UNIVERSITA' DEGLI STUDI DI SIENA

Doctorate in Biomedicine and Immunological Science

Clinical Pharmacology section

Chronic protection against ischemia and reperfusion injury during therapy with different organic nitrates

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PART 1

General Introduction:

MOLECULAR BASIS OF ISCHEMIA-REPERFUSION INJURY AND OF ISCHEMIC PRECONDITION

1.1. Theories about ischemia and reperfusion injury

Ischemic heart disease is the leading cause of death worldwide. As such, novel therapeutic strategies for protecting the heart against ischemia–reperfusion injury are urgently needed to reduce myocardial injury, preserve cardiac function, prevent the development of heart failure and improve clinical outcomes in patients with ischemic heart disease. It was estimated that 3.8 million men and 3.4 million women die each year from these diseases ([1, 2]). Because of the progression of epidemic diseases such as diabetes mellitus, hypertension, hypercholesterolemia and increasing average age of population, the incidence of such diseases is growing. For this reason, the research that deals with the treatment of ischemic syndromes and in particular heart disease is of primary importance.

Because cell death is a partially reversible event or, otherwise, it can be postponed even after a prolonged ischemia, all strategies aimed at preserving the vitality of cells during both primary and secondary prevention should be considered as a therapeutic goal of primary importance ([3]).

At nowadays, modern intervention strategies in this field are based on two key observations: first one is that the extension of necrosis caused by ischemia and therefore the prognosis of the disease are time-dependent, and second one that acting on specific metabolism pathways, it is possible to modulate the resistance of the tissue against ischemic injury.

It is possible to draw an hypothetical graph of an ischemic event with time on the abscissa and the amount of the injury on the ordinates; well therapeutic

interventions may induce the reduction of its spreading during time with early reperfusion of ischemic areas and on the other hand they can decrease the magnitude of the insult modulating the ischemic resistance of the heart muscle.
From these two different but complementary approaches to the problem originate different strategies. In fact the aim of interventional cardiology is the reduction in duration of ischemia allowing reperfusion of ischemic myocardium in urgency, while the reduction of ischemic damage regardless of the time is leaded by research in cardioprotection.

In 1971 Maroko was the first to face the problem of reduction of infarct necrosis. The idea was that, since the infarct extension is directly proportional to the clinical severity of ischemia, any strategy that reduces the infarct size would have improved the prognosis of the disease ([4]).
Reimer and Jennings for first demonstrated that the reduction of duration of ischemia could reduce total infarct ([5]). This observation led directly to the concept of reperfusion therapy, which now, through the use of thrombolysis or primary percutaneous angioplasty is the most effective strategy for the treatment of acute coronary syndromes.
As soon as reperfusion therapy has became an ordinary intervention, it was clear that the extension of the ischemic area was determined not only by the degeneration due to lack of substrates during ischemia but also by an injury that acts during reperfusion.
It was therefore hypothesized for the first time the existence of damage not only due to ischemia by itself but due to both ischemia and reperfusion (IR) ([6, 7]).

In fact there are evidences in animal models that reperfusion induces the death of cardiomyocytes that were still viable during ischemia. This phenomenon may partly explain why, despite a sudden revascularization, the amount of myocardial necrosis that undergoes after myocardial infarction is still above 10%. Studies in animal models suggest that reperfusion injury is responsible for more than 50% of the final extension of the infarcted area.

Hearse, to explain this phenomenon, coined the term "oxygen paradox" ([8]). Indeed, he noted that the reperfusion of rat's heart with a low oxygen pressure buffer had little effect on the extent of the infarct but the complete re-oxygenation after a period of anoxia caused immediate cell necrosis. It appears clear that although oxygen was necessary to resolve ischemic event, was at the same time responsible for reperfusion injury. Hence it was born the idea of ischemia and reperfusion injury induced by oxygen free radicals (ROS), which to date has not yet been fully explained. According to this theory the reintroduction of oxygen leads to a sudden increase in the production of ROS that could lead the damage of cell membranes, the dysfunction of enzymes essential to cell life and to interfere with ion pumps and with the regulation of cell volume. With the advancement of knowledge and research to date we know certainly that ROS are responsible for cell death but in the other hand there are many other factors involved as effectors (1).

In the field of cardioprotection the first step was made in 1986 when Murry found that subjecting myocardial tissue to 4 cycles of 5 minutes of occlusion and 5 minutes of reperfusion of coronary arteries rendered the heart more resistant to a subsequent prolonged ischemia. For the first time and in different

animal models it was possible to reduce the extent of ischemic damage by more than 50%. Murry named this effect "ischemic preconditioning" (9).

The important new was that for the first time the infarct size could be changed independently of the time of reperfusion.

To date with the advancement of knowledge and research we know that oxygen radicals are responsible for cell death but for sure there are many other factors involved as effectors.

Numerous studies have highlighted the possibility that the phenomenon of preconditioning could also acting in the human myocardium ([10]). In fact, in vitro experiments with trabecular myocytes or tissue taken from human individuals have demonstrated the possibility to induce this phenomenon ([11]). Numerous experimental in vivo studies have shown this phenomenon, even if indirectly.

In fact it was proved that repeated inflations of a balloon angioplasty results in less angina, less ST segment elevation and less lactate production compared to a single inflaction, and even the onset of pre-infarction angina reduces the size of a subsequent infarct and is associated with better prognosis.

Although there are so very strong evidence that this phenomenon is also inducible in human myocardial tissue and that it clearly represents a reproducible and effective method able to reduce the area of necrosis, in practice it can not be simply applied to clinical routine because this method requires that the preconditioning is applied before ischemia and unfortunately the onset of the infarct is not in any way predictable.

It is therefore logical that so many efforts have been made to understand the mechanisms that mediate this tolerance to ischemia in order to develop drugs or methods that can artificially induce the preconditioning effect.

1.2. Molecular mechanisms of ischemia and reperfusion injury

A limited supply of oxygen and metabolic substrates as occurs during induction of an ischemia determines metabolic, functional and structural changes in vascular and myocardial cells. In addition to the lack of substrates, the shift between aerobic and anaerobic metabolism determine the accumulation of toxic metabolites and ions. Inhibition of cell metabolism is associated with rapid depletion of ATP and creatinine phosphate. Since myocytes are trying to correct the load of acidosis by extruding H^+ ions through the Na^+/H^+ exchanger, then this induce the accumulation of Na^+, which in turn can not be removed from the cytosol through the Na^+/K^+-ATP pumps because of the lack of ATP itself. The subsequent activation of Na^+/Ca^{++} exchanger in reversed mode helps to exchange Na^+ with the extracellular environment but it determines accumulation of intracytosolic Ca^{++}. All these changes, combined with the accumulation of toxic catabolites, oxygen free radicals and reduced pH, have important effects on membrane potential, contractility and cell activation status of several Ca^{++} dependent protease that with ongoing ischemia they are able to induce cell death ([12, 13]).

The end of ischemia is marked by the beginning of reperfusion but itself leads to further cell damage. This phenomenon, known as myocardial reperfusion injury, can paradoxically reduce the benefits of reperfusion itself ([14]). To date we know that cell death is basically determined by two main factors: (1) hypercontracture of the myofilaments due to Ca^{++} overload that mechanically induces the rupture of the cell membrane and (2) the opening of the mitochondrial permeability transient pores (mPTP), which instead leads to the activation of enzymatic cascades that produce several proapoptotic mediators ([15]).

1.2.1 The hypercontracture

There is strong evidence that excessive activation of contraction (so named hypercontracture) play an important role in cell death as the main responsible of the breakage of the cell plasma membrane ([16]). The Ca^{++} overload during ischemia due to dysfunction of the exchanger Na^{+}-Ca^{++} and the sudden fluctuations of Ca^{++} during reperfusion are the effectors of this phenomenon ([17, 18]). The rupture of sarcolemma is also facilitated by the cytoskeletal fragility induced by calpain-dependent hydrolysis of filamentous proteins (such as alpha-fodrin) which form a proteinaceous network responsible for the mechanical strength of the sarcolemma. In turn, calpain is activated by high concentrations of Ca^{++}. The calpain is also able to hydrolyse the Na^{+} pumps that are essential for the safe storage of Ca^{++}. This mechanism represents a vicious

circle where the increase of Ca^{++} increases the activation of calpain, which in turn leads to dysfunction of the Na^{+} pump exacerbating the overload of Ca^{++} ([19]). Finally we know that the influence of massive intracellular Na^{+} due to the rupture of the sarcolemma in a cell can also spread to adjacent cells through gap junctions leading to a cytonecrotic chain effect ([20]).

1.2.2. The opening of mPTP

The mitochondrial permeability transition pore (mPTP) are the other mediators of reperfusion injury ([21]).

Such structures, located in the inner mitochondrial membrane, are made by the linking of an adenine nucleotide translocase (ANT) with a voltage-dependent anionic channel (VDAC or so called “porine”). Cyclophilin D represents the regulatory portion of the pore and it is inhibited by cyclosporine (figure 1.7 and 1.8).

Under physiological conditions the inner mitochondrial membrane is impermeable to almost all the metabolites and ions as the mPTP are in closed conformation. During certain conditions of stress their temporary opening allows the rebalancing of matrix molecules.

Subsequently to a prolonged ischemia during reperfusion the oxidative stress, the increase of Ca^{++}, alkalinization and reduced concentration of ATP are able to determine prolonged mPTP opening. This molecular phenomenon is associated with irreversible damage to these organelles, evidenced firstly by the

loss of transmembrane polarization, the subsequent swelling of the matrix and finally by the rupture of mitochondrial membranes with subsequent release into the cytosol of proapoptotic factors such as cytochrome C. These findings are incompatible with life and lead rapidly to cell necrosis or apoptosis ([22]).

1.2.3. The coeffettors of IR injury

Of great importance is that both these mechanisms, ie the opening of MPTP and the hypercontracture, are closely related to each other and are dependent on other factors that play a role as coeffectors. These effectors are represented by (1) the ROS, (2) the cytosolic overload of Ca^{++}, (3) the activation of the inflammation mediators, (3) the lack of ATP and (4) the changes in pH ([23]).

During myocardial reperfusion, ROS are mainly produced by xanthine oxidase (from endothelial cells), by NADPH oxidase (from neutrophils), by nitric oxide synthase (eNOS) and by uncoupling of oxidative phosphorylation in the mitochondrial respiratory chain ([24]). Indeed ROS are also responsible of myocardial damage due to the opening of mPTP, the activation of chemotaxis of neutrophils and finally they mediate the dysfunction of the sarcoplasmic reticulum (contributing to the overload of Ca^{++}) and the damage of the cell membrane through the peroxidation of lipids and denaturation of cytosolic enzymes. Finally they cause direct oxidative damage to DNA.

During reperfusion myocardial Ca^{++}, already accumulated in cytosol during the ischemic phase, is further accumulated due to the dysfunction of the

sarcoplasmic reticulum and sarcolemma mainly due to oxidative stress. The generation of ATP by oxidative phosphorylation caused by the reintroduction of substrates, together with the overload of Ca^{++} induce the phenomenon of hypercontracture. Furthermore, the recovery of mitochondrial membrane potential drives the partial shift of the overloaded intracytosolic Ca^{++} within the mitochondrial matrix and that in concert with the production of ROS, the alkalinization of the matrix and the lack of ATP determines the opening of mPTP ([25]).

The rapid increase of pH during reperfusion is another co-factor. Alkalinization by Na^{+}/H^{+} and $Na^{+}/HCO3$- exchangers and the rapid washout of lactic acid both facilitate the opening of the mPTP and the binding of Ca^{++} with troponin favouring the hypercontracture.

Finally, hours after the end of ischemia, the accumulation of neutrophils in areas of necrosis in response to the release of mediators (ROS, adenosine, complement fragments and cytokines) induces a further damage indirectly through the occlusion of the microvasculature and directly through the production of ROS and lytic enzymes.

1.3. The ischemic preconditioning

In 1986 Murry et al. demonstrated for the first time a phenomenon they called ischemic preconditioning ([7, 9]). In their study in dogs, the authors showed that 4

cycles of myocardial ischemia (lasting 5 minutes each) interspersed with cycles of the same duration of reperfusion, were able to limit the size of necrosis by 75% after a prolonged occlusion (45 minutes) of the same coronary artery.

Essentially the induction of ischemic preconditioning determines a state of resistance to prolonged ischemia. This phenotype appears to be experimentally induced by brief periods of sublethal ischemia followed by reperfusion. This effect, which is now widely reproduced in various human and animal experimental models, was defined as "the most powerful form of protection against in vivo myocardial ischemia together with the early reperfusion" ([26]). Other studies have also shown that the effects of preconditioning are not limited to the reduction of the area of necrosis, but are also extended to the prevention of arrhythmias ([27]) and myocardial stunning ([28]), although it is not clear if these effects arise from the reduction of cell death or rather represent another separate processes.

The study of the mechanism of induction of preconditioning has potential applications not only in cardiology, but also in the field of transplantation (especially kidney and liver) and in prevention of cerebrovascular diseases. In fact in many of these studies has been demonstrated that the mediators involved in the protection of the myocardium are the same that can induce preconditioning in other organs.

To increase the interest of researchers for a possible clinical application of this phenomenon was the observation that the state of protection induced by ischemic preconditioning is sustained over time ([27, 29]). In fact the preconditioning effect is operative for 1 to 2 h after sustained coronary

occlusion, then is lost for several hours (refractory period), but redevelops when sustained coronary occlusion is induced approximately 24 h after the preconditioning. The first phase is known as early or classical preconditioning and the delayed phase as the second window of protection (SWOP) (Figure 1.1).

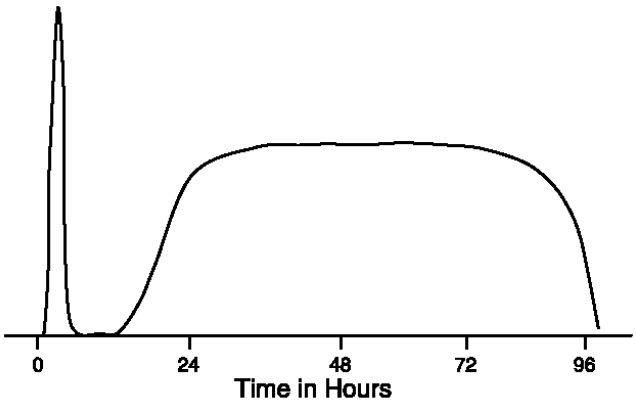


Figure 1.1. A standard ischemic preconditioning (IPC) stimulus of one or more brief episodes of non-lethal myocardial ischemia and reperfusion elicits a bi-phasic pattern of cardioprotection. The first phase manifests almost immedi-ately following the IPC stimulus and lasts for 1–2 h, after which its effect disappears (termed classical or early IPC). The second phase of cardioprotection appears 12–24 h later and lasts for 48–72 h (termed the Second Window of Protection [SWOP] or delayed or late IPC).

Early or classical preconditioning is triggered by activation of adenosine and other agonist receptors, and it is mediated by activation of proteinkinase C coupled to G proteins and the opening of ATP-dependent potassium channels in the sarcolemma and mitochondria, with the activation of the mitochondrial ATP-dependent potassium channels as the key event ([17]). There is uncertainty about the immediate effectors of preconditioning that are induced by the

activation of the ATP-dependent potassium channels. However, recent work has identified a prosurvival pathway involving the activation of ERK and Akt kinases linked to the prevention of the opening of the mitochondrial permeability transition pore (mPTP).

While the early preconditioning seems to depend mainly on the activation of ionic currents by different enzymatic mediators, the latter (SWOP) appears to be mediated by the protein neosynthesis resulting in genomic induction, post-translational protein modifications, different compartmentalization of synthesized proteins and inhibition of proapoptotic enzymes. The SWOP is mediated by the activation of a kinase cascade, including mitogen-activated proteins (MAP) kinases and nuclear factor kappa B (NfnB), leading to the activation of a gene program. Significant gene products implicated in protection include superoxide dismutase, nitric oxide synthase, cyclooxygenase 2 (COX2), and heat shock proteins. These enzymes create a protective enviroment for the cardiomyocyte, but again, the exact mechanisms of the protective effect are uncertain.

1.3.1. Mediators of preconditioning.

The stimuli able to induce the two windows of preconditioning seem to be the same and they include adenosine, endogenous opioids, sympathetic activation, bradykinin, ROS and nitric oxide (NO) ([29, 31]).

The first evidence that preconditioning is a phenomenon mediated by receptors (and thus potentially inducible by agonists of these receptors), came from a study in rabbit hearts of Downey, where A_1 adenosine receptor blockers prevent the development of preconditioning and in the other hand agonists of these receptors induced it ([32]). A short time later, similar observations were reported in cardiac receptors for nor-epinephrine and opioids ([33]). To date we know that all Gi protein-coupled receptors are capable of inducing preconditioning and that different receptors are able to cooperate to provide an adequate stimulus in this direction. Therefore, we suppose that during ischemia, mediators such as adenosine, epinephrine, opioids, and bradykinin ([34]) are released and they mediate the induction of preconditioning through links with specific receptors.

From an evolutionary point of view, the redundancy of this system, as noted above, seems to confirm the importance of itself: nature seems to want to ensure that the preconditioning can develop even in the event that one of these ways is missing or does not produce sufficient protection (figure 1.12)

1.3.2. The role of ROS and NO.

In addition to the mediators mentioned above, there are numerous scientific evidence suggesting that reactive species of oxygen (ROS) are able to induce preconditioning ([24]). ROS refers to a family of molecules that are heterogeneous in size with different chemical qualities and biological potential. O_2^- appears to be the principal representative of these compounds. The O_2^- is formed by all cells from the univalent reduction of molecular oxygen ([35]), a reaction mediated by a variety of enzymes, including nitric oxide synthase, NAD (P) H, xanthine oxidase and the mitochondrial respiratory chain. As discussed above, there is evidence that the long-established ischemia and especially reperfusion induce a sudden increase in the bioavailability of O_2^-. H_2O_2 is formed by dismutation of superoxide dismutase enzyme, while reacting with nitric oxide (NO) it forms the powerful oxidant peroxynitrite. Although H_2O_2 is considered the most stable free radicals of oxygen among all, it can react with transition metals in reduced state (such as heme groups of many proteins) oxidizing and forming the hydroxyl radical (OH^-), in turn highly reactive ([36]).

There are many evidences that show a role of ROS in ischemic preconditioning. In fact, pretreatment with antioxidants such as superoxide dismutase and vitamin E (even if it reduces the direct damage caused by peroxidation during reperfusion) can inhibit the development of preconditioning ([37, 38]).

Recent evidence also highlights the role of another free radical in the induction of preconditioning, the nitric oxide (NO), ([39]).

NO is actually a crucial mediator capable of regulating many processes, primarily in the vasculature, but also in central nervous system and in the heart. In fact, the endogenous NO, produced by the vascular endothelium, is one of the main physiological mediators involved in the regulation of vascular homeostasis. The NO is also the active catabolite of nitrates that are still the most widely used drugs in the treatment of ischemic heart disease.

1.3.3 Endogenous production and donors of NO

The vascular endothelium is a single layer of cells lining in the inner surface of all vessels. Over the past 20 years numerous studies have shown that this tissue has a fundamental role in the regulation of a variety of cardiovascular processes, including control of vascular tone, cell proliferation, coagulation, extracellular matrix deposition, hemostasis and inflammatory responses ([40-42]). NO is the best characterized substance among all released by the endothelium ([42]). NO is an highly reactive free radical that it is produced by endothelial cells through the activity of nitric oxide synthase (NOS). Normally, the NOS produces NO through two steps ([43]):the first is the reduction of molecular O_2 to form O_2^- which, in the second step, oxidizes L-arginine to form L-citrulline and NO. NO is able to freely pass cell membranes and acts as an autocrine and paracrine mediator ([44]).

More specifically, this molecule acts directly in cytoplasm of smooth muscle cells through activation of guanylate cyclase (sGC), which formed as a second

messenger cGMP by GDP and finally this is able to activate specific protein kinase (PKC). In turn, these enzymes induce vasodilation (and other effects as inhibition of platelet aggregation and leukocyte actraction) through phosphorylation of channel proteins involved in the homeostasis of $Ca2^+$ (sarcoplasmic $Ca2^+$-ATPase) and K^+([45]). The reduction in the amount of calcium ions into the cytosol of myocytes and activation of K^+ currents hyperpolarize the membrane (sarcolemma) determining relaxation of the myocyte and globally resulting in vessels vaso-dilation ([46,47]).

There are three different isoforms of the enzyme responsible for NO synthesis: the constitutive endothelial nitric oxide synthase (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS) ([43, 47]). The red-ox of tetrahydrobiopterin plays a role key in the function of this enzyme ([48]): when this cofactor is oxidized to dihydrobiopterin, the NOS is "uncoupled", ie it is able to catalyze only the first of the two reactions. The same happens in deficiency states of the substrate L-arginine ([48]). In fact the transfer of electrons from NADPH stops to the reduction of molecular oxygen into O_2^- and subsequently due to the lack of substrate and / or cofactor, the process stop at this radical instead at NO production. Thus, an excess of ROS from any intra or extracellular source triggers a vicious feed-forward cycle, in which the activity of NOS is shifted to production of ROS instead of the more stable NO.

Ultimately, ROS and NOS uncoupled, would seem to be the cause of the cytotoxic damage at the time of reperfusion, as well as the development of tolerance.

The endogenous production of NO can be mimicked by certain drugs such as nitrates. In fact, organic nitrates undergo a process of biotransformation leading to production of NO in vascular endothelium. Since a long time we know that the administration of nitrates induces significant dilation of the venous vessels and an increase in blood volume in capacitance beds ([50, 51]). In fact, nitrates promote a blood self-transfusion from the heart and lungs into the splanchnic and peripheral circulation. This promote a lower venous return to right heart, resulting in decreasing in preload, cardiac output and stroke volume. For this reason the use of nitrates is absolutely contraindicated in myocardial right infarction.

Another effect of considerable clinical impact is the increase in coronary blood flow. The dilatory effect in this vascular district promotes the use of nitrates in the prevention and treatment of myocardial ischemia in patients with coronary stenoses.

1.3.4. The role of NO in the induction of preconditioning

In the past 10 years a substantial number of studies have evaluated the role of NO in the modulation of ischemia-reperfusion injury independently of its hemodynamical activity. It has been hypothised that these molecules could act as triggers and mediators of preconditioning ([44, 52]).

Increasing evidences show that administration of NO donors is able to induce the phenomenon of preconditioning unlike the endogenous role of NO (ie the role of NOS) is debated.

In this regard Nakano et al. ([53]) have shown in an ex vivo model that pretreatment with SNAP, an inorganic nitrate, was able to reduce the amount of myocardial infarction induced by coronary occlusion lasting 30 minutes and followed by 2 hours of reperfusion . The effect size was similar in magnitude to that induced by ischemic preconditioning.

More clear is the role of NO in the induction of delayed preconditioning (the one that appears after 24 hours from an ischemic stimulus or exogenous administration of a given drug). This finding was confirmed in several studies ([44, 53, 54]). In one of those, Bolli's group has demonstrated that administration of nitroglycerin for 30 minutes the day before a coronary angioplasty procedure was able to reduce the elevation of ST segment during intracoronary balloon inflation ([55]). Moreover in animal studies nitroglycerin, can reduce the size of the ischemic area when administered within 72 after the onset of a heart attack.

1.3.5. The effectors of preconditioning

A brief ischemia or administration of nitrates could promote the formation of ROS (superoxide anion in particular) in such quantities as not to cause oxidative damage ([39]). The NO derived from the biotransformation of nitrates (in addition to the one formed by NOS) react rapidly with O_2^- released from mitochondria during administration of nitrates to form peroxynitrite ([56]). The increase in sub-cytotoxic concentrations of peroxynitrite and superoxide anion would be able to activate in animal models the ε isoform of protein kinase C ([57, 58]) or inactivate the specific inhibitor of the δ isoform of protein kinase C and,

through the recruitment of other protein kinase cascades leads to the expression of some genes, including aldose reductase, cyclooxygenase, iNOS, the mitochondrial manganese superoxide dismutase (MnSOD) and some heat shock proteins (HSP).

Recently, the preliminary results of a clinical trial in patients undergoing primary angioplasty in humans have shown that intra-coronaric administration of KAI-9803, an inhibitor of protein kinase C δ, during reperfusion can reduce myocardial infarction.

Recently,it has been highlighted the role of a new effector of preconditioning. In fact, the oxidative burst would be able, at the mitochondrial level, to act on mPTP, determining their transient opening ([59, 60]). Even if it is not yet clarified the exact mechanism, this event is associated with activation of an enzymatic cascade (RISK kinase) that mediates the induction of a program of cell survival ([61]). Many drugs, such as glucagon-like peptide-1 ([62, 63]), erythropoietin ([64]), atorvastatin ([65]) and atrial natriuretic peptide ([66]), seem able to reduce the extent of necrosis by activation of these kinases. Even in this case, a recent clinical trial has confirmed that the administration of high dose atorvastatin in patients with acute non-ST-elevations during primary angioplasty has been able to reduce the amount of ischemic area ([64]).

Therefore, ROS would act in two completely opposite sides, depending on the concentrations of these molecules. At high concentrations, during ischemia-reperfusion, they determine cytotoxic damage, whereas at low concentrations before ischemia-reperfusion injury, they are able to induce and likely to mediate

the ischemic preconditioning. The molecular mechanisms by which this phenomenon is determined are still unknown, but it is thought that many end-effectors may be able to act primarily on the two mechanisms responsible for cell death: hypercontracture and prolonged opening of mPTP ([67]). We know that after IR injury the cascade cGMP / PKG is severely depressed in myocytes and endothelial cells, but oppositely when this pathway is preserved as in the preconditioned myocardium, exerts its protective effect against ischemia ([68]). The cascade cGMP / PKG could indeed specifically protect cardiomyocytes against hypercontracture and rupture of the sarcolemma through several mechanisms. The main one is mediated by the reduction of the oscillations of Ca^{++} in the sarcoplasmic reticulum. One recent study showed that administration of analogs of cGMP during reoxygenation determined the phosphorylation of fosfolambdano dependent PKG and the increasing in the reuptake of Ca^{++} in the sarcoplasmic reticulum, and in turn speeding up the normalization of fluctuations in the Ca^{++} and reducing the likelihood of hyperconctracture ([14 ; 57]). A second mechanism by which the cGMP / PKG can prevent hypercontracture is through inhibition of the sensitivity of myocytes against Ca^{++}. In addition, the preconditioning acts by attenuating the activation of calpain-mediated Ca^{++} during reperfusion. Armstrong's group in this regard showed that preconditioning increases the mechanical strength of the sarcoplasmic reticulum ([69]). Calpain interacts with mitochondria and has been shown that at this level is able to determine the release of cytochrome C and other proapoptotic molecules ([70]).

Another important mechanism involves gap junctions. The intercellular communication mediated by gap junctions allow intercellular propagation of hyperconctracture and subsequent cell death during reperfusion. This phenomenon appears to be mediated by the passage of Na^+ from the damaged cells to the adjacents. Because both the preconditioning and the communications mediated by gap junctions are tightly regulated by PKC and RISK, it was hypothesized that gap junctions may be the end-effectors of preconditioning ([20, 71]).

Studies done in the past 8 years have shown that preconditioning also acts predominantly by attenuating the stimuli that lead to the opening of mPTP. At this time, we still have no valid assumptions about what might be the exact mechanism that acts at the level of mitochondrial pores.

From the clinical point of view this hypothesis about the molecular mechanisms of preconditioning are of great importance because when they will be understood we could design drugs that may act triggering this protective phenomenon. Because of their high ability to produce NO and O2-, for their spread in clinical practice and for the reduced incidence of side effects, nitrates seem to date the most useful drugs with preconditioning effect when administered acutely during ischemia.

PART 2

NITRATES AND TOLERANCE

2.1. Introduction

Organic nitrates, as already mentioned in previous sections, are a family of drugs used in the treatment of ischemic heart disease and for heart failure both because of their effectiveness in alleviating signs and symptoms and their proven safety ([72]).

The representatives of this class of drug are nitroglycerin (NTG), isosorbide dinitrate (ISDN), isosorbide-5-Mononitrate (ISMN) and other preparations of minor clinical relevance (as Nitroprusside, Molsidomine, Pentaerithrityl tetranitrate PETN).

Every compounds have the same mechanism of action which acts through the formation of nitric oxide (NO).

Someone as Nitroprusside and Molsidomine produce NO directly, others through a process of enzymatic degradation. The model to study the action of nitrates is represented by nitroglycerin (NTG). NTG is a fast-acting drug with a half-life of 7 minutes, and, for that reason, it represents the gold standard for the symptomatic treatment of angina. It is converted to NO by an enzyme, not yet fully typed ([72, 73]), forming NO directly or a compound named nitrosothiols ([74]).

These molecules act directly on the membrane of smooth muscle cells through activation of cytoplasmic guanylate cyclase, (GC) which formed as a second messenger cGMP by GDP. The latter reduces the levels of calcium ions into the cytosol of myocytes and, regulating the currents of K, it hyperpolarizes the membrane. The effect of both these mechanisms is to promote the relaxation of the myocyte, resulting in vasodilation([75, 76]).

From an hemodynamic viewpoint, nitrates are vasodilators, which act primarily on the venous capacitance vessels; this effect appears to depend on increased sensitivity toward NO of venous myocites.

Nitrates could indeed dilate veis, arteries and arterioles through the previous described cascade of intracellular events leading to NO production and activation of cGMP. In the smaller , distal vessels of the microcirculation, NTG and organic nitrates appear to have little vasodilatory capacity, perhaps because the enzyme responsible for nitrate bioconversion to NO is not present in these vessels. Veins take up nitrate more avidly than arteries, and venodilation is more pronounced at lower plasma concentration of nitrates.

In effect, nitrates induce a self-trasfusion of blood away from the heart and lungs to the splanchnic and peripheral circulations. This effect results in a lower venous return to right heart, resulting in preload fall and decreasing of cardiac output and stroke volume.

Nitrates are potent and reliable agents for decreasing left and right ventricular filling pressure in the normal heart as well as in patients with congestive heart failure and pulmonary capillary hypertension. Nitrates also act as hypotensive drugs since they also reduce the aortic and pulmonary compliance and impedance; typically hypotensive effect is more significant in systolic blood pressure. In the normal heart decreased left and right ventricular pre-load contribute to the decrease in cardiac output and blood pressure. The reduction of pre-load and, subsequently of after-load due to lowering of intra-ventricular pressure determines a lower left ventricular wall stress in systole and diastole and then a lower energy demand of the myocardium ([77]).

In the presence of normal left ventricular systolic function, stroke volume and cardiac output usually decline following NTG administration. However, in the setting of chronic hearth desease or generally in increased systemic vascualr resistance nitrate allows the left ventricle to empty more efficiently so stroke volume may increase due to nitrate ”un-loading” effects ([78]).

Another effect of considerable clinical impact is the increase in coronary blood flow. The vaso-dilatory effect in this vascular district, promotes the use of nitrates in the prevention and treatment of myocardial ischemia in patients with chronic coronary artery disease. A corollary of this action is the role of primary importance that have nitrates in recruitment of collateral circulation, in dilation of critical stenosis in atherosclerotic coronary and in increasing blood flow toward the healthy myocardium.

So the organic nitrates are used in the treatment of myocardial ischemia for two important effects: on one hand the objective of inducing a reduction in the consumption of O_2 and the other to increase blood flow and its supply of nutrients downstream of the ischemic myocardium ([79]).

Therefore, the clinical situations in which the nitrate is primarily used are the treatment and prophylaxis of myocardial ischemia in patients with coronary atherosclerosis, the treatment of symptoms of congestive heart failure, left ventricular contractile dysfunction, the prevention of cardiac remodeling after infarct and pulmonary edema; a particular use of nitroglycerin is related to the treatment of hypertension and, in the intravenous formulation, control of blood flow in surgical procedures. The use of nitrates is limited by the onset of headache and, above all, tolerance, the phenomenon of tachyphylaxis for which

nitrates lose their hemo-dynamic and clinical effects when administered for a period, depending on the nitrate, which exceeds the 12-24 hours ([80, 81]). Tolerance represents a critical limit of nitrate therapy and it severely limits their therapeutic potential. To date, the nitrate is usually administered to maintain a window of at least 10 hours daily.

2.2 Differences between nitrates

Structurally different organic nitrates exhibit different therapeutic profiles with regard to unwanted reactions and the benefit provided to the patients in specific disease states. This is not only because of different pharmacokinetics, pharmacodynamics, dosing regimen and routes of application, but also because of different bioactivation mechanisms.

As described above all nitrates, except nitroprusside and molsidomine, pass a series of complex enzymatic bio-transformations until they generate NO or related molecules such as S-nitrosothiols. The enzymes that catalyze these reactions have not yet been fully identified but it seems that the differences in action between the various nitrates should be found at this level.

In a recent article by Munzel, he hypothises a number of possible enzymes catalyzing this reaction, these include: glutathione S-transferase, cytochrome P450, xanthine oxidoreductase and mitochondrial aldehyde dehydrogenase. All these enzymes are involved the formation of ROS (reactive oxygen species), molecules that seem also to mediate the state of tolerance ([82]). Recently, among the enzymes that could metabolize GTN has been identified

the mitochondrial isoform of aldehyde dehydrogenase (ALDH-2), an enzyme that is subject to inactivation by oxidative mechanisms. The group Mhunzel et all sought to determine if the biotransformation of GTN mediated by ALDH-2, represents a common pathway for bioactivation of organic nitrates and if all organic nitrates induce oxidative stress during bioactivation. So ISMN, ISDN, GTN and PETN were tested for their effects on the formation of mitochondrial ROS and on activity of ALDH-2. It was also evaluated the effect of Benomyl, an inhibitor dell'ALDH-2, on the vasodilator ability of these different organic nitrates in endothelium of rat aorta rings.

In particular in intact endothelium the potency of different organic nitrates correlates with the number of groups $-ONO_2$ (nitrous) present in the molecule. While as regards the role of ALDH-2 bioactivation in organic nitrates highlighted by the addition of benomyl, it was found that the ALDH-2 is involved in the bio-activation of GTN and PETN, but does not reduce significantly the vaso-dilating power of ISMN and ISDN. It was also observed that GTN and PETN induce a greater production of mitochondrial ROS and consequently a greater ALDH-2 inhibition, whereas ISMN and ISDN cause only a slight increase in production of ROS.

In general, the vasodilator potency, dependence on ALDH-2-catalyzed bioactivation, stimulation of mitochondrial ROS, and inhibition of ALDH-2 dehydrogenase activity increase with the number of $-ONO_2$ groups present in the organic nitrate molecule. A remarkable deviation from this general behavior was the finding that PETN did not inhibit ALDH-2 esterase activity, whereas GTN did.

In nitrate-tolerant vessels, benomyl was unable to further decrease vasodilator responses to GTN ([83]) (figure 2.3), suggesting that both benomyl and nitrate tolerance interfere with the same bioactivation step specifically required for GTN/PETN.

In many vascular beds, the vasodilator-response curve to GTN consists of a high- and a low-potency component, suggesting that bioactivation may not be uniform for a given nitrate and between different nitrates. In contrast, the much less potent ISDN and ISMN exhibit a monophasic dose-response curve. These observations supports the hypothesis that exists at least two pathways of bioactivation for these organic nitrates. The high-potency pathway may be identified as the ALDH-2-dependent bioactivation step, and the low –potency pathway seems to be dependent of cytochrome P450.

It is interesting that the potency of organic nitrates to generate mitochondrial superoxide and peroxynitrite, as well as to inhibit ALDH-2 dehydrogenase activity, increased with their vasodilator potency. This is not contradictory, because ALDH-2 contains three cysteine residues in the catalytic center (Figure 2.4), rendering the dehydrogenase activity highly sensitive toward oxidative inactivation ([84]). In addition to its dehydrogenase activity, ALDH-2 also exhibits esterase activity, which has been proposed to be essential for the bioactivation of GTN. This activity also involves oxidation-sensitive cysteine residues ([84]). So the bioactivation of GTN by ALDH-2 could lead to oxidative inactivation of the enzyme.

Although the highly potent nitrates share a common bioactivation pathway, there are some remarkable differences between GTN and PETN. First, PETN elicited significantly less mitochondrial superoxide formation at similar concentrations compared with GTN. Second, PETN caused less inactivation of ALDH-2 dehydrogenase activity compared with GTN. These results may explain the previous findings that PETN lacks not only in vivo tolerance ([85]).

This hypothesis was tested in animal studies on dogs and rabbits, which have shown that chronic therapy with PETN does not cause tolerance and oxidative stress and also PETN has beneficial effects in the progression of atherosclerosis in hyperlipidemic rabbits ([92, 93]).

The mechanism that justifies this effect is the ability of PETN to induce the formation of ferritin and heme oxygenase-1, as demonstrated in cultured endothelial cells ([90]); the increase of ferritin lowers the levels of free iron in order to reduce the formation of free radicals. Add to that the protective effect of heme oxygenase-1 is known to be secondary to increase degradation of the porphyrins to bilirubin, a potent antioxidant ([91]).

2.3. Nitrates and pharmacological preconditioning

Oxidative stress associated with the acute administration of NTG may be able to induce a cascade of biochemical events similar to what happens during a short period of ischemia, the so called ‘pharmacological preconditioning’.

Multiple studies in animal models have shown how the pre-treatment with organic nitrates or nitroglycerin 24 hours before myocardial damage by IR is associated with a significant reduction in the infarcted area. Recently, studies in

humans have shown that nitroglycerin induces a delayed preconditioning effect in models of low flow ischemia, through the coronary vessel ligament, or high consumption of oxygen, obtained by pacing ([38, 92, 93]).

Therefore, there is sufficient evidence to support the hypothesis that brief exposure to NTG induce a protective phenotype similar to the late preconditioning. The protection induced by nitroglycerin is mediated by protein kinase Cε and the simultaneous induction of the expression of several genes involved in protective preconditioning. In any case, the subsequent genomic activation leads to increased expression of several genes, such as inducible NOS, superoxide dismutase, cyclo-oxygenase-2, the emo-oxygenase-1 and HSP (heat shock protein) ([94]). The ultimate target of this complex protective mechanism would be modulation of the opening of mPTP.

Our group has recently published a work which shows that in humans, the preconditioning effect induced by nitroglycerin is mediated through the ROS production and the mPTP transient opening ([95]).

With regard to organic nitrates, in reference to pharmacological preconditioning, it should be noted that it is impossible to speak of class effect. In fact our group has recently published that among the organic nitrates, nitroglycerin (NTG) and PETN, are able to induce pharmacological preconditioning, in contrast to isosorbide mononitrate, unable to generate this effect ([96]). Probably the different routes of bioactivation followed by different organic nitrates may play a major role in the induction of preconditioning and in its maintenance over time, as we shall see later.

Although the preconditioning induced by acute NTG may have a significant clinical importance, it is unclear whether this effect persists during chronic administration. The results reported in the literature in this regard are conflicting. In a model of myocardial ischemia-reperfusion injury in rabbits, Hill has shown that continuous administration of nitroglycerin for 28 days in doses able to induce hemodynamic tolerance maintain drug's ability to reduce the area of infarct due to coronary ligament ([28]). The analysis of these data, however, was limited by the fact that the treatment was discontinued 72 hours before starting ischemia.

In contrast, Szlivassy, in a study in rabbits showed that, using a continuous administration of nitroglycerin lasting 7 days, and inducing ischemia after only 6 hours from its suspension, the treatment had no protective effect. In a subsequent study, the same author showed that intermittent treatment with nitroglycerin (12 hours / day) for 7 days prevented the development of tolerance and preserved vascular preconditioning effect ([97]).

It should be emphasized that the presence of nitrate tolerance prevents the development of ischemic preconditioning. This observation is of considerable importance, since the long-term therapy with nitrates interfere with the beneficial effects brought about by ischemic preconditioning. Although it has not been elucidated the patho-physiological mechanism underlying this interaction, this observation agrees with that observed in other diseases characterized by vascular oxidative stress, such as hypercholesterolemia and advanced age, which are also associated with the loss of the ability to induce ischemic preconditioning ([98]).

2.4. Tolerance to nitrates and cross-tolerance

The efficacy of nitrates in chronic is greatly limited by the development of tolerance ([99]). Nitrate tolerance is a complex phenomenon, which involves neurohormonal counter-regulation, collectively classified as pseudotolerance, as well as intrinsic vascular processes, defined as vascular tolerance. GTN-induced desensitization of vasodilator responses to NO donors and endothelium-derived NO is termed cross-tolerance.

From a clinical point of view a typical phenomenon associated with vascular tolerance is the worsening of anginal symptoms compared with pretreatment state after cessation of nitrate therapy, the so-called withdrawal or rebound effect.

All of these states have to be discerned from an acute loss of GTN efficacy at intermediate to high concentrations in in vitro experiments, the so-called tachyphylaxis.

GTN therapy is associated with activation of neuro-hormonal vasoconstrictor forces. This has been demonstrated for intra-venous GTN therapy and with GTN patches in patients with coronary artery disease, ([35,36]) heart failure, ([37]) and control subjects. GTN-induced drops in blood pressure cause a baroreflex stimulation leading to a variety of neuro-hormonal adjustments. These include increases in catecholamine levels and their release rates, ([38,39]) increases in plasma vasopressin, plasma renin activity (reflecting increased circulating angiotensin II), and aldosterone levels ([36,39]) (Figure 2). These changes are not

GTN specific and have also been observed during therapy with other vasodilators. The degree of neurohormonal stimulation depends on the dose of GTN.

GTN therapy is also associated with a marked increase in intravascular volume, which may attenuate the preload effect of GTN. A decrease in hematocrit during long-term GTN treatment ([36,44]) very likely reflects intravascular volume expansion secondary to a transvascular shift of fluid caused by an alteration in Starling forces and/or aldosterone-mediated salt and water retention. All of these neurohormonal counter-regulatory mechanisms are regarded as pseudotolerance.

A further mechanism contributing to vascular tolerance is the increased sensitivity to receptor-dependent vasoconstrictors. This has been shown in rabbits treated with GTN for a 3-day period in a clinically relevant concentration of 1.5 ug/kg per minute and in rats chronically infused with GTN. Reports from patients with coronary artery disease also indicate that this observation may have clinical significance. Heitzer et al observed that reductions in forearm blood flow in response to intraarterial (brachial artery) angiotensin II and phenylephrine were markedly enhanced in patients pretreated with GTN for a 48-hour period (0.5 ug/kg per minute). These responses could be blocked by concomitant treatment with the angiotensin-converting enzyme inhibitor captopril, suggesting that an activated renin angiotensin system is responsible, in part, for this phenomenon. Therefore, an increase in sensitivity to vasoconstrictors represents a major mechanism responsible for the attenuation of the vasodilator effects of GTN.

In nitrate tolerant rabbits, we could normalize vasoconstrictor responses in vitro by inhibitors of protein kinase C (PKC), an important enzyme for maintenance of agonist-induced smooth muscle contraction. Then this is associated with upregulation of ex vivo platelet function and release of vasoconstrictor molecules ([102, 103]), the activation of the autonomic sympathetic and renin-angiotensin system and a lower release of NO. The effect of this combination of modifications is a paradoxical increase ("rebound") of vascular tone. In clinical practice, therefore, that we observe at first is that the vasodilator ability of drug is progressively lost during treatment longer than 12-24 hours. At the same time, increased vascular tone determine episodes of "rebound angina".

Endothelial dysfunction (ED) can be observed in humans during prolonged GTN therapy. In large coronary arteries, Caramori et al found that continuous treatment (5 days) with GTN patches leads to enhanced acetylcholine (ACh)-induced paradoxical constriction, instead of endothelium-dependent vasodilation, which was taken as a surrogate parameter for ED. By using strain gauge plethysmography our group showed that chronic (6-day) GTN treatment (0.6 mg/h, GTN patches) resulted in a marked reduction of Ach-infusion–induced increases in forearm blood flow of healthy volunteers. Likewise, the vasoconstriction elicited in control subjects by L-NMMA (NOS inhibitor) infusion, which unmasks a tonic reduction in vascular tone by basal NOSIII-derived NO, was significantly blunted in volunteers treated with GTN. In the lowest concentration, L-NMMA even caused a paradoxical dilation. The authors concluded that GTN treatment reduces basal as well as agonist-stimulated

vascular NO bioavailabiliy and that this may, at least in part, be attributable to abnormalities in NOS function.

Taken together, we believe that chronic GTN treatment causes ED, which may have important clinical implications, because ED has been shown to be a predictor on adverse long-term outcome in patients with coronary artery disease. It has been demonstrated that GTN prolonged treatment stimulates the vascular production of peroxynitrite and ROS generated from a rapid reaction of NO with superoxide. A stable metabolite of peroxynitrite, nitrotyrosine, is formed by nitration of tyrosine, either free or protein bound. In vitro and in vivo data indicated that GTN treatment increased vascular and urinary nitrotyrosine levels, which can be taken as a semiquantitative indicator of increased peroxynitrite formation. Increased vascular peroxynitrite formation may affect the proper function of NOS and thus induce ED by different mechanisms.

The exact mechanisms underlying this phenomenon are still unclear, but in any case, recent data indicate that the increased production of ROS induced by NTG inhibits the enzymes responsible for its bioactivation ([100, 101]), thus leading to the loss of vasodilatation cGMP-mediated.

The bioactivation of organic nitrates has different pathways, dependent also on the conditions of low (therapeutic) or high (pharmacological) concentrations ([104]). The high-affinity way favors the development of rapid tachyphylaxis induced by NTG, as demonstrated by the loss of vasodilator capacity of NTG after incubation of aortic rings with a high bolus of NTG itself ([105]).Chen et al suggested that the mitochondrial isoform of aldehyde dehydrogenase (ALDH-2) is responsible for this kind of bioactivation of GTN when applied in low

concentrations (Figure 1), because inhibitors of this enzyme in vitro and in vivo and depletion of mitochondria reduced the bioactivity of GTN. So ALDH-2 forms inorganic nitrite from GTN, but the bioactive intermediate (NOx), which activates sGC has not yet been identified.

In any case, it should be noted that inhibition of ALDH-2 by benomyl did not reduce the vasodilation induced by acetylcholine, while the dose-response curve of NTG is not abolished completely, but moves to the right, this feature suggests and confirms the existence of a second pathway with a lower affinity as the cytochrome P 450. By the way, the role of ALDH-2 has been confirmed by experiments in which, using knock-out animal models ALDH-2-/-, or by administering disulfiram, an inhibitor drug, we witnessed a reduction of NTG vasodilator potency.

As previously highlighted ALDH-2 is able to metabolize only organic nitrates containing more than two nitrate groups ($-NO_2$), which are attacked by the nucleophilic thiol groups of ALDH-2, with subsequent formation of an intermediate thio-nitrate product. In a second step we see the release of the de-nitrate metabolite and the formation of an intermediate with a tionitrato-cysteine group, follow by the formation of a disulfide bond and release of NO. The restoration of dell'ALDH reductase-2 is achieved after the reduction thiol-oxidized enzyme by a thiol donor such as dithiothreitol. The disulfide can also be induced by ROS direct oxidation of thiols.

At an higher level of oxidation there is the formation of sulfonic acid ($-SO_3H$) associate with irreversible ALDH-2 inhibition. At this point to turn this pathway on the enzyme must be synthesised de novo.

The considerable importance played by the reducing power of thiol groups, essential for the bioactivation of NTG, can thus be reduced by the attack of free radicals of oxygen and nitrate itself, confirming the initial "thiol theory" supported by Needlemann, that more than 30 years ago had suggested an interaction between nitrate tolerance and depletion of the pool of thiols in mitochondria ([107]).

From this point of view we must emphasize once again that organic nitrates behave at a heterogeneous class, in fact it seems that PETN does not cause tolerance, or endothelial dysfunction. In vitro studies ([108]), recently confirmed by our research group ([96]), have shown that PETN is able to induce gene expression of emo-ossigensi-1 and ferritin. These enzymes have antioxidant activity and induce a protective effect against ROS- toxicity. At the same time it was shown that PETN does not affect the activity of ALDH esterase-2 activity and does not induce the production of ROS, another mechanism that would explain the lack of development of tachyphylaxis (105).

In conclusion, there are several experimental evidences that emphasize the central role in the bioactivation of PETN and NTG by ALDH-2 ; it seems to decide the pathway followed by bioactivation is a threshold of about 1-10 μM: below this concentration the drug is metabolized mainly through ALDH-2, above the same bioactivation depends on less specific pathways like that of cytochrome p450.

Thus NTG would appear to induce ALDH-2 inhibition with a sort of negative feedback mechanism, mediated by the production of ROS and peroxynitrite and

then whit the shifting of its dose-response curve on the lower affinity molecular way.

This results in a reduced release of NO at the same dose, requiring an increase dosage of the drug.

The complex pathophysiology described above is able to explain the classical tolerance developed by nitrates in the chronic continuous treatment.

PART 3

Scientific protocol:

CHRONIC PROTECTION AGAINST ISCHEMIA AND REPERFUSION INJURY DURING THERAPY WITH DIFFERENT ORGANIC NITRATES

3.1 Introduction

Organic nitrates have long been used for their vasodilator effects, but their clinical utility is limited by the development of tolerance, ie the loss of hemodynamic effects that is observed upon prolonged administration (reviewed in previously chapters)([109]). Beyond their hemodynamic properties, it is now recognized that organic nitrates also have potent protective effects that are similar to those of ischemic preconditioning. Single, short-term exposure to nitroglycerin (GTN) has been associated with reduced evidence of ischemia in vitro([92]), in animals([97]), in patients during both exercise stress test and angioplasty ([93,110]) as well as with a reduced endothelial dysfunction after ischemia and reperfusion (IR) injury ([95,96]).

Alike to hemodynamic tolerance, also the preconditioning effects of GTN appear to be attenuated during repeated, 2 hours-daily, administration ([111,112]). This phenomenon, whose mechanism remain unknown, appear to depend on oxidative stress, as administration of the antioxidant vitamin C was able to recapture the preconditioning effect of GTN ([111]). Importantly, the development of such “tolerance” to the preconditioning effects of GTN would limit any therapeutic plan designed to exploit a preconditioning effect of daily exposure to this organic nitrate.

intrinsic antioxidant properties of this molecule and to the induction of heme-oxygenase-1, an enzyme involved in both antioxidant protection and ischemic preconditioning ([87]). While it is known that PETN, like GTN, possess acute preconditioning properties, the present study was designed to test, in humans, whether differences across these two nitrates exist upon repeated administration, and whether prolonged PETN administration is associated with preserved preconditioning protection. In human isolated endothelial cells, we studied the role of the aldehyde dehydrogenase 2 (ALDH-2), a mitochondrial enzyme that appears to be involved in both the bioactivation of organic nitrates ([114]) and in the protection conferred by ischemic and pharmacologic preconditioning ([115]).

3.2 Methods

The Ethics Committee of the University Hospitals of Siena, Italy and Mainz, Germany, approved the protocols. Written informed consent was obtained from all participants.

In an investigator-blinded, parallel trial, 30 healthy (age range 25-32) volunteers were randomised to one of 3 groups: 1) transdermal GTN (0.6 mg/hour) administered for 2-hours a day for 6 days; 2) oral PETN 80mg o.d. for 6 days; 3) no therapy. Seven days after the randomization (24 hours after the last administration of the study medications), all subjects underwent measurement of endothelium-dependent flow-mediated dilation (FMD) before and after

induction of local IR (15 minutes of ischemia followed by 15 minutes of reperfusion) .

The measurements were performed in a quiet, comfortable room and at a controlled temperature of 23 ˚ C. The subjects were asked to abstain from food or coffee and take the last dose of the drug 24 hours before the experiment. It has been asked to patients to lie on a couch and holding their left arm at heart level on a depression pillow, to ensure adequate immobilization. For each patient we applied 3 electrodes to monitor heart activity and a pulse-oximeter to check the ischemia caused by distal cuff inflation. The latter was subsequently placed at the wrist and distally (at least 5cm away) to the territory of the radial artery explored. For image acquisition we used a vascular 15 MHz probe, connected to an ultrasound ACUSON SEQUOIA 512 system. To keep the probe firm and make any adjustments to its position, we used an adequate mechanical support, custom-built (figure 3.1)

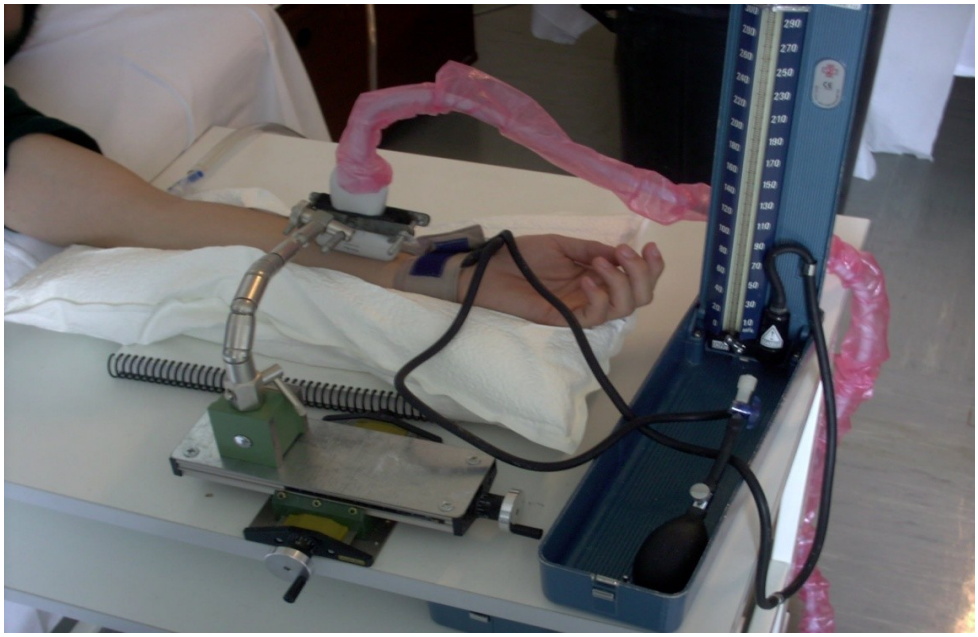


Figure 3.1. Equipment used in our laboratory for measuring the FMC and FMD

The artery was explored in the longitudinal projection, and the images was enlarged by using the "ZOOM" function of the machine. The outer diameter was measured at the peak of the R wave on ECG. As soon as the operator had reached the best possible definition of the images, these have been acquired in digital format using a dedicated computer. The basal diameter was recorded for one minute, after which the cuff was inflated at a pressure sufficient to surmount the patient's systolic blood pressure of 50 mmHg. Distal ischemia, confirmed by the pulse oximeter, was maintained for 4'30 ". After this time the cuff was released. Registration continued for the next 4'30", during which it was calculated the diameter of the artery, expression of the degree of vaso-dilation.

The time taken for each study was usually about 20-30 minutes. The measurement of flow mediated vasodilation (FMD) is an highly reproducible and not invasive method used for assessment of NO-mediated vaso-dilation. The increased flow at the radial artery following the ischemia (reactive hyperemia) produces an increase in shear stress that serves as a stimulus for the synthesis of NO through the activation of eNOS. Finally endothelial NO determine the vasodilatation. Changes in radial artery diameter are detected by ultrasound images and measured as % increase in radial artery diameter compared to baseline measurement.

Because changes in arterial diameter are the critical endpoints of the study, it is necessary that such measurements are made with highly accurate instruments. To this end we have developed, in collaboration with the Department of Information Engineering of the University of Siena, a software called splines

(Figure 3.2). This software is based on MATLAB platform, which enables the direct analysis of ultrasound images ([116]).

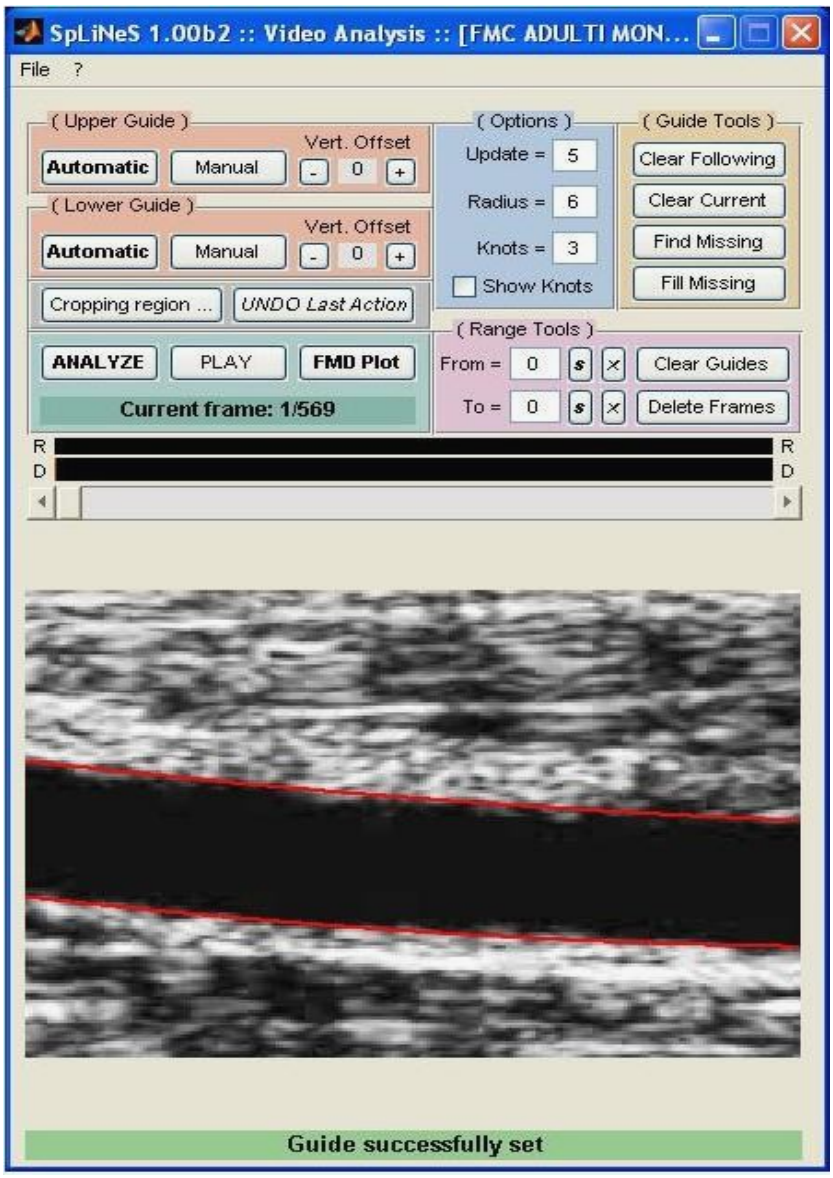


Figure 3.2 Splines

This human in vivo forearm model is a well-consolidated method to specifically study IR-induced endothelial dysfunction while leaving smooth muscle responses unaltered; the impairment in endothelial responsiveness observed in

these studies can be prevented by ischemic and pharmacologic (including GTN-mediated) preconditioning ([96]).

3.2.1 Cell culture and treatment

Human endothelial cells EA.hy 926 cells were a kind gift of C.J. Edgell (University of North Carolina at Chapel Hill, USA) and were grown in Dulbecco's modified Eagle's medium (Sigma) with 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 1× HAT (hypoxanthine, amethopterin/methotrexate, thymine). For experiments, the cells were seeded in 6-well plates at a density of 2×10^5 cells/well and grown to 60–70 % confluence until the experiment was started. Cells were kept in the medium and exposed to GTN or PETN (5 µM for 24 hours) or no therapy for four consecutive days. Twenty-four hours after the last exposure to the nitrates, the medium was removed and cells were scraped in some plates in 0.4 ml Laemmli buffer and frozen at -80 °C until Western blot analysis. Cells in other plates were used for determination of ALDH-2 activity.

3.2.2 Western blot analysis for ALDH-2 expression

Western blot analysis was performed as previously described ([117]). Laemmli cell samples were subjected to Western blot analysis. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes. After blocking, immunoblotting was performed with antibodies against β-actin (42kDa) (1:2500,

Sigma-Aldrich) as controls for loading and transfer and polyclonal rabbit ALDH-2 (1:2000, kindly provided by K.K. Ho and H. Weiner, Purdue University, West Lafayette, USA). Detection was performed by enhanced chemiluminescence with peroxidase conjugated anti–rabbit (1:10000, Vector Lab., Burlingame, CA) secondary antibody. The antibody-specific bands were quantified by densitometry as described.

3.2.3 HPLC (high-performance liquid chromatography) analysis for ALDH-2 activity

Cells were incubated for 45 min with Monal 62 (50 µM) in phosphate buffered saline (PBS) and 50 µl of the supernatant was used for HPLC analysis. The oxidation of 6-methoxy-2-naphthylaldehyde (Monal 62) to the fluorescent naphthoic acid product ([118]) was traced by HPLC analysis. The system consisted of a control unit, two pumps, mixer, detectors, column oven, degasser, and an autosampler (AS-2057 plus) from Jasco (Groß-Umstadt, Germany) and a C_{18}-Nucleosil 100-3 (125×4) column from Macherey& Nagel (Düren, Germany). A high pressure gradient was employed with acetonitrile/water (90/10 v/v%) and 25 mM citrate buffer, pH 2.2, as mobile phases with the following percentages of the organic solvent: 0 min, 40%; 8 min, 60%; 8–10 min, 100%; 11 min, 40%. The flow was 1 ml/min and naphthoic acid was detected by fluorescence (Ex. 310 nm/Em. 360 nm).

3.2.4 Statistical analysis

Results are expressed as mean±SD. Within- and between-group differences for all variables were evaluated with 2- or 1-way ANOVA for separate or repeated measures, as appropriate. The Bonferroni correction was used for post hoc comparisons. P<0.05 was set as the threshold for significance.

3.3 Results

3.3.1 Hemodynamic data

Data are presented in table 3.1 and figure 3.3.

				Radial artery diameter (mm)			
	Blood pressure		Heart rate	Before IR		After IR	
	SBP	DBP		Basal	ΔFMD	Basal	ΔFMD
No	115±3	73±3	78±2	2.06±0.11	0.13±0.01	2.12±0.11	0.02±0.02*
GTN	116±3	68±2	70±3	2.13±0.12	0.16±0.02	2.23±0.13	0.10±0.02*
PETN	110±3	70±3	68±4	2.24±0.11	0.18±0.02	2.27±0.12	0.14±0.04

P<0.01, ANOVA

Table 1. The effect of IR and nitrate therapy on hemodynamic parameters and IR-induced endothelial dysfunction. Hemodynamic parameters were similar across before IR and were not affected by IR. FMD was significantly blunted by IR in the placebo and (although to a lesser extent) in the GTN group. SBP: systolic blood pressure, in mmHg; DBP: diastolic blood pressure. ΔFMD = diameter increase during reactive hyperaemia, * = P<0.01 compared to corresponding ΔFMD before IR.

Before IR, there was no difference across groups in heart rate, blood pressure, blood flow, resting radial artery diameter or FMD (P=ns for all).

IR caused a significant blunting of the endothelial responses in the control group (Figure 1, P<0.01 versus before IR).This effect was partially prevented in the group that received treatment with GTN (Figure 1, P=ns compared to control group on ANOVA). Confirming our previous observation that the preconditioning-mimetic properties of GTN partially wane upon repeated administration(8), FMD after IR was significantly blunted in the GTN group (P<0.01 compared to before IR, within group). In contrast, FMD was not modified by IR in the PETN group (P=ns compared to before IR). Differences across groups were confirmed by ANOVA analysis (P<0.01) (figure 3.3)

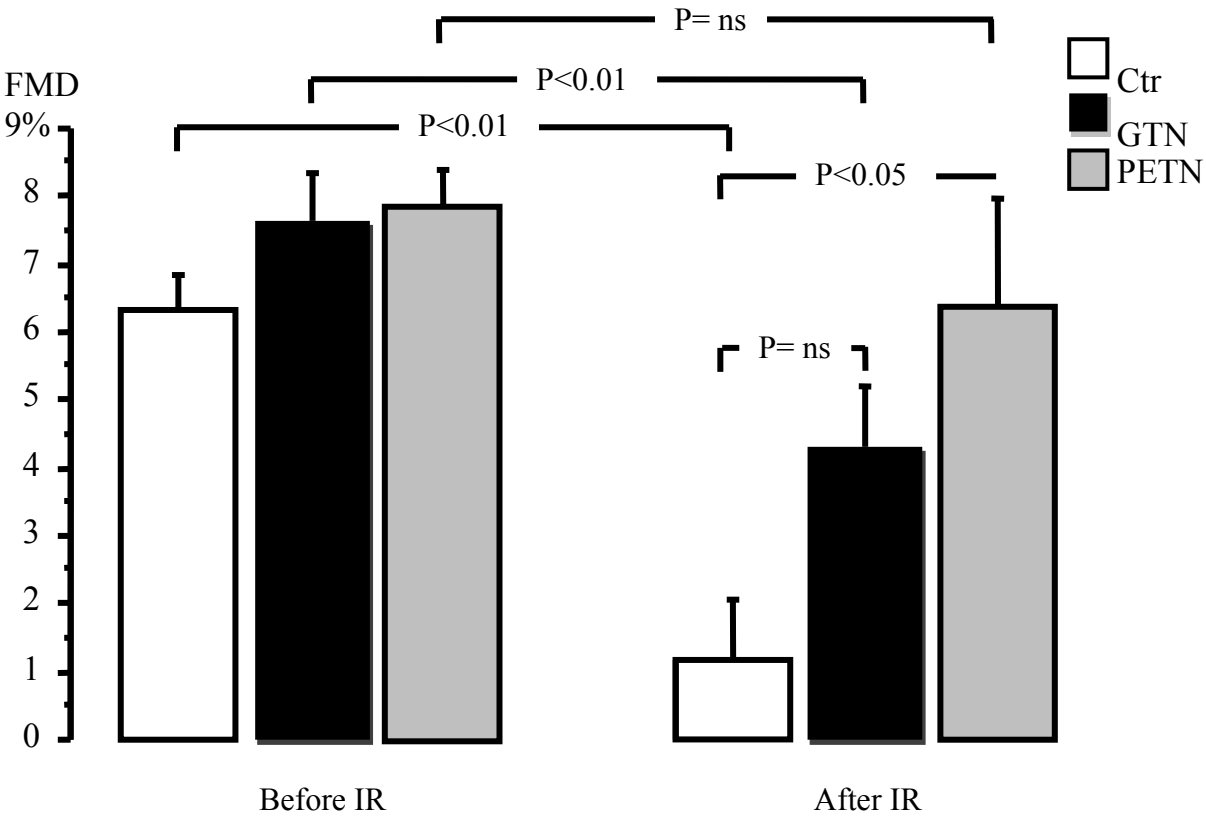
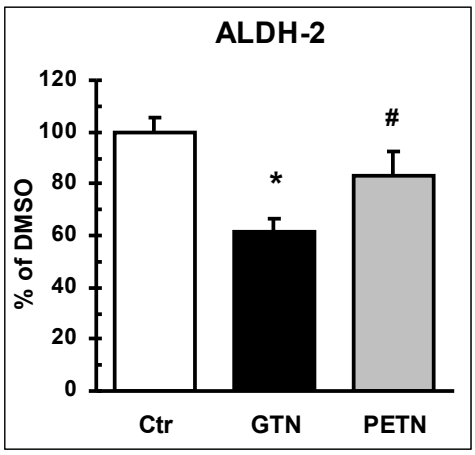


Figure 3.3.

3.3.2 ALDH-2 protein expression and activity data

Data are presented in Figure 3.4 and 3.5. Incubation with repeated dosages of GTN (5 µM) was associated with a decrease in the expression of the protective enzyme ALDH-2 ($P<0.05$ vs control). In contrast, repeated PETN incubations (each 5 µM) were not associated with a loss of the expression of ALDH-2(P=NS vs control; $P<0.05$ vs GTN). These observations were mirrored by the ALDH-2 activity, which was markedly decreased in response to chronic GTN treatment and only marginally altered by PETN incubations.



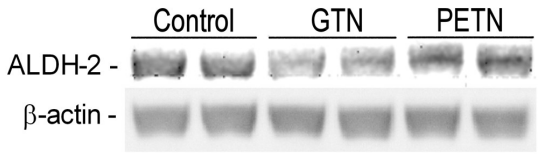


Figure 3.4. expression of the protective enzyme ALDH-2

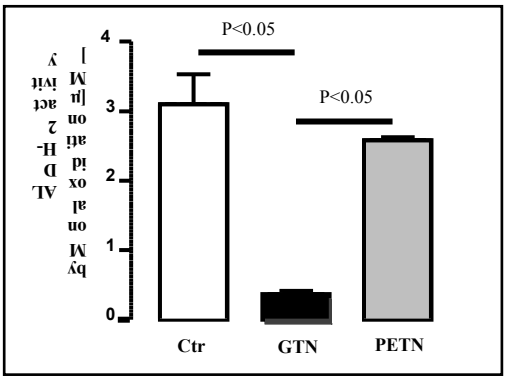


Figure 3.5. Activity of enzyme ALDH-2

3.4 Discussion

A number of studies have investigated the existence and mechanism of preconditioning protection induced by a variety of pharmacological stimuli (including a single exposure to organic nitrates). Both the extent of protection and the molecular mechanisms of pharmacologic preconditioning appear to be similar to those induced by classical ischemic preconditioning ([92]); as such, pharmacologic preconditioning might represent a practical approach to long-term protection in patients at risk. Similar to the endothelial effect of other drugs([119]), however, the clinical relevance of this approach is dependent on whether the protection induced can be maintained over prolonged or repeated exposure to the preconditioning stimulus. Of note, few studies have investigated whether such “chronic” preconditioning actually exists. Emphasizing the existence of differences between “short-term” and “chronic” preconditioning, a recent animal model found that, despite similar protection from IR, the pattern of

genomic activation induced by a single preconditioning stimulus is profoundly different from that induced by the repetition of the same stimulus ([120, 121]). Thus, while this protective phenomenon may in certain cases be preserved, it should not be assumed that “short-term” preconditioning can in all cases be translated into long-lasting (clinically more relevant) protection.

In the present study, repeated administration of PETN resulted in continued protection against IR-induced endothelial dysfunction. In line with our previous findings ([108]), the protection induced by repeated daily GTN administration was attenuated, with an FMD post-IR that was intermediate between that in the placebo and the PETN groups, a situation that is analogous to the hemodynamic effects of the two drugs during prolonged continuous therapy. The present data also suggest a possible mechanism for this observation. PETN is an organic nitrate that, like GTN, undergoes biotransformation catalyzed by the mitochondrial ALDH-2 to release its active metabolite, nitric oxide or a nitric oxide adjunct ([122]). This drug is used in the therapy of stable angina but a benefit has also been shown in heart failure ([123]). Due to pharmacodynamic differences and to intrinsic antioxidant properties, PETN appears to be devoid of hemodynamic tolerance (reviewed previously). While evidence of GTN-induced (but not PETN-induced) oxidative inhibition and reduced expression of the ALDH-2 in whole vessels and isolated smooth muscle cells has been reported previously ([125, 126]), our findings emphasize the impact of organic nitrates on endothelial homeostasis. In the present paper, this ALDH-2 inhibition provided a mechanism for the loss of GTN’s preconditioning-like effects, and for our previous evidence that administration of an antioxidant

is able to recapture this protective effect. Indeed, animal studies have recently shown that both physical and pharmacological preconditioning stimuli converge in the activation of the ALDH-2, and that pharmacological inhibition of ALDH-2 inhibits effective preconditioning ([127]). Although the specific function of ALDH-2 remains unclear, the importance of this enzyme appears to be unquestionable, as preserved ALDH-2 activity is required for both ischemic and pharmacologic preconditioning. Compatible with this concept, the inhibition of this enzyme observed in endothelial cells in our in vitro studies might lead to loss of the protective molecular cascade initially triggered by GTN, resulting in a loss of endothelial protection (i.e., in an increased susceptibility to IR-induced endothelial dysfunction). Notably, we previously showed that a mitochondrial production of reactive oxygen species plays a key role in the GTN-induced preconditioning. The present data are compatible with the hypothesis that, upon repeated administration, oxidative modifications in the ALDH-2 might cause loss of function of this enzyme, leading to reduced GTN biotransformation and inhibition of the end-effector of both ischemic and pharmacologic preconditioning.

Some limitations need to be acknowledged: first, healthy subjects were enrolled, and the peripheral circulation was tested in this experimental model. Although abnormalities in FMD have been shown in many conditions ([129-133]), like for any model, the translation of the present data to the clinical setting (patients with coronary artery disease, coronary or cerebral circulation) needs to be further investigated. Similarly, the data from isolated endothelial cells should not be automatically transferred to the clinical/human setting, even though we

previously showed human in vivo ALDH-2 inhibition in response to GTN ([134]). As well, the two drugs have different pharmacodynamic properties, and PETN has a longer half-life than GTN (8-10hours versus 20-30 minutes)([135]). Although no hemodynamic effect was present at 24 hours, it cannot be excluded that the effect of PETN observed here might be at least in part mediated by antiischemic properties that are independent of traditional preconditioning mechanisms.

In sum, while the present data provide further mechanistic evidence for the preconditioning effect of organic nitrates, they also emphasize the analogies between the mechanisms leading to “hemodynamic” nitrate tolerance and nitrate-induced preconditioning. At the level of whole vessels, previous studies emphasized the possible role of an oxidative ALDH-2 inhibition in determining tolerance to the hemodynamic effects of GTN (but not PETN). We show that inhibition of the ALDH-2 by GTN also occurs in isolated endothelial cells and that, *in vivo in humans*, this phenomenon determines the susceptibility to IR-induced endothelial dysfunction. Thus, the functional status of the ALDH-2 appears to be important not only for the metabolism and bioactivation of organic nitrates, but also for tissue recovery in the setting of ischemia. This evidence should be seen in light of preliminary studies reporting an increased incidence of cardiovascular events in patients undergoing nitrate therapy ([133]), and emphasizes the need of a prospective, randomized trial on the clinical long-term impact of these drugs. Importantly, preconditioning might have clinical implications ([137]), but our data emphasize the complexity of this phenomenon, and support the concept that the protection observed in response to a single exposure to a preconditioning stimulus should not be assumed to imply that the

same degree of protection would be observed in the chronic setting. Future studies testing the applicability of preconditioning as a clinical tool should also test whether this effect is maintained over prolonged time.

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