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Mild mitochondrial uncoupling in mice affects energy metabolism, redox balance and longevity

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Summary

Caloric restriction is the most effective non-genetic intervention to enhance lifespan known to date. A major research interest has been the development of therapeutic strategies capable of promoting the beneficial results of this dietary regimen. In this sense, we propose that compounds that decrease the efficiency of energy conversion, such as mitochondrial uncouplers, can be caloric restriction mimetics. Treatment of mice with low doses of the protonophore 2,4-dinitrophenol promotes enhanced tissue respiratory rates, improved serological glucose, triglyceride and insulin levels, decrease of reactive oxygen species levels and tissue DNA and protein oxidation, as well as reduced body weight. Importantly, 2,4-dinitrophenol-treated animals also presented enhanced longevity. Our results demonstrate that mild mitochondrial uncoupling is a highly effective *in vivo* antioxidant strategy, and describe the first therapeutic intervention capable of effectively reproducing the physiological, metabolic and lifespan effects of caloric restriction in healthy mammals.
Key words: caloric restriction; 2,4-dinitrophenol; energy conversion; free radicals; life span.

Introduction

Caloric restriction, or the limitation of dietary calories without lack of essential nutrients, extends lifespan in a variety of species, including yeast, worms, flies, mice, rats and, probably, nonhuman primates (Sohal & Weindruch, 1996; Partridge & Gems, 2002; Roth *et al*., 2004). In humans, caloric restriction leads to improvements in blood glucose and plasma lipid levels similar to those seen in other animals (Walford *et al*., 2002).

One of the central effects of caloric restriction in many models is to promote changes in mitochondrial respiratory rates (Lin *et al*., 2002; Merry, 2004; Barros *et al*., 2004; Bonawitz *et al*., 2007; Guarente, 2008). In *Saccharomyces cerevisiae*, caloric restriction augments replicative and chronological lifespan by increasing mitochondrial respiration (Lin *et al*., 2002; Barros *et al*., 2004; Fabrizio *et al*., 2005; Tahara *et al*., 2007), enhancing the activity of the Sir2p histone deacetylase (Lin *et al*., 2000) and preventing the build-up of mitochondrially generated reactive oxygen species (ROS; Barros *et al*., 2004; Tahara *et al*., 2007). Indeed, a variety of interventions that enhance or inhibit mitochondrial respiration in yeast augment or decrease lifespan, respectively (Lin *et al*., 2002; Barros *et al*., 2004; Bonawitz *et al*., 2007). Dietary restriction also leads to increased respiration and longevity in *Caenorhabditis elegans* (Bishop & Guarente, 2007). In *Drosophila melanogaster*, *NF1* gene mutants have shortened lifespans associated with decreased respiratory rates and elevated ROS formation, while flies overexpressing NF1 present increased lifespan and respiration, along with lower ROS production (Tong *et al*., 2007). Increasing respiration in flies by expression of uncoupling protein decreases ROS production and enhances lifespan (Fridell *et al*., 2005). In addition, treating larvae with the chemical uncoupler 2,4-dinitrophenol (DNP) enhances average lifespan (Padalko, 2005).

In mammals, many studies (but not all, see Lambert & Merry, 2005; Ferguson *et al*., 2007) demonstrate that caloric restriction stimulates respiratory rates (see Guarente, 2008, for a review). Increases in respiration involve enhanced biogenesis and increases in mitochondrial density in tissues (Lambert *et al*., 2004; Nisoli *et al*., 2005) as well as decreases in coupling between oxygen consumption and oxidative phosphorylation (Lambert & Merry, 2004; Merry, 2004; Xiao *et al*., 2004). In addition, Speakman *et al*. (2004) elegantly demonstrated that mice which spontaneously exhibit enhanced lifespans present higher oxygen consumption rates, strongly suggesting a direct association between mitochondrial respiration and the aging process.

Respiratory rates are well known to affect mitochondrial ROS production (Korshunov *et al*., 1997; Skulachev, 1998; Balaban *et al*., 2005) and caloric restriction is widely associated with a decrease in oxidative damage (see Sohal & Weindruch, 1996; Merry, 2004, for reviews). In addition, antioxidants targeted to mitochondria increase lifespan in mice (Schriner *et al*., 2005; Skulachev, 2007), suggesting that mitochondrially generated ROS are a cause of lifespan limitation. On the other hand, decreases in ROS release measured in mitochondria from calorically restricted animals (Sohal & Weindruch, 1996; Merry, 2004) are not consistently found when using intact cells (Lambert & Merry, 2005), and accumulation of oxidative damage in mitochondria is not necessarily associated to functional defects and enhanced aging (Stuart *et al*., 2005).

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Altogether, although the role of ROS in aging is still not directly determined, a large body of results indicate a strong correlation between caloric restriction, mitochondrial respiration and lifespan. However, a direct causative role of respiratory increments in lifespan extension still remains to be established in mammals. To determine if enhanced mitochondrial respiratory rates were causative of beneficial effects of caloric restriction, we treated mice with low doses of the classic mitochondrial protonophore DNP. DNP allows protons to cross the inner mitochondrial membrane in a manner not coupled to oxidative phosphorylation (Parascandola, 1974; Harper *et al.*, 2001), resulting in increased electron transport and oxygen consumption rates. Due to its uncoupling properties, DNP was used as a diet pill in the 1930s, although its toxicity at doses close to therapeutic made it an undesirable clinical tool (Parascandola, 1974). Here, we used doses 10–100 times lower than those used therapeutically in the past, and over 1000 times lower than the lethal dose for mice (Parascandola, 1974; De Felice & Ferreira, 2006), as a tool to investigate the effects of systemic mild mitochondrial uncoupling on animal energy metabolism, redox state and lifespan. We found that treatment with low doses of DNP promoted the beneficial effects of caloric restriction, including reducing tissue markers of oxidative stress, body weight, serum glucose and triglyceride levels. More importantly, mild mitochondrial uncoupling significantly increased lifespan.

Results

In order to assess if mitochondrial uncoupling could effectively mimic caloric restriction, we treated adult mice chronically with the uncoupler DNP. A DNP concentration (1 mg L^{-1} of drinking water) that promoted mild decreases in weight gain was chosen from preliminary doses tested (results not shown) and was given to adult, female, Swiss mice starting at 18 weeks of age (see the Experimental procedures). Although hyperthermia occurs with toxic DNP doses (Parascandola, 1974), the dosage used here was low enough not to affect body temperatures or water intake (Fig. 1a,b). It is important to note that, because animals were housed at 22 °C, extra heat generated due to uncoupling promoted by DNP could easily dissipate, avoiding hyperthermia. Food intake was also equal in both groups (Fig. 1c), while DNP-treated animals presented significantly lower body mass (Fig. 1d). The lower weight gain in the DNP group in the presence of equal food ingestion indicates a lower efficiency of conversion of ingested food. Indeed, weight gain/ingestion was decreased in DNP-treated animals (Fig. 1e). This effect is typical of mild mitochondrial uncoupling (Harper *et al.*, 2001), and is also found in calorie-restricted animals (Lambert & Merry, 2004; Nisoli *et al.*, 2005).

The uncoupling effect of DNP treatment on mitochondrial respiration was confirmed by measuring tissue oxygen consumption (Fig. 2a). Interestingly, systemic DNP administration

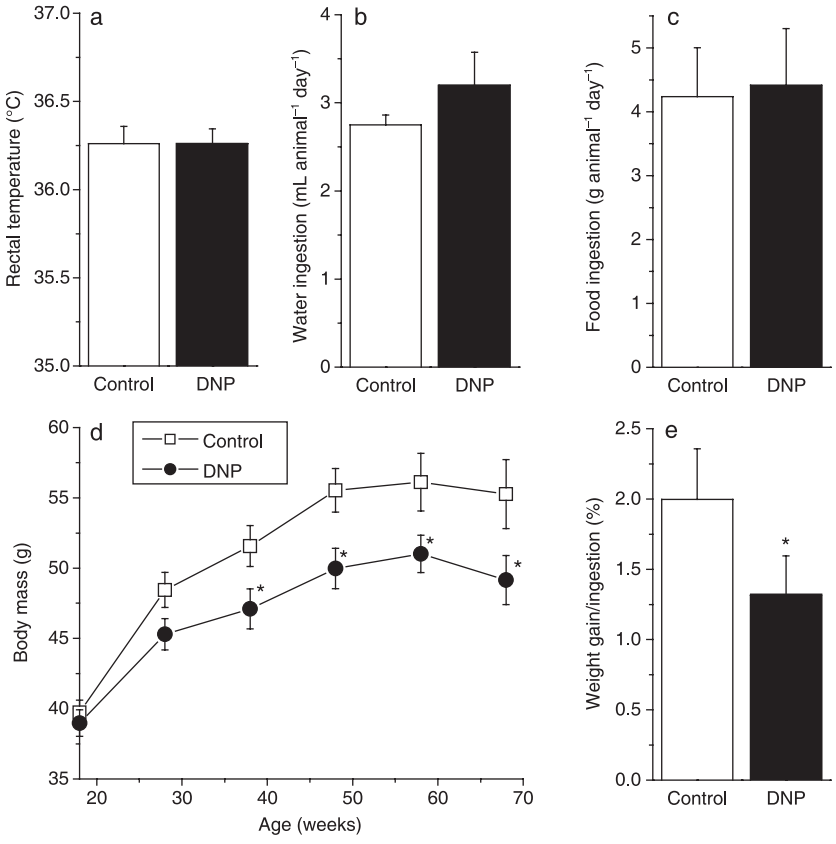

Fig. 1 Mice treated with 2,4-dinitrophenol (DNP) present lower efficiency of energy conversion. Rectal body temperatures (a), water ingestion (b), food ingestion (c), body mass (d), and efficiency of energy conversion (e) were determined as described in the Experimental procedures for control (empty bars/symbols) and DNP-treated (full bars/symbols) animals. *$p < 0.05$ vs. control.

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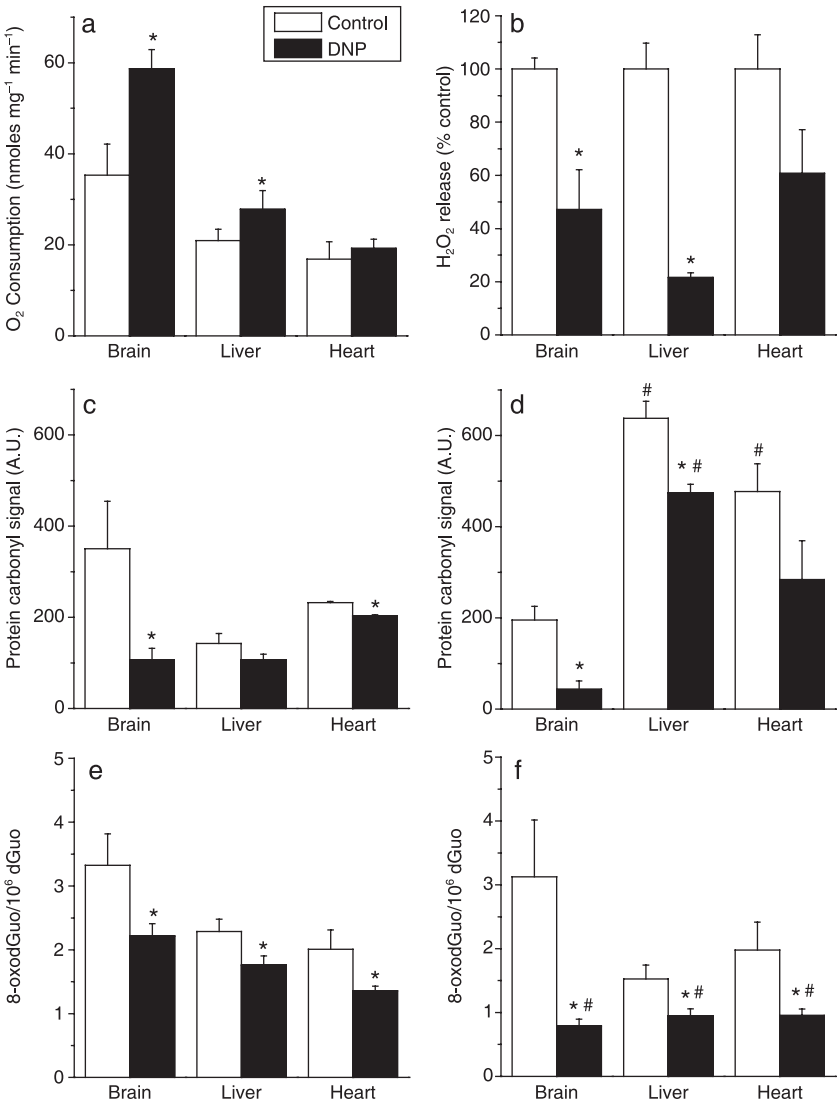

Fig. 2 2,4-Dinitrophenol (DNP) increases respiratory rates and prevents oxidative stress. Tissue oxygen consumption (a), H_2O_2 release (b), protein carbonyls (after 1 month, c; or 5 months, d) and 8-oxodGuo (after 1 month, e; or 5 months, f) were determined as described in the Experimental procedures for control (empty bars) or DNP-treated (full bars) animals.*$p < 0.05$ vs. control; #$p < 0.05$ vs. 1 month.

lead to tissue-selective respiratory effects: a small change was observed in heart, a mild increment was observed in liver, and considerable respiratory enhancement was measured in brain samples. We believe that DNP may accumulate more substantially in the brain due to enhanced lipid contents.

We focussed next on measuring the impact of respiratory changes on redox state in DNP-treated animals. Caloric restriction increases respiration and prevents the production of oxidants in isolated mitochondrial preparations, explaining lower damage to tissues in aged calorie-restricted animals (Sohal & Weindruch, 1996; Lambert & Merry, 2004; Merry, 2004). Indeed, higher respiratory rates and mitochondrial uncoupling have been extensively shown to be associated with a decrease in ROS production in isolated mitochondrial preparations (Korshunov *et al*., 1997; Skulachev, 1998; Balaban *et al*., 2005). However, studies using intact cells report conflicting results and have not universally uncovered a decrease in ROS formation with caloric restriction or mitochondrial uncoupling (Nègre-Salvayre *et al*., 1997; Lambert & Merry, 2005; MacLellan *et al*., 2005; Johnson-Cadwell *et al*., 2007). In our animals systemically treated with uncoupler, a lower release of oxidants was observed (Fig. 2b). In order to confirm that the prevention of ROS formation under our conditions was accompanied by an improvement in redox state, we measured oxidation levels of DNA and proteins in the brain, liver and heart. After 1 month of treatment with DNP, animals presented lower protein carbonylation (Fig. 2c), a pattern that showed stronger significance after 5 months of treatment (Fig. 2d). In addition, levels of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) in the brain, liver and heart were significantly decreased after 1 month of DNP (Fig. 2e), a result indicative of lower levels of DNA oxidation. Strikingly, continued DNP treatment for 5 months lead to very significant reductions in 8-oxodGuo levels in all tissues tested (Fig. 2f), and further decreased oxidation levels relative to animals treated for 1 month.

In addition to evaluating redox state, serological data were collected to check the impact of mild mitochondrial uncoupling

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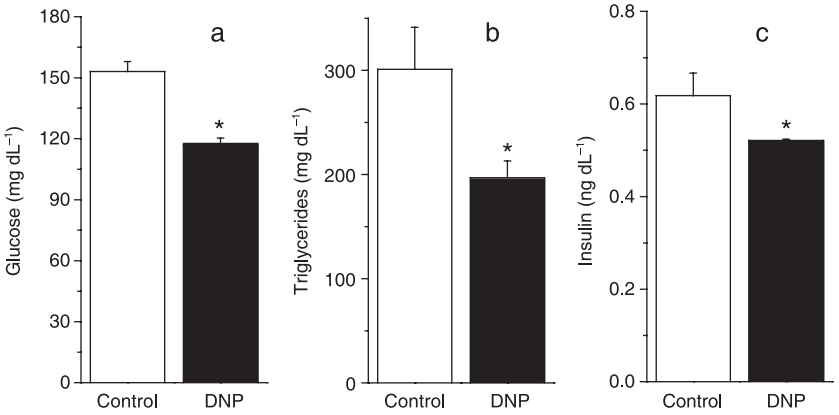

Fig. 3 Mice treated with 2,4-dinitrophenol (DNP) present improved serological parameters. Serum glucose (a), triglycerides (b) and insulin (c) levels were determined as described in the Experimental procedures for control (empty bars) and DNP-treated (full bars) animals. *$p < 0.05$ vs. control.

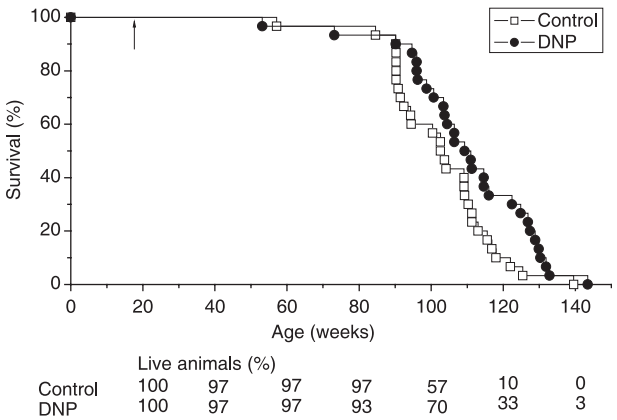

Fig. 4 2,4-Dinitrophenol (DNP) extends lifespan. Mice were treated with DNP (full symbols) starting at the 18th week of age (as indicated by the arrow), and lodged under standard conditions (see the Experimental procedures). DNP-treated animals (full symbols) presented a significant increment in mean lifespan ($p = 0.038$) when compared to controls (empty symbols).

on carbohydrate and lipid metabolism. Uncoupling with DNP significantly decreased serum glucose (Fig. 3a) and triglyceride levels (Fig. 3b). Furthermore, insulin levels were lower in DNP-treated animals (Fig. 3c). Again, the results observed are similar to those seen in caloric restriction (Masoro et al., 1992; Lev-Ran, 1998).

Because many of the characteristics of caloric restriction were found in DNP-treated mice, we evaluated whether long-term DNP treatment could extend lifespan. We found (Fig. 4) that DNP treatment in itself was capable of promoting an extension in murine longevity. Median longevity in the control group was 722 days, vs. 771 days in the DNP group, while mean lifespan was 718.8 days in the control and 769.7 days in the treated group. Although the change in lifespan promoted by DNP was not large, it is statistically significant ($p = 0.038$). It should also be noted that, although lifespans observed with female Swiss mice under control conditions in our facilities were slightly longer than those reported in the literature (Guayerbas & De La Fuente, 2003), this outbred strain has a lower life expectancy than long-lived inbred strains often used in longevity studies (Van Zant & de Haan, 1999). Altogether, our survival curves support the idea that artificially increasing mitochondrial respiration in mice leads the prevention of aging.

Discussion

Our study was designed to verify if therapeutic interventions promoting enhanced oxygen consumption through mild mitochondrial uncoupling were sufficient to have an impact on aging in mammals. Increases in mitochondrial respiratory rates are observed under a variety of conditions that are associated with enhanced longevity, including caloric restriction (see Guarente, 2008, for review). Indeed, mild mitochondrial uncoupling increases both replicative and chronological longevity in yeast (Barros *et al.*, 2004) and treatment with DNP or uncoupling protein expression increases lifespan in flies (Fridell *et al.*, 2005; Padalko, 2005). Unfortunately uncoupling proteins in mammals are tissue-selective and poorly active, as indicated by the lack of obesity in knockout animals (Arsenijevic *et al.*, 2000; Gong *et al.*, 2000). Furthermore, no effective pharmacological uncoupling protein agonist has been identified to date. Thus, we used a more direct approach to promote uncoupling and chronically treated mice with the protonophore DNP.

We identified a DNP dose (1 mg L^{-1} drinking water) that decreases the efficiency of energy conversion moderately (Fig. 1e), reaching levels similar to those observed in caloric restriction (Nisoli *et al.*, 2005). This treatment promotes a slow decrease in weight gain over time, but not weight loss (Fig. 1d) or hyperthermia (Fig. 1a). We found that systemic treatment with such low doses of DNP leads to enhanced tissue oxygen consumption (Fig. 2a), associated with a decrease in ROS release from these tissues (Fig. 1b), indicative of lower levels of formation of these species.

In isolated mitochondria, small changes in respiratory rates are typically accompanied by very substantial decreases in ROS release (Korshunov *et al.*, 1997; Skulachev, 1998). Although the efficacy of mitochondrial uncoupling as a mechanism to control ROS release is not universal, and may not be applicable to some *in vivo* systems (Johnson-Cadwell *et al.*, 2007), mild uncoupling is believed to be one of the central mechanisms through which

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oxidant production is controlled in mitochondria (Skulachev, 1996; Balaban *et al*., 2005). Indeed, based on the concept of mild uncoupling introduced by Skulachev (1996), Brand (2000) proposed the 'uncoupling to survive' hypothesis, which states that regulated mitochondrial uncoupling occurs as a mechanism to reduce oxidative stress, prevent oxidative damage and aging.

Uncoupling decreases mitochondrial ROS release through many mechanisms (Skulachev, 1998). First, higher respiratory rates increase oxygen consumption, possibly resulting in lower oxygen tensions in the mitochondrial microenvironment. This decreases the probability of one electron reduction of oxygen at the electron transport chain, generating the superoxide radical anion, the primary ROS produced by mitochondria (Balaban *et al*., 2005). A second effect of enhanced respiratory rates on ROS release is the maintenance of electron transport intermediates, in particular complexes I and III, at more oxidized states which cannot donate an electron to oxygen, producing superoxide radicals. Lower mitochondrial inner membrane potentials also decrease the occurrence of reverse electron transfer from complex II to complex I, a major source of ROS in many tissues (Turrens, 2003). Reverse electron transfer is thermodynamically feasible at high inner membrane potentials, which compensate for the differences in redox potential between complexes I and II. Finally, increased respiratory rates decrease ROS release by pyruvate and α-ketoglutarate dehydrogenases in mitochondria, due to increments in the availability of NAD^+ (Starkov *et al*., 2004; Tretter & Adam-Vizi, 2004; Tahara *et al*., 2007).

In all, preventing mitochondrial generation of ROS by uncoupling may be a far more effective antioxidant strategy than attempting to remove these species by supplementing antioxidants. Indeed, although antioxidant supplementation effectively decreases oxidative stress under pathological conditions and/or dietary limitations, there is little evidence of any effectiveness under physiological conditions (Møller & Loft, 2006; Hwang & Bowen, 2007). On the other hand, we show here that *in vivo* uncoupling (Fig. 2c–e), similarly to caloric restriction (Sohal & Weindruch, 1996; Merry, 2004), has a marked effect preventing physiological oxidative modifications to proteins and DNA. Our results thus demonstrate that chronic systemic mitochondrial uncoupling is a highly effective antioxidant strategy.

In addition to improving redox state, DNP treatment presented a positive effect on energy metabolism. Animals presented lower glucose and insulin levels, and significantly reduced serum triglycerides (Fig. 3). These changes are most probably a result of the decreased efficiency of energy conversion, leading to higher catabolic fluxes. Interestingly, similar effects are observed in animals submitted to caloric restriction, which exhibit mitochondria with higher proton leaks (Lambert & Merry, 2004). The reasons for this effect are not easily understood, since uncoupling further hampers energy metabolism in animals with limited access to calories. It is possible that the resulting increase in metabolic rates compensates for the decrease in efficiency (Nisoli *et al*., 2005; Guarente, 2008). Indeed, the occurrence of mitochondrial biogenesis under these conditions may help compensate for the decrease in efficiency of ATP production by enhancing the number of functional respiratory chains (Lambert *et al*., 2004; Nisoli *et al*., 2005). Irrespective of the reason for this uncoupling, it involves hormonal signalling, since infusion of insulin into calorie-restricted animals reverses uncoupling (Lambert & Merry, 2004). Lower insulin levels in calorie-restricted animals may induce nitric oxide synthase (Nisoli *et al*., 2005) and increase uncoupling protein expression (Nisoli *et al*., 2003). Interestingly, nitric oxide increases promoted by caloric restriction also promote mitochondrial biogenesis (Nisoli *et al*., 2005). In all, literature data strongly support the concept that caloric restriction is accompanied by insulin and nitric oxide-signalled enhancement in respiratory rates.

Overall, our results indicate that increased respiration and oxidative phosphorylation inefficiency are central modulators of the beneficial effects of caloric restriction, since the mitochondrial uncoupler DNP acts as a highly effective caloric restriction mimetic. Much attention has been placed in recent years on the development of pharmaceutical interventions capable of promoting the beneficial effects of caloric restriction (Ingram *et al*., 2006). The classes of drugs studied to date include glycolytic inhibitors, drugs that affect signalling pathways involving insulin and/or IGF-1, sirtuin-activating compounds and agonists of peroxisome proliferator-activated receptors. Among these drugs, to our knowledge, only resveratrol (3,5,4′-trihydroxystilbene) has been shown to have an impact on mammalian lifespan without decreasing caloric intake (i.e. without promoting caloric restriction). This compound protected mice from the harmful effects of a high fat diet, when evaluated at the median lifespan (Baur *et al*., 2006). Resveratrol is a polyphenol found in red wine that extends the lifespan of diverse species, including *S. cerevisiae*, *C. elegans* and *D. melanogaster*. It is believed to act by increasing the activity of Sir2 and its mammalian orthologue SIRT1 (Howitz *et al*., 2003), although this activation has not been directly demonstrated (Kaeberlein *et al*., 2005). In mammals, SIRT1 is involved in fat mobilization, insulin secretion and mitochondrial biogenesis, among other metabolically linked activities (Chen & Guarente, 2007), and resveratrol may modulate these functions. Indeed, resveratrol changes the physiology of mice on high-calorie diets to a healthier state (Baur *et al*., 2006).

However, the beneficial effects of resveratrol were only observed in animals fed an exceptionally high-fat (60%), high-calorie diet with a significant impact on life expectancy (Baur *et al*., 2006). Our data showing that mild mitochondrial uncoupling not only promotes serological changes typical of caloric restriction, but also prevents tissue oxidative damage and enhances lifespan in mice fed standard laboratory chow containing 4% fat, establishes a novel class of effective caloric restriction mimetics: drugs that promote mild mitochondrial uncoupling. Further studies will hopefully determine whether the lifespan extending effects are linked to the bioenergetic, metabolic and redox changes promoted by DNP. Although the notoriety and toxicity of DNP still precludes its direct clinical use, we believe that modifications of DNP, use of uncouplers with very controlled and mild effects (Lou *et al*., 2007) or the use of drugs targeting natural mitochondrial mild uncoupling pathways, including

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uncoupling proteins (Nègre-Salvayre *et al.*, 1997; Ricquier & Bouillaud, 2000) and ATP-sensitive K^+ channels (Kowaltowski *et al.*, 2001; Ferranti *et al.*, 2003), will prove useful in controlling redox state, triglyceride levels, glycemia, insulinemia and lifespan.

Experimental procedures

Animal care

Experiments were approved by the Comitê de Ética em Cuidados e Uso Animal, and follow NIH guidelines. Female Swiss Webster outbred albino mice, purchased originally from Taconic Farms, were bred and lodged at the Biotério de Produção e Experimentação da Faculdade de Ciências Farmacêuticas e Instituto de Química, with HEPA-filtered air and under controlled temperature (22 °C), humidity, light (12-h light/dark cycles) and pressure. The colony is specific pathogen free submitted to sanitary controls thrice a year. After weaning, animals were allowed free access to standard irradiated laboratory rodent diet (Nuvital CR1, Colombo, Brazil) containing 21.6% protein and 4.0% lipid. Starting at 18 weeks, the DNP group continuously received drinking water containing 1 mg L^{-1} DNP, prepared biweekly, in light-protected bottles. DNP did not alter water ingestion at any time point measured (data shown represent animals at 75 weeks). Based on water ingestion and body weight, DNP doses ranged between 30 and 105 μg kg^{-1} day^{-1}. A group of 60 animals (30 treated with DNP and 30 controls) was kept in the animal facilities until the end of their natural lifespan. These animals were submitted to weekly weighing, temperature measurements, and food and water ingestion analysis. Other DNP-treated and control animals were sacrificed at 22 or 32 weeks (1 month and 5 months treatment, respectively) to collect organs and serum.

Body temperatures

Rectal temperatures were measured using a digital thermometer (BD Basic, Becton Dickinson, São Paulo, Brazil). Animals were adapted to rapid comfortable immobilization and measurements. Temperatures were recorded between 14:00 and 15:00 hours. No differences in temperatures were noted at any time point measured. Data shown are averages of 5 days of measurements per animal, at 75 weeks of age.

Water and food ingestion

Mice were kept in groups of two in metabolic cages (Beira-Mar, São Paulo, Brazil), with free access to food and water. After 24-h adaptation, food ingestion and water intake were measured over a further 24 h. No differences between the treated and untreated groups were noted at any time point measured. Data shown were collected at 75 weeks of age. Fecal and urinary quantities were also measured, and standard urinalysis was conducted, presenting no significant differences (results not shown).

Body weight and efficiency of energy conversion

Animals were individually weighed weekly throughout their natural lifespan. The data shown represent averages of measurements made every 10 weeks, for sake of clarity. Weight gain/ingestion was calculated over weeks 18 through 38.

Oxygen consumption

Brain, liver and heart tissues were collected and segmented into fine (~1 mm) pieces in phosphate-buffered saline (PBS) (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4). The suspension was added to the temperature-controlled (37 °C) chamber of a Clark-type electrode (Hansatech Instruments, Norfolk, UK). Oxygen consumption was recorded over 20 min, assuming solubility at 37 °C was 210 μmol mL^{-1}. Samples were homogenized for protein content determination.

H_2O_2 release

Tissues were processed as described for oxygen consumption measurements, and incubated for 20 min in 37 °C PBS. Fifty micromolar Amplex Red plus 1 U mL^{-1} horseradish peroxidase were added to the supernatant to determine H_2O_2 concentrations. Baseline fluorescence in the same media was subtracted from all measurements. Amplex Red reacts with peroxidase-H_2O_2 complexes with 1 : 1 stoichiometry to produce fluorescent resorufin (Zhou *et al.*, 1997). Fluorescence was measured at Ex = 563 and Em = 587 nm, using 5 nm slits on an Hitachi 4500 fluorescence spectrophotometer. Data are expressed as percentages of control fluorescence since different tissues promoted changes in baseline fluorescence, rendering calibrations imprecise.

Protein carbonyl detection

Protein carbonylation was quantified by derivatization with 2,4-dinitrophenylhydrazine followed by immunological detection (Shacter *et al.*, 1994). Organs were homogenized in buffer containing 320 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris-HCl, 0.1 mM desferroxamine and protease inhibitors (1 mM sodium orthovanadate, 1 mM phenylmethanesulphonylfluoride, 2 μg mL^{-1} aprotinin, 2 μg mL^{-1} pepstanin, 2 μg mL^{-1} leupeptin). The homogenates were stored at – 80 °C. Thawed samples (4 mg protein mL^{-1}) were incubated with 5 mM 2,4-dinitrophenylhydrazine and 6% sodium dodecyl sulphate (SDS) for 30 min, followed by neutralization with 25% β-mercaptoethanol. Eighteen micrograms of protein were then applied to an SDS-PAGE (5% stacking, 8% running gel). Proteins were transferred to a nitrocellulose membrane and Western blot was developed using primary anti-dinitrophenylhydrazine rabbit antibody (Calbiochem, Darmstadt, Germany) at 1 : 5000 dilution and secondary peroxidase-linked anti-rabbit IgG from Pierce KLP (Rockford, IL, USA). Images were analyzed using ImageQuant software, integrating intensities of all visible bands in a fixed selected area, relative to the background. Decreases in carbonylation in DNP samples were

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widespread, and did not significantly differ between specific protein bands.

DNA extraction and enzymatic hydrolysis

DNA was isolated from tissues kept at –80 °C using the method described by Wang *et al*. (1994), with some modifications. The tissue (300 mg) was suspended and washed in 2 mL of a lysis solution (1% w/v Triton X-100, 320 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5). After centrifugation at 1500 *g* for 10 min, the nucleus pellets were suspended in 3 mL of 10 mM Tris-HCl buffer, pH 8.0, containing 5 mM EDTA and 0.15 mM deferoxamine mesylate salt. RNAse A (30 μL of a 10 g L^{-1} solution in 10 mM sodium-acetate pH 5.2, heated for 15 min at 100 °C) and RNAse T1 (4 μL of a 20 U μL^{-1} solution in 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 2.5 mM deferoxamine mesylate salt) were added with 200 μL of 10% (w/v) SDS. The reaction mixture was incubated at 37 °C for 1 h. Thirty microliters of proteinase K (20 g L^{-1}) were added, followed by additional incubation at 37 °C for 1 h. For brain samples, 1 : 1 chloroform was added, followed by homogenization and phase separation to remove excess lipid. The aqueous phase followed the same subsequent steps as all tissues. After centrifugation at 5000 *g* for 15 min, the liquid phase was collected and 4 mL of isopropanol were added. The content was homogenized by inversion until a whitish precipitate appeared. The precipitate was collected by centrifugation at 5000 *g* for 15 min and washed with 1 mL of 60% (v/v) isopropanol followed by 1 mL of 70% (v/v) ethanol. After additional centrifugation at 5000 *g* for 15 min, the DNA pellet was solubilized in 0.1 mM deferoxamine mesylate. The DNA concentration was measured spectrophotometrically at 260 nm and its purity was assessed by ensuring $A_{260}/A_{280} \geq 1.7$. DNA hydrolysis was performed as described by Fiala *et al*. (1989) with some adaptations. For the hydrolysis of 100 μg of DNA, 2.0 μL of 1 M sodium acetate buffer pH 5.0 and 1 U of nuclease P_1 were added to the sample, which was incubated at 37 °C for 30 min. Four microliters of 1 M Tris-HCl buffer (pH 7.4) and 4 μL of 500 mM potassium acetate buffer (pH 7.0) containing 100 mM of Tris-acetate and 100 mM magnesium acetate were added, followed by the addition of 3 U of alkaline phosphatase. The final volume of the sample was adjusted to 100 μL with water. The reaction mixture was incubated at 37 °C for 1 h.

High performance liquid chromatography–electrochemical detection (HPLC/EC) of 8-oxodGuo

Analysis was performed as described by Fiala *et al*. (1989) with some adaptations. Samples (100 μg) of digested DNA were injected into the HPLC/EC system, consisting of a Shimadzu LC-10 AD pump (Shimadzu, Tokyo, Japan) connected to a Luna C18 analytical column (250 mm × 4.6 mm i.d., 5 μm; Phenomenex, Torrance, CA, USA), kept at 18 °C by a Shimadzu CTO-10AS VP column oven. The isocratic eluent was 25 mM potassium phosphate buffer (pH 5.5) and 8% methanol at a flow rate of 1 mL min^{-1}. Coulometric detection was provided by a Coulochem II detector (ESA, Chelmsford, MA, USA) and spectrophotometric detection by Shimadzu SPD-10 A. The potential of the electrode was set at 280 mV. Elution of unmodified nucleosides was simultaneously monitored by a Shimadzu SPD-10AV/VP UV detector set at 254 nm. Shimadzu Class-LC10 1.6 software was used to calculate the peak areas. The molar ratio of 8-oxodGuo to dGuo in each DNA sample was determined based on coulometric detection at 280 mV for 8-oxodGuo and absorbance at 254 nm for dGuo in each injection.

Glycemia, triglyceridemia and insulinemia

Blood was collected from fasted 32-week animals and centrifuged at 780 *g* for 15 min. Serum was analyzed for glucose and triglyceride levels by the Laboratório de Análises Clínicas, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, using enzymatic colorimetric assays (Labtest Diagnóstica, Lagoa Santa, Brazil). Insulin levels were determined in frozen serum samples using an ELISA kit from Linco Research (St. Charles, MO, USA).

Data analysis

Data were analyzed using GraphPad Prism and Origin Software. Figures 1–3 represent averages ± SEM of measurements from 7–30 different animals. DNP and control groups were compared using unpaired *t*-tests. Two-tailed *p*-values under 0.05 were considered significant. Survival curves (Fig. 4) were compared by log rank tests.

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