 1 h regional ischemia followed by 6, 24, 48 or 72 h of reperfusion [37]. As discussed above, the extent of necrosis peaked at 24 h of reperfusion, and remained constant thereafter. In contrast, the appearance of apoptotic cells in the peri-necrotic area progressively increased up to 72 h of reperfusion, which was confirmed by increased intensity of DNA ladders (Fig. 1). These data suggest that necrosis and apoptosis occur simultaneously during reperfusion, with a relatively rapidly developing necrotic cell death during the early phase of reperfusion followed by a slower appearance of apoptosis during the late phase of reperfusion (Fig. 2).

2.3. Role of apoptosis in extension of myocardial injury during reperfusion

The appearance of apoptotic cells in the peri-necrotic zone during reperfusion suggests that apoptosis may be in part responsible for extending infarction over time after the onset of reperfusion [37]. To determine the relative contribution of apoptosis to infarct extension and contractile dysfunction during reperfusion, we used aurantricarboxylic acid (ATA), an endonuclease inhibitor, to examine whether ATA at reperfusion reduces extension of infarction and improves regional contractile function by inhibiting apoptosis [38]. In a closed-chest canine model of 1 h of coronary occlusion followed by 24 h of reperfusion, ATA treatment at the onset of reperfusion significantly reduced the number of apoptotic cells in the peri-necrotic myocardium, consistent with the absence of DNA laddering. This decrease in the extent of apoptosis was associated with a significant increase in Bcl-2 and the decrease in Bax and caspase 3 activity. Importantly, the inhibition of myocardial apoptosis by ATA significantly reduced the area of necrosis. This observation suggests a link between apoptosis and necrosis (in the absence of a direct effect of ATA on pathogenesis of necrosis, such as an anti-inflammatory effect). Some studies have suggested a cross-over of apoptosis to necrosis [16,17]. This hypothesis is supported by some recent reports showing a reduction in infarct size in an in vivo rat model by inhibiting apoptosis acutely [39,40]. It has been demonstrated that the treatment with anti-inflammation at early reperfusion failed to attenuate extension of necrosis seen at late reperfusion [19,41]. It is possible, therefore, that a short period of anti-apoptotic treatment at early reperfusion may also temporally delay, but not permanently reduce apoptosis seen at the late stage of reperfusion due to its dynamic nature. There is no evidence, however, showing that anti-apoptotic therapy alters the time course of apoptosis during early versus late phase of reperfusion at the present time.

3. Possible mechanisms underlying the development of apoptosis during reperfusion

Cell–cell interactions between blood cells and vascular endothelial cells and the release of cytokines and generation of reactive oxygen species from activated neutrophils (PMNs), endothelial cells and myocytes during
reperfusion have been proposed as triggers in the induction of apoptosis. These interactions are initiated within the early movements of reperfusion, and may continue during the ensuing hours and days.

3.1. Apoptosis potentially triggered by infiltrated PMNs

In the inflammatory component of reperfusion-induced myocardial injury, the PMN activation and expression of
Although there was one study showing that infarct size through production of cytokines (i.e. TNF-PMNs have migrated into the interstitium [5,44–46]. of being activated, thereby contributing to tissue injury cell–cell contact between PMNs and myocytes once the PMNs and necrotic debris, macrophages have the potential time increases, injury to myocytes may occur due to direct 62]. In addition to performing phagocytosis by eliminating enzymes from activated PMNs [42–44]. As the reperfusion nant inflammatory cell type in the target tissue [37,52,59–48 h of reperfusion, macrophages constitute the predomi-

adhesion molecules on vascular endothelial cells and myocytes have been linked with reperfusion-induced necrosis. The injurious effects of PMNs on reperfused myocardium during early reperfusion (4–6 h) is related to their adherence to vascular endothelial cells, and to the generation of superoxide free radicals and proteolytic enzymes from activated PMNs [42–44]. As the reperfusion time increases, injury to myocytes may occur due to direct cell–cell contact between PMNs and myocytes once the PMNs have migrated into the interstitium [5,44–46]. Although there was one study showing that infarct size was not altered by reducing PMN accumulation with monoclonal anti-PMN antibody [47], abundant evidence has demonstrated a reduction in necrosis by blocking cell–cell interactions using anti-adhesion molecule antibo-

dynamic progression of cell death. All values are expressed as mean±SEM. *P<0.05 vs. 6 h of reperfusion (data from Zhao et al. [5,37]).

Fig. 2. The time course of development of infarct size (An/Ar, area of necrosis expressed as a percentage of area at risk) and appearance of apoptotic cells (TUNEL positive cells expressed as a percentage of total normal nuclei) after 1 h coronary occlusion followed by 6, 24, 48 and 72 h of reperfusion (R), respectively. Extension of infarct size peaked at 24 h of reperfusion, while the population of apoptotic cells in the peri-necrotic zone from transmural tissue sections progressively increased over the course of reperfusion, indicating a dynamic progression of cell death. All values are expressed as mean±SEM. *P<0.05 vs. 6 h of reperfusion (data from Zhao et al. [5,37]).

Macrophages originate from progenitor cells in the bone marrow and circulate in the blood as monocytes before migrating into tissues. Under normal conditions, the half life of monocytes in the circulation is relatively short and only lasts ~1 day, but the life span of macrophages in [36–58] tissues is several months [52]. In pathological states, there is a greater production of monocytes derived from bone marrow, causing enormous extravasation and accumulation in the affected tissue [59]. Following extravasation from bone marrow, monocytes emigrate in tissue toward the site of injury and undergo transformation into a larger phagocytic cell, the macrophage. Compared with the time course of PMN migration during reperfusion [5], monocytes begin to emigrate relatively slowly. Within 48 h of reperfusion, macrophages constitute the predomin-

apoptotic cells (TUNEL positive cells expressed as a percentage of total apoptotic cells) after 1 h coronary occlusion followed by 6, 24, 48 and 72 h of reperfusion (R), respectively. Extension of infarct size peaked at 24 h of reperfusion, while the population of apoptotic cells in the peri-necrotic zone from transmural tissue sections progressively increased. Data are expressed as mean±SEM. *P<0.05 vs. 6 h of reperfusion (data from Zhao et al. [5,37]).

In contrast to the pathogenesis of necrosis, the precise role of PMN activation in development of apoptosis still remains unclear. One study showed that granulocytopenia did not prevent reperfusion-induced apoptosis in a rabbit model of occlusion–reperfusion [6], while two studies in a rat infarct model showed that increased PMN accumulation in reperfused myocardium is associated with an augmentation of apoptosis [7,24]. In our canine and rat models of regional ischemia and reperfusion, a linear relationship between PMN accumulation in the area at risk and the number of apoptotic cells is consistent with a role of PMN in development of apoptosis [24,37]. In this connection, proinflammatory mediators such as reactive oxygen species and various cytokines released from activated PMNs have been proposed as triggers in the induction of apoptosis [7,9,51]. However, more direct experimental data are necessary to establish a causative role of PMN in reperfusion-induced apoptosis, and to determine the potential mechanisms involved. Therefore, anti-PMN treatment ad-

3.2. Apoptosis potentially triggered by infiltrated monocytes/macrophages

More recently, much attention has been placed on the role of macrophages in the induction of apoptosis. A number of in vitro studies using cultured cell lines have demonstrated macrophage-induced apoptosis through the release of cytokines and cytotoxic agents such as TNFα and nitric oxide [64,66–68]. In isolated neonatal cardiac myocytes [69], macrophage-derived cytokines caused a time-dependent induction of myocardial apoptosis. Recent data from our laboratory showed that most of the macrophages within ischemic/reperfused myocardium main-

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model of 1 h ischemia followed by 48 h of reperfusion showing a role of migrated macrophages in the induction of cerebral apoptosis [70]. However, further study is necessary to elucidate a direct role of macrophages in the pathogenesis of apoptosis.

3.3. Non-inflammatory cell-triggered apoptosis during reperfusion

Soluble inflammatory mediators (i.e. cytokines and reactive oxygen substances) arising locally from vascular endothelium, the myocytes or microcirculatory compartments may play individual roles in developing the short- and long-term responses to reperfusion-elicited injury through the receptor-dependent pathway. These mediators released from the activated endothelium and myocytes in addition to inflammatory cells, may contribute to the induction of apoptosis in isolated-perfused heart after ischemia and reperfusion (see below for detail).

3.4. Some considerations of methods used to detect apoptosis

Demonstration of apoptotic myocytes after ischemia and reperfusion in most current studies is primarily based on TUNEL staining. Limited by the specificity of the TUNEL method as reported previously [10,71], it is very possible that infiltrated inflammatory cells, necrotic cells and damaged endothelial cells may occasionally positively be stained in a TUNEL assay. Therefore, the number of TUNEL positive cells in whole tissue may be over-estimated. For this reason, differentiation of cell types in tissue section by using double staining and monitoring duration of apoptotic process by a combination of the methods with microscopic evaluation, analysis of pro- and anti-apoptotic proteins and down-stream caspase activation, detection of mitochondrial function as well as DNA fragmentation are highly recommended [10,71]. Lack of TUNEL positive cells in necrotic tissue, absence of TUNEL positive neutrophils confirmed by double staining with monoclonal anti-neutrophil antibody and TUNEL stain in the same tissue section as shown in Fig. 3, time-dependent changes in pro- and anti-apoptotic proteins such as Bcl-2, p53 and Bax, and progressively increased intensity of DNA fragmentation (not smear pattern) during reperfusion from our previous studies [12,37] supported that the dead cells detected from the peri-necrotic zone are mainly apoptotic cells. Since reduction in infarct size can be achieved by anti-apoptotic therapy, therefore, apoptosis developed during reperfusion may participate in extension of infarct size [38].

4. Signal transduction pathways involved in the induction of apoptosis

It is generally accepted that the process of apoptosis involves the activation of death receptor-dependent and -independent signal transduction pathways [8,26]. The binding of pro-apoptotic ligands to their receptors initiates a process that results in an imbalance in regulating proteins (i.e. Bcl-2 family) and an activation of cytosolic proteases (i.e. caspase family). The direct stimulation of the receptor-independent pathway such as activation of mitochondria induces the release of cytochrome C and forms a complex with Apaf-1 (apoptosis protease activating factor-1) and procaspase 9, and further co-induces the activation of downstream caspases. It has been confirmed that the change in status of caspases from the inactive to the active form by both stimulating pathways is the key step to induction of apoptosis [10].

4.1. Initiation of cell death by receptor-dependent and -independent pathways

Death receptors located on the cell surface include TNFR1 (also called p55 or CD120a), Fas (CD95 or Apo1), DR3, DR4 and DR5. The binding of TNFα, FasL and TRAIL (TNF-related apoptosis-inducing ligand) to their respective receptors initiates signal transduction pathways that result in the induction of apoptosis (Fig. 4). The intracellular portion of these death receptors includes Fas-associated death domain (FADD) and TNFR-associated death domain (TRADD). FADD reacts with Fas and DR3, whereas TRADD interacts with TNFR1 and DR4 after stimulation of the respective receptors. In addition, the stimulating signal from TNFR1 is also linked to the Fas signaling pathway through the interaction between TRADD with FADD [72,73].

One of the best characterized pathways involved in the execution phase of apoptosis after stimulation of death
Fig. 4. Mechanisms of receptor-dependent and -independent caspase activation. Binding of TNFα and Fas-L to their receptors leads to activation of procaspase 8 and 10 through the TNF-receptor and fas-associated death domain (TRADD and FADD, respectively). Activated caspase 8 and 10 then cleave caspases 3, 6, and 7 to their activated forms, eliciting subsequent apoptotic cell death. Binding of TRAIL (TNF-related apoptosis-inducing ligand) with death receptor 4 (DR4) can also be initiated via TRADD signaling pathway. Furthermore, direct stimulation by cellular stress increases mitochondrial permeability transition pore and induces cytochrome C release through receptor-independent signaling pathway. Cytochrome C then forms a trimeric complex with apaf-1 (apoptosis protease activating factor-1) and procaspase-9 and further activates caspase 3 initiating a cascade of apoptotic cell death. Alternatively, TNFα-receptor binding may lead to activation of the c-jun N-terminal kinase (JNK) from p38 mitogen-activated protein kinase (MAPK) family and formation of the nuclear transcription factor KB (NF-kB), respectively, to induce apoptotic cell death in the heart. AIF, apoptosis-inducing factor.

Receptors primarily depend on the sequential activation of a series of cytosolic proteases, termed caspases [10]. Under normal conditions, caspases are synthesized as inactive pro-caspases. During the induction of apoptosis, procaspases are activated through a proteolytic process leading to a self-amplifying cascade of proteolysis and cleavage of many cellular proteins. The Fas-FADD and TNFα-TRADD-D-elicited execution pathways initially involve the recruitment of procaspases (i.e. caspase 8 and caspase 10), followed by activation of downstream caspases (i.e. caspases 3, 6, and 7). Caspase 3 has now been implicated as a key protease that promotes the cleavage of cytoskeletal and nuclear proteins, resulting in the biochemical and morphological hallmarks of apoptosis after ischemia and reperfusion (Fig. 4).

The receptor-independent apoptotic pathway involves the release of cytochrome C and AIF (apoptosis-inducing factor) into the cytoplasm from the intermembrane space of mitochondria in response to cell stimulation (Fig. 4). Upon release from the mitochondria, cytochrome C directly forms a trimeric complex with Apaf-1 in an ATP-dependent manner. This complex activates procaspase 9 resulting in cleavage into caspase 9, the most upstream caspase in the mitochondrial apoptotic pathway [74]. The release of activated caspase 9 subsequently cleaves procaspase 3 into its activated form, caspase 3. AIF may independently exert its apoptotic action by directly stimulating caspases.

Several studies have provided evidence of mitochondrial dysfunction and caspase activation in ischemia/reperfusion-induced myocardial apoptosis. The release of cytochrome C and the cleavage of caspases 3 and 9 have been demonstrated in cultured apoptotic cardiomyocytes exposed to hypoxia and reoxygenation [75], in vivo rat model of regional myocardial ischemia and reperfusion [76] and in oxidative stress-mediated ventricular dysfunction and failure in canine models [77]. Furthermore, apoptosis detected in human cardiomyopathy has also been associated with activation of caspase 3 through the release of cytochrome C from mitochondria [78]. In a recent study, we found that activated caspase 3 was highly expressed in the ischemic myocardium in a canine model of ischemia and reperfusion [38]. Accordingly, inhibition of caspase activation has been associated with the attenuation of apoptosis and reduction in infarct size [76,79]. However, it is not clear whether caspase activation is involved in the time-dependent development of apoptosis during reperfusion, or whether a short period of anti-
caspase treatment at early reperfusion permanently inhibits upregulation of caspase activity in the late phase of reperfusion.

4.2. Regulation of cell death by intracellular proteins

Among intracellular apoptosis-regulating proteins that have been reported, the Bcl-2 family (B-cell leukemia/lymphoma 2-like proteins) plays a pivotal role in regulating the responses of cells to a wide variety of apoptotic signals [15,80,81]. The Bcl-2 family is composed of a large group of anti-apoptotic proteins (Bcl-2, Bcl-xl, Bcl-w, Bag-1 and BI-1) that, when overexpressed, prevent apoptosis and attenuate expression of a large group of pro-apoptotic proteins (Bax, Bak, Bad, Bid and Bim). A number of possible mechanisms in which anti-apoptotic proteins such as Bcl-2 and Bcl-xl regulate apoptosis have been widely reported, including inhibiting the generation of reactive oxygen species [8,9], preventing intracellular acidification and elevation of intracellular calcium [82,83], attenuating the cytotoxic effect of pro-apoptotic Bcl-2 family proteins (i.e. Bax and Bak) [17] and reducing the release of cytochrome C and AIF from mitochondria [84,85]. In transgenic mice in which Bcl-2 is overexpressed, improved cardiac function has been correlated with a reduction of cardiomyocyte apoptosis after ischemia and reperfusion [86]. Furthermore, a clinical study reported that positive Bcl-2 protein expression was detected in salvaged myocytes surrounding infarcted tissues, while Bax was clearly overexpressed in the infarcted area [15].

The tumor suppressor DNA-binding protein p53, functioning as a transcription factor, is the most frequently mutated protein in human tumors. Much evidence suggests that p53 is an intermediate effector of apoptosis, for example, working either by activating the transcription of death proteins (Bax) or suppressing the transcription of survival proteins (Bcl-2), or both [26]. Exposure of cardiomyocytes to reactive oxygen substances has resulted in increased levels of p53 proteins [87]. In a recent study using isolated working rat hearts, downregulated Bcl-2 and upregulated p53 have been associated with induction of apoptosis after ischemia and reperfusion [88]. To illustrate the time course of expression of these anti-apoptotic and pro-apoptotic proteins and correlate their changes with the development of apoptosis during ischemia and reperfusion, we recently demonstrated that ischemia without reperfusion did not induce apoptosis or alter the appearance of apoptotic regulating proteins [12]. However, a short period of ischemia followed by reperfusion induced a time-dependent reduction in the expression of Bcl-2 protein and increase in the expression of Bax and p53 proteins (Fig. 5) [5,37], consistent with the development of apoptosis by TUNEL assay. Taken together, therefore, an imbalance in anti- and pro-apoptotic proteins expressed during reperfusion may be involved in initiating myocyte cell death. Persistently enhanced expression in p53 in tissue during the late stage of reperfusion following ischemia supports this hypothesis [89].

5. Reduction in apoptosis by IP

IP refers to a process in which a brief, reversible period of ischemia followed by reperfusion enhances myocardial resistance to a subsequent longer period of ischemia. In every animal species studied, IP has produced the most reproducible reduction in experimental necrosis [90–92]. Numerous studies have been undertaken to confirm its protective effect and possible mechanisms involved since Murry et al. first described this endogenous protective mechanism in limiting infarct size [21].

5.1. Inhibition of apoptosis by ‘classic’ or ‘early’ IP

‘Classic’ or ‘early’ IP occurs immediately (within minutes), lasts ~1 to 2 h after the preconditioning stimulus, and is then lost [93,94]. Murry et al. showed that IP is ineffective when the sustained ischemia is extended to 3 h [21]. Gross et al. demonstrated that IP reduced infarct size after 60 min of coronary occlusion, but not after 90 min, indicating that timely reperfusion is still a beneficial
strategy in limiting necrosis after ischemia [95]. It is now generally accepted that in the in vivo acute model of ischemia and reperfusion, a profound myocardial salvage from infarction is found if the duration of sustained ischemia is equal to or less than 60 min. Murry et al. reported that early IP consistently reduced infarct size after 4 days of reperfusion, suggesting a permanent reduction in necrosis [21]. Since the development of apoptosis showed a delayed fashion during reperfusion [37], it is unknown whether the level of apoptotic cells seen at late reperfusion could be inhibited by early IP.

Consistent with the inhibition of acute necrosis by early IP, data from in vivo and in vitro studies have shown that IP can also attenuate apoptosis [7,24,96]. In the open-chest rat model, one to five cycles of 5 min IP markedly attenuated the appearance of positive apoptotic cells in conjunction with a reduction in infract size [7]. We have also found that in a rat model of ischemia and reperfusion, a single 5 min of IP preceding 30 min ischemia significantly decreased the population of positive apoptotic cells in the area at risk from 28.6±2.8% in control to 3.4±0.9 in preconditioned hearts (Fig. 6), and reduced the intensity of DNA ladders. In addition, IP decreased the expression of the pro-apoptotic protein, Bax (Fig. 7) [24]. A reduction in apoptosis by IP was also reported in isolated-perfused rat hearts [97,98]. A short period of antecedent IP significantly reduced the number of apoptotic cells and increased the expression of the anti-apoptotic protein Bcl-2 after ischemia followed by reperfusion. It is clear from these studies that attenuation of apoptosis by IP is associated with a reduction in acute necrosis analysed early (<6 h) after reperfusion. It is unknown, however, whether this represents a reduction in overall infarction (necrosis plus apoptosis) or a reduction in the conversion of apoptosis to necrosis. In addition, although apoptosis progresses after reperfusion over a period of days, and IP attenuates apoptosis acutely, it is not known whether a reduction in infarct size measured days after reperfusion is achieved by inhibiting apoptosis.

5.2. Inhibition of apoptosis by delayed IP

IP induces a biphasic pattern of myocardial protection. Following the acute phase of protection by early IP, a delayed phase, termed the ‘second window of protection’ appears between 12 and 24 h after the initial IP stimulus, which lasts up to 72 h [99–101]. Inconsistent results regarding the effect of delayed IP on infarct size have been reported [101–103]. However, there are few studies that have determined the effect of delayed IP on apoptosis. Recently, Baghelai et al. has reported that in isolated-perfused rat heart 24 h after α1-adrenoceptor stimulation with phenylephrine, the number of apoptotic cells in ischemic myocardium was significantly reduced accompanying an increased Bclx/Bax ratio [104]. Although some studies have shown that reduction of apoptosis in delayed phase is associated with activation of protein kinase C isoforms and opening of mitochondrial ATP-sensitive K⁺ channels (see below), no direct evidence at this point suggests that delayed IP inhibits apoptosis.

6. Potential mechanisms involved in attenuation of apoptosis by IP

The potential mechanisms mediating the two windows of protection by IP have been widely explored since the first description of IP by Murry et al. [21]. The results obtained to date suggest that the adaptive cardioprotective achieved by IP is both a receptor- as well as non-receptor-mediated phenomenon, depending upon the type of substances released during ischemia. As shown in Fig. 8, following a brief period of antecedent ischemia, a number of endogenous substances are released that may be involved in early and delayed protection by IP. These endogenously released substances include adenosine [91], bradykinin [105], catecholamines [106], angiotensin II [107], nitric oxide [108,109], free radicals [110], opioids [111,112] and heat shock protein [100,113]. Activation of the corresponding receptors on the cell surface or direct interaction with cells may trigger one or more signal transduction pathways that culminate in the activation of protein kinase C, phosphorylation and/or gene transcription of proteins, and opening of mitochondrial K⁺ channels [113–115]. Since detailed reviews describing the various signaling mechanisms in preventing necrosis appear elsewhere [90,99,110,114], we will focus on a discussion of the possible mechanisms leading to attenuation of apoptosis with IP by targeting its effects on triggers, mediators and end-effectors during the early and delayed windows of protection.

6.1. Protective mechanisms that attenuate the triggers of apoptosis

6.1.1. Inhibition in generation of reactive oxygen substances

As discussed previously, data from in vivo and in vitro animal studies have shown that oxygen free radicals released from inflammatory cells, activated endothelial cells and myocytes after ischemia and reperfusion, may trigger both necrosis and apoptosis [23,116–118]. Several studies have reported that early IP inhibits apoptosis by reducing this burst of reactive oxygen species generated from inflammatory cells (i.e. PMNs and macrophages) and non-inflammatory cell types (i.e. endothelial cells and myocytes). In this regard, a number of antioxidants and free radical scavengers have been reported to inhibit the appearance of apoptosis, which supports reactive oxygen species as triggers of apoptosis [51,95,119]. In an in vivo canine model, Ambrosio et al. reported that recombinant human superoxide dismutase significantly reduced the
Fig. 6. Detection of apoptotic myocytes using TUNEL method and neutrophil accumulation using immunohistochemistry with anti-CD18 antibody (WT3) in ischemic zone in control group (A and C) and ischemic preconditioning (IP) group (B and D), respectively. IP significantly reduced the number of TUNEL positive cells (arrows) and CD18 positive cells (arrow heads) vs. control group after ischemia and reperfusion. Red and brown staining indicate TUNEL positive and CD18 positive cells. (E) Detection of DNA fragmentation using agarose gel electrophoresis from the same study. Numbers at the bottom of the figure indicate the non-ischemic zone (lanes 1 and 3 in the control group and lanes 5 and 7 in the IP group) and ischemic zone (lanes 2 and 4 in the control group and lanes 6 and 8 in the IP group) (data from Nakamura et al. [24]).
number of TUNEL positive cells after 90 min ischemia followed by 48 h of reperfusion [95]. In contrast, Baines et al. found that the elevated levels of reactive oxygen species induced by IP contribute to a reduction in necrosis in the rabbit heart after regional ischemia followed by reperfusion. Since the protection was blocked by an oxygen radical scavenger, MPG administered before IP, the authors concluded that, in contrast to oxygen radical-induced reperfusion injury, increased, rather than decreased generation of reactive oxygen substances might be a factor contributing to the protection rendered by IP [110]. In addition, in cultured cardiomyocytes, Hoek et al. have also found that hypoxic preconditioning induces a transient increase in reactive oxygen species, but attenuates their generation at reperfusion [120]. Apparently, the timing, source and local concentration achieved during release of oxygen radicals during IP and the subsequent longer ischemia/reperfusion may help to explain the protective effect of IP [121]. Transient generation of free radicals at a lower concentration and sub-threshold level for inducing damage seen during IP may act as a trigger for a cascade of intracellular signaling leading to induction of IP. However, preventing the generation of a high concentration of free radicals during the longer period of ischemia and reperfusion by IP may be a mechanism of protecting the myocytes from apoptosis. To link this seemingly paradoxical relationship, Zahler et al. [122] demonstrated in human umbilical vein endothelial cells that transient endothelial cell stimulation with the reactive oxidant H$_2$O$_2$ significantly attenuated TNFα-induced expression of adhesion molecules (i.e. E-selectin and ICAM-1) and release of cytokines (i.e. IL 6 and 8). Since these mediators have been suggested to play a role in eliciting PMN and endothelial cell interactions, the authors conclude that stimulation of a transient sub-threshold (i.e. non-damaging) oxidative stress may awake a kind of anti-inflammatory ‘endothelial preconditioning’ that then reduces endothelial responsiveness to inflammatory mediators. However, in vivo studies are needed to demonstrate such an inhibition after IP on reactive oxygen species generation during IP and the subsequent longer ischemia and reperfusion to confirm this hypothesis.

6.1.2. Direct preservation of vascular endothelium

In isolated Langendorff-perfused rat heart, Scarabelli et al. recently reported that reperfusion-induced vascular endothelial apoptosis precedes myocyte cell apoptosis, suggesting that the release of soluble pro-apoptotic mediators from activated endothelial cells may promote myocyte apoptosis [123]. Therefore, preservation of endothelial integrity by IP against inflammatory cell adhesion may potentially reduce myocyte apoptosis. A report by Thourani et al. [23] from our laboratory found that one cycle of 5 min IP significantly attenuated endothelial dysfunction, expressed as reduced PMN adherence to ischemic/reperfused coronary endothelium, and preserved

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Fig. 7. Expression of Bcl-2 and Bax proteins in control (I/R) and ischemic preconditioning (IPC) groups was visualized by Western blot analysis. Densitometrically, there was no significant difference between the groups in expression of Bcl-2 protein (A). However, overexpressed Bax protein was significantly attenuated in the IP group (B). Immunodetection of actin (top) with a monoclonal anti-actin antibody was performed as an internal control. Nor, normal tissue; *P<0.05 IPC vs. I/R group (data from Nakamura et al. [24]).
and pro-apoptotic proteins, an increased release of cytochrome C from mitochondria, increased caspase activation, and activation of protein kinase C isozymes have been proposed as primary signal pathways involved in the induction of apoptosis after initiation of the death signal stimulus. Through altering these pathways, early IP has shown a profound effect on reducing myocardial apoptosis.

6.2.1. Bcl-2 family and p53

Several studies have shown a reduction of apoptosis by IP via altering the expression of anti- and pro-apoptotic proteins after ischemia and reperfusion. In the isolated-perfused rat heart preparation, Maulik et al. found that four episodes of 5 min IP significantly reduced apoptosis by upregulating Bcl-2 and downregulating p53 expression [88,97]. These effects are independent of action on inflammatory cells. In vivo data from our laboratory showed that early IP did not alter Bcl-2 expression, but significantly reduced the expression of Bax protein in ischemic / reperfused myocardium (Fig. 7), which increases the ratio of Bcl-2 to Bax proteins in preventing the progression of apoptosis in myocardium [24]. In phenylephrine-induced delayed IP, Baghelai et al. [104] also found that an increased Bclx / Bax ratio is associated with a reduction in apoptosis in rabbit heart. An increase in the Bcl-2/Bax ratio prevents cytochrome C release from mitochondria after an apoptotic stimulus [84,85]. Thus far, an attenuation of cytochrome C release from mitochondria by altering Bcl-2/Bax ratio may be an important mechanism of inhibiting apoptosis by IP. In conjunction with progressively developed apoptosis during the late phase of reperfusion, we have previously shown a time-dependent reduction in expression of Bcl-2 and an increase in expression of Bax and p53 proteins after reversible coronary occlusion (Fig. 5) [37]. However, an interrelationship between changes in expression of Bcl-2 family proteins, mitochondrial activity and induction of apoptosis after ischemia and reperfusion by IP in vivo needs to be investigated further in vivo models.

6.2.2. Cytochrome C

Since the release of cytochrome C from mitochondria after ischemia and reperfusion has been suggested as a major player in the induction of the receptor-independent apoptotic pathway, the stabilization of mitochondria may reflect a possible mechanism involved in inhibition of apoptosis by IP. Xu et al. [133] found in cultured myocytes that IP reduced cytochrome C release and the appearance of apoptosis by inhibiting mitochondrial permeability transition. In Langendorff-perfused rat hearts, Wang et al. [134] showed that IP preserved normal mitochondrial function and prevented mitochondrial calcium overload during reperfusion, confirmed by improved cardiac function. Laclau et al. [135] also found that IP maintains normal mitochondrial integrity reflected by decreased permeability to exogenous cytochrome C and preserved agonist-stimulated vascular endothelial relaxation in both coronary macrovessels and microvessels in a canine model of coronary occlusion and reperfusion. This observation is consistent with an attenuation of PMN accumulation in ischemic/reperfused area at risk observed in another study from our laboratory with the same model [124]. These data are in good agreement with previous observations concerning inhibition of PMN-vascular endothelial cell interactions and PMN accumulation by early IP [22,125,126]. Furthermore, in addition to a protective effect of IP in the heart, recent studies have reported similar reduction in PMN-endothelial cell interactions by IP in experimental ischemic/reperfused models in liver [127,128], skeletal muscle [129], lung [130], as well as in humans [131]. To explain possible mechanisms for inhibition of this cell–cell interaction by IP, the downregulation of intercellular adhesion molecules (i.e. CD11/CD18 on neutrophils and ICAM-1 on endothelium) has been proposed [131,132].

6.2. Mechanisms aimed at altering the signal pathways of apoptosis

As previously mentioned, an imbalance between anti-
6.2.3. Caspase

Attenuation of apoptosis after ischemia and reperfusion by inhibiting caspase activity by early IP was initially reported by Piot et al. [136]. In rats subjected to 30 min coronary occlusion followed by 180 min of reperfusion, one cycle of 5 min IP significantly reduced internucleosomal DNA fragmentation and cleavage of caspase 1 and 3. In liver with persistent ischemia, Yadav et al. [137] demonstrated that IP reduced the number of apoptotic cells and improved the animal survival rate through downregulation of caspase 3 activity. Furthermore, using in vivo rat and rabbit models of regional myocardial ischemia and reperfusion, data from our laboratory and others have reported a reduction in apoptosis through inhibition of caspase activation when the inhibitors were administered either before, during or after ischemia, providing indirect evidence of a role for caspase in key signaling pathways controlling apoptosis, and intervention at the level of caspase activation will interrupt the progression of apoptosis [38,76,138–140]. It has been recently demonstrated that caspase inhibitors also significantly reduced both necrosis and apoptosis when the drugs were only delivered at reperfusion, again suggesting cross-over between apoptosis and necrosis [79,140].

6.2.4. Protein kinase C

It is recognized that the activation and subsequent translocation of protein kinase C (PKC) isozymes from the cytosol to the mitochondria is a primary signaling pathway in eliciting cardioprotection by early IP [141–143]. At present, 12 PKC isozymes have been identified based on cofactors required for maximal activation; these isozymes include the classical isozymes α, β1, β2 and γ, which require calcium and diacylglycerol for activation, the novel isozymes δ, ε, η, θ and μ, which only require diacylglycerol for activation, and the atypical isozymes ζ, ι, and λ, which do not require calcium and diacylglycerol, but require phosphatidylserine for their activation [142,144]. The role of PKC in apoptosis was initially demonstrated by using phorbol esters that imitate diacylglycerol and activate both the classical and novel PKC isozymes. Activation of PKC has been shown to induce apoptosis in some cells and prevent cell death in others, the response being largely dependent on the subtype of PKC isozymes involved [142,145,146]. Stimulation of PKCα, PKCβ2 and PKCε, as well as the atypical isozymes PKCζ, PKCι and PKCλ, appears to be anti-apoptotic, whereas overexpression of PKCδ and PKCθ is pro-apoptotic.

Although the effect of IP on necrosis through activation of PKC isozymes has been intensively investigated in vivo and in vitro [69,115,120,143,147,148], only a few studies have shown that IP reduces apoptosis by a PKC-dependent pathway. Okamura et al. demonstrated that the protective effect of IP on apoptosis in vivo was blocked by using a non-isozyme selective PKC antagonist [149]. In cultured neonatal chick cardiomyocytes, Liu et al. have found that PKCe, but not PKCδ, is involved in inhibition of apoptosis by IP after simulated ischemia and reoxygenation, thereby suggesting involvement of a PKC-dependent pathway in early IP [150]. Furthermore, stimulation of PKC isoforms has also been found to be involved in cardioprotection mediated by the opening of mitochondrial ATP-sensitive K+ channels (mito-K_ATP channels) [115]. However, it is not clear at the present time whether this delayed IP inhibits apoptosis through PKC activation.

6.3. Mechanisms aimed at the end effector of apoptosis

Observations from a large body of experimental data using in vivo and in vitro models (primarily based on indirect pharmacological evidence), suggests the opening of mito-K_ATP channels as the potential end effector in both early and delayed IP. The selective mito-K_ATP channel antagonist, sodium 5-hydroxydecanoate (5-HD), blocks the protective effect of IP, and the agonist, diazoxide can mimic the protective effect of IP [114,151–153]. However, there are thus far limited data regarding whether opening of mito-K_ATP channels specifically by IP prevents myocardial apoptosis. Conflicting results have been reported from in vitro studies using mito-K_ATP channel openers. Holmuhamedov et al. [154] demonstrated that in Ca2+-overloaded mitochondria, treatment with diazoxide induced mitochondrial swelling, Ca2+-leakage and release of cytochrome C. In contrast, in cultured neonatal rat cardiomyocytes stimulated by oxidative stress, Akao et al. [152] reported the opposite effect with opening of mito-K_ATP channels; induction of apoptosis in myocytes (assessed by mitochondrial membrane integrity and potential loss, translocation of cytochrome C, activation of caspase and ADP-ribose polymerase cleavage) was significantly suppressed by treatment with diazoxide. The protection was blunted by 5-HD, suggesting that opening of mito-K_ATP channels may be a potential mechanism for attenuation of apoptosis. In this connection, Takashi et al. found that pretreatment with diazoxide reduced both necrosis and apoptosis observed at 24 h after intervention when rats were subjected to 30 min of ischemia followed by 120 min of reperfusion, suggesting a role for the opening of K_ATP channels in delayed myocardial protection [153]. However, the reduction of apoptosis through opening of mito-K_ATP channels during the two windows of IP protection needs to be confirmed further.

7. Summary remarks

Apoptosis is a programmed process that develops simultaneously with necrosis principally during reperfusion, but...
with a time-course that is slower (days) than the development of necrosis (hours) [37,155]. Several mechanisms trigger apoptosis after ischemia and reperfusion: (1) the generation of cytokines and reactive oxygen substances from endothelium, myocytes or cell–cell interactions between inflammatory and endothelial cells; (2) imbalance in regulation of anti-apoptotic and pro-apoptotic proteins; (3) activation of downstream caspases; and (4) the release of cytochrome C from mitochondria. Stimulation of PKC isoymes and the opening of mito-KATP channels have been shown to be associated with a reduction in apoptosis in addition to necrosis. Most of the current studies have provided evidence showing that early IP reduces apoptosis and necrosis by mechanisms as summarized in the present review [37,155]. Therefore, therapeutic strategies must take into account the pathodynamics of apoptosis in reperfusion injury, as well as the numerous pathways involved. From a clinical standpoint, it must be determined whether these interventions delay or permanently reduce apoptosis. However, there are no studies so far showing that early IP permanently reduces apoptosis after a longer period of reperfusion due to the short period of observation in acute experimental studies. Furthermore, it is not clear whether a reduction in apoptosis contributes to the overall reduction in infarct size after prolonged reperfusion. In addition, it is also unknown whether a short treatment with a caspase inhibitor permanently attenuates apoptosis in the later phase of reperfusion. The more basic question remains whether a reduction in apoptosis translates into improvement of clinically relevant outcomes such as infarction, incidence of arrhythmias, global contractile performance, or survival. Initial studies hold promise of such a translational benefit. If physiological outcomes are improved, then a limitation of apoptosis may offer an opportunity for treatment of ischemic heart disease, heart failure and other cardiac diseases. Although most studies to date have shown that ‘classic’ or ‘early’ IP reduces necrotic and apoptotic cell death, more studies are needed to clarify the protective effect of delayed IP on apoptosis and related mechanisms.

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