RESEARCH ARTICLE

Effects of plant-derived polyphenols on TNF-α and nitric oxide production induced by advanced glycation endproducts

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Advanced glycation endproducts (AGEs) accumulate on protein deposits including the β-amyloid plaques in Alzheimer's disease. AGEs interact with the "receptor for advanced glycation endproducts", and transmit their signals using intracellular reactive oxygen species as second messengers. Ultimately, AGEs induce the expression of a variety of pro-inflammatory markers including the tumor necrosis factor (TNF-α) and inducible nitric oxide (NO) synthase. Antioxidants that act intracellularly, including polyphenols, have been shown to scavenge these "signaling" reactive oxygen species, and thus perform in an anti-inflammatory capacity. This study tested the pure compounds apigenin and diosmetin as well as extracts from silymarin, uva ursi (bearberry) and green olive leaf for their ability to attenuate AGE-induced NO and TNF-α production. All five tested samples inhibited BSA-AGE-induced NO production in a dose-dependent manner. Apigenin and diosmetin were most potent, and exhibited EC_{50} values ~10 μM. In contrast, TNF-α expression was only reduced by apigenin, diosmetin and silymarin; not by the bearberry and green olive leaf extracts. In addition, the silymarin and bearberry extracts caused significant cell death at concentrations ≥10 μg/mL and ≥50 μg/mL, respectively. In conclusion, we suggest that plant-derived polyphenols might offer therapeutic opportunities to delay the progression of AGE-mediated and receptor for advanced glycation endproducts-mediated neuro-inflammatory diseases including Alzheimer's disease.

Keywords:
Antioxidants / Cytokines / Glycation / Nitric oxide / Polyphenols

1 Introduction

The glycation hypothesis of aging suggests that modification of proteins by glucose (the "Maillard reaction") leads to the development of "advanced glycation end-products" (AGEs) [1]. In the human brain, AGEs accumulate in neuronal perikarya of the hippocampus and parahippocampus, as well as in reactive astroglia in brains after the third decade of age [2]. In Alzheimer's disease (AD), this effect is twofold: AGEs accumulate extracellularly on β-amyloid plaques and intracellulary in neurons and astrocytes [3]. The binding of AGEs to its receptor, RAGE (receptor for advanced glycation endproducts), activates NADPH-oxidase, a central participant in the production of superoxide radicals [4]. Superoxide and its conversion product, hydrogen peroxide, were shown to activate redox-sensitive transcription factors such as nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1) [5], resulting in the upregulation of cytokines such as interleukin (IL)-1, IL-6,

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Abbreviations: AGEs, advanced glycation endproducts; **AD,** Alzheimer's disease; **iNOS,** inducible nitric oxide synthase; **MTT,** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **NF-κB,** nuclear factor kappa B; **NO,** nitric oxide; **RAGE,** receptor for advanced glycation endproducts; **TNF,** tumor necrosis factor

tumor necrosis factor (TNF-α) and inducible nitric oxide synthase (iNOS) [5, 6]. Interestingly, RAGE is also activated by Aβ, the major pro-inflammatory peptide present in amyloid plaques in AD and HMGB1/amphoterin, a novel pro-inflammatory ligand released from dying cells [7]. This suggests that despite the controversy regarding the relative importance of Aβ, AGEs or HMGB1 as a pro-inflammatory ligand in AD *in vivo*, the Aβ/AGE/HMGB1-RAGE-NF-κB-cytokine/iNOS system presents an interesting drug target for AD [8]. TNF-α and NO were chosen as relevant biomolecules due to their role in augmenting inflammation and/or inducing cell death. TNF-α (cachexin or cachectin) s a cytokine involved in systemic inflammation that upregulates other NF-κB-regulated cytokines and is also a member of a group of cytokines that stimulate the acute phase reaction [9]. TNF-α is also able to induce apoptotic cell death [10]. NO is generated by phagocytes (microglia, monocytes, macrophages and neutrophils) as part of the human immune response. Phagocytes are armed with iNOS, which is activated by interferon-γ (IFN-γ) as a single signal or by TNF-α as part of a dual signal response [11]. NO has been implicated in the neuronal injury caused by activated microglia, presumably by causing dysfunction of enzyme complexes in mitochondria. NO can react with thiol groups, iron-sulfur clusters and heme proteins following diffusion across the cell and mitochondrial membranes. Through these mechanisms NO is able to inactivate enzymes including complex I, complex II–III and complex IV of the mitochondrial electron transport chain [12].

A number of drug types that disrupt this pathway at the receptor level have been identified. These include RAGE-antagonists, neutralizing antibodies against RAGE, the decoy receptor soluble RAGE, or AGE-modifying drugs that might change the structure of agonistic AGEs and decrease or abolish RAGE binding affinity [13]. Downstream from RAGE membrane permeable antioxidants such as α-lipoic acid and 17β-estradiol have previously been shown to scavenge AGE-induced oxygen free radicals, which act as second messengers in redox-sensitive signal transduction pathways [6].

We have recently shown that apigenin and diosmetin are effective in the attenuation of LPS-induced NO and TNF-α release at concentrations in the low μM range, and subsequently proposed their use in the treatment of septic shock [14]. In this present study, we have selected apigenin (found in chamomile, Labiatae plants and grapefruit), diosmetin (abundant in citrus fruits mainly in sweet orange and lemon), silymarin extract (prepared from milk thistle seeds), uva ursi (bearberry) extract (prepared from the leaves of the common bearberry *(Arctostaphylos uva ursi*) and green olive leaf extract. The aim of this study was to test the effect of these polyphenols or polyphenol-rich preparations on AGE-induced NO production and TNF-α release in microglia in order to evaluate their potential as inhibitors of the AGE-RAGE-NF-κB-cytokine/iNOS signaling pathway.

2 Materials and methods

2.1 Materials

Cell culture reagents were purchased from Invitrogen, Mulgrave (Vic), Australia. TNF-α ''Quantakine'' ELISA kit was purchased from PeproTech (Rocky Hill, NJ, USA). Dialysis membranes and all other chemicals were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). Plant polyphenols (extracts and pure compounds) were supplied by Nutrafur, Alcantarilla, Spain. The RAGE antibody (Cat ♯sc8229) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The endotoxin determination kit was from HyCult Biotechnology (Uden, The Netherlands).

2.2 Production of BSA-AGE

BSA-AGE was generated by incubation of 10 mg/mL BSA with 100 mM glucose in 200 mM phosphate buffer at pH 7.4, at 60°C for 6 wk as described previously [6]. In previous pilot experiments, AGE formation (''browning'') was monitored by an increase in OD at 400 nm and AGE-related fluorescence at 370/440 nm (excitation/emission) as described previously [15, 16]. Since no increase in both parameters were found after 6 wk, BSA-AGE was assumed to be maximally modified. Control BSA was produced under the same conditions except that glucose was omitted. Unbound glucose and glucose degradation products were removed by dialysis at 4°C in 0.9% NaCl. BSA-AGE preparations (10 mg/mL) displayed an OD_{400} of 1.2 ± 0.2, control BSA (10 mg/mL) of 0.2 ± 0.05. BSA-AGE and control solutions were aliquoted and stored at −20°C.

2.3 Endotoxin assay

BSA-AGEs were tested for endotoxin content using the LAL Chromogenic Endpoint assay, according to the manufacturer's specifications (♯ HIT302, Hycult Biotechnology). Endotoxin levels for BSA-AGEs were determined to be lower than 1 U/mg protein.

2.4 Preparation of stock solutions for flavonoids and plant extracts

Pure flavonoids (>95%) and complex plant extracts were dissolved in DMSO at concentrations of 100 mM and 100 mg/mL, respectively. Dilutions were then prepared in DMEM. All stock solutions were stored at −20°C; dilutions in DMEM were stored at 4°C for not longer than 1 wk before use.

2.5 Cell culture

N-11 murine microglia were seeded into 96-well tissue culture plates at a density of 5×10^4 cells *per* well. Cultures

were grown for 24 h in DMEM containing 5% fetal calf serum, supplemented with penicillin (200 U/mL), streptomycin (200 μg/mL) and fungizone (2.6 μg/mL). Prior to activation, the cell culture medium was then replaced with medium containing 0.1% fetal calf serum for another 24 h. N-11 cells were then pre-incubated with various concentrations of polyphenols or the RAGE antibody (Cat♯ sc8229, Santa Cruz) for 2 h before addition of BSA-AGE (750 μg/mL). Supernatants were collected after 24 h for the determination of NO and TNF-α. All supernatants were stored at −80°C.

2.6 Nitrite and TNF-α determination in cell culture supernatant

Nitrite is the major stable degradation product of NO that accumulates in the supernatant of NO-releasing cells [17]. Nitrite concentration was measured by transferring 50 μL of supernatant to a fresh 96-well plate and adding 25 μL of a solution of 1% sulfanilamide in water and 25 μL of 0.1% napthyethylene-diamine in 5% HCl (Griess Reagent). The violet azo dye reaction was allowed to develop for 15 min at room temperature. The absorbance was measured at 540 nm and nitrite concentration was calculated by comparison to a standard curve of sodium nitrite from 0 to 100 μM in DMEM (coefficient of variation: 9.26%). If indicated, the absorbance background was further adjusted by subtracting the absorbance of AGEs and polyphenols at 540 nm. The concentration of TNF-α was determined by a commercial sandwich ELISA (Cytolab/Peprotech Asia ♯900-K54) according to the manufacturer's instructions, which include a standard curve for TNF-α ranging from 15 pg/mL to 4 ng/mL (coefficient of variation: 5.64%).

2.7 Determination of cell viability by the MTT and Alamar Blue (rezazurin) assays

For the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, 100 μL of MTT solution (0.2 mg/mL MTT in 0.9% NaCl) was added and incubated for 1 h at 37°C (5% CO_2). MTT solution was then removed and 100 μL of 95% ethanol was added. The plate was then shaken for a minimum of 30 min to dissolve the crystals, before absorbance was measured at 595 nm. For the Alamar Blue assay, the remaining media was removed by aspiration and 200 μL of DMEM containing 1.25 mg/L Alamar Blue (resazurin) was added and incubated for 2 h at 37°C. The formation of the conversion product resorufin was determined by fluorescence spectroscopy (excitation at 545 nm, emission at 595 nm). The percentage of viable cells was calculated as follows. The absorbance (MTT) or fluorescence (Alamar Blue, AB) of the solutions without cells was set to 0% (background, negative control) and the solutions including cells as 100% (positive control), and the following formula used: % viable cells = ((MTT or AB value obtained in experiment background)/(MTT or AB value of the positive control background)) × 100).

2.8 AGE-RAGE binding assay

Binding of sRAGE to AGEs was determined by a direct ELISA. All steps were performed at room temperature if not indicated otherwise. In detail, a Nunc MaxisorpTM 96-well plate was coated with 100 μL of a 15 mg/mL solution of BSA-AGE. The plate was covered and incubated overnight at 4°C, before being washed three times with 300 μL ice-cold PBS+0.05% Tween-20. Blocking of non-specific binding sites was achieved by adding 300 μL PBS+1% w/v BSA (pH 7.4) (reagent buffer) for 2 h at room temperature. The plate was then aspirated, and 100 μL of serially diluted human sRAGE (initial concentration of 200 ng/mL) was added. The plate was covered and incubated again overnight at 4°C before being washed three times with ice-cold reagent buffer. To each well, 100 μL of reconstituted biotinylated anti-human RAGE antibody (R&D Systems) was added. The plate was incubated for 8 h at 4°C and then washed three times with reagent buffer. One hundred microliter streptavidin-horseradish peroxidase (R&D Systems, Catalog ♯ DY998) was added and incubated for 1 h at 4°C. The plate was washed three times with ice-cold reagent buffer before adding 100 μL of TMB+horseradish peroxidase substrates to each well. The plate was left to develop at room temperature for 20 min before adding 50 μL of 1 M H_2SO_4 stop buffer. Absorbance was measured at 450 nm.

2.9 Data presentation and analysis

All results are expressed as the mean ± SD of two independent experiments, performed in duplicate (TNF-α, AGE binding to RAGE) or triplicate (NO). In some cases, error bars were so small that they are contained within the symbol. Data sets were analyzed, and statistically significant differences from control or reference values were determined using a one-way ANOVA, followed by Tukey's test for multiple comparisons. p values <0.05 were considered to be statistically different, and this is indicated by an asterisk in the relevant figures.

3 Results

We have previously demonstrated that N-11 microglia secrete a variety of cytokines including TNF-α, IL-1, IL-6 and monocyte chemoattractant protein-1 upon stimulation with AGEs [18, 19]. Since AGEs display considerable batch-to-batch differences in their pro-inflammatory activity, the first set of experiments were performed to confirm that (i) our BSA-AGE batch would bind to RAGE and (ii) BSA-AGE signaling occurs *via* RAGE and yields dose-dependent NO and TNF-α production.

3.1 Binding of BSA-AGE to RAGE

Binding of BSA-AGE to RAGE was confirmed *via* direct ELISA, in which BSA-AGE was immobilized on a microtiter

plate and incubated with different concentrations of sRAGE. sRAGE levels were then quantified. A dose-dependent binding of sRAGE to BSA-AGE could be detected up to sRAGE concentrations of 25 ng/mL. At higher concentrations, no additional binding was observed indicating saturation of the RAGE binding sites. Unspecific binding between BSA and sRAGE was minimal (Fig. 1).

3.2 NO production induced by BSA-AGE

BSA-AGE was applied in concentrations between 25 μg/mL and 750 μg/mL. AGE-induced iNOS protein expression is not induced before 6 h after AGE exposure [6], therefore NO production was allowed to proceed for 24 h, after which cell culture supernatant was collected NO production was induced by BSA-AGE in a concentration-dependent manner, reaching an average maximum nitrite concentration of 23.4 μM at a BSA-AGE concentration of 750 μg/mL (Fig. 2). The working concentration of BSA-AGE chosen for all subsequent activation assays was 750 μg/mL (~12 μM), as this generated an easily detectable amount of nitrite (> 20 μM nitrite after 24 h) without causing more than 20% cell death within 24 h (data not shown).

3.3 TNF-α production induced by BSA-AGE

To determine the best time points for measuring BSA-AGE induced TNF-α release, N-11 cells were incubated with BSA-AGEs (750 μg/mL) for 0–24 h. After defining time points, supernatant samples were collected and the TNF-α content in the cell culture supernatant was determined using ELISA. The TNF-α concentration in the medium increased steadily up to 24 h, reaching approximately 2.5 ng/mL; therefore, this time point was chosen for all further experiments (Fig. 3).

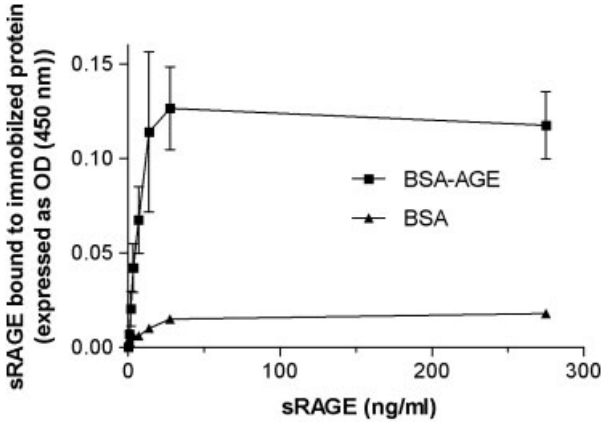

Figure 1. Binding of BSA-AGE to sRAGE. In a direct ELISA, specific binding of sRAGE (visualized by a RAGE-HRP-TMB detection method using a TMB reaction product with an absorbance maximum at 450 nm after acidification) to immobilized BSA-AGE was demonstrated. A dose-dependent binding of sRAGE to BSA-AGE (but not to unmodified BSA) could be detected up to 25 ng/mL concentrations of sRAGE. At higher concentrations, no additional binding was observed.

3.4 Inhibition of BSA-AGE induced NO and TNF-α production by a neutralizing RAGE antibody

To prove that BSA-AGE is linked to NO and TNF-α production by RAGE, the addition of a neutralizing RAGE antibody (raised against a C-terminal peptide of RAGE) was tested for inhibitory effects on this pathway. For this purpose, N-11 microglia were pre-incubated with various concentrations of a neutralizing RAGE-antibody for 2 h, then stimulated with BSA-AGE (750 μg/mL) for 24 h, after which the supernatant medium was

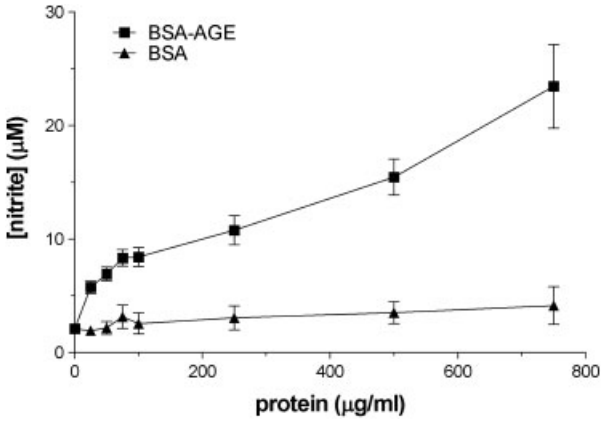

Figure 2. Dose-dependent NO production induced by BSA-AGE. For analysis of NO production (measured as nitrite), N-11 microglia were stimulated with BSA-AGE (25–750 μg/mL) for 24 h. For determination of cell viability, N-11 microglia were incubated with MTT or resazurin for 2 h at 37°C.

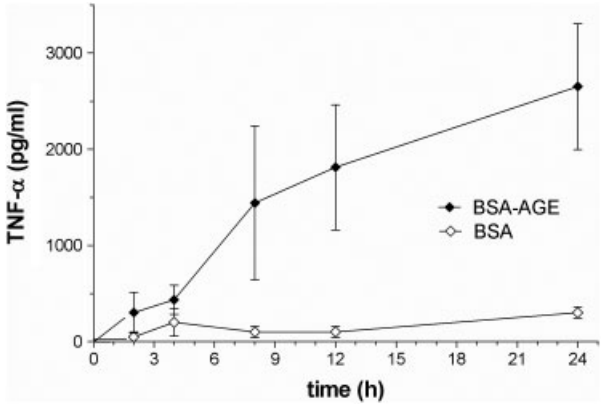

Figure 3. Time dependent TNF-α production induced by BSA-AGE. N-11 cells were incubated with BSA-AGE (750 μg/mL) for 0–24 h. After 0, 2, 4, 6, 12 and 24 h, cell culture supernatant was removed. TNF-α concentration was determined by a commercial ELISA (Peprotech).

Table 1. Effect of five tested compounds/plant extracts on nitric oxide (NO) and TNF-α production and on cell viability in N-11 microglia activated with 750 μg/mL BSA-AGEs

Compound/extract	NO		TNF-α		Cell viability in % (at EC_{50} for NO)	
	EC_{50}		EC_{50}		Alamar Blue (%)	MTT (%)
	(μM)	(μg/mL)	(μM)	(μg/mL)		
Apigenin	14	–	8	–	>85	>85
Diosmetin	19	–	39	–	>85	>85
Silymarin extract	–	8	–	10	>85	>85
Green olive leaf extract	–	65	–	NA	>85	>85
Uva ursi extract	–	NA	–	NA	>85	70

Concentrations of samples that contained one major compound with a minimum purity of 95% are expressed in μM. Plant extracts that are complex mixtures are expressed in μg/mL.

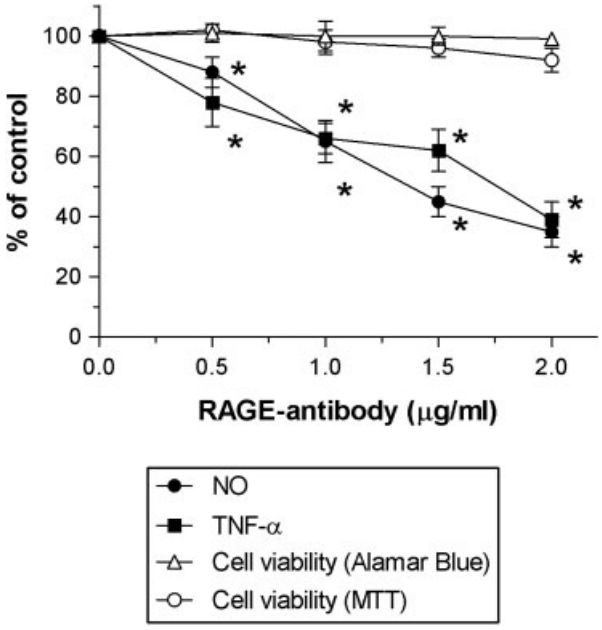

Figure 4. Dose-dependent inhibition of AGE-BSA induced NO and TNF-α production by a neutralizing RAGE antibody. To prove that BSA-AGE is linked to NO production by RAGE-mediated signaling, a neutralizing RAGE antibody was tested to determine whether this pathway would be blocked. For this purpose, N-11 microglia were pre-incubated with various concentrations of a neutralizing RAGE-antibody for 2 h, then stimulated with BSA-AGE (750 μg/mL) for 24 h, after which the medium was removed for nitrite determination. A dose-dependent inhibition of NO and TNF-α production could be observed.

assessed for nitrite concentration. A dose-dependent inhibition of NO and TNF-α production could be observed (Fig. 4).

3.5 Effect of selected polyphenols on BSA-AGE-induced NO and TNF-α production

Apigenin: BSA-AGE-induced NO and TNF-α production were both inhibited by apigenin (5,7,4′-trihydroxyflavone) in a dose-dependent manner, with EC_{50} values of approximately 14 and 8 μM, respectively (Fig. 5A). Apigenin appeared to be non-toxic, as only the MTT assay showed a small decrease in cell viability at 100 μM (Fig. 5A).

Diosmetin: The flavonoid diosmetin (5,7,3′-trihydroxy-4′-methoxy flavone) also dose dependently down-regulated BSA-AGE induced NO and TNF-α production, with EC_{50} values of 18 μM and 39 μM, respectively (Fig. 5B). The number of viable cells remained above 90% at diosmetin concentrations up to 100 μM (Fig. 5B).

Silymarin extract: Silymarin extract with its main ingredient silybin (*ca.* 60%) reduced AGE-BSA-induced NO production with an EC_{50} value of around 8 μg/mL. Silymarin also decreased TNF-α production in response to BSA-AGE, with an EC_{50} value of 10 μg/mL (Fig. 5C). Cell viability decreased at silymarin extract concentrations above 20 μg/mL, at which cell viability dropped below 70% (Fig. 5C).

Uva ursi (bearberry) extract: The main ingredient, arbutin, is a glycosylated benzoquinone (2R,3S,4S,5R,6S)-2-hydroxymethyl-6-(4-hydroxyphenoxy)oxane-3,4,5-triol). The bearberry extract inhibited BSA-AGE induced NO production with an EC_{50} of 25 μg/mL (Fig. 5D). Quite unexpectedly, the bearberry extract increased the TNF-α production, reaching 145% of control values at 50 μg/mL (Fig. 5D).

Green olive leaf extract: The extract from green olive leaf contains 20% oleuropein. Green olive leaf extract decreased BSA-AGE induced NO production in a dose-dependent manner with an EC_{50} value of 65 μg/mL (Fig. 5E). The green olive leaf extract also up-regulated TNF-α production, reaching a maximum of 150% at concentrations of 60 μg/mL (Fig. 5E) The EC_{50} values for all compounds are summarized in Table 1.

4 Discussion

In this study, we show that the two pure compounds apigenin and diosmetin as well as the extracts of silymarin, uva ursi (bearberry) and, to a lesser degree, green olive leaf inhibit AGE-induced NO production. In contrast, TNF-α

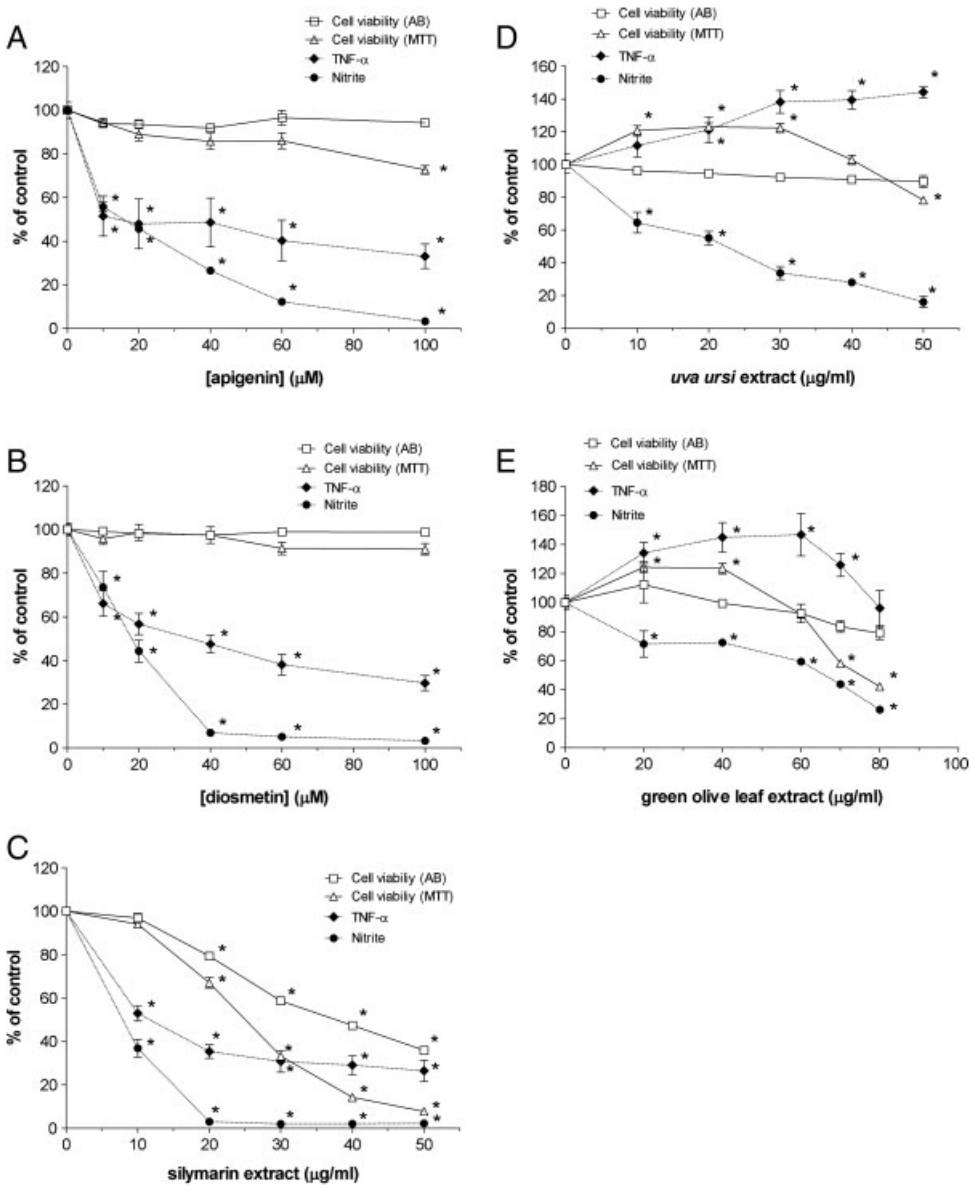

Figure 5. Dose effect of plant extracts and plant-derived polyphenolic compounds on BSA-AGE-induced NO and TNF-α production. N-11 microglia were pre-incubated with various concentrations of the plant extracts and pure polyphenolic compounds including apigenin (A), diosmetin (B) as well as silymarin (C), uva ursi (D) and green olive leaf extract (E) for 2 h before addition of 750 μg/mL BSA-AGE. After 24 h of incubation, conditioned media was collected. TNF-α was measured by a commercial ELISA, NO production was analyzed by determination of the nitrite concentration using Griess reagent. Cell viability was determined with the MTT and Alamar Blue Assays. Statistically significant changes ($p<0.05$) in NO and TNF-α production (compared to the values without polyphenols) are indicated by asterisks.

expression was only reduced by apigenin, diosmetin and silymarin, but not by the bearberry and green olive leaf extracts. In addition, the silymarin and uva ursi extracts caused a significant degree of cell death at concentrations $\geq 10\,\mu g/mL$ and $\geq 50\,\mu g/mL$, respectively, which was most evident in the MTT assay. Since both assays reflect mitochondrial respiratory activity, changes in cell number or metabolism might be responsible for the lower NO production (which depends on the expression of iNOS) and TNF-α expression. On the contrary, green olive leaf (at concentrations of 20–40 μg/mL) and uva ursi extract (at concentrations of 10–30 μg/mL) increased cell viability by about 20%, and it is possible that this might be, at least partly, responsible for the increase in TNF-α production. Very interestingly, the two cell viability assays show differential results for some of the toxic compounds, with a stronger decrease in the MTT than Alamar Blue assay, particularly at higher concentrations of the compounds. This difference might be explained by the fact that MTT forms precipitating formazan crystals inside the cell, which might impair the function of an already stressed cell.

The proposed mechanism(s) by which these five different agents exert their anti-inflammatory activities are discussed below. Transcriptional regulation of iNOS and TNF-α is a complex process, involving a number of transcription factors including NF-κB, AP-1, and various members of the C/EBR, ATF/CREB and STAT family [20]. Although the exact target of the polyphenols along the pro-inflammatory signal cascades is not known in detail, it is most likely they

interfere with the NF-κB pathway somewhere upstream of kinases phosphorylating the inhibitor of κB, IkB. There are two signaling pathways leading to the activation of NF-κB known as the canonical pathway (or classical) and the non-canonical pathway (or alternative pathway) [21]. The common regulatory step in both of these cascades is activation of an IkB kinase (IKK) complex consisting of catalytic kinase subunits (IKKα and/or IKKβ) and the regulatory non-enzymatic scaffold protein NEMO. Activation of NF-κB dimers is due to IKK-mediated phosphorylation-induced proteasomal degradation of the IkB inhibitor enabling the active NF-κB transcription factor subunits to translocate to the nucleus and induce target gene expression. In the canonical (or classical) activation pathway, the complex consisting of IKK-β and IKK-γ/NEMO phosphorylates two critical serine residues in IκB-α. IκB-α can then be targeted for ubiquitination and degradation. Some non-canonical pathways of IKK-independent activation of NF-κB stipulate the selective activation of NF-κB subunits [22]. It is quite likely that the general anti-inflammatory activity of polyphenols is, at least partly, based on their ability to suppress the activation of the transcription factor NF-κB along, which we will discuss for each polyphenol (or extract) in detail below.

Similar to our observations, apigenin has been shown to inhibit iNOS and COX-2 expression in LPS-activated RAW 264 macrophages with an IC_{50} of approximately 15 μM [23], as well as the synthesis of inflammatory cytokines including TNF-α and IL-1β in J774 macrophages [24] and human monocytes [25]. Comalada *et al*. suggested that apigenin inhibits the phosphorylation of Ser 32 on IκB-α in macrophages exposed to LPS [26]. A detailed mechanistic study in human primary monocytes suggested that apigenin did not affect the LPS-induced phosphorylation and degradation of IκB-α, or the DNA-binding activity of NF-κB in these cells. In this study, apigenin suppressed the LPS-induced phosphorylation of Ser536 on the p65 subunit of NF-κB in mouse macrophages by inhibiting IKK kinase activity. In addition, it was shown that apigenin neither directly affected the activity nor decreased the level of IKK protein [23], suggesting that it inhibits IKK activity through an indirect upstream mechanism [25].

Diosmetin has also been reported to significantly prevent iNOS induction and TNF-α release in splenocytes challenged with concanavalin A [27]. Similarly to apigenin, diosmetin also decreases phosphorylation of IκB-α in LPS-activated macrophages [26]. In addition, it has been shown that apigenin and diosmetin do not directly inhibit the catalytic activity of the iNOS enzyme [28] nor significantly scavenge NO on their own [14], suggesting that it might also act upstream of IκB along the signaling cascade.

In concordance with our data, Lu *et al*. reported [29] that silybin (silibinin) attenuated the expression of iNOS and TNF-α mRNA when it was induced by Aβ (25–35), a further ligand of RAGE [30]. By and large, it is proposed that silymarin's anti-inflammatory activity is also mediated through its inhibitory effects on NF-κB activation. Silymarin also demonstrated abrogation of NF-κB activation when the cells induced by a wide variety of inflammatory agents such as TNF-α, phorbol esters, LPS, okadaic acid, and ceramide [31]. The antagonistic effect of silymarin/silybin on TNF-α induced activation of NF-κB were suggested to be mediated by the inhibition of the phosphorylation and degradation of IκB-α thus impeding the translocation of p65 and p50 subunits to the nucleus [32].

Uva ursi (bearberry) extract, prepared from the leaves of the common bearberry (*Arctostaphylos uva ursi*), contains up to 20% arbutin (a glycosylated hydroquinone), ursolic acid, tannic acid, gallic acid, some essential oils and resins, tannins, phenolic glycosides and flavonoids. The lack of inhibition of bearberry extracts on TNF-α expression in our experiments is reflected by the literature, where no reports about such anti-inflammatory effects have been published so far. On the other hand, non-glycosylated hydroquinones have been reported to be excellent inhibitors of inflammatory responses including TNF-α production [33]. The reason for this apparent discrepancy could be that arbutin is unable to penetrate the cell membrane because of its hydrophilic sugar moiety. The inhibitory effects of the uva ursi extract on NO levels could be caused by the tannin and procyanidin oligomers, which have been shown to be direct NO scavengers [34].

Green olive leaf extract contains 20% oleuropein, a powerful antioxidant capable of scavenging superoxide radicals and inhibiting the respiratory burst in neutrophils [35]. The extract also contains small quantities (around 1–2%) of flavone-glucosides (luteolin-7-glucoside, apigenin-7-glucoside, diosmetin-7-glucoside) and the secoiridoid verbascoside [36]. In septic rabbits, injected with *Pseudomonas aeruginosa*, administration of oleuropein decreased TNF-α and IL-6 levels [37]. However, no cell-based experiments investigating a possible reduction in TNF-α and NO production by green olive leaf extract have been reported so far.

In summary, we suggest that the general anti-inflammatory activity of cell membrane permeable polyphenols such as apigenin, diosmetin and silymarin is mainly based on their ability to suppress the activation of the transcription factor NF-κB.

The approach to the use of bioflavonoids as a general anti-inflammatory and anti-aging therapy for humans is still controversial. Low bioavailability and loss of function due to metabolic processing are the two main arguments against the efficacy of dietary supplementation with plant bioflavonoids [38]. However, there is now sufficient interest to investigate their use as an alternative to anti-inflammatory drugs [39]. A common daily diet usually includes up to a few hundred milligrams *per* day of flavonoids [40]. Significant bioavailability has also been shown for some flavonoids, such as hesperetin, naringenin and quercetin [41, 42]. To be physiologically effective, plasma

concentrations of these compounds should be of the same order as the maximum plasma concentrations attained after a polyphenol-rich meal. The literature describes similar levels for all these compounds at around 5–60 μM [43, 44], which suggest that apigenin and diosmetin could be delivered and maintained in plasma at a therapeutically relevant concentration.

Since polyphenols are not very lipophilic compounds, how much of the polyphenols (or their metabolites) can permeate through the blood brain barrier to reach the brain is still debatable. Furthermore, whether the level of particular polyphenolic compounds in the brain can reach relevant concentrations to exert an anti-inflammatory and/or neuroprotective effect is also uncertain. In addition, the question of whether polyphenols are actually required to reach the brain to exert a neuroprotective effect can also be asked.

Although the concentrations of orally given polyphenols in the brain are usually less than 1 nmol/g tissue [45], there is more and more evidence that consumption of polyphenols protects against neurotoxic insults, indicating that they can exert (by whatever mechanism) central neuroprotective effects *in vivo*. For example, in an animal model of stroke, dietary consumption of resveratrol (3 days) reduced infarct volume by 36% in 24 h in mice [46]. In an animal model of familial AD (APP(Swe)/PS1dE9 transgenic mice), polyphenol rich grape seed extract was shown to inhibit Aβ aggregation, reduce Aβ production and protect against Aβ neurotoxicity *in vitro*. Amyloid plaques and microgliosis were also reduced by 49 and 70%, respectively [47].

There is also a substantial amount of epidemiological evidence indicating the positive health effects of dietary polyphenols in age- (and AGE)-related degenerative diseases including vascular dementia and AD [48]. In a population-based prospective study (the ''Kame Project''), fruit and vegetable juices significantly decreased the risk of AD. The hazard ratio for AD, comparing subjects who drank fruit and vegetable juices >3 times *per* week with those who drank <1 *per* week was 0.24 [49].

We believe that it might be worth investigating these plant-derived polyphenolic antioxidants as novel cost-effective anti-inflammatory drugs in future studies, first in animal models of AD and finally in humans, most likely as preventative drugs for patients with mild cognitive impairment, which is often a prodrome of early stage AD.

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