Mediterranean diet and cardioprotection: the role of nitrite, polyunsaturated fatty acids and polyphenols

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Abstract

The continually increasing rate of myocardial infarction (MI) in the Western world at least partly can be explained by a poor diet lacking in green vegetables, fruits, and fish, and enriched in food that contains saturated fat. In contrast, a number of epidemiological studies provide strong evidence highlighting the cardioprotective benefits of the Mediterranean diet enriched in green vegetables, fruits, fish and grape wine. Regular consumption of these products leads to an accumulation of nitrate/nitrite/NO$^\bullet$, polyunsaturated fatty acids (PUFA), and polyphenolic compounds, such as resveratrol, in the human body. Studies have confirmed that these constituents are bioactive exogenous mediators, which induce strong protection against MI. The aim of this review is to provide a critical, in-depth analysis of the cardioprotective pathways mediated by nitrite/NO$^\bullet$, PUFA, and phenolic compounds of grape wines discovered in the recent years, including cross-talk between different mechanisms and compounds. Overall, these findings may facilitate the design and synthesis of novel therapeutic tools for the treatment of MI.

1. Introduction

Myocardial infarction (MI) remains a major clinical problem in the western world. Acute MI takes about 140,000 lives every year in the USA alone [1]. In general, MI is a consequence of a long ischemic insult, which initiates irreversible intracellular events including shortage of ATP supply, collapse of ionic homeostasis, and massive cardiomyocyte death. Although restoration of the blood flow through the ischemic zone is absolutely required for survival, most of the intracellular damage occurs during reperfusion. Thus, the problems associated with MI are largely attributed to ischemic-reperfusion (IR) injury [2]. IR injury results in impaired contractile function and depression of mitochondrial bioenergetics, as the consequences of imbalanced ionic homeostasis [3] and functional disturbances due to protein/lipid modifications [4].

Ischemic preconditioning (IPC) is an effective strategy to protect the model heart from MI. IPC usually comprises one or more short ischemic insults intercepted with reperfusion prior to a prolonged period of IR [4,5]. While the signaling mechanisms of IPC are still unclear, important roles for a number of associated events have been proposed; this includes, but is

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not limited to, modifications and translocations of intracellular kinases [6], activation of mitochondrial ATP sensitive potassium (mK_{ATP}) channels [6], and mild mitochondrial uncoupling [7]. However, clinical application of IPC is difficult. For this reason, another strategy in the development of MI treatments entails investigation of the mechanisms of IPC and development of pharmacological tools to mimic these signaling pathways. Unfortunately, today not even a single FDA approved drug exists for the lowering of cardiac infarct size [8].

Based on several epidemiological, clinical and experimental studies, it has been established that certain types of diet may have beneficial effects for the cardiovascular (CV) system in general and be effective therapeutic tools for the prevention or treatment of MI [9,10,11,12]. For example, the Mediterranean diet, which typically includes a bolus of green vegetables, fruits, fish and grape wines, is associated with decreased concentrations of inflammatory markers such as C-reactive protein and interleukin-6 in MI survivors [13]. Although great success has been made in the accumulation of solid scientific background underlying these phenomena, the full mechanisms of protection are far from being elucidated. Recent studies show several components of the Mediterranean diet can trigger cardioprotection. This review focuses on a number of these components that have attracted attention over the past few years: (a) nitrite, (b) polyunsaturated fatty acids (PUFA), and (c) wine polyphenols. The interplay between these components is also discussed.

2. Nitrite as a promising therapeutic tool against MI

2.1. Accumulation of nitrate/nitrite in the human body: impact of the Mediterranean diet

Although 80% of the basal plasma nitrite (NO_2^-) level derives from oxidation of $NO^\bullet$ [14], although reduction of nitrate (NO_3^-) may also contribute to elevation of NO_2^- [15]. It has been reported that exogenous NO_3^- intake (10mg/kg in humans) may increase plasma NO_2^- concentration up to 4–5 fold in 30 min [16]. Notably, plasma NO_2 concentration was decreased by 50% in mice that were placed on a dieting lacking in both NO_3^-/NO_2^- [17].

The largest dietary sources of NO_3^- for the human body include green vegetables such as spinach, lettuce, and collard greens and also radishes, beets [18,19], and meat [20]. Furthermore, NO_2^- itself can be found in cured meats [21]. Raat *et al* meta-analyzed a number of studies and based on the approximate amount of consumed vegetables reported average daily NO_3^- ingestion as 77 mg for US diet and 400 mg for Mediterranean diet [17]. A different study revealed that an exemplary Mediterranean meal contains approximately 325mg/serving of combined NO_3^-/NO_2^-, significantly higher in comparison to Western meals (only ~20mg/serving) [22]. These discrepancies indicate that the actual amount of daily NO_3^-/NO_2^- intake should be considered with caution because the data may vary depending on study design or targeted population [19,23]. Nevertheless, despite slight variation in the magnitude of the difference, the Mediterranean diet appears to provide greater NO_3^- supplementation than a regular Western diet.

2.2. NO_2^- as a stable precursor of $NO^\bullet$ in the body

After being accumulated in the saliva, NO_3^- can be reduced to NO_2^- by several bacterial organisms including *S. epidermidis*, *Veillonella*, *Nocardia*, and *S. aureus* [24,25]. Among other functions, NO_2^- can be converted into $NO^\bullet$ gas, which mediates numerous signaling events in the cell [26]. Accordingly, the following sections will be mostly devoted to the cardioprotective effects of $NO_2^-/NO^\bullet$.

The bio-efficacy of NO_2^- has been demonstrated in many studies, for example where consumption of nitrate-enriched beetroot resulted in a 2-fold elevation of the plasma NO_2^- level, which was also correlated with a reduction in blood pressure [27]. Further reduction of

NO_2^- to $NO^\bullet$ may occur under acidic conditions both in the oral cavity and in the stomach [28,29,30]. Additionally, several studies have indicated that bioconversion of NO_2^--to-$NO^\bullet$ is stimulated by polyphenols [29,31,32].

Until recently, NO_2^- had been considered primarily as a stable precursor of $NO^\bullet$, and thus, investigation of the $NO^\bullet$-dependent activity of NO_2^- dominated the field. In recent years, several studies have shown that NO_2^- itself can be recruited by cells and reduced to $NO^\bullet$ when the latter is depleted [33,34,35]. Since NOS function requires O_2 to make $NO^\bullet$, but the reduction of NO_2^- to $NO^\bullet$ is not O_2-dependent, this process may replace regular NOS function under ischemic conditions. This reduction can be mediated by several different proteins including hemo- and myoglobins [33,34], the mitochondrial respiratory chain [36], and xanthine oxidoreductase [37].

Both recent and ongoing studies have revealed significant therapeutic potential regarding the use of NO_2^-/$NO^\bullet$ in the treatment of MI [35,38,39,40,41,42,43]. The mechanisms of cardioprotection by NO_2^- have been extensively investigated. Its cardioprotective efficacy has been confirmed at subcellular, cellular, organ and organism levels and in different models of IR injury, including isolated cardiac mitochondria [41], isolated cardiomyocytes [40], perfused heart [35] and in vivo left coronary artery occlusion [41,44].

2.3. Cardioprotection via nitrosylation: role of NO_2^- reduction

It is widely accepted that acute effects of NO_2^-/$NO^\bullet$ may be mediated via post-translational modifications of proteins (for review see [45]). *Nitrosylation* is the reaction of $NO^\bullet$ with metal centers such as heme. Classic $NO^\bullet$-dependent vasorelaxation is mediated via nitrosylation of soluble guanylate cyclase (sGC), causing subsequent regulation of vascular tone and blood flow [46]. Consistent with a role for sGC signaling in cardioprotection, pharmacological activation of sGC elicits protection against IR injury [47,48,49]. However, it was reported that NO_2^- caused hypoxic vasodilation via both sGC-dependent and independent pathways [50]. On the organ level, NO_2^--dependent vasorelaxation may play a role in hypoxic blood flow regulation [51]. Mechanisms of NO_2^- reduction to $NO^\bullet$ in the vessels have not been fully elucidated, but several studies demonstrated that this might include deoxygenated hemoglobin [52]. Importantly, Gladwin's group found that deoxymyoglobin was a far more efficient NO_2^- reductase in comparison to deoxyhemoglobin, which indicated that myocardium contains a powerful NO_2^- reductase system [34]. The ability of NO_2^- to prevent myocardial infarction was completely abolished in myoglobin$^{-/-}$ mice [40]. It is odd, however, that other NO_2^- reductase systems, such as xanthine oxidoreductase, did not partake in the reduction of NO_2^- in this case, as even partial myoglobin-independent cardioprotection was not observed [40]. Nevertheless, this signifies that the existence of a deoxymyoglobin/NO_2^- reductase system may be exceptionally important for the cardioprotection afforded by NO_2^-.

While the role of myoglobin as an oxygen transporter in cardiomyocytes is widely acknowledged [53,54], novel evidence has uncovered deoxymyoglobin's important role in the regulation of mitochondrial function, in which it facilitates NO_2^- reduction to $NO^\bullet$ [34].

2.4. $NO^\bullet$ signaling in mitochondria and cardioprotection

It is well-established that direct interaction of $NO^\bullet$ with the electron-transport chain causes reversible inhibition of mitochondrial respiration [55]. Primarily, this effect may be associated with nitrosylation of complex IV or *S-nitrosation* of complex I (a reversible $NO^\bullet$-dependent modification of thiols) [56]. The mechanisms and consequences of $NO^\bullet$ interaction with mitochondria have been extensively studied [45], especially in the context of the complex roles of both $NO^\bullet$ and mitochondria in cardiac pathophysiology.

As a note, the mitochondrial bioenergetic machinery is a primary target for damage during IR injury [4]. Mitochondrial damage due to reactive oxygen species (ROS) generation and Ca^{2+} overload and its consequences, particularly the opening of the permeability transition (PT) pore, are key features of IR injury [57]. Effects of the PT pore opening include massive mitochondrial swelling, inner membrane disruption and loss of mitochondrial function [58]. In addition, IR results in irreversible damage of the mitochondrial respiratory chain primarily because of multiple structural modifications of proteins and lipids via oxidation [59], carbonylation [60] or alkylation [61].

It is widely accepted now that $NO_2^-/NO^{\bullet}$ may preserve mitochondrial function during IR injury, eliciting strong cardioprotective effects [42]. In general, reversible inhibition of mitochondrial respiration has been considered as a potential therapeutic strategy for treatment of MI [55], and has been successfully tested in varied models of IR injury [55,62,63,64]. Reversible inhibition facilitates the slow re-introduction of electron flow through the respiratory chain at the beginning of reperfusion, which substantially prevents the burst of ROS and delays full restoration of the membrane potential, a driving force for Ca^{2+} uptake [55]. Hence, ROS generation and Ca^{2+} overload, the key factors in cardiac IR injury, are avoided [55].

A substantial amount of experimental evidence has revealed that reversible S-nitrosation of complex I may inhibit ROS generation and Ca^{2+} overload by mitochondria during early reperfusion, which is critical for cardioprotection [41,42,62,63,65,66]. We demonstrated that low-molecular weight S-nitrosothiols (e.g. S-nitroso-mercaptopropionyl-glycine) protected isolated cardiomyocytes and perfused rat hearts against IR injury and that this effect was accompanied by S-nitrosation and inhibition of complex I [62,63]. These results were recapitulated in an in-vivo mouse model of MI [63].

There is consensus that the mitochondrion is a central focus for IPC signaling, and the most commonly recognized mitochondrial signaling mechanisms in IPC are mediated via $NO^{\bullet}$ [26]. We reported that endogenous S-nitrosation of mitochondrial proteins was detected during IPC [62,66]; furthermore Sun *et al.* found that a number of mitochondrial proteins were S-nitrosated during IPC, including the 75kDa subunit of complex I [62,66]. Reversible inhibition of mitochondrial respiration due to S-nitrosation of complex I may serve as a protective endogenouse mechamism during IPC [55,56,62].

Perhaps the best evidence for the mitochondrion as a terminal site for the cardioprotective effects of NO, is that a mitochondrially-targeted NO donor (which accumulates inside mitochondria at several-hundred-fold greater concentration relative to the cytosol), was cardioprotective at a concentration of only 100 nM in isolated perfused hearts, and at 100 ng/kg in vivo [65,67].

Further establishing a connection between NO_2^-, S-nitrosation and cardioprotection, it should be noted that administration of exogenous NO_2^- caused S-nitrosation and inhibition of complex I, and resulted in protection against MI [41]. Reversible inhibition of complex I has also been claimed to be a mechanism of NO_2^--induced ardioprotection in a model of cardiac arrest [39]. It is important to note that intravenous injection of NO_2^- during the last 5 minutes of ischemia significantly reduced MI injury [68], which makes NO_2^- therapy clinically relevant. In this study S-nitrosothiols and iron-nitrosyl-protein complexes were not elevated in the whole blood or plasma after NO_2^- administration. This led the authors to conclude that NO_2^- itself likely accounted for the cardioprotection observed; however, the question remains whether S-nitrosation occurred in the myocardial tissue where protection actually took place [68].

Additionally, Hogg's group found that NO_2^- may mediate protection against IR injury via mitochondrial ATP sensitive potassium (mK_{ATP}) channels [69]. Although the mechanism of NO_2^- interaction with mK_{ATP} channels was not revealed, several other groups have demonstrated that mK_{ATP} channels can be activated by S-nitrosothiols [70,71,72].

2.5. NO•-independent mechanisms of cardioprotection

Although NO•-independent mechanisms of cardioprotection by NO_2^- are less investigated, several important effects have recently been discovered. The discovery of *nitrated lipids (or nitroalkenes)* is one of the most novel and exciting findings in the field of reactive lipid research [73]. Nitrated lipids can be formed by several different mechanisms, such as through a reaction of NO_2^- with unsaturated fatty acid derivatives at low pH (≤ 4) [74]. Nitroalkenes are a novel class of cell-signaling molecules that possess potent anti-inflammatory properties via inhibition of both NF-kB activity and cytokine secretion [75]. As potent peroxisome proliferator-activated receptor-γ (PPARγ) ligands, nitroalkenes regulate gene expression and cell metabolism [76]. Due to their electrophilic nature, nitroalkenes may cause post-translational modifications of proteins that contain nucleophilic residues (Cys, Lys, His) [77]. Although the full therapeutic potential of nitroalkenes has not been fully elucidated thus far, strong evidence has emerged that low concentrations of nitrated linoleic acid elicit potent acute protection in isolated cardiomyocytes as a model of IR injury [78]. Further, Freeman's group demonstrated that nitrated oleic acid induces significant protection against MI in the in-vivo IR injury model of left anterior descending (LAD) coronary artery occlusion [79]. Notably, we have found that different nitroalkenes possess varying efficacies in the reduction of infarct size (unpublished data). Although the mechanism underlying this has not yet been evaluated, it might be due to the inherent stability of nitroalkenes [76] or to the properties of their parent fatty acids, which are discussed below. Markedly, the Mediteranian diet rich in NO_2^- and PUFA, and supplemented with acidic vinegar, may favor intra-gastric generation of nitrated lipids. Indeed, d'Ischia's group has shown nitration of unsaturated fatty acids from extra virgin olive oil under exposure to NO_2^- in mild acidic conditions [80].

In summary, in the past several years a number of fundamental studies have revealed NO_2^- as a bioactive molecule that may possess the capacity to diminish or even prevent the detrimental consequences of MI in diverse animal models. Moreover, promising preclinical results anticipate forthcoming clinical trials (www.clinicaltrials.gov/ct2/show/NCT00924118) of nitrite therapy for patients with anterior ST-segment elevation MI [81].

3. PUFA and their derivatives against MI

3.1. Cardioprotective efficacies of PUFA

Mediterranean diet traditionally includes an abundance of vegetables and fish, both of which contain a substantial amount of diverse PUFA (ω-3, 6, 9). Epidemiological studies demonstrate that there is a direct correlation between PUFA consumption (especiallyω-3) and low levels of CV diseases in some populations [82,83]. The scientific nomenclature of PUFA is well described (see for review [84]), and will not be discussed in detail herein. Briefly, PUFA are divided into 3 classes based on the position of the first double bond from the methyl carbon, labeled "ω": (1) ω-3, i.e. DHA-docosahexaenoic, EPA-eicosapentaenoic, ALA-α-linolenic; (2) ω-6, i.e. LA-linoleic, GLA-γ-linolenic, AA-arachidonic; and (3) ω-9, i.e. OA-oleic. Several large studies [9,11] have drawn a correlation between regular PUFA consumption and reduced risk of CV diseases. Extending this knowledge, comparative studies have revealed that mixed ω-3s have a greater cardioprotective effect than any other PUFA [85]. It was shown that ω-3 [86,87,88], and to a lesser extent ω-6 [89,90], PUFA

protected the heart from MI, while ω-9 and saturated FA have minor or no effect [85,91]. Although a recent study demonstrated that ALA offers significant protection against acute MI in humans [92], further investigations clarified that EPA and DHA take the most responsibility for ω-3-mediated cardioprotection [93]. Moreover, it has been discussed that the EPA-to-DHA ratio is a significant factor in determining the degree of protection (for review see [94]). Thus, the cardioprotective efficacies of PUFA may be determined by at least two factors: (i) structural specificities, i.e. position of the first double bond, and (ii) the ratio of acids within one individual "ω" group.

Until recently, much of the scientific research and even clinical trials were conducted without a clear understanding of the molecular mechanisms of cardioprotection afforded by ω-3. Early studies suggested that this cardioprotection was mediated via replacement of ω-6 with ω-3. Thereby an increased ratio of ω-3/ω-6 in cell membranes diminished the detrimental effects of pro-inflammatory AA-derived eicosanoids, prostaglandins and leukotrienes [95,96,97].

3.2. Cardioprotection by EPA and DHA derivatives

Extensive studies have revealed that the protective effects of EPA and DHA may be mediated through the formation of reactive lipid molecules called resolvins [98]. Biosynthesis of these molecules is a multi-step process and involves participation of several enzymes, such as acetylated cyclooxygenase-2, cytochrome P450, and lypoxygenases [98,99,100,101]. A more detailed description of the synthetic process, nomenclature and generic properties of resolvins is beyond the scope of this review and can be found elsewhere [101,102,103,104]. Due to their potent anti-inflammatory properties [105,106,107,108] these molecules may serve as promising therapeutic tools for MI treatment. Indeed, resolvins (E1, D1) prevent polymorphonuclear neutrophil (PMN) activation and translocation into the tissue [99,101], which may reduce ROS production and inflammation during reperfusion. Resolvin E1 regulates cytokine/chemokine production [108] and inhibits TNFα-induced nuclear translocation of NF-kB [109]. One can predict that based on their anti-inflammatory properties, resolvins might be particularly effective in the prevention of secondary inflammation initiated by PMN infiltration into ischemic tissue. Consequently, an ideal model to test these molecules would be an in vivo model of IR injury. Indeed, Keyes *et al* highlighted that administering resolvin E1 robustly decreased infarct size in the model of left coronary artery occlusion in rats [110]. Notably, in this study resolvin E1 was able to prevent IR injury when added 2 min before reperfusion at a concentration of 100nM, suggesting that this protocol might be clinically relevant [110]. Future studies will uncover the ability of other resolvins to prevent MI. In addition to resolvins, there are two other major classes of anti-inflammatory DHA-derivatives called protectins [111,112] and maresins [113]. Protectins are principally associated with anti-apoptotic effects in the brain and neuro-protection [114], while maresins' function remains to be elucidated.

Recently, Freeman *et al*. characterized several electrophilic oxo-derivatives of DHA and EPA, which were synthesized in activated macrophages via the cyclooxygenase-2 dependent pathway. Similar to resolvins, these also were found to possess strong anti-inflammatory properties [115]. Furthermore, at physiological intracellular concentrations (28 –190nM), it has been shown that these molecules are potent PPARγ activators [115]. Therefore, it would be reasonable to propose that these electrophiles may elicit protection against MI by a means comparable to those of nitroalkenes and other PPAR ligands [76,116,117]. Nevertheless, these results should be tempered with recent findings that commercial PPARγ ligands (thiazolidinediones) are associated with increased adverse cardiac events in humans (discussed below).

3.3. Cardioprotection by ω-6 derivatives

Although investigation of the cardioprotective events of PUFAs has been considerably shifted toward ω-3, it should be noted that several ω-6 AA derivatives, including 12(S)-hydroperoxyeicosatetraenoic acid [118], epoxyeicosatrienoic acids [119,120] and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 [116,117], have already been shown to induce protection against MI. It was reported that one of the mechanisms against MI afforded by 12(S)-hydroperoxyeicosatetraenoic acid may be through the activation of transient receptor potential (TRP) channels (e.g. vanilloid receptor 1) [118]. These channels can be activated by electrophiles through covalent modification of critical Cys residues [121], and may be responsible for activation of cardiac nociceptors in response to myocardial ischemia [122]. Notably, capsaicin (an endogenous ligand of these receptors) is also cardioprotective against IR injury [123].

The significance of epoxyeicosatrienoic acids for cardioprotection was demonstrated in hearts overexpressing human cytochrome P450 AA epoxygenase CYP2J2 [124]. Transgenic mice elicited greater endogenous protection against IR injury compared with wild-type hearts [124]. Cardioprotection provided by epoxyeicosatrienoic acids may be mediated via activation of mK_{ATP} channels or p42/p44 mitogen-activated protein kinase (MAPK) [119,124]. Furthermore, since epoxyeicosatrienoic acid administration significantly prevented MI in dogs when administered at the time of reperfusion [120], these molecules may be effective, clinically-relevant therapeutic tools.

15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 provides cardioprotection by several independent mechanisms. For example, the cardioprotective effects of this molecule can be mediated by activation of heme-oxygenase-1, regulation of heat shock protein 70, or reduction of the expression of adhesion molecules ICAM-1 and P-selectin [116,117]. In addition, similar to nitroalkenes and oxo-derivatives of ω-3, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 is a potent activator of PPARγ [125], and thereby possesses metabolic and anti-inflammatory properties which might contribute to protection against MI [76,116,117].

Arachidonic acid is a precursor for another class of anti-inflammatory molecules called lipoxins [126]. Biosynthesis of these molecules is achieved by 15-lypoxygenase at the moment of inflammation, with ensuing regulation of PMN infiltration into the tissue [127]. Gavins *et al.* demonstrated a potential role of lipoxin A_4 receptors in cardioprotection against MI in mice [128]. Prior to this, the protective role of lipoxin A_4 had already been reported in cerebral IR injury [129], IR-induced gastric mucosal damage [130], and kidney IR [131]. Anti-inflammatory properties of lipoxin A_4 may be mediated via PPARγ activation [132] suggesting that this mechanism may be common for many ω-3 and ω-6 lipid derivatives.

3.4. Potential pitfalls and new directions with regard to the clinical application of PUFA and their derivatives

Upon translating these findings into humans, several important limitations should be mentioned regarding PPARγ activators and reactive lipids. Despite their cardioprotective efficacy, some PPARγ ligands, including rosiglitazone, may increase the risk of acute MI, stroke, heart failure and mortality in elder patients [133]. Regarding reactive lipids, it should be noted that the desired cardioprotection afforded by them is strictly dose-dependent. For example, while high concentrations (>20μM) of α,β-unsaturated aldehyde 4-hydroxy-2-nonenal caused cardiomyocyte death, low dosage initiated Nrf2-Keap1 (nuclear factor erythroid-2 related factor 2 - Kelch-like ECH - associated protein 1)-mediated stimulation of antioxidant machinery and subsequent cardioprotection against IR injury [134]. Similar to

this, high dosages (>20μM) of nitrated linoleic acid induced mitochondrial swelling and PT pore formation, however low concentrations (<1μM) elicited strong cardioprotection [78].

In addition, a major limiting factor which may determine the clinical applicability of molecules such as nitroalkenes, lipoxins, resolvins, and other electrophilic species, is their stability and chemical reactivity. Virtually most of these compounds are light sensitive and decompose rapidly in aqueous media. Thus, their "druggability" will depend on successful formulation for human clinical administration (e.g. lipid emulsions, liposomal ecapsulation, or stabilization in a suitable vehicle).

Overall, intensive studies of PUFA derivatives in recent years have revealed several classes of reactive lipid molecules possessing potent anti-inflammatory cardioprotective properties. Moreover, these findings have made possible a reconsideration of some existing postulates and the drawing of future perspectives for the field. For example, while regular OA (ω-9) and LA (ω-6) were not protective, the corresponding nitrated PUFA robustly prevented MI injury in mice [79] and IR injury in cardiomyocytes [78]. Likewise, Trostchansky *et al.* were able to synthesize several isomers of nitrated arachidonic acid and showed they possessed strong anti-inflammatory properties that have potential cardioprotective effects [135]. These studies demonstrate that nitroalkenes are a superb example of how structural manipulations of certain PUFA may dramatically enhance or create de-novo cardioprotective properties. Furthermore, it is possible that these structural and functional metamorphoses may be facilitated as a part of the Mediterranean diet, in which cells procure an additional supply of PUFAs and nitrite.

4. Cardioprotection by phenolic components of red and white wines: cross-talk with Sirtuins, caloric restriction and IPC

About 500 years ago Alvise Cornaro, who died at age 102, explained his longevity and good health in the book "THE ART OF LIVING LONG". Based on his experiences, regular wine consumption and caloric restriction (CR) were two critical components of a long, healthy life.

Regular consumption of grape wine is an integral element of the Mediterranean diet. Epidemiological studies have shown the beneficial effects of moderate consumption of wine on the CV system [10,12]. Although the cardioprotective effects of red and white wines against IR injury have been known for some time and have been well described [136,137,138], it has not been until recently that the actual protection mechanisms have been extensively studied.

The cardioprotective benefits of grape wine are usually attributed to their phenolic components. In mammalian hearts, red wine polyphenols have been shown to elicit strong cardioprotective effects against IR injury [139,140,141,142]. Polyphenolic compounds such as quercetin, resveratrol, or catechins are potent antioxidants [141,142,143,144]; thus, one of the mechanism of protection they provide might be the inhibition of oxidative stress upon reperfusion. Brookes *et al.* demonstrated that quercetin-mediated protection against IR injury was associated with activation of manganese superoxide dismutase (MnSOD) and preservation of mitochondrial function [141]. The most studied polyphenol, resveratrol, may trigger a broad spectrum of cardioprotective pathways, including inhibition of glycogen synthase kinase-3β (GSK-3β) [145], activation of mK$_{ATP}$ and large conductance Ca^{2+} - activated K$^+$ channel (BK$_{Ca}$) channels [143], induction of autophagy via the mammalian target of rapamycin (mTOR) pathway [146], activation of transcription factor Nrf2 [147], expression of heme oxygenase 1 [148], and upregulation of eNOS [149]. In addition, polyphenols stimulate in vivo NO$_2$$^-$ reduction to NO$^•$ [31,32], providing cardioprotection

via NO•-mediated pathways. Interestingly, resveratrol is synthesized by plants in response to fungal infection [150], and so the cardioprotective efficacy of red wines may vary based upon external influences on the grape berries during vegetation.

Extensive studies of the phenolic components of white wine (n-tyrosol and hydroxytyrosol) revealed that they also possessed strong protection against IR injury [151,152]. Although cardioprotective mechanisms of white wine constituents are less studied in comparison to red wine polyphenols, several pathways have been revealed. Thus, in addition to being potent antioxidants [153], n-tyrosol and hydroxytyrosol activate eNOS [151], replicating one of the cardioprotective pathways described above for resveratrol.

Further elucidating the cardioprotective mechanisms of phenolic compounds derived from grape wine, herein we draw attention to the possible role of the sirtuin family of proteins. Sirtuins (SIRTs 1–7) are a family of mammalian NAD$^+$-dependent lysine deacetylases, which are orthologs of yeast silent information regulator (Sir2P) histone deacetylase. In model organisms, SIRTs may replicate some signaling events triggered by CR [154] and can be activated by several components of red and white wine [137,154,155]. Although direct implication of SIRTs in the treatment of MI have not been fully elucidated, recent studies revealed that SIRTs activation may be a promising strategy for the treatment of several CV diseases, including hypertrophy [156] and chronic heart failure [157].

SIRTs have attracted much attention since the discovery that SIRT1 may be activated by resveratrol [155]. Sinclair and co-authors found that resveratrol stimulated Sir2 (an analog of human SIRT1) in *S. cerevisiae* and extended their lifespan [155]. In mammals, regulation of SIRT1 function by resveratrol was confirmed in many tissues [158], including myocardium [137]. Several other polyphenols were shown to stimulate SIRT1 activity, although to a lesser extent than resveratrol [155].

Having established the cardioprotective efficacy of n-tyrosol and hydroxytyrosol against IR injury, Das and colleagues found that these two constituents of white wine also activated SIRT1 in rat myocardium [137,152]. Furthermore, white wine was found to be an even more potent activator of SIRT1 than the resveratrol in red wine, although resveratrol showed greater cardioprotective efficacy [137]. These results indicated that the magnitude of cardioprotection provided by white wine or resveratrol does not exactly correlate with the degree of SIRT1 activity [137]. Therefore activation of SIRT1 may be necessary, but alone is not sufficient in providing the observed cardioprotection against IR injury. Nevertheless, these observations enable the conclusion to be drawn that the cardioprotection rendered by the components of grape wine is principally accompanied by myocardial SIRT1 activation.

In light of these findings, a number of important limitations should be mentioned. Foremost, since SIRT1 is the best-characterized of the sirtuin family proteins, most studies are focused on its activation, while information about the other 6 members of this family is very limited. Even so, activation of SIRTs 3, 4, and 7 has been demonstrated in rat cardiac H9C2 cells after exposure to resveratrol [159]. Second, phenolic compounds mediate multiple cardioprotective pathways (see above). Consequently, SIRT1 is not the only target [160], and thus at least a portion of the cardioprotective effect afforded by these compounds may, in reality, be SIRT1-independent. Because of this, significant efforts have been recently made toward the synthesis and testing of resveratrol analogs, with the hope that they might be more selective activators of SIRT1. Already, several novel compounds have been generated [154,161]. It was demonstrated that the synthetic SIRT1 activator SRT1720 was 1000 times more potent than resveratrol and could suppress aged-related disturbances in model animals [162]. However, these results concerning the ability of SIRT1720 to directly activate SIRT1 were recently questioned because of methodological artifacts [163]. Most of

these inconsistencies between different laboratories are due to the lack of precise methods to measure SIRT activity. In the future, both the synthesis of specific activators and development of reliable methods for determination of SIRT1 activity will clarify the role of these proteins in cardiac pathologies, including MI.

In addition to exogenous activators, SIRTs can be upregulated and activated by several endogenous mechanisms initiated by CR [164], IPC [165,166] and mild stress [157,165]. One study found that CR upregulated and activated SIRT1 in various types of tissues, including myocardium [167]. Concurrently, CR reduced infarct size, improved left ventricular function and attenuated inflammatory response after cardiac ischemic insult [168,169,170,171]. Moreover, it is well-documented that CR has the ability to prevent aged-related intolerance to cardiac ischemia in model animals [167,169,172,173]. It was demonstrated that phosphorylated AMP-activated protein kinase and elevated serum level of adiponectin were critical for CR-mediated cardioprotection [170,171]. Intriguingly, AMP-activated protein kinase regulates SIRT1 activity via modulation of NAD^+ level, and thus observed protection may be regulated via SIRT1-dependent mechanism [174]. Investigating this apparent connection between CR and SIRT1, Bolli's group has reported that 6 months of CR caused upregulation of nuclear SIRT1 and improved cardiac recovery after myocardial ischemia in middle-aged rats [167].

It has been shown that cardioprotection afforded by IPC may be associated with upregulation or activation of SIRT1 [165,175]. Recently, a role for SIRT1 in IPC was demonstrated in the brain [176]. Although direct involvement of SIRTs in cardiac IPC has not been completely proven, SIRT1 protein expression was found to be increased in porcine myocardium after IPC [165]. In isolated perfused and in vivo mouse hearts, we demonstrated that IPC stimulated SIRT1 activity and caused deacetylation of cytosolic proteins without increasing the level of SIRT1 protein [166]. Furthermore, SIRT1 inhibition blocked the cardioprotection afforded by IPC [166]. Alcendor *et al.* demonstrated that cardiac-specific over-expression of SIRT1 elicited resistance to oxidative stress, and that SIRT1 was upregulated in response to mild oxidative stress [157]. Therefore it seems reasonable to propose that SIRT1 might be activated in response to IPC mediated non-lethal oxidative stress. These findings support the idea that SIRT1 may regulate endogenous protective mechanisms against myocardial stress and may be involved in IPC signaling.

Intriguingly, it has been demonstrated that aging hearts fail to undergo IPC cardioprotection [177,178,179]. The mechanism of this resistance is yet to be elucidated, but it may include chronic oxidative stress, DNA mutations, or loss of protein function. Fortunately, CR preserves the ability of aging heart to be preconditioned [172,180,181]. Having established that CR stimulates SIRT1 activity, it is possible that CR can reverse the loss of IPC efficacy with aging via mechanisms involving SIRT1. Future studies should clarify if these events are mechanistically related.

5. Downstream mechanisms for SIRTs mediated cardioprotection

Because SIRT1 has been shown to be activated by phenolic compounds [137,154,167], the downstream signaling cascades with account for the effects of this protein in cardioprotection are of great interest. SIRT1 deacetylates and activates eNOS, thereby increasing $NO^\bullet$ production [182] which is known to be protective in several CV diseases, including MI [26]. Furthermore, SIRT1 can be upregulated and activated by $NO^\bullet$ [183], forming a positive feed-back loop which enhances $NO^\bullet$ availability. Thus, consumption of green vegetables enriched with nitrite may indirectly cause SIRT1 upregulation.

SIRT1 mediated deacetylation can inhibit several potentially detrimental mechanisms associated with MI. One of these mechanisms of SIRT1 protection may be inhibition of poly

(ADP) ribose polymerase-1 (PARP1) activity, and subsequent prevention of PARP-dependent cell death [184]. This inhibition may occur either directly via deacetylation and deactivation of PARP1 [185], or indirectly via concurrent binding to the common substrate NAD^+ [186]. In addition, autophagy, an essential process in self-cleaning and cardioprotection [187], is regulated by SIRT1 via deacetylation of Atg-5, 7 and 8 [188]. Indeed, SIRT1$^{-/-}$ embryonic heart tissue accumulates damaged mitochondria, indicating failure of autophagy [188]. Stimulation of autophagy has been shown to enhance anti-apoptotic mechanisms in cardiomyocytes after acute MI [189].

Furthermore, SIRT1 is known to regulate the activity of several important transcription factors and coactivators that are essential for the protection of numerous types of CV disturbances. For example, SIRT1 is able to induce the deacetylation and activation of Forkhead box O (FOXO1, 3 &4) [190,191], which regulates the expression of antioxidant enzymes and induces cardiac resistance to oxidative stress [192]. SIRT1 also deacetylates and stimulates Hif-2α [193], which is known to regulate cardiomyocyte resistance to ischemia [194] and PGC-1α [195], which promotes mitochondrial biogenesis and may be beneficial for recovery after MI [196]. Furthermore, SIRT1 inhibits the transcriptional activity of NF-kB via deacetylation of subunit RelA/p65 [197], which may prevent inflammatory responses during MI [198].

Although the cardiac expression and activation of SIRTs 2 to 7 by CR and exogenous nutrients have not been fully elucidated, recent studies have shown that several SIRTs are critical for heart development and normal cardiac function [199,200,201]. Among them SIRT3 is one of the best-characterized proteins, after SIRT1, and is the major mitochondrial class III deacetylase [202]. SIRT3 may be exceptionally important for cardiac tissue because one important downstream target of SIRT3 signaling is the regulation of mitochondrial metabolism [203]. For instance, SIRT3 deacetylates and activates isocitrate dehydrogenase 2 [204], increasing the oxidative decarboxylation of isocitrate and elevating the NADPH level, which may keep antioxidant enzymes in reduced states and provides efficient machinery for ROS scavenging upon reperfusion [205]. Furthermore, SIRT3 regulates OX-Phos by interacting with the 39kDa subunit of complex I (NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 9) and modulating complex I activity through deacetylation [200]. Importantly, complex I activity in SRT3$^{-/-}$ mice is endogenously inhibited, and we found that intact, functional complex I is required for the cardioprotection afforded by IPC [63].

A number of seminal studies have demonstrated the direct involvement of SIRT3 in several anti-apoptotic mechanistic pathways. It has been shown that SIRT3 may elicit anti-apoptotic functions by deacetylating Ku70 and promoting its interaction with BAX [206]. This prevents BAX translocation into mitochondria and consequent cell death [207]. As mentioned above (section 2.4), another mechanism that mediates cell death during myocardial reperfusion is activation of the mitochondrial PT pore [58]. Cyclophilin D is a structural component of the PT pore, and deleting this enzyme or preventing it from binding to ANT significantly reduces IR injury [208,209]. Shulga *et al.* demonstrated that SIRT3 deacetylated cyclophilin D and prevented its interaction with ANT in HeLa cells [210].

Overall, despite the fact that SIRTs have not been shown to function directly in cardioprotection against IR injury it is likely that SIRTs may orchestrate the regulation of multiple signaling mechanisms responsible for downstream protective events. Therefore, the role of SIRTs in Mediterranean diet-mediated cardioprotection remains to be more fully elucidated.

5. Concluding remarks: possible interplay between $NO_2^-/NO^{\bullet}$, PUFAs and polyphenols

The amount of food and the type of food we consume are two important issues that have a great impact on normal CV function. They may also determine the level of risk for CV pathology, including MI. Large portions of green fruits, vegetables, fish and grape wine enrich the Mediterranean diet with nitrite, ω-3/ω-6 PUFAs, and polyphenols. This review describes the potential cardioprotective roles of these substances against MI, as well as a discussion of protective mechanisms. As highlighted in multiple parts of this review, it is exceptionally worthwhile to propose that some sort of cross linking interactions exist between $NO_2^-/NO^{\bullet}$, PUFAs and polyphenols. In fact, having established that grape wine stimulates SIRTs activity, several research projects involving human volunteers revealed that wine consumption may also increase the level of PUFAs. For example, a study of a cohort of 1604 volunteers revealed that moderate wine consumption was associated with increased blood concentration of ω-3 PUFAs (EPA and DHA) [10]. Additionally, in concurrence with this study, red wine supplementation improved Mediterranean diet by increasing PUFAs and balancing ω-3/ω-6 ratio [12]. Wine-dependent reduction of NO_2^- to $NO^{\bullet}$ both in the stomach and the oral cavity [29,31] was demonstrated as well.

Further supporting a possible interplay between NO_2^- and PUFAs, it should be noted that PUFAs enhanced endothelial $NO^{\bullet}$ generation in human endothelial cells [211]. As was mentioned above (section 2), the interaction of $NO_2^-/NO^{\bullet}$ and PUFAs yields the formation of nitroalkenes [76,212]. Besides being potent cardioprotective mediators, nitroalkenes may also stimulate $NO^{\bullet}$ production via expression of eNOS [213], forming a positive feed back loop. Further including SIRT1 in this chain of events, $NO^{\bullet}$ stimulates SIRT1 activity [182] and vice versa, SIRT1 stimulates $NO^{\bullet}$ production [183]. Therefore, aside from its exogenous supplementation, $NO_2^-/NO^{\bullet}$ may also be produced endogenously, recruiting PUFAs- and SIRT1-dependent pathways (see figure 1).

In conclusion, the cardioprotective effects of the Mediterranean diet may arise through a variety of both distinct and convergent mechanisms summarized in figure 1. Interestingly, several of the events described above for the Mediterranean diet and illustrated in Figure 1 may also be implicated in the endogenous protection afforded by IPC. Indeed, IPC may be triggered via $NO_2^-/NO^{\bullet}$ generation [214], PUFAs elevation [215], endogenous synthesis of electrophilic lipid derivatives [78,79], or SIRTs activation [165,175]. Taken as a whole, although IPC itself is impractical to administer in humans, through the Mediterranean diet it may be possible to obtain similar benefits and build up strong endogenous protection in an effort to prevent MI.

Acknowledgments

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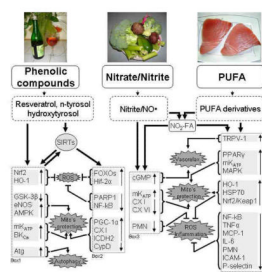
Figure 1. Mechanisms of cardioprotection provided by the components of the Mediterranean diet

Phenolic compounds, nitrite/NO•, and PUFA are originated from the components of the Mediterranean diet. Mechanisms triggered by polyphenols and shown in box 1 & 2 can be mediated via SIRTs dependent (box 2) and independent (box 1) pathways (see section 3). The backward arrow from box 1 indicates that SIRTs can be activated by some of these events (for example cross talk between SIRTs and NO• [182,183]; AMPK [174] and autophagy [188]). Nitrite/NO•· are bioactive molecules, which provide cardioprotection via mechanisms described in section 2 and shown in box 3. PUFAs' cardioprotective pathway involves biosynthesis of several classes of anti-inflammatory lipid derivatives (see section 3). Interaction of Nitrite/NO• with PUFA or its derivatives generates nitroalkenes, cell-signaling molecules, that possess strong protection against MI. Box 4 shows the mechanisms that mediate PUFAs' derivatives' cardioprotection. Nitroalkenes possess some NO•-dependent and independent properties (from box 3 & 4; see explanations in the text).

Abbreviations: PUFA – polyunsaturated fatty acids; NO• – nitric oxide; NO_2-FA – nitroalkenes; ROS – reactive oxygen species; Nrf2 - Nuclear factor erythroid 2-related factor 2; HO-1 – heme oxygenase 1; GSK-3β - glycogen synthase kinase-3beta; eNOS - endothelial nitric oxide synthase; AMPK - AMP-activated protein kinase; mK_{ATP} - mitochondrial ATP-sensitive K^+ channel; BKCa - large conductance Ca^{2+} -activated K^+ channel; Atg – autophagy proteins; FOXOs - forkhead box O transcription factors; Hif-2α - hypoxia-inducible factor 2-alpha; PARP1 - Poly (ADP-ribose) polymerase 1; NF-kB - nuclear factor-kappa B; PGC-1α - peroxisome proliferator-activated receptor-γ coactivator 1alpha; CX I – complex I; ICDH2 - isocitrate dehydrogenase 2; CypD – cyclophilin D; cGMP - cyclic guanosine monophosphate; CX VI – complex IV; PMN - polymorphonuclear neutrophil; TRPV-1 - transient receptor potential cation channel, subfamily V, member 1; PPARγ - Peroxisome proliferator-activated receptor γ; MAPK - mitogen-activated protein kinase; HSP70 – heat shock protein 70; Nrf2/Keap1 - nuclear factor erythroid-2 related factor 2 - Kelch-like ECH - associated protein 1; TNFα - Tumor necrosis factor-alpha; MCP-1 - monocyte chemoattractant protein-1; IL-6 - interleukin-6; ICAM-1 - Inter-Cellular Adhesion Molecule 1.