Supplemental Information for:

Resveratrol ameliorates aging-related metabolic phenotypes by inhibiting cAMP phosphodiesterases

EXTENDED EXPERIMENTAL PROCEDURES

Resveratrol treatment

Approximately 16 hr prior to treating Hela cells with resveratrol, the concentration of fetal calf serum was reduced from 10% to 0.2%. For C2C12 myotubes, the horse serum concentration (2%) was not changed prior to treatment with resveratrol. A stock solution (50 mM) of resveratrol (Calbiochem) was generated by dissolving it in DMSO. Although only studies using 50 μM resveratrol are shown here, we have obtained highly similar results with 25 μM resveratrol.

SiRNA

The human Epac1 siRNA and PKAc (α) siRNA were purchased from Dharmacon. Control siRNA was also from Dharmacon. Epac1 and PKAc (α) were knocked down by transfecting Hela cells with siRNA by using Lipofectamine2000 according to the manufacturer's protocol. Four days after transfection, Hela cells were treated with resveratrol as described above.

Cyclic AMP measurement

The cyclic AMP complete enzyme immunoassay kit from Assay Designs was used as directed by the manufacturer.

ROS measurements

ROS levels were determined in muscle extracts using the ROS sensitive fluorescent dye dichlorodihydrofluorescein (DCF). Briefly, oxidation-insensitive dye (carboxy-DCFDA) was used as a control to ensure that changes in the fluorescence seen with the oxidation sensitive dye (H2DCFDA) were due to changes in ROS production. Oxidation insensitive and oxidation-sensitive dyes were first dissolved at a concentration of 12.5 mM and diluted with homogenization buffer to 125 μM immediately before use. Diluted dyes were added to tissue extract (100 μg) in a 96- well plate to achieve a final concentration of 25 μM. The change in fluorescence intensity was monitored at two time points (0 and 30 min) by using a microplate

fluorescence reader (Bio-Tek Instruments), with excitation set at 485 nm and emission set at 530 nm.

Photoaffinity-labeling

Photoaffinity labeling of PDE3 with the fluorescent cAMP analog 8-azido-[DY-547]-cAMP was carried out in 0.1 ml total volume containing 50 mM HEPES (pH7.5), 250 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, 0.1 mM DTT, 5 μg ovalbumin, 0.2 μM 8-azido-[DY-547]-cAMP (Axxora), 150 μM IBMX, protease inhibitor cocktail (Roche), and with the indicated concentrations of cAMP or resveratrol. After incubation (96 well cell culture plates, 30 min on ice, in darkness), samples were irradiated (15 min) with a mineral lamp (Ultraviolet products, 254 nM) positioned 6 cm above the top of the plates. Reactions were terminated by boiling in Laemmli SDS sample buffer and subjected to SDS-PAGE. Binding of fluorescent 8-azido-cAMP was detected by scanning with Typhoon (Amersham).

Body temperature measurement

Temperature was determined between 9 am and 11 am (room temperature was 22 ± 1°C) with a rectal probe (Physitemp Instruments) connected to a portable thermometer (TH-5 Physitemp instruments).

Fat index calculation

Fat mass was first measured by NMR spectroscopy by using a Minispec (Bruker Biospin Corporation, Houston, TX). Fat index was calculated by dividing the fat mass by total body weight.

Docking analysis

The crystal structure of PDE3B catalytic domain (PDE entry code of 1SOJ) and AutoDock 4.2 (Huey et al., 2007) was used for docking of resveratrol. A grid box with 90 x 90 x 90 points equally spaced at 0.375 Å was generated using AutoGrid. Parameters used for Lamarckian genetic algorithm (LGA) were as follows: random initial orientation and position, population size (150), maximum number of energy evaluations (25 million), maximum number of generations (27000), mutation rate (0.02), crossover rate (0.8), and 100 docking runs. The final

100 conformations produced by this docking method were clustered if their root-mean-square deviations differed by less than 2.0 Å.

Mitochondrial DNA (mtDNA) Quantification

Relative amounts of nuclear DNA and mtDNA were determined by quantitative Real-Time PCR. The ratio of mtDNA to nuclear DNA reflects the mitochondrial content in a cell. Muscle tissue (gastrocnemius) was homogenized and digested with Proteinase K overnight in a lysis buffer for DNA extraction by using the DNeasy kit (QIAGEN). Quantitative PCR was performed by using the following primers (mtDNA specific PCR, forward 5'- CCGCAAGGGAAAGATGAAAGA-3', reverse 5'-TCGTTTGGTTTCGGGGTTTC- 3'; and nuclear DNA specific PCR, forward 5'-GCCAGCCTCTCCTGATGT-3', reverse 5'-GGGAACACAAAAGACCTCTTCTGG- 3' and SYBR Green PCR kit in a prism 7500HT sequence detector (Applied Biosystem) with a program of 20 minutes at 95°C, followed by 50 to 60 cycles of 15 seconds at 95°C, 20 seconds at 58°C and 20 seconds at 72°C. Mitochondrial DNA content was normalized with nuclear DNA content.

Transfection of full-length and catalytic domain PDE4

Expression vectors for full-length PDE4 and the catalytic domain of PDE4 were constructed by inserting the cDNA encoding His-tagged full-length PDE4D7 or the catalytic domain of PDE4D (Burgin et al., 2010) into the mammalian expression vector pCDNA3. Subconfluent C2C12 myocytes (80%) were transfected by using the Lipofectamine 2000 transfection reagent with expression vectors for full-length PDE4 or the catalytic domain of PDE4. Myocytes were then differentiated into myotubes according to the standard protocol by using DMEM containing 2% horse serum.

Oxygen consumption rate measurements in C2C12 myotubes

C2C12 cells were seeded in XF 24-well cell culture microplates (Seahorse Bioscience, North Billerica, MA) at 2.0–3.0 x 10^4 cells/well (0.32 cm^2) in 250 µl high glucose DMEM growth medium supplemented with 10% fetal bovine serum and 1% pen/strep. Following 24h, cells were switched to low serum media (2% horse serum, 1% pen/strep) to induce differentiation. Cells were fed every 24h for 4 days. On the day of testing, 10µM of 007, 50 µM of resveratrol, 25 µM of rolipram or DMSO as a vehicle control were suspended in fresh DMEM media and the cells

were returned to the incubator for 6 hr. After 6 hr, cells were washed twice with 500 µl assay medium (unbuffered low glucose DMEM supplemented with pyruvate and glutamine, pH 7.4). A final volume of 600 µl of assay medium was added to each well prior to the experimental protocol. Cells were then transferred to a CO_2 free incubator, maintained at 37C for one hour before the start of the assay. Following assay calibration, measurements of oxygen consumption rate (OCR) were performed every 10 minutes for 2.5 hours. At 30, 60, 90, and 120 minutes, 75 µl of palmitoleate (100 µM)/2% BSA was injected sequentially into each well and the effect on OCR was determined. At the end of the assay, media was aspirated, cells were washed twice with cold PBS and cells were collected from each plate in 50 µl lysis buffer containing protease inhibitors (50mM Tris pH 7.5; 250mM Sucrose; 1mM EDTA; 1mM EGTA; 1% Triton X-100; 1mM NaVO4; 50mM NaF; 0.10% DTT; 0.50% protease inhibitor cocktail) and protein content was determined by using the DC protein assay (Biorad, Hercules, CA). The oxygen consumption rate was normalized to protein content.

Reagents

[^{3}H]cAMP was from Amersham. Purified recombinant PDE1, PDE2, PDE4 and PDE5 were obtained from Signalchem. Flag-PDE3B was purified from Sf21 cell lysates using a Flag-agarose (Sigma) affinity column. Recombinant PKA-R2 and recombinant PKA holoenzyme were obtained from Sigma and Biaffin (Germany), respectively. Rolipram, ryanodine and U73122 were purchased from Calbiochem.

Immunoblotting

Cells were lysed in RIPA buffer and subjected to immunoblotting. For tissue extraction, samples were pulverized in liquid nitrogen and homogenized in a lysis buffer. The following antibodies were used: AMPK, p-AMPK (T172), phospho-ACC, which recognizes phosphorylated Ser 79 in ACC1 or phosphorylated Ser 22 in ACC2 and ACC (Cell Signaling Technology); Sirt1 (Upstate Biotechology), V5 (Invitrogen) and Actin (Santa Cruz). PGC-1α acetylation was visualized by immunoprecipitation from the cell extract (500 µg) using PGC-1α antibody (Santa Cruz) followed by immunoblotting with antibody specific for acetylated lysine (Cell Signaling Technology) or for PGC-1α. Levels of PGC-1α acetylation were then quantified by scanning densitometry.

Ca^{2+} signal measurements

C2C12 myoblasts were seeded on a 96 well plate (Perkin Elmer). After differentiation, they were preincubated with 20 μM U73122 for 1 hr. Ca^{2+} release was measured using the fluorescent calcium indicator Fluo-4AM (Molecular Probes) according to manufacturer`s suggestions. Ca^{2+} increases are reported as $\Delta F/F$ $((F-F_{basal})/F_{basal})$, where F indicates fluorescence.

Real-time PCR

Total RNA was isolated by using the TRIzol reagent extraction kit (Invitrogen), according to the manufacturer's instructions. RNA was subsequently reverse transcribed to cDNA by using the high capacity cDNA archive kit (ABI). The mRNA levels were measured by real time PCR using the ABI PRISMTM 7900HT Sequence Detection System (Applied Biosystem).

NAD^+/NADH ratio measurements

The NAD^+/NADH ratio was measured from whole cell extracts of C2C12 myotubes using the NAD^+/NADH quantification kit from Biovision based on an enzymatic cycling reaction, according to the manufacturer's instructions.

Adenylyl cyclase activity measurements

Adenylyl cyclase was expressed in Sf9 cells and Sf9 membranes containing individual adenylyl cyclase isoforms were prepared as described elsewhere (Taussig et al., 1994). Adenylyl cyclase activity was measured using the procedure previously described (Smigel, 1986). All assays were performed for 10 min at 30°C in a final volume of 100 μl containing 10 μg of adenylyl cyclase expressing Sf9 membrane protein and a final concentration of 10 mm $MgCl_2$. Forskolin and resveratrol were added to the assay tube to a final concentration of 100 uM when tested. Assays were performed in duplicate and the results are represented as mean±S.D. of two experiments.

EPAC Assay

Measurements were performed essentially as previously described (Rehmann, 2006), but with the use of the fluorescence GDP analogue GDP-BODIPY (Molecular Probes) instead of mant-GDP. Briefly, 200 nM Rap1B preloaded with GDP-BODIPY was incubated in the presence of 100 nM Epac1 (residues 149-881) and an excess of 20 μM GDP and in the presence chemicals as indicated. Nucleotide exchange is monitored as a decrease in the fluorescence signal over time, since Rap bound GDP-BODIPY displays an approximate 5 times higher fluorescence intensity than does free GDP-BODIPY in the buffer solution.

Serum GLP-1 measurement

Serum GLP-1 levels were measured using GLP-1 (active) ELISA (Millipore), according to the manufacturer's directions.

Rap1 pull down assay

pGEX Ral GDS-RA, an expression vector for GST-RalGDS-RBD, was transformed into *Escherichia coli* (strain BL21). Protein production was initiated by addition of isopropyl β-D-thiogalactopyranoside (IPTG) to the culture. The fusion protein was affinity purified on a glutathione sepharose 4B column (Amersham Bioscience) from the supernatant of bacteria lysed by sonication. GST-RalGDS-RBD precoupled to a glutathione sepharose 4B column was added to the cell lysates and incubated at 4°C for 60–180 min with slight agitation. Beads were washed four times in lysis buffer and subjected to immunoblotting.

SUPPLEMENTAL REFERENCES

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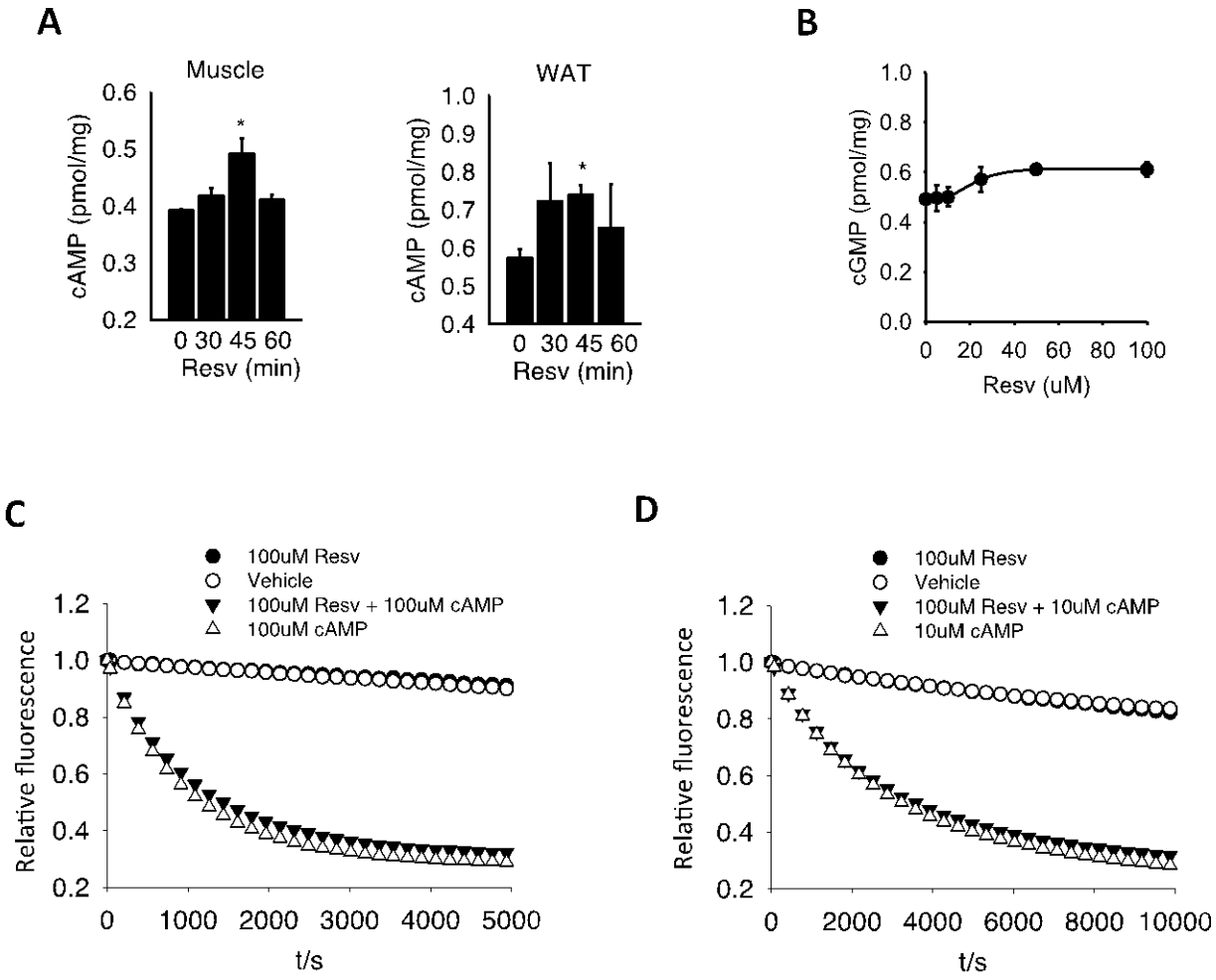


Figure S1, related to Figure 1. The effect of resveratrol on cAMP levels.
(A) Cyclic AMP levels in mouse skeletal muscle and white adipose tissue (WAT) after administration of resveratrol by oral gavage (100 mg/kg) (n=4). *, $p<0.05$ compared to vehicle-treated (0 min).
(B) Cyclic GMP levels in C2C12 myotubes 30 min after treatment with the indicated concentrations of resveratrol.
(C, D) Resveratrol does not affect EPAC activity. The effect of resveratrol (100 μM) or vehicle on EPAC activity in the absence or presence of 100 μM (C) or 10 μM (D) cAMP.

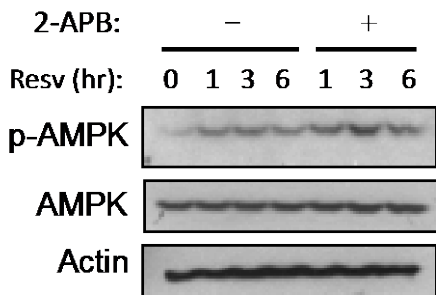
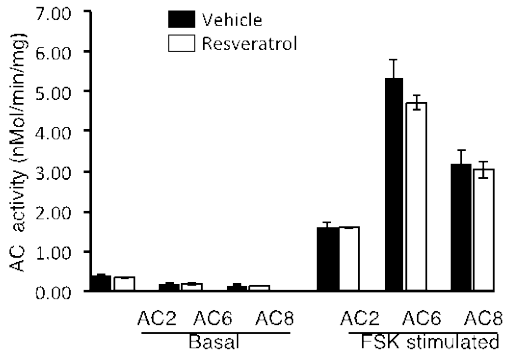


Figure S2, related Figure 3. The IP3 receptor is not essential for resveratrol to activate AMPK. C2C12 myotubes were treated with resveratrol either in the presence (+) or absence (-) of the IP3 receptor antagonist 2-APB.

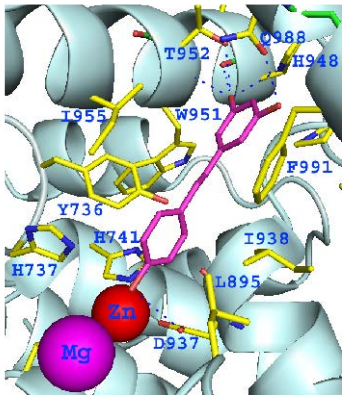
A



B

Cluster	Models in cluster	Binding energy (−kcal/mol)	*Kd* (μmol)
1	32	6.92 – 7.64	2.5 – 8.47
2	2	7.13 – 7.15	5.7 – 5.9
3	64	6.76 – 7.04	6.89 – 11.14
4	1	6.99	7.55
5	1	6.7	12.22

C



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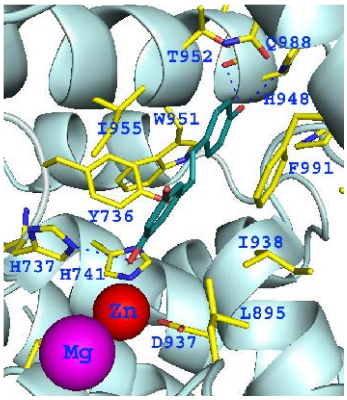

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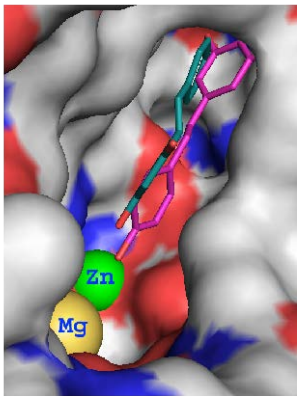

Figure S3, related to Figure 4. Resveratrol is a PDE inhibitor.

(A) Resveratrol does not affect adenylyl cyclase activity. Adenylyl cyclase activity of AC2, AC6 and AC8 was measured in the presence of resveratrol (100 μM) or DMSO both before (basal) and after stimulation with forskolin (FSK) (n=2). Results are expressed as the mean±S.D.

(B) Simulated binding of resveratrol to PDE3B. The binding energy and K_d are shown for each cluster.

(C) Simulated binding of the representative of docked cluster #1 resveratrol (pink sticks) to the PDE3B active site. Interacting residues are shown in yellow sticks. The hydrogen bonds are indicated with dotted lines.

(D) Simulated binding of the representative of docked cluster #3 resveratrol (green sticks) to the PDE3B active site.

(E) A surface model of the overlays of the two main docked conformations of resveratrol (sticks) into the pocket of PDE3B. Colors red and blue represent oxygen and nitrogen, respectively.

Explanation of the Docking results: To investigate how resveratrol may bind to the active site of PDE3, the crystal structure of the PDE3B catalytic domain (Scapin et al., 2004) was used as the template for docking of resveratrol. The hundred docked conformations output from program

AutoDock 4.2 (Huey et al., 2007) can be clustered into two major groups that account for 32 and 64% of the total population (Figure S3B). Cluster #1 has the lowest binding energy and the simulated dissociation constant (K_d) of 3-9 μM. The oxygen atom of the tyrosyl group forms a hydrogen bond with Asp937 on one end of resveratrol while the oxygens of the 2,4-dihydroxylbenzenyl group on the other end forms three hydrogen bonds with His948, Thr952, and Gln988 (panels C and E). In addition, the models in cluster #1 contact via van der Waals force with residues of Tyr736, Leu895, Ile938, Pro941, Trp951, Ile955, Gln988, and Phe991. Cluster #3 has a larger K_d at 7-11 μM and forms only two hydrogen bonds with His948 on one end of resveratrol and with His737 on another end, respectively (panel D and E). The models in cluster #3 form van der Waals interactions with the similar set of residues as cluster #1 does, although the interactions of individual atoms in two clusters are different. An invariant glutamine, Gln988 in PDE3B, has been shown to form hydrogen bonds with substrates and inhibitors in all PDE families (Ke and Wang, 2007). However, the resveratrol model shows that cluster #1, but not cluster #3, forms a hydrogen bond with Gln988.

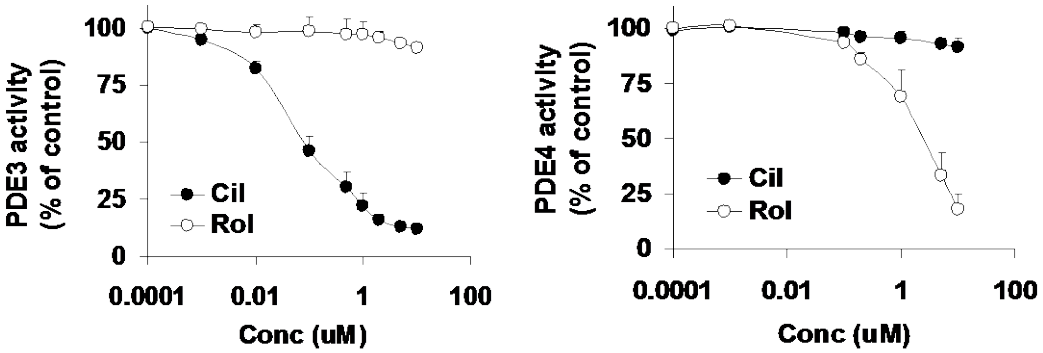

Figure S4, related to Figure 5. Inhibitors of PDE3 and PDE4 are highly specific.
Phosphodiesterase activities of recombinant PDE3 and PDE4 were measured in the presence of PDE3 inhibitor cilostamide (Cil) and PDE4 inhibitor rolipram (Rol).