Balance of nitric oxide and reactive oxygen species in myocardial reperfusion injury and protection.

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BALANCE OF NITRIC OXIDE AND REACTIVE OXYGEN SPECIES IN MYOCARDIAL REPERFUSION INJURY AND PROTECTION

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Running title
Myocardial reperfusion injury and protection

Abstract:

Depending on their concentrations, both nitric oxide (NO) and reactive oxygen species (ROS) take part either in myocardial ischemia reperfusion injury or in protection by ischemic and pharmacological pre- (Ipre) and postconditioning (Ipost). At the beginning of reperfusion a transient release of NO is promptly scavenged by ROS to form the highly toxic peroxynitrite which is responsible for a further increase of ROS via eNOS uncoupling.

The protective role of NO has suggested the use of NO donors to mimic Ipre and Ipost. However NO donors have not always given the expected protection, possibly because they are responsible for the production of different amounts of ROS which depend on the amount of released NO.

The present review is focused on the role of the balance of NO and ROS in myocardial injury and its prevention by Ipre and Ipost, as well as after the use of NO-donors given with or without antioxidant compounds to mimic Ipre and Ipost.

Keywords: nitric oxide, NO-donors, reactive oxygen species, antioxidants, ischemia-reperfusion injury, myocardial protection.

Postischemic myocardial reperfusion is responsible for about 50% of the ischemia-reperfusion (I/R) injury (1). In early reperfusion an increased production of reactive oxygen species (ROS) is accompanied by a reduced availability of nitric oxide (NO). For the above reasons, either NO-donors or antioxidant compounds (AOX) have been tested to reduce infarct size and the incidence of arrhythmias, as well as to improve the postischemic mechanical recovery. Unfortunately, not always the expected results were obtained.

After an excursus on I/R injury and myocardial protection, the present review deals with the difference between the activity of endogenous and exogenous NO as well as with the interaction of NO-donors and AOX. It will be also discussed why an impairment of the protective effect can occur if an excessive amount of NO is released.

NO production in the heart

Nitric oxide synthases (NOSs) cause the production of NO by acting on L-arginine in the presence of molecular oxygen. The cofactor tetra-hydro-biopterin (BH_4) is needed for the reaction. In fact, if BH_4 or L-arginine are absent, NOSs produce superoxide anion ($O_2^{-\bullet}$) instead of NO. This phenomenon is the so called *NOS uncoupling* (2).

Three isoforms of NOS have been classified, two constitutive, neuronal NOS (nNOS) and endothelial NOS (eNOS), and one inducible (iNOS). In the heart, nNOS has been targeted to the sarcoplasmic reticulum (3, 4) and in non-adrenergic non-cholinergic (NANC) autonomic vasodilator fibers, while eNOS has been found in the endothelial cells of both coronary vasculature and endocardium and in cardiomyocytes (5, 6). Finally, i-NOS is present in ventricular cardiomyocytes and fibroblasts, as well as in vascular smooth muscles and in endothelial cells (7, 8, 9).

A NOS has also been found in the inner membrane of cardiomyocyte mitochondria. Although initially eNOS or iNOS were both candidates for this mitochondrial NOS (mt-NOS), further studies identified it as a nNOS (10).

The activation of the constitutive NOSs is Ca^{2+}-calmodulin dependent. As it will be seen below, a number of factors leading to myocardial protection require the Ca^{2+}-induced activation of eNOS. However, the increase in intracellular Ca^{2+} concentration can also activate cellular phospholipase A_2 which can inhibit nNOS via the release of arachidonic acid (11). Unlike constitutive NOS, i-NOS is Ca^{2+}-calmodulin independent and is activated by nuclear factor kappa B (NF-κB), as well as by lipopolysaccharide, cytokines and other agents (12, 13). A negative interaction exists between the activity of the constitutive and inducible NOS. In fact, the release of nitric oxide by iNOS may be impaired if an increase of NO availability by constitutive NOS downregulates iNOS expression via NF-κB inhibition (11, 14). Conversely, nNOS inhibition results in a large delayed NO production by iNOS (11).

Nitric oxide may be also generated via NOS-independent pathways. In fact nitrite is not only a product, but also a source of NO in various reduction mechanisms. These mechanisms include the reduction of nitrite in the ischemic heart under acidotic and highly reduced conditions, or in normoxia, when an adequate concentration of nitrite is available (15). Moreover, nitrite reduction to NO can also be due to the catalytic activity of xanthine oxidase (XO) in early reperfusion (16, 17) or to the nitrite reductase activity of myoglobin in ischemia and reperfusion (18).

As regards the non-enzymatic production in the ischemic acidotic heart, it has been reported that NO release can increase 100-fold when pH falls from 7.4 to 5.5. Due to this abundant production, which can exceed the amount generated by tissue NOS, nitric oxide may be responsible for cell death with reduction of cardiac contractility via peroxynitrite ($ONOO^-$) production (19).

Ischemia-reperfusion injury

Myocardial I/R injury occurs when 30 or more minutes of ischemia are followed by reperfusion. The injury consists of cell necrosis and apoptosis plus a period of hypocontractility or myocardial stunning. Also arrhythmias, included ventricular tachycardia and fibrillation, may appear during I/R.

During ischemia, the re-synthesis of ATP by oxidative phosphorylation is compromised. A partial compensatory mechanism is provided by the enhancement of anaerobic glycolysis which however allows the re-synthesis of a very small amount of ATP and causes the production of hydrogen ions (20). The limited ATP re-synthesis favors the accumulation of metabolites and phosphate which is responsible for an increase of the cell osmotic load (21).

The shortage of ATP induces an increase of Na^+ and Ca^{2+} cellular concentrations due to the impairment of Na^+/K^+- and Ca^{2+}-ATPase activities. Also the intracellular accumulation of H^+ contributes to the final cellular Ca^+ overload by activating the H^+/Na^+ and Na^+/Ca^{2+} sarcolemmal exchanger (21, 22). This overload enhances cell hyperosmolarity and leads to cell swelling.

Rather than a worsening of ischemic injury, reperfusion is *per se* the source of specific components of cardiac damage (23) which are mediated by the lack of NO and the production of ROS (1, 24, 25).

The reperfusion-induced increase of Ca^{2+} overload in cardiomyocytes leads to other detrimental effects. These effects are the inhibition of mitochondrial respiration, the activation of phopsholipases A2 and C and proteases (26, 27), the induction of arrhythmias during ischemia and reperfusion (28) and the contracture of parts of myocardium not affected by cell death (29). While the phospholipases lead to the degradation of membrane phospholipids, the proteases alter the attachment of the sarcolemma to the cytoskeleton (27). This loss of cell integrity, together with the hyperosmolarity and the abnormal mechanical forces developed by the contracture, can result in the disruption of the sarcolemma (27).

The duration of ischemia plays a very important role in the genesis of the injury. In fact in the isolated rat heart, a time-dependent reduction of BH_4 content occurs during ischemia, so that in 30 and 60 min, BH_4 degradation reaches 58% and 92% respectively of the control (30)

Nitric oxide and reactive oxygen species in reperfusion injury

The lack of NO is preceded by a transient increase. In fact, in the early stage of reperfusion, a further increase in Ca^{2+} level takes place in cardiomyocytes and endothelial cells leading to a brief activation of nNOS, eNOS and mtNOS (31, 32). Moreover, upon the sudden arrival of a large amount of oxygen, a burst of $O_2^{-\bullet}$ occurs in response to the activity of XO on hypoxanthine, a product of ATP catabolism, as well as of NADPH-oxidase on molecular oxygen. However, as said above, the activation of XO may be responsible for the biotransformation of organic nitrates to NO. Thus, an enzyme involved in the oxidative stress might also limit the lack of NO and counteract another aspect of I/R injury.

The transient activation of NOS involves the rapid consumption of L-arginine and BH_4, so that NOS uncoupling causes a further production of $O_2^{-\bullet}$ (2). Superoxide anion rapidly removes NO to form peroxynitrite ($ONOO^-$) which can oxidize that amount of BH_4 which has not been removed during ischemia (30, 33), and make self-sustaining the production of $O_2^{-\bullet}$. On the other hand, $ONOO^-$ can stop NO production by destabilizing the eNOS dimmer.

ROS production, together with Ca^{2+} overload, can also cause the opening of mitochondrial permeability transition pores (mPTP), which release cytochrome-c in cytosol and trigger a cascade to myocardial apoptosis and necrosis (34, 35). Moreover, the opening of mPTP inhibits the respiratory chain thus inducing a further abundant release of ROS (36). This vicious cycle is the so-called ROS-induced ROS release (RIRR), which amplifies the oxidative stress (37). As a matter of fact, reperfusion-induced cell death plays a pivotal role in determining the final extension of infarct size.

Endothelial dysfunction and no-reflow phenomenon

Also coronary vasculature may be affected by reperfusion. In the vessels, the impaired synthesis of NO and the increased production of ROS are components of the *acute endothelial dysfunction* occurring after an ischemia (38, 39). In fact, in early reperfusion, the lack of NO, which follows the

initial burst, favors platelet aggregation directly and induces neutrophil adhesion through the activation of cellular adhesion molecules (40).

Platelet aggregation and neutrophil adhesion reduce the equivalent lumen of the capillary bed. Since the lack of NO implies vasoconstriction, after an initial postischemic hyperemia, acute endothelial dysfunction may reduce or arrest the blood flow, thus causing the so called *no-reflow* phenomenon (38, 41). A contribution to this phenomenon can be provided by the mechanical compression exerted by the reperfusion-related tissue oedema (42). One of the major determinants of the extent of no-reflow is represented by the duration of ischemia (43). It is likely that the dependence of no-reflow on the duration of ischemia is the consequence of the progressive reduction of BH_4 during ischemia (30).

Protection of the heart against ischemia-reperfusion injury: ischemic pre- and postconditioning.

Reduction of infarct size and incidence of myocardial arrhythmias were initially obtained with IPre (44, 45). Later on, IPre was also seen to ameliorate the postischemic contractile recovery (53) and to prevent coronary endothelial dysfunction (41, 47, 48).

Ipre is obtained with one or more brief (2-5 min) coronary occlusions before the beginning of an ischemia long enough to produce infarction (44). This procedure induces two periods, or windows, of protection. The first window lasts 2-3 hours after the end of the preconditioning manoeuvres, whereas the second one appears about 20-24 hours after the end of the first one and lasts 70 hours or longer (48). In addition to this classical procedure, Ipre may also be obtained in cardiac surgery with the use of volatile anesthetics such as that isoflurane, sevoflurane and desflurane, to prevent the effect of perioperative myocardial ischemia (49). As a mean and long term strategy against cardiovascular attacks, regular physical exercise may also represent a sort of preconditioning.

Apart from the above peculiar kinds of cardiac protection, Ipre is of little, if any, use in limiting the effects of an unpredictable ischemic insult. Thus, the possibility to intervene after ischemia was

considered. In the dog, the group of Vinten-Johansen (25, 50) set up the technique of IPost, in which very short (about 10 s) coronary occlusions are performed starting a few seconds after the onset of reperfusion.

Endogenous NO in myocardial protection

Initially the limitation of the infarct size was attributed to the release of adenosine, which was seen to trigger a pathway leading to the opening of mitochondrial K^+-ATP-dependent channels (mito K_{ATP}) (46, 51). More recently, it has been demonstrated that mito K_{ATP} opening is followed by mitochondrial release of ROS which, as explained below, intervenes in myocardial protection (52, 53).

At the same time, the IPre-induced limitation of the infarct size and prevention of arrhythmias during and after a prolonged coronary occlusion began to be attributed to NO (27, 54-56). This hypothesis was confirmed by the administration of either NOS inhibitors or NO-donors in various animal species (48, 57, 58). It is note-worthy that also the myocardial protection by volatile anesthetics has been attributed to NOS activation (49).

Initially the release of NO was attributed to the endothelial cells in response to the binding of bradykinin with B_2 receptors. Bradykinin production was considered to depend on the reduction of tissue pH in response to preconditioning ischemia (27, 54, 55). Later on, the activation of eNOS in the vascular endothelium was also attributed to adenosine (59, 60).

Adenosine and bradykinin can activate eNOS not only in the endothelial cells but also in cardiomyocytes via sarcolemmal G-protein coupled receptors (GPCR) (61). Independently of its origin, NO leads to the opening of both sarcolemmal and mito K_{ATP} channels in cardiomyocytes via GS-cGMP-PKG pathway (62). While the opening of the former limits Ca^{2+} flux into the cell, the activation of the latter prevents the opening of mPTP. Both these effects result in the limitation of infarct size (62, 63).

As regards the prevention of arrhythmias by NO, the beneficial effect may be due to the reduction of Ca^{2+} intracellular concentration (64) by S-nitrosylation of various proteins involved in Ca^{2+} transport. In fact, in response to nitrosylation, L-type Ca^{2+} channels and mitochondrial F1-ATPase are inactivated, while sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2) increases its own activity (65, 66). Moreover, also the NO-induced phosphorylation of sarcolemmal K_{ATP} channels limits Ca^{2+}entrance by shortening action potential (62).

ROS in myocardial protection

In addition to their injuring effect, a limited properly timed release ROS from mitochondria participate to the cascade that connects the opening of mito K_{ATP} channels to the inhibition of mPTP. In this cascade, after cGMP-PKG-induced activation of PKCε1 has caused the opening of mito K_{ATP} channels, the production of a small amount of ROS occurs and results in the inhibition of mPTP opening (67, 68). The involvement of ROS in the signalling pathway to myocardial protection is supported by other investigations (52, 67-71).

Independently of the cascade to inhibition of mPTP, since 1993 in isolated rabbit hearts it has been found that mitochondrial respiratory chain is a source of ROS during a reperfusion that follows 30 min of ischemia (72). It was seen that such a release displayed a detrimental role which was suppressed with the blockade of the respiratory chain at the level of NADH dehydrogenase.. The difference between these findings and the intervention of ROS in RISK cascade might be attributed to the different release of free radicals after a long- and short (preconditioning) lasting hyperemia.

Common pathways of IPre and IPost

A common pathway involving NO has been proposed for both IPre and IPost (69, 73) (fig. 1). It has been suggested that in both procedures adenosine and bradykinin link to GPCR which activates PKB/Akt via tyrosin-kinase and phosphoinositol-3-kinase (PI3K). In turn, PKB/Akt stimulates

myocardial eNOS to produce NO which inhibits mPTP directly by protein nitrosylation (74) or through the opening of mito K_{ATP} channels via the cGMP-PKG cascade (69, 70).

This common pathway is a component of the more complex *Reperfusion Injury Salvage Kinase (RISK) cascade* (69). In this multiple-way cascade, the inhibition of mPTP can also occur without the involvement of NO, i.e. through either Akt-induced inhibition of proapoptotic BAX-BAD or phosphorylation/inhibition of glycogen synthase kinase-3β (GSK 3β). A contribution to the inhibition of BAX-BAD is provided by the sequential GPCR-induced activation of Ras, mitogen-activated protein kinase kinase 1/2 (MEK 1/2) and extracellular regulated kinase 1/2 (Erk 1/2).

A signalling cascade called *Survivor Activating Factor Enhancement (SAFE) Pathway* has been proposed as alternative to RISK cascade (75, 76). SAFE pathway starts with the linkage of tumor necrosis factor α (TNF-α) to the specific receptor TNF-R_2 followed by the subsequent activation of Janus kinase (JAK) and of the signal transducer and activator of transcription-3 (STAT3). Finally, it results in the inhibition of mPTP via phosphorilation/inhibition of GSK 3β (77-79). In addition, phosphorylated STAT3 migrates into the nucleus and promotes the transcription of some genes, among which iNOS gene has been included (80), suggesting a role of NO in SAFE**,** at least in the second window of protection.

The question arises whether any interaction between RISK and SAFE pathways exists also in the first window of protection and whether NO plays a role in this interaction.

Lecour (76) underlines that SAFE excludes the intervention of PKB/Akt and Erk 1/2 which characterises RISK cascade. This opinion has been strengthened by the results of Lacerda *et al*. (81), who report that IPost-induced protection persists after inhibition of either PI3K or Erk1/2, but does not occur in $TNF^{-/-}$ and $TNFR2^{-/-}$ mice.

On the other hand, an upstream NO production by eNOS has been suggested to be responsible for the activation of TNF-α (80,82). Although this hypothesis has been proposed in an investigation not finalized to the study of myocardial protection, we cannot completely exclude that, at least in part,

SAFE might be triggered after eNOS activation in the RISK cascade. However, to our knowledge, so far no evidence has confirmed this hypothesis.

A partial interaction between SAFE and RISK cascades has been proposed by Goodman *et al.* (83). They observed that in mice STAT3 inhibition decreases the phosphorylation of eNOS-activator Akt, while PI3K inhibition attenuates postconditioning protection without affecting STAT3 phosphorylation. They concluded that effectiveness of SAFE requires the contribution of the RISK cascade. Nevertheless, the real interaction between the two pathways needs further studies.

At present, the only possible statement is that NO might be involved in both pathways and that, due to its various modes of action, it is at the same time "*trigger, mediator, potential effector of cardioprotection"* (80).

Antioxidant compounds in myocardial protection

The production of ROS and $ONOO^-$ upon reperfusion (72, 84) is considered responsible for the oxidative stress and the triggering of reperfusion injury. In particular, while $O_2^{-\bullet}$ has a very short half life and can be rapidly scavenged in cardiomyocytes, $ONOO^-$ has persistent cytotoxic effects (34, 85). In fact, in addition to its own oxidative property, $ONOO^-$ can decompose in two more toxic reactive species like hydroxyl ($OH^{-\bullet}$) and nitrogen dioxide ($NO_2^{\bullet}$) radicals (34).

Owing to the role of ROS in reperfusion injury, the administration of AOX has been proposed for cardioprotection. However, clinical investigations provided controversial effects (86-88). Thus, the administration of AOX at the beginning of reperfusion has also been seen to abolish the protection by ischemic pre- or postconditiong (70, 89). The explanation of this paradoxical effect may be found in the observation that in early reperfusion the mitochondrial release of ROS is involved in myocardial protection (52, 68-70).

NO-donors in myocardial protection

Due to the role of NO in myocardial protection, the use of NO-donors has been proposed for the prevention of I/R injury and the treatment of chronic cardiovascular diseases.

So far several NO-donors have been used for experimental and clinical purposes. Nitroglycerin (NTG), sodium nitroprusside, NO metal complexes, and organic esters of nitrous acid had already been used in various cardiovascular diseases before it was clear that their activity depends on NO release. For this reason these donors have been considered "accidental NO-donors" (90). Other classes of NO-donors, such as nitrosothiols and NONOates, are widely used in research. All these compounds release NO spontaneously by self-decomposition. Other compounds, i.e. hydroxyguanidine derivates, release NO in response to enzymatic oxidation (90, 91).

Nitroglycerin, which is generally included among the NO releasing organic nitrates, has also been found, to possess a highly limited NO releasing potency (92-94).

An interesting group of NO donors is represented by nitroaspirine, i.e. nitro-esthers of ASA, that in the cardiac environment of the stomach release nitrogen dioxide which in turn decomposes to NO. (95).

Recently, derivatives of the furoxan ring, indicated as *furoxans*, have been studied. These compounds, as well as nitrates and nitrites, release NO by reactions with acids, alkali, metals and thiols (90).

Among the various donors, inorganic nitrite can avoid the systemic effect of other donors, because its XO-catalised conversion to NO occurs prevalently within the acidotic ischemic region (16).

In mimicking IPre and IPost, exogenous NO can replace the endogenous one that in reperfusion is scavenged by $O_2^{-\bullet}$ with $ONOO^-$ production. In addition, exogenous NO attenuates cardiac preload and afterload, prevents platelet aggregation, contrasts the no-reflow phenomenon and, with the contribution of coronary dilatation, ameliorates the balance between myocardial oxygen consumption and supply (96).

Excess of NO released from donors can blunt the activity of the various NOS**s** (2,97). Since constitutive NOSs are more sensitive than iNOS to the NO inhibitory activity, this negative

feedback may be important in the presence of NOS uncoupling, when it can counteract the production of $O_2^{-\bullet}$ during I/R (2).

NO-donors can be used with acute and chronic administrations.

Acute administration is that commonly used to mimic IPre and IPost. Significant reductions of infarct size and/or improvements of mechanical recovery have been observed in various species when NO-donors were given before or after regional or global ischemia (16, 98-102). The use of NO donors to mimic preconditioning has been shown to be successful in either experimental studies (71, 95) or clinical trials (103-105).

Although in clinical practice, as in the case of an unpredictable heart attack, NO-donors may be administered prevalently to mimic IPost, pretreatment was seen to be successful for surgical interventions. In humans, Leesar *et al*. (104) report that NTG infusion 24 hours before coronary angioplastic reduces ST segment shift during the ischemia induced by inflating the angioplastic balloon.

In spite of what observed in an overwhelming majority of investigations, sometimes exogenous NO was seen to fail in limiting I/R injury (106-110). Occasionally, NO donor can even suppress an otherwise obtained protection (111). To explain this adverse role of NO, it has been speculated that its excess can inhibit neuronal signals induced by adenosine, a necessary element of the cascade leading to the release of cardioprotective factors (111).

Chronic administration of NO-donors is mainly based on the use of nitrates in the therapy of ischemic heart diseases, heart failure and pulmonary hypertension (112-116). In chronic ischemic heart diseases, nitrates are given because of their anti-anginal and anti-ischemic properties.

Interaction of NO-donors with antioxidant compounds

The removal of NO by $O_2^{-\bullet}$ in reperfusion should be overcome if exogenous NO is given with an AOX that increases NO bioavailability and prevents the injury. The synergy of NO-donors and

AOX was studied by Kutala *et al*. (100). In perfused rat hearts undergone I/R, they found that pre-treatment with a NO-releasing aspirin-derivative, *2-(acetyloxy)benzoic acid 3-(nitrooxymethyl)phenyl ester* (NCX 4016), reduced infarct size and improved postischemic mechanical recovery. They obtained similar positive effects after pre-treatment with AOXs such as urate, SOD and *4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl* (Tempol).

The protection was greater if one of these antioxidants was give in a mixture with NCX-4016. Interestingly, Tempol increased the availability of NO and reduced ROS and peroxynitrite concentration (100).

Rastaldo *et al.*, (106) tried to mimic IPost by infusing for 20 min after ischemia a furoxan NO-donor *4-[(dimethylamino) methyl]furoxan-3-carbamide* and an antioxidant substructure of Vitamin E *2,2,5,7,8-pentamethylchroman-6-ol*. The protection occurred only when the two compounds were integrated in a hybrid molecule, but not if they were given as a mixture. The hybrid molecule allows them to enter the cell simultaneously and to ameliorate their synergy. Moreover, while Kutala group used a *per se* effective antioxidant, Rastaldo and coworkers used a concentration of AOX (1 μM) which preliminary experiments had showed to be *per se* too low to protect the heart. In contrast with the protocol of Kutala group, protection was not obtained if the hybrid was used to mimic IPre instead of IPost. It is possible that in trying to mimic IPre, the concentration of AOX component was too small to be still active to prevent the oxidative stress occurring in reperfusion after 30 min of ischemia.

Paradoxical effect of a large release of NO from donors

As regards NO and myocardial protection, it has been reported (117) that while moderate concentration of NO lead to myocardial protection, high concentration are responsible for the opening of mPTP. Recently, experiments on isolated permeabilized myocytes revealed that, in a range of 0.5-500 μM, high concentrations of NO-donors impair mitochondrial respiration and induce apoptosis (74).

As a matter of fact, the protective effect of NO-donors in reperfusion is expected to inversely depend on the concentration of the released NO. In the experiments of Rastaldo et al. (106, 107) the hybrid molecule failed to attenuate I/R injury either if the concentration was increased to 10 µM, or if the weak potency furoxan was replaced with a strong potency one without increasing hybrid concentration. It has been supposed that a moderate intracellular release of NO triggers a cascade in which the production of a limited amount of mitochondrial ROS leads to myocardial protection by mPTP inactivation without producing oxidative stress (fig. 2). On the contrary, if the release of NO is excessive, a high production of $ONOO^-$ occurs, so that the oxidative stress prevails on the protection and causes mPTP opening (109, 118).

Although moderate and high NO concentrations are not easy to define, it has been proposed that high concentrations should exceed 10 mM (119). However, the levels are difficult to foresee when exogenous NO is added to a pre-existing unknown concentration of the same compound.

The reverse concentration-dependent protective activity of NO is consistent with the unexpected effect of L-NAME and L-NMMA, which, at the concentration of 3 and 30 µM respectively, were seen to protect the isolated working rabbit heart, possibly by reducing the intracellular level of NO with a reduction in the final formation of $ONOO^-$. The hypothesis was confirmed by the possibility to abolish the protection with L-arginine (120). Interestingly, the protection took place only if the inhibitors were administered before but not after ischemia. It is likely that after ischemia NOS-inhibition worsens the NO shortage that, after the transient increase, is already present as a cause of reperfusion injury.

A double-edge sword effect by NO donors has also been seen in the mechanical recovery after ischemia (108, 121-123). This might be related to the fact that low concentrations of NO improve myocardial contractility, whereas the opposite occurs with high concentrations, either in the presence or in the absence of an infarcted area (122, 124). In fact, while low concentrations of NO lead to direct and PKA-mediated enhancement of Ca^{2+} handling (123, 125), high concentrations

induce the generation of a large amount of cGMP which reduces L-type Ca^{2+} channels current and Ca^{2+} responsiveness of Troponin C via PKG (123, 125-127).

While the effect of NO donors alone has been ascertained to be beneficial in a large amount of investigations (71, 95; 103-105), in the experiments of Rastaldo *et al.* (106, 107) they were effective only in the presence of AOX. Although there is no sure explanation of these diverging results, it may be argued that a role was played by the difference in the experimental model, such as isolated hearts *vs.* intact animals, different timing of the donor administration with respect to the ischemia, type of the donor. However, independently on whether or not AOX is required by NO donors to induce protection, there is evidence enough that low concentrations of NO are more effective than high concentrations.

Conclusions

An interplay between NO and ROS is responsible for either reperfusion injury or myocardial protection. In reperfusion, a transient initial release of NO in the presence of $O_2^{-\bullet}$ leads to the formation of $ONOO^-$, whose injuring effect is stronger than that of $O_2^{-\bullet}$, also because it uncouples eNOS leading to a further production of $O_2^{-\bullet}$**.** In brief, the initial transient release of NO triggers a negative feedback by preventing NO release by eNOS, and a feedforward pathway that exalts oxidative damage. The detrimental role of $ONOO^-$ resulting from the reaction of NO and $O_2^{-\bullet}$ must not be underestimated.

The balance between NO and ROS explains the diverging results obtained with the use of NO donors in the prevention of I/R injury. It is likely that a small release of exogenous NO induces a limited production of ROS in mitochondria, leading to mPTP inactivation. On the contrary, a large release of NO produces ROS in excess and favours the predominance of oxidative stress.

Nitrate tolerance is somehow similar to the lack of protection when an excess of exogenous NO combines with an incremental production of ROS.

The results reported in this article are mainly from animals experiments. From a therapeutic point of view, they suggest that a proper balance between NO and AOX must be taken into account. A critical point is that exogenous NO is added to endogenous NO, whose concentration and kinetics are difficult, if not impossible, to assess *in vivo*. Since AOX, exogenous and endogenous NO contribute to this balance, time of administration, potency and intracellular delivery of the active molecules together with their concentration should be properly selected. Due to the complexity of these variables, further study are required to set up adequate therapeutic protocols.

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References:

1. Yellon DM, Hausenloy DJ. Myocardial reperfusion injury. N Engl J Med. 2007 Sep;357(11):1121-35.
2. Podesser BK, Hallström S. Nitric oxide homeostasis as a target for drug to cardioplegia. Br J Pharmacol. 2007 Aug;151(7):930-40.
3. Xu KY, Huso DL, Dawson TM, Bredt DS, Becker LC. Nitric oxide synthase in cardiac sarcoplasmic reticulum. Proc Natl Acad Sci U S A. 1999 Jan;96(2):657-62.
4. Sears CE, Bryant SM, Ashley EA, Lygate CA, Rakovic S, Wallis HL, Neubauer S, Terrar DA, Casadei B. Cardiac neuronal nitric oxide synthase isoform regulates myocardial contraction and calcium handling. Circ Res. 2003 Mar;92(5):e52-9.
5. Schulz R, Smith JA, Lewis MJ, Moncada S. Nitric oxide synthase in cultured endocardial cells of the pig. Br J Pharmacol. 1991 Sep;104(1):21-4.
6. Danson EJ, Paterson DJ. Cardiac neurobiology of nitric oxide synthases. Ann N Y Acad Sci. 2005 Jun;1047:183-96.
7. Schulz R, Nava E, Moncada S. Induction and potential biological relevance of a Ca(2+)-independent nitric oxide synthase in the myocardium. Br J Pharmacol. 1992 Mar;105(3):575-80.
8. Spink J, Cohen J, Evans TJ. The cytokine responsive vascular smooth muscle cell enhancer of inducible nitric oxide synthase. Activation by nuclear factor-kappa B. J Biol Chem. 1995 Dec;270(49):29541-7.
9. Balligand JL, Ungureanu-Longrois D, Simmons WW, Kobzik L, Lowenstein CJ, Lamas S, Kelly RA, Smith TW, Michel T. Induction of NO synthase in rat cardiac microvascular endothelial cells by IL-1 beta and IFN-gamma. Am J Physiol. 1995 Mar;268(3 Pt 2):H1293-303.

10. Kanai AJ, Pearce LL, Clemens PR, Birder LA, VanBibber MM, Choi SY, de Groat WC, Peterson J. Identification of a neuronal nitric oxide synthase in isolated cardiac mitochondria using electrochemical detection. Proc Natl Acad Sci U S A. 2001 Nov 20;98(24):14126-31.
11. Palomba L, Persichini T, Mazzone V, Colasanti M, Cantoni O. Inhibition of nitric-oxide synthase-I (NOS-I)-dependent nitric oxide production by lipopolysaccharide plus interferon-gamma is mediated by arachidonic acid. Effects on NFkappaB activation and late inducible NOS expression. J Biol Chem. 2004 Jul;279(29):29895-901.
12. Muller B, Kleschyov AL, Gyorgy K, Stoclet JC. Inducible NO synthase activity in blood vessels and heart: new insight into cell origin and consequences. Physiol Res. 2000;49(1):19-26.
13. Mariotto S, Cavalieri E, Amelio E, Ciampa AR, de Prati AC, Marlinghaus E, Russo S, Suzuki H. Extracorporeal shock waves: from lithotripsy to anti-inflammatory action by NO production. Nitric Oxide. 2005 Mar;12(2):89-96.
14. Förstermann U, Sessa WC. Nitric oxide synthases: regulation and function. Eur Heart J. 2012 Apr;33(7):829-37.
15. Zweier JL, Li H, Samouilov A, Liu X. Mechanisms of nitrite reduction to nitric oxide in the heart and vessel wall. Nitric Oxide. 2010 Feb;22(2):83-90.
16. Webb A, Bond R, McLean P, Uppal R, Benjamin N, Ahluwalia A. Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia-reperfusion damage. Proc Natl Acad Sci U S A. 2004 Sep;101(37):13683-8.
17. Li H, Cui H, Liu X, Zweier JL. Xanthine oxidase catalyzes anaerobic transformation of organic nitrates to nitric oxide and nitrosothiols: characterization of this mechanism and the link between organic nitrate and guanylyl cyclase activation. J Biol Chem. 2005 Apr;280(17):16594-600.
18. Hendgen-Cotta UB, Merx MW, Shiva S, Schmitz J, Becher S, Klare JP, Steinhoff HJ, Goedecke A, Schrader J, Gladwin MT, Kelm M, Rassaf T. Nitrite reductase activity of

myoglobin regulates respiration and cellular viability in myocardial ischemia-reperfusion injury. Proc Natl Acad Sci U S A. 2008 Jul;105(29):10256-61.

19. Zweier JL, Samouilov A, Kuppusamy P. Non-enzymatic nitric oxide synthesis in biological systems. Biochim Biophys Acta. 1999 May 5;1411(2-3):250-62.

20. Buja LM. Myocardial ischemia and reperfusion injury. Cardiovasc Pathol. 2005 Jul-Aug;14(4):170-5.

21. Vandenberg JI, Rees SA, Wright AR, Powell T. Cell swelling and ion transport pathways in cardiac myocytes. Cardiovasc Res. 1996 Jul;32(1):85-97.

22. Xiao XH, Allen DG. Activity of the Na(+)/H(+) exchanger is critical to reperfusion damage and preconditioning in the isolated rat heart. Cardiovasc Res. 2000 Nov;48(2):244-53.

23. Kloner RA. Does reperfusion injury exist in humans? J Am Coll Cardiol. 1993 Feb;21(2):537-45.

24. Tsao PS, Aoki N, Lefer DJ, Johnson G 3rd, Lefer AM. Time course of endothelial dysfunction and myocardial injury during myocardial ischemia and reperfusion in the cat. Circulation. 1990 Oct;82(4):1402-12.

25. Kin H, Zhao ZQ, Sun HY, Wang NP, Corvera JS, Halkos ME, Kerendi F, Guyton RA, Vinten-Johansen J. Postconditioning attenuates myocardial ischemia-reperfusion injury by inhibiting events in the early minutes of reperfusion. Cardiovasc Res. 2004 Apr;62(1):74-85.

26. Prasad MR, Popescu LM, Moraru II, Liu XK, Maity S, Engelman RM, Das DK.Role of phospholipases A2 and C in myocardial ischemic reperfusion injury. Am J Physiol. 1991 Mar;260(3 Pt 2):H877-83.

27. Van der Vusse GJ, van Bilsen M, Reneman RS. Ischemia and reperfusion induced alterations in membrane phospholipids: an overview. Ann N Y Acad Sci. 1994 Jun;723:1-14.

28. Opie LH, Coetzee WA, Dennis SC, Thandroyen FT. A potential role of calcium ions in early ischemic and reperfusion arrhythmias. Ann N Y Acad Sci.1988;522:464-77.

29. Inserte J, Garcia-Dorado D, Ruiz-Meana M, Padilla F, Barrabés JA, Pina P, Agulló L, Piper HM, Soler-Soler J. Effect of inhibition of Na(+)/Ca(2+) exchanger at the time of myocardial reperfusion on hypercontracture and cell death. Cardiovasc Res. 2002 Sep;55(4):739-48.

30. Dumitrescu C, Biondi R, Xia Y, Cardounel AJ, Druhan LJ, Ambrosio G, Zweier JL. Myocardial ischemia results in tetrahydrobiopterin (BH4) oxidation with impaired endothelial function ameliorated by BH4. Proc Natl Acad Sci U S A. 2007 Sep 18;104(38):15081-6..

31. Zweier JL, Wang P, Kuppusamy P. Direct measurement of nitric oxide generation in the ischemic heart using electron paramagnetic resonance spectroscopy. J Biol Chem. 1995 Jan;270(1):304-7.

32. .Zenebe WJ, Nazarewicz RR, Parihar MS, Ghafourifar P. Hypoxia/reoxygenation of isolated rat heart mitochondria causes cytochrome c release and oxidative stress; evidence for involvement of mitochondrial nitric oxide synthase. J Mol Cell Cardiol. 2007 Oct;43(4):411-9

33. Chen W, Druhan LJ, Chen CA, Hemann C, Chen YR, Berka V, Tsai AL, Zweier JL. Peroxynitrite induces destruction of the tetrahydrobiopterin and heme in endothelial nitric oxide synthase: transition from reversible to irreversible enzyme inhibition. Biochemistry. 2010 Apr 13;49(14):3129-37.

34. Ferdinandy P, Schulz R. Nitric oxide, superoxide, and peroxynitrite in myocardial ischaemia-reperfusion injury and preconditioning. Br J Pharmacol. 2003 Feb;138(4):532-43.

35. Halestrap AP, Clarke SJ, Javadov SA. Mitochondrial permeability transition pore opening during myocardial reperfusion--a target for cardioprotection. Cardiovasc Res. 2004 Feb 15;61(3):372-85.

36. Di Lisa F, Canton M, Carpi A, Kaludercic N, Menabò R, Menazza S, Semenzato M. Mitochondrial injury and protection in ischemic pre- and postconditioning. Antioxid Redox Signal. 2011 Mar 1;14(5):881-91.

37. Zorov DB, Filburn CR, Klotz LO, Zweier JL, Sollott SJ. Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. J Exp Med. 2000 Oct;192(7):1001-14.

38. Pagliaro P, Chiribiri A, Mancardi D, Rastaldo R, Gattullo D, Losano G. Coronary endothelial dysfunction after ischemia and reperfusion and its prevention by ischemic preconditioning. Ital Heart J. 2003 Jun;4(6):383-94.

39. Pagliaro P, Moro F, Tullio F, Perrelli MG, Penna C. Cardioprotective pathways during reperfusion: focus on redox signaling and other modalities of cell signaling. Antioxid Redox Signal. 2011 Mar 1;14(5):833-50.

40. Langer HF, Chavakis T.Leukocyte-endothelial interactions in inflammation. J Cell Mol Med. 2009 Jul;13(7):1211-20.

41. Schwartz BG, Kloner RA. Coronary no reflow. J Mol Cell Cardiol. 2012 Apr;52(4):873-82.

42. Vrints CJ. Pathophysiology of the no-reflow phenomenon. Acute Card Care. 2009;11(2):69-76.

43. Reffelmann T, Kloner RA.The no-reflow phenomenon: A basic mechanism of myocardial ischemia and reperfusion. Basic Res Cardiol. 2006 Sep;101(5):359-72.

44. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation. 1986 Nov;74(5):1124-36.

45. Shiki K, Hearse DJ. Preconditioning of ischemic myocardium: reperfusion-induced arrhythmias. Am J Physiol. 1987 Dec;253(6 Pt 2):H1470-6.

46. Cohen MV, Liu GS, Downey JM. Preconditioning causes improved wall motion as well as smaller infarcts after transient coronary occlusion in rabbits. Circulation. 1991 Jul;84(1):341-9.

47. Kaeffer N, Richard V, François A, Lallemand F, Henry JP, Thuillez C. Preconditioning prevents chronic reperfusion-induced coronary endothelial dysfunction in rats. Am J Physiol. 1996 Sep;271(3 Pt 2):H842-9.

48. Pagliaro P, Gattullo D, Rastaldo R, Losano G. Ischemic preconditioning: from the first to the second window of protection. Life Sci. 2001 May 25;69(1):1-15.

49. Loveridge R, Schroeder F. Anaesthetic preconditioning. Contin Educ Anaesth Crit Care Pain 2010;10(2):38-42.

50. Zhao ZQ, Corvera JS, Halkos ME, Kerendi F, Wang NP, Guyton RA, Vinten-Johansen J. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. Am J Physiol Heart Circ Physiol. 2003 Aug;285(2):H579-88.

51. Auchampach JA, Gross GJ. Adenosine A1 receptors, KATP channels, and ischemic preconditioning in dogs. Am J Physiol. 1993 May;264(5 Pt 2):H1327-36.

52. Andrukhiv A, Costa AD, West IC, Garlid KD. Opening mitoKATP increases superoxide generation from complex I of the electron transport chain. Am J Physiol Heart Circ Physiol. 2006 Nov;291(5):H2067-74.

53. Oldenburg O, Cohen MV, Yellon DM, Downey JM. Mitochondrial K(ATP) channels: role in cardioprotection. Cardiovasc Res. 2002 Aug 15;55(3):429-37.

54. Parratt J, Vegh A. Pronounced antiarrhythmic effects of ischemic preconditioning. Cardioscience. 1994 Mar;5(1):9-18.

55. Vegh A, Papp JG, Parratt J. Attenuation of the antiarrhythmic effects of ischaemic preconditioning by blockade of bradykinin B2 receptors. Br J Pharmacol. 1994 Dec;113(4):1167-72.

56. Jones SP, Bolli R. The ubiquitous role of nitric oxide in cardioprotection. J Mol Cell Cardiol. 2006 Jan;40(1):16-23.

57. Vinten-Johansen J, Zhao ZQ, Zatta AJ, Kin H, Halkos ME, Kerendi F. Postconditioning--A new link in nature's armor against myocardial ischemia-reperfusion injury. Basic Res Cardiol. 2005 Jul;100(4):295-310.

58. Faghihi M, Alizadeh AM, Khori V, Latifpour M, Khodayari S. The role of nitric oxide, reactive oxygen species, and protein kinase C in oxytocin-induced cardioprotection in ischemic rat heart. Peptides. 2012 Oct;37(2):314-9.

59. Xu Z, Mueller RA, Park SS, Boysen PG, Cohen MV, Downey JM. Cardioprotection with adenosine A2 receptor activation at reperfusion. J Cardiovasc Pharmacol. 2005 Dec;46(6):794-802.

60. Ray CJ, Marshall JM. The cellular mechanisms by which adenosine evokes release of nitric oxide from rat aortic endothelium. J Physiol. 2006 Jan;570(Pt1):85-96.

61. Busija AR, Fridolfsson HN, Patel HH. A new sense of protection: role of the Ca2+-sensing receptor in ischemic preconditioning. Am J Physiol Heart Circ Physiol. 2010 Nov;299(5):H1300-1

62. Niwano S, Hirasawa S, Niwano H, Sasaki S, Masuda R, Sato K, Masuda T, Izumi T. Cardioprotective effects of sarcolemmal and mitochondrial K-ATP channel openers in an experimental model of autoimmune myocarditis. Role of the reduction in calcium overload during acute heart failure. Int Heart J. 2012;53(2):139-45.

63. Han J, Kim N, Joo H, Kim E, Earm YE. ATP-sensitive K(+) channel activation by nitric oxide and protein kinase G in rabbit ventricular myocytes. Am J Physiol Heart Circ Physiol. 2002 Oct;283(4):H1545-54.

64. Wang H, Kohr MJ, Wheeler DG, Ziolo MT. Endothelial nitric oxide synthase decreases beta-adrenergic responsiveness via inhibition of the L-type Ca2+ current. Am J Physiol Heart Circ Physiol. 2008 Mar;294(3):H1473-80.

65. Sun J, Morgan M, Shen RF, Steenbergen C, Murphy E. Preconditioning results in S-nitrosylation of proteins involved in regulation of mitochondrial energetics and calcium transport. Circ Res. 2007 Nov 26;101(11):1155-63.

66. Tamargo J, Caballero R, Gómez R, Delpón E. Cardiac electrophysiological effects of nitric oxide. Cardiovasc Res. 2010 Sep 1;87(4):593-600.

67. Costa AD, Jakob R, Costa CL, Andrukhiv K, West IC, Garlid KD. The mechanism by which the mitochondrial ATP-sensitive K+ channel opening and H2O2 inhibit the mitochondrial permeability transition. J Biol Chem. 2006 Jul 28;281(30):20801-8.

68. Pain T, Yang XM, Critz SD, Yue Y, Nakano A, Liu GS, Heusch G, Cohen MV, Downey JM. Opening of mitochondrial K(ATP) channels triggers the preconditioned state by generating free radicals. Circ Res. 2000 Sep 15;87(6):460-6.

69. Hausenloy DJ, Yellon DM. Preconditioning and postconditioning: united at reperfusion. Pharmacol Ther. 2007 Nov;116(2):173-91.

70. Penna C, Rastaldo R, Mancardi D, Raimondo S, Cappello S, Gattullo D, Losano G, Pagliaro P. Post-conditioning induced cardioprotection requires signaling through a redox-sensitive mechanism, mitochondrial ATP-sensitive K+ channel and protein kinase C activation. Basic Res Cardiol. 2006 Mar;101(2):180-9.

71. Tritto I, D'Andrea D, Eramo N, Scognamiglio A, De Simone C, Violante A, Esposito A, Chiariello M, Ambrosio G. Oxygen radicals can induce preconditioning in rabbit hearts. Circ Res. 1997 May;80(5):743-8.

72. Ambrosio G, Zweier JL, Duilio C, Kuppusamy P, Santoro G, Elia PP, Tritto I, Cirillo P, Condorelli M, Chiariello M, et al. Evidence that mitochondrial respiration is a source of potentially toxic oxygen free radicals in intact rabbit hearts subjected to ischemia and reflow. J Biol Chem. 1993 Sep 5;268(25):18532-41.

73. Yang XM, Proctor JB, Cui L, Krieg T, Downey JM, Cohen MV. Multiple, brief coronary occlusions during early reperfusion protect rabbit hearts by targeting cell signaling pathways. J Am Coll Cardiol. 2004 Sep 1;44(5):1103-10.

74. Ohtani H, Katoh H, Tanaka T, Saotome M, Urushida T, Satoh H, Hayashi H. Effects of nitric oxide on mitochondrial permeability transition pore and thiol-mediated responses in cardiac myocytes. Nitric Oxide. 2012 Feb;26(2):95-101.

75. Lecour S, Smith RM, Woodward B, Opie LH, Rochette L, Sack MN. Identification of a novel role for sphingolipid signaling in TNF alpha and ischemic preconditioning mediated cardioprotection. J Mol Cell Cardiol. 2002 May;34(5):509-18.

76. Lecour S. Activation of the protective Survivor Activating Factor Enhancement (SAFE) pathway against reperfusion injury: Does it go beyond the RISK pathway? J Mol Cell Cardiol. 2009 Jul;47(1):32-40.

77. Miura T, Miki T. GSK-3beta, a therapeutic target for cardiomyocyte protection. Circ J. 2009 Jul;73(7):1184-92.

78. Negoro S, Kunisada K, Tone E, Funamoto M, Oh H, Kishimoto T, Yamauchi-Takihara K. Activation of JAK/STAT pathway transduces cytoprotective signal in rat acute myocardial infarction. Cardiovasc Res. 2000;47(4):797-805.

79. Gross ER, Hsu AK, Gross GJ. The JAK/STAT pathway is essential for opioid-induced cardioprotection: JAK2 as a mediator of STAT3, Akt, and GSK-3 beta. Am J Physiol Heart Circ Physiol. 2006;291(2):H827-34.

80. Heusch G, Boengler K, Schulz R. Cardioprotection: nitric oxide, protein kinases, and mitochondria. Circulation 2008;118(19):1915-9.

81. Lacerda L, Somers S, Opie LH, Lecour S. Ischaemic postconditioning protects against reperfusion injury via the SAFE pathway. Cardiovasc Res 2009;84(2):201-8.

82. Thielmann M, Dörge H, Martin C, Belosjorow S, Schwanke U, van De Sand A, Konietzka I, Büchert A, Krüger A, Schulz R, Heusch G. Myocardial dysfunction with coronary

microembolization: signal transduction through a sequence of nitric oxide, tumor necrosis factor-alpha, and sphingosine. Circ Res. 2002;90(7):807-13.

83. Goodman MD, Koch SE, Fuller-Bicer GA, Butler KL. Regulating RISK: a role for JAK-STAT signaling in postconditioning? Am J Physiol Heart Circ Physiol. 2008;295(4):H1649-56.

84. Zweier JL, Flaherty JT, Weisfeldt ML. Direct measurement of free radical generation following reperfusion of ischemic myocardium. Proc Natl Acad Sci U S A. 1987;84(5):1404-7.

85. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev. 2007;87(1):315-424.

86. Flaherty JT, Pitt B, Gruber JW, Heuser RR, Rothbaum DA, Burwell LR, George BS, Kereiakes DJ, Deitchman D, Gustafson N,et al. Recombinant human superoxide dismutase (h-SOD) fails to improve recovery of ventricular function in patients undergoing coronary angioplasty for acute myocardial infarction. Circulation 1994;89(5): 1982-1991.

87. Vinten-Johansen J, Zhao ZQ, Zatta AJ, Kin H, Halkos ME, Kerendi F. Postconditioning--A new link in nature's armor against myocardial ischemia-reperfusion injury. Basic Res Cardiol. 2005;100(4):295-310.

88. Marchioli R, Levantesi G, Macchia A, Marfisi RM, Nicolosi GL, Tavazzi L, Tognoni G, Valagussa F; GISSI-Prevenzione Investigators. Vitamin E increases the risk of developing heart failure after myocardial infarction: Results from the GISSI-Prevenzione trial. J Cardiovasc Med (Hagerstown). 2006;7(5):347-50.

89. Vanden Hoek TL, Becker LB, Shao Z, Li C, Schumacker PT. Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. J Biol Chem. 1998;273(29):18092–8.

90. Gasco A, Fruttero R, Sorga G, Di Stilo A, Calvino R. NO donors: focus on furoxan derivatives. Pure Appl Chem. 2004;76:973-81.

91. Lefèvre-Groboillot D, Boucher JL, Stuehr DJ, Mansuy D. Relationship between the structure of guanidines and N-hydroxyguanidines, their binding to inducible nitric oxide synthase (iNOS) and their iNOS-catalysed oxidation to NO. FEBS J. 2005;272(12):3172-83.

92. Kleschyov AL, Oelze M, Daiber A, Huang Y, Mollnau H, Schulz E, Sydow K, Fichtlscherer B, Mulsch A, Munzel T. Does nitric oxide mediate the vasodilator activity of nitroglycerin? Circ Res. 2003;93(9):e104–e112.

93. Nunez C, Victor VM, Tur R, Alvarez-Barrientos A, Moncada S, Esplugues JV, D'Ocon P. Discrepancies between nitroglycerin and NO-releasing drugs on mitochondrial oxygen consumption, vasoactivity, and the release of NO. Circ Res. 2005;97(10):1063–1069.

94. Münzel T, Daiber A, Gori T. Nitrate therapy: new aspects concerning molecular action and tolerance. Circulation. 2011;123(19):2132-44.

95. Cohen MV, Yang XM, Downey JM. Nitric oxide is a preconditioning mimetic and cardioprotectant and is the basis of many available infarct-sparing strategies. Cardiovasc Res. 2006 May 1;70(2):231-9.

96. Miller MR, Megson IL. Recent developments in nitric oxide donor drugs. Br J Pharmacol. 2007;151(3):305-21.

97. Griscavage JM, Hobbs AJ, Ignarro LJ. Negative modulation of nitric oxide synthase by nitric oxide and nitroso compounds. Adv Pharmacol. 1995;34:215-34.

98. Nakano A, Liu GS, Heusch G, Downey JM, Cohen MV. Exogenous nitric oxide can trigger a preconditioned state through a free radical mechanism, but endogenous nitric oxide is not a trigger of classical ischemic preconditioning. J Mol Cell Cardiol. 2000;32(7):1159-67.

99. Qin Q, Yang XM, Cui L, Critz SD, Cohen MV, Browner NC, Lincoln TM, Downey JM. Exogenous NO triggers preconditioning via a cGMP- and mitoKATP-dependent mechanism. Am J Physiol Heart Circ Physiol 2004;287(2):H712-8.

100. Kutala VK, Khan M, Mandal R, Potaraju V, Colantuono G, Kumbala D, Kuppusamy P. Prevention of postischemic myocardial reperfusion injury by the combined treatment of NCX-4016 and Tempol. J Cardiovasc Pharmacol 2006;48(3):79-87.

101. Yui H, Imaizumi U, Beppu H, Ito M, Furuya M, Arisaka H, Yoshida K. Comparative effects of verapamil, nicardipine, and nitroglycerin on myocardial ischemia/reperfusion injury. Anesthesiol Res Pract 2011: 521084

102. Wakeno-Takahashi M, Otani H, Nakao S, Uchiyama Y, Imamura H, Shingu K. Adenosine and a nitric oxide donor enhances cardioprotection by preconditioning with isoflurane through mitochondrial adenosine triphosphate-sensitive K+ channel-dependent and -independent mechanisms. Anesthesiology. 2004;100(3):515-24.

103. Cohen MV, Yang XM, Downey JM. Nitric oxide is a preconditioning mimetic and cardioprotectant and is the basis of many available infarct-sparing strategies. Cardiovasc Res. 2006 May 1;70(2):231-9.

104. Leesar MA, Stoddard MF, Dawn B, Jasti VG, Masden R, Bolli R. Delayed preconditioning-mimetic action of nitroglycerin in patients undergoing coronary angioplasty. Circulation. 2001;103(24):2935-41.

105. Ambrosio G, Del Pinto M, Tritto I, Agnelli G, Bentivoglio M, Zuchi C, Anderson FA, Gore JM, López-Sendón J, Wyman A, Kennelly BM, Fox KA; GRACE Investigators. Chronic nitrate therapy is associated with different presentation and evolution of acute coronary syndromes: insights from 52,693 patients in the Global Registry of Acute Coronary Events. Eur Heart J. 2010 Feb;31(4):430-8.

106. Rastaldo R, Cappello S, Folino A, Di Stilo A, Chegaev K, Tritto I, Pagliaro P, Losano G. Low concentrations of a nitric oxide-donor combined with a liposoluble antioxidant compound enhance protection against reperfusion injury in isolated rat hearts. J Physiol Pharmacol. 2010;61(1):21-7.

107. Rastaldo R, Cappello S, Di Stilo A, Folino A, Losano G, Pagliaro P. A lipophilic nitric oxide-donor and a lipophilic antioxidant compound protect rat heart against ischemia-reperfusion injury if given as hybrid molecule but not as a mixture. J Cardiovasc Pharmacol. 2012;59(3):241-8.

108. Bolli R. Cardioprotective function of inducible nitric oxide synthase and role of nitric oxide in myocardial ischemia and preconditioning: an overview of a decade of research. J Mol Cell Cardiol. 2001;33(11):1897-918.

109. Salloum FN, Takenoshita Y, Ockaili RA, Daoud VP, Chou E, Yoshida K, Kukreja RC. Sildenafil and vardenafil but not nitroglycerin limit myocardial infarction through opening of mitochondrial K(ATP) channels when administered at reperfusion following ischemia in rabbits. J Mol Cell Cardiol 2007;42(2):453-8.

110. Schulz R, Kelm M, Heusch G. Nitric oxide in myocardial ischemia/reperfusion injury. Cardiovasc Res. 2004;61(3): 402-13.

111. .Steensrud T, Li J, Dai X, Manlhiot C, Kharbanda RK, Tropak M, Redington A. Pretreatment with the nitric oxide donor SNAP or nerve transection blocks humoral preconditioning by remote limb ischemia or intra-arterial adenosine. Am J Physiol Heart Circ Physiol. 2010 Nov;299(5):H1598-603

112. Gibbons RJ, Abrams J, Chatterjee K, Daley J, Deedwania PC, Douglas JS, et al.; American College of Cardiology; American Heart Association Task Force on practice guidelines (Committee on the Management of Patients With Chronic Stable Angina). ACC/AHA 2002 guideline update for the management of patients with chronic stable angina--summary article: a report of the American College of Cardiology/American Heart Association Task Force on practice guidelines (Committee on the Management of Patients With Chronic Stable Angina). J Am Coll Cardiol 2003;41(1):159-68.

113. Yurtseven N, Karaca P, Kaplan M, Ozkul V, Tuygun AK, Aksoy T Canik S, Kopman. Effect of nitroglycerin inhalation on patients with pulmonary hypertension undergoing mitral valve replacement surgery. Anesthesiology. 2003;99(4):855–8.

114. Hunt SA, Abraham WT, Chin MH, Feldman AM, Francis GS, Ganiats TG, et al.; American College of Cardiology; American Heart Association Task Force on Practice Guidelines; American College of Chest Physicians; International Society for Heart and Lung Transplantation; Heart Rhythm Society. ACC/AHA 2005 Guideline Update for the Diagnosis and Management of Chronic Heart Failure in the Adult: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Update the 2001 Guidelines for the Evaluation and Management of Heart Failure): developed in collaboration with the American College of Chest Physicians and the International Society for Heart and Lung Transplantation: endorsed by the Heart Rhythm Society. Circulation. 2005;112(12):e154-e235

115. Fox K, Garcia MA, Ardissino D, Buszman P, Camici PG, Crea F, et al.;Task Force on the Management of Stable Angina Pectoris of the European Society of Cardiology; ESC Committee for Practice Guidelines (CPG). Guidelines on the management of stable angina pectoris: executive summary: The Task Force on the Management of Stable Angina Pectoris of the European Society of Cardiology. Eur Heart J 2006;27(11):1341-81

116. Dickstein K, Cohen-Solal A, Filippatos G, McMurray JJ, Ponikowski P, Poole-Wilson PA, et al. ESC Committee for Practice Guidelines (CPG). ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2008: the Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2008 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association of the ESC (HFA)and endorsed by the European Society of Intensive Care Medicine (ESICM). Eur Heart J. 2008;29(19):2388-442.

117. Patel HH, Tsutsumi YM, Roth DM. Mito-controversies: mitochondrial permeabilità transition pore and myocardial reperfusion injury. Anesthesiology. 2008 Feb;108(2):182-4.

118. Brown GC, Borutaite V. Nitric oxide and mitochondrial respiration in the heart. Cardiovasc Res. 2007 Jul 15;75(2):283-90.

119. Kojda G. Mechanisms of inotropic effects induced by nitric oxide. Ital Heart J. 2001;2(3):48S-49S.

120. Schulz R, Wambolt R. Inhibition of nitric oxide synthesis protects the isolated working rabbit heart from ischaemia-reperfusion injury. Cardiovasc Res 1995;30(3):432-9.

121. Ohba M, Kawata H. Biphasic nature of inotropic action of nitric oxide donor NOC7 in guinea-pig ventricular trabeculae. Jpn J Physio. 1999;49(4):389-94.

122. Hui Y, Mochizuki T, Kondo K, Umemura K, Sato S. Nitric oxide donor, NOC7,reveals biphasic effect on contractile force of isolated rat heart after global ischemia. J Anesth. 2008;22(3):229-35.

123. González DR, Fernández IC, Ordenes PP, Treuer AV, Eller G, Boric MP. Differential role of S-nitrosylation and the NO-cGMP-PKG pathway in cardiac contractility. Nitric Oxide. 2008;18(3):157-67.

124. Mohan P, Sys SU, Brutsaert DL. Positive inotropic effect of nitric oxide in myocardium. Int J Cardiol. 1995;50(3):233-7.

125. Massion PB, Balligand JL. Modulation of cardiac contraction, relaxation and rate by the endothelial nitric oxide synthase (eNOS): lessons from genetically modified mice. J Physiol. 2003;546(Pt 1): 63-75.

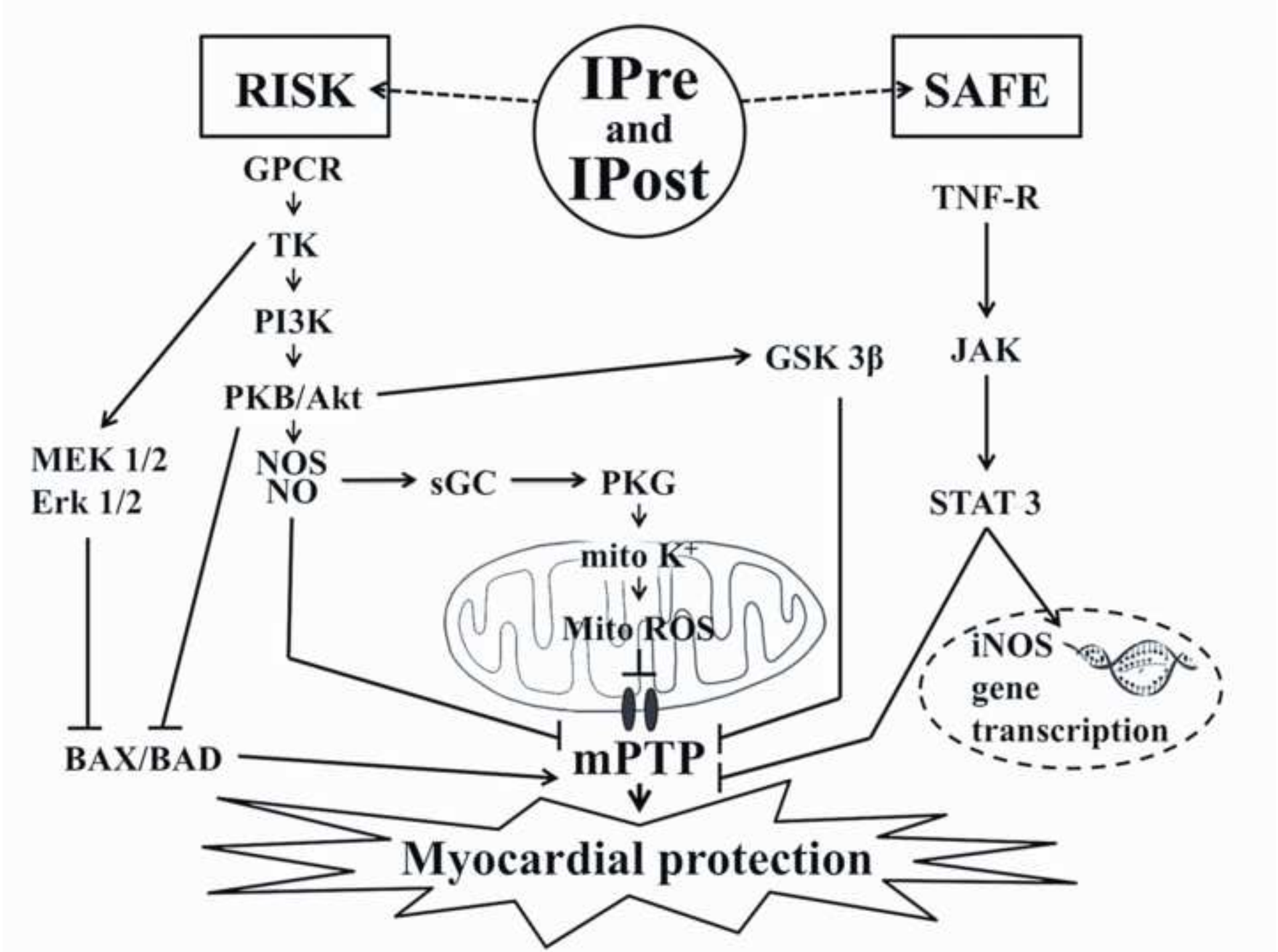
126. Jiang LH, Gawler DJ, Hodson N, Milligan CJ, Pearson HA, Porter V, Wray D. Regulation of cloned cardiac L-type calcium channels by cGMP-dependent protein kinase. J Biol Chem 2000;275(9):6135-43.

127. Layland J, Li JM, Shah AM. Role of cyclic GMP-dependent protein kinase in the contractile response to exogenous nitric oxide in rat cardiac myocytes. J Physiol. 2002;540(Pt 2):457-67.

Figure Legends

Figure 1 Reperfusion injury salvage kinase (RISK) and survivor activating factor enhancement (SAFE) protective pathways elicited by ischemic pre (IPre) and postconditioning (IPost). RISK and SAFE pathways begin with the binding of protective agents to G-protein coupled receptors (GPCR) and tumor necrosis factor receptors (TNF-R) respectively. In both cases the protection is achieved by the inactivation of mitochondrial permeability transition pores (mPTP). TK = tyrosine-kinase; PI3K = phospho-inositol-3-kinase; PKB/Akt = protein kinase B; NOS = nitric oxide synthase; NOS = nitric oxide synthase; NO = Nitric oxide; MEK 1/2 = mitogen-activated protein kinase kinase 1/2; Erk 1/2=. extracellular regulated kinase 1/2; BAX/BAD = proapoptotic proteins; sGC = soluble guanylate-cyclase; PKG = protein kinase G; mito K = mitochondrial K^+ ATP-dependent channel; Mito ROS = mitochondrial reactive oxygen species; mPTP = mitochondrial permeability transition pores; GSK 3β = glycogen synthase kinase-3β; JAK = Janus kinase; STAT3 = signal transducer and activator of transcription-3; iNOS = inducible nitric-oxide synthase.

Figura 2 Different effects of low (A) and high (B) NO concentrations. The density of the circles represents nitric oxide (NO) concentration. If its concentration is low, NO can induce a limited production of reactive oxygen species (ROS) which inhibits the activation of mitochondrial permeability transition pores (mPTP) through a signalling cascade thus inducing myocardial protection. On the contrary, if the release of NO is high, there is such a production of ROS that the oxidative stress prevails on the protective cascade and causes mPTP opening thus inducing myocardial injury.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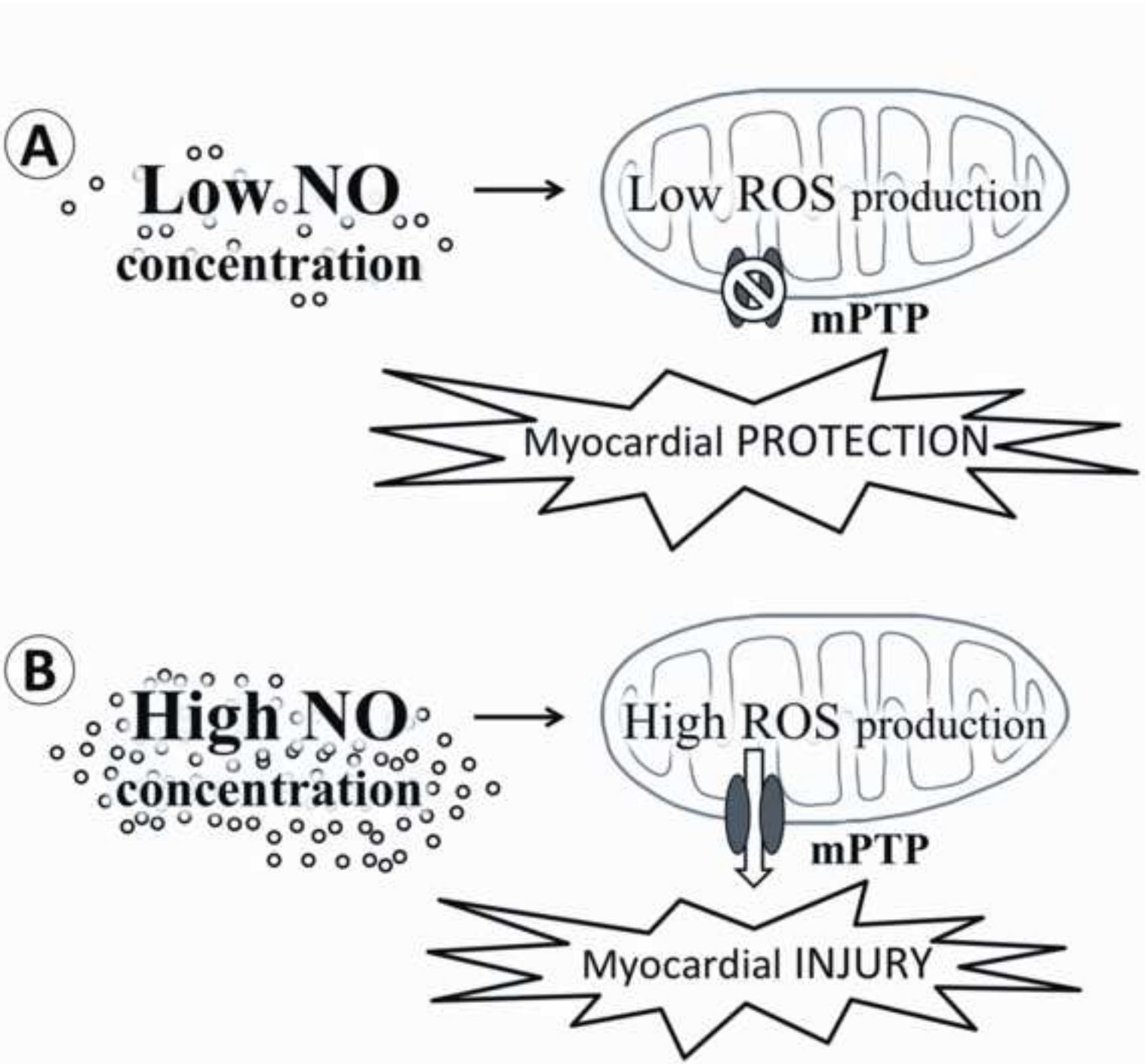
gene
transcription
BAX/BAD
mPTP
Myocardial protection


A
Low NO
concentration
Low ROS production
mPTP
Myocardial PROTECTION
B
High NO
concentration
High ROS production
mPTP
Myocardial INJURY