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SIRT1 stimulation by polyphenols is affected by their stability and metabolism

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Abstract

Silent information regulator two ortholog 1 (SIRT1) is the human ortholog of the yeast sir2 protein; one of the most important regulators of lifespan extension by caloric restriction in several organisms. Dietary polyphenols, abundant in vegetables, fruits, cereals, wine and tea, were reported to stimulate the deacetylase activity of recombinant SIRT1 protein and could therefore be potential regulators of aging associated processes. However, inconsistent data between effects of polyphenols on the recombinant SIRT1 and on in vivo SIRT1, led us to investigate the influence of (1) stability of polyphenols under experimental conditions and (2) metabolism of polyphenols in human HT29 cells, on stimulation of SIRT1. With an improved SIRT1 deacetylation assay we found three new polyphenolic stimulators. Epigallocatechin galate (EGCg, 1.76-fold), epicatechin galate (ECg, 1.85-fold) and myricetin (3.19-fold) stimulated SIRT1 under stabilizing conditions, whereas without stabilization, these polyphenols strongly inhibited SIRT1, probably due to H_2O_2 formation. Using metabolically active HT29 cells we were able to show that quercetin (a stimulator of recombinant SIRT1) could not stimulate intracellular SIRT1. The major quercetin metabolite in humans, quercetin 3-*O*-glucuronide, slightly inhibited the recombinant SIRT1 activity which explains the lack of stimulatory action of quercetin in HT29 cells. This study shows that the stimulation of SIRT1 is strongly affected by polyphenol stability and metabolism, therefore extrapolation of in vitro SIRT1 stimulation results to physiological effects should be done with caution.
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Keywords: SIRT1; Sir2; Polyphenols; Quercetin; Resveratrol; EGCg

1. Introduction

Silent information regulator two ortholog 1 (SIRT1) is the human ortholog of the yeast sir2 protein. It belongs to a class of proteins called sirtuins that possess a NAD^+-dependent deacetylase activity. Sir2 most likely is one of the key proteins in mediating the caloric restriction-dependent lifespan extension in *S. cerevisiae*, *C. elegans* and *D. melanogaster* (Guarente, 2005). Lifespan extension by caloric restriction is not seen in yeast, *C. elegans* and drosophila mutants that do not express the sir2-gene (Lin et al., 2002; Rogina and Helfand, 2004; Tissenbaum and Guarente, 2001). Because sir2 is conserved from prokaryotes to mammals, the human SIRT1 protein could also be involved in regulating life extending processes in humans. Recent discoveries have shown that SIRT1 regulates several stress related processes (Brunet et al., 2004; Cohen et al., 2004; Luo et al., 2001; Motta et al., 2004; Vaziri et al., 2001; Yeung et al., 2004), fatty acid metabolism and adipogenesis (Picard et al., 2004), axonal neurodegeneration (Araki et al., 2004) and muscle cell differentiation (Fulco et al., 2003). SIRT1 controls these processes by NAD^+-dependent deacetylation of acetylated lysine groups of several transcription factors and other proteins. Histones (Imai et al., 2000; Vaquero et al., 2004), p53 (Luo et al., 2000; Vaziri et al., 2001), FOXO transcription factors (Brunet et al., 2004; Motta et al., 2004; van der Horst et al., 2004), ku70 (Cohen et al., 2004), TAFI168 (Muth et al., 2001), myoD (Fulco et al., 2003), p300 (Bouras et al., 2005) and PGC1α (Nemoto et al.,

Abbreviations: AMC, 7-amino-4-methylcoumarin; EC, (−)-epicatechin; ECg, (−)-epicatechin gallate; EGC, (−)-epigallocatechin; EGCg, (−)-epigallocatechin gallate; NAD, nicotinamide adenine dinucleotide; sir2, silent information regulator 2; SIRT1, silent information regulator two ortholog 1; TSA, trichostatin A

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2005; Rodgers et al., 2005) are all substrates for SIRT1 deacetylation.

An interesting finding was that the deacetylation activity of SIRT1 can be stimulated by several polyphenolic compounds (Howitz et al., 2003). Polyphenols are a wide group of dietary compounds from plants, occurring in high amounts in fruits, vegetables, cereals, wine and tea. Epidemiological studies suggest that a diet rich in polyphenols may protect against cardiovascular diseases (Arts and Hollman, 2005; Hertog et al., 1993), and mechanistic studies in cells and animals have shown that polyphenols have a wide range of properties that also may play a role in the prevention of other diseases, such as cancer (Lambert et al., 2005; Yang et al., 2001) and neurodisfunctions (Schmitt-Schillig et al., 2005; Youdim et al., 2004). Polyphenols are strong antioxidants, but are also known to interfere in signal transduction pathways (Hou et al., 2004; Mandel et al., 2004; Williams et al., 2004), inflammation (Kris-Etherton et al., 2004; Middleton et al., 2000; Nijveldt et al., 2001), and can interact with a number of proteins involved in cell proliferation (Brusselmans et al., 2005; Middleton et al., 2000; van der Woude et al., 2005). The stimulation of SIRT1 could possibly be an additional process that may explain the mechanisms by which dietary polyphenols exert their beneficial effect in humans.

Howitz et al. showed that the deacetylation activity of SIRT1 could be enhanced by the following polyphenols: resveratrol (up to 13-fold), butein (8.5-fold), picaetannol (7.9-fold), isoliquiritigenin (7.6-fold), fisetin (6.6-fold) and quercetin (4.6-fold). Stimulation of deacetylation activity by resveratrol resulted in regulation of several SIRT1 mediated physiological processes. Resveratrol treatment in experimental model systems expressing SIRT1 or its homologous genes increased the lifespan of *S. cerevisiae*, *C. elegans* and *D. melanogaster* (Howitz et al., 2003; Wood et al., 2004), suggesting that resveratrol could mimic the effects of caloric restriction in model organisms. In mammalian cell culture models resveratrol reduced fat storage and triglyceride release in differentiated 3T3-L1 cells (Picard et al., 2004), increased p53-mediated cell survival in HEK293 cells (Howitz et al., 2003) and inhibited NFκB-dependent transcription in NSCLC cells (Yeung et al., 2004). Regulation of these effects by resveratrol was abolished in the analogous SIRT1 knockdown model. Although other polyphenols (quercetin and piceatannol) were shown to have a marked effect on SIRT1 activity, they did not have any effect on lifespan in yeast. Only resveratrol and fisetin were shown to have a physiological effect that was mediated by sir2 (Howitz et al., 2003; Wood et al., 2004).

We hypothesize that stability of polyphenols under experimental conditions and metabolism of polyphenols in cells eliminates the stimulatory action of polyphenols on SIRT1 activity. Firstly, it is well known that after ingestion, polyphenols are metabolized by phase II enzymes in the intestine and liver. As a result, all tissues, except those of the gastro-intestinal tract, are only exposed to glucuronidated and sulfated metabolites of polyphenols. This has a profound effect on their bioactivity (Williamson et al., 2005). Secondly, most polyphenols are readily oxidized in aqueous media with a pH higher than 7, resulting in the formation of polyphenolic oxidation products and H_2O_2, which can lead to misinterpretation of experimental in vitro results (Halliwell, 2003).

Previous studies that determined sir2 deacetylase activity used radioactive methods with ^{14}C-NAD$^+$, ^{14}C-acetylated p53 or ^{3}H-acetylated histone groups as radioactive substrate (Bedalov et al., 2001; Borra et al., 2005; Luo et al., 2001; McDonagh et al., 2005), HPLC methods to analyze substrate conversion/product formation (Hoffmann et al., 1999; Jackson and Denu, 2002; Tanner et al., 2000) or spectrophotometric plate reader methods with a synthetic substrate containing a specifically cleaved fluorochrome (Heltweg et al., 2003; Marcotte et al., 2004; Wegener et al., 2003). These methods are either difficult to perform or not very specific. Spectrophotometric analysis of fluorochromes is hampered by autofluorescence of polyphenols and inherent background of fluorescent synthetic substrates. Therefore, we developed a more specific HPLC method. With this method we investigated whether stability and metabolism affect the polyphenolic stimulation of SIRT1. We used recombinant SIRT1 as well as metabolically active HT29 colon carcinoma cells, because colonic cells are exposed to unconjugated polyphenols from the diet via the lumen of the gastro-intestinal tract. Several polyphenols (Fig. 1), including resveratrol, catechins, quercetin and its major metabolite in humans, quercetin 3-*O*-glucuronide, were tested.

2. Experimental procedures

2.1. Materials

Quercetin, resveratrol, myricetin, nicotinamide, trichostatin A, catalase, 7-amino-4-methylcoumarin (AMC), (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECg), (−)-epigallocatechin gallate (EGCg) and gallic acid were purchased from Sigma–Aldrich. Isorhamnetin was purchased from Roth, tamarixetin from Extrasynthese and quercetin 3-*O*-β-glucuronide from Apin Chemicals. Human recombinant SIRT1, Fluor de Lys-SIRT1 deacetylase substrate, Fluor de Lys Developer II 5× concentrate, NAD$^+$ and Fluor de Lys deacetylated standard were purchased from Biomol. All chemicals used were of analytical grade.

2.2. Cell culture

HT29 colon carcinoma cells (ATCC) were cultured in DMEM (D5648, Sigma–Aldrich) with 5% fetal bovine serum, 25 mM HEPES, penicillin, streptomycin and non-essential amino acids in a humidified 37 °C incubator with 5% CO_2. Cells were subcultured once a week and medium was refreshed once a week. For SIRT1 incubation experiments, $\sim 5 \times 10^3$ cells/well were plated in a 96-well tissue culture plate one day before the start of the incubation. For quercetin uptake experiments cells were plated at $\sim 3 \times 10^4$ cells/well in a six-well tissue culture plate.

2.3. SIRT1 activity: overview

The Biomol SIRT1-assay is based on the deacetylation by SIRT1 of a synthetic substrate (Fluor de Lys-SIRT1 substrate), consisting of four amino acids with one acetylated lysine group (Arg-His-Lys-Lys(Ac)) and a fluorochrome (7-amino-4-methylcoumarin, AMC). After deacetylation, the fluorochrome is specifically released only from the deacetylated substrate by adding Developer II. To analyze the effects of polyphenols on deacetylation by the recombinant SIRT1 protein and intracellular deacetylation, we adjusted the Biomol method. We substituted the original detection with a fluorescence plate reader, with a more specific HPLC-fluorescence method to quantify AMC release.

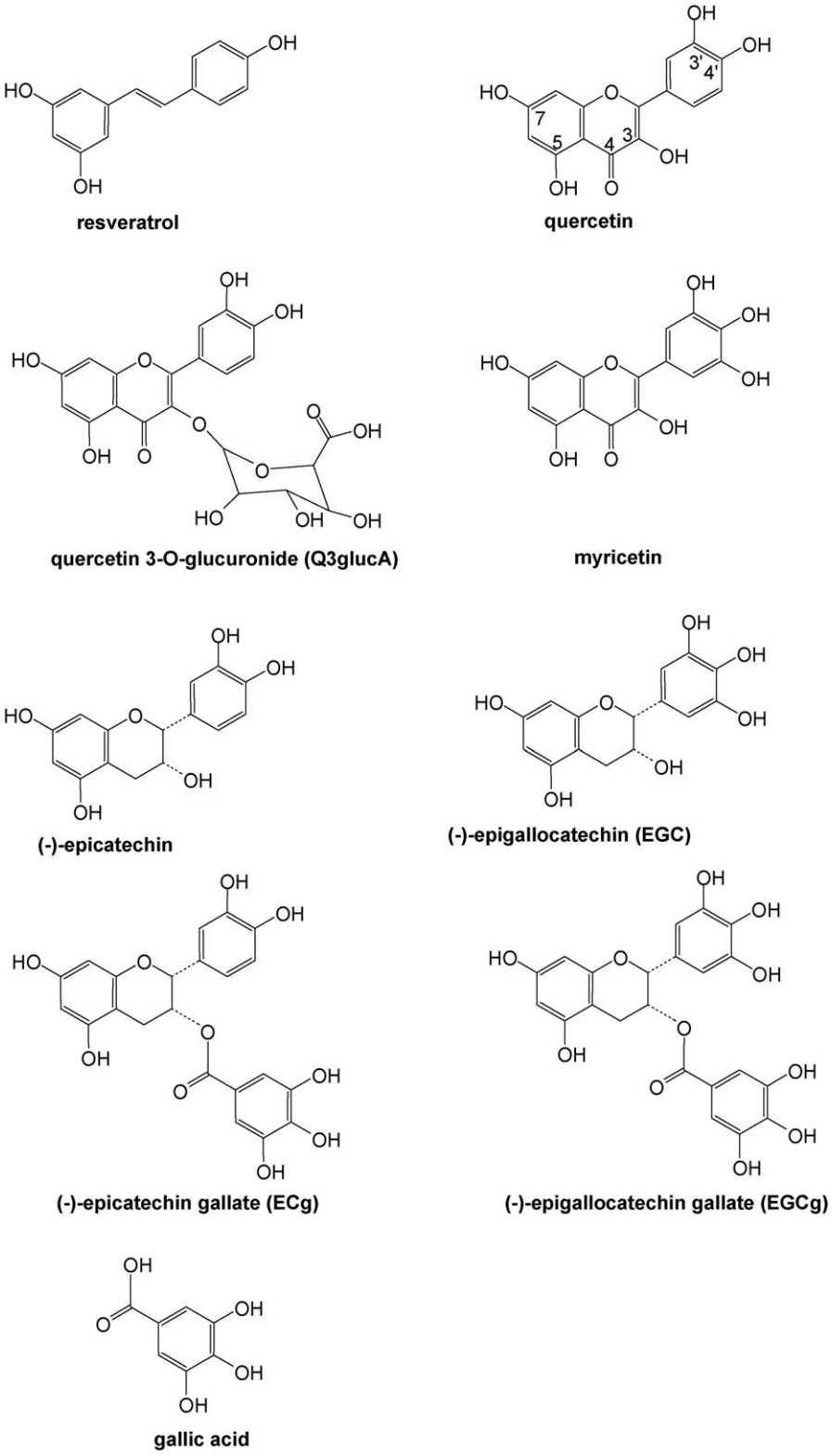

Fig. 1. Molecular structures of analyzed polyphenols.

2.4. SIRT1 activity: recombinant SIRT1

The assay was performed in a total volume of 50 μl in assay buffer (25 mM Tris/HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mg/ml BSA) with 0.5–1 U of SIRT1 protein (depending on different activities of different protein batches). The concentration of the synthetic substrate was 25 μM and the NAD^+ concentration was 500 μM. Final concentrations of all polyphenols were 100 μM (levels of organic solvent (DMSO or methanol) did not exceed 1%). Assays were performed with or without 1 mM ascorbic acid, 100 U/ml catalase or a combination of both. Before incubation, polyphenols were mixed with SIRT1 and were preincubated at 37 °C for 10 min. Substrate solution was

also prewarmed for 10 min at 37 °C. Incubation was started by combining the substrate with the SIRT1 and polyphenol solution. Control samples were incubated with 1% solvent and without polyphenols. Blank samples (without SIRT1 protein and polyphenols and with 1% solvent) were taken along as well. Three replicates of each incubation were performed in each experiment. After 15 min the incubation was stopped by addition of 50 μl 0.5× Developer II concentrate with 2 mM of the SIRT1 inhibitor nicotinamide in assay buffer. The samples were allowed to develop for 15 min at room temperature with additional shaking (∼500 rpm). Samples were further processed for HPLC analysis by adding 200 μl acetonitrile and 100 μl 20% H_3PO_4 after which the samples were centrifuged for 10 min at 10,000 rpm. The supernatant was injected on the HPLC system.

2.5. SIRT1 activity: intracellular SIRT1

For analysis of intracellular SIRT1 activity, HT29 cells were incubated with 25 μM substrate, 1 μM trichostatin A (TSA, to inhibit class I and II histone deacetylases) and 1 mM ascorbic acid in DMEM with 25 mM HEPES, 1% non-essential amino acids and 1% penicillin/streptomycin but without FBS. Expression of SIRT1 in HT29 cells was confirmed by PCR (data not shown). Polyphenols were incubated at a concentration of 100 μM. Incubations were performed for 4 h in a 37 °C tissue culture incubator. After incubation, the conversion of substrate to deacetylated substrate was analyzed in the exposure medium. Intracellular levels of deacetylated substrate were analyzed as well (cell processing was similar to the intracellular quercetin analyses, see below), and were about 40 times lower than medium concentrations. Apparently the deacetylated substrate is efficiently transported out of HT29 colon cells. Therefore medium concentrations were taken as a marker for intracellular substrate conversion. Control incubations were performed without cells and 8–32 replicates of each incubation were performed in each experiment. The same developer reaction to release the fluorochrome was performed as in the recombinant SIRT1 assay with slight modifications. Twenty-five microlitres of medium sample was incubated in a 96 well per plate (preplugged tubes plate, Bioplastics) with 25 μl 0.5× Developer II concentrate with 2 mM nicotinamide in assay buffer for 15 min on a well plate shaker at 600 rpm at room temperature. With the addition of 100 μl acetonitrile and 50 μl 20% H_3PO_4 the developer reaction was stopped and the protein was precipitated by centrifugation at 1780 × g. The supernatant was transferred to a 96-well Collection Plate (Captiva, Varian) and injected on the HPLC system. Total protein levels of all wells were analyzed with the Bio-Rad protein assay reagent, based on the Lowry method. No differences in protein levels between control and polyphenol incubations were seen after 4 h of exposure.

2.6. Analysis of quercetin uptake and metabolism

HT29 cells were exposed to 10 μM quercetin in a six-well tissue culture plate in the presence of 1 mM ascorbic acid in DMEM with 25 mM HEPES, 1% penicillin/streptomycin and 1% non-essential amino acids and 5% fetal bovine serum and incubated at 37 °C. A time course of exposure was performed up to exposures of 24 h. After exposure, cells were washed with HBSS, trypsinized and the cell pellet was dissolved in 100 μl Milli-Q water. The cells were lysed with four freeze–thaw cycles in liquid nitrogen and a 37 °C water bath. The method for further processing of samples for HPLC injection was based on the analysis of quercetin in plasma (Arts et al., 2004, 2003).

2.7. HPLC analysis of AMC

The HPLC system consisted of two Merck Hitachi LaChrom Elite L2130 pumps (Hitachi) and a LaChrom Elite L2200 injector, with an insert for injection of 96-well plates. Separation was achieved by injecting 20 μl sample onto an Inertsil column (150 mm × 4.6 mm, 5 μm, Alltech), protected by a NewGuard RP18 guard column (15.0 mm × 3.2 mm, 5 μm, Perkin-Elmer) with a flow of 1 ml/min. The solvent for elution was 30% acetonitrile in 10 mmol/l phosphate buffer, pH 6.9. Total run time was 7.5 min. Peaks were detected with a fluorescence detector (FP-920, Jasco) set at 347 nm excitation and 440 nm emission wave lengths. Samples were compared to a standard curve of AMC in 50% acetonitrile and 5% H_3PO_4 and corrected by subtracting the AMC levels in blank samples. Fold stimulation by polyphenols was calculated by comparing the AMC levels in the polyphenol treated samples with the control samples.

2.8. HPLC analysis of quercetin

The same HPLC system was used for quercetin analysis as for AMC analysis. Separation was achieved by injecting 20 μl sample onto a Chromolith RP-18e column (100.0 mm × 4.6 mm, Merck), protected by a NewGuard RP18 guard column (15.0 mm × 3.2 mm, 5 μm, Perkin-Elmer). Columns were housed in a column heater (ESA) set at 30 °C. The solvents for elution were 5% acetonitrile in 25 mmol/l citrate buffer, pH 3.7 (solvent A) and 70% acetonitrile in 25 mmol/l citrate buffer, pH 3.7 (solvent B). The elution program at a flow rate of 2.5 ml/min was as follows: 0–12 min, linear gradient from 0 to 43% B; 12–13 min, from 43 to 100%; 13–14.5 min, isocratic at 100% B; 14.5–15.5 min, linear return to 0% B. Total run time was 17 min. Peaks were detected with a coulometric array detector (Coularray detector model 6210; ESA) set at 75, 250 and 500 mV (Pd as reference). Quercetin was quantified at the lowest potential (75 mV).

2.9. Statistical tests

Power calculations were performed to calculate the number of samples needed to detect a significant difference in SIRT1 deacetylation activity between quercetin treated cells and control cells. The expected intracellular fold stimulation of SIRT1 by quercetin (1.19) was calculated by using the intracellular fold stimulation of SIRT1 by resveratrol (1.65), and the ratio of the fold changes found in the recombinant assay. A significant change ($\beta = 0.8$, $\alpha = 0.05$) between control samples and quercetin treated cells could have been found with a sample size of $n = 32$. p-Values were calculated with a t-test. All error bars in figures represent standard deviations.

3. Results

3.1. Specific HPLC method for analyzing SIRT1 activity

Polyphenols and synthetic fluorescent substrates can potentially interfere with the analysis of a fluorochrome in a deacetylation assay. We therefore developed a method that analyzed the deacetylase activity of SIRT1 more specifically than other methods that are based on the release of a fluorochrome. In the published Biomol assay (Howitz et al., 2003), we added a protein precipitation step and analyzed the samples by HPLC with fluorescence detection. With this method we could separate resveratrol and the fluorescent substrates from the fluorochrome (AMC). Resveratrol clearly had a higher retention on the column than AMC (Fig. 2A and B), therefore interference by resveratrol was eliminated. The identity of the first peaks in the chromatograms (Fig. 2A and B) was confirmed by injecting substrate or deacetylated substrate standards without any reaction. The other polyphenols also did not interfere with AMC analysis (data not shown). Recovery of spiked AMC in the recombinant and intracellular assay was 98–100%. This method allowed us to analyze the influence of polyphenols on the deacetylation activity of recombinant SIRT1 and intracellular SIRT1, without interference by autofluorescence from the polyphenols and without high background fluorescence from the synthetic substrate.

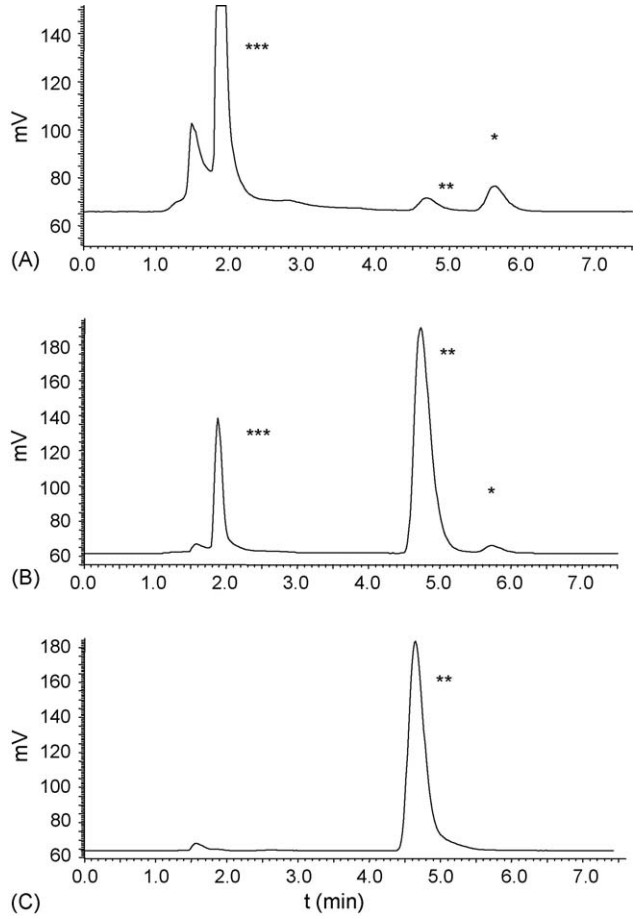

Fig. 2. HPLC chromatograms of a typical recombinant SIRT1 protein assay. Blank sample 15 min after start of incubation with substrates and resveratrol but without SIRT1 (A), resveratrol stimulated SIRT1 sample 15 min after start of incubation with SIRT1, substrates and resveratrol (B) and standard of 62.5 nM AMC (C). The samples were taken from a reaction where SIRT1 (1 U) was incubated with 100 μM resveratrol with 25 μM substrate and 500 μM NAD^+. (*) Resveratrol, (**) AMC and (***) substrate and deacetylated substrate peaks.

3.2. Polyphenols influence recombinant SIRT1 activity directly and indirectly

To determine the effects of polyphenols on SIRT1 deacetylase activity, we used the recombinant SIRT1 protein. Because several of the tested polyphenols are known to be unstable in aqueous solution we conducted experiments with and without addition of Vitamin C or catalase. Addition of Vitamin C did not change the effect on SIRT1 activity significantly for resveratrol, quercetin, EC and ECg (Table 1). However, a striking finding was that EGCg, EGC, gallic acid and myricetin inhibited the activity of SIRT1 when the compounds were incubated without any stabilizing agents (Table 1). For example, the substrate deacetylation was approximately 25 times lower in the presence of EGCg than in the control reaction. Addition of 100 U/ml catalase negated the inhibitory effect of EGCg, whereas addition of 1 mM Vitamin C resulted in stimulation of SIRT1 (Fig. 3A). This indicates that the inhibition probably is an indirect effect caused by oxidation products of EGCg or by H_2O_2. To investigate whether formation of oxidation products of EGCg or H_2O_2 formation was responsible for the inhibition of SIRT1 activity, we preincubated EGCg for half an hour without Vitamin C, catalase, SIRT1 or substrate. SIRT1 and substrate were incubated afterwards with the preincubated solution with and without addition of stabilizing agents. Stabilizing agents again eliminated the inhibitory effects of polyphenols on SIRT1 activity (Fig. 3B). This suggests that H_2O_2 formation is the major process that caused the activity of the protein to decrease. H_2O_2 formation during EGCg incubations did not influence the developer reaction or AMC stability (data not shown). Myricetin, gallic acid and EGC also inhibited SIRT1 activity when no Vitamin C was added. With addition of Vitamin C, myricetin stimulated SIRT1 3.19 ± 0.6 fold (average ± S.D., Fig. 3C). All further incubations were done with the addition of 1 mM Vitamin C.

Several tested polyphenols influenced the deacetylation activity of the recombinant SIRT1 protein. Resveratrol was the best stimulator of SIRT1 activity; it increased the deacetylation activity 5.03 ± 0.7 fold (Fig. 4). The two flavonols, quercetin and myricetin, stimulated SIRT1 activity by 2.15 ± 0.62 and 3.19 ± 0.61 fold, respectively. With the proper incubation conditions, catechins conjugated to a gallic acid group (EGCg and ECg) stimulated SIRT1 activity significantly (EGCg: 1.76 ± 0.42 fold, ECg: 1.85 ± 0.35 fold). Epicatechin (EC) and epigallocatechin (EGC) had no effect on SIRT1 activity. Also

Table 1
Modulation of SIRT1 activity by polyphenols (100 μM) with and without addition of 1 mM Vitamin C

	+Vitamin C				−Vitamin C			
	Fold change	S.D.	*n*	Effect	Fold change	S.D.	*n*	Effect
Polyphenols where addition of Vitamin C does not change the SIRT1 effect								
Resveratrol	4.66	0.60	5	+	5.18	0.70	12	+
Quercetin	2.15	0.62	11	+	2.54	0.11	3	+
ECg	1.85	0.35	6	+	1.91	0.26	6	+
EC	0.99	0.06	3	o	1.09	0.25	6	o
Polyphenols where addition of Vitamin C does change the SIRT1 effect								
EGCg	1.90	0.40	6	+	0.04	0.02	12	−
EGC	0.91	0.20	5	o	0.41	0.11	6	−
Myricetin	3.19	0.61	9	+	0.09	0.03	3	−
Gallic acid	1.02	0.07	6	o	0.44	0.06	6	−

(+) Stimulation, (o) no effect and (−) inhibition.

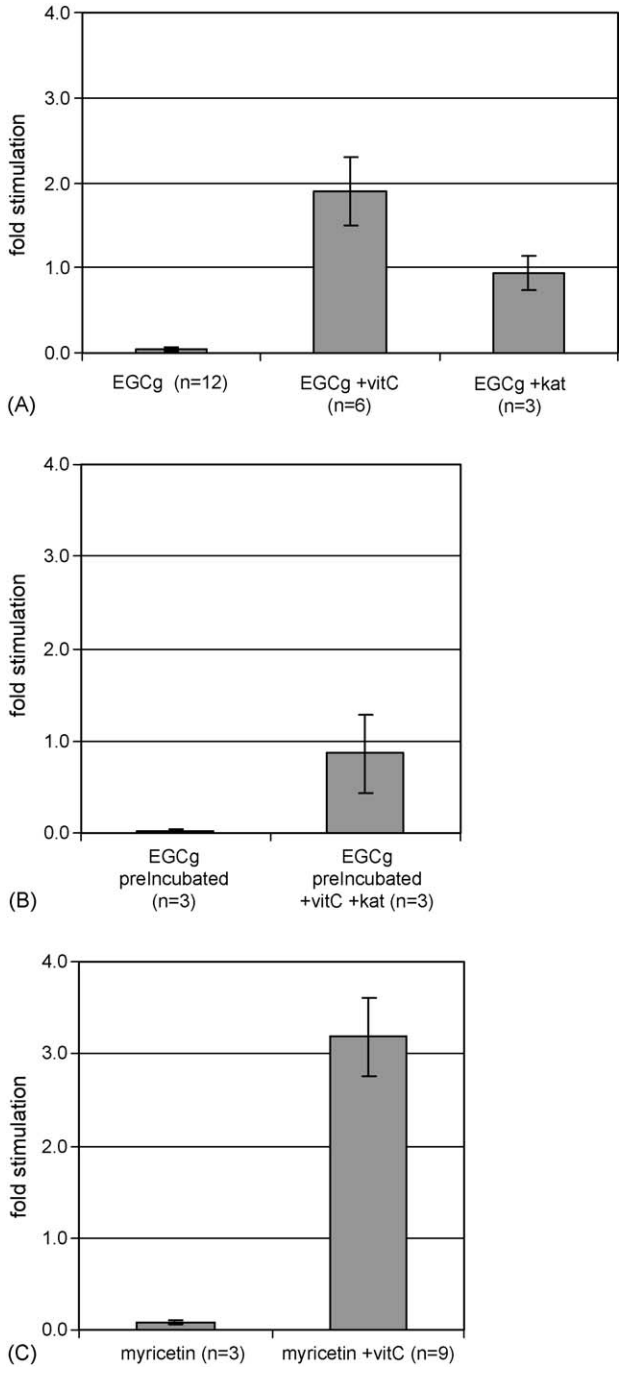

Fig. 3. Fold stimulation of the activity of the recombinant SIRT1 protein incubated under several conditions with polyphenols. SIRT1 incubated with and without stabilizing agents and EGCg (A), preincubated EGCg (B) and myricetin (C). All reactions were performed for 15 min with 0.5–1 U SIRT1, 25 μM substrate, 500 μM NAD^+, 100 μM polyphenol and with or without 1 mM Vitamin C (+vitC) or 100 U/ml catalase (+kat). EGCg preIncubated = preincubation (30 min) with 100 μM EGCg without addition of stabilizing agents, substrates and SIRT1 protein. n = Number of replicates.

gallic acid itself had no effect on SIRT1 activity. Because most polyphenols are rapidly metabolized in epithelial cells before entering the blood, it is of relevance to test metabolites of these compounds to better simulate the effects of polyphenols in an in vivo system. In contrast to the stimulating effect of quercetin, one of the metabolites of quercetin (quercetin 3-*O*-β-glucuronide) did not show a stimulatory effect. In fact, a slight, but significant, inhibition of SIRT1 activity was seen for quercetin 3-*O*-β-glucuronide (Fig. 4).

3.3. Polyphenol effects on SIRT1 activity in HT29 cells

The stimulatory effects of polyphenols on the isolated SIRT1 protein are only relevant if these effects can be reproduced in an in vivo situation. Therefore we chose to analyze the effects of one of the stimulators (quercetin) in a cellular system (HT29 colon carcinoma cell line). Before determining the effects on SIRT1, we first analyzed the uptake kinetics and intracellular levels of quercetin. For this, HT29 cells were incubated with 10 μM quercetin and 1 mM Vitamin C for up to 24 h. We analyzed quercetin and quercetin metabolites in cellular extracts and incubation medium with and without enzymatic hydrolysis of the samples with a *Helix pomatia* enzyme mixture containing both sulfatase and glucuronidase activity. In this way, we could distinguish between aglycone forms and conjugated forms of quercetin, isorhamnetin (3′-*O*-methoxy quercetin) and tamarixetin (4′-*O*-methoxy quercetin). Quercetin was quickly taken up by HT29 cells and immediately metabolized to methoxylated (isorhamnetin and tamarixetin) and glucuronidated/sulfated conjugates (Fig. 5A). The intracellular concentrations of total quercetin metabolites reached a maximum at 2–4 h after start of exposure. After 24 h only low levels of conjugated quercetin were left inside the cell (Fig. 5A). The levels of intracellular quercetin aglycone were much lower than conjugated quercetin and also peaked at 4 h after start of exposure (Fig. 5C). The concentrations of free quercetin aglycone in the incubation medium quickly decreased (Fig. 5D) and the levels of quercetin metabolites in the medium increased till a steady-state was reached approximately 8 h after start of exposure (Fig. 5B).

Because the maximum intracellular levels of quercetin aglycone and quercetin conjugates were reached 2–4 h after start of exposure we chose to incubate HT29 cells for 4 h with polyphenol and SIRT1 substrate. Resveratrol was taken as a positive control. Incubations of HT29 cells with resveratrol showed that the deacetylation activity was increased 1.65-fold in cells exposed to resveratrol (Fig. 6), indicating that the stimulation of deacetylation activity by resveratrol could also be reproduced in HT29 cells. In contrast, quercetin did not show any effect on the deacetylation activity of SIRT1 in HT29 cells (Fig. 6).

4. Discussion

The inconsistent finding that polyphenols could stimulate the deacetylase activity of SIRT1 on the protein level, but not in a cellular system, prompted us to compare the effects of polyphenols on recombinant SIRT1 with the effects on SIRT1 deacetylase activity in metabolically active HT29 colon carcinoma cells. We show that intracellular activity of SIRT1 in HT29 cells is only stimulated by resveratrol and not by quercetin. This is probably due to rapid metabolism of

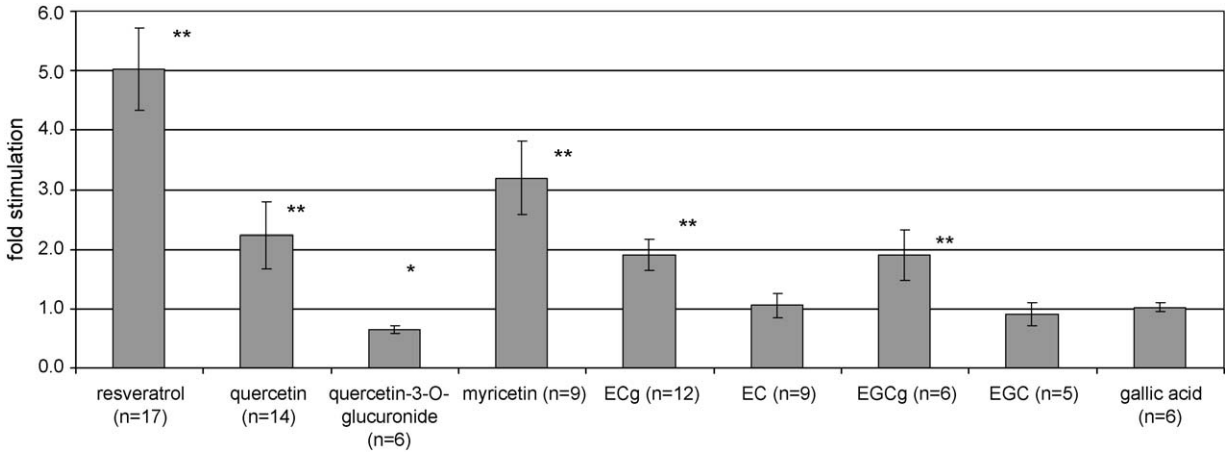

Fig. 4. Fold stimulation of the recombinant SIRT1 deacetylase activity by polyphenols. For the polyphenols where addition of stabilizing agents did not result in a significant change in SIRT1 stimulation, both results from experiments without and with stabilizing agents were used. All reactions were performed for 15 min with 0.5–1 U SIRT1, 25 μM substrate, 500 μM NAD^+, 100 μM polyphenol and 1 mM Vitamin C. n = Number of replicates. $^{*}p < 0.05$, $^{**}p < 0.01$ meaning a significant difference from control incubations without polyphenols.

quercetin because quercetin 3-*O*-glucuronide did not stimulate recombinant SIRT1. In this paper, we also show for the first time that the catechins EGCg and ECg and the flavonol myricetin can stimulate the activity of the recombinant SIRT1 protein, but only under the proper incubation conditions.

The stimulating effect of polyphenols on SIRT1 has been shown in Hela cells (Howitz et al., 2003) and in drosophila S2 cells (Wood et al., 2004). However, large differences were seen between polyphenolic effects on intracellular SIRT1 and on the isolated SIRT1 protein. For example, butein showed a 8.5-fold stimulation on the isolated protein, but did not have any effect in Hela cells (Howitz et al., 2003). On the other hand, butein showed a stimulating effect both on the drosophila sir2 protein and in drosophila S2 cells (Wood et al., 2004). Intracellullar effects of quercetin on SIRT1 were not found, whereas effects on isolated sir2 proteins were high; 4.6-fold on human SIRT1 (Howitz et al., 2003) and 1.25-fold on *C. elegans* sir2.1 (Wood et al., 2004). We found a 2.15-fold stimulation of recombinant SIRT1 deacetylase activity by quercetin (Fig. 4). However, no effect was seen on intracellular SIRT1 activity in quercetin exposed HT29 cells. We used colon cells because these cells are readily exposed to polyphenols via the diet. Quercetin was conjugated in HT29 to glucuronides and immediately transported out of the cell, as was shown by high concentrations

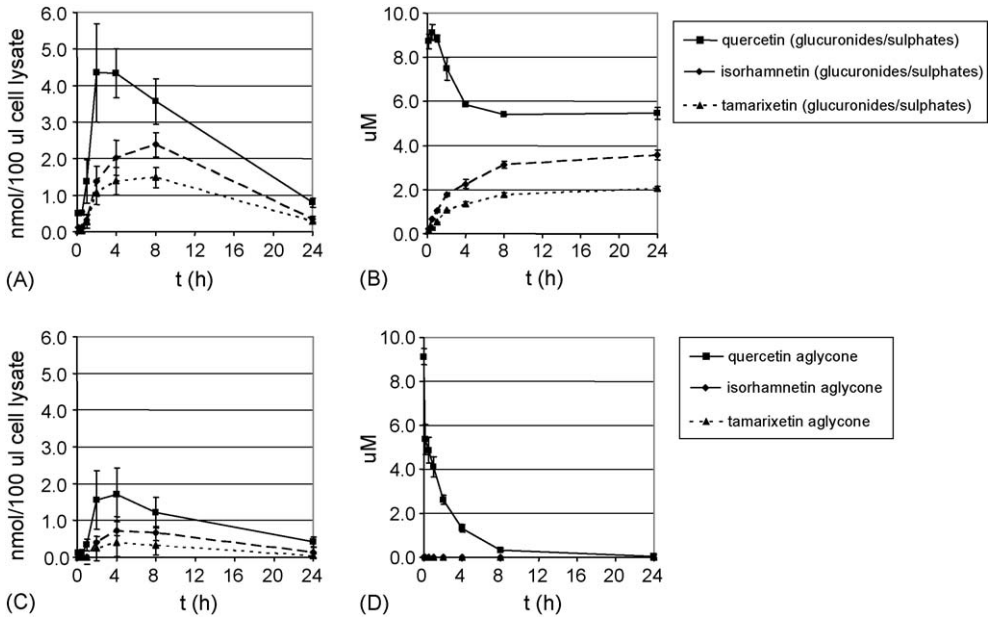

Fig. 5. Quercetin uptake in HT29 colon carcinoma cells. Quercetin uptake was analyzed in incubation medium (B and D) and cell lysate (A and C) after exposure up to 24 h to 10 μM quercetin with 1 mM Vitamin C. Panels 'A' and 'B' show levels of quercetin, isorhamnetin (3′-OCH_3-quercetin) and tamarixetin (4′-OCH_3-quercetin) aglycone and glucuronides/sulfates in cell lysate and medium. Samples are enzymatically hydrolyzed with glucronidase/sulfatase before extraction. Panels 'C' and 'D' show levels of quercetin, isorhamnetin and tamarixetin aglycones in cell lysate and medium. Samples are not hydrolyzed before extraction.

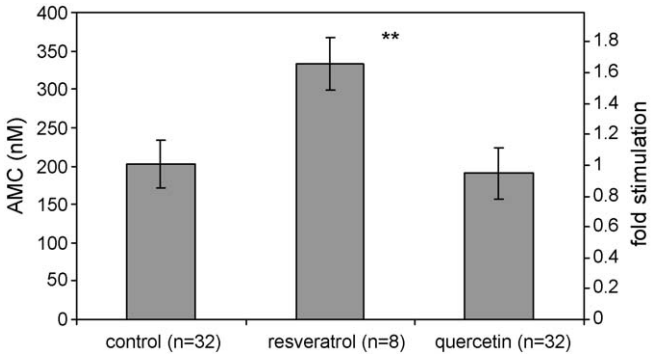

Fig. 6. AMC levels and fold stimulation after exposure of HT29 cells for 4 h to 25 μM substrate, 1 μM TSA and 100 μM polyphenol. AMC levels represent the deacetylase activity of intracellular SIRT1 protein. n = Number of replicates. $^{**}p < 0.01$.

of quercetin glucuronides in the medium. Additional metabolites of quercetin found in the medium of quercetin exposed HT29 cells were methoxylated quercetin (isorhamnetin and tamarixetin), these metabolites were also conjugated to glucuronides. van der Woude et al. (2004) showed that the major metabolites formed by HT29 cells were quercetin 3-*O*-glucuronide and quercetin 4′-*O*-glucuronide. Quercetin 3-*O*-glucuronide is also one of the major quercetin metabolites in human plasma (Mullen et al., 2004) and is formed in the intestine and liver by UDP glucuronosyl transferases (Boersma et al., 2002). In contrast to the stimulating effect of quercetin on SIRT1, quercetin 3-*O*-glucuronide did not have a stimulating effect on the deacetylase activity of SIRT1, even a slight inhibition of activity was seen. This difference between the effects of a major quercetin metabolite and quercetin itself may explain the lack of stimulating effect of quercetin in a cellular system.

Resveratrol was able to stimulate the activity of SIRT1 in HT29 cells by 1.65-fold. This relatively low stimulation of intracellular SIRT1 activity as compared to the effects of resveratrol on the recombinant protein, points to an effective metabolism or low uptake of resveratrol in HT29 cells. Uptake and metabolism studies of resveratrol in hepatocytes and Caco-2 cells (Kaldas et al., 2003; Lancon et al., 2004) showed that resveratrol was conjugated to sulfates and glucuronides and effectively transported out of the cell. However, some accumulation of resveratrol in Caco-2 cells was also found (Kaldas et al., 2003). A higher potency and higher accumulation than quercetin probably explains why resveratrol is able to stimulate intracellular SIRT1, whereas quercetin is not.

Apart from the metabolism of polyphenols, stability of polyphenols in incubation medium can influence the effects of polyphenols on SIRT1 activity. Polyphenols are antioxidants and are readily oxidized in aqueous media (Halliwell, 2003). Several polyphenols oxidize and form hydrogen peroxide. EGCg and gallic acid (200 μM) produce up to ∼650 μM H_2O_2 when incubated for 1 h in HEPES buffered DMEM medium. When these compounds are incubated in combination with an excess Vitamin C, the H_2O_2 production is decreased to ∼100 μM (Wee et al., 2003). In our experiments EGC and gallic acid inhibited the activity of SIRT1 slightly when no Vitamin C was added, whereas after stabilization with Vitamin C, EGC and gallic acid did not have an effect (Table 1). The two other compounds with three hydroxyl groups in the B-ring, EGCg and myricetin, almost completely inhibited the activity of SIRT1 without Vitamin C. When stabilized with Vitamin C, EGCg and myricetin stimulated the activity of the recombinant SIRT1 protein (Fig. 3). Wee et al. (2003) showed that Vitamin C could prevent the breakdown of gallic acid in culture medium, whereas catalase could not. We found similar results with EGCg (Fig. 3A); EGCg in combination with catalase did not stimulate recombinant SIRT1, whereas EGCg stabilized with Vitamin C stimulated SIRT1. Howitz et al. (2003) found a slight inhibition of SIRT1 activity by myricetin (0.89-fold) and a more pronounced inbition by EGC (0.41-fold) and EGCg (0.32-fold), indicating that Howitz did not use stabilizing conditions when incubating polyphenols with SIRT1.

Both ECg and EGCg stimulated the activity of recombinant SIRT1, while EC and EGC were not able to stimulate SIRT1. Thus the attached gallic acid group seems to be necessary for a stimulating activity of catechins. An advantage of this gallolyation of the polyphenolic backbone is that these polyphenols are hardly conjugated to glucuronides and sulfates in the human body. Therefore, EGCg is present in human plasma for 77–90% in its free form (Manach et al., 2005), whereas other polyphenols, like resveratrol, myricetin and quercetin, are conjugated to glucuronides and sulfates. Tissues, other than gastro-intestinal tract tissues, are also mainly exposed to conjugates of resveratrol (Wenzel et al., 2005) and quercetin (de Boer et al., 2005), while EGCg was found in tissues in its unconjugated form (Meng et al., 2002). This may have a significant impact on the bioactivity of EGCg in the human body.

Recent studies have shown that the activation of recombinant SIRT1 by resveratrol only occurs when a fluorochrome is attached to the synthetic substrate, whereas without the fluorochrome resveratrol did not stimulate SIRT1 (Borra et al., 2005; Kaeberlein et al., 2005). Nevertheless, resveratrol has been shown to be a stimulator in sirtuin-dependent processes extensively (Howitz et al., 2003; Picard et al., 2004; Wood et al., 2004; Yeung et al., 2004). Secondly, Sir2 proteins do not seem to discriminate their substrates on the basis of sequence, but by conformation (Khan and Lewis, 2005). The conformation of the fluorescently tagged substrate may resemble the conformation of an in vivo substrate better than substrates without a fluorochrome. This suggests, in combination with the fact that only SIRT1 and not the other human sirtuins deacetylate p53-based substrates in vitro (Michishita et al., 2005), that the Biomol substrate may be well suited for analysis of SIRT1 activity. Resveratrol increases the affinity of the substrate for the protein probably by inducing a conformational change in the SIRT1 protein (Borra et al., 2005). Whether other polyphenols, like EGCg and quercetin, act in the same way as resveratrol is not yet established, but is likely.

With our improved method for determining polyphenolic modulation of recombinant and intracellular SIRT1 deacetylase activity, we were able to identify three new polyphenolic

stimulators of SIRT1 deacetyase activity, ECGg, ECg and myricetin. Furthermore, we found that probably the metabolism of polyphenols is responsible for diminished or absent SIRT1 modulating activity in a human cellular system as compared to the effect of the polyphenol on the isolated protein. Therefore, extrapolation of in vitro SIRT1 stimulation results to physiological effects should be done with caution.

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