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Dietary resveratrol administration increases MnSOD expression and activity in mouse brain

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ABSTRACT

trans-Resveratrol (3,4′,5-trihydroxystilbene; RES) is of interest for its reported protective effects in a variety of pathologies, including neurodegeneration. Many of these protective properties have been attributed to the ability of RES to reduce oxidative stress. *In vitro* studies have shown an increase in antioxidant enzyme activities following exposure to RES, including upregulation of mitochondrial superoxide dismutase, an enzyme that is capable of reducing both oxidative stress and cell death. We sought to determine if a similar increase in endogenous antioxidant enzymes is observed with RES treatment *in vivo*. Three separate modes of RES delivery were utilized; in a standard diet, a high fat diet and through a subcutaneous osmotic minipump. RES given in a high fat diet proved to be effective in elevating antioxidant capacity in brain resulting in an increase in both MnSOD protein level (140%) and activity (75%). The increase in MnSOD was not due to a substantial proliferation of mitochondria, as RES treatment induced a 10% increase in mitochondrial abundance (Citrate Synthase activity). The potential neuroprotective properties of MnSOD have been well established, and we demonstrate that a dietary delivery of RES is able to increase the expression and activity of this enzyme *in vivo*.

trans-Resveratrol (3,4′,5-trihydroxystilbene; RES), a bioactive component of red wine, has become well known for its reported ability to extend lifespan in model organisms ranging from yeast to vertebrates [1–4]. In addition to lifespan extension, RES has also shown putative protective actions against neurodegeneration, cancer, cardiovascular disease, diabetes, and the detrimental effects associated with high fat diets [5,6]. Oxidative stress is a shared observation in many of these pathologies and resistance to oxidative stress is a strong correlate of lifespan potential [7]. A RES induced decrease in cellular oxidative stress may provide a mechanism by which this polyphenol is able to exert a wide range of beneficial effects. Although RES has antioxidant properties related to the presence of its phenolic hydroxyl groups, low bioavailability, and a weak ability to directly scavenge reactive oxygen species, (ROS), makes cytoprotection via direct chemical reactions unlikely [8,9]. A more plausible hypothesis is that RES initiates a cascade of intracellular events that lead to an upregulation of cellular defense systems, which in turn protect against oxidative stress.

Interactions between RES and intracellular signalling molecules including sirtuins and the fork head family of transcription factors have been reported both *in vivo* and *in vitro* [6,10,11]. Recent studies suggest that activation of SIRT1, and its target PGC-1α, by RES in mice leads to changes in mitochondrial number and function [11]. As the primary source of ROS production in most cell types [7], mitochondria are important components of responses aimed at decreasing oxidative stress. The mitochondrial isoform of superoxide dismutase, MnSOD, is therefore a downstream target of many signalling pathways proposed to mediate cellular stress resistance [12]. Previous work by our laboratory has shown that RES is able to induce MnSOD in a human lung fibroblast cell line (MRC-5) [13]. An elevation of MnSOD *in vivo* in response to RES would be a significant finding given the enzyme's importance in various models of disease [14]. Many of RESs reported *in vivo* effects are consistent with an increase in mitochondrial ROS metabolism; however, observation of antioxidant enzyme activities, including MnSOD, in normal mice following chronic RES treatment has not yet been reported. We hypothesize that an important action of RES may be to reduce intracellular oxidative stress by increasing mitochondrial ROS metabolism.

The aim of this study was to examine antioxidant enzymes in the brain, heart, and liver of mice administered RES for 4

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consecutive weeks. To assess the influence of delivery method three routes of RES administration were tested: incorporation into a standard laboratory diet, incorporation into a high fat diet or delivery via a subcutaneous osmotic minipump. Here, we report that both dietary and subcutaneous RES delivery methods are capable of altering the activities of key antioxidant enzymes GPx, CAT, and MnSOD, and increasing mitochondrial content in heart, brain and liver.

Materials and methods

Materials. C57 BL6 mice were obtained from Charles River Laboratories (Charles River, Canada). Alzet 2004 minipumps were purchased from Alzet (Cupertino, USA). *trans*-Resveratrol (purity >95%) was purchased from ChromaDex Inc. (Irvine, USA). Mouse chow, AIN-93G and AIN-93G modified (60% calories from fat), was purchased from DYETS (Bethlehem, USA). Chemicals and materials used in Western blotting were as in Robb et al. [13].

Animal care conditions. C57 BL6 mice (Charles River, Canada) were housed in groups of three in a temperature and humidity controlled environment subject to a 12 h light/dark cycle. Standard mouse chow and water were available ad libitum to the minipump treatment groups. Dietary delivery groups were given controlled access to food, water was available ad libitum. All treatment protocols adhered to CCAC guidelines.

Resveratrol treatment. At 5 weeks, mice were introduced to RES by one of three delivery methods; in a high fat diet, a standard laboratory diet or through a subcutaneous osmotic minipump (MP). RES was incorporated into mouse chow of both standard and high fat diets at a concentration of 0.1% (w/w), such that mice obtained a RES intake of approximately 200 mg/kg/day. Alzet 2004 minipumps were preloaded with 50% degassed DMSO or 1.825 M RES prepared in 50% degassed DMSO. The minipumps were implanted subcutaneously under isoflorane anesthesia and released vehicle or RES at a flow rate of 0.25 μL/h to give a dosage of 100 mg/kg/day. The standard diet (SD) group contained 8 control mice and 8 RES-treated mice. The high fat diet (HFD) group contained 6 control mice and 8 RES-treated mice. The osmotic minipump group contained 5 control mice and 8 RES-treated mice.

Tissue harvesting. Animals were sacrificed by cervical dislocation at the end of 4 weeks of treatment. Brain, liver, and heart were removed and immediately frozen on dry ice.

Tissue homogenization. Frozen tissues were homogenized in two volumes of ice-cold buffer containing 10 mM KH_2PO_4 (pH 7.3), 30 mM KCl, 20 μM EDTA, and 0.1% Triton X-100 using a polytron homogenizer. The homogenates were centrifuged at 500 g for 10 min. The resulting supernatant was collected and protein concentration was determined by the Bradford method.

Enzyme activities. Citrate synthase, catalase, and glutathione peroxidase activities were assayed as in Robb et al. [13]. The activities of MnSOD and CuZnSOD were measured using an in-gel assay

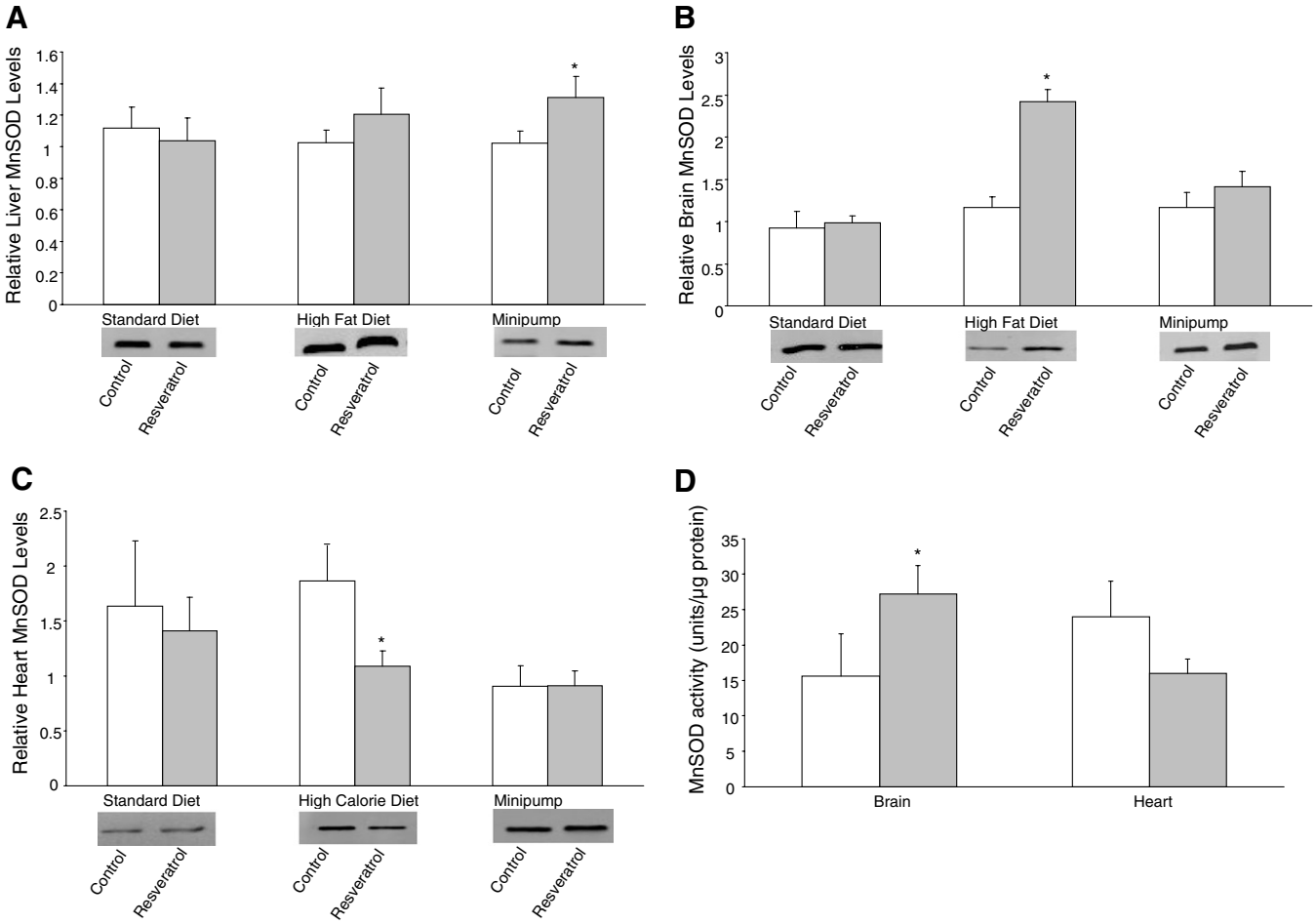

Fig. 1. MnSOD protein level and activity in brain, heart, and liver tissue of control (open bars) and resveratrol (solid bars) groups of three treatment methods ($n = 5–8$). Relative changes in MnSOD protein level in tissue homogenates and representative Western blots showing MnSOD protein band in (A) liver homogenates (B) brain homogenates and (C) heart homogenates. Relative change was measured using an internal standard as a reference and values were interpolated form a standard curve. Values shown are means ± SEM of duplicate Western blots for each homogenate. (D) Activity of MnSOD in brain and heart of high fat diet group. Values shown are means ± SEM of duplicate measurements of four homogenates per group. *Significantly different from control group ($P < 0.05$).

as described [15,13]. Quantification was achieved using an in-gel standard curve constructed from a dilution series of pure bovine liver SOD.

Western blotting. Immunodetection of MnSOD and CuZnSOD was done as described in Robb et al. [13]. Briefly, 20 μg crude homogenate protein was separated by SDS–PAGE and electrotransferred to a PVDF membrane. MnSOD and CuZnSOD were visualized using an Odyssey infrared imaging system (LI-COR Biosciences). The arbitrary luminescence values of MnSOD bands in each Western were normalized to an internal standard.

Statistical analysis. Data collected were analyzed by ANOVA, followed by Tukey's honestly significant difference post hoc test. Statistical analysis was performed using SYSTAT. $P < 0.05$ was taken as significant. Error bars represent SEM.

Results

The effects of RES on the cytosolic and mitochondrial isoforms of superoxide dismutase were determined by Western blotting for CuZnSOD and MnSOD. CuZnSOD protein levels were unchanged by RES in all tissues and experimental groups (data not shown). In contrast, the mitochondrial isoform, MnSOD, protein level was found to vary in response to RES administration and delivery method. Administration of RES in a standard diet was ineffective in modulating MnSOD protein levels in all examined tissues (Fig. 1). In liver of the minipump delivery group, MnSOD was elevated with RES treatment when compared to the DMSO vehicle control (Fig. 1A). A substantial increase in MnSOD protein level in brain was observed when RES was given in a high fat diet. Interestingly, administration of RES through an osmotic minipump, which was hypothesized to provide the highest circulating levels of RES, was ineffective in raising MnSOD protein levels above control values in brain (Fig. 1B). In contrast to observations made in brain tissue, MnSOD levels decreased in heart tissue of mice fed RES in a high fat diet (Fig. 1C). To ascertain whether changes in protein level were reflective of enzyme activity, SOD activity was measured. Changes in MnSOD protein levels in the high fat treatment method corresponded to a parallel increase, or decrease, in enzyme activity (Fig. 1D).

Administration of RES in a high calorie diet has been reported to increase mitochondrial number in liver, skeletal muscle and brown adipose tissue [6,11]. Therefore, to determine if any observed changes in MnSOD, a mitochondria specific antioxidant, were a result of a change in the number of mitochondria, citrate synthase (CS) activity was measured. CS is a citric acid cycle enzyme whose activity is commonly measured as a proxy of mitochondrial number. CS activity was unchanged in liver of RES-treated mice in all three delivery methods (Fig. 2A). CS activity was increased in brain tissue of mice given RES in high fat diet and through an osmotic minipump (Fig. 2B). An increase in CS activity was also observed in heart tissue of mice given RES in high fat diet, while a decrease in mitochondrial number was observed in the minipump treatment group (Fig. 2C). MnSOD protein levels were normalized to CS activity to account for any changes in protein level due to increased mitochondrial number. With mitochondrial number accounted for, changes in MnSOD protein level continued to follow the same trends (data not shown).

The effect of RES on cellular antioxidant enzyme capacity was further analyzed by measuring the activities of catalase and glutathione peroxidase, two enzymes that participate in the removal of hydrogen peroxide. RES delivery though a standard laboratory diet failed to change the activity of catalase in any of the examined tissues. An induction of catalase activity in heart tissue of mice administered RES through a high fat diet or osmotic minipump was observed (Fig. 3B), while this effect was absent in liver (data not shown) and brain tissue (Fig. 3A).

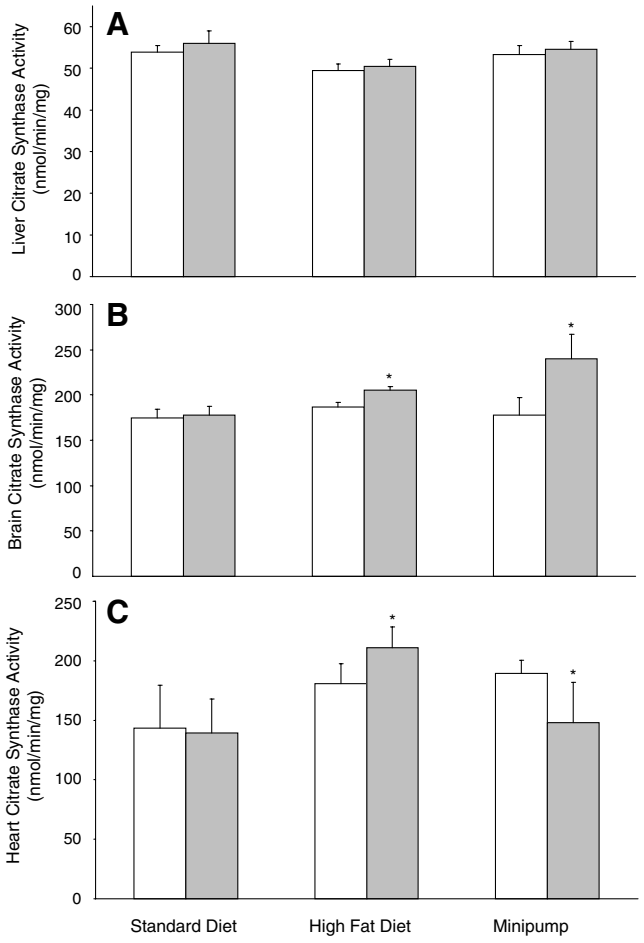

Fig. 2. Citrate synthase activity in brain, heart, and liver tissue of control (open bars) and resveratrol (solid bars) groups of three treatment methods ($n = 5–8$). (A) CS activity in liver homogenates (B) CS activity in brain homogenates and (C) CS activity in heart homogenates. Values shown are means ± SEM of duplicate measurements of each homogenate. *Significantly different from control group ($P < 0.05$).

A subtle decrease in glutathione peroxidase activity was observed in brain tissue of mice treated with RES through high fat diet or osmotic minipump (Fig. 4A). In contrast, GPx activity was increased in heart cells of mice given RES through a high fat diet. A trend toward increasing GPx activity in the heart tissue of the RES minipump treatment group was also observed, although the effect did not reach statistical significance (Fig. 4B).

Discussion

Recently, RES has been reported to improve mitochondrial function of mice on a high calorie diet [6,11]. We hypothesized that an improved ability to metabolize mitochondrial ROS may play an important role in this observation, based on our previous finding that chronic RES treatment dramatically upregulates MnSOD in human cells *in vitro* [13]. MnSOD is the only SOD present in the mitochondrial matrix, and is capable of reducing intracellular oxidative stress. Overexpression of MnSOD increases resistance to mitochondrial dysfunction, permeability transition, and apoptotic death invoked by oxidative stress in various disease contexts [16–20].

It is therefore interesting that RES administered in a high fat diet induced a significant increase in MnSOD protein level (140%) and activity (75%) in brain tissue that could not be explained simply by proliferation of mitochondria (10%). This is an important observation given that MnSOD overexpression alone is neuropro-

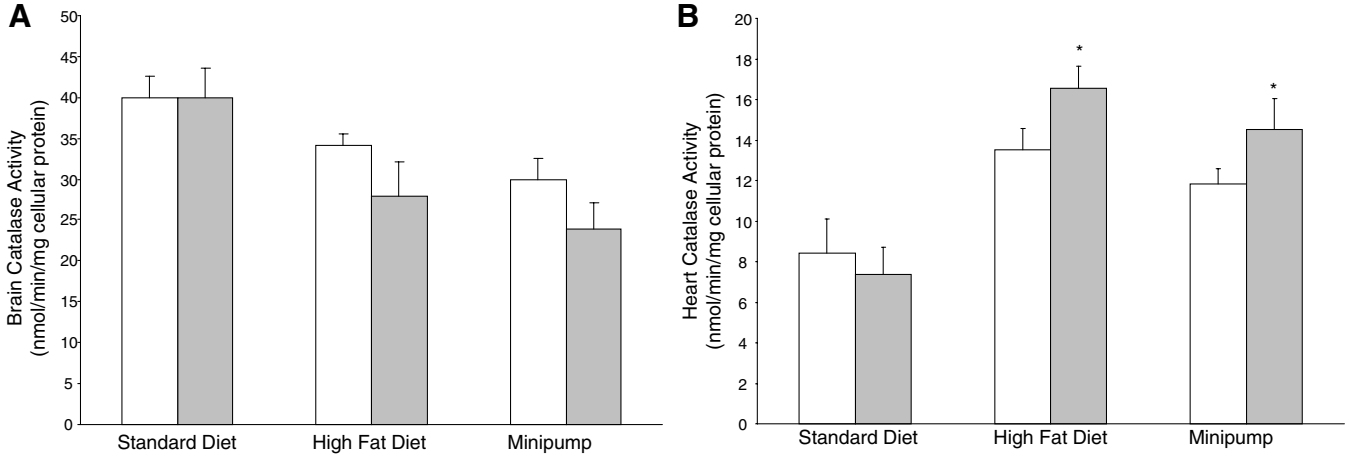

Fig. 3. Catalase activity in brain and heart tissue of control (open bars) and resveratrol (solid bars) groups of three treatment methods ($n = 5–8$). (A) CAT activity in brain homogenates (B) CAT activity in heart homogenates.

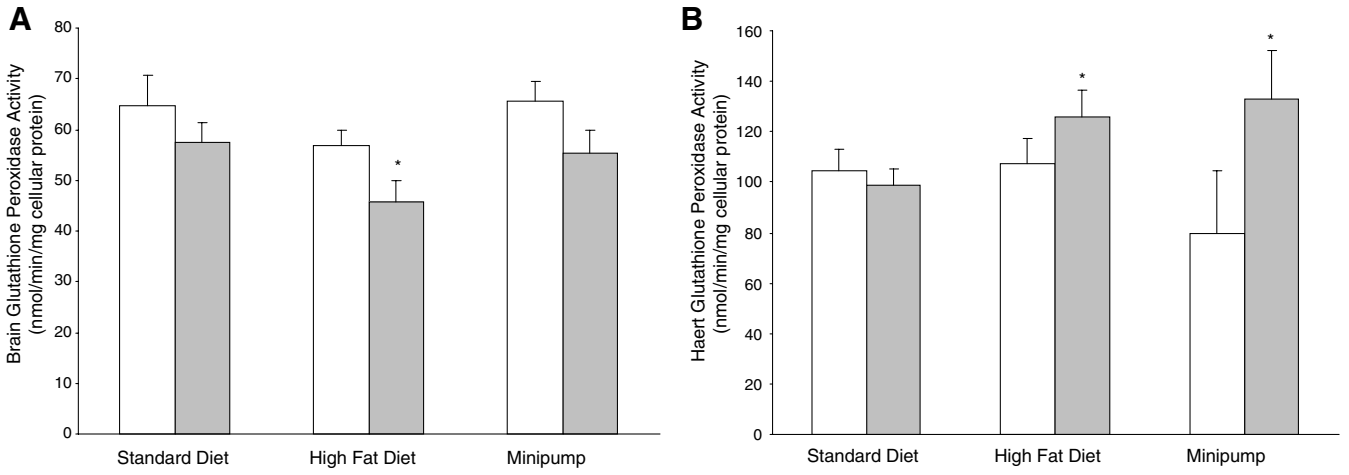

Fig. 4. Glutathione peroxidase activity in brain and heart tissue of control (open bars) and resveratrol (solid bars) groups of three treatment methods ($n = 5–8$). (A) GPx activity in brain homogenates (B) GPx activity in heart homogenates.

tective, ameliorating oxidative damage in response to ischemic events and chemical stressors such as MPTP [19–22]. RES has also been shown to protect against neuronal death [23–25] and is therefore of interest for its potential ability to protect against neurodegeneration [26]. We thus suggest that MnSOD is a downstream target of RES that plays a role in the neuroprotective effects of this polyphenol. From the present study, it is clear that the mode of delivery is important in determining RES's effects. Improved delivery methods, or longer term treatments may allow for further elevation of MnSOD expression in the brain. In any event, it appears that RES could represent a safe dietary means by which increased MnSOD expression can be achieved in brain, and neuroprotective benefit realized.

MnSOD protein level has been shown to vary between different regions of the brain [27], however, whether this reflects differences in MnSOD per mitochondrion, or simply differences in the mitochondrial content of neurons within different brain regions is unclear. While evidence of MnSOD induction with RES may support its use to prevent oxidative stress associated with neurodegenerative disease, this experiment was conducted with whole tissue homogenates, and it is therefore not possible to determine whether specific areas of the brain are influenced more than others. It will be interesting to examine specific brain regions, such as the substantia nigra, to determine whether the effects of RES are highly localized.

RES has many cardio-protective properties [5] and interacts with a number of antioxidants in the heart, such as thioredoxin [28]. Previous studies have reported increased levels of SOD in cultured cardiomyocytes (H9C2 cells) treated with micromolar concentrations of RES, as well as in the myocardium of diabetic hearts [29,30]. Nonetheless, under the conditions used in this study a decrease in MnSOD protein level and activity was observed. RES did, however, induce subtle increases in the activities of glutathione peroxidase and catalase in heart tissue of both the high calorie and minipump groups. This agrees with previous observations made in vascular tissue treated with RES. In these experiments, GPx and CAT were found to be critical in RES's protective effects against oxidative stressors in cultured aortic preparations [31]. It is therefore interesting that changes in these enzymes were also observed *in vivo*. The net result of reduced MnSOD activity concomitant with increased capacity to remove H_2O_2 should be a reduction in $[H_2O_2]$, which could also be protective in cardiomyocytes.

In addition to altering MnSOD levels, RES has been reported to interact directly with mitochondrial oxidative phosphorylation [32–34] and biogenesis [6,11,35]. One observation that has been

made is that RES treatment in a high fat diet increases mitochondrial abundance in liver, brown adipose tissue and skeletal muscle of mice [6,11]. Interestingly, we did not observe an increase in CS activity (a proxy of mitochondrial number) in liver, despite using a comparable dose of RES in the same strain of mice. This may be due to differences in the length of treatment time and the age of the mice at the onset of RES treatment. However, significant increases in CS activity were observed in brain and heart tissue of the high fat RES group. Therefore, it seems that oral administration of RES is capable of inducing mitochondrial proliferation in a variety of highly oxidative tissues. The mechanism and significance of this potentially important observation remain to be determined. It may represent a general shift toward a more oxidative metabolism, as is observed in caloric restriction [6]. However, genetic manipulations that increase MnSOD have also been shown to increase mitochondrial oxidative capacity [16]. Therefore, the improved mitochondrial and physiological function observed in rodents treated with RES administered in high fat diets may be directly linked to the induction MnSOD or other antioxidant enzymes in addition to mitochondrial proliferation.

While we do not know the circulating levels of RES in each treatment group, it is interesting that the extent to which RES was able to induce changes in antioxidant enzymes and mitochondrial number was dependent on its route of administration. This perhaps suggests that the delivery modes were able to augment RES's transport and accumulation in the body. RES undergoes extensive chemical modifications in the small intestine following its ingestion, and is quickly metabolized [9]. Despite its apparent low bioavailability, 4 weeks of chronic exposure to RES was sufficient to induce a number of changes in the observed enzymes. RES is a hydrophobic molecule, and it may be that interactions with serum proteins and lipids are able to increase localized concentrations, while circulating plasma levels remain low. In any case, administering RES in a high fat diet was highly effective in modifying antioxidant enzyme activities, and mitochondrial number. This property may be exploited to further increase the bioavailability of this polyphenol following ingestion.

In conclusion, RES in a high fat vehicle may represent a dietary means of achieving the protective effects of increased MnSOD levels in the brain.

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