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Neuroprotective effects of hesperetin in mouse primary neurones are independent of CREB activation

Stephanie Rainey-Smith[a], Lars-Wilhelm Schroetke[a], Parmvir Bahia[a], Ahmed Fahmi[a], Rachel Skilton[a], Jeremy P.E. Spencer[a,1], Catherine Rice-Evans[a], Marcus Rattray[a,b,*], Robert J. Williams[a]

[a] King's College London, The Wolfson Centre for Age-Related Diseases, Guy's Hospital Campus, London SE1 1UL, UK
[b] University of Reading, Reading School of Pharmacy, PO Box 228, Whiteknights, Reading RG6 6AJ, UK

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ABSTRACT

Dietary flavonoids, including the citrus flavanone hesperetin, may have stimulatory effects on cytoprotective intracellular signalling pathways. In primary mouse cortical neurone cultures, but not SH-SY5Y human neuroblastoma cells or human primary dermal fibroblasts (Promocells), hesperetin (100–300 nM, 15 min) caused significant increases in the level of ERK1/2 phosphorylation, but did not increase CREB phosphorylation. Administration of hesperetin for 18 h did not alter gene expression driven by the cyclic AMP response element (CRE), assessed using a luciferase reporter system, but 300 nM hesperetin partially reversed staurosporine-induced cell death in primary neurones. Our data show that hesperetin is a neuroprotective compound at concentrations where antioxidant effects are unlikely to predominate. The effects of hesperetin are cell-type dependent and, unlike the flavanol (−)epicatechin, neuroprotection *in vitro* is not associated with enhanced CREB phosphorylation or CRE-mediated gene expression.

Consumption of dietary flavonoids has been associated with reduced risk of neurodegenerative diseases in man [15,26,31], neuroprotection against disease-relevant insults in mice or rats [8,12,13,17,34] and improvements in cognition and learning in animal models [7,8,14,27,30]. The mechanisms are often assumed to involve the hydrogen-donating antioxidant activities of flavonoids but, in addition to their antioxidant effects, flavonoids at low concentrations can also modulate intracellular signalling pathways [32]. Several flavonoids have been shown to influence one or more components of cytoprotective intracellular signalling cascades including phosphatidylinositol 3-kinase (PI 3-kinase), tyrosine kinases and MAP kinases [1,21,23,25,28,29,33].

(−)Epicatechin is one of the most important bioactive flavonoids derived from a flavanol-rich diet [22]. When applied to primary cortical neurones at concentrations of 100–300 nM for 15 min, (−)epicatechin produces rapid phosphorylation of ERK and CREB, two components of the pro-survival mitogen activated protein kinase signalling cascade and enhances cyclic AMP response element (CRE)-mediated gene expression in neurones [23]. However, there are five other major families of flavonoids and phenolics in the human diet, and relatively little information to date as to whether flavonoids of different classes act through similar or distinct intracellular cellular mechanisms, and whether those mechanisms are universal or found only in some cell-types. The flavanone hesperetin is the major circulating aglycone metabolite of hesperidin, abundant in citrus fruit and drinks [6,9]. Hesperetin *in vitro* is known to act as an antioxidant and scavenger of peroxynitrite [3,18,19] and might modulate signalling pathways in an analogous way to (−)epicatechin. For example, in fibroblasts and neurones *in vitro*, hesperetin can promote ERK phosphorylation [18,28]. *In vivo*, hesperetin feeding to rats suppressed kidney NfκB-mediated gene expression through modulation of ERK, JNK and p38 pathways [10].

Recently, hesperetin has been reported to afford neuroprotection *in vitro* through an Akt- and ERK-dependent mechanism [28], but whether this involves the activation of CREB, as previously shown for (−)epicatechin, is not known. The main aim of the study was to test this possibility and to characterise whether the effects of hesperetin extend to other cell types as well as cortical neurones.

All reagents, unless specified, were from Sigma or Merck.

Primary cortical neurones were prepared from 15 to 16-day-old Swiss mouse embryos (NIH, Harlan, UK) as described previously [4]. The SH-SY5Y human neuroblastoma cell line was obtained from Dr Peter Vaughan. SH-SY5Y neuroblastoma were cultured in 1:1 Ham's F12 medium: Eagle's MEM supplemented with 10% foetal calf

* Corresponding author at: University of Reading, Reading School of Pharmacy, PO Box 228, Whiteknights, Reading RG6 6AJ, UK. Tel.: +44 118 378 7892; fax: +44 118 378 4703.
E-mail address: m.a.n.rattray@reading.ac.uk (M. Rattray).
[1] Current address: Molecular Nutrition Group, University of Reading, School of Chemistry, Food Biosciences and Pharmacy, UK.

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serum, 1% non-essential amino acids and 0.1% gentamicin. Human dermal fibroblasts were obtained from PromoCell (Heidelberg, Germany), cultured in fibroblast basal medium (PromoCell) supplemented with insulin (5 μg/ml), bFGF (1 ng/ml), amphotericin (50 ng/ml) and gentamicin (50 μg/ml). Promocells were switched to medium without bFGF 18 h before flavonoid treatment to reduce basal protein kinase activity (not shown).

(−)Epicatechin and hesperetin (Extrasynthase, Genay, France) were dissolved in 100% methanol and stored in sealed aliquots at −70 °C. Cells were washed twice with HEPES-buffered medium (HBM: 20 mM HEPES, 140 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 6 mM glucose, 5 mM $NaHCO_3$, 1.2 mM Na_2HPO_4 pH 7.4) before exposure to fresh (−)epicatechin or hesperetin (10 nM–10 μM) in HBM for 15 min at 37 °C or overnight directly into the growth medium for neuroprotection studies.

Cells were washed once with ice-cold PBS, pH 7.4, containing EGTA (200 μM) and lysed at 4 °C in lysis buffer (50 mM Tris, 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.5 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml antipain, 2 μg/ml pepstatin A, 1 μg/ml chymostatin, 5 mM sodium pyrophosphate, 1 mM Na_3VO_4 and 50 mM NaF). Lysates were collected and centrifuged at 2000 × *g* (5 min, 4 °C), protein concentration determined by the BioRad Bradford protein assay. Samples were treated for 3 min at 95 °C in boiling buffer (62.5 mM Tris, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.0025% bromophenol blue). Samples (30 or 40 μg/lane) were run on 9% SDS-polyacrylamide gels and proteins transferred to nitrocellulose membranes (Hybond-ECL; GE Healthcare) by semi-dry electroblotting. Membranes were blocked in TBS (20 mM Tris, pH 7.5, 0.5 M NaCl) containing 4% skimmed milk powder (25 min) then washed twice in TBS containing 0.05% Tween 20 (TTBS). The blots were incubated overnight with antibodies in TTBS containing 1% skimmed milk powder. Antisera were anti-ACTIVE MAPK (ERK1/2, Promega, 1:2500 dilution), anti-ERK 2 (C14, Santa Cruz Biotechnology, 1:1000), anti-phospho-CREB(Ser^{133}) (Cell Signaling Technology 1:1000) or anti-CREB (Cell Signaling Technology 1:1000). Blots were washed twice in TTBS and then incubated with goat anti-rabbit IgG peroxidase conjugate (1:1000) in antibody buffer for 45 min. Blots were washed twice in TTBS and once in TBS before exposure to ECL Western blotting detection reagents and autoradiography (GE Healthcare). Bands were analysed using BioImage Intelligent Quantifier software (Bio Image Systems Inc.).

Cortical neurones cultured in 24-well plates (5×10^5 /well) were transfected overnight with 0.25 μg of the pCRE-luc *cis* reporter plasmid (Stratagene) and 0.25 μg of the transfection efficiency *Renilla* luciferase pRL-TK plasmid (Promega) using 1 μl LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. The growth medium was removed 24 h later and neurones stimulated for 15 min at 37 °C in HBM containing hesperetin (100 and 300 nM) or 50 ng/ml brain-derived neurotrophic factor (BDNF, Peprotech). After 15 min the compounds were removed and replaced with conditioned medium and the cells returned to the CO_2 incubator for a further 18 h. Luciferase assays were performed using a Dual-Glo luciferase assay system as described in the manufacturer's instructions (Promega). Lysates were read in a 96-well luminometer plate (Sigma) and luciferase activities produced by the pCRE luciferase reporter plasmid measured using a Veritas microplate luminometer (Turner Biosystems Inc.). *Renilla* luciferase activities produced by the pRL-TK control plasmid were then assayed by adding an equal volume of Dual-Glo Stop & Glo substrate and measuring again in the luminometer. All treatments were performed in quadruplicate on 5–6 independent cultures. Firefly luciferase activities were standardized to the corresponding *Renilla* luciferase activities.

Hesperetin was added directly to the culture medium for 24 h before neurones were exposed to staurosporine (10 μM) for a further 24 h. Cell viability was then assessed by Propidium Iodide-Hoe 33342 double staining and by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. For double staining the medium was removed and neurones were incubated with Hoe 33342 (10 μM, all cells) and propidium iodide (10 μM, dead cells) in HBM for 15 min. Cell images were captured and the numbers of dead cells relative to total cells were quantified using an *In Cell Analyser* 1000 (GE Healthcare). MTT turnover was used as an indirect measure of cell viability and mitochondrial function. Following exposure to staurosporine, cells were washed twice in PBS before the addition of MTT (0.5 mg/ml) in HBM for 60 min at 37 °C. HBM was removed, formazan solubilised in DMSO (100%) and absorbance read at 505 nm.

Mouse primary neurones, human SH-SY5Y neuroblastoma cells or human primary dermal fibroblasts (PromoCells) were exposed to a range of concentrations of hesperetin (10 nM–10 μM) or (−)epicatechin (10 nM–10 μM) for 15 min. Primary neurones, SH-SY5Y neuroblastoma and human primary dermal fibroblasts when exposed to hesperetin all showed increased pERK over a broad concentration range in a concentration-related manner. The concentration-dependency responses were bell-shaped which is typical for these compounds (Fig. 1A). Table 1 shows some of the data from these experiments (results using 100 nM, 300 nM and

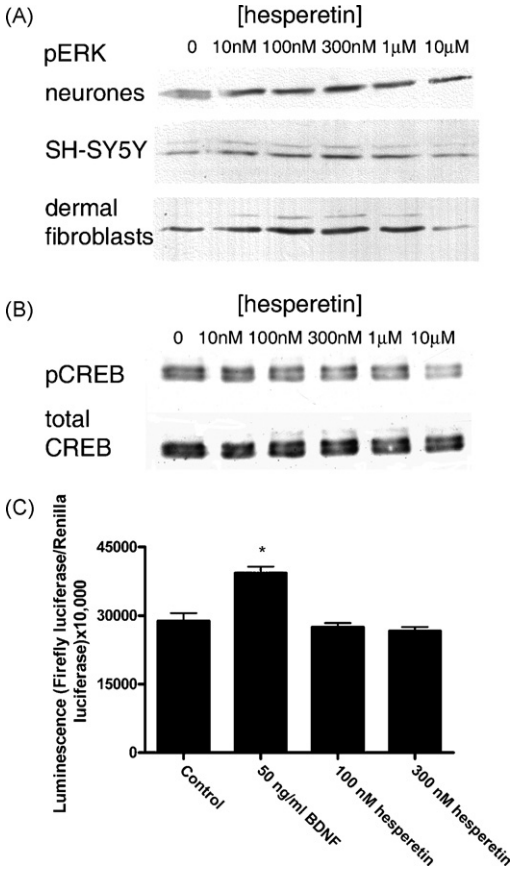

Fig. 1. Panel A shows representative Western blots probed for phosphorylated ERK1/2 for primary neurones, SH-SY5Y neuroblastoma and human dermal fibroblasts (Promocells) exposed to hesperetin (10 nM–10 μM) for 15 min before protein extraction. Panel B shows the effect of hesperetin (10 nM–10 μM) for 15 min on phosphorylated and total CREB. Panel C shows the effect of overnight incubations with 100 nM or 300 nM hesperetin on CRE-mediated luciferase expression in neurones co-transfected with a CRE-firefly luciferase reporter plasmid and an internal control plasmid which expressed renilla luciferase. Brain-derived neurotrophic factor (50 ng/ml) was used as a positive control to drive CRE-mediated gene expression. Data show mean luminescence levels, i.e. firefly luciferase/*Renilla* luciferase activities ×10,000. Error bars are SEM, $n = 3$. $^*p < 0.05$.

Table 1
Effect of hesperetin or (−)epicatechin on ERK phosphorylation in three different cell types

	Mouse primary cortical neurones	Human neuroblastoma SH-SY5Y	Human primary dermal fibroblasts
Hesperetin			
100 nM	265 ± 80 (4)**	166 ± 63 (5)	238 ± 78 (4)
300 nM	187 ± 37 (4)*	219 ± 86 (5)	251 ± 50 (4)
1 μM	175 ± 47 (4)	151 ± 44 (5)	192 ± 40 (4)
(−)Epicatechin			
100 nM	378 ± 40 (4)**	109 ± 21 (5)	155 ± 22 (4)
300 nM	260 ± 28 (4)**	189 ± 91 (5)	160 ± 41 (4)
1 μM	160 ± 26 (4)	129 ± 21 (5)	187 ± 50 (4)*

Numbers represent means ± S.E.M. of phosphorylated ERK band intensity relative to that found in untreated controls. Number of experiments indicated in brackets. *$p < 0.05$; **$p < 0.01$ (ANOVA followed by Dunnett's Multiple Comparison Test).

1 μM are shown). While hesperetin enhanced pERK in each of the three cell types tested, only in primary neurones were the increases statistically significant. In primary neurones, 100 nM hesperetin, caused a greater than twofold increase in pERK levels compared to controls, and while pERK levels were increased in dermal fibroblasts and SH-SY5Y cells, these increases were not significant (Table 1). At 10 nM and 10 μM, hesperetin did not enhance pERK levels (data not shown) in primary neurones or any of the other cell types tested. In primary neurones, 100 nM (−)epicatechin caused a greater than threefold increase in pERK (Table 1), and, like hesperetin, produced modest increases of pERK in primary dermal fibroblasts and SH-SY5Y neuroblastoma cells.

We have previously reported that (−)epicatechin stimulates CREB phosphorylation and recruitment of CRE-mediated gene expression in primary neurones via a mechanism that is partially dependent on ERK and Akt activation [23]. Since hesperetin stimulates pERK in neurones, we tested hesperetin (10 nM–10 μM, 15 min) for its ability to alter the levels of Ser^{133}-phosphorylated CREB (pCREB). Hesperetin caused no increase in the levels of pCREB relative to total CREB (Fig. 1B), although interestingly at the highest concentration tested (10 μM) hesperetin reduced the levels of pCREB relative to total CREB. To assess whether hesperetin caused gene expression driven by the CRE, primary mouse neurones were co-transfected with a CRE-reporter plasmid, which contains firefly luciferase, and as an internal standard control, *Renilla* luciferase plasmid. Hesperetin at 100 nM and 300 nM (18 h) did not alter the levels of CRE-mediated luciferase expression compared to untreated controls (Fig. 1C).

Hesperetin signalling through ERK may be neuroprotective even in the absence of changes in CREB-mediated gene expression. In order to test this possibility, primary neurones were subjected to a pro-apoptotic insult by exposing them to staurosporine (100 nM–100 μM) for 24 h. Staurosporine induced a concentration-dependent cell death as assessed by an MTT endpoint assay (Fig. 2A; EC_{50} = 3.2 μM) or by Propidium Iodide Hoe 33342 double staining (not shown). In order to test whether hesperetin could protect neurones against staurosporine-induced death, cells were incubated with hesperetin (100 nM–10 μM) for 24 h before exposure to staurosporine (10 μM) for a further 24 h. Neuronal cell death was then measured by propidium iodide and Hoe 33342 double staining. Staurosporine caused substantial neuronal damage that was inhibited by low but not high concentrations of hesperetin (Fig. 2B and C). Maximum protection was observed at 300 nM hesperetin (~40% protection), but at higher concentrations protection was lost (Fig. 2C) and at the highest concentration tested there was evidence of a toxic gain of function. To determine which intracellular pathways mediated the hesperetin-induced

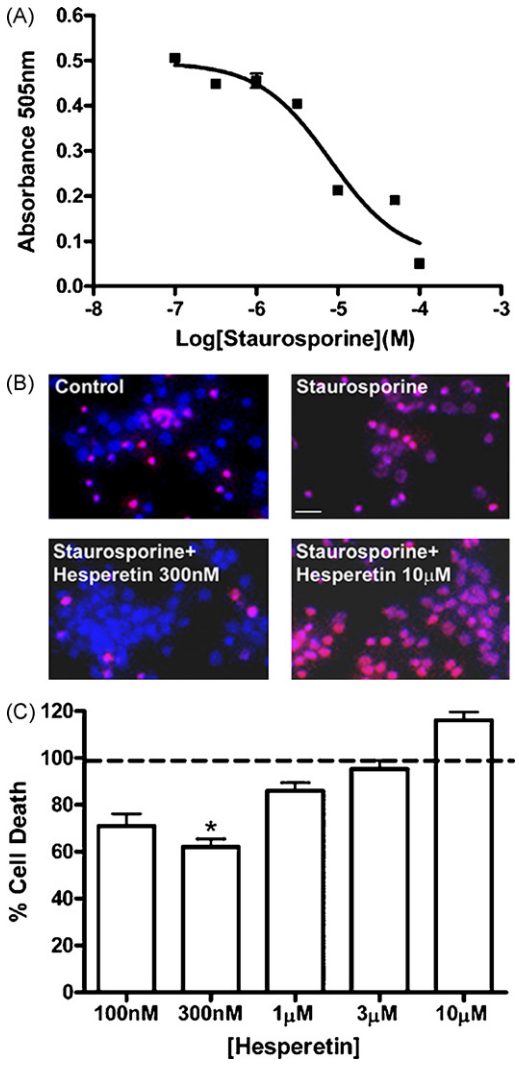

Fig. 2. Effect of staurosporine on cell death was assessed by measurement of MTT turnover (Panel A) or (Panel B) by measuring the number of propidum iodide positive cells (pink = dead cells) relative to Hoe 33342-stained cells (blue = total cells). Panel C shows quantification of protection by hesperetin against staurosporine-induced cell death, % Cell death (*y*-axis) is calculated from the number of propidum iodide positive cells compared to the number of propidium iodide positive cells in the group treated with staurosporine (10 μM) alone. Data are presented as mean ± SEM. of three independent experiments, each carried out in quadruplicate. Comparisons to staurosporine (hatched line = 100%) were analysed using one-way analysis of variance (ANOVA), followed by post hoc Student–Newman–Keuls multiple range test. *$p < 0.05$.

neuroprotection we administered either a PI 3-kinase inhibitor (Wortmannin, 150 nM) or a MEK1/2 inhibitor (U0126, 10 μM), both completely and significantly blocked the cytoprotective effects of hesperetin on staurosporine-induced cell death (reducing cell survival in these experiments from 71 ± 3% to 47 ± 4% or 52 ± 3%, respectively, $n = 3$), suggesting a functional role of the MAPK and PI 3-kinase pathways in neuroprotective effects of hesperetin.

These results show that hesperetin, an aglycone flavonoid of the flavanone class derived from the diet, enhanced ERK phosphorylation in each of the three different cell types used, but only in primary neurones were these effects statistically significant. In neurones, hesperetin had potent effects on ERK phosphorylation, i.e. was effective at concentrations between 100 nM and 300 nM and caused increases in ERK phosphorylation about twofold of control levels. In primary mouse neurones the maximal enhancement of ERK phosphorylation by hesperetin was similar to (−)epicatechin, but with

a lower peak effect, in agreement with previous reports in primary neurones [28]. The concentration range in which hesperetin produced activation of ERK is notable, i.e. it is concentrations in the nanomolar range which are the most active. These concentrations are relevant to the levels which can be obtained from the diet, and contrast to the high concentrations of flavonoids that are used in many studies of flavonoid biology. Hesperetin *in vitro* is known to act as an antioxidant and scavenger of peroxynitrite [3,18,19], however, at the effective concentrations in this study, it is unlikely that antioxidant effects or generation of peroxides mediate ERK phosphorylation. Hesperetin may be activating ERK and other kinases through direct interactions with the proteins themselves or through receptor-mediated signalling processes. Indeed, hesperetin has recently been suggested to bind directly to CNS and cardiac potassium channels [5,20], and inhibit phosphodiesterase activity [11]. In contrast to the stimulatory actions of low concentrations of hesperetin on ERK phosphorylation, at the highest concentrations used (10 μM), the effects of hesperetin were reduced. This is likely to be due to the effect of flavonoids at these higher concentrations activating protein phosphatases or by direct inhibition of effector kinases and appears to be a common feature of quercetin [25], (−)epicatechin [23] and hesperetin [28] signalling in neurones.

While hesperetin is similar to (−)epicatechin in neurones in stimulating ERK phosphorylation, the maximal effect of hesperetin on ERK phosphorylation is lower than (−)epicatechin and we show here, that unlike (−)epicatechin, hesperetin neither induces CREB phosphorylation nor CRE-mediated luciferase expression in neurones. Despite this inability to activate CREB-mediated protective pathways, low concentrations of hesperetin exerted protective effects against staurosporine-induced cell death. The mechanism underlying this protection is independent of CRE-mediated gene expression and, we show, involve MAPK and PI 3-kinase dependent regulation of anti-apoptotic signalling pathways. The exact details of the signalling pathways remain to be elucidated but are likely to involve inhibition of the mitochondrial-associated pro-apoptotic protein, Bad, and the apoptosis signal-regulating kinase 1, ASK1 [28]. Thus the study reveals significant differences between two flavonoids of different classes, despite an overlap in their ability to stimulate phosphorylation of ERK and afford neuroprotection.

The differences between cell responses to hesperetin may relate to the entry of flavonoids generally into cells. Flavanones are relatively non-polar (more lipophilic) compared to other flavonoid subgroups and therefore access cells to a greater degree [24]. The differences in activity between cell types demonstrated here are most likely be explained by differences in flavanone metabolism in different cell types. We think it likely that in SH-SY5Y and fibroblasts, flavanones are metabolized and cleared from cells more quickly that in the primary neurons, thus explaining their relative lack of activity.

The bioactive concentrations of hesperetin identified in our study are likely achievable *in vivo* following feeding. Indeed, hesperidin and/or its metabolite hesperetin are bioactive *in vivo*, producing sedative [16] and anticonvulsive effects [5] and enhancing recovery of retinal function after ischaemia in the rat [2]. Overall our data suggests that hesperetin can be added to the growing list of dietary-derived polyphenolic compounds that exert beneficial actions in the central nervous system.

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