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Para-hormesis: An innovative mechanism for the health protection brought by antioxidants in wine

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Abstract. We have recently proposed a new paradigm for understanding how antioxidants in fruit and vegetables provide protective effects for health [1]. Here we describe how this new paradigm is relevant to explaining how polyphenols in wine can provide health benefits. The new paradigm of "para-hormesis" is based on the reality that in cells, the by far major antioxidant mechanism is enzyme-catalyzed reduction of hydroperoxides rather than non-enzymatic scavenging of free radicals and other oxidants and that dietary antioxidants increase antioxidant enzymes and their substrates. Indeed, non-enzymatic scavenging by wine polyphenols may be restricted to the intestinal lumen as kinetic considerations rule out a significant contribution of non-enzymatic scavenging in cells. Indeed, antioxidants function through their metabolism in cells to electrophiles that induce antioxidant enzymes and elevate the concentrations of nucleophiles, particular NADPH, glutathione and thioredoxin that are the substrates for these enzymes. This maintenance of "nucleophilic tone" provides the means for "antioxidant defense"

1. Introduction

We provide here a brief summary of our recent proposal for how polyphenols present in wine, fruit and vegetables provide antioxidant defense in cells. We will also briefly review how wine polyphenols can function in the intestinal lumen in post-prandial consumption. The primary focus however, is on a new paradigm for antioxidant function that we have named para-hormesis.

First, we will define here how we use the terms 'antioxidants,' 'reductants,' 'nucleophiles,' 'oxidants,' and 'electrophiles.' Nucleophiles are molecules that give electrons to other molecules, called electrophiles. Reductants are then nucleophiles that give one or two electrons to an electrophile, without forming covalent bond. The kind of electrophile that receives electrons from a reductant without forming a covalent bond is then called an oxidant. There are different functional definitions for the word, 'antioxidant.' In chemistry, antioxidants are nucleophilic reductants that directly react with oxidants, thus preventing the oxidation of a third molecule. In biology, antioxidants may act directly or indirectly to increase the capacity to remove electrophiles.

Approximately ten millennia ago humans developed agriculture, which provided availability of nutrients required for maintenance of metabolic energy. Recognition of micronutrients including vitamins that prevented deficiency diseases arose about a century ago although the observation that fruits, vegetables and wine contained substances that enhanced health

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existed in folk traditions well before the advent of modern chemistry. But, the recognition that vegetal foods contain specific phytochemicals that may generally reduce the incidence of disease is quite recent [2–4]. Although their effectiveness was supported by epidemiological, animal and *in vitro* studies, understanding of how specific phytochemicals provide defense against oxidants developed under some major misconceptions.

Increasing realization that free radical scavenging cannot explain the effects of the beneficial effects of phytochemicals (with the exception of vitamin E) led some investigators to begin investigation into other explanations for the apparent antioxidant activity of these compounds. But, as this was developing, failed clinical trials for antioxidants and controversy surrounding the effects on aging of resveratrol, the polyphenol in wine receiving the most attention, diminished enthusiasm for antioxidants even among scientists in the field of free radical research. Nonetheless, looking at all the studies, including those with negative findings, suggested to some that these compounds have a maximal effectiveness similar to that of vitamins so that supplemental polyphenols and other phytochemicals only provide a benefit to those whose diet is suboptimal in fruits and vegetable consumption. As is clear from increasing obesity and incidence of metabolic syndrome, far too many people consume suboptimal diets.

Wine phytochemicals can be part of the solution, although it may be less common to have a suboptimal diet among moderate wine consumers. Regardless, proposing the use of wine as a supplement to food has been controversial as the contribution of alcohol to both beneficial and deleterious consequences for health provides a whole other set of issues. Here however, we will restrict our discussion to the phytochemicals.

We will describe how the phytochemicals found in wine and vegetal foods activate a signal transduction pathway that results in an increase in the antioxidant enzymes and their substrates, glutathione, thioredoxin and NADPH, which we have named 'nucleophilic tone' [1]. This involves an apparently paradoxical effect in which the phytochemicals are metabolically converted to electrophiles that drive the activation of the Nrf2/EpRE pathway. The literature has many examples of electrophilic compounds producing cellular injury that triggers a hormetic response that provides subsequent protection [5]. Here however, we propose that the phytochemicals produce a "parahormetic" effect in which there is no toxicity to overcome in increasing nucleophilic tone.

2. Antioxidants and free radicals

The ability of a very large number of organic compounds to react rapidly with free radicals has been recognized for almost two centuries and their use in preventing oxidation of polymers and food is of great importance [6]. There is also no argument about the involvement of free radicals in many biological processes both "good" and "bad" [7]. Important examples of the bad side were the observations by Gershman of the involvement of free radicals in radiation damage and its enhancement by oxygen [8] and the development of the free radical theory of aging by Harman [9]. Thus, it was assumed that scavenging with antioxidants could prevent oxidative damage from excessive free radical production and lead to a longer and healthier lifespan. Unfortunately, the syllogism that the more antioxidants one could pack into cells and tissues, the greater would be the resistance to pathology caused by free radicals has run into the hard reality of failure at least in higher animals and in human clinical trials [10–13]. As described below, while the logic seemed reasonable, limited physiological uptake and the kinetics of competition with biological targets cause free radical scavenging by antioxidants to be a negligible component of antioxidant defense in cells. Although still controversial, long term, high dose α-tocopherol administration for cardiovascular disease prevention may be the sole exception [14].

Despite the kinetic realities that antioxidants do not work in cells by scavenging free radicals, some investigators persist in reporting studies in which a newly discovered antioxidant is demonstrated to scavenge radicals *in vitro*, thereby providing the next 'great breakthrough' (sarcasm intended) in antioxidant defense. With the exception of vitamin E and enzymatic dismutation catalyzed by superoxide dismutase, there is actually no evidence of significant defense in cells by free radical scavenging. An explanation for why vitamin E is an exception in physiologically relevant free radical scavenging, a history of free radical production in carbon tetrachloride toxicity and lipid peroxidation, antioxidant defense, and a review of the chemistry of oxygen that promotes free radical production by phagocytes and mitochondrial metabolism are provided in our recent publication [1].

3. Phytochemical antioxidants versus antioxidant enzymes in antioxidant defense

Scavenging by antioxidants is a second order reaction in which *Reaction rate = k[A][B]*, where [A] is antioxidant concentration, [B] the free radical concentration, and k is the second order rate constant. The most powerful free radical, hydroxyl radical ($HO^{\cdot}$) reacts with almost all organic molecules with rate constants approaching the rate of their diffusion ($>10^9\,M^{-1}s^{-1}$) [15, 16]. Thus, no scavenger can be protective by reacting with $HO^{\cdot}$. Put another way, to be 50% effective, the concentration of a scavenger would need to be equal to the concentration of all the other molecules combined. Alkoxyl radicals ($RO^{\cdot}$) that derive from decomposition of lipid hydroperoxides are produced in membranes [17, 18]. While they react somewhat more slowly with organic molecules than $HO^{\cdot}$, for both $RO^{\cdot}$ and $HO^{\cdot}$ the only efficient protection against them is to prevention of their formation.

The rates of absorption, transport and competition kinetics that limit antioxidant effectiveness in cells do not apply everywhere. Such an exception is in the intestinal lumen where oxidative degradation of food can leading to post-prandial oxidative stress, which has been demonstrated to be clearly prevented by consumption of wine polyphenols with the food [19].

Although intracellular scavenging of free radicals is not an effective mechanism of protection, antioxidant defense can effectively remove the less reactive, but still reactive species hydrogen peroxide (H_2O_2) and lipid hydroperoxides by enzymatically reducing them to their corresponding alcohols [20, 21]. Catalase can dismutate H_2O_2 to H_2O and O_2, while peroxidases and peroxiredoxins catalyze the reduction of hydroperoxides using the electrons of nucleophilic thiols, glutathione (GSH) or thioredoxin (Trx) [22]. Cells maintain GSH and Trx in their reduced forms using enzyme-catalyzed reduction by NADPH primarily. The pentose shunt is the primary source of NADPH making glucose the principal "antioxidant" in cells. Thus, by putting the burden of antioxidant defense on two-electron reduction catalyzed by enzymes, nature evolved a far more effective means of protection than one-electron free radical scavenging. Indeed, as H_2O_2 and lipid hydroperoxides are the source of $HO^{\cdot}$ and $RO^{\cdot}$, respectively, their removal by the antioxidant enzyme/nucleophilic substrate-dependent system is where two-electron biochemistry prevents one-electron chemistry in cells.

Another misconception is that antioxidants can reduce hydroperoxides in non-enzymatic two electron reactions, efficiently. Indeed thiols, like glutathione have reaction rates with hydroperoxides that are relatively fast for a non-enzymatic reaction, up to $10\,M^{-1}s^{-1}$. But, this rate is trivial in comparison with enzymatic reactions catalyzed by various peroxidases and peroxiredoxins that may be 10^5 times or more faster [22]. Therefore, peroxidase- or peroxiredoxin-catalyzed reactions using nucleophilic thiols, rather than non-enzymatic two-electron reactions are biologically significant.

4. Increasing the cellular adaptive response to oxidative challenge brought by electrophiles

We have defined nucleophilic tone as "the capacity to remove electrophiles through enzyme catalyzed, dynamic flow of reducing equivalents from NADPH, GSH and Trx" [1]. Increasing nucleophilic tone therefore involves increasing the overall potential for cells to respond to an oxidative challenge from electrophiles. Here we will describe the principal means through which cells react to the production of oxidants and how the phytochemicals in wine, fruit and vegetables produce this response.

5. The paradoxical action of phytochemical antioxidants: Activation of the Electrophile Response element

So, if the phytochemicals in wine and other edibles do not act as scavengers of oxidants, should we be asking what the epidemiologists have been smoking? The short answer is 'No, because the phytochemicals induce nucleophilic tone.' Indeed, a large number of phytochemicals, including wine polyphenols, induce antioxidant enzymatic systems.

There are two mechanisms through which phytochemicals induce nucleophilic tone. In one, the chemistry is the generation of superoxide ($O_2^{\cdot -}$) and H_2O_2, while in the other, the molecule is, or is metabolized to an electrophile. In other words, paradoxically, phytochemical antioxidants increases nucleophilic tone by increasing electrophile concentration. Fortunately, and the reason we refer to this phenomenon as 'para-hormesis,' the concentrations of electrophiles reached in mammals are well below toxic.

Amusingly, it is the inability to reach the high concentrations needed to scavenge free radicals that accounts for the lack of toxicity by the phytochemical itself. An example of pathogen-induced hormesis is observed in plants where bacteria and fungi cause a significant increase in the concentrations of phytochemicals so that they become lethal against the parasites and protective of the host [23]; however, the pathways through which phytochemicals kill pathogens versus those that protect mammals are markedly different.

The realization that it was actually the generation of electrophiles that caused the increase in nucleophilic tone came from a consideration of how some compounds considered as planar aromatic antioxidants were able to induce transcription of several genes in the group called of antioxidant enzymes called Phase II enzymes. As we will describe briefly, several studies [24–40]. Demonstrated that phenolic compounds, along with sulforaphane, an isothiocyanate in cruciferous vegetables, increased endogenous antioxidant protection through activation of transcription mediated by what was first called the Antioxidant Response Element (ARE) but is more accurately called the Electrophile Response Element (EpRE).

When polyphenols and other planar aromatic ortho and para hydroquinones are oxidized to quinones (Fig. 1), they generate $O_2^{\cdot -}$ and H_2O_2. Thus, it was reasonably concluded that these species were responsible for gene induction by planar aromatic compounds that were oxidized to phenolic compounds [40]. We now understand that high concentrations, H_2O_2 can activate the EpRE [41], but twenty years ago the labs of Talalay [37] and Daniel [39] clearly demonstrated that it was electrophiles, including quinones generated from planar aromatic phenolic compounds that were more likely responsible than $O_2^{\cdot -}$ or H_2O_2 for Phase II enzyme induction.

Once the ARE/EpRE was recognized, the transcription factor(s) that activated it were sought. Nrf2 (NF-E2-related factor 2) was found to be the principal transcription factor that binds to the EpRE in response to activation by electrophiles [42]. Nrf2 is a rapidly turning over protein in the cytosol. This rapid turnover is mediated by Keap1 (Kelch-like ECH-associated protein 1), [43] (also called called INrf2 (inhibitor of Nrf2) [44]), that assists in Nrf2 ubiquitinylation that marks the protein for proteasomal degradation [43]. If critical cysteine residues in Keap1 are alkylated or oxidized, Keap1 cannot facilitate Nrf2 degradation, which allows Nrf2 to translocate to the nucleus [45].

The activation of EpRE by high concentrations of H_2O_2 referred to above begins with the formation of a disulfide between two Keap1 molecules [41]. It is however, alkylation of a critical cysteine residue on Keap1 by an electrophile that permits Nrf2 to escape degradation. There are many cysteines in Keap1 but which of these is the critical target appears to depend upon the concentration of the particular electrophile [46–51].

In the nucleus, Nrf2 forms a heterodimer with a partner protein in binding to EpRE. Several studies suggest this is c-Jun [52] while others claim it is a small Maf protein [53]. Furthermore, phosphorylation of Nrf2 is required for translocation to the nucleus and activation of EpRE-regulated gene transcription [54–60].

6. Wine polyphenols in para-hormesis

Depending upon the type of grape and many other factors, the polyphenol content of wine varies widely. Rather than reviewing the individual chemistries of hundreds of compounds, we will review the general chemical issues and focus on resveratrol, which has attracted considerable attention and is even being used as a supplement in some wine and as a nutraceutical in capsule form.

A principle consideration for the chemistry shown in Fig. 1 is that the polyphenol must have two hydroxyl groups in ortho or para relationship in order to redox cycle to produce $O_2^{\cdot -}$ and H_2O_2 and form the quinone. The general structure that acts as the electrophile is the α,β-unsaturated carbonyl (RHC = CH-CR = O) moiety. This undergoes a Michael addition with a cysteine that is in the thiolate (S^-) form (Fig. 2). It has been suggested that in Keap1, zinc coordinating to cysteine, delocalizes the proton and thus increases the nucleophilicity, accounts for its greatly enhanced sensitivity to electrophiles than protein cysteines in general [61]. Thus, in a sea of cysteines, the zinc-coordinated cysteine residues of Keap1 can act as a sensor.

While many of the polyphenols in wine would be expected to act through the chemistry in 2, resveratrol cannot! This is because resveratrol (Fig. 3) is has a meta-hydroquinone and a phenol structure and cannot be oxidized to form the α,β-unsaturated carbonyl structure. Nonetheless, as also shown in Fig. 3, enzymatic oxidation of the phenol moiety by tyrosinase [62] can form the ortho hydroquinone. The 3-hydroxyresveratrol can be a precursor to form-

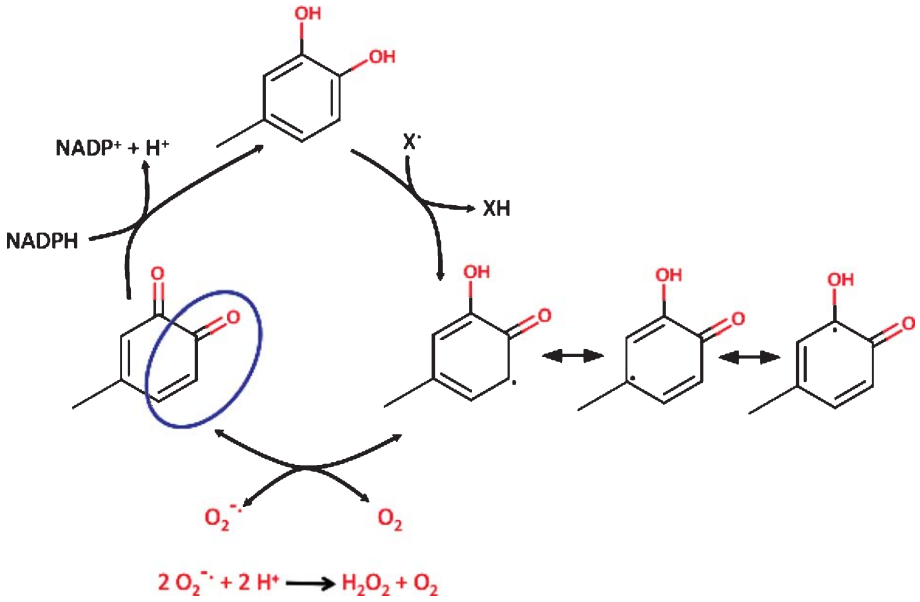

Fig. 1. General scheme for redox cycling and the generation of electrophilic quinones.

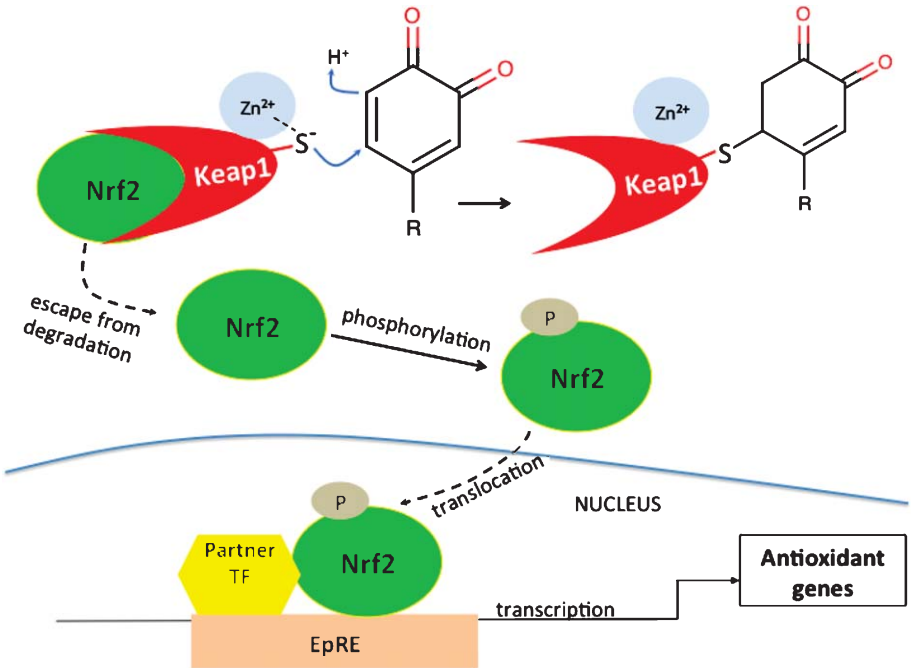

Fig. 2. Keap1 reacts with α,β-carbonyl moiety in a Michael addition. Enhancement by Zn.

ing the α,β-unsaturated carbonyl structure that then reacts with Keap1 (Fig. 2). As a caveat to this entire discussion, it should be noted that resveratrol, as well as other polyphenols affect other signaling pathways besides the Nrf2/EpRE pathway; however, the effect on nucleophilic tone through the Nrf2/EpRE pathway can account at least for much of these effects.

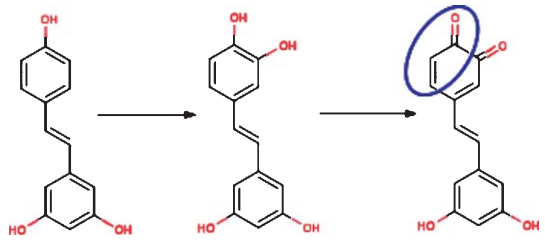

Fig. 3. Resveratrol oxidation.

6.1. Nucleophilic tone, inflammation and health

Life is self-protecting and reactions to agents that produce injury vary with the ability of cells to defend themselves [63]. The basic elements of inflammation as a "reaction to an injury" consist of an array of mechanisms designed to eliminate stimuli, repair tissues through elimination of the most damaged cells, and proliferate. Remarkably, a large number of these events operate through oxidative reactions in a network of redox signaling [63, 64]. Indeed, in a general view, inflammation as response to injury evolves with the formation of electrophiles. This deviation from redox homeostasis is often referred to as 'oxidative stress.'

An excessive or inappropriate response to a given physiological challenge can lead to the prolonged alteration of homeostasis we classify as disease. Cancer, liver and lung fibrosis, neurodegeneration, and other diseases of aging., can all be seen as the outcome of excessive activation of responses to injury. Surveying the enormous amount of literature on nutraceutical effects of natural compounds and recognition of the value of these compounds in folk medicine around the world, produces a leitmotif relative to regulation of inflammation and protection from chronic degenerative diseases [3] and cancer [65, 66] by nutritional phytochemicals.

In conclusion, we propose the name 'Para-Hormesis' to describe the process by which nutritional antioxidants optimize the cellular defense system by mimicking electrophiles and increasing the Nucleophilic Tone, preventing in this way diseases generated by an excess of inflammatory response [63]. While presenting 'Para-Hormesis' as a paradigm shift in understanding physiological mechanisms of action by nutritional antioxidants we are happy to recall, besides modern epidemiology, the ancient wisdom describing the health protective effect of wine which is certainly one of the major sources of "nutritional antioxidants".

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