Unique Properties of Polyphenol Stilbenes in the Brain: More than Direct Antioxidant Actions; Gene/Protein Regulatory Activity

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Key Words

Bilirubin · Biliverdin · Blood flow · Carbon monoxide · Hemin · Iron

Abstract

The 'French Paradox' has been typically associated with moderate consumption of wine, especially red wine. A polyphenol 3,4',5-trihydroxy-trans-stilbene (a member of the non-flavonoids family), better known as resveratrol, has been purported to have many health benefits. A number of these valuable properties have been attributed to its intrinsic antioxidant capabilities, although the potential level of resveratrol in the circulation is likely not enough to neutralize free radical scavenging. The brain and the heart are uniquely vulnerable to hypoxic conditions and oxidative stress injuries. Recently, evidence suggests that resveratrol could act as a signaling molecule within tissues and cells to modulate the expression of genes and proteins. Stimulation of such proteins and enzymes could explain some of the intracellular antioxidative properties. The modulation of genes could suffice as an explanation of some of resveratrol's cytoprotective actions, as well as its influence on blood flow, cell death, and inflammatory cascades. Resveratrol stimulation of the expression of heme oxygenase is one example. Increased heme oxygenase activity has led to significant

protection against models of in vitro and in vivo oxidative stress injury. Resveratrol could provide cellular resistance against insults; although more work is necessary before it is prescribed as a potential prophylactic in models of either acute or chronic conditions, such as stroke, amyotrophic lateral sclerosis, Parkinson, Alzheimer, and a variety of age-related vascular disorders.

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The 'French Paradox,' so named after a population studied in France, is defined by a low incidence of cardiovascular problems despite a diet that is relatively high in saturated fat [1]. Because of the typical levels of wine intake of the French, it has also been postulated that a moderate consumption of red wine could be associated with this paradox. A vast amount of recent literature proposes that it is the polyphenols in red wine that provide the beneficial effect, mainly attributable to their potential to act as antioxidants. These polyphenols are divided into two main categories: flavonoids and non-flavonoids. The flavonoids, found in natural extracts of plants and fruits, are considered to be the most abundant polyphenols, whereas the non-flavonoid stilbenes are considered to be a minor class. The naturally occurring polyphenol stilbene, resveratrol (3,4',5-trihydroxy-trans-stilbene), has been proposed to possess most of the beneficial health ef-

Fig. 1. Experimental neurologic benefits of resveratrol.

fects of red wine, considering that resveratrol is mainly found in the skin of the grapes, and the skin and seeds are generally not used in processing white wines. Consequently, it has been proposed that resveratrol would potentially be the most active ingredient [2–5]. Given all of this, the antioxidant properties of red wine, then, must be associated with the actions of resveratrol, and we propose that the protective properties are likely due to a unique cascade of intracellular events leading to activation of unique antioxidant pathways. This hypothesis is being proposed, taking into consideration that the modest amount of resveratrol in the blood is not likely to reach a high enough plasmatic level to neutralize free radicals. Consequently, resveratrol is likely to stimulate an intracellular signaling pathway, leading to cytoprotection.

As represented here, several genes and proteins have been shown to be potential targets for resveratrol modulation. Heme oxygenase (HO) is a possible candidate. We have shown that resveratrol is a potent inducer of HO protein levels, activity, and cytoprotection [6]. HO's main function is to cleave heme (Iron-Protoporphyrin-IX), which then liberates iron, generating carbon monoxide and biliverdin, which is rapidly converted to bilirubin.

By degradation of heme, a prooxidant, into biliverdin/bilirubin, antioxidants, modulation of HO activity and levels would seem to be cytoprotective against free radical damage. Using in vitro and in vivo models, we have also shown that HO can be neuroprotective [7]. In addition, HO and its metabolites have been associated with antiapoptotic and antiinflammatory actions and are known to have a vasodilatory effect.

Several other enzymatic systems have been suggested to be either directly or indirectly modified by different members of the stilbene family. Here, we propose that protective properties of HO are the mechanisms that provide the brain's resistance to a variety of neurologic stresses. Resveratrol has been shown to have a unique effect on neuronal cell death and inflammatory processes, which are all important therapeutic targets in the development of either acute and/or chronic neurodegenerative diseases [8–10]. These vascular properties become especially important when considering that reduction in cerebral blood flow (CBF), followed by a reperfusion phase, is likely to affect specific neurons and/or the cell types that are especially vulnerable to free radical damage. Consequently, preventing cell death is likely to have a beneficial

effect on the rate of neuroinflammation and its consequences. Considering all of this together, one can make a valid hypothesis that polyphenol stilbenes can precondition neurons against induced stress damage.

Figure 1 contains a list of potential neurologic benefits associated with models of neurologic diseases treated with resveratrol. Of importance is the fact that resveratrol has been shown to be protective in several species. Following publication in *Science* and *Nature* of initial reports that suggested a potential biologic role of resveratrol [24, 25], more than 900 original research articles have been published to support the beneficial effect of resveratrol.

It is somewhat paradoxical that such a simple, natural component, extracted from plants and fruits, can have such a variety of actions. The flavonoids constitute the majority of phenols in red wine and are mainly divided into three classes: the flavanols, the flavonols, and the anthocyanins. The known flavonoids (hydroxycinnamic acids, benzoic acids, and stilbenes) are less abundant, and the stilbenes are only a minor class of the known flavonoids.

Interestingly, during the production of red wine, complex sugars from the grapes are fermented into alcohol, and it is believed that alcohol would be considered a good solvent for extracting polyphenols from the skins and seeds. Considering that resveratrol is mainly found in the skin of the grapes, it has been reported that the amount of phenols within white wine would be much less than in red wine. Although the reported amounts of resveratrol in wine vary, they suggest that approximately 7 mg/l are present in most reds, as compared to 0.5 mg/l in whites [26, 27]. For purposes of comparison, the flavonoid concentrations in red wine have been in the range of 1,300–1,500 mg/l. Once again, considering all of the beneficial properties of resveratrol, its minimal amount available in wine, if active, is likely to be mediated via activation of intracellular pathways.

It is interesting to note, also, that resveratrol exists in two isomers, the *cis* and the *trans*, as shown in figure 2. Both of these are found in wine, and, although it appears that only the *trans* isomer is found in grapes, direct light could cause its isomerization from *trans* to *cis*. In our preliminary observations in neurons, we indicated that the active isoform would be mainly the *trans* resveratrol.

The fact that resveratrol, a simple active ingredient, which has been proposed to be responsible for the rationale behind the French Paradox, is present in such small amounts in wine or juice, and the theory concerning its bioactivity by its direct antioxidant capacity deserve reconsideration. Thus, it led us to hypothesize the activa-

Fig. 2. UV-light-induced isomerization of *trans*- to *cis*-resveratrol.

tion of an antioxidant intracellular pathway. Considering the direct antioxidant properties associated with resveratrol and taking into account the absorption rate and modifications/conjugations, one would have to consume liters of these drinks in order to achieve the necessary plasmatic molar ratio to neutralize the free radicals and achieve the beneficial health effects. To highlight the variability of resveratrol's effects, figure 3 shows the intracellular consequences of cells treated with the maximal concentration of 25 μM , although the list is not exhaustive. We eliminated experiments in which the concentration was higher than 25 μM . We believe that it is reasonable to think that a level $\leq 25 \mu M$ could potentially be achieved under normal conditions and not typically under pharmacologic conditions.

Figure 3 shows the expression regulated by resveratrol of different genes and proteins and describes some of the potential functions. Living organisms under aerobic conditions are continuously exposed to potential damage caused by reactive oxygen species (ROS). ROS are produced naturally during normal cellular activity and mitochondrial function. In addition, induced stress is likely to modify these normal functions and simulate the generation of free radical damage. Many of the studies using polyphenols have required pretreatment with resveratrol to exert these beneficial biologic functions. Such pretreatment would require either an increase in the concentration of this polyphenol or stimulate a cascade leading to activation of an endogenous antioxidant system. This activation is critically important to cytoprotection in tissue with a weak, endogenous, antioxidant system. The heart and the brain are two unique examples of tissues with weak defenses, as evidenced by infarct damage following ischemia/reperfusion.

Considering the antioxidant properties associated with resveratrol, we have concentrated on the neuroprotective effect of resveratrol and the possibility that this

Fig. 3. Example of resveratrol-regulated genes/proteins affected in cells (<25 μM). HO1 = Heme oxygenase 1; COX-2 = cyclooxygenase 2; eNOS = endothelial nitric oxide synthase; iNOS = inducible nitric oxide synthase; ET-1 = endothelin-1; GADD45 = growth-arrest- and DNA-damage-induced protein 45; TNF α = tumor necrosis factor- α ; ATF3 = activating transcription factor 3; IGFBP = insulin-like growth factor-binding protein.

Fig. 4. Example of potential outcomes from gene/protein regulation (i.e., HO1) and potential enzymatic role in the brain. Note: This list of potential actions would occur at physiologic levels, while abnormal or pharmacologic levels of these compounds could have deleterious effects.

Fig. 5. Resveratrol mediates heme degradation by induction of heme oxygenase levels.

pathway could involve the induction of an antioxidant system, such as heme oxygenase. Regulation of HO activity has been shown to be protective in acute and/or chronic neurodegenerative conditions. Figure 4 summarizes some of the potential outcomes of regulation of the HO protein and its activity.

As mentioned above, HO catalyzes the degradation of heme, which is mainly a prooxidant, into iron, biliverdin/bilirubin, and carbon monoxide (fig. 5). Two isoforms of HO have been isolated and characterized [7, 33–35]. A third isoform has been reported, although it does not seem to be translated into a protein [36]. HO1, the first to be isolated [34], is the inducible enzyme and appears to be concentrated mainly in the liver and spleen, an understandable observation considering the high turnover of hemoglobin, which has heme in its core. Under basal conditions, HO1 is barely detectable in the brain, although several reports suggest that, under a variety of stimuli, it can be induced within brain tissue [37–48]. HO2 is an isoform that is constitutively expressed and appears to be concentrated mainly in the brain and testis [48]. Its protein expression level appears to be extremely stable, which is likely to respond to normal cellular homeostasis.

Regulation of the HO1 protein and its activity has elicited a great deal of interest. Regulation of HO1 has been suggested to be in response to many cellular and organ

stresses, in order to defend against disruption of any system homeostasis. HO1 has been suggested to have different functions in the brain. It is one of the heat shock proteins, which are stress proteins that are induced in different cells and following different stimuli [37], including hypothermia [38], global ischemia [41], subarachnoid hemorrhage [42], Parkinson disease [47], Alzheimer disease [39, 40], and several other acute and chronic neurologic conditions [43–46].

In a model of transient ischemia, we and others have shown that HO mRNA and proteins are induced [43]. Reports have indicated that induction of HO proteins in neurons would be protective [49], and our preliminary data indicate that resveratrol could induce HO levels within neurons, potentially affording neuroprotection. Consequently, we believe that modulation of HO levels and its activity could be a pathway by which resveratrol present in red wine or other concentrated extracts could potentially protect the nervous system against induced oxidative stress damage. Many heme-containing enzymes are located in the mitochondria, the cytosol, and the endoplasmic reticulum; and they presumably undergo rapid turnover during oxidative stress. HO is the main enzyme, which can degrade free heme, a prooxidant, and maintain levels that would not reach toxicity. Following an ischemic event, tissue injury has been proposed to be mainly due to increased oxidative stress by free radicals

generated during the reperfusion phase. We have previously shown that the infarct volume in HO2 knockout (HO2^{-/-}) mice versus wild-type (WT) mice after stroke is approximately double. HO2 is the isoform present under normal basal conditions. Consequently, increasing the activity of heme oxygenase is likely to be protective in stroke.

Interestingly, the group led by Maines has demonstrated significant reduction in infarct size after stroke in a transgenic mouse model on which HO1 was over-expressed using a neuron-specific promoter [50]. In addition, we have recently accumulated evidence suggesting that pre-treatment with resveratrol would be a most potent inducer of HO1 in mouse primary neuronal cultures, that it would be sufficient to prevent induced neurotoxicity, and that this neuroprotection was significantly attenuated by the use of a heme oxygenase inhibitor [51]. All together, these results suggest that an increase in HO1 protein levels and its activities within neurons is likely to provide neuroprotection and promote cell survival.

Heme

Heme metabolism is a crucial metabolic process. It has been postulated that free heme can rapidly be generated from an induced turnover of heme-containing proteins/enzymes, for example, catalase, glutathione peroxidase, superoxide dismutase, cytochrome, guanylate cyclase, nitric oxide synthase, etc. It has been further suggested that during hypoxia, ischemic injury could trigger significant amounts of heme being released into the intracellular pool. When stimulation of these hemoproteins is degraded, heme becomes free; probably not salvaged, it should be rapidly degraded. HO is the enzyme that can rapidly cleave prooxidant through heme and limit its capacity to enter into generation of a free radical cycle – notably, through its iron molecule. Consequently, HO could be considered an antioxidant enzyme by degradation of the prooxidant heme. Our previous observations indicate that resveratrol could specifically induce HO1 within neurons and potentially protect cells against oxidative stress injury. Such a pathway is an interesting target for resveratrol, considering that it can regulate the redox state of the cell and prevent cells from dying through an oxidative stress-induced cascade.

Iron

Degradation of heme by HO1 also generates a molecule of iron. Evidence suggests that regulation of HO1 protein levels could modulate the level of intracellular heme. It is postulated that HO can stimulate the efflux of

iron outside the cell [52]. Homeostasis of iron is a key factor in controlling cell toxicity. As one example, free iron has been considered to be a key ingredient in the Fenton reaction, in which by reacting with H₂O₂, it generates free radicals. Regulation of cellular homeostasis of iron is a complex and tightly regulated system. It is regulated by many proteins, a number of which are still under extensive characterization. Rapid upregulation of HO1 by resveratrol in neurons could potentially directly affect the intracellular iron level.

It has been previously demonstrated that decrease in HO1 activity would be sufficient to change its iron level within the cell. For example, by using HO^{-/-} mice, it has been shown that iron accumulates in several organs [53]. In addition, numerous iron-binding proteins are regulated by intracellular levels of free iron [54]. As an example, ferritin in the cell can sequester numerous molecules of iron, and its intracellular level is tightly regulated to free iron.

Therapeutic implications of controlling iron levels within tissues or within cells are numerous. As an example, administration of desferoxamine, a trivalent iron chelator, over a 2-year period slows the clinical progression of symptoms associated with Alzheimer disease [55]. Further study may bear out that the regulation of HO1 levels also regulates the cellular iron homeostasis. Interestingly, very little has been investigated regarding the potential role of resveratrol in regulating iron and determining its potential effect in neurologic disorders.

Carbon Monoxide

Carbon monoxide (CO) is a gas that is almost exclusively generated in cells by degradation of heme by heme oxygenase [56, 57]. In that it is a gas, carbon monoxide can travel freely through intracellular and extracellular compartments. The CO literature is vast and complex with many controversies that have yet to be resolved [58–63]. CO is better known to be toxic at high levels. It can also cause death [64]. Interestingly, in the recent literature, low concentrations of CO have been suggested to be protective. Although CO has an affinity slightly lower to its homologue, nitric oxide (NO), which is another gas, it appears that its half-life would be significantly longer. Such an observation could allow carbon monoxide, by binding to heme moiety present on several key enzymes, to modulate their function [65]. Within the cell, physiologic/normal levels of CO generated from degradation of intracellular heme are likely to have multiple biologic functions on several heme-containing proteins. For example, CO has been shown to act as a vasodilator by po-

tentially binding with soluble guanylate cyclase (sGC) and modulating its vasoactive activities. It can act on calcium-activated potassium channels (K_{Ca} channels) and regulate their opening [66, 67]. CO has also been recently reported to have specific antiapoptotic and antiinflammatory actions [68, 69]. Rapid induction of HO1 could be a means by which resveratrol can increase CO levels within physiologic concentrations and allow cells and tissue to benefit from many of CO's biologic actions, especially in scenarios in which blood flow is reduced and cell survival is compromised.

Bilirubin

Bilirubin is known for its toxicity, at high micromolar concentrations, in the central nervous system (CNS), especially in neonates. Although under physiologic concentrations, bilirubin can act as an endogenous antioxidant [70, 71]. In testing a series of antioxidants, bilirubin was shown to have significant superoxide and hydroxyl radical scavenger activity [72]. Using an animal model of hyperbilirubinemia, a protective effect against cerebral ischemia was demonstrated. We have also observed, using primary hippocampal and cortical neuronal cultures, that bilirubin can be protective at low concentrations [73, 74]. Moreover, in previous observations on the potential role of HO in Alzheimer disease pathology [39, 75], levels of bilirubin derivatives in the cerebral spinal fluid were reported to be significantly increased in brains of AD patients as compared to control [76]. An increase in HO1 within AD brains was reported. Increased HO1 levels could potentially increase the bilirubin level within a physiologic range, and such a pathway could explain some of the antioxidant properties associated with resveratrol in relation to neurologic deficits in age-related dementia.

Figure 6 briefly summarizes some of the neurologic disorders that can be beneficially affected by the regulation of HO activity. Regulation of genes by resveratrol, such as in the case of HO1, presents a potential mechanism by which its prophylactic use might be considered, under either acute or chronic neurologic disorders. For example, under ischemic stroke conditions, reperfusion frequently occurs after focal ischemia, particularly in the case of cerebral embolism and transient ischemic attack. These can be warning signs of impending stroke. Recurrence is a prevalent phenomenon in patients who have suffered one episode of stroke. Therefore, availability of a prophylactic approach in these patients is an important goal of preventive medicine. Reports that coronary heart disease appears to be reduced in patients who chronically

Fig. 6. Non-exhaustive list of potential neurologic benefits of resveratrol-regulating HO1.

cally consume moderate amounts of red wine support the prospect of a prophylactic role for resveratrol. Consequently, a better understanding of the mechanisms by which polyphenols and red wine can protect against ischemic and toxic insults would be of great interest.

Acknowledgements

This work is supported in part by the NIH grants (AA014911 and AT002113), the Wine Institute, and the ABMR Foundation. The author would like to thank Tzipora Sofare, MA, for her assistance in preparing the manuscript.

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