

TECHNIQUES FOR QUANTIFYING EFFECTS OF DIETARY ANTIOXIDANTS ON TRANSCRIPTION FACTOR TRANSLOCATION AND NITRIC OXIDE PRODUCTION IN CULTURED CELLS

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ABSTRACT: *Dietary antioxidants can affect cellular processes relevant to chronic inflammatory diseases such as atherosclerosis. We have used non-standard techniques to quantify effects of the antioxidant soy isoflavones genistein and daidzein on translocation of Nuclear Factor-KB (NF-KB) and nitric oxide (NO) production, which are important in these diseases. Translocation was quantified using confocal immunofluorescence microscopy and ratiometric image analysis. NO was quantified by an electrochemical method after reduction of its oxidation products in cell culture supernatants. Activation of the RAW 264.7 murine monocyte/macrophage cell line increased the ratio of nuclear to cytoplasmic immunostaining for NF-kB. The increase was exacerbated by pre-treatment with genistein or daidzein. To show that decreases could also be detected, pre-treatment with the pine bark extract Pycnogenol® was examined, and found to reduce translocation. NO production was also increased by activation, but was reduced by pre-treatment with genistein or daidzein. In the EA.hy926 human endothelial cell line, constitutive production was detectable and was increased by thrombin. The confocal and electrochemical methods gave data that agreed with results obtained using the established electromobility shift and Griess assays, but were more sensitive, more convenient, gave more detailed information and avoided the use of radioisotopes.*

KEY WORDS: Confocal Microscopy, Daidzein, Endothelium, Genistein, Macrophage, Microelectrode, Nuclear Factor Kappa-B

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INTRODUCTION

There has been considerable interest in the use of dietary antioxidants to slow the development of atherosclerosis, the disease

underlying most heart attacks and strokes. Key roles in atherogenesis are played by (i) lipoprotein oxidation, (ii) endothelial cell dysfunction—including loss of constitutive nitric oxide (NO) bioactivity—and (iii) inflammation, including nuclear translocation of the pro-inflammatory transcription factor Nuclear Factor-kB (NF-kB) and induction of NO production by macrophages. All three are driven by reactive oxygen species and might be amenable to antioxidant prophylaxis (Steinberg et al., 1989; Griendling et al., 2000; Zeihar and Schachinger, 1994).

Despite their potential importance, NO production and NF-kB translocation, and consequently the effects of dietary antioxidants on them, are difficult to study. NO is labile ($t_{1/2} < 30$ s in physiological solutions [Griffith, 1985]) and is constitutively produced only at low levels. Concentrations in the vicinity of unstimulated endothelial cells in culture are typically subnanomolar (Guo et al., 1996) and the rapid degradation ensures that bulk concentrations are lower still. Such concentrations are well below the sensitivity of the established Griess reaction assay (~ 1 $\mu\text{mol/L}$), which is consequently used for assessing the much higher output that can be induced in macrophages, rather than the constitutive output by endothelial cells. The sensitivity of the assay can be improved 50-fold by the use of fluorimetric detection methods involving 2-3 diamionaphthalene, but with this technique non-linearities emerge above 3 $\mu\text{mol/L}$ (Marzinzig et al., 1997). Other techniques have problems of specificity, or require expensive, specialised equipment.

NF-kB activation can be assessed in a number of ways, including measurement of NF-kB transactivation of transiently transfected or endogenous genes. Each method has advantages and disadvantages (see review by Janssen and Sen [1999]). Of most relevance to the present study, NF-kB nuclear translocation is usually studied by the electromobility shift assay (EMSA) but this method is not ideal since it relies on nuclear extraction and involves the use of radioactive ^{32}P . Furthermore, the assay is only semi-quantitative and this makes the interpretation of small differences difficult.

Here we demonstrate that simple quantitative techniques, based on confocal microscopy of NF-kB translocation and electrochemical

detection of NO, overcome these limitations, give good agreement with the established methods and can detect influences of dietary antioxidants.

METHODS

1. Quantification of NF- κ B nuclear translocation by confocal immunofluorescence microscopy

Cell culture

Cells of the RAW 264.7 murine macrophage line were obtained from the European Collection of Cell Culture (ECACC, Salisbury, UK). They were seeded onto coverslips (5×10^4 cells/cm²) and cultured in DMEM for 48 h. They were then incubated with 50 μ M genistein, 50 μ M daidzein, Pycnogenol® (a standardised maritime pine extract) at the same mass concentration (13.5 μ g/ml), or vehicle (DMSO) alone, for a further 24 h. After washing with phosphate buffered saline (PBS, 0.15M, pH 7.4), they were activated with lipopolysaccharide (LPS; 500 ng/ml) and interferon gamma (INF- γ ; 100 U/ml) for 30 min, before being fixed with 4% paraformaldehyde for 15 min.

Staining

Cells were permeabilised with methanol at -20°C for 10 min, blocked with 1% BSA, and incubated with 5 μ g/ml goat anti-mouse p65 polyclonal antibody (sc-372g; Santa Cruz) for 1 h. After washing, they were incubated with 5 mg/ml FITC-conjugated donkey anti-goat IgG (sc-2024, Santa Cruz) for a further 1 h, and mounted in Vectashield (Vector Laboratories, UK).

Microscopy

Coverslips were scanned with a laser scanning confocal microscope (TCS AOBs, Leica; 495 nm excitation, 519 nm emission). 3 fields of view per treatment were imaged in each experiment.

Image analysis

Using a grid, 12 cells in each field of view were selected for quantification of immunostaining. For each cell, regions of interest (ROIs) were drawn around the nucleus and the cytoplasm with the confocal software. A region of coverslip, devoid of staining but close to the cell, was also imaged, to correct for background fluorescence and other offsets. A mean pixel intensity for each ROI was obtained from the software. The ratio described below was calculated for each cell to indicate the relative levels of antibody staining in the nucleus and cytoplasm.

$$\text{Ratio} = (\text{Nuclear intensity} - \text{background intensity}) \div (\text{Cytoplasmic intensity} - \text{background intensity})$$

2. NF- κ B-DNA binding assessed by EMSA

Cell culture

RAW 264.7 seeded into 6 well plates were cultured in DMEM before being activated for 1 h, with or without isoflavone pretreatment, as described above. Nuclear extraction and the EMSA were performed as described by Lin et al. (2001) using the NF- κ B consensus oligonucleotide 5'-AGTTG AGGGG ACTTT CCCAG GC-3'. In preliminary experiments, supershift polyclonal antibodies for p65 and p50 were incubated with activated cell nuclear extracts. NF- κ B-

DNA bands were quantified by densitometry using Quantity One software.

3. Electrochemical detection of NO

Cell culture

RAW 264.7 were cultured and treated in 6 well plates as above. Supernatants were frozen 24 h after activation. Cells of the EA.hy926 human endothelial line were seeded into 6 well plates at 2.5×10^5 well⁻¹ and allowed to adhere for 24 h. Serum was withdrawn 24 h before stimulation with thrombin (100 U/well) dissolved in fresh medium.

Nitrate reduction

Following thawing, deproteinisation (2:1:1 supernatant: 0.3M NaOH: 0.3M ZnO₄) and centrifugation (7,000g for 5 min), 250 μ l of each supernatant were incubated with 25 ml of NADPH/FAD (1.5:0.03 mg/ml), and 7.5 μ l (10 u/ml) of nitrate reductase (from *Aspergillus niger*; Sigma, UK) for 30 min, to reduce nitrate to nitrite.

Electrochemistry

An ISO-NOP electrode (WPI) was equilibrated in 10 ml of acidified KI (6.25 μ M) for 10 min. Each sample was added to this solution as a single 250 μ l bolus. The acidified KI reduced the nitrite to NO. The resulting change in redox current from the electrode was continuously recorded using DUO 18 data software (WPI). NO concentrations were calculated from the maximum current, using a calibration curve obtained with KNO₂.

4. Colorimetric detection of NO

Equal volumes of Griess reagent (Sigma, UK) and either RAW 264.7 supernatants (described above) or NaNO₂ calibration standards were mixed in flat-bottomed, optically-clear 96 well plates and incubated in the dark for 10 min. Absorbance at 540 nm was determined using culture medium as a blank.

RESULTS

1. NF- κ B translocation

Confocal immunofluorescence microscopy in conjunction with ratiometric image analysis was easily able to detect translocation of NF- κ B when RAW 264.7 cells were activated: nuclear immunostaining was 40% of cytoplasmic staining in nonactivated cells but 3 times cytoplasmic staining in activated cells (Fig 1 A-C). Contrary to expectation, pretreatment with the antioxidant soy isoflavones genistein or daidzein increased nuclear:cytoplasmic ratios on activation by 40% (Fig 1C). Microscopy after staining cells with primary antibody alone or secondary antibody alone, as controls for non-specific binding, gave negligible intensities (data not shown). To ensure that reductions in activation could be detected with the confocal technique, Pycnogenol®, a pine bark extract with antioxidant properties (Virgili et al., 1998), was used as a positive control; as expected, a reduction in nuclear translocation on activation was observed (Fig 1C).

Comparable results were obtained by EMSA (Fig 2A, B). Nuclear extracts gave two distinct bands. Preliminary experiments showed that addition of the specific competitor (50-fold excess of NF-kB oligonucleotide) completely removed both bands. The preliminary experiments also showed that addition of the anti-p65 antibody resulted in a single supershift of the top band

while addition of the anti-p50 antibody resulted in a double supershift, suggesting that the top band is a p65/p50 heterodimer and the bottom band is a p50/p50 homodimer. Densitometry showed a more than 3-fold increase in DNA binding on activation, and this increase was further enhanced approximately 20-25% by pre-treatment with 50 μ M genistein or daidzein.

Figure 1. Non-activated (A) and activated (B) macrophages treated with anti-p65 primary antibody and an FITC-conjugated secondary antibody. ROIs, described above, are indicated on the image. Translocation of NF-kB from cytoplasm to nucleus on activation is evident. (Width of each image = 50 μ m before magnification.) C, effect of pretreatment with antioxidants on translocation. Ratios of nuclear:cytoplasmic immunofluorescence were assessed by confocal microscopy; values <1 indicate brighter cytoplasmic staining for NF-kB, ratios >1 indicate brighter nuclear staining. Means + SEMs of two independent experiments, each in triplicate. *P<0.05 compared to activated

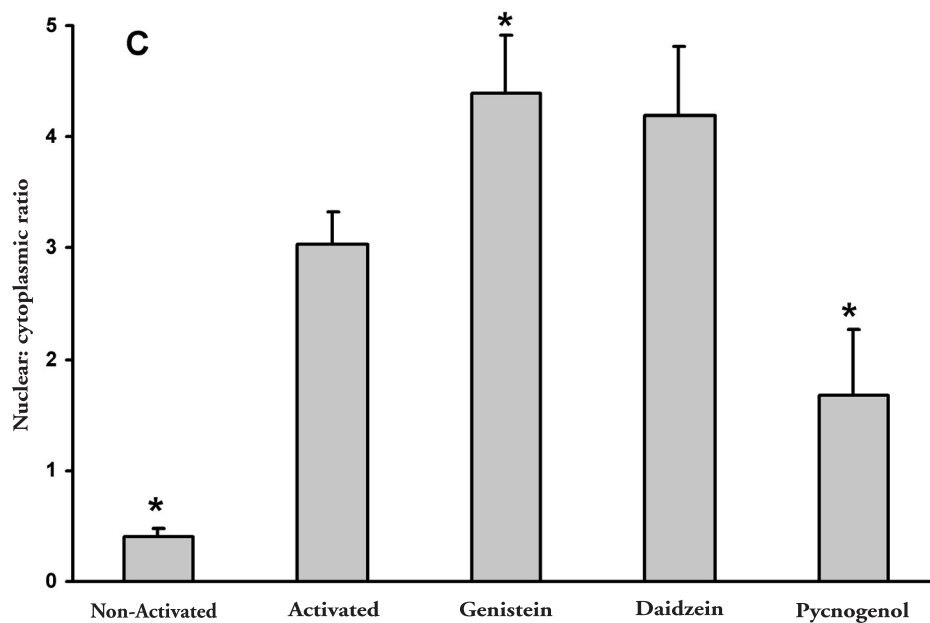
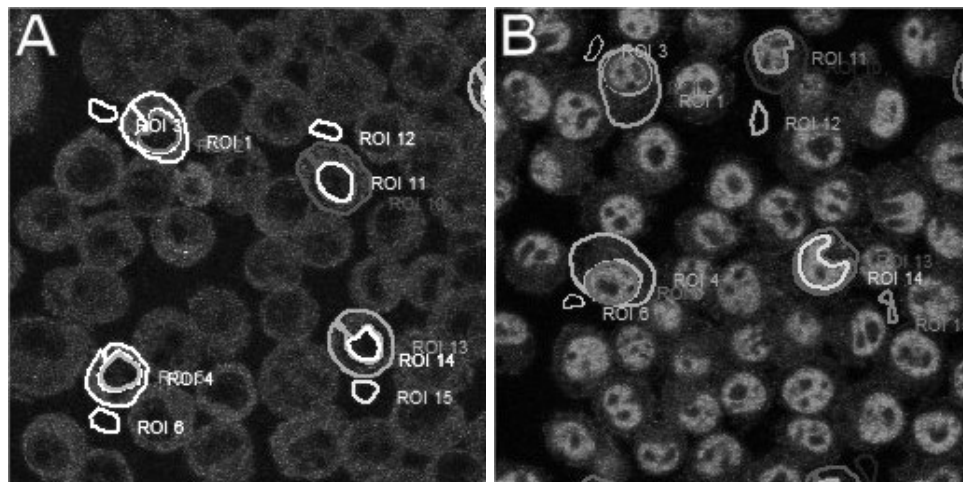


Figure 2. A, an EMSA gel illustrating p50/p50 and p50/p65 DNA binding in RAW 264.7 nuclear extracts. NA, non-activated; A, activated; G50, genistein-treated, and D50, daidzein-treated, before activation. B, effect of genistein and daidzein on DNA binding, assessed by densitometry of EMSA gels. Means + SEMs of three independent experiments, each in triplicate. *P<0.05 compared to activated

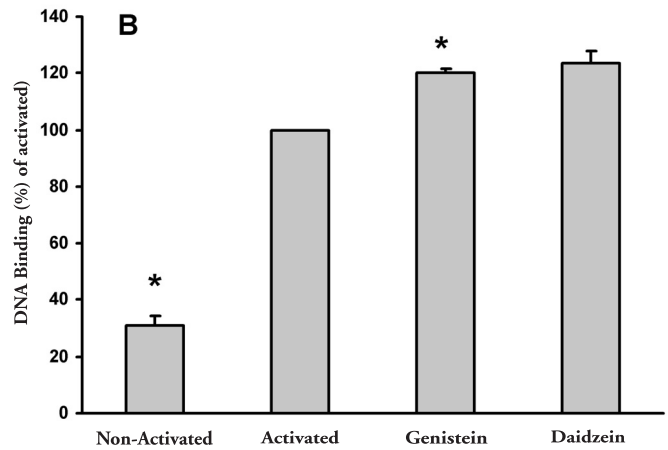
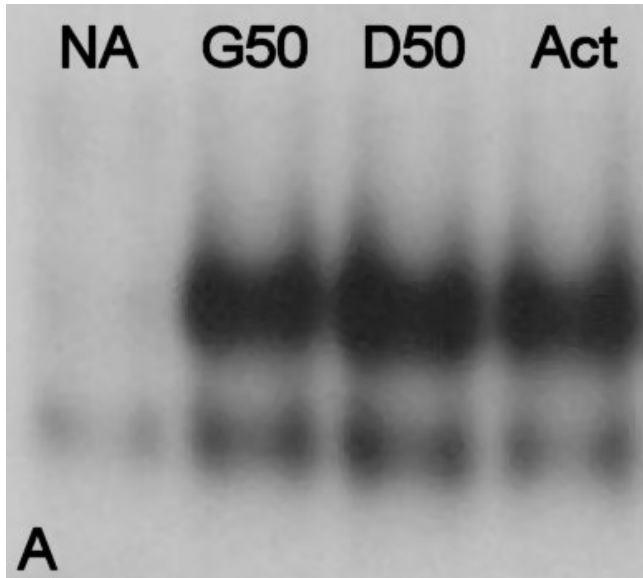


Figure 3. Electrochemical detection (A) and detection by Griess reaction (B) of effects of genistein and daidzein on NO production in RAW 264.7 macrophages activated with INF- γ and LPS. Means + SEMs of two independent experiments, each in triplicate. * P<0.05 compared to activated

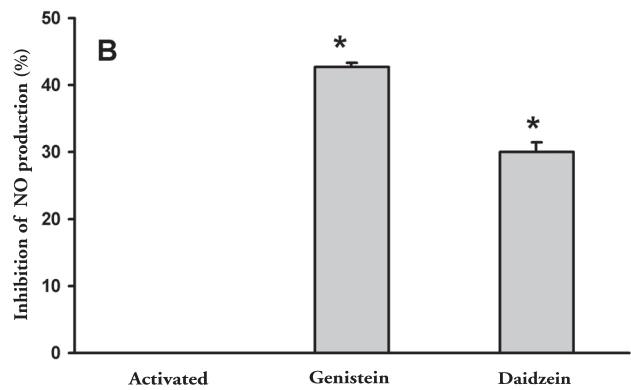
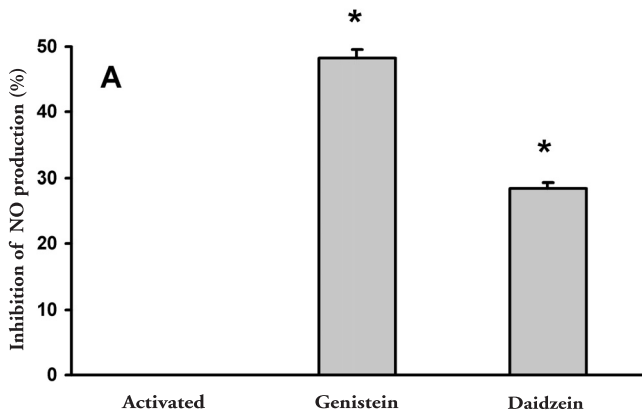
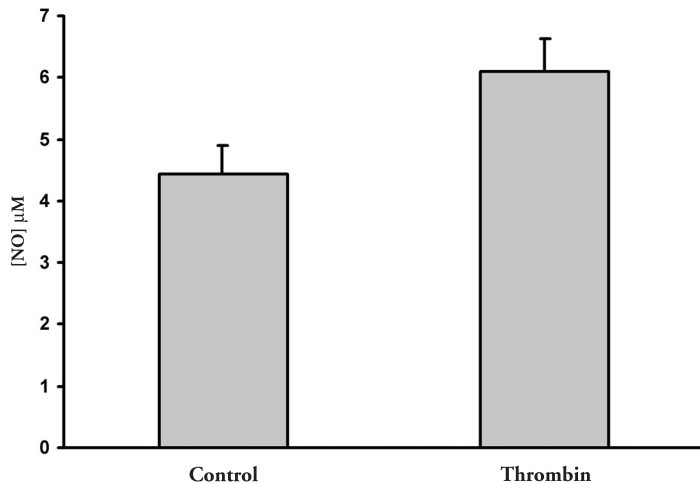


Figure 4. Electrochemical detection of constitutive NO production by endothelial cells and the increase caused by thrombin. Means + SEMs of two independent experiments, each in duplicate.



2. NO concentration

The NO-specific microelectrode was easily able to measure concentrations of NO in reduced samples of the medium used to culture RAW 264.7 cells, and could detect a large increase after the cells were activated: mean values were 6 (SEM = 0.4) μM in wells containing nonactivated cells and 115 (SEM = 1.3) μM in wells containing activated cells. The Griess reaction gave 78 ± 6 μM for activated cells and could not detect the NO produced by nonactivated cells. The lower value for activated cells probably reflects the use of nitrate reductase in the electrochemical but not the Griess assays; only some oxidation products of NO were assessed in the latter. Pretreatment with genistein or daidzein reduced electrochemically-detected NO production 48% and 28%, respectively, after activation (Fig 3A). The Griess reaction gave nearly identical values (42% and 31% inhibition, respectively; Fig 3B).

The electrochemical technique, unlike the Griess reaction, was sufficiently sensitive to detect constitutive NO production by the endothelial cell line EA.hy926. A 40% increase in NO production by these cells was detected after treatment with thrombin, a classical agonist for the production of NO by the endothelial form of nitric oxide synthase (eNOS) (Fig. 4).

DISCUSSION

Understanding the mechanisms by which dietary antioxidants might retard the development of atherosclerosis requires a molecular approach. In this study we focused on NO and NF- κB as target molecules because there is substantial evidence that they play key roles in atherogenesis (see Naseem, 2005; de Winther et al., 2005). We examined whether these molecules are influenced by the soy isoflavones genistein and daidzein. The relatively low prevalence of cardiovascular disease in Japan has been attributed to the consumption of soy. The FDA has approved

a health claim for soy based on recognition that soy protein lowers serum lipid levels (Anderson et al., 1995) but the isoflavones may also have beneficial effects (Clarkson, 2002). We applied confocal and electrochemical techniques to assess their influence on NO production and NF- κB translocation.

Confocal immunofluorescence microscopy allows specific proteins to be localised in 3 dimensions with submicron resolution. This makes it particularly useful for studying the nuclear translocation of transcription factors, where it is the subcellular localisation itself that is of functional importance. (Conventional immunofluorescence microscopy does not allow protein in the nucleus to be distinguished from that in overlying cytoplasm.) We combined confocal immunofluorescence with image analysis in order to quantify the relative concentrations of antibody – in this case, raised against NF- κB – in nucleus and cytoplasm. We used a ratiometric analysis coupled with the subtraction of local background readings; this obviated the need to allow for spatial and temporal variation in the efficiency of fluorophore excitation and detection. Direct visualisation enhances confidence in results compared with indirect methods involving nuclear extraction and allows damaged cells to be avoided. The technique is convenient and does not involve the use of radiolabels.

The confocal method, like the EMSA, does not give a direct measure of transactivation. Ideally, therefore, it should be combined with an assay for transcription. However, such assays suffer from a number of problems (Janssen and Sen, 1999). Reporter gene assays are accurate and sensitive, but the transfection conditions need to be optimised for every cell type and considerable chemical or physical manipulation may be required. The most significant drawback is that transient transfection results in an unphysiologically high level of target sequence in the nucleus, which can lead to an overestimate of the regulatory effect of NF- κB . This can be avoided by measuring the production of native proteins whose genes require NF- κB for transcription. However, other transcription factors are likely also to be involved in the expression of such genes and this assay is consequently not specific to NF- κB .

Several electrochemical methods have previously been developed for the direct measurement of NO production by cells. Although the use of electrochemical detection confers high specificity and sensitivity (detection limit c. 1 nM), these methods are technically demanding and have not been routinely used. The rapid oxygenation of NO (to nitrite and nitrate) in physiological buffers and cell culture media means that the electrode has to be placed almost in contact with the cells. This is difficult to arrange, potentially damaging to the cells and electrode, and reduces the area sampled to the point where aberrant cells can influence the result. Furthermore, the detection efficiency critically depends on the separation between electrode and cell, and can also be affected by factors such as thermally-induced convection currents. Unless real-time measurements are required, a better indirect method, pioneered by Berkels et al. (2001) and used here, is to reduce cell culture supernatants so that NO is regenerated from its oxidation products during the assay. Because the electrode is more sensitive than the Griess reaction, and because cumulative

production is being assessed, constitutive NO production from unstimulated endothelium can easily be detected. The method is more convenient than techniques based on chemiluminescence and has higher throughput than those based on bioassays.

The electrochemical and confocal techniques gave remarkably close agreement with the EMSAs and Griess reaction, respectively. As expected, the confocal technique and EMSA both showed a substantial nuclear translocation of NF- κ B in RAW 264.7 cells on activation, whilst the electrochemical technique and Griess reaction both showed an increase in NO production. The electrochemical technique was also able to detect constitutive NO production from the human endothelial cell line Ea.hy926 and its increase by thrombin, which were below the detection threshold of the Griess reaction in our hands.

There was also good agreement when examining the effects of genistein and daidzein on the responses of RAW 264.7 cells to activation, but the results were unexpected: the dietary antioxidants did not reduce – and even increased – nuclear translocation of NF- κ B, a key pro-inflammatory transcription factor. However, despite the presence of an NF- κ B binding site in the promoter of the gene encoding iNOS, they did reduce NO production.

The detection of an increase in NF- κ B translocation after pre-treatment with the isoflavones is unlikely to have resulted from a problem with the confocal technique. First, comparable results were obtained with the EMSA. Second, a decrease was observed when using Pycnogenol® as a positive control. Previous studies of the effects of Pycnogenol® on NF- κ B translocation in RAW 264.7 cells have given apparently contradictory effects – Park et al. (2000) found it increased translocation, Cho et al. (2000) found a decrease, and Virgili et al. (1998) found no effect. However, these discrepancies can be explained by the use of different agents to activate the cells. Park et al. used INF- γ , Cho et al. used LPS, and Virgili et al. used a mixture of the two. We also used a mixture of the two, but although we used the same INF- γ concentration (100 U/ml) as Virgili et al., we (like Cho et al.) used a 50-fold higher LPS concentration (500 v. 10 ng/ml). We therefore expected Pycnogenol® to decrease activation, and this was observed.

Several previous studies (e.g. Choi et al., 2003) have shown that genistein reduces LPS-induced NF- κ B translocation; however, in these studies genistein and LPS were co-incubated. A direct interaction between the two compounds may therefore have been responsible. It is widely assumed that the translocation of NF- κ B is redox sensitive, but recent evidence throws doubt on this view (Nier et al., 2005). Furthermore, although genistein and daidzein are antioxidants, they also have other properties, including oestrogenicity (e.g. Biggers and Curnow, 1954), that can exert influences on cells. The increase in NF- κ B translocation may have been caused by such properties. We have previously shown that the reduction by genistein of induced NO production does not depend on iNOS mRNA expression (Gottstein et al., 2003), which may account for the apparently contradictory effects of genistein on NF- κ B translocation and NO production. The techniques described above will allow further investigation of

these phenomena and could be applied in many other studies of the effects of dietary antioxidants and their metabolites (Rimbach et al., 2003; 2004) on key processes in inflammatory diseases.

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CONFLICTS OF INTEREST: None declared

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