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Abstract

Chronic inflammation is a hallmark of neurodegenerative disease and cytotoxic levels of nitric oxide (NO) and pro-inflammatory cytokines can initiate neuronal death pathways. A range of cellular assays were used to assess the anti-inflammatory and neuroprotective action of resveratrol using murine microglial (C8-B4), macrophage (RAW264.7) and neuronal-like (Neuro2a) cell lines. We examined the release of NO by Griess assay and used a Bioplex array to measure a panel of pro- and anti-inflammatory cytokines and chemokines, in response to the inflammatory stimuli lipopolysaccharide (LPS) and interferon-γ (IFN-γ). Resveratrol was a potent inhibitor of NO and cytokine release in activated macrophages and microglia. The activity of resveratrol increased marginally in potency with longer pre-incubation times in cell culture that was not due to cytotoxicity. Using an NO donor we show that resveratrol can protect Neuro2a cells from cytotoxic concentrations of NO. The protective effect of resveratrol from pro-inflammatory signalling in RAW264.7 cells was confirmed in co-culture experiments leading to increased survival of Neuro2a cells. Together our data are indicative of the potential neuroprotective effect of resveratrol during nitrosative stress and neuroinflammation.

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Neuroprotection of Neuro2a cells and the cytokine suppressive and anti-inflammatory mode of action of resveratrol in activated RAW264.7 macrophages and C8–B4 microglia

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ABSTRACT

Chronic inflammation is a hallmark of neurodegenerative disease and cytotoxic levels of nitric oxide (NO) and pro-inflammatory cytokines can initiate neuronal death pathways. A range of cellular assays were used to assess the anti-inflammatory and neuroprotective action of resveratrol using murine microglial (C8–B4), macrophage (RAW264.7) and neuronal-like (Neuro2a) cell lines. We examined the release of NO by Griess assay and used a Bioplex array to measure a panel of pro- and anti-inflammatory cytokines and chemokines, in response to the inflammatory stimuli lipopolysaccharide (LPS) and interferon-γ (IFN-γ).

Resveratrol was a potent inhibitor of NO and cytokine release in activated macrophages and microglia. The activity of resveratrol increased marginally in potency with longer pre-incubation times in cell culture that was not due to cytotoxicity. Using an NO donor we show that resveratrol can protect Neuro2a cells from cytotoxic concentrations of NO. The protective effect of resveratrol from pro-inflammatory signalling in RAW264.7 cells was confirmed in co-culture experiments leading to increased survival of Neuro2a cells. Together our data are indicative of the potential neuroprotective effect of resveratrol during nitrosative stress and neuroinflammation.

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1. Introduction

Chronic inflammation is known to be an important etiological condition for various chronic diseases, including atherosclerosis, diabetes, and arthritis. Furthermore, increasing evidence suggests that innate immune activation is a major component of age-related neurodegenerative diseases, such as Alzheimer's and Parkinson's disease (for recent reviews see (Heneka et al., 2014; Norden et al., 2015)).

Inflammatory cells, including macrophages and microglia, play a major role in the body's response to immunogenic challenges, by re-establishing tissue homeostasis, producing large amounts of superoxide, nitric oxide (NO) and pro-inflammatory cytokines that aggravate and propagate inflammation and disrupt the normal function of cells. For example, the pro-inflammatory cytokine, tumour necrosis factor-α (TNF-α), binds to two tumour necrosis factor receptors TNFR1 and TNFR2 and, depending on the adaptor protein, activates various signalling pathways including nuclear factor-κB (NF-κB), and the mitogen activated protein kinases (MAPK), p38 and c-Jun N-terminal kinase (JNK), which can lead to

Abbreviations: COX-2, cyclooxygenase-2; CSAIDs, cytokine suppressing anti-inflammatory drugs; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; Mip-1α, macrophage inflammatory protein-1α; NF-κB, nuclear factor-kB; NO, nitric oxide; NSAIDs, non-steroidal anti-inflammatory drugs; SNAP, S-Nitroso-N-acetyl-DL-penicillamine.

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proliferation, cell migration, apoptosis and necrosis (McCoy and Tansey, 2008).

NO is a signalling molecule with diverse cellular roles; it is released by macrophages, neurons and endothelial cells and can be either protective or toxic to cells depending on the cellular context (Ooi et al., 2013a; Gamper and Ooi, 2015). The toxicity of NO is attributed to its ability to bind to proteins that contain heme, iron, copper or organic side groups, such as thiols, resulting in protein disruption and alterations in cellular activities. As a result of reactivity with a wide range of cellular components, high levels of NO are toxic to neurons, while low levels can be neuroprotective (Balez and Ooi, 2015). Similarly there is evidence that activation of microglia could have a protective role in neurodegenerative diseases (Luo and Chen, 2012; Kan et al., 2015). Therefore promoting anti-inflammatory cytokines or limiting pro-inflammatory cytokine and NO production by activated macrophages/microglia should be beneficial for prevention of systemic and local inflammation and would be potentially neuroprotective.

To date, pharmacotherapy of inflammatory conditions is based on the use of non-steroidal anti-inflammatory drugs (NSAIDs). However, NSAIDs can cause serious gastrointestinal toxicity such as gastric bleeding and the formation of stomach ulcers. Some NSAIDs, particularly cyclooxygenase-2 (COX-2) inhibitors, have been linked to increased blood pressure, greatly increased risk of congestive heart failure, thrombosis and myocardial infarction (Mitchell and Warner, 2006). NSAIDs are inhibitors of cyclooxygenase and specifically suppress the production of prostaglandins (Vane et al., 1998). Thus the identification of anti-inflammatory treatments with fewer adverse effects and drugs with broader anti-inflammatory properties is an important task. One such therapeutic approach involves suppressing the production of interleukin-1 (IL-1), TNF-α and other NF-kB regulated cytokines at the level of transcription, translation and/or secretion. The activities associated with certain of pyridinyl imidazoles involve a class of compounds referred to as cytokine suppressing anti-inflammatory drugs (CSAIDs) (Karunaweera et al., 2015).

The stilbene resveratrol (3,4′,5-trihydroxy-trans-stilbene) is a polyphenol CSAID found in grapes, red wine, mulberries knotweed, peanuts and other plants. Resveratrol dampens inflammation in arthritis (Elmali et al., 2007) and immune responsiveness in autoimmune disease (Singh et al., 2010). Resveratrol can also penetrate the blood brain barrier in vivo (Baur et al., 2006), however the effect of resveratrol on a broad range of pro- and anti-inflammatory factors has not previously been investigated.

In the present study, we first examined the expression of various pro- and anti-inflammatory cytokines, chemokines and NO by murine microglia (C8–B4) and macrophages (RAW264.7) in response to the inflammatory stimuli lipopolysaccharide (LPS) and interferon-γ (IFN-γ). We then determined the effects of resveratrol on cytokines and chemokines in activated C8–B4 microglia and RAW264.7 macrophages and investigated the effect of the pre-incubation time in cell culture on the calculated potency. Resveratrol potently inhibited the release of NO and a number of cytokines from activated macrophages and microglia and was able to protect neuronal-like Neuro2a cells from cytotoxic concentrations of NO. Conditioned media from activated RAW264.7 cells reduced the viability of Neuro2a cells, an effect that was prevented by pre-treatment of the activated RAW264.7 cells with resveratrol. Together our results highlight the anti-inflammatory effects of resveratrol and its potential as a neuroprotective agent against neuroinflammation.

2. Materials and methods

2.1. Materials

The 23-multiplex cytokine assay was from Bio-rad (Gladesville, Australia). All cell culture materials were from Invitrogen (Mulgrave, Australia). Cell culture plasticware including Primaria plates was from Greiner Bio-One (Frickenhausen, Germany), Protease inhibitor cocktail, naphthyethylene-diamine, sulfanilamide, Corning® Costar® Spin-X® spin filter, LPS (from Salmonella enteric serotype thyphimurium), resazurin and S-Nitroso-N-acetyl-DL-penicillamine (SNAP) were from Sigma–Aldrich (Castle Hill, Australia). Interferon-γ was from Pepro Tech (Rocky Hill, USA). TrypLE Express™ was purchased from Life Technologies (Mulgrave, Australia). Cell lines were obtained from the ATCC.

2.2. Cell maintenance

RAW264.7, C8–B4 and Neuro2a cells were maintained in DMEM containing 25 mM glucose, supplemented with 200 U/ml penicillin, 200 μg/ml streptomycin, 200 mM glutamine (GlutaMax, Gibco) and 10% fetal bovine serum. Cells were grown in 75 cm^2 tissue culture flasks and incubated at 37 °C in 5% CO_2. RAW264.7 and Neuro2a cells were detached from the flask with a rubber policeman and split 1:10 every 5 days. C8–B4 cells were split 1:2 every 2 weeks and partial medium changes were performed twice a week.

2.3. Resveratrol treatment of LPS and IFN-γ activated C8–B4 and RAW264.7 cells and SNAP-treated Neuro2a cells

Cells were washed twice with PBS and harvested with TrypLE Express. Cells were then plated in a 96-well plate at a density of 60,000 cells/well and incubated for 24 h before treatment. Cells were pre-treated with resveratrol for 1 h before adding the activation mix containing 12.5 μg/ml LPS and 5 Units IFN-γ for 24 h. The total volume of medium in each well was 100 μl and to obtain enough supernatant for further analysis, the supernatant from 3 wells was combined. The supernatant was spin filtered (0.2 μm Corning spin filter) and 0.4 μl protease inhibitor cocktail containing Aprotinin, Bestatin, E–64, Leupeptin and Pepstatin A (Sigma) was added. Fifty microlitres of supernatant was immediately used for measuring NO and the remainder was stored at −80 °C for later analysis by cytokine array. Neuro2a cells were pre-treated with resveratrol for 1 h before treatment with the NO donor, SNAP, at concentrations ranging from 0.1 to 1000 μM or the vehicle control (DMSO). Cells were harvested for cell viability assays and the supernatant collected for Griess assay to measure nitrite concentration.

2.4. Neuro2a incubation with conditioned media from activated RAW264.7 cells

As in Section 2.3 RAW264.7 cells were plated at a density of 60,000 cells/well and incubated for 24 h before treatment. RAW264.7 cells were activated with 12.5 μg/ml LPS and 5 Units IFN-γ for 24 h. Cells were pre-treated with 100 μM resveratrol or vehicle control (DMSO) for 1 h prior to inflammatory activation. Neuro2a cells were seeded at a density of 60,000 cells per well in a 96-well plate, and incubated for 24 h. Media from RAW264.7 cells were added to Neuro2a cells. As a further control, Neuro2a cells were also pre-treated with 100 μM resveratrol or vehicle control (DMSO) for 1 h prior to application of RAW264.7 conditioned media. After 24 h of incubation with conditioned media from RAW264.7 cells, the Neuro2a cells were assessed by cell viability and Griess assay. The

same measurements were simultaneously taken for the RAW264.7 cells.

2.5. Resazurin-based assay for determination of cell viability

Cell viability was assessed in terms of the metabolic capability of cells to convert the non-fluorescent redox indicator, resazurin, into its highly fluorescent product, resorufin. DMEM (100 μl) containing 0.125 mg/l resazurin was added to each well of a 96-well plate and incubated for 90 min a 37 °C. Fluorescence was measured at 560 nm excitation/590 nm emission using a BMG Labtech POLARstar Omega fluorescent plate reader (BMG LABTECH, Ortenberg, Germany).

2.6. Nitrite determination by Griess assay

The nitrite concentration in the culture medium was measured as an indicator of NO production using the Griess reaction. Supernatant from each well (50 μl) was transferred to a fresh 96 well plate and 25 μl of 1% sulfanilamide plus 25 μl of 0.1% napthyethylene-diamine in 5% HCl were added. When combined, these compounds (known as Griess reagent), form a violet colour in the presence of nitrite. After 10 min incubation at room temperature, the absorbance of each well was measured at 540 nm using a Bio-Rad Model 680 Microplate reader (Bio-Rad, Gladesville, Australia). Nitrite concentration was calculated with reference to a standard curve of sodium nitrite generated by known concentrations, ranging from 0 to 100 μM.

2.7. Determination of cytokine levels using a Bioplex cytokine assay

A panel of 23 cytokines was measured, using a Bioplex Pro™ mouse cytokine 23-plex kit (Bio-Rad, Gladesville, Australia), according to the manufacturer's protocol.

2.8. Data analysis

The standard error of the mean (SEM) and all other values were calculated from at least three independent experiments. Significant differences were assessed by one-way ANOVA with Tukey's multiple comparisons test. IC_{50} values for resveratrol were calculated using the sigmoidal dose–response function.

3. Results

3.1. LPS and IFN-γ activation increases NO and cytokine/chemokine release in murine RAW264.7 and C8–B4 cells

For the determination of the potency of a CSAID, cell lines with a broad range of cytokine and chemokine response are needed. These cell lines should react upon stimulation with pro-inflammatory stimuli, such as LPS and IFN-γ, with up-regulation of inducible nitric oxide synthase (with resulting NO production and release), as well as increased expression and release of pro-inflammatory cytokines such as TNF-α, interleukin-1α (IL-1α), IL-1β, IL-6, IL-12, as well as chemokines such as granulocyte colony-stimulating factor (G-CSF).

When both cell lines were activated with a combination of LPS and IFN-γ, a significant increase in NO production (determined as nitrite) was observed. The activated macrophage cell line RAW264.7 released approximately four times more NO compared to C8–B4 cells (Table 1). In addition, 17 different inflammatory markers were determined using the Bioplex Pro™ mouse cytokine 23-plex immunoassay (6 markers were below detection threshold; Table 1).

In both cell lines, activation with LPS and IFN-γ for 24 h led to a significant up-regulation of the pro-inflammatory cytokines IL-1α, IL-6, TNF-α, IL-12(p40) and the chemokines G-CSF and C–C motif chemokine 5 (CCL5, also known as RANTES). Activation also up-regulated the anti-inflammatory cytokines IL-10 and IL-13 in RAW264.7 macrophage cells but not in C8–B4 microglia. The chemokines monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein-1α (Mip-1α), Mip-1β, were also only up-regulated in RAW 264.7, but not in C8–B4 microglia. In general, the C8–B4 cells showed a more limited cytokine/chemokine release repertoire than the RAW264.7 cells under the same activation conditions (Table 1).

3.2. Resveratrol prevents NO and cytokine release in RAW264.7 and C8–B4 cells but has a limited effect on chemokine release

To answer the question as to whether resveratrol possesses broad anti-inflammatory actions, cells were pre-incubated with resveratrol at concentrations up to 100 μM for 1 h prior to activation by LPS and IFN-γ in RAW264.7 and C8–B4 cells, after which the release of NO, cytokines and chemokines was measured. Only those pro-inflammatory markers that had shown an up-regulation of >1.5 fold in the previous experiment were analysed. An anti-inflammatory effect of resveratrol (defined as an IC_{50} value < 100 μM) could be demonstrated in both cell lines. Resveratrol down-regulated the pro-inflammatory cytokines IL-1α, IL-1β, IL-6 and TNF-α in both RAW264.7 and C8–B4 cells. However, it also down-regulated the anti-inflammatory cytokine IL-10 in RAW264.7 cells only (Table 2). Resveratrol effects on chemokines were more limited, leading to down-regulation of only MCP-1 and RANTES in RAW264.7 (Table 3).

3.3. Increasing pre-incubation time marginally increases potency of resveratrol

Since the effects of inflammatory activation were more potent in the RAW264.7 cells and since resveratrol was more effective in limiting inflammatory activation of RAW264.7 cells, we continued our experiments using this cell line only. One of the most fundamental questions to judge the clinical efficacy of a compound is whether the concentration of the compound in the human body is in the same order of magnitude as its IC_{50} value. However, it might be possible that some cell culture experiments are also conducted under suboptimal conditions, and effective concentrations are higher than needed. For example, a very short pre-incubation time might make it impossible or difficult for the compound to reach an intracellular target, whereas long pre-incubation times might lead to substantial degradation of the compound.

We wanted to determine whether our experimental setup of 1 h pre-incubation time affected potency, resulting in limited diffusion of resveratrol into the cell. We hypothesized that the calculated potency might increase when the incubation time was extended. To answer this question, RAW264.7 cells were pre-incubated with resveratrol for 1, 3, 6, 9 and 24 h before activation with LPS and IFN-γ. NO was measured after 24 h to assess the potency of resveratrol. In these experiments the calculated IC_{50} decreased from 44.7 ± 7.1 μM after 1 h pre-incubation to 26.7 ± 3.5 μM after 6 h pre-incubation, increasing after 24 h pre-incubation (Fig. 1). To exclude the possibility that the decrease in the release of the pro-inflammatory markers were caused by cell death, cytotoxicity of resveratrol was measured in RAW264.7 and C8–B4 cells. At pre-incubation times of 1 h, and concentrations below 100 μM, resveratrol did not show cytotoxicity in RAW264.7 cells. Only at a concentration of 110 μM did resveratrol cause a decrease in cell viability by 13% in C8–B4 cells (data not shown). The LC_{50} values

Table 1
Comparison of cytokine & chemokine responses to activation with LPS and IFN-γ in RAW264.7 and C8-B4 cells. Data are shown as mean concentration (pg/ml) ± SEM from 3 independent experiments and fold change. Underline denotes anti-inflammatory cytokines. Significant difference between unactivated and activated cells denoted as * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = not significant.

Inflammatory response marker	Concentration in RAW 264.7 macrophages (pg/ml)		Upregulation (fold)	Concentration in C8-B4 microglia (pg/ml)		Upregulation (fold)
	Unactivated	Activated		Unactivated	Activated	
Nitric oxide (μM)	1.0 ± 0.4	60 ± 4	60***	1.3 ± 0.3	16.4 ± 0.8	13***
IL-1 α	46.0 ± 1.4	331 ± 21	7***	17 ± 2	158 ± 37	9.3**
IL-1 β	2585 ± 149	3203 ± 81	1.2*	1502 ± 48	1459 ± 277	ns
IL-6	12 ± 3	10522 ± 822	877***	21 ± 5	572 ± 117	27**
IL-9	1195 ± 133	2132 ± 224	1.8**	140 ± 3	154 ± 11	ns
IL-12(p40)	44 ± 6	88 ± 6	2.0**	129 ± 11	1051 ± 234	8.1**
IL-12(p70)	413 ± 15	1252 ± 22	3.0***	314 ± 29	286 ± 74	ns
TNF-α	237 ± 5	142870 ± 8619	603***	304 ± 60	9395 ± 2085	31**
G-CSF	25 ± 1	113944 ± 12150	4558***	155 ± 64	7070 ± 2191	46**
GM-CSF	417 ± 9	716 ± 22	1.7***	302 ± 25	375 ± 46	ns
IL-10	91 ± 3	10358 ± 765	114***	122 ± 7	117 ± 23	ns
IL-13	749 ± 19	2105 ± 50	3.0***	876 ± 42	915 ± 196	ns
Eotaxin	1430 ± 63	4234 ± 414	3.0***	1928 ± 134	2399 ± 260	ns
KC	23 ± 2	55 ± 2	2.4***	20 ± 1	47 ± 7	2.3**
MCP-1	534 ± 69	45626 ± 5286	85***	23226 ± 1794	26115 ± 8948	ns
Mip-1α	66979 ± 6675	177204 ± 22988	2.6*	4559 ± 582	5526 ± 3711	ns
Mip-1β	13336 ± 1089	538430 ± 3531	40***	12933 ± 622	9336 ± 2573	ns
RANTES	150 ± 3	35472 ± 3226	236***	92 ± 41	752 ± 226	8*

Table 2
Effect of resveratrol on cytokine levels in RAW264.7 and C8–B4 cells. Data shown are mean IC_{50} of resveratrol (μM) ± SEM from 3 independent experiments, n.d. = not determined. Underline denotes anti-inflammatory cytokines.

Cytokine	Cells	IC_{50} (μM)
IL-1α	RAW264.7	95 ± 11
	C8–B4	68 ± 15
IL-6	RAW264.7	53 ± 14
	C8–B4	99 ± 21
IL-9	RAW264.7	>136
	C8–B4	>110
IL-12(p40)	RAW264.7	>136
	C8–B4	>110
IL-12(p70)	RAW264.7	>136
	C8–B4	n.d.
TNF-α	RAW264.7	62 ± 8
	C8–B4	42 ± 6
G-CSF	RAW264.7	>136
	C8–B4	50 ± 6
GM-CSF	RAW264.7	>136
	C8–B4	60 ± 11
IL-10	RAW264.7	37 ± 2
	C8–B4	>110

(lethal concentration at which 50% of the cells were killed) remained the same in all pre-treatment ranges but increased only after 24 h pre-treatment (Table 4). The increase in NO production at

Table 3
Effect of resveratrol on chemokine levels in RAW264.7 and C8–B4 cells. Data shown are mean IC_{50} of resveratrol (μM) ± SEM from 3 independent experiments, n.d. = not determined.

Chemokine	Cells	IC_{50} (μM)
Eotaxin	RAW264.7	>136
	C8–B4	n.d.
KC	RAW264.7	>136
	C8–B4	>110
MCP-1	RAW264.7	77 ± 7
	C8–B4	>110
Mip-1α	RAW264.7	>136
	C8–B4	>110
Mip-1β	RAW264.7	>136
	C8–B4	>110
RANTES	RAW264.7	63 ± 7
	C8–B4	>110

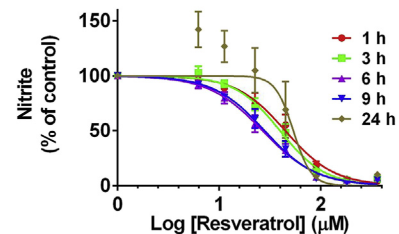

Fig. 1. The effect of resveratrol pre-incubation time on inhibition of NO release. Dose response curves for resveratrol induced down-regulation of nitrite in RAW264.7 cells with different pre-incubation times prior to inflammatory activation with LPS and IFN-γ. Data shown are mean ± SEM, n = 3.

24 h pre-treatment was therefore likely due to an increase in cell number following 24 h pre-incubation. Our data suggest that the calculated potency of resveratrol increases 1.6 fold with increasing pre-incubation times and this is not due to an alteration in cell viability.

3.4. *Resveratrol protects Neuro2a cells from cytotoxic levels of NO*

The neuroprotective and neurodegenerative capacity of inflammatory cells are central to understanding pathways pertinent to neuronal survival. Identifying molecules that promote neuronal survival is central to preventing neurodegeneration. The simplest way to study these effects is to remove contributing factors and study one component of the system. To determine whether resveratrol could be neuroprotective against cytotoxic concentrations of NO, Neuro2a cells were treated with 1 μM resveratrol for 6 h prior to incubation with the NO donor, SNAP. This concentration was chosen based on our previous experiments in Neuro2a cells and because resveratrol reaches nanomolar to micromolar concentrations in the plasma and brain (Nguyen et al., 2013; Walker et al., 2014). SNAP incubation at concentrations of 0.1 and 1 μM did not increase nitrite levels above vehicle control, as measured by Griess assay (no significant difference from control). SNAP at concentrations of 10, 100 and 1000 μM increased nitrite levels in Neuro2a media by 4, 15 and 100 fold, respectively (Fig. 2), with no significant difference in nitrite levels measured in the media from

Table 4
IC_{50} and LC_{50} values for resveratrol at different pre-incubation times in RAW264.7. Data shown are mean ± SEM, n = 3.

Pre-incubation time (h)	IC_{50} for NO inhibition (μM)	LC_{50} (μM)
1	44.7 ± 7.1	143.7 ± 18.8
3	39.7 ± 5.7	153.8 ± 17.0
6	26.7 ± 3.5	152.6 ± 20.5
9	28.0 ± 3.6	141.9 ± 29.8
24	53.6 ± 17.4	249.4 ± 165.4

cells treated with resveratrol compared to control. Concentrations ranging from 0.1 to 100 μM SNAP had no effect on Neuro2a cell viability, while 1000 μM SNAP was neurotoxic, reducing cell viability by 22 ± 5% ($p < 0.05$, n = 3; Fig. 3) compared to vehicle control. Even at the lowest concentration of resveratrol tested in macrophages and microglia (1 μM) resveratrol was able to protect Neuro2a against cytotoxic concentrations of NO and nitrosative stress (Fig. 3). Resveratrol increased cell viability in the presence of 10, 100 and 1000 μM SNAP by 2.7, 2.9 and 2.1 fold, respectively, and was thus able to prevent the cytotoxic effects of 1000 μM SNAP.

Since resveratrol was able to protect cells against cytotoxic concentrations of NO, we further investigated its potential as a neuroprotective agent. The neuroprotective effect of resveratrol was tested in a macrophage-neuron co-culture experiment using the transfer of conditioned media, as described previously (Münch et al., 2003). Media were incubated with RAW264.7 cells for 24 h and then incubated with Neuro2a cells. RAW264.7 cells were pretreated with 100 μM resveratrol or vehicle control (DMSO) for 1 h prior to inflammatory activation and activated with 12.5 μg/ml LPS and 5 Units IFN-γ for 24 h, as in the previous experiments. Media from RAW264.7 cells were added to Neuro2a cells and after 24 h incubation cell viability and Griess assays were performed for the RAW264.7 and Neuro2a cells. As expected, inflammatory activation of RAW264.7 cells led to a dramatic up-regulation of nitrite in the media of RAW264.7 cells (Fig. 4A). Nitrite remained at similar levels 24 h after transfer of the media to the Neuro2a cells (Fig. 4B). Pre-incubation of the RAW264.7 cells with 100 μM resveratrol prevented increases in nitrite that remained present in the media that was transferred to the Neuro2a cells and incubated for 24 h (Fig. 4A, B). Resveratrol treatment alone on RAW264.7 (without inflammatory activation) had no effect on the viability of Neuro2a cells (Fig. 5A). Conversely, inflammatory activation of RAW264.7 led to a reduction in Neuro2a viability that was reduced by pre-incubation of RAW264.7 with resveratrol. As further controls, media containing the inflammatory mixes, with or without resveratrol were added direct to the Neuro2a cells in the absence of incubation with RAW264.7 cells (Fig. 5B). Application of this media had no significant effect on viability of Neuro2a cells in any of the conditions, showing that the reduction in Neuro2a viability was caused by molecules secreted by activated RAW264.7 cells. In addition, Neuro2a cells were pre-treated with 100 μM resveratrol or vehicle control (DMSO) for 1 h prior to application of RAW264.7 conditioned media (Figs. 4 and 5). This treatment had no further effect on nitrite levels or Neuro2a viability, suggesting that the neuroprotective effects observed were specifically due to the anti-inflammatory effects of resveratrol on RAW264.7 cells. Together these data highlight the potential neuroprotective role for the action of resveratrol via its anti-inflammatory actions, in addition to its ability to protect against cytotoxic levels of NO.

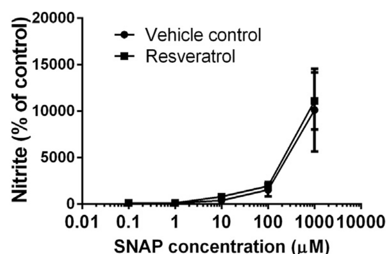

Fig. 2. Quantitation of nitrite following incubation with the NO donor SNAP. Neuro2a cells were incubated with 1 μM resveratrol or vehicle control for 6 h prior to treatment with vehicle or 0.1, 1, 10, 100 or 1000 μM of the NO donor SNAP and incubated for 24 h. Nitrite levels in the cell media were measured by Griess assay. Data shown are mean ± SEM, n = 3.

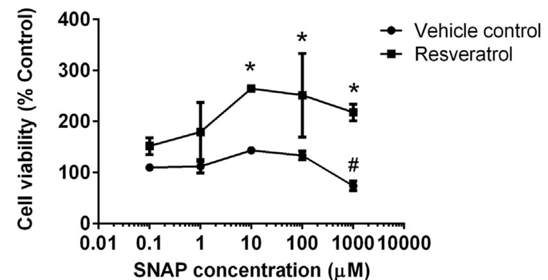

Fig. 3. The effects of the NO donor SNAP on cell viability following pre-treatment with resveratrol. Neuro2a cells were incubated with 1 μM resveratrol or vehicle control for 6 h prior to treatment with vehicle or 0.1, 1, 10, 100 or 1000 μM of the NO donor SNAP and incubated for 24 h. Cell viability was measured by resazurin assay. Data shown are mean ± SEM, n = 3. Significant interactions were determined by two-way ANOVA; # denotes $p < 0.05$ SNAP treatment compared to vehicle control; * denotes $p < 0.05$ resveratrol treatment compared to vehicle control.

4. Discussion

4.1. RAW264.7 macrophages exhibited a more reactive phenotype than C8–B4 microglia, releasing higher levels of nitric oxide and pro-inflammatory cytokines

During brain inflammation, the microglia proliferate and migrate to the inflammatory lesion (Kettenmann et al., 2011). Blood derived macrophages may also enter the brain via the blood brain barrier. (Guillemin and Brew, 2004). In the central nervous system two different macrophage populations have a role to play in supporting neurons, and these are often described as CNS-resident microglia and CNS-infiltrating peripheral macrophages. In neurodegenerative diseases, such as Alzheimer's disease, CNS-infiltrating peripheral macrophages may contribute to neurodegeneration, however activated microglia may also protect neurons by removing toxic aggregates and cellular debris (Kraft and Harry, 2011). Functional differences in CNS-resident microglia and acutely infiltrating inflammatory macrophages have been identified (Carson et al., 2007). Thus an understanding of cell-type specific effects and the differences in commonly used cellular models of macrophages and microglia is important to identify the roles these cells play in neuroprotection/neurodegeneration (Simard et al., 2006).

Activated microglia behave similarly to macrophages by inducing cytotoxicity in a variety of cells through the release of NO (Brantley et al., 2010). It is likely that some microglia develop from a pool of blood monocytes and colonize the brain early in development (Lawson et al., 1990, 1992). Evidence suggests that LPS and IFN-γ-activated microglia isolated from the adult brain are more similar to macrophages infiltrating the CNS than naive microglia of the brain (Schmid et al., 2009). Therefore it is important to consider the responses of each of these cell types to activation and the effects of any neuroprotective agents in preventing these responses. To address this we have carried out a comprehensive study on the anti-inflammatory effects of resveratrol, a polyphenol

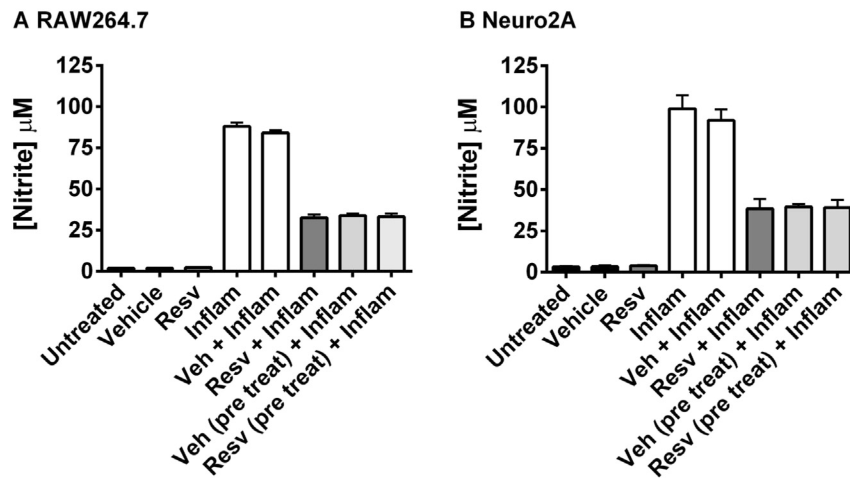

Fig. 4. Inflammatory activation of RAW264.7 cells caused increases in nitrite measured by Griess assay. (A) Griess assay on RAW264.7 cells treated with LPS and IFN-γ for 24 h ± 1 h pre-incubation with resveratrol (100 μM) in RAW264.7 cells. Samples were untreated, treated with vehicle (veh) or resveratrol (resv) ± inflammatory activation (inflam; LPS and IFN-γ). (B) The media was transferred from RAW264.7 cells to Neuro2A cells and incubated with Neuro2A cells for 24 h prior to Griess assay. To confirm the effects were driven by resveratrol on the RAW264.7 cells rather than on Neuro2A cells, Neuro2A cells were also pre-treated with vehicle or resveratrol prior to application of the RAW264.7 media (Veh (pre-treat) + inflam; Resv (pretreat) + Inflam). Data shown are mean ± SEM, n = 4.

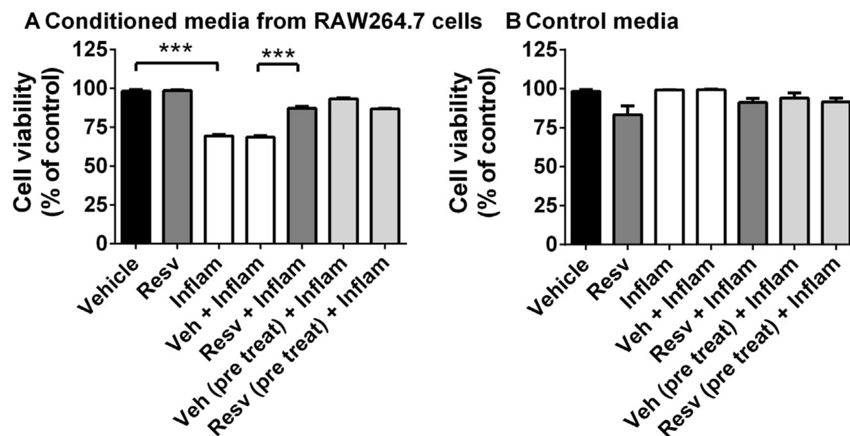

Fig. 5. Conditioned media from inflammatory activated RAW264.7 cells reduced viability of Neuro2A cells, which could be prevented with resveratrol treatment of RAW264.7 cells. (A) Cell viability of RAW264.7 cells 24 h following treatment with inflammatory activators (inflam; LPS and IFN-γ) ± pre-incubation for 1 h with vehicle (veh) or resveratrol (resv) as in Fig. 4. To confirm the effects were driven by resveratrol on the RAW264.7 cells rather than on Neuro2A cells, Neuro2A cells were also pre-treated with vehicle or resveratrol prior to application of the RAW264.7 media (Veh (pre-treat) + inflam; Resv (pretreat) + Inflam). (B) Cell viability of Neuro2A cells was measured following transfer of the same media mixes as in (A) but these were added directly to the Neuro2a cells in the absence of incubation with RAW264.7 cells. Data shown are mean ± SEM, n=4.

present in grape skin, artichoke and basil, with purportedly neuroprotective actions (reviewed in (Jayasena et al., 2013)). To study the effects of resveratrol we compared macrophage and microglial cell lines during activation by LPS and IFN-γ. Both cell lines expressed a panel of pro-inflammatory cytokines following activation, though C8–B4 microglial cells were more limited in their repertoire. RAW264.7 macrophages released higher levels of 15 out of 17 cytokines tested and four times as much NO (measured as nitrite) compared to C8–B4 microglial cells. Therefore RAW264.7 macrophages show a more reactive phenotype than C8-B4 cells following activation with the same concentrations of LPS and IFN-γ.

4.2. Resveratrol exhibits anti-inflammatory properties

Resveratrol reduced 7 out of 9 pro-inflammatory cytokines from C8–B4 cells and 5 out of 9 pro-inflammatory cytokines from RAW264.7 cells. It also significantly reduced nitrite levels in both lines, but had a more limited effect on chemokine release, reducing only MCP-1 and RANTES in macrophages. Additionally, resveratrol treatment did also reduce the anti-inflammatory cytokine IL-10. However our experiments also showed that resveratrol protects against pro-inflammatory or cytotoxic molecules released into the media by activated macrophages. Importantly the concentrations of resveratrol that had an identifiable anti-inflammatory effect are consistent with concentrations that can be found in the plasma (μM levels) (Walker et al., 2014). In addition it is likely that resveratrol metabolites, including resveratrol-3-sulfate also possess anti-inflammatory activities that could provide further anti-inflammatory activity (Walker et al., 2014).

Consistent with our findings, resveratrol reduced the levels of IL-6, NO, and TNF-α in RAW264.7 cells (Yang et al., 2014). In this study, the expression levels of high mobility group protein B1 and toll-like receptor 4 were reduced following resveratrol treatment, hinting at potential mechanisms involved in these anti-inflammatory effects. Our study builds on these findings providing a comprehensive view of the down-regulation of cytokines by resveratrol. A previous study identified that resveratrol

similarly down-regulated MCP-1 mRNA, to reduce foam cell formation, potentially through up-regulation of AMP-activated protein kinase (AMPK) and sirtuin-1 (Dong et al., 2014). Our data together with that of others is therefore generating a picture of the broad anti-inflammatory actions of resveratrol. Such broad and potent anti-inflammatory activities have led to proposals of resveratrol treatment for diverse conditions, including cancer (Jang et al., 1997), pain (Gentilli et al., 2001), neurodegenerative disease (Virgili and Contestabile, 2000), obesity (Baur et al., 2006), diabetes (Su et al., 2006), rheumatoid arthritis (Elmali et al., 2007) and depression (Xu et al., 2010). However, there are also examples in which resveratrol dietary supplementation worsened clinical severity, for example in viral and autoimmune mouse models of multiple sclerosis (Sato et al., 2013), suggesting that resveratrol would make a poor option for therapeutic treatment of demyelinating diseases. In addition, our data suggest that there is a narrow therapeutic window for resveratrol with higher levels contributing to cytotoxicity. The design of the study focuses on the protective effects of resveratrol in reducing pro-inflammatory cytokines and the release of soluble cytotoxic factors but it must also be noted that there are many putative beneficial biological roles of activation of these cells. For example, activation of microglia allows the removal of toxic aggregates and cellular debris and the effects of resveratrol in vivo must be tempered with the possibility that local immune suppression contributes to neurodegeneration (Kan et al., 2015). Similarly, as a counter to the reductions in pro-inflammatory cytokines by resveratrol treatment, it must be noted that resveratrol also inhibited the release of the anti-inflammatory cytokine, IL-10, and had a limited effect on chemokine release. A recent study using hippocampal astrocytes identified a role for resveratrol in down-regulating TNF-α, IL-1β, IL-6 and IL-10 in astrocytes following LPS activation (Bellaver et al., 2015). The mechanisms identified were similar to those in neurons and involved NF-κB, p38 and MAPK signalling (Bellaver et al., 2015). Other mechanisms proposed for the protective effects of resveratrol in primary astrocytes are reviewed in (Quincozes-Santos and Gottfried, 2011). For example, changes in glutamate handling (Vieira de Almeida et al., 2007) and protection via the activation of nuclear factor erythroid 2-related factor 2 (Erlank et al., 2011), a transcription factor that protects astrocytes against glutathione reduction and oxidative stress (Steele et al., 2013a, 2013b) have been implicated. Future in vivo studies and co-culture experiments will help to delineate the specific roles of individual cytokines and chemokines in distinct cell types in mediating neurodegenerative and neuroprotective mechanisms.

4.3. Effects of resveratrol in vivo and its potential as a neuroprotective agent

An in-depth study of the levels of inflammatory markers in humans following resveratrol ingestion has not yet been carried out. However, consistent with our findings of a large down-regulation of pro-inflammatory molecules, a small study using an extract of *Polygonum Cuspidatum* (containing 40 mg resveratrol) taken daily for 6 weeks did lead to the reduction of plasma concentrations of TNF-α and IL-6 (Ghanim et al., 2010). In a recent study in a mouse model of familial Alzheimer's disease, long-term resveratrol treatment reduced amyloid and prevented memory loss, potentially via activation of sirtuin-1 and AMPK signalling pathways (Porquet et al., 2014). Surprisingly this occurred even following an increase in IL-1β and TNF-α. Potent anti-inflammatory activity for resveratrol has now been identified in a number of disease models in mice, including reduced lung inflammation through increasing superoxide dismutase, glutathione peroxidase, and catalase activity by enhancing nuclear translocation of NF-κB and heme oxygenase-1 activity (Liu et al., 2014). Multiple mechanisms for the neuroprotective activity of resveratrol have been identified (reviewed in (Renaud and Martinoli, 2014)). Suggested mechanisms for resveratrol in protecting neurons against Alzheimer's disease include by preventing oxidative stress and toxic amyloid aggregation (Ladiwala et al., 2010; Shariatizi et al., 2015) and this could occur by augmentation in the expression and activity of anti-oxidant enzymes, such as catalase and superoxide dismutase (Sadi and Konat, 2015). Other findings have highlighted a mechanism of action for resveratrol in activating sirtuin-1 to inhibit p53 transcription and enhance neuroprotection (Feng et al., 2015) or via MAPKs, NF-κB and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) signalling pathways (Zhang et al., 2010).

We have previously shown that resveratrol has pro-energetic effects in Neuro2a cells with the ability to increase ATP and GTP levels in the cells (Nguyen et al., 2013). In line with this, resveratrol increased cell viability, an effect that was most evident in cells treated with high levels of the NO donor but not in the absence of NO (Figs. 2, 4 and 5). NO is a potent activator of AMPK and in turn AMPK protects cells against the damaging effects of NO; in pancreatic β cells this mechanism involves activation of inositol-requiring enzyme 1 (Meares et al., 2011). During disruption of energy homeostasis AMPK is activated by an increased AMP/ATP ratio (Sanders et al., 2007). In pancreatic β cells AMPK activation by NO improved metabolic function of β-cells to prevent cell death following NO treatment (Sanders et al., 2007). Resveratrol did not increase the energy charge potential (a measure of energy status of the cell) of Neuro2a cells in conditions of sufficient nutrition but only during starvation conditions (Nguyen et al., 2013). Our current results suggest that resveratrol is also protective under conditions of nitrosative stress in Neuro2a cells. Our data do not support a role for resveratrol in direct scavenging of nitric oxide (Figs. 2, 4 and 5) but rather suggest it promotes a cytoprotective pathway. Thus it is possible that resveratrol promotes cell survival of Neuro2a cells against NO-mediated cell death via the activation of AMPK, in addition to its anti-inflammatory effects.

An important issue in considering resveratrol as a potential neuroprotective agent is whether it can exert functional effects in the brain following peripheral administration or ingestion. In line with this consideration, brain inflammation caused by streptozotocin injections induced cognitive impairment in rats, yet memory performance was enhanced following peripheral resveratrol administration (Sharma and Gupta, 2002). In mice, resveratrol administration upregulated sirtuin-1 to protect neurons against neurodegeneration in models of Alzheimer's disease and motor neurone disease (Kim et al., 2007). These findings have now been confirmed in adult primates, in which dietary resveratrol treatment (200 mg/kg/day) for 18 months increased working memory and cognitive performance (Dal-Pan et al., 2011). Importantly, these studies have recently been extended to humans and a picture of the neuroprotective effects of resveratrol in aging adults is emerging. Following 26 weeks of resveratrol intake (200 mg/day) older adults showed significant increases in hippocampal functional connectivity and memory performance, demonstrating an increased retention of words (Witte et al., 2014). We have studied the effects of nitric oxide and inflammation on Neuro2a cell viability. Technical developments in generating the many distinct cell types of the brain using induced pluripotent stem cells will provide a more accurate model of the human nervous system in vitro (Ooi et al., 2013b). Future studies will capitalise on these advancements to identify the contribution of distinct cell types and molecular mechanisms involved in neuroprotection and neurodegeneration.

5. Conclusions

The findings from our study suggest that resveratrol has broad anti-inflammatory effects and is capable of down-regulating pro-inflammatory responses in cellular models of both CNS-resident microglia and CNS-infiltrating peripheral macrophages. Our findings provide further evidence of the neuroprotective potential of resveratrol during neuroinflammation.

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References

Balez, R., Ooi, L., 2015. Getting to NO Alzheimer's disease: neuroprotection versus neurotoxicity mediated by nitric oxide. Oxidative Med. Cell. Longev. Article ID 278164, (in press).
Baur, J.A., Pearson, K.J., Price, N.L., Jamieson, H.A., Lerin, C., Kalra, A., Prabhu, V.V., Allard, J.S., Lopez-Lluch, G., Lewis, K., Pistell, P.J., Poosala, S., Becker, K.G., Boss, O., Gwinn, D., Wang, M., Ramaswamy, S., Fishbein, K.W., Spencer, R.G., Lakatta, E.G., Le Couteur, D., Shaw, R.J., Navas, P., Puigserver, P., Ingram, D.K., de Cabo, R., Sinclair, D.A., 2006. Resveratrol improves health and survival of mice on a high-calorie diet. Nature 444, 337–342.
Bellaver, B., Souza, D., Bobermin, L., Souza, D., Gonçalves, C.-A., Quincozes-Santos, A., 2015. Resveratrol protects hippocampal astrocytes against LPS-induced neurotoxicity through HO-1, p38 and ERK pathways. Neurochem. Res. 40, 1600–1608.
Brantley, E.C., Guo, L., Zhang, C., Lin, Q., Yokoi, K., Langley, R.R., Kruzel, E., Maya, M., Kim, S.W., Kim, S.-J., Fan, D., Fidler, I.J., 2010. Nitric oxide-mediated Tumoricidal activity of murine microglial cells. Transl. Oncol. 3, 380–388.
Carson, M., Bilousova, T., Puntambekar, S., Melchior, B., Doose, J., Ethell, I., 2007. A rose by any other name? the potential consequences of microglial heterogeneity during CNS health and disease. Neurotherapeutics 4, 571–579.
Dal-Pan, A., Pifferi, F., Marchal, J., Picq, J.-L., Aujard, F., on behalf of RC, 2011. Cognitive performances are selectively enhanced during chronic caloric restriction or resveratrol supplementation in a primate. PLoS ONE 6, e16581.
Dong, W., Wang, X., Bi, S., Pan, Z., Liu, S., Yu, H., Lu, H., Lin, X., Wang, X., Ma, T., Zhang, W., 2014. Inhibitory effects of resveratrol on foam cell formation are mediated through monocyte chemotactic protein-1 and lipid metabolism-related proteins. Int. J. Mol. Med. 33, 1161–1168.
Elmali, N., Baysal, O., Harma, A., Esenkaya, I., Mizrak, B., 2007. Effects of resveratrol in inflammatory arthritis. Inflammation 30, 1–6.
Erlank, H., Elmann, A., Kohen, R., Kanner, J., 2011. Polyphenols activate Nrf2 in astrocytes via H2O2, semiquinones, and quinones. Free Radic. Biol. Med. 51, 2319–2327.
Feng, Y., Liu, T., Dong, S.-Y., Guo, Y.-J., Jankovic, J., Xu, H., Wu, Y.-C., 2015. Rotenone affects p53 transcriptional activity and apoptosis via targeting SIRT1 and H3K9 acetylation in SH-SY5Y cells. J. Neurochem. 134, 668–676.
Gamper, N., Ooi, L., 2015 Feb 20. Redox and nitric oxide-mediated regulation of sensory neuron ion channel function. Antioxid. Redox Signal. 22 (6), 486–504.
Gentilli, M., Mazoit, J.X., Bouaziz, H., Fletcher, D., Casper, R.F., Benhamou, D., Savouret, J.-F., 2001. Resveratrol decreases hyperalgesia induced by carrageenan in the rat hind paw. Life Sci. 68, 1317–1321.
Ghanim, H., Sia, C.L., Abuaysheh, S., Korzeniewski, K., Patnaik, P., Marumganti, A., Chaudhuri, A., Dandona, P., 2010. An Antiinflammatory and reactive oxygen species suppressive effects of an extract of polygonum cuspidatum containing resveratrol. J. Clin. Endocrinol. Metabolism 95, E1–E8.
Guillemin, G.J., Brew, B.J., 2004. Microglia, macrophages, perivascular macrophages, and pericytes: a review of function and identification. J. Leukoc. Biol. 75, 388–397.
Heneka, M.T., Kummer, M.P., Latz, E., 2014. Innate immune activation in neurodegenerative disease. Nat. Rev. Immunol. 14, 463–477.
Jang, M., Cai, L., Udeani, G.O., Slowing, K.V., Thomas, C.F., Beecher, C.W.W., Fong, H.H.S., Farnsworth, N.R., Kinghorn, A.D., Mehta, R.G., Moon, R.C., Pezzuto, J.M., 1997. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science 275, 218–220.
Jayasena, T., Poljak, A., Smythe, G., Braidy, N., Münch, G., Sachdev, P., 2013. The role of polyphenols in the modulation of sirtuins and other pathways involved in Alzheimer's disease. Ageing Res. Rev. 12, 867–883.
Kan, M.J., Lee, J.E., Wilson, J.G., Everhart, A.L., Brown, C.M., Hoofnagle, A.N., Jansen, M., Vitek, M.P., Gunn, M.D., Colton, C.A., 2015. Arginine deprivation and immune suppression in a mouse model of Alzheimer's disease. J. Neurosci. 35, 5969–5982.
Karunaweera, N., Raju, R., Gyengesi, E., Muench, G., 2015. Plant polyphenols as inhibitors of NF-κB induced cytokine production — a potential anti-inflammatory treatment for Alzheimer's disease? Front. Mol. Neurosci. 8.
Kettenmann, H., Hanisch, U.K., Noda, M., Verkhratsky, A., 2011. Physiology of microglia. Physiol. Rev. 91, 461–553.
Kim, D., Nguyen, M.D., Dobbin, M.M., Fischer, A., Sananbenesi, F., Rodgers, J.T., Delalle, I., Baur, J.A., Sui, G., Armour, S.M., Puigserver, P., Sinclair, D.A., Tsai, L.H., 2007. SIRT1 Deacetylase Protects against Neurodegeneration in Models for Alzheimer's Disease and Amyotrophic Lateral Sclerosis.
Kraft, A.D., Harry, G.J., 2011. Features of microglia and neuroinflammation relevant to environmental exposure and neurotoxicity. Int. J. Environ. Res. Public Health 8, 2980–3018.
Ladiwala, A.R.A., Lin, J.C., Bale, S.S., Marcelino-Cruz, A.M., Bhattacharya, M., Dordick, J.S., Tessier, P.M., 2010. Resveratrol selectively remodels soluble Oligomers and Fibrils of amyloid Aβ into Off-pathway conformers. J. Biol. Chem. 285, 24228–24237.
Lawson, L.J., Perry, V.H., Dri, P., Gordon, S., 1990. Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. Neuroscience 39, 151–170.
Lawson, L.J., Perry, V.H., Gordon, S., 1992. Turnover of resident microglia in the normal adult mouse brain. Neuroscience 48, 405–415.
Liu, H., Ren, J., Chen, H., Huang, Y., Li, H., Zhang, Z., Wang, J., 2014. Resveratrol protects against cigarette smoke—induced oxidative damage and Pulmonary inflammation. J. Biochem. Mol. Toxicol. 28, 465–471.
Luo, X.-G., Chen, S.-D., 2012. The changing phenotype of microglia from homeostasis to disease. Transl. Neurodegener. 1, 9.
McCoy, M.K., Tansey, M.G., 2008. TNF signaling inhibition in the CNS: implications for normal brain function and neurodegenerative disease. J. Neuroinflammation 5, 45.
Meares, G.P., Hughes, K.J., Naatz, A., Papa, F.R., Urano, F., Hansen, P.A., Benveniste, E.N., Corbett, J.A., 2011. IRE1-Dependent activation of AMPK in response to nitric oxide. Mol. Cell. Biol. 31, 4286–4297.
Mitchell, J.A., Warner, T.D., 2006. COX isoforms in the cardiovascular system: understanding the activities of non-steroidal anti-inflammatory drugs. Nat. Rev. Drug Discov. 5, 75–86.
Münch, G., Gasic-Milenkovic, J., Dukic-Stefanovic, S., Kuhla, B., Heinrich, K., Riederer, P., Huttunen, H., Founds, H., Sajithlal, G., 2003. Microglial activation induces cell death, inhibits neurite outgrowth and causes neurite retraction of differentiated neuroblastoma cells. Exp. Brain Res. 150, 1–8.
Nguyen, N.T.Q., Ooi, L., Piller, S.C., Münch, G., 2013. Proenergetic effects of resveratrol in the murine neuronal cell line Neuro2a. Mol. Nutr. Food Res. 57, 1901–1907.
Norden, D.M., Muccigrosso, M.M., Godbout, J.P., 2015 Sep. Microglial priming and enhanced reactivity to secondary insult in aging, and traumatic CNS injury, and neurodegenerative disease. Neuropharmacology 96 (Pt A), 29–41.
Ooi, L., Gigout, S., Pettinger, L., Gamper, N., 2013a. Triple cysteine module within M-type K(+) channels mediates reciprocal channel modulation by nitric oxide and reactive oxygen species. J. Neurosci. official J. Soc. Neurosci. 33, 6041–6046.
Ooi, L., Sidhu, K., Poljak, A., Sutherland, G., O'Connor, M., Sachdev, P., Münch, G., 2013b. Induced pluripotent stem cells as tools for disease modelling and drug discovery in Alzheimer's disease. J. Neural Transm. 120, 103–111.
Porquet, D., Griñán-Ferré, C., Ferrer, I., Camins, A., Sanfeliu, C., Del Valle, J., Pallàs, M., 2014. Neuroprotective role of trans-resveratrol in a murine model of familial Alzheimer's disease. J. Alzheimers Dis. 42, 1209–1220.
Quincozes-Santos, A., Gottfried, C., 2011. Resveratrol modulates astroglial functions: neuroprotective hypothesis. Ann. N. Y. Acad. Sci. 1215, 72–78.
Renaud, J., Martinoli, M.G., 2014. Resveratrol as a protective molecule for neuroinflammation: a review of mechanisms. Curr. Pharm. Biotechnol. 15, 318–329.
Sadi, G., Konat, D., 2015. Resveratrol regulates oxidative biomarkers and antioxidant enzymes in the brain of streptozotocin-induced diabetic rats. Pharm. Biol. 1–8.
Sanders, Matthew J., Grondin, Pascal O., Hegarty, Bronwyn D., Snowden, Michael A., Carling, D., 2007. Investigating the mechanism for AMP activation of the AMP-activated protein kinase cascade. Biochem. J. 403, 139–148.
Sato, F., Martinez, N.E., Shahid, M., Rose, J.W., Carlson, N.G., Tsunoda, I., 2013. Resveratrol exacerbates both autoimmune and viral models of multiple sclerosis. Am. J. Pathology 183, 1390–1396.
Schmid, C.D., Melchior, B., Masek, K., Puntambekar, S.S., Danielson, P.E., Lo, D.D., Gregor Sutcliffe, J., Carson, M.J., 2009. Differential gene expression in LPS/IFNγ activated microglia and macrophages: in vitro versus in vivo. J. Neurochem. 109, 117–125.
Shariatizi, S., Meratan, A.A., Ghasemi, A., Nemat-Gorgani, M., 2015. Inhibition of amyloid fibrillation and cytotoxicity of lysozyme fibrillation products by polyphenols. Int. J. Biol. Macromol. 80, 95–106.
Sharma, M., Gupta, Y.K., 2002. Chronic treatment with trans resveratrol prevents intracerebroventricular streptozotocin induced cognitive impairment and oxidative stress in rats. Life Sci. 71, 2489–2498.
Simard, A.R., Soulet, D., Gowing, G., Julien, J.-P., Rivest, S., 2006. Bone Marrow-derived microglia play a critical role in restricting senile Plaque formation in Alzheimer's disease. Neuron 49, 489–502.
Singh, U.P., Singh, N.P., Singh, B., Hofseth, L.J., Price, R.L., Nagarkatti, M., Nagarkatti, P.S., 2010. Resveratrol (Trans-3,5,4′-trihydroxystilbene) induces silent mating type information Regulation-1 and down-regulates nuclear transcription factor-κB activation to Abrogate Dextran sulfate sodium-induced colitis. J. Pharmacol. Exp. Ther. 332, 829–839.
Steele, M., Fuller, S., Maczurek, A., Kersaitis, C., Ooi, L., Münch, G., 2013a. Chronic inflammation Alters production and release of glutathione and related thiols in human U373 astroglial cells. Cell Mol. Neurobiol. 33, 19–30.
Steele, M.L., Fuller, S., Patel, M., Kersaitis, C., Ooi, L., Münch, G., 2013b. Effect of Nrf2 activators on release of glutathione, cysteinylglycine and homocysteine by

human U373 astroglial cells. Redox Biol. 1, 441–445.

Su, H.-C., Hung, L.-M., Chen, J.-K., 2006. Resveratrol, a Red Wine Antioxidant, Possesses an Insulin-like Effect in Streptozotocin-induced Diabetic Rats.

Vane, J.R., Bakhle, Y.S., Botting, R.M., 1998. Cycloygenases1 and 2. Annu. Rev. Pharmacol. Toxicol. 38, 97–120.

Vieira de Almeida, L., Piñeiro, C., Leite, M., Brolese, G., Tramontina, F., Feoli, A., Gottfried, C., Gonçalves, C.-A., 2007. Resveratrol increases glutamate uptake, glutathione content, and S100B secretion in cortical astrocyte cultures. Cell Mol. Neurobiol. 27, 661–668.

Virgili, M., Contestabile, A., 2000. Partial neuroprotection of in vivo excitotoxic brain damage by chronic administration of the red wine antioxidant agent, trans-resveratrol in rats. Neurosci. Lett. 281, 123–126.

Walker, J., Schueller, K., Schaefer, L.-M., Pignitter, M., Esefelder, L., Somoza, V., 2014. Resveratrol and its metabolites inhibit pro-inflammatory effects of lipopolysaccharides in U-937 macrophages in plasma-representative concentrations. Food Funct. 5, 74–84.

Witte, A.V., Kerti, L., Margulies, D.S., Flöel, A., 2014. Effects of resveratrol on memory performance, hippocampal functional connectivity, and glucose metabolism in healthy older adults. J. Neurosci. 34, 7862–7870.

Xu, Y., Wang, Z., You, W., Zhang, X., Li, S., Barish, P.A., Vernon, M.M., Du, X., Li, G., Pan, J., Ogle, W.O., 2010. Antidepressant-like effect of trans-resveratrol: involvement of serotonin and noradrenaline system. Eur. Neuropsychopharmacol. 20, 405–413.

Yang, Y., Li, S., Yang, Q., Shi, Y., Zheng, M., Liu, Y., Chen, F., Song, G., Xu, H., Wan, T., He, J., Chen, Z., 2014. Resveratrol reduces the proinflammatory effects and lipopolysaccharide- induced expression of HMGB1 and TLR4 in RAW264.7 cells. Cell. Physiol. Biochem. 33, 1283–1292.

Zhang, F., Liu, J., Shi, J.S., 2010. Anti-inflammatory activities of resveratrol in the brain: role of resveratrol in microglial activation. Eur. J. Pharmacol. 636, 1–7.