Nitric oxide scavenging by food: implications for *in vivo* effects of diet

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Accepted: 4 January 2010

ABSTRACT

Recent advice to the general public is to increase intake of fresh fruit and vegetables, a message based on the assumed benefits of the antioxidant content of plant substances. Although there have been numerous studies on the reactive oxide species scavenging of fresh food products, few studies have focused on whether or not compounds in the diet can modulate the levels of nitric oxide (NO). Nitric oxide is a key signalling molecule that controls vasodilation and blood pressure, along with a range of other physiological events. Here, it is shown that commonly used food substances, such as cabbage, broccoli, kidney bean and oranges, all have the capacity to scavenge NO from solution, and therefore can potentially alter the level of NO in humans, with ramifications for the physiological systems that NO regulates. Using spinach, at least one element of the NO scavenging ability was shown to be heat-unstable, although heat-treating of other leaf materials had little effect, showing that NO scavenging will still occur after cooking. It is proposed that the NO scavenging of dietary components needs to be investigated more thoroughly before the full effects of increasing antioxidants through increased intake of fresh fruit and vegetables can truly be understood.

KEYWORDS: Antioxidants.
Diet.
Nitric oxide.
Reactive oxygen species.
Scavenging.

Introduction

It is now well established that for a healthy lifestyle a good balanced diet is recommended. Certain types of diet, such as the Mediterranean diet, are thought to be particularly beneficial.[1] In the UK, government advice is to eat five portions of fruit and vegetables a day, preferably of different colours. One of the major health benefits of fresh produce is thought to be from the fact that they contain antioxidants.

As aerobic organisms, humans use the reducing power of NADH to generate a proton gradient across the inner membrane of mitochondria. This resulting electrochemical potential is used subsequently for the production of ATP. The by-product is the production of water, from the 4 electron reduction of molecular oxygen. However, the electron transport chain, which undertakes this activity, has a small capacity to leak, and the total reduction of oxygen does not always occur. If single electrons are used to reduce the molecular oxygen that needs to be present, the end result is the superoxide anion.[2] This will rapidly dismute to hydrogen peroxide in the presence of protons, and a further cascade of reactions can generate more reactive species, usually referred to as reactive oxygen species (ROS).

Furthermore, superoxide anions are generated in a directed way by phagocytic cells, using the enzyme NADPH oxidase, in a mechanism to kill invading organisms. Such enzymes are not restricted to phagocytes, and it is well established that in humans there are several isoforms in many cell types.[3] Other enzymes, such as xanthine oxidoreductase, can also produce ROS.

In a similar manner, nitric oxide (NO) is produced by a variety of cells, and has a range of physiological effects.[4] It is a relatively labile, small gaseous signalling molecule which is also used in the host defence armoury. In mammals it is produced by nitric oxide synthase (NOS), which is encoded by three genes (*eNOS*, *nNOS* and *iNOS*). Therefore, factors that can affect the presence of NO may have serious effects not only on the control of blood flow but also on other cellular functions.

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Reactive oxygen species are relatively reactive and can undergo a variety of reactions with biologically important molecules such as DNA, proteins and lipids. Such reactions result in DNA damage, lipid peroxidation and protein dysfunction. Antioxidants are compounds that react with ROS and prevent them from causing such cellular damage. Antioxidants are molecules that range from enzymes such as superoxide dismutase and catalase to more simple compounds such as vitamin C (ascorbate) and vitamin E (α-tocopherol).[5]

It is the presence and ROS-scavenging activities of compounds and proteins such as these that typically is the focus of studies to show the benefits of particular diets. However, ROS can be used in cell signalling cascades,[6] and have been shown to alter the rates of cell proliferation, alter the activity of intracellular signalling proteins (e.g., mitogen-activated protein kinases and tyrosine phosphatases) and alter gene expression.

It can be seen, therefore, that reactive compounds can also be beneficial to the organism, and this is certainly true for

NO. Nitric oxide was first identified as endothelial-derived relaxing factor (EDRF) with profound effects on the vasodilation of blood vessels, but is known to be involved in the control of a range of other physiologies.[4] In mammals, NO has many effects through the activation of guanylyl cyclase, the generation of cGMP and the resultant downstream effects.[7,8] However, NO can also compete with ROS for the reactivity of thiol groups on proteins, which results in the formation of nitro-thiols (so-called S-nitrosylation). This is a reversible event, leading to conformational changes in the topology and hence activity or functioning of a protein, and is in many ways similar to phosphorylation.[9,10]

Furthermore, ROS and NO can react together. They are often produced at the same time, under the same conditions, and so they may modulate the activity of each other. Potentially, they may also generate new signalling molecules such as peroxynitrite.[11]

Despite the number of studies undertaken on dietary antioxidants, there have been some reports on work carried out to investigate the effect of dietary compounds on NO, but they have been limited. Heller *et al*.[12] suggested that α-tocopherol may decrease *in vivo* NO, but together with ascorbic acid may cause an increase in NO production via a mechanism that leads to the activation of endothelial nitric oxide synthase. Others have looked at the NO scavenging effect of green tea.[13]

This study aims to show that a range of fruit and vegetables, common in the diet, can scavenge NO, and suggests that this could have an effect *in vivo*.

Materials and methods

Materials

Plant samples studied included spinach (*Spinacea oleracea*), using old leaf and young leaf samples, broccoli (*Brassica oleracea* var. *italica*), orange (*Citrus aurantium*), white cabbage (*Brassica oleracea* var. *capitata*) and kidney beans (*Phaseolus vulgaris*). These were chosen because they are commonly consumed in the UK, are known to show high antioxidant activity or have a high iron content.

Preparation of plant extracts

Plant materials (5 g) were frozen using liquid nitrogen and then ground to a fine powder using a sterile pestle and mortar. The resultant powder was added to 15 mL 1% (v/v) Tween 20 (polysorbate 20) in phosphate buffered saline (PBS; 10 mmol/L phosphate [pH 7.4] containing 137 mmol/L NaCl, 2.7 mmol/L KCl). The homogenate was centrifuged at 10,000 x*g* for 30 min at 4°C and the clear supernatant frozen immediately for preservation.

For use in NO-scavenging assays, the clarified extracts were either used directly (not heated) or were placed in a water bath at 85°C (heat-treated) for 15 min prior to use.

NO-scavenging activity

The NO-scavenging assay was based on the sodium nitroprusside (SNP)-mediated increase in fluorescence of the NO-specific dye diaminofluorescein-2 (DAF-2), measured using a fluorescence spectrophotometer (F-2500, DIGLAB Hitachi). The fluorescence of each sample (excitation: 500 nm, emission: 515 nm) was recorded for 600 sec. As a control, the fluorescence of a sample consisting of 1 mL PBS, 2 µL 5 mmol/L DAF-2 and 50 µL 1mmol/L SNP was used.

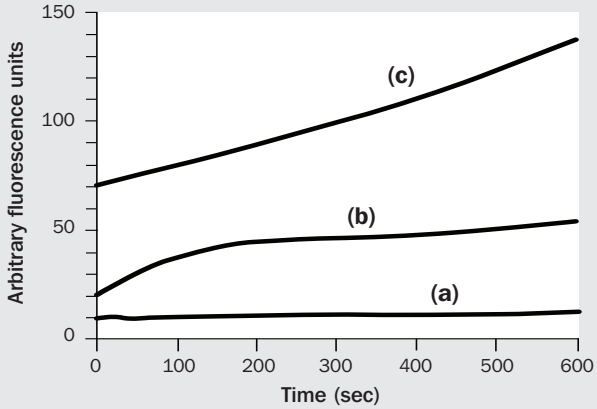

Fig. 1. Plant extract-attenuated SNP-mediated DAF-2 fluorescence. Plant extracts (broccoli) in PBS with Tween-20 attenuated an NO-dependent increase in DAF-2 fluorescence with time. The emission fluorescence of l mL 1xPBS samples containing (a) 50 µL plant extract, 10 µm DAF-2 and no SNP, (b) 50 µL plant extract, 10 µm DAF-2 and 50 µm SNP, and (c) 10 µm DAF-2, 50 µm SNP and no plant extract were measured at 515 nm for 600 sec.

For each plant extract sample, an aliquot was added to this assay protocol. Briefly, 10 µL diluted plant extract (not heated or heat-treated) was added to 990 µL PBS in an Eppendorf tube. Then, 2 µL 5mmol/L DAF-2 and 50 µL 1 mmol/L SNP was added. This solution was mixed thoroughly and transferred immediately to a quartz cuvette (Hellma Worldwide Precision cells, synthetic far-UV quartz) for measurements.

The average rate of increase in fluorescence of each replicate was measured between 200 and 400 sec and presented as a percentage of this rate determined for the control (no extract). Each extract was tested five times with two replicate sample extracts from each type of plant-based food product, which resulted in a total of 10 replicates of each treatment. Data are presented as mean±standard error.

Results

It is well established that the dye DAF-2 and its derivative, DAF-2DA, can be used for the measurement of extracellular and intracellular NO, respectively. As such, it has been used to measure the production of NO from plant materials.[14] However, by the chemical production of reactive nitrogen species (RNS) such as NO by donors, the same assay can be used to measure the presence of RNS in solution, and then be used to estimate the scavenging of those RNS by added materials (e.g., plant extracts). Such a strategy has been adopted previously by others.[15]

As can be seen in Figure 1, when no NO donor, in this case SNP, is added, there is no increase in DAF-2-mediated fluorescence. Addition of SNP led to a steady increase in fluorescence over the period of the assay (600 sec). Addition of a known NO scavenger, 2-carboxyphenyl-4,4-5, 5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO),[16] indicated

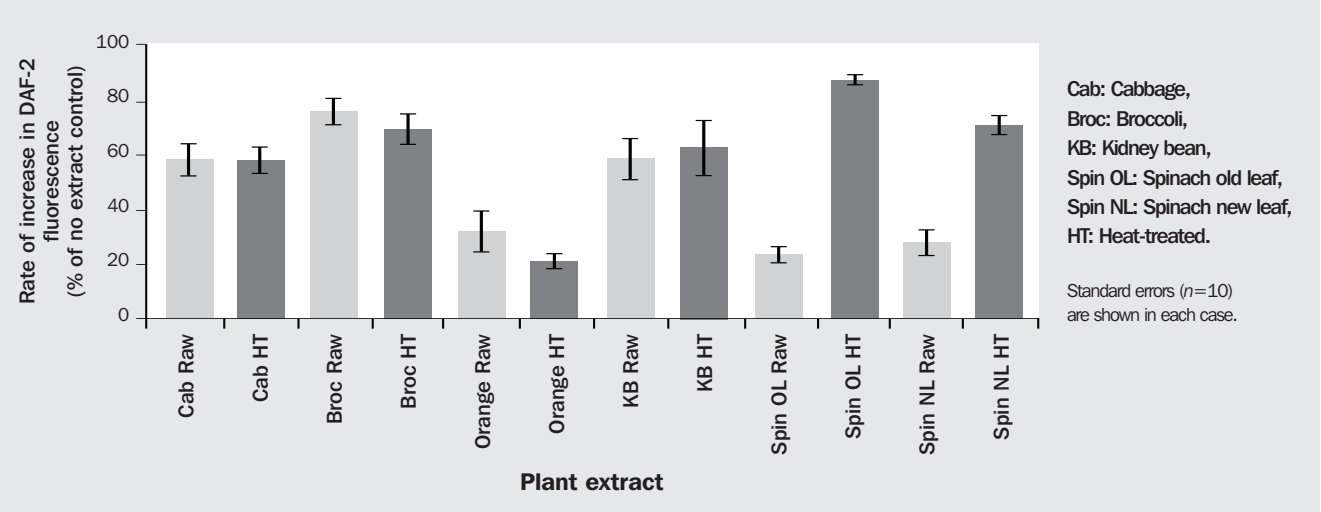

Fig. 2. The inter-species differential ability of plant extracts to scavenge NO before and after heat treatment. Standardised fluorometric assays were used to monitor the ability of extracts of different food plants to attenuate the NO-associated fluorescence of the dye DAF-2. Here, the measured rate of increase in DAF-2 fluorescence is inversely proportional to the ability of the extract to attenuate NO.

that the SNP-mediated DAF-2 signal was likely to result from the presence of NO (data not shown).

Addition of 5 μL broccoli extract had a profound effect on fluorescence production, indicating that NO-mediated fluorescence was reduced by the presence of this plant material. Interestingly, with this amount of added plant material, the attenuation of the fluorescence was temporarily limited, as the fluorescence appeared to increase towards the end of the assay. This suggests that the scavenging material in the plant extract was running out, allowing SNP/NO-mediated DAF-2 fluorescence to increase once again.

Having established this as a viable assay for measuring the effect of plant materials on NO in solution, a range of plant materials was used in the assay. As can be seen in Figure 2, all those tested had an effect. The largest effect was seen with orange and spinach, and far less with broccoli. Although no attempt was made to determine the exact components responsible in the plant extracts, heat treatment of the extracts was used. Heat-treating cabbage, orange, broccoli and kidney bean had little or no effect, suggesting that the active substance was probably not protein-based. However, heat-treating extracts of young and old spinach leaves had a considerable effect on their ability to reduce NO in solution, indicating that the active compounds here were probably not the same as those found in other food substances.

Discussion

Most studies of the effects of diet on antioxidants have concentrated on their modulation of ROS. Therefore, most assays have concentrated on the ability of dietary constituents to scavenge ROS directly or to modulate what are thought to be the downstream effects of too much ROS, such as oxidation of biomolecules, particularly lipids.[17] However, few studies have investigated whether or not dietary components can directly alter NO levels. Nitric oxide came to prominence as a cell signalling molecule in the late 1980s when it was realised that it was the compound that caused vasodilation (i.e., EDFR). Therefore, realisation that alterations of NO by the diet are possible suggests that the diet may have an effect on vasodilation and other physiological events mediated by NO.[4]

It has already been suggested that some constituents of the diet may stimulate vasorelaxation. This may be by an effect on K^+ channels,[18] but it has also been reported that it might be mediated by increasing the production of NO, or at least having an impact on an NO-mediated signalling pathway.[19,20] On the other hand, the polyphenols quercetin and resveratrol from grapes were found to reduce *iNOS* gene expression and NO production.[15]

Work has also been performed on NO scavenging. Polyphenols from grapes were reported to be scavengers of NO in an *in vitro* system using sodium nitroprusside,[15] as used in the present study. Furthermore, green tea was found to have NO-scavenging capacity.[13]

Here, it is reported that a range of dietary plants commonly used in the UK can have an effect on the presence of NO. Leaf materials such as cabbage, broccoli and spinach all scavenge NO, and, interestingly, heat-treating some of them has little or no effect. Oranges, too, can reduce NO. Thus, it would in interesting to extend this study to include similar plants that are usually eaten raw, such as lettuce, tomatoes and other salad foods.

One of the main known reactions of NO is with iron and iron-containing complexes, hence its activation of guanylyl cyclase through interaction with the haem group.[8] Some plants are known to have a relatively high iron content, and are used in the diet for this reason. Therefore, the high NO scavenging seen by plants such as spinach is not surprising. The present study investigated whether or not younger leaves may have more NO scavenging than older leaves, but there appeared to be little difference. However, the NO scavenging in this plant extract was heat-labile, suggesting the involvement of an iron-protein complex.

Here, the assay is based on the ability of DAF to measure NO, and there has been some debate about its specificity. However, compounds which could be found in plant

materials, such as ascorbate, which are known to react with DAF-2 are also seen to create fluorescent compounds with the same optical characteristics as the formation of the DAF-NO adduct.[21] The presence and reactions of compounds such as ascorbate would increase the fluorescence seen, not reduce it, and therefore the effects recorded here may be an underestimate of the true NO scavenging of the plant extracts used. Other probes, such as rhodamine-based ones,[22] or more expensive and complex assays for NO, such as electron paramagnetic resonance,[23] could be used instead to confirm such results.

Clearly, modulation of *in vivo* NO is important, and many pharmaceuticals target the signalling mediated by NO. Some drugs release NO to increase levels *in vivo*,[24] but some drugs may exert effects through NO scavenging.[25] Inhibitors of NOS, thus preventing the endogenous production of NO, have been shown to increase blood pressure[26] and cause vasoconstriction.[27] Therefore, exogenous modulation of NO metabolism clearly has profound effects. If NO-scavenging compounds, which could have an effect on *in vivo* NO levels, are available in the diet, in common foods used, then ramifications of their intake in the diet need to be appreciated. This could be particularly pertinent to cardiovascular disease.[28] It has been reported that lower NO generation may increase coronary artery disease, mediated, for example, by altering the ability of platelets to interact with vessel walls.[29]

Nitric oxide is a relatively reactive compound and may be expected to have a short life in blood. It is known to react with haem-containing compounds such as haemoglobin,[30,31] and therefore NO may not have a long-ranging effect. However, this view is challenged[32] and it is thought that circulating NO is important, and in fact persists for 100–500 sec, which is long enough for effects in tissues.[33] To this end, the NO levels in plasma have been measured.[34] It was found, using dogs, that acetylcholine increased the measureable NO in a dose-dependent manner and that administration of a NOS inhibitor, in this case L-NAME, reduced circulating NO levels. Therefore, other compounds, too, may have similar effects on the levels of plasma NO, which could have important effects on a range of tissues, or on the blood vessels themselves.

This investigation is far from complete and poses a series of questions, many of which must be addressed if the full effects of diet on oxidative and nitrosative stress are to be understood. Clearly, in these data, some of the compounds which are active in scavenging NO are heat-sensitive and some are not (Fig. 2). So, identifying these compounds will be important. Of course, NO can also lead to the formation of other nitrogen-based signalling compounds such as nitrite,[35] which can react with dietary compounds such as ascorbic acid,[36] and the impact of this on the physiological effects of NO needs to be understood.

Of immense importance is the understanding of how any of the NO-scavenging compounds are taken up into the bloodstream, and in what form. They can only be bioactive if in the correct place, and if not taken up then they may not have any effect. However, NO is known to have effects in the intestine itself,[37] and therefore diet may have a direct effect on NO-mediated intestinal events.

Of particular importance is the ability to assess whether or not compounds from the diet have any physiological effects mediated by NO scavenging. In the assay used here, an artificial donor is placed in the presence of an artificial NO target, and therefore true biological effects clearly need to be studied.

Using animal models and technologies to measure NO in plasma, as developed by Neishi *et al.*,[34] it should be possible to determine if diet has an impact on circulating NO levels, and to glean the effects this might have, either locally or systemically. This might be of particular relevance to those who are already suffering from hypertension. If NO is reduced, vasorelaxation may be compromised, leading to higher blood pressure and exacerbation of disease symptoms.

At a time when pressure is being put to bear on people to consume five portions of fruit and vegetables per day, and the perceived wisdom is to increase antioxidant levels as much as possible, is it possible to have too much of a good thing? In some cases perhaps caution should be considered. It certainly would be important to know what the true effects of diet are on *in vivo* NO and its metabolism and physiology.

It has been suggested that positive dietary effects may be mediated by antioxidants and their influence on ROS metabolism,[1] but here it is suggested that diet may have a profound effect on NO metabolism and the expected downstream effects. In the future, more widespread screening of fresh food products should be used to assess the NO-scavenging capacity of components of the human diet, with studies undertaken to show the true physiological effects of dietary components. □

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