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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.

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.