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CARDIAC

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 (BH₄) is needed for the reaction. In fact, if BH₄ or L-arginine are absent, NOSs produce superoxide anion (O₂^{•-}) 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, iNOS is Ca^{2+} -calmodulin independent and is activated by nuclear factor kappa B (NF- κ B), 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- κ B 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 $\text{Na}^+/\text{Ca}^{2+}$ 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 phospholipases 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 $\text{O}_2^{\bullet-}$ 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 $\text{O}_2^{\bullet-}$ (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 $\text{O}_2^{\bullet-}$. On the other hand, ONOO^- can stop NO production by destabilizing the eNOS dimer.

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 BH₄ 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 $\text{PKC}\epsilon 1$ 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-3 β (GSK 3 β). 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-R₂ 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 phosphorylation/inhibition of GSK 3 β (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 O₂[•] 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[•]) and nitrogen dioxide (NO₂[•]) 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 postconditioning (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 derivatives, 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-esters 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-catalysed 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 NOSs (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, Leeser *et al.* (104) report that NTG infusion 24 hours before coronary angioplasty reduces ST segment shift during the ischemia induced by inflating the angioplasty 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 μ M) 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 μ M, 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 μ M, 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 μ M 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 Ca²⁺ 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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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 3 β = glycogen synthase kinase-3 β ; 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.





