Three model systems measure oxidation/nitration damage caused by peroxynitrite

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Diseases activate the innate immune response which causes ancillary damage to the human body. Peroxynitrite (OONO–) or its carbon dioxide derivatives cause oxidation/nitration and hence mutation to various body polymers e.g. DNA, RNA, protein, lipids and sugars. The control of the ancillary damage can come from antioxidants which inhibit control the amount of peroxynitrite available for damage. In this paper we have developed three different levels of antioxidant screening: (i) Peroxynitrite or SIN-1 reaction with luminol to produce light, and the inhibition of light by substances therefore represents antioxidation. (ii) Nicking of plasmid DNA occurs via oxidants: and is prevented by antioxidants. (iii) Detection of plasmid luciferase activity post-oxidation and infection indicates either prevention or repair of damage: via antioxidants. We found green tea and a number of its polyphenolic constituents effective only at the first level of antioxidation, while extracts of various fruit help at all levels antioxidation. In the final analysis, a combination of green tea extracts and fruits is suggested to produce more complete antioxidant protection.

[McConnell P S, Reasor M J and Van Dyke K 2003 Three model systems measure oxidation/nitration damage caused by peroxynitrite; *J. Biosci*. **28** 71–76]

1. Introduction

Oxidative and nitrosative damage to biological systems from peroxynitrite or its carbon dioxide derivative is now recognized as a key system(s) to be controlled in chronic diseases (Van Dyke 2002). Although strong oxidants protect us against disease, if made in excessive amounts continuously and in the wrong place, chronic disease results. How can we control excessive amounts of oxidation and/or nitration? The obvious solution is to use a variety of antioxidants. However, we need model systems to test the potency of these various antioxidants. We designed three different chemical systems which could be used to test the efficacy of the various antioxidants. The first system uses SIN-1 (linsidomine) to generate peroxynitrite in the presence of luminol. This produces oxidant-dependent chemiluminesence which can be inhibited by water-soluble antioxdants. We found that green tea and its polyphenols actively inhibit the light produced by SIN-1 generation of peroxynitrite (Van Dyke *et al* 2002). The second test system was to measure the effects of peroxynitrite on the knicking of the DNA from a g-Wiz-luciferase plasmid (6372 base pairs) (McConnell 2001). Green tea or its polyphenols proved not to be very effective in preventing damage of the supercoiled DNA conversion to the knicked form. The third test system was to measure the effects of green tea against the infectivity of the peroxynitrite-treated – g-Wiz-luciferase plasmid into a susceptible strain of *Escherichia coli* (Van Dyke *et al* 2002). There was no evidence of infection displayed as luciferase activity. This indicates the damage was too extensive for DNA repair. The green tea and/or its polyphenols inhibit the production of peroxynitrite but not its activity once it is formed. However, extracts of coloured fruits

Keywords. Antioxidant; peroxynitrite; polyphenols

Abbreviations used: AUC, Area under the light curve; EGCG, epigallocatechin gallate.

inhibit at all three levels and therefore are more complete antioxidants, suggesting that it may be helpful to combine coloured fruits and green tea to get the most complete antioxidant coverage.

2. Materials and methods

2.1 *Reagents for chemiluminescence*

The sydnonimine congener (SIN-1), known to produce peroxynitrite (ONOO–), formed the basis of our experiments and was obtained from Dr Karl Schönafinger, Frankfurt, Germany. SIN-1 produces peroxynitrite at a rate of about 1% min (based on the total amount of compound). For our experiments (figures 2 and 3) SIN-1 at a concentration of 15 mg/ml was dissolved in 0·1 mol/l PBS buffer (pH 7·4). It was kept on ice and maintained its activity for several hours. For details on SIN-1 see Van Dyke *et al* (1999, 2002a).

Luminol and green tea polyphenolic constituents were obtained from Sigma, St. Louis, MO, USA. Luminol was dissolved in 1 ml DMSO, and subsequently diluted to produce a stock solution (10^{-4} M) in 0·1 M PBS at pH 7·4.

Two hundred fifty mg of green tea powdered extract (Pharmanex, Simi Valley, CA, USA) was diluted in 10 ml of PBS at pH 7·4 and used as a stock solution (dilutions are made using PBS, and antioxidant samples from this original stock solution were diluted every 10-fold from through six dilutions).

2.2 *Chemiluminescence assay*

The following volumes were used: 100 µl of luminol solution [(0·6 mmol/l final concentration, 100 µl dilutions of green tea in 0·1 M PBS buffer (pH 7·4) and 200 µl buffer alone and 100 µl of the solution of SIN-1 (5·8 mM final concentration)] were pipetted into a 3 ml round-bottomed luminometer tube, resulting in a total volume of 500 µl. A control solution was also used to provide a reference to the light stimulation produced by the combination of SIN-1 and luminol. The final volumes of this solution consisted of 100 µl of luminol, 300 µl of buffer and 100 µl of SIN-1. SIN-1 was kept on ice prior to pipetting and was added last because of its instability at room temperature. When the green tea solutions are diluted for assay, all colour which might inhibit light assay via quenching is eliminated.

The luminometer tube was placed in a Berthold luminometer, model LB9505C six-channel luminometer with the temperature control set at 37°C and the light reaction was measured for a duration of 20 min. The SIN-1 assay ingredients were mixed prior to assay, with SIN-1 added last. The assay was reported as counts per minute integrated over the 20 min period. We measured the area under the light curve (AUC) because it reproduces better and is generally more meaningful than peak height. AUC measurements are accomplished by utilizing a KINB program supplied with the instrument (Berthold). This program utilizes trapezoidal approximation as the method of measurement for AUC.

2.3 *Statistics*

Chemiluminescence was integrated over the initial 20 min of each reaction with SIN-1. Reactions were repeated in separate experiments a minimum of three times. All bar graphs depict averages and the associated error bars represent standard deviations. Significance level is set at $P = 0{\cdot}05$ and is calculated from the appropriate Student's *t*-test where appropriate. Blanks were run without SIN-1 or peroxynitrite and produced a minimal light signal that was ignored in assays.

2.4 *Comments on detailed previous work*

The work described in this paper is quite brief due to extended abstract form of the paper. A major portion of the work has been published elsewhere except for the DNA knicking work and the cross comparison of epigallocatechin gallate (EGCG). In other words the how and the why of how these systems are assembled are discussed in great detail (see Van Dyke *et al* 1999, 2002a,b). We have used purified systems in the comparisons at all three levels and find that ECGC only inhibits effectively at the level where peroxynitrite is assembled, or where it is there very transiently with bonafide peroxynitrite itself. Green tea extract is a complex mixture but it too only inhibits at the first or primary level. However certain fruit extracts work at all three levels of antioxidation, i.e. peroxynitrite level, knicking of DNA level and gene action and repair level. It appears even if we do not know the exact composition of the fruit antioxidants they produce a more complete antioxidant protection than either green tea extract or EGCG. However since there is no exact way to balance the dose of antioxidants in mixtures versus single substances, one could argue that maybe the doses were not the same. Fruit extracts were highly diluted and that the green tea extract was fairly concentrated so we are inclined to believe that fruit extracts are more complete source of antioxidants.

3. Nicking experiments

3.1 *Plasmid DNA*

Plasmid DNA was obtained from Aldveron of Fargo, North Dakota (email address is DNA@aldveron.com) at

10 mg per 10 ml TBE (1 μg plasmid DNA/ml TBE) quantities. The DNA was supplied in a sterile vial in sterile buffered solution at a concentration of 1 mg/ml Tris-EDTA pH 8·0 buffer. The plasmid we used was the g-Wiz™-luciferase high expression plasmid. There are 6372 base pairs in the plasmid which contains a kanamycin resistance marker as well as the luciferase gene. The plasmid can infect Gram negative bacteria and mammalian cells with a cytomegalovirus infectious mechanism. A plasmid gene map with relevant sites for our work is shown in figure 1.

3.2 *Nicking plasmid DNA with peroxynitrite*

Plasmid DNA was incubated for 10 min at room temperature with various buffer-diluted concentrations of peroxynitrite (P) which was purchased from Upstate Biotechnology at Lake Placid, NY, USA (techserv@upstatebiotech.com). The original concentration of peroxynitrite is 138 mM. The solution contains 0·3 M NaCl and 0·3 M NaOH.

Peroxynitrite (approx. 50 mmol/l) was diluted 1/1000 with water. The original solution was supplied in 0·3 mol/l NaOH, and this stabilizes the compound. Once diluted, it must be kept on ice and used quickly, especially after dilution. The diluted basic OONO– is then rapidly injected into the 1x TBE buffer at pH 7·4. Under these neutralizing conditions the half-life of OONO– is less than 1 s. Peroxynitrite is stored at – 80°C until use.

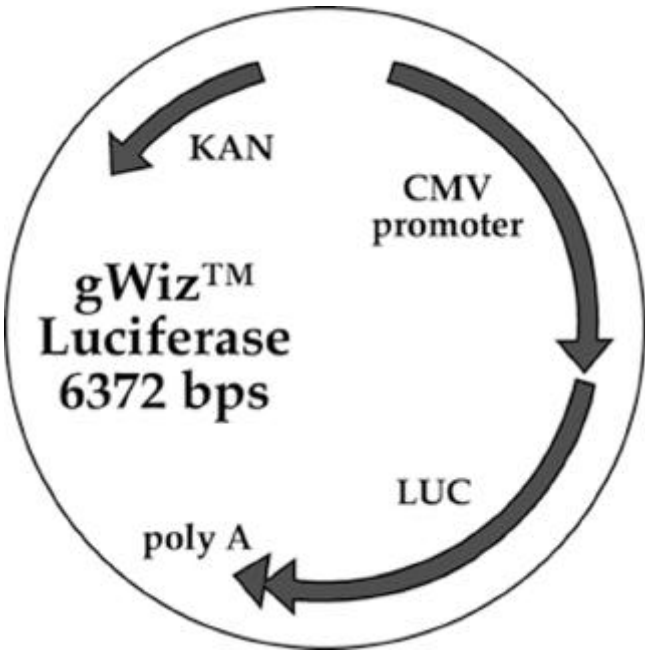

Figure 1. An incomplete gene map of the g-Wiz-luciferase plasmid.

3.3 *Electrophoresis of plasmid DNA*

DNA strand breaks in supercoiled DNA were analysed after agarose gel electrophoresis. The agarose was purchased from Sigma Chemical Co, St. Louis, MO, USA. Electrophoresis was accomplished using 0·7% agarose. The amount of 1·05 g of the agarose was measured and dissolved in 150 ml of 1x TBE buffer. The solution was microwaved in a Sanyo conventional microwave oven for ca. 2·5 min or until agarose had completely dissolved. The 1x solution of TBE buffer was diluted from a 10x solution of TBE buffer that was prepared by thoroughly mixing 54 g Tris, 27·5 g Boric acid, and 2 ml 0·5 M EDTA, pH 8·0. Deionized water was added to raise the total volume to 500 ml.

The first gels were run in order to determine the necessary amount of plasmid DNA. Volumes of 2 μl, 5 μl, 10 μl, 15 μl, 20 μl, 30 μl, 40 μl, and 50 μl of the original 1 mg/ml plasmid solution were loaded into the gels with 3 μl of loading buffer. From figure 2 it was determined that 2 μl of plasmid solution sufficiently showed a high percentage of the plasmid in the supercoiled DNA as opposed to a smaller percentage in the nicked DNA form.

A dose-response experiment with peroxynitrite was performed with the agarose gels to determine the proper dosage and length of incubation. Peroxynitrite was diluted with TBE buffer in a range of 2 : 1 to 4 : 1 (see starting concentrations of peroxynitrite above). The 50 μl of the various dilutions of peroxynitrite were added to the solutions of plasmid DNA with TBE buffer, and loading buffer electrophoresed and placed in an incubator for 0·5 h–1 h. From our previous work, it was determined that a 2 : 1 dilution of peroxynitrite caused the highest percentage of the plasmid DNA to be in the nicked form.

To determine whether or not antioxidants are able to protect the plasmid DNA from the oxidative damage caused by peroxynitrite, green tea and/or its constituents were

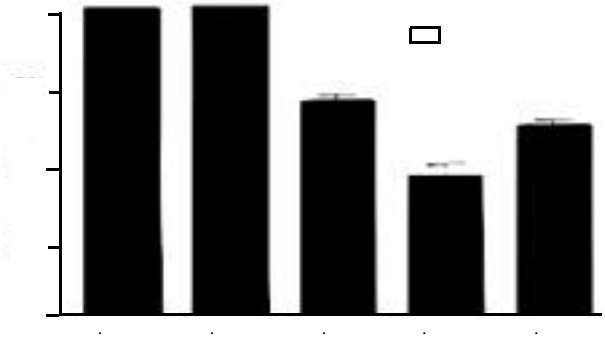

Figure 2. Effects of green tea extract against SIN-1 (peroxynitrite generator) measured by luminol luminescence.

incubated with the solution mix at doses ranging from full strength 10^{-3} M to 10^{-5} M.

The electrophoresis was carried out for 1·5 h at 50 mV. Gels were stained with ethidium bromide solution (700 ml dH_2O + 70 µl EtBr 10 mg/ml) for approximately 15 min. DNA when viewed under ultraviolet light (302 nm) cause the ethidium-stained DNA to fluoresce orange. The nicked plasmid is retarded in its movement due to oxidative damage and runs a shorter distance from the origin than the native supercoiled DNA form of the plasmid. Aldevron states that less than 5% of the plasmid is supplied in the relaxed or nicked form.

3.4 *Stastistics*

Using the FluoroChem imaging device for stained plasmid DNA, we quantitated fluorescent spots with the ultraviolet box set (302) on ethidium settings for the agarose gels using AlphaEase™ software 2D densitometry (Alpha Innotech Corporation, San Leandro, CA, USA). The densitometry was done using the software and percent protection calculated by comparing the density of ethidium-based fluorescent signal from the nicked DNA to the density of the signal from the supercoiled plasmid DNA designated as 100%. Therefore, if a given peroxynitrite concentration nicked the DNA and produced 80% nicking, this would be subtracted from the 100% to give 20% nonnicked DNA. However, in the presence of the green tea and/or its constituents, produced 90% nonnicked DNA.

4. Infection of competent *E. coli* with plasmids to test for infectivity or peroxynitrite-induced damage to plasmid DNA

E. coli strain DH1 α was made competent for infection with the g-Wiz-plasmid DNA using protocol SOP 6 from Massey Cancer Center. Briefly, *E. coli* is cultured in Lacto Bacillus broth to the log phase, which produces a turbidity of 0·2 to 0·3 absorbance at 600 nm in 4 h. The culture is placed on ice for 15 min. The solution is centrifuged in 50 ml tubes at 3000 rpm at 2°C for 10 min. The supernatant solution is discarded and the pellet is resuspended in 100 mM $CaCl_2$, using half the original culture volume. This solution is incubated on ice for 10 min and centrifuged and pellet is retained. The resulting supernatant is once again discarded. For every 20 ml of the original culture solution, 1 ml of 100 mM $CaCl_2$ and 0·43 ml of 1 : 1 glycerol : sterile water is added to the cell pellet. The *E. coli* is resuspended in this solution, split into 0·5 ml aliquots, and frozen at – 80°C.

Competent *E. coli* are thawed on ice for 10 min. DMSO (1·5 µl) is added and mixed gently. Plasmid DNA (100 to 500 ng) that had been incubated with peroxynitrite with or without fruit extracts (at a 1 : 2·5 dilution) is added. The mixture in small tubes is incubated on ice for 30 min. The tubes with the mixture of competent bacteria and plasmids is heat-shocked at 42°C for 90 s. The mixture is chilled on ice for 5 min. The entire tube of cells is added to 20 ml Luria broth without any antibiotics and incubated for 1 h at 37°C in a CO_2 incubator with shaking to allow antibiotic resistance to be expressed. Kanamycin is added at a final concentration of 5 µg/ml final solution of LB broth. The 50 ml of solution is incubated for 1 to 2 days to produce enough bacteria to test for the luciferase gene product.

4.1 *Assay for luciferase gene product*

Bacterial solution (40 ml, with or without infected plasmids) is placed in a 50 ml centrifuge tube and centrifuged at 3000 rpm at 2°C for 10 min. The supernatant solution is decanted. A solution (200 µl) of 1% Triton X-100 with DTT and 0·1 M Tris-EDTA at pH 8 is added and the lysed mixture sonicated briefly. This solution is added to 200 µl of a luciferin/ATP glow solution purchased from Promega (Madison, WI, USA). The assay solution is placed in 3 ml round-bottom Berthold cuvettes and assayed for 50 s at room temperature in a LB9505 C Berthold luminometer. The integrated light signal is assessed using KINB software. The blank signal when no luciferase is present is usually in the order of $1{\cdot}2 \times 10^3$ counts and when luciferase is present the signal is $1{\cdot}7 \times 10^7$ counts, which is more than a 10,000 fold difference. The luminescence end point produces a clear difference. Either there is no luciferase acitvity in the bacteria or there is good luciferase activity. Therefore, when luciferase activity appears in a preparation, it means that the plasmid infected the *E. coli* and was intact or repaired enough by the *E. coli* to be functional. If there is no activity, it means that the plasmid could not infect and be repaired by the *E. coli* DNA-repair system. This approach evaluates whether the antioxidants from the different preparations are effective in preventing the damage to the DNA to the extent the antioxidants and/or *E. coli* repair system could produce an infective and genetically active plasmid.

4.2 *Extraction of fruit*

The different fruits, blueberry, grapes, etc. were homogenized (60 g of fruit per 200 ml) in a solution of 50% alcohol using a Tekmar homogenizer for 5 min.

4.3 *Plasmid DNA*

The plasmid DNA was obtained as described previously in § 3.1.

5. Results

In figure 2, the effect of an extract of green tea made of 250 mg of original powder and 10 ml PBS buffer is noted. The extract is effective at inhibiting peroxynitrite-based chemiluminescence over multiple orders of magnitude, as were the fruits extracts (Van Dyke *et al* 2002a,b). In figure 3, one of the components of green tea, EGCG, is shown to be very effective similar to the green tea extract at high dose, as well as several other extracts (data not shown). In figure 4 we observe the effect of the same compound, EGCG, as well as another green tea constitiuent epicatechin, on knicking of plasmid DNA caused by peroxynitrite. At a different doses it was ineffective as an inhibitor whereas fruit extracts have been shown to be effective (Van Dyke *et al* 2002a,b). Figure 5 shows the effect of green tea extract itself as well as the polyphenolic constituent catechin on knicking of plasmid DNA caused by peroxynitrite. In table 1, green tea extract was ineffective in preventing damage to plasmid DNA so that luciferase was not expressed. However, also in table 1, we see that various dark coloured fruit extracts were effective in protecting against DNA damage from peroxynitrite of the luciferase plasmid so that it can be expressed in susceptible *E. coli*.

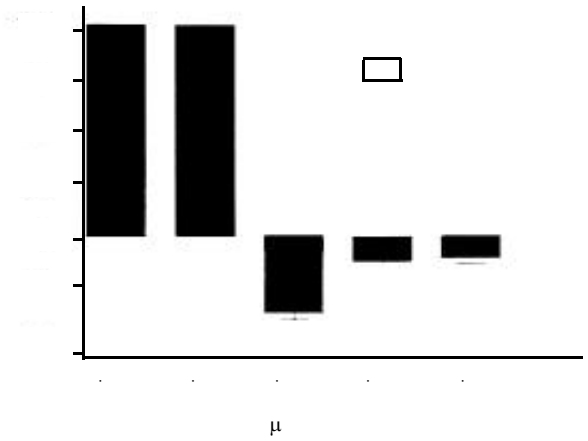

Figure 3. Effect of EGCG against SIN-1 (peroxynitrite generator) measured by luminol luminescence.

6. Discussion

We have developed a screening system that works at three different levels of antioxidation. First, the SIN-1 generates superoxide and nitric oxide which combine to produce peroxynitrite. The peroxynitrite reacts with luminol to produce light. If a drug or substance interferes with any part of the mechanism by quenching superoxide or

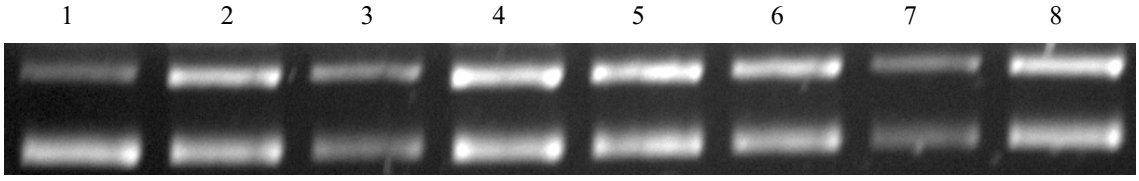

Figure 4. The minimal protection by various dilutions of green tea constituent polyphenols. Lane 1 represents plasmid DNA alone. Lane 2 represents plasmid DNA with peroxynitrite. Lanes 3–5 represent plasmid DNA incubated with peroxynitrite and (–) epicatechin beginning with full strength and diluted to 1 : 100 (see starting concentrations of various green tea constituent polyphenols above). Lanes 6–8 represent plasmid DNA incubated with peroxynitrite and (–) EGCG beginning with full strength and diluted to 1 : 100.

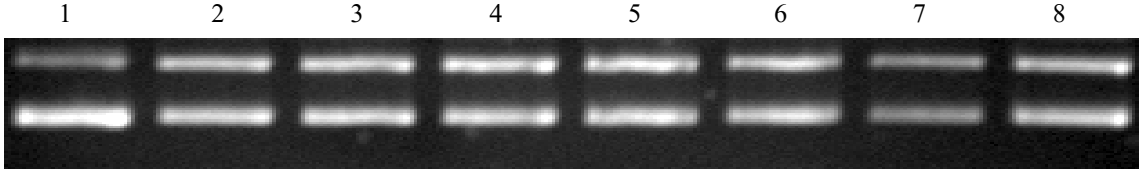

Figure 5. The minimal protection by green tea extract along with an additional polyphenolic constituent. Lane 1 represents plasmid DNA alone. Lane 2 represents plasmid DNA with peroxynitrite. Lanes 3–5 represent plasmid DNA incubated with peroxynitrite and green tea extract (see initial concentrations above) beginning with full strength and diluted to 1 : 100. Lanes 6–8 represent plasmid DNA incubated with peroxynitrite and catechin beginning with full strength and diluted to 1 : 100 (see starting concentrations of various green tea constituent polyphenols above).

Table 1. Luciferase expression from *E. coli*.

	Preparation						
	Control	Peroxynitrite	Raspberry	Blueberry	White grape	Green tea	EGCG
Luciferaseactivity	Yes	No	Yes	Yes	No	No	No

Note: Undamaged plasmid infects *E. coli* and creates luciferase activity. All samples contained plasmid, bacteria, and peroxynitrite (except control which contained buffer instead of peroxynitrite) and were tested a minimum of three times. Luciferase activity was 10,000 fold greater than peroxynitrite and plasmid.

nitric oxide or peroxynitrite, or interferes with peroxynitrite, reacting with luminol, the substance acts as an early-step-1 antioxidant.

We know that peroxynitrite can react with DNA by attacking the sugar backbone causing chain scission. This phenomenon is known as knicking. The green tea or its extracts could not prevent knicking to any great extent, but we have shown previously that certain fruit extracts are protective (Van Dyke *et al* 2002a,b). This is the second stage of antioxidant protection.

The third stage is the actual production of intact gene product which is biologically active. Since this is a plasmid that carries the luciferase gene from fireflies it can produce light in the presence of oxygen, ATP and magnesium ion. Therefore, after infection of the plasmids treated with oxidant, antioxidants or buffer (control) we infect the susceptible *E. coli* that have been heat shocked and grow these *E. coli* infected by the plasmids. Then we lyse the *E. coli* and measure luciferase activity. None of the green tea extracts or the polyphenolic constituents tested produced activity, while extracts of dark fruits were effective. Since the system with the plasmid is complex, there is a combination of events taking place. The antioxidant which is effective, prevents plasmid damage so that when infected into the *E. coli*, its gene is expressed; but the repair mechanism from *E. coli* probably plays a role, as well. If the plasmid too is not damaged by the peroxynitrite, maybe the repair by *E. coli* is enough so that luciferase is expressed. Fruit antioxidants appear to be more effective than green tea at the tested doses. One problem is that it is difficult to adjust the doses of two complex mixtures so that they are similar. However, in any case, the third step of our antioxidant screening is important because it displays real gene production which is measured by biochemical activity of the gene product (luciferase).

A recent paper from the lab of Helmut Sies may explain why green tea polyphenols are only protective at the first level of damage when they interfere with SIN-1 activated luminol luminescence (Schroeder *et al* 2001). Schroeder *et al* (2001) demonstrated that epicatechin, one of the major green tea polyphenols selectively prevents nitration but not oxidation reactions with peroxynitrite. If the nitration pathway of peroxynitrite were blocked and the oxidation pathway was allowed, maybe this is why knicking, and knicking and repair were not inhibited by EGCG which we have shown to have similar antioxidant activity.

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