BCL2-regulated apoptotic process in myocardial ischemia-reperfusion injury (Review)

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Abstract. The leading cause of death in developed countries is cardiovascular disease, where coronary heart disease is the main cause of death. Myocardial reperfusion is the most significant method to prevent cell death after ischemia. However, restoration of blood flow may paradoxically lead to myocardial ischemia-reperfusion injury (MI/RI) accompanied by metabolic disturbances and cardiomyocyte death. As the myocardium has an extremely limited ability to regenerate, the mechanisms of regulated cell death, including apoptosis, are the most significant for contemporary research due to their reversibility. BCL2 is a key anti-apoptotic protein. There are several signaling pathways and compounds regulating BCL2, including PI3K/AKT and MEK1/ERK1/2, JAK2/STAT3, endothelial nitric oxide synthase, PTEN, cardiac ankyrin repeat protein and microRNA, which can serve as targets for modern methods of cardioprotective therapy inhibiting intrinsic apoptosis and saving viable cardiomyocytes after MI/RI. The present review considers the mechanisms of Bcl2-regulated apoptosis in the development and treatment of MI/RI.

Contents

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1. Introduction

In 1972, the Austrian pathologist J.F. Kerr, in cooperation with his Scottish colleagues A.H. Wyllie and A.R. Currie, introduced the concept of 'apoptosis' (after the ancient Greek ἀπόπτωσις-leaf fall) to describe a morphologically stereotypical form of cell death characterized by cytoplasmic volume depletion, chromatin condensation and margination, shrinkage of the nucleus (pyknosis), fragmentation of the nucleus (karyorhexis), blebbing of the membranes and formation of discrete apoptotic bodies with an undamaged cell membrane (1,2).

According to the contemporary biochemical classification of Nomenclature Committee on Cell Death, apoptosis is considered to be one of the morphological signs typical for different types of regulated cell death (RCD) (3). One of the forms of RCD is intrinsic apoptosis. Intrinsic apoptosis, initiated by the cell itself in response to intracellular damage, is also known as mitochondrial apoptosis, as the mitochondria performs the key role in this process (4). The trigger event is the increase in mitochondrial outer membrane permeabilization (MOMP) and release of proteins that are normally sequestered between the two mitochondrial membranes (5,6). The MOMP and thus the entire process of intrinsic apoptosis is regulated by members of the BCL2 protein family that are embedded in the outer membrane (6,7).

BCL2 is an acronym for B-cell lymphoma/leukemia-2. As its name suggests, the gene expressing BCL2 was for the first time found in B-cell malignant neoplasms. This acronym is also used for the designation of the entire family of homological proteins (8). Different proteins of this family contain BCL2 homology domains (BH: BH1, BH2, BH3 and BH4) (Fig. 1) (9) and can be divided into two groups: Pro-apoptotic and anti-apoptotic. Pro-apoptotic proteins include BCL2-associated X protein (BAX), (BCL2 antagonist/killer (BAK), BCL2-related ovarian killer, BH3 interacting domain death agonist, BCL2-associated agonist of cell death, BCL2-interacting killer, BCL2-interacting

mediator of cell death), BCL2-modifying factor, activator of apoptosis harakiri, BCL2-interacting protein 3 (ANIP3), NIX (BNIP3-like), phorbol-12-myristate-13-acetate-induced protein 1 (NOXA) and p53 upregulated modulator of apoptosis (PUMA). Meanwhile, anti-apoptotic proteins include BCL2, BCL2 X-linked protein (BCL-X_L), myeloid cell leukemia 1 and BCL-w and A1/BFL-1 (10-12). The BCL2 family proteins are capable of interacting with each other, whereby their different partnerships result in different outcome of the cell fate (9). In response to apoptosis stimulation, BAX and BAK proteins are exposed to oligomerization on the mitochondrial outer membrane (13,14). This process is blocked by BCL2 protein, which inhibits mitochondrial permeabilization and cell death by interacting with BAX and BAK (9,15). Enhanced expression of *Bcl2* may increase cell resistance to apoptosis in cells, such as tumor cells. The BCL2/BAX ratio is a type of 'rheostat' regulating cell death depending on the balance between BCL2 and BAX in cells (16).

Cardiomyocyte apoptosis is a well-known key process during the development of ischemia (17) During apoptosis inhibition, the BCL2/BAX ratio is increased, which contributes to cardiomyocyte survival in the peri-infarct area (18). Previous investigations revealed a significant role of abnormal *Bcl2* expression in cardiomyocyte apoptosis modulation in MI/RI, as its expression rate has a direct effect on cardiomyocyte apoptosis and cardiac function (19,20).

The key object in the clinical treatment of MI/RI based on molecular mechanisms of the injury progression is decreasing the rate of cardiomyocyte apoptosis. BCL2 is the key protein in the entire BCL2 family that is responsible for the anti-apoptotic process and promotion of cell survival. Therefore, the present review focused mainly on this protein and aimed to investigate how it is regulated during MI/RI and how this can be exploited for clinical use. The present review also assessed possible ways of using BCL2 as a target for pharmacological correction.

2. Pathogenesis of myocardial ischemia-reperfusion injury (MI/RI)

Cardiovascular diseases are the main cause of death worldwide. In 2016, 85% of cases resulted from myocardial infarction or cerebral stroke (21,22). Coronary heart disease is the main cause of death and disabilities (23). Myocardial infarction is tissue necrosis following acute ischemia, which is characterized by absolute insufficiency of coronary blood circulation (24).

Ischemia is a complex pathological mechanism resulting from a decrease in the local blood flow in a tissue or organ (25). Ischemia occurs commonly in the myocardium due to occlusion of the coronary arteries responsible for myocardial perfusion (25). The heart is a constantly contracting organ, requiring a high rate of metabolic activity, which makes it extremely susceptible to any disorders of oxygen supply. Under normal conditions, mitochondria consume oxygen and generate ATP. A decrease in oxygen supply leads to the inhibition of mitochondrial oxidative phosphorylation and, consequentially, the switch from aerobic to anaerobic metabolism (26). Anaerobic glycolysis causes a reduction of intracellular pH (26). The combination of enhanced sodium and calcium influx into cells, due to Na^+-H^+ and Na^+-Ca^{2+} exchange, correspondingly increases acidity and intracellular calcium levels (27). Moreover, a rapid elevation in intracellular Ca^{2+} leads to a pathological increase in mitochondrial permeability transition; however, a reduction of intracellular pH inhibits this process (27). Disordered ion homeostasis is followed by osmotic gradient formation, which is accompanied by water inflow into the cell with a subsequent swelling and disturbance in intracellular ion balance (28). If blood supply is not properly restored after ischemia, the absence of sufficient ATP levels and high levels of Ca^{2+} lead to myocyte atrophy and eventually apoptosis and necrosis (28). The activation of caspase-3 and maximal activity of pro-apoptotic proteins BAX, Noxa and PUMA are observed on the 1st day post-coronary artery occlusion; however, anti-apoptotic proteins BCL2 and BCL-XL remain relatively unchanged, which indicated that the pro-apoptotic pathways are activated rapidly in MI/RI while cell protective pathways remain inactive (29).

Reperfusion of the stunned myocardium during percutaneous coronary intervention is necessary to minimize myocardial damage. For patients with myocardial infarction accompanied by elevation of ST-segment, the timely reperfusion of the myocardium using either thrombolytic therapy or primary percutaneous coronary intervention, is the most effective method of treatment to restrict the size of infarction area, support systolic function and reduce manifestations of heart failure (30). Reperfusion therapy of coronary insufficiency after myocardial infarction is also the most effective method to save cardiomyocytes suffering from hypoxia, support cardiac function and save patients' lives (31). Reperfusion is the most significant method to prevent tissue death after ischemia. However, restoration of blood flow can paradoxically lead to MI/RI, characterized by metabolic disturbances, local inflammatory response, cell death and a consequent cardiac remodeling and dysfunction, contributing to adverse cardiac events after myocardial ischemia (25,32,33). Although reperfusion is necessary for the restoration of oxygen and nutrient influx, which supports cellular metabolism, it may paradoxically cause consequent pathological processes aggravating tissue damage (34,35). MI/RI may exacerbate structural and functional disturbances of the myocardium and cause a strong effect on the restoration of cardiac function after recurrent reperfusion (35-37).

The phenomenon of paradoxical aggravation after oxygen flux restoration was described for the first time >50 years ago when it was shown that reperfusion caused several pathological changes in heart exposed to coronary occlusion (26). MI/RI is associated with different pathophysiological mechanisms, including calcium overload, production of oxygen free radicals, endothelial dysfunction, immune response, mitochondrial dysfunction, cardiomyocyte apoptosis and autophagy and platelet aggregation (38-41). During this process, apoptosis is the main pathological mechanism, which plays a critical role in cardiac remodeling after myocardial infarction (42).

Cardiomyocyte apoptosis and necrosis caused by MI/RI are the most critical pathological processes in cases of cardiac dysfunction after previous myocardial infarction (43). Myocardial necrosis is predominantly observed at the late stages of MI/RI while cell apoptosis is observed throughout the whole process (43). Apoptosis is one of the most important mechanisms of MI/RI and it has a considerable effect on the degree of damage and

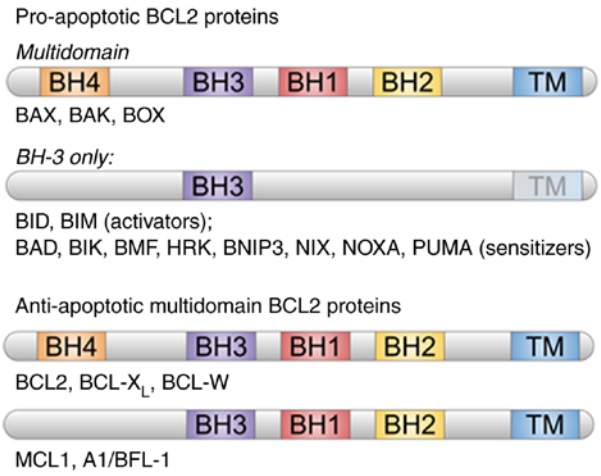

Figure 1. BCL2 proteins and homology domains. The BCL-2 family of proteins is divided into three groups based on their functional role in the regulation of apoptosis and the number of BH domains they bear. Pro-apoptotic BCL2 proteins include: Multidomain proteins BAX, BAK, BOX and BH3-only proteins BID, BIM, BAD, BIK, BMF, HRK, BNIP3, NIX, NOXA and PUMA; anti-apoptotic multidomain BCL2 proteins BCL2, BCL-X_L, BCL-W, MCL1 and A1/BFL-1. BH, BCL2 homology; BAK, BCL2 antagonist/killer; BOX, BCL2-related ovarian killer; BID, BH3-interacting domain death agonist; BIM, BCL2-interacting mediator of cell death; BAD, BCL2-associated agonist of cell death; BIK, BCL2-interacting killer; BMF, BCL2-modifying factor; HRK, activator of apoptosis hara-kiri; BNIP3, BCL2-interacting protein 3; NIX, BNIP3-like; NOXA, phorbol-12-myristate-13-acetate-induced protein 1; PUMA, p53-upregulated modulator of apoptosis; BCL-X_L; BCL2 X-linked protein; BCL-w, BCL2-like protein 2; MCL1, myeloid cell leukemia 1; A1/BFL-1, BCL2-related protein A1.

consequently on the prognosis of heart failure development (42). Thus, effective inhibition of apoptosis caused by MI/RI is one of the important lines of research and it is of great importance for cardiac function improvement after myocardial infarction and for preventing myocardial remodeling.

Apoptosis plays a critical role in the pathogenesis of MI/RI (44). Inhibition of apoptosis may decrease the degree of myocardial damage and prevent injury caused by MI/RI (45). Mitochondrial injury accompanying hypoxia contributes to a decrease in BCL2 content and opening the mitochondrial permeability transition pore (MPTP) (46,47). Reverse blockade of electron transport in ischemia supports high levels of BCL2 accompanied by a decrease in susceptibility to MPTP opening after ischemia (5). Functional inhibition of BCL2 using its low-molecular antagonist HA14-1 sensitizes MPTP opening in mitochondria under normal physiological conditions (46). These results indicated a potential link between decreased or inhibited function of BCL2 and MPTP opening in MI/RI. It can be hypothesized that the BCL2 protein family governs cells undergoing apoptosis. In this context, investigating the regulation of BCL2 during MI/RI may be beneficial in revealing pathways with a potential for possible clinical application.

3. The main pathways of BCL2 regulation in MI/RI

The BCL2 protein family regulates cardiomyocyte death in MI/RI (48,49). The synthesis of BAX and caspase-3 is significantly enhanced and production of BCL2 is inhibited during MI/RI (50). Studies have shown that the key role in apoptotic initiation is due to oxidative stress (51,52).

Two main types of protein activity regulation are known: Fast regulation via post-translational modification (usually phosphorylation/dephosphorylation) and slow regulation via gene expression regulation. BCL2 was shown to have three basic sites of phosphorylation (T69, S70 and S87) which results in changes in its anti-apoptotic activity (11). The modulating role of BCL2 phosphorylation remains to be fully elucidated, moreover, there are contradicting facts described in literature which can derive from the feasibility of single phosphorylation of different amino acids or triple phosphorylation of all three amino acids in the structure of BCL2 (10,46,53). Moreover, BCL2 phosphorylation of the same type in normal and cancer cells can lead to different effects. An attempt to clarify these contradictions was undertaken by Song *et al* (53), who managed to build a mathematical model for BCL2 phosphorylation in different types of cancer cells and revealed that the turning point was 50% triple phosphorylation (T69, S70 and S87) that switched BCL2 from apoptotic to anti-apoptotic action.

The limitation of this conclusion is that it can only be reliably applied to cancer cells.

Several kinases that have BCL2 as a target for phosphorylation are well described in literature: Protein kinase C α, JNK, p38/MAPK, ERK and pyruvate kinase isoform M2 (PKM2). Dephosphorylation of phosphorylated (p)-BCL2 is performed by protein phosphatase A2. BCL2 phosphorylation mediated by JNK, p38/MAPK and PKM2 was shown to occur in cardiomyocytes. JNK and p38/MAPK inactivate BCL2 by phosphorylating and inducing apoptosis, causing cardiomyocyte injury after ischemia and during oxidative stress (54,55). By contrast, PKM2 phosphorylates BCL2 with the aid of heat shock protein 90 to prevent its degradation, thus enhancing its stability and promoting its anti-apoptotic properties (56). Several publications link the degree between BCL2 triple phosphorylation with the crosstalk between autophagy and apoptosis (57-59). This switch point is feasible due to different affinities of BCL2 and p-BCL2 to beclin-1 as the main autophagy inducer (57). Thus, phosphorylation of BCL2 leads to the dissociation of beclin-1 from the BCL2-beclin-1 complex with consequent phosphorylation of beclin-1 and the formation of an active PI3K III complex and autophagy induction (57). The lower degree of BCL2 phosphorylation resulted in autophagy induction, while more extensive BCL2 phosphorylation reduced its affinity to BAX, causing its dissociation and thus resulting in apoptosis induction (58).

Other mechanisms of BCL2 regulation involve gene expression and result in changes in BCL2 intracellular levels. Several signaling pathways are known to regulate the rate of intrinsic apoptosis including PI3K/AKT and MEK1-ERK1/2, endothelial nitric oxide synthase (eNOS), PTEN and JAK2/STAT3 (59-65) (Fig. 2).

The reperfusion injury salvage kinase (RISK) pathway was described for the first time by Schulman *et al* (59) in 2002, while they were studying the mechanisms underlying the cardioprotective effect caused by urocortin. The RISK pathway is a combination of two parallel cascades: PI3K/AKT and MEK1/ERK1/2. The pathways were analyzed in detail in a series of subsequent pharmacological experiments in which the protective effect of several interventions was blocked by a simultaneous administration of PI3K and ERK inhibitors at different times (60). In the broadest term, RISK refers to the

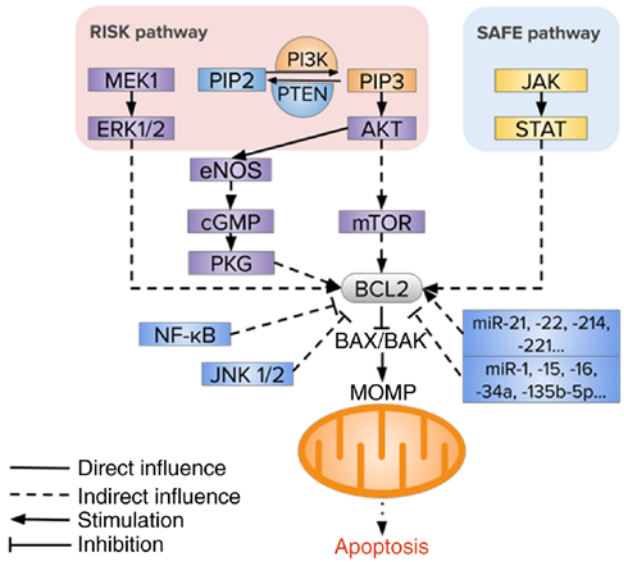

Figure 2. Main pathways of BCL2 regulation in MI/RI. The figure shows the simplified scheme of BCL2 signal transduction regulation in MI/RI. The RISK pathway is in red and the SAFE pathway is in blue. MI/RI, myocardial ischemia-reperfusion injury; RISK, reperfusion injury salvage kinase; SAFE, survivor activating factor enhancement; PIP3, phosphatidylinositol-triphosphate 3; PIP2, phosphatidylinositoltriphosphate 2; eNOS, endothelial nitric oxide synthase; PKG, protein kinase G; miR, microRNA; MOMP, mitochondrial outer membrane permeabilization.

group of pro-survival protein kinases responsible for cardioprotection via specific activation during reperfusion.

The PI3K/AKT/mTOR signaling pathway is an important regulatory mechanism for protein synthesis and is closely associated with intracellular oxidation and reduction in the mitochondria (61). It was found that stress *in vitro* and *in vivo* may lead to an increase in the rate of tyrosine receptor phosphorylation which activates PI3K, indirectly stimulating AKT phosphorylation, increasing the rate of p-mTOR and activating the expression of the anti-apoptotic factor *Bcl2* (61,62). It was also shown that the levels of *PI3K*, p-AKT and p-mTOR in rat myocardial cells after MI/RI were significantly lower compared with the controls (62). Following MI/RI, expression levels of *caspase-3* and *Bax* were significantly increased in myocardial cells whereas *Bcl2* expression significantly decreased (63).

The conformation of PI3K can be changed and activated by the action of growth factors and mitogens, which convert phosphatidylinositoldiphospate 2 (PIP2) into phosphatidylinositoltriphospate 3 (PIP3) (63). Several studies demonstrated that the PI3K/AKT signaling pathway may facilitate cell apoptosis in case of MI/RI by influencing the BCL2/BAX ratio (64,65). Zhang *et al* (66) showed that the PI3K/AKT/mTOR signaling pathway is inhibited in the cardiomyocytes of rats with myocardial infarction, which leads to significant activation of cardiomyocyte apoptosis.

A significant role in the regulation of the PI3K/AKT signaling pathway in MI/RI belongs to PTEN which dephosphorylates PIP3 back into PIP2, thus inhibiting the PI3K/AKT signaling pathway (Fig. 2). This protein plays an important role in apoptosis (67). Nevertheless, only a few studies evaluated the role of PTEN in MI/RI experimental models. In particular, it was shown that PTEN inhibition protected the myocardium from MI/RI by activating the PI3K/AKT/eNOS/ERK pathway, which is one of the variants of pro-apoptotic pathway induction (67). An increase of PTEN levels may suppress the activity of the PI3K/AKT signaling pathway, which may cause myocardial cell apoptosis during MI/RI (68). It was also shown that expression of PTEN and BAX levels in myocardial cells in the MI/RI group were markedly higher compared with sham-operated animals, but phosphorylation of AKT and BCL2 levels were significantly lower (69).

ERK1/2 plays a key role in the transduction of extracellular stimuli (70). ERK1/2 acts as an important protein kinase in reperfusion damage (71). Mitogen-activated protein kinase (MEK1 or MAP2K) was shown to hyperactivate the ERK1/2 signaling pathway (72). The ERK1/2 signaling cascade acts as the main regulator of intracellular apoptosis (73). Although the function of ERK1/2 in apoptosis is controversial (74), inhibition of this pathway is associated with a reduction in the number of apoptotic cells and the BAX/BCL2 ratio as well as a decrease in mitochondrial membrane potential and cell viability in MI/RI (75,76).

The anti-apoptotic effects of nitrogen oxide (NO) mediated-cGMP/ protein kinase G (PKG) signaling can be associated with increased synthesis of anti-apoptotic BCL2 and inhibition of MPTP formation (77,78). Moreover, NO and natriuretic peptides may prevent cardiomyocyte apoptosis via cGMP/PKG-dependent inhibition of intracellular calcium overload (79).

The JAK/STAT signaling pathway is a key component of the survivor activating factor enhancement (SAFE) pathway, which can transmit cell signals from the plasmalemma to the nucleus, providing regulation of gene expression (80-85). The JAK/STAT pathway plays an important role in different mechanisms in the myocardium, including apoptosis (81,86), MI/RI (87,88), preconditioning (89) and postconditioning (90,91). In 2009, Lecour (92) showed that in addition to the RISK pathway, SAFE can be an alternative pathway mediating signaling activated by postconditioning. The JAK/STAT pathway consists of the family of receptor-associated cytosol tyrosine kinases, which phosphorylate tyrosine (93). Phosphorylation and activation of signal transducer and activator of transcription (STAT) in response to ischemic preconditioning (IPC) contribute to cardioprotection by means of signaling cascades and inhibition of pro-apoptotic factors (94). STAT3 is a central component of cardioprotection (95,96). Subsequent studies showed that the JAK2/STAT3 signaling pathway takes part in the anti-apoptotic effect of preconditioning, which is realized by increasing the synthesis of anti-apoptotic BCL2 and suppressing the pro-apoptotic protein BAX (90,97).

The inhibition of pathways that increase the BCL2/Bax ratio and enhancement of pathways leading to its lowering is typically observed in MI/RI, which is associated with hypoxic conditions (84). *In vitro* MI/RI modeling in cardiac myoblasts revealed an increase in BCL2 protein levels accompanied by an increase in p-PI3K and p-AKT levels after antioxidant treatment (94). Cell survival was also increased while the expression of pro-apoptotic BAX was downregulated (98). These results supported the idea that hypoxia-induced oxidative stress acts as a main downregulatory factor for BCL2 and BCL2-family controlled intrinsic apoptosis.

Koeppen *et al* (99) found that expression of serum pro-inflammatory substances is significantly higher in patients with myocardial infarction compared with healthy people. This is an important factor contributing to disease progression due to apoptosis activation. It was also revealed that the toll-like receptor 4 (TLR4)/NF-κB signaling pathway was a potential therapeutic target for MI/RI treatment (100,101). Several studies showed that the TLR4/NF-κB signaling pathway plays a critical role in the regulation of the inflammatory response and cardiomyocyte apoptosis during MI/RI (102,103).

Cardiac ankyrin repeat protein (CARP), a transcription co-factor regulating gene expression in cardiomyocytes, inhibits apoptosis induced by MI/RI increasing *Bcl2* gene expression (104). CARP is linked with the promotor site of the gene *Bcl2* through formation of a complex with transcription factor GATA-4 which regulates transcription and enhances cardioprotection (104).

Hyperlipidemia can stimulate the activation of cardiomyocyte apoptosis in MI/RI. Immunocytochemical analysis revealed an increase in the expression of pro-apoptotic *Bax* and inhibition anti-apoptotic *Bcl2* expression in the myocardium of rats exposed to a hypercholesterol diet (105). These results are in agreement with the data obtained by Guo *et al* (106) and Kuo *et al* (107). In this model, the levels of pro-apoptotic proteins BAK and BAX are significantly increased, which is a sign of induction of intrinsic apoptosis (108). Hypercholesterinemia is associated with an increase in the BCL2/BAX ratio in the myocardium which leads to the aggravation of myocardial damage after its reperfusion due to the activation of cardiomyocyte apoptosis rate (107). It was also shown in the experiments in *Oryctolagus* (rabbits) that *Bcl2* expression is increased in the myocardium during hypercholesterolemia by 50% compared with the controls (109). In *Oryctolagus* with hypercholesterolemia and myocardial ischemia, a marked reduction of *Bcl2* expression and similar degree of the increase in *Bax* expression were observed (109).

MicroRNAs (miRs) are one of the most important epigenetic regulators (110). In recent years, several studies revealed the role of miRs in the process of MI/RI (111-119). miRs change the key signaling mechanisms which makes them potential therapeutic targets (111,112). miRs act as transcription regulators in a wide range of biological processes underlying the response to stress, cell proliferation and cell death (113,114). miRs may bind to the 3'-untranslated region of the mRNA of a target gene, hence destroying mRNA or preventing mRNA translation and negatively regulating the expression of the target gene at the post-transcriptional level (115). Disturbances in miR expression or function are closely associated with cardiovascular diseases; miRNAs take part in different pathophysiological processes including myocardial infarction (116), MI/RI (37,117) or cardiac remodeling (118) with a possible role as aggravating (20) or neutralizing agents (37).

For example, miR-1 is predominantly expressed in cardiac myocytes and closely associated with MI/RI in rats as its levels inversely correlate with BCL2 protein synthesis in cardiomyocytes in MI/RI (119). Mice studies also showed that enhancement of miR-135b-5p expression in MI/RI leads to activation of the JAK2/STAT3 signaling pathway, *Bax* expression and *Bcl2* inhibition (120). Hullinger *et al* (121) demonstrated that miR-15b, a member of the miR-15 family, aggravated myocardial damage caused by MI/RI via affecting BCL2. miR-16 expression is activated during MI/RI and has an inhibiting effect on *Bcl2* expression, which contributed to the enhancement of cardiomyocyte apoptosis after MI/RI (122). Inhibition of miR-16 expression may suppress cardiomyocyte apoptosis after MI/RI, resulting in a reduction of infarction area (122). miR-221 is involved in the pathogenesis of MI/RI by regulating the PTEN/AKT signaling pathway, along with *Bax* and *Bcl2* expression (123-125). Expression of *Bcl2* and microtubule-associated proteins 1A/1B light chain 3B II in cardiomyocytes of newly born rats is significantly decreased, which is accompanied by enhanced expression of miR-497 in anoxia-reoxygenation (126). Another study revealed the cardioprotective role of mir-21 in MI/RI via the activation of the PTEN/AKT signaling pathway and BCL2 (127). miRNA-22 may inhibit cardiomyocyte apoptosis by inhibiting *p53* acetylation and decreasing the levels of pro-apoptotic genes *Bax* and *p21* by affecting one of its targets-cAMP response element-binding protein (128-130). miR-214 reduced myocardial damage caused by MI/RI via the PI3K/AKT signaling pathway, accompanied by a decrease in BAX levels and an increase in BCL2 levels (131). miR-34a, activated in rats with MI/RI, repressed *Bcl2 in vivo* and *in vitro* (132).

The regulation of BCL2-dependent apoptosis in MI/RI is quite versatile and depends on a large number of factors, including activation of emergency genetic programs, changes in metabolic processes and the involvement of additional signaling pathways protecting the myocardium from the negative effects of hypoxia. The ability to influence these mechanisms makes it possible to reduce cardiomyocyte damage, also via induction of BCL2.

4. Therapy of MI/RI

Various forms of cell death may occur during acute MI/RI including necrosis, apoptosis, autophagy, necroptosis and pyroptosis, which may influence the terminal size of the myocardial infarction area after MI/RI (3). This may be used as a new target for cardioprotection, which may include the activation of endogenous cardioprotective signaling pathways: Cascade NO/cGMP/PKG, RISK and SAFE pathways, mitochondrial morphology, cardiomyocyte apoptosis and others (77-79).

Cardiomyocytes of adult humans are characterized by an extremely limited regeneration capacity (133). As a result, there is a continuous process of renewal and reparation of cells mediated by different mechanisms, including apoptosis (134).

In the 1990s, studies focused on the role of different types of cell death in cardioprotection after MI/RI (135). Pro-apoptotic proteins were the main subjects of research at the time, where they were considered to be new targets in MI/RI (135). This was based on a hypothesis suggesting a possibility of saving viable cardiomyocytes when the signaling pathway of regulated cell death was potentially interrupted (135). For example, caspase inhibition during reperfusion restricted the size of the myocardial infarction area in animal models (136). Besides preventing cell death by inhibition of pro-apoptotic caspases, the focus was also given to the use of growth factors that prevented apoptotic processes via activation of proteins contributing to cell survival, such as kinases responsible for the survival

associated with PI3K and ERK1/2 activation. This method was suggested to be protective against MI/RI (137,138).

However, there are still no effective methods for prevention of MI/RI in patients with myocardial infarction (139). Previous attempts to perform cardioprotective treatment of MI/RI (antioxidants, calcium blockers and anti-inflammatory drugs) were not successful (140). The advantages of growth factors (137,138) was restricted because the signaling pathways they were involved in lead simultaneously to activation of apoptosis and induction of fibrosis (141).

Oxidative stress, Ca^{2+} overload, pH changes and inflammation during early reperfusion are the main mediators of tissue alteration, which emphasizes the importance of this period for the pathogenesis of MI/RI (142, 143). In canine experiments, the size of the infarction area significantly increased on the 6th to 24th h after reperfusion. However, Argaud *et al* (144) revealed no difference in the size of the myocardial infarction area between the 4th and 72nd h after reperfusion in *Oryctolagus cuniculus*. Species differences and particular methods of MI/RI modeling can be referred to as the reasons for such different results (143,144).

Regardless of the success in the research of cardioprotective methods on animals, their use in clinical practice still present with severe difficulties (145-147). Some pharmaceutical approaches faced just little success, and although the suggested methods of ischemic conditioning seem promising, their effects may be minor and, in some cases, even controversial (148). Differences between preclinical models of transient myocardial ischemia and coronary heart disease with specific characteristics in patients including age, concomitant diseases and drug therapy may help explain the difficulties in introducing the potential cardioprotective techniques into clinical practice (149).

Numerous different methods of cardioprotective therapy of MI/RI have been suggested in the past three decades (150). These approaches are commonly based on the controlled use of short-term ischemia and reperfusion (ischemic conditioning), pharmacotherapy or physiotherapy including hypothermia or electric stimulation of nerve terminals (30,140).

Therapeutic methods of MI/RI based on ischemic conditioning include local IPC and ischemic post-conditioning (IPostC) as well as remote ischemic conditioning (140), which delays pH restoration, prevent NOS decomposition and consequent formation of reactive forms of oxygen and nitrogen and also increase the content of PKG, a component of the RISK pathway, and cause enhancement of the SAFE pathway in reperfused cardiomyocytes (151-153). As aforementioned, all these factors regulate BCL2 in MI/RI, indicating cardioprotective effects of ischemic conditioning due to inhibition of BCL2-family dependent apoptosis (Fig. 3). The following part of the review details the exploration of the mechanisms underlying these strategies of MI/RI therapy.

Effects of IPC on BCL2 regulated apoptosis in MI/RI. Murry *et al* (154) published an original study showing that IPC (several short-term cycles of ischemia and reperfusion) protected tissues from subsequent ischemic stroke. This discovery, described in *Canis* experiments, was afterwards reproduced in numerous preclinical studies in other animals and other organs besides the heart (155,156) and then in humans (157). The concept of IPC was then transformed into 'ischemic conditioning'-a wide term including a number of associated cardioprotective methods used either directly towards the heart (IPC or IPostC) or distantly (remote ischemic pre-, per- or postconditioning) (157). Thus, effective methods providing the reduction of MI/RI have become an important field of research.

The potential of IPC is inevitably restricted by the necessity to use it before ischemia, which is of great difficulty for patients with myocardial infarction (158). However, this method initiated a number of subsequent studies, which have brought considerable success in understanding the mechanisms underlying MI/RI and IPC as a result of the potential development of cardioprotective therapy (159).

The cardioprotective effect of IPC is evidenced by a decrease in the size of the myocardial infarction area and a reduction in the number of apoptotic cardiomyocytes (157). Activation of the JAK2/STAT3 signaling pathway in response to IPC contributed to cardioprotection via signaling cascades responsible for the inhibition of pro-apoptotic factors (160). Early phase of IPC enhanced JAK/STAT signal transduction by activation of STAT3, which is nearly neutralized by AG490, a JAK2 inhibitor (161). Constitutive deletion of STAT3 stimulated apoptosis, increased the size of infarction area and caused a reduction in cardioprotective effects after pharmacological preconditioning (162).

Studies showed that IPC increased the activity of cyclooxygenase-2 and inducible NOS 24 h after intervention, which depends on transcriptional regulation via the JAK/STAT signaling pathway (163,164). Taken together, these observations lead to the conclusion that IPC activated the SAFE pathway (Figs. 2 and 3).

Chen *et al* (165) investigated the cardioprotective action of exercise preconditioning on periodic cardiomyocyte apoptosis caused by hypoxia in rats. The results of this study showed that 5 days of exercise on a treadmill may decrease the apoptotic index of the myocardium and *caspase-3* expression and increase the BCL2/BAX ratio, which indicated cardioprotective effects based on suppression of hypoxia-induced cardiomyocyte apoptosis. Based on previous studies, exercise preconditioning significantly reduced myocardial damage caused by physical load during ischemia, which is associated with lower levels of cardiac troponin I (cTnI) in the serum, a decrease in the size of the myocardial infarction area, suppression of cardiomyocyte apoptosis, an increase in the levels of anti-apoptotic protein BCL2 and a decrease in the activity of caspase-3 (165). These results are evidence of the cardioprotective action of preconditioning from MI/RI and accompanying apoptosis (160).

Effects of IPostC on BCL2 regulated apoptosis in MI/RI. IPostC was first described Zhao *et al* (166). IPostC, which is induced by short-term episodes of ischemia-reperfusion at the beginning of reperfusion can restrict MI/RI by the activation of intrinsic signaling cascade reactions.

Restoration of myocardial blood circulation caused by postconditioning improved the contractile function of the myocardium and also restricts the size of infarction area, which is confirmed by a lower serum concentration of creatine kinase (CK) and the activity of lactate dehydrogenase compared with the data obtained after MI/RI without previous postconditioning (167,168).

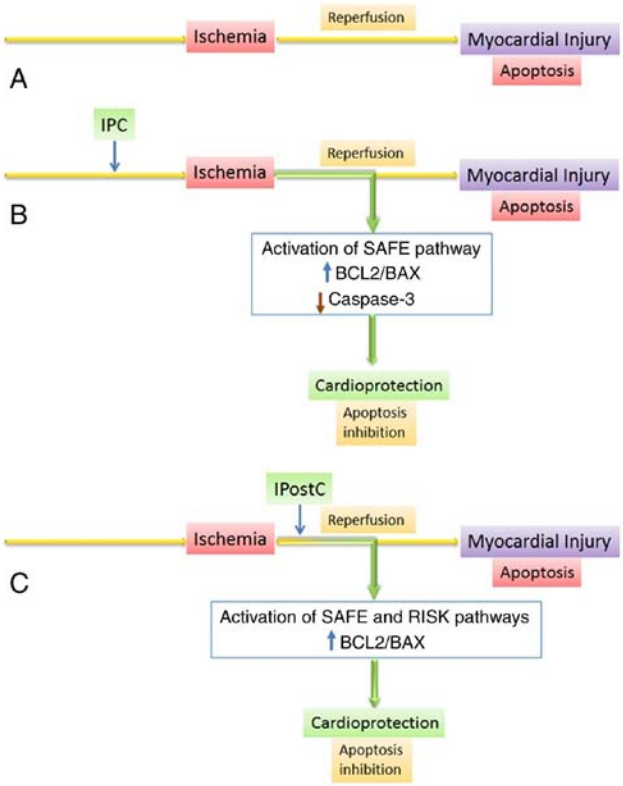

Figure 3. Effects of IPC and IPostC on BCL2-regulated apoptosis in MI/RI. (A) The progress of MI/RI without any treatment. (B) The effects of IPC on BCL2-regulated apoptosis in MI/RI. (C) The effects of IPostC on BCL2-regulated apoptosis in MI/RI. IPC, ischemic preconditioning; IPostC, ischemic postconditioning; MI/RI, myocardial ischemia-reperfusion injury; RISK, reperfusion injury salvage kinase; SAFE, survivor activating factor enhancement.

The effectiveness of IPostC as a method of myocardial protection from MI/RI was also confirmed in several other studies. IPostC does not only decrease the size of the infarction area (143,167) but also limits cardiomyocyte apoptosis after reperfusion. Budhram-Mahadeo *et al* (29) showed that IPostC stimulated BCL2 synthesis and inhibited BAX production. Another study demonstrated the ability of IPostC, similar to IPC, to restrict cardiomyocyte apoptosis after reperfusion via the SAFE pathway (169). IPostC activated STAT3 after reperfusion, and a JAK2 inhibitor (AG490) suppressed the anti-apoptotic effects of IPostC (170). The anti-apoptotic effects of the JAK2-STAT3 signaling pathway were demonstrated in several studies performed on tumors (171). Several genes encoding proteins mediating apoptosis, such as *Bcl2* and *Bcl-x*l, were identified as target genes for STAT3 (170,171). Notably, an increase in BCL2 levels is typical for the period between the 2nd and 24th h after reperfusion in IPostC (166). IPostC might inhibit cardiomyocyte apoptosis during long-term reperfusion via regulation of anti-apoptotic factors such as BCL2 (167). A long-term anti-apoptotic effect of IPostC may be associated with an increase in BCL2 levels 24 h after reperfusion, which is controlled by JAK2/STAT3 (167). Moreover, the PI3K/AKT signaling pathway, regulated by JAK2 signaling, is necessary for cardioprotection of IPostC (169).

An increase in the expression of AKT and BCL2 proteins is accompanied by inhibition of BAX synthesis, which is a sign of activation of the PI3K/AKT signaling pathway and inhibition of cardiomyocyte apoptosis (44). Activation of this pathway, as the main component of the RISK pathway, prevented cardiomyocyte apoptosis, protected the myocardium from MI/RI and plays a critical role in IPostC effects (172-174). Goodman *et al* (175) demonstrated that JAK/STAT signaling may contribute to the initiation of RISK signal transduction via activation of PI3K/AKT, and JAK/STAT signaling alone, without subsequent activation of RISK, is not sufficient for cardioprotection after IPostC. Other studies showed that JAK2 signaling regulated the activation of the PI3K/AKT pathway after IPostC (169). Blocking the PI3K/AKT pathway decreased the cardioprotective effects of IPostC at every timepoint (169). Activation of the JAK2/STAT3/BCL2 pathway without activation of the PI3K/AKT pathway may be insufficient for apoptosis limitation (169).

The positive effects of IPostC may be inhibited by a high-cholesterol diet (109). Moreover, hypercholesterolemia inhibited the phosphorylation of AKT and ERK1/2, which were activated by IPostC in the myocardium and also caused excessive apoptosis due to inhibition of BCL2, increased levels of cytochrome *c* and enhanced activities of caspases 9 and 3 (176).

Effects of pharmacotherapy on BCL2-regulated apoptosis in MI/RI. In recent years, there is an increasing interest in studying the pharmacological methods of cardioprotection (150). The ultimate objectives of cardioprotection strategies include molecular targets mainly involved in signaling pathways of regulated cell death such as ion channels, proteases, reactive oxygen species, contractile elements or components of MPTP (141). As a rule, these strategies are based on existing medicines and they rarely undergo pre-clinical trials (140). The only exclusion is cyclosporine A, which is targeted at MPTP. However, cyclosporine A showed controversial results and failed in clinical trials (140).

Although pharmacotherapy is not commonly included in cardioprotective strategies, several investigations have shown that a number of medicines are capable of cushioning the effects of MI/RI (176,177,179-182,186-190). The present review briefly reviews those that promote cell survival and reduce apoptosis by affecting the BCL2/Bax ratio through specific signaling pathways. Most of the agents provide pleiotropic effects and activate several pathways simultaneously, leading to an increase of BCL2 expression (179-182,186-190). The comparative data on these medicines is summarized in Table I.

Metformin, which is widely used for the treatment of carbohydrate metabolism disorders, inhibits apoptosis in culture (H9c2 cells) and rat cardiomyocytes following injury caused by hypoxia-reoxygenation or ischemia-reoxygenation by increasing the BCL2/BAX ratio with the involvement of metalloreductase STEAP4 (177). These results *in vitro* and *in vivo* affirmed the hypothetical effects of metformin on MI/RI produced by cellular apoptosis inhibition. The molecular mechanisms of this anti-apoptotic function of metformin are still poorly understood, though it was earlier reported that they include activation of AMPK (178). AMPK is considered to be a key molecule for cardioprotection based on the modulation of several signaling pathways involved in glucose metabolism and energy homeostasis (179). AMPKs are proteins that promote cell protection in ischemic conditions as the AMP/ATP ratio

Table I. Effects of several pharmaceuticals on apoptosis via affecting BCL2 expression.

Name of compound	Molecular target	References
Metformin	STEAP4 AMPK activation	176,177
Berberine	JAK/STAT activation	179
Escitalopram	ND	180
Ilexsaponin A	ND	181
Salvianolic acid	Caspase-3 inhibition	50
Sevoflurane	MicroRNA-135b-5p suppression	119
Dexmedetomidine	α2-adrenoreceptors, PI3K/AKT/GSK-3β	182
Rapamycin	MAPK, JAK2/STAT3 activation	183-186
Melatonin	AMPK/PGC-1α/SIRT3, Notch1/Hes1 activation	187-190,193

STEAP4, metalloreductase STEAP4; ND, not determined; GSK-3β, glycogen synthase kinase-3β; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator-1α; SIRT3, NAD-dependent protein deacetylase sirtuin-3.

indicates intercellular energetic status and is increased in ischemic tissues (191).

A considerable cardioprotective effect of berberine, an alkaloid from *Berberis vulgaris*, was revealed. This plant alkaloid was shown to reduce serum levels of heart injury markers such as CK-MB, LDG and cTnI with simultaneous upregulation of BCL2 expression and mitochondrial cytochrome *c* and downregulation of BAX (180).

The antidepressant escitalopram was shown to suppress cardiomyocyte apoptosis in patients with previous myocardial infarction compared with the controls, which was accompanied by a decrease in the BAX/BCL2 ratio (181).

Preliminary administration of Ilexsaponin A increased the levels of anti-apoptotic protein BCL2 and decreased pro-apoptotic protein BAX. These results confirmed that Ilexsaponin could suppress cardiomyocyte apoptosis in MI/RI, being a new potential cardioprotective agent which may be used for MI/RI treatment (182).

Preliminary introduction of Salvianolic acid (10, 20 or 30 mg/kg/day) effectively decreased myocardial synthesis of BAX and caspase-3 and increased BCL2 levels (50).

Inhaled administration of sevoflurane (halogenated anesthetic) inhibited BAX expression and enhanced *Bcl2* expression in mice, which was mediated by suppression of miRNA-135b-5p, whereby drug prevented MI/RI by activating the JAK2/STAT3 signaling pathway (120).

Postconditioning with dexmedetomidine (high-selective agonist of α2-adrenoreceptors), which is widely used in anesthesiology and resuscitation, significantly increased the BCL2/BAX ratio in the rat myocardium with modeled diabetes mellitus and MI/RI via the PI3K/AKT/GSK-3β signaling pathway (183).

Rapamycin (an inhibitor of mTOR) is used for coating coronary stents containing special drugs to prevent in-stent restenosis after coronary angioplasty (184,185). Rapamycin induces unique cardioprotective signal transduction that includes phosphorylation of ERK, STAT3, eNOS and GSK-3β in association with increased BCL2/BAX ratio (184). JAK2/STAT3 signal transduction plays a critical role in cardioprotection induced by rapamycin, which is associated with an increase in BCL2/BAX (185). BCL2 expression was enhanced after STAT3 activation via ERK-dependent phosphorylation caused by rapamycin administration (186). Introduction of rapamycin before reperfusion is a promising method that might be capable of considerable restriction of the myocardial infarction area and inhibition of cardiomyocyte apoptosis after MI/RI via signaling pathways involving MAP kinases and PI3K/AKT (187).

Interestingly, a study showed an evident role of melatonin in cardioprotection through the enhancement of *Bcl-xl* and *Bcl2* expression and inhibition of *Bax* gene expression by reduction of oxidative stress via the activation of the NAD-dependent protein deacetylase sirtuin-3 (SIRT3) signaling pathway (188-190). SIRT3 is localized in the mitochondria and regulates several mitochondrial metabolic pathways (192). Moreover, during MI/RI and type 1 diabetes, melatonin significantly inhibited apoptosis by suppression of caspase-3 and BAX production, cleavage of caspase-3 and an increase in BCL2 levels (192). These effects were also inhibited by a specific blocker of AMPK signal transduction (compound C) which determines that this signaling pathway plays a key role in the cardioprotective action of melatonin (192). Firstly, it was demonstrated that melatonin treatment is a potential strategy for prevention of MI/RI injury in cases of type 1 diabetes mellitus as it could enhance mitochondrial biogenesis and support normal functions of the mitochondria (192). Secondly, it was also shown that the AMPK/peroxisome proliferator-activated receptor γ coactivator 1α/SIRT3 signaling pathway played a key role in the cardioprotective action of melatonin (193). Melatonin also showed a strong protective effect via Notch1/Hes1 signal transduction in a receptor-dependent manner (193). The PTEN/AKT signaling pathway is a key consequent mediator of BCL2 expression enhancement in rats (*in vivo*) and cultivated H9C2 cardiomyocytes (*in vitro*) (194).

It is important to note that the mechanisms of metabolic cardioprotection of most preparations have been poorly investigated to date (177,180-184). The data of different randomized controlled trials often do not prove the effectiveness of the suggested methods (180-184). Clinical data is available for metformin, rapamycin, dexmedetomidine, berberine and sevoflurane, but sufficient evidence of effective cardioprotection is

still missing (195). Considerable appending of new theoretical data is required that would include information concerning molecular and cellular mechanisms which this therapy would be targeted at.

5. Conclusions and perspectives

In recent years, focus on apoptosis has become a promising direction in the research of cardiovascular pathology since there is an opportunity to control this process and to protect the functional reserve of the myocardium. The studies mentioned in this review have demonstrated a number of effective methods for inhibiting cell apoptosis. Conclusions based on these results, unfortunately, did not lead to a final solution to the problem of prevention and treatment of MI/RI. There is still a lack of data to recommend or to introduce these results into clinical practice. This is predominantly explained by the fact that there is no consensus for common biological and pathogenetic significance of BCL2 associated processes: Is it cardioprotective or only a pathological mechanism leading to cardiomyocyte death and aggravation of myocardial degradation?

Proteins of the BCL2 family play main roles in intrinsic apoptosis, and regulation of their activity allows significantly reduced cell death. In addition to the influence of BCL2 protein on apoptosis development, it is worth paying attention to its non-apoptotic functions in MI/RI development. For example, BCL2 regulation features mitochondrial, nuclear and endoplasmic reticulum processes (including calcium homeostasis) and glucose and lipid metabolism (196-198).

The preservation of functionally active cardiomyocytes is a priority in the development of new algorithms for MI/RI treatment. A wider research of BCL2 integration into cellular processes in MI/RI is likely to result in building a more complete signaling network that can be targeted at for preventing reperfusion injury of cardiomyocytes.

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Authors' contributions

AYK and MLB conceptualized the study; AYK and MLB wrote the original draft; AYK, MLB, EVN, SPS and EA participated in writing and edited the review; EVN and APS prepared the figures; SMS edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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