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Abstract: Mitochondrial dysfunction is observed in a broad range of human diseases including rare genetic disorders and complex acquired pathologies. For this reason, there is increasing interest in identifying safe and effective strategies to mitigate mitochondrial impairments. Natural compounds are widely used for multiple indications, and their broad healing properties suggest that several may act by improving mitochondrial function. This review focuses on (–)-epicatechin, a monomeric flavanol, and its effects on mitochondria. The review summarises the available data on the effects of acute and chronic (–)-epicatechin supplementation on mitochondrial function, outlines the potential mechanisms involved in (–)-epicatechin-supplementation-induced mitochondrial biogenesis and discusses some future therapeutic applications.

Key words: mitochondria, epicatechin, flavanols, mitochondrial biogenesis

Introduction

Mitochondria are subcellular organelles that play a central role in providing ATP to sustain the bioenergetic needs of cells. Furthermore, these organelles act as vital hubs responsible for sensing, integrating and generating multiple signals and metabolites required for the short- and long-term adaptability of the organism to a changing environment. Not surprisingly, mitochondrial dysfunctions are observed in many human diseases, including genetic mitochondrial diseases and complex pathologies such as neurodegenerative diseases, cardiovascular diseases, metabolic diseases and cancer.[1,2] In many of these diseases, reduced oxidative capacity, increased generation of reactive oxygen species (ROS) and propensity to mitochondria-mediated apoptosis are among the frequently reported factors that contribute to the pathogenesis. For these reasons, identification of strategies to mitigate these abnormalities is viewed as an opportunity to prevent or at least limit the progression of many disorders.

In this regard, there is a growing interest in identifying safe natural compounds that enhance mitochondrial function and have limited or no side effects. Among the natural compounds, polyphenols appear to be of interest to modulate mitochondrial function. For example, resveratrol has been repeatedly shown to improve mitochondrial function and protect against metabolic diseases by activating mitochondrial biogenesis through Sirtuin 1 (SIRT1) and Peroxisome Proliferator activated receptor Gamma coactivator 1 alpha (PGC1α).[3] More recently, (−)-epicatechin (EPI), a member of the flavonoids chemical family, has received attention.[4–6] This low molecular weight (290.27 g/mol) compound has been associated with mitochondrial improvements in animal and human studies on different organs or tissues, such as the heart, skeletal muscle and neurons.[4–6] Despite this evidence, the mechanisms that underlie the effects of EPI on mitochondrial function and biogenesis remain unclear.

In this review, we provide a brief overview of the chemical structure and metabolism of EPI. We then describe the known effects of EPI on mitochondrial functions in key tissues and organs affected by mitochondrial dysfunction, and then discuss the potential underlying mechanisms. Finally, we discuss the therapeutic interest of EPI supplementation to improve mitochondrial function, including mitochondrial energy production, ROS production and calcium retention capacity. Better knowledge of the effects of EPI on the mitochondrial phenotype, as well as of the fundamental underlying mechanisms, is important for the design of future EPI-based nutraceutical strategies.

1. Epicatechin

Catechins are ubiquitous polyphenolic compounds that can be found in high concentrations in certain fruits and vegetables. The molecular formula is $C_{15}H_{14}O_6$; it contains two asymmetric carbon atoms for a total of four diastereoisomers (Figure 1): (+)-catechin (2R, 3S), (−)-catechin (2S, 3R), (−)-epicatechin (2R, 3R) and (+)-epicatechin (2S,3S). Catechins are isomers in a *trans* configuration, whereas EPI are isomers in a *cis* configuration. These monomers can form links that allow them to assemble as dimers, oligomers and polymers of catechins called procyanidins.[7] The oral bioavailability of the stereoisomers and their metabolites differs from each other in plasma and urine after a consumption of equal amount of each stereoisomer (1.5mg/kg body weight (bw)): (−)-epicatechin > (+)-epicatechin > (+)-catechin > (−)-catechin.[8]

Cocoa contains more phenolic compounds compared to black tea, green tea and red wine, which explains the growing interest in evaluating the effect of cocoa on human health.[9] Although cocoa beans contain a mixture of monomeric, oligomeric and polymeric flavanols, EPI is the most common polyphenolic monomer found in cacao and cacao products such as dark chocolate; it represents up to 35% of the polyphenol content in the cocoa bean.[10] Moreover, EPI is predominant, with a ratio of 1:0.1 compared to catechin, and the EPI content ranges from 12 to 16 mg/g in unfermented cocoa beans.[11] Whereas (+)-catechin and (−)-epicatechin are commonly found in cocoa, their enantiomers, namely (−)-catechin and (+)-epicatechin, are not commonly found in nature.[12] Furthermore, it has been established that EPI is the main bioactive molecule that underlies the vascular health benefit associated with cocoa and chocolate.[13] However, both fermentation and roasting have a significant negative impact on EPI content. Fermentation reduces the content of EPI and the soluble polyphenol content by 10–20%, and roasting decreases EPI levels in a temperature-dependent manner.[10,11] For instance, an increase in roasting temperature from 127 to 181°C is associated with an approximately 50% reduction in total polyphenols.[10] As a result, specific attention should be paid to EPI content when evaluating the potential benefits of EPI supplementation on human health, as marked variations can exist depending on the sources and/or the manufacturing process.[14]

2. Bioavailability

Knowledge about the absorption, distribution, metabolism and excretion (ADME) of EPI is key to designing and executing dietary intervention studies and interpreting the results.

2.a. Bioavailability of EPI from dark chocolate

Following consumption of dark chocolate, plasma EPI levels rise rapidly and peak after 2–3 h.[15,16] Generally, in humans studies, the amounts of EPI consumed range between 20 and 200mg, which lead to maximum plasma EPI concentrations ranging between 30 and 5000 nM, at 0.9 to 3.2h after consumption.

The bioactivity of EPI is also linked to the appearance of its metabolites in the blood circulation. Once ingested, EPI can be: *i)* absorbed by the gastrointestinal tract and exert its effects as the parent compound; *ii)* absorbed and conjugated by phase II enzymes as glucuronidated and/or methylated and/or sulfonated metabolites; or *iii)* metabolised by the microbiota to compounds that can be absorbed.[17,18] For example, following ingestion of a drink containing 1.8 mg EPI per kg bw, the major phase II metabolites quantified were (−)-epicatechin-3′-β-D-glucuronide, (−)-epicatechin-3′-sulfate, and a 3′-O-methyl-(−)-epicatechin-5/7-sulfate, with a peak values ranging from 214 to 377 nM 2h after ingestion.[18] Therefore, the biological effects of EPI observed in *in vivo* studies could be attributed to EPI itself or its metabolites.

It should be noted that the amount of EPI and its metabolites reported in a portion of the literature have been underestimated for methodological reasons. Indeed, studies that had used a ß-glucuronidase/sulfatase treatment prior to analysis by reverse phase high-performance liquid chromatography (HPLC) did not consider the initial phase II metabolites present prior to treatment. Furthermore, commercial ß-glucuronidase/sulfatase enzymes usually fail to fully hydrolyse all the EPI metabolites (EPIm).[19] This limitation has been overcome with the development of new methods to detect structurally related EPIm and 5C-ring fission metabolites.[20,21] Using radiolabeled [^{14}C]EPI in a diet-controlled study, Ottaviani et al. investigated the ADME of EPI and reported that: *i)* EPI is very well absorbed, as 82% of radiolabeled EPI is recovered in urine within 48-h following ingestion; and *ii)* ~70% of the radiolabeled EPI ingested is absorbed in the lower intestine following catabolism by the gut microbiome.[22] These results highlight the importance of also considering EPIm in studies that aim to elucidate the molecular mechanisms underlying the benefits of EPI supplementation.

Recent studies have revealed that plasma levels of EPIm reach their maximal concentration 2–3 h after the ingestion of cocoa. Most of the phase II metabolites (95%)

generated by the gut microbiome are absorbed in the small intestine and appear in the circulatory system.[18,23] Some bioactive EPIm can even reach plasma concentrations that are higher and may exerts effects that are different from EPI itself. For example, (–)-epicatechin-3'-glucuronide inhibits NADPH oxidase at low concentration (Hhalf maximal inhibitory concentration (IC_{50}) = 7.9±2.6 µM *vs.* IC_{50} > 100 µM for EPI); it reaches a concentration of 359 nM 2 h after an acute supplementation with 60 mg EPI.[22,24] Based on these recent technical improvements, the concentrations of EPI and its phase II metabolites reached in the plasma following ingestion of dark chocolate are within the accepted range to exert a biological effect.[25] Overall, the data available clearly indicate that EPI is a highly bioavailable compound.[26]

Several factors affect the ADME of EPI following cocoa ingestion.[27] One is the food matrix, which has a significant impact on EPI bioavailability. For instance EPI absorption occurs more rapidly from liquid food matrices compared to solid chocolate.[27] Moreover, the presence of carbohydrates in the food matrix may increase the absorption of EPI, while in general the presence of lipids, proteins or milk does not appear to affect the absorption of flavanols.[28]

The amount of EPI administrated is also an obvious determinant of EPI and EPIm levels reached in the plasma. A survey of 22 studies available in the literature indicates that the concentration of EPI and its phase II metabolites in the blood circulation per milligram of EPI consumed is 9.4 nM, which suggests that the absorption rate is well below saturation.[27] However, the impact of increasing the amount of EPI intake on its metabolism remains to be determined.

Genetic polymorphisms that alter phase II enzyme activities may also result in variable rates of EPI absorption/metabolism across individuals.[27] However, this aspect is poorly understood and needs to be studied further. Age does not appear to be a major factor because there were only slight differences in EPI's between young and elderly subjects.[29] Finally, there were major differences in EPI's ADME across species. More specifically, 80% of EPIm present in humans were not detected in rats, while the similarity between mice and humans was slightly more favourable because the two major human phase II metabolites were detected.[22] These data are of relevance and should be seriously considered given that rodents are commonly used to study the safety, and the physiological benefits of EPI as well as the underlying mechanisms of action.

As mentioned previously, cocoa beans contain a complex of monomeric, oligomeric and polymeric flavan-3-ols, which can display different biological activities. Hence, Rowley et al.10/20/20 2:21:00 PM employed fractionation methods to purify and test serial dilutions of

monomeric catechins, oligomeric procyanidins or polymerics procyanidins in cultured 832/13 ß-cells (concentrations of individual compounds ranged from not detectable to 2.64μM). These experiments revealed that the monomeric fraction is the only effective bioactive form that can enhance glucose-stimulated insulin secretion with a concomitant mitochondrial respiration increase.[30] Of note, EPI was the predominant plasma flavanol absorbed in humans following consumption of a cocoa beverage containing equal amounts of EPI and catechin, which are the two monomeric flavanols, with plasma catechin levels reaching less than 10% of the EPI concentrations.[31]

Several studies have reported the effects of EPI on mitochondria in cell culture.[4,6,30,32,33] While supraphysiological concentrations (10–100 μM) have been used in some cases, clear evidence of positive effects have been observed using EPI concentrations in the low molecular range (down to 1 μM), which is close to the concentrations encountered *in vivo*.[33] These data thus suggest that physiological EPI concentrations up to 5 μM can promote mitochondrial adaptations.[4,6,32] Moreover, comparison of EPI and its gut microbial metabolites demonstrated that EPI is the most potent stimulator of glucose oxidation and mitochondrial function; these data further illustrate the interest in studying the EPI monomeric form and its metabolites.[5]

2.b. Bioavailability of pure EPI

Comparative studies have established that cocoa extracts display a greater biological activity than EPI alone, a finding that suggests a synergistic effect between EPI and other cocoa compounds.[34] A recent diet-controlled study evaluated EPI's ADME using radiolabeled and stereochemically pure [2-^{14}C](−)-epicatechin ([^{14}C]EPI).[22] Subjects ingested a drink that contained radiolabeled [^{14}C]EPI (300 μCi, 60 mg), and kinetic profiles were measured over a 48-h period. The authors demonstrated that 82 ± 5% of ingested [^{14}C]EPI was absorbed. The plasma radioactivity kinetic profile was biphasic, with peaks at 1 and 6 h. The first peak reflected the kinetic profile of EPIm and methyl-(−)-epicatechin metabolites, whereas the second peak corresponded to 5C-ring fission microbiotal metabolites that are absorbed at more distal points in the gastrointestinal tract. Overall excretion of [^{14}C]EPI reached 94.8% of the total ingested, suggesting that tissue deposition of compounds derived from the acute intake of EPI is < 5.2%. Tissue distribution and uptake of EPI in specific cell types have not been extensively studied. However, studies available for cell cultures and rodent models have indicated that glial cells and the liver can to uptake EPI.[35,36] Nevertheless, EPI metabolites do not appear to be taken up by cells to a significant extent.[35,37,38] To the best of our knowledge,

the ability of mitochondria to uptake EPI has never been assessed and should be explored in future studies.

3. Effects of EPI on mitochondrial content, membrane fluidity and protein composition

Several studies have reported that chronic EPI supplementation is associated with an increase in tissue mitochondrial content. As illustrated in Table 1, most of these studies have explored the effect of EPI supplementation on human and murine muscle.[39–46] However, a number of studies have investigated the EPI-induced adaptations *in vitro* in various cell lines.[6,30,32,33,47] While physiological concentrations ranging from 1 to 5 μM have been used in some cases, others have used supraphysiological doses up to 10 μM (indicated in bold type in Table 1). This broad range of EPI concentrations, combined with the fact that pure EPI, as opposed to a complex cocoa extract or a combination of EPI and EPIm, have been used, most likely explain some of the discrepancies between *in vitro* and *in vivo* studies.[5] In addition, diet and lifestyle have not always been rigorously controlled rigorously in human studies. This factor may have affected EPI's ADME, and in turn the impact on the mitochondrial phenotype within tissues.

In mice, Watanabe et al.[41] reported an increase in the mitochondrial DNA (mtDNA):nuclear DNA ratio in the gastrocnemius and soleus muscles after 14 days of EPI supplementation. In cell culture, Moreno-Ulloa et al.[32] reported an increase in mitochondrial content following exposure to 3 μM EPI these data suggest that a physiological concentration of EPI is sufficient to stimulate mitochondrial biogenesis. This amelioration was associated with increased expression of inner and outer mitochondrial membrane marker proteins (e.g. mitofilin and voltage-dependent anion channel (VDAC), respectively) after a chronic supplementation of at least 10 weeks in various species and tissues.[6,39,40,42,47–49] It is intriguing that the increase in cristae perimeter was associated with a greater abundance of mitofilin,[40,49,51] which maintains mitochondrial cristae structure and remodelling.[50] For example, oral EPI supplementation for 30 days in mice increased the cristae:outer membrane ratio by > 30%, a finding that suggests a great cristae density per unit of mitochondrial surface.[51] These EPI-induced adaptations were maintained at least 15 days after supplementation was stopped; thus, EPI supplementation seems to produce long lasting morphological adaptations.

Evidences has also suggested that the membrane lipid composition is modified following treatment with EPI.[43] Using electron paramagnetic resonance on purified membrane from mouse heart (after the mice were fed with EPI for 10 days), Panneerselvam et al.[43] assessed the membrane fluidity as a proxy for changes in the membrane lipid microenvironment near the

bilayer surface. These authors reported an increase in the mitochondrial rigidity/fluidity ratio, suggesting that EPI supplementation induces a shift in membrane lipid composition. However, it remains unclear to what extent concomitant changes in the protein density within the cristae can also affect the rigidity/fluidity ratio reported in this study. Based on the relatively short duration of treatment used in the study (e.g. 10 days), one may argue that significant increases in protein abundance may not yet be present. This hypothesis implies that changes in lipid composition are mainly responsible, but this eventuality remains to be established.

The electron transport chain is composed of four large membrane-embedded complexes localised in the inner mitochondrial membrane. Electrons flow through these proton-pumping complexes in a series of redox reactions, which allows the generation of a proton gradient that is subsequently used by ATP synthase to drive ATP formation. In humans as well as rodents or cell culture models, EPI supplementation significantly impacts on respiratory chain complex content in skeletal and cardiac muscles,[4,30,39,40,47,49,51] endothelial cells[4,47] and cancer cells.[52] For example, in patients with heart failure or type 2 diabetes, EPI supplementation (50 mg twice a day (b.i.d.)) over 3 months increased complex I content in the *vastus lateralis* muscle of all patients.[49] In rodents, there was a significant increase in protein content for all complexes after 14–15 days of EPI treatment.[39,40,51] These effects appeared to be independent of muscle type. Indeed, after 2 weeks of oral gavage, there was increased complex II content in the heart and quadriceps muscle, which express different types of myosin heavy chain isoforms and broadly different metabolic machinery.[40] Moreover, the beneficial effect of EPI supplementation were observed in both young and old mice.[39,40,44]

Several studies performed over the last decade have convincingly shown that respiratory chain complexes do not solely exist as individual entities within the inner membrane. With the exception of complex II, all other respiratory chain proteins can form dynamic supramolecular assemblies called supercomplexes (SCs). In mammalian cells, SCs exist in varying stoichiometries and are mainly composed of the so-called respirasome ($CI/CIII_2/CIV_{1-4}$), assemblies composed of monomeric CI and CII dimers ($CI/CIII_2$) as well as dimeric CIII and mono- or dimeric CIV ($CIII_2/CIV_{1-2}$).[53–55] These supramolecular structures are thought to decrease ROS production, stabilise or assist in the assembly of individual complexes, regulate respiratory chain activity and prevent protein aggregation in the protein rich inner mitochondrial membrane.[53–55] To our knowledge, the impact of EPI supplementation on the steady state levels of various SC has not been established and warrants investigation.

A recent study tested the association between EPI supplementation (100 mg b.i.d. for 4 weeks) and endurance training in recreationally active humans.[45] The authors reported that EPI

supplementation blunted the positive effect of training on muscle succinate dehydrogenase content and whole body aerobic fitness. However, EPI did not prevent the rise in cytochrome *c* and citrate synthase (CS) contents observed in response to training. These results contrast with other studies that have reported elevated CS activity and complex I and ATP synthase contents in humans following 3 months of EPI supplementation (26 or 100mg per day).[49,56] These discrepancies may result from differences in individual characteristics between the studies, the antioxidant properties of flavanols, or the concentration of EPI used. In fact, in the study of Taub et al.,[56] the subjects had a ~30% lower maximal oxygen consumption (VO_{2max}) compared with those included in the study of Schwarz et al.[45] This finding is noteworthy since previous studies that have examined the effect of cocoa flavanol intake in active subjects have failed to observe an increase in performance.[57] This is in accordance with a recent systematic review from our group suggesting that cocoa flavanol intake may induce biological adaptations without obligatorily affecting exercise performance.[58] While acute antioxidant supplementation may have a beneficial impact on fatigue and exercise performance, long term supplementation blunted the exercise ROS-induced signal, which may also apply to EPI.[59] Moreover, the positive effects of EPI on performance have been mainly obtained in studies involving diseased patients or sedentary subjects. Therefore, the ability of EPI to improve mitochondrial content should be further explored in humans with various levels of fitness. Furthermore, the interaction between EPI supplementation and endurance training should be tested carefully in order to determine the potential advantage of combining the two strategies. Finally, differences in EPI dosage across studies may explain some of the discrepancies because changes in mitochondrial respiration appear to vary depending on the concentration of EPI reached. Indeed, a previous study reported that maximal respiration is stimulated at physiological concentrations of EPI (< 0.7 μM), while higher concentrations can alter mitochondrial respiration (> 1 μM).[60]

Mitochondrial fatty acid β-oxidation represents a major source of carbon and reduced equivalents (NADH and $FADH_2$) that donate electrons to support oxidative phosphorylation in the mitochondrial electron transport chain. An important rate-limiting step in this biochemical pathway is the import of fatty acids across the inner membrane by the carnitine palmitoyl transferases 1 and 2 shuttle system (CPT1-2). Flavanol supplementation reportedly increased this capacity both in oxidative and glycolytic muscle.[41] More specifically, CPT-2 protein content was increased in the gastrocnemius and soleus muscles after treating mice with the flavan-3-ol fraction, comprised mostly of EPI, during 14 days.[41] However, the underlying mechanism remains unknown, and the impact of EPI supplementation on transporters for other substrates has not yet been investigated.

Collectively, these data support the idea that EPI supplementation increases mitochondrial content in tissues, enhances cristae density, and augments the abundance of major inner membrane proteins involved in oxidative phosphorylation, and metabolite transport across the double membrane system.

4. Effect of EPI on mitochondrial function

Beyond changes in the mitochondrial content and membrane protein/lipid composition, several studies have focused on the impact of acute and chronic EPI supplementation on mitochondrial functions (Table 2).

4.a. Direct effect of EPI on isolated mitochondria

The direct effects of an acute EPI supplementation on isolated mitochondria have not been widely studied (Table 2). However, the available data indicate that short-term exposure to EPI has a direct impact on respiration in mitochondria isolated from striated muscle. Incubation of isolated cardiac mitochondria with EPI concentrations within the physiological range (0.3-2.4 μM) increased basal rates of respiration in the presence of various substrates (pyruvate+malate, succinate+amytal or palmitoyl-L-carnitine+malate); these data suggest that EPI exerts an uncoupling.[60] At low concentrations (< 0.7 μM) EPI, increased maximal ADP-stimulated respiration, while the opposite occurred at higher concentrations (> 1μM).[60] Moreover, following acute EPI exposure, the ability of exogenous cytochrome c to stimulate mitochondrial respiration was reduced; this finding suggests a better preservation of mitochondrial membrane integrity with an EPI concentration lower than 0.8 μM.[60]

Interestingly, there are apparent beneficial effects of an acute exposure at EPI concentrations that are lower or similar to the peak plasma levels achieved following oral supplementation, whereas the deleterious effects are reported with EPI concentrations that are 1.5-fold greater than the peak plasma concentration reached *in vivo*.[60]

Collectively, these data support the notion that EPI has the capacity to acutely modulate mitochondrial respiration through direct effects on complex IV, and possibly other respiratory chain components. The significance of these observations, however, remains difficult to establish because the local concentration of EPI reached at the vicinity or within mitochondria following *in vivo* supplementation currently remains unknown.

In addition to these *in vitro* experiments, a limited number of studies have examined the effect of acute EPI supplementation on whole-body aerobic metabolism. In humans, acute consumption of dark chocolate was found to altered muscle carbohydrate partitioning without

affecting cycling time trial performance.[61] More specifically, this study reported a concomitant decrease in plasma glucose oxidation in skeletal muscle and an increase in muscle glycogen utilisation during a submaximal steady state exercise 2 h after ingestion of dark chocolate containing 89 mg EPI. However, it is currently unclear to what extent these changes are related to the acute effects of EPI on mitochondrial respiration. Further mechanistic studies should be conducted in humans to tease out the role of mitochondria versus other factors, such as changes in the regulation of muscle microcirculation.

4.b. Effects of EPI supplementation in intact cells or *in vivo*

A consistent body of evidence indicates that EPI supplementation - directly in the cell culture media, or *in vivo* in rodents and humans - affects multiple mitochondrial functions, including: *i)* mitochondrial respiration and electron transport chain enzyme activities; *ii)* mitochondrial hydrogen peroxide production, which reflects mitochondrial ROS production; and *iii)* mitochondrial calcium retention capacity, which reflects the susceptibility to mitochondrial permeability transition pore (mPTP) opening.

Mitochondrial respiration

Studies performed in cell culture or *in vivo* in mice have shown that EPI supplementation increases maximal ADP-stimulated respiration (e.g. state 3 respiration) when mitochondria are energised with combinations of energy substrates that feed various sites along the respiratory chain.[5,30,43,52,62] Consistent with these results, previous studies performed on isolated mitochondria have reported an increase in maximal respiration following chronic EPI supplementation *in vivo* in rodents. These data indicates that, beyond changes in tissue mitochondrial content, intrinsic changes within mitochondria occur following EPI supplementation.[43,60] Although limited data are available on the longer-term impact of EPI supplementation, recent results suggest that it is associated with benefits.[56] In that study, daily consumption of dark chocolate (representing 26 mg/day of EPI) for 3 months increased the activity of the marker enzyme citrate synthase by 2.5 fold in *vastus lateralis* muscle of sedentary subjects. This change suggests an increase in tissue mitochondrial content.[56] Interestingly, this effect was associated with an improvement in aerobic exercise capacity.[56] Although diet was not controlled and may have influenced mitochondrial function, these data suggest that chronic EPI supplementation could contribute to the maintenance, recovery or improvement of muscle oxidative capacity in sedentary individuals.

ROS production and antioxidant defence

Within cells, mitochondria represent an important source of ROS, which can either act as signalling molecules or source of injury depending on the amount being produced.[59] In mitochondria, there are currently 11 sites within these organelles that are known to generate ROS, each with different capacities and properties.[63] The predominant and most reactive molecules generated at these sites are superoxide anions, which are rapidly converted to the less harmful molecule hydrogen peroxide by superoxide dismutase (SOD2). Hydrogen peroxide is in turn converted to water by various antioxidant systems within the mitochondrial matrix, including catalase as well as the peroxiredoxin, glutathione and thioredoxin systems. Studies performed *in vivo* in rodents and humans and *in vitro* in cell culture models have indicated that EPI supplementation can alter the activity of these systems.[6,30,33,39,42,43,56,64–66] For instance, in sedentary humans, consumption of dark chocolate (26 mg/day EPI) for 3 months was associated with a 20% increase in skeletal muscle glutathione levels, which was similar to the improvement observed in response to exercise training.[56,67] Similarly, in patients with heart failure or type 2 diabetes, EPI supplementation boosted the activity of SOD2 and catalase in the *vastus lateralis* muscle.[66] Conversely, EPI supplementation for10 days increased superoxide levels, measured using electron paramagnetic resonance, in mouse heart.[43]

While these data indicate that the antioxidant capacity is increased following EPI supplementation, it is still unclear whether this treatment results in an overall reduction in mitochondrial ROS emission in tissues. Given that EPI supplementation concomitantly increases mitochondrial content and the abundance of several ROS-generating enzymes within tissues, the upregulation of key antioxidant systems could take place mainly to preserve the normal balance between ROS emission and scavenging. This would be compatible with the fact that the expression of several antioxidant genes, such as nuclear factor erythroid 2-related factor 2 (NRF2) or PGC1α, are regulated by transcription factors involved in mitochondrial biogenesis.[68] More mechanistic studies that examine the impact of EPI on the balance between ROS emission and scavenging are required to determine whether EPI supplementation, beyond improving mitochondrial oxidative capacity, is also associated with a net increase in the antioxidant capacity.[69]

Calcium metabolism

Beyond generating ATP and ROS, mitochondria play a central role in cellular Ca^{2+} dynamics. Due to their highly negative membrane potential, mitochondria actively take up Ca^{2+} when cytosolic levels increase. Under physiological conditions, the influx of Ca^{2+} in

mitochondria stimulates metabolism by activating several Ca^{2+}-sensitive enzymes of the Krebs cycle and respiratory chain.[70] However, excessive accumulation of Ca^{2+} within mitochondria can have deleterious effects, the most severe one being the opening of the mPTP, which is well known for its central role in triggering mitochondria-mediated cell death.[71] To date, only one study has evaluated the effect of EPI supplementation on the susceptibility of mitochondria to undergo mPTP opening.[43] After treating mice with EPI (1 mg/kg by oral gavage) for 10 days, isolated cardiac mitochondria were submitted to an *in vitro* Ca^{2+} challenge, which is a common method to assess sensitivity to mPTP opening.[43] The authors reported that EPI supplementation increased the resistance to Ca^{2+}-induced PTP opening and noted that this effect was abolished by treating mice with naltrindole. These data suggest that EPI might confer this protective effect through ∂-opioid receptor signalling.[43] Further studies are required to confirm and better understand the effect of EPI supplementation on pore regulation. It will be particularly interesting to evaluate the effect of EPI supplementation in a pathophysiological model of mPTP sensitivity to determine the effectiveness of this compound as a therapeutic agent.

5. Mitochondrial biogenesis

From the available biochemical and functional studies, it is clear that one of the most important effects of EPI is to enhance tissue oxidative capacity through an increase in mitochondrial content and a densification of cristae. These adaptations imply that EPI stimulates the formation and assembly of mitochondria through activation of mitochondrial biogenesis, a complex and highly regulated process that involves several transcription factors, nuclear hormone receptors, and transcriptional co-activators. These molecules act collectively to coordinate changes in the expression of nuclear and mitochondrial DNA encoded genes. Although the exact mechanisms remain incompletely understood, evidence to date suggests that EPI probably enhances mitochondrial biogenesis through multiple and partly overlapping mechanisms.[6,30,39,40,42,46–49,51]

5.a. Effect of EPI on mitochondrial biogenesis transcriptional complexes:

Several studies have focused on PGC1α-NRF1/2-mitochondrial transcription factor A (TFAM) signalling axis (Table 3),[6,30,39,40,42,46–49,51] which plays a major role in the transcription of several nuclear encoded genes, activation of mitochondrial protein import and mtDNA replication.[72] In geriatric mice, two weeks of oral supplementation with EPI reportedly increased PGC1α, NRF-2 and TFAM protein levels in skeletal muscle, heart, kidney and brain.[39] Consistent with these observations, studies in mouse liver and ß-cells showed that EPI

supplementation promotes nuclear NRF2 translocation.[30,73] EPI or EPIm may interact with intracellular Kelch-like-ECH-associated protein 1 (Keap1), which is a cytoplasmic repressor of NRF2 that inhibits translocation of NRF2 to the nucleus.[74] Within the nucleus, NRF2 binds to NRF promoter regions on TFAM, the main factor that regulates mtDNA replication.[30,73] Similarly, in humans, supplementation with chocolate squares and cocoa beverages containing EPI 3 months increased PGC1α and TFAM protein expression in the *vastus lateralis.*[49,56] An increase in the expression of NRF factors, PGC1α and TFAM has also been reported in various cell lines exposed to a range of EPI concentrations for 12-48 h.[4,6,30,32,33,47] These data further confirm the stimulatory effect of EPI on one of the main pathways coordinating changes in the expression of nuclear and mitochondrial DNA encoded genes.

Beyond NRFs, other core transcription factors of the mitochondrial biogenesis pathway are activated in response to EPI (Table 3).[72] Nichols et al. reported that the activity of the cAMP-response-element-binding protein (CREB), which is known to directly promote the expression of β-oxidation and respiratory chain genes containing CREB response elements, was increased in cells exposed for 24h to physiological concentrations of EPI (0.1-0.3 μM).[75] In addition to having direct effects on mitochondrial genes, activation of CREB by EPI can also indirectly potentiate mitochondrial biogenesis by enhancing the expression of PGC1, resulting in the activation of multiple PGC1α-sensitive transcriptional complexes.[72] Similarly, in mice, 14-days oral EPI supplementation was shown to increased the expression of peroxisome proliferator activated receptor beta (PPARβ), which works in concert with PGC1α to regulate the expression of several β-oxidation and oxidative phosphorylation genes.[46]

5.b. Effect of EPI on mitochondrial biogenesis signalling pathways:

Multiple signalling pathways converge on the core mitochondrial biogenesis transcriptional complexes to ensure that mitochondrial metabolism adequately responds to changes in the environmental and cellular state. As shown in Table 3, several studies have attempted to identify which pathway(s) could underlie(s) the beneficial effect of EPI on mitochondrial biogenesis and function. Although much remains to be discovered, converging evidence indicates that at least two important mechanisms are likely involved, namely the stimulation of NO-dependent signalling, and activation of sirtuins.

Stimulation of nitric oxide (NO)-dependent signalling:

Several studies have established that EPI directly enhances NO generation. Dose response studies in bovine coronary artery endothelial cells have shown that NO production is potently stimulated in the presence of nanomolar concentrations of EPI (half effective concentration (EC_{50}) = 2 nM).[46] Mechanistically, this phenomenon can be explained by the inhibitory effect of cocoa flavanols on the arginine degrading enzyme arginase, which in turn increases the availability of L-arginine for NO synthesis.[76] In addition, evidence indicates that supplementation with EPI increases the activity of endothelial nitric oxide synthase (eNOS) in tissues and cells under normal and diabetic conditions.[6] These direct effects of EPI on NO bioavailability can account for the vasodilatory effect of EPI.[77] In addition, the eNOS inhibitor L-NAME blunts the stimulatory effect of EPI on mitochondrial biogenesis;[6] these data indicate that enhanced NO signaling at least partially underlies the effect of this flavanol on mitochondrial biomass.

NO enhances mitochondrial biogenesis through several mechanisms. The first one is the production of cGMP by guanylate cyclase (GC), which in turn activates protein kinase A (PKA), ultimately resulting in the phosphorylation and nuclear translocation of CREB.[78] NO also stimulates AMP-activated protein kinase (AMPK), which is known to phosphorylate and activate PGC-1α.[79] Furthermore, NO promotes the dissociation of NRF2 from Keap1, the cytosolic anchoring protein that normally acts as a repressor, thus promoting the nuclear translocation of NRF2, and subsequent stimulation of mitochondrial biogenesis as well as the expression of several antioxidant genes containing NRF binding elements.[80] Importantly, evidences has suggested that all three mechanisms are stimulated in response to EPI. In primary cortical neurons, exposure to low EPI concentrations for 24 h increased CREB phosphorylation.[75] A study reported activation of AMPK, assessed using phospho-specific AMPK antibodies, in human muscle following 3-month supplementation with chocolate squares and cocoa beverages.[56] In addition, (−)-epicatechin-3-gallate disrupts the NRF2-Keap1 interaction.[81] Taken together, these data clearly indicate that NO plays an important role in activating mitochondrial biogenesis following supplementation with EPI.

Activation of sirtuins:

The sirtuins are a family of proteins that act predominantly as nicotinamide adenine dinucleotide (NAD)-dependent deacetylases.[82] In mammals, three members of this family predominantly localise to the nucleus (Sirt1, Sirt6, Sirt7), one is exclusively cytosolic (Sirt2) and three are only found in mitochondria (Sirt3, Sirt4, Sirt5). In recent years, sirtuins have

emerged as key metabolic sensors; they use intracellular metabolites such as NAD and short-chain carbon fragments such as acetyl coenzyme A to modulate mitochondrial turnover and function. Most mammalian sirtuins appear to act predominantly as deacetylases, with targets that include histones and several proteins located in the cytosol and mitochondria.[82]

Sirt1, the most extensively studied member of this family, plays an important role in promoting mitochondrial biogenesis by promoting deacetylation of PGC1α, thereby enhancing its transcriptional activity.[83] Several studies have reported that EPI can stimulate Sirt1.[4,6,39,49,66,84] In humans, EPI supplementation increased activated phospho-SIRT1 or total SIRT1 content in muscle, a phenomenon that was associated with a decrease in PGC1α acetylation.[49,66]

Interestingly, proanthocyanidins, which contain EPI, caused a dose-dependent increase in tissue NAD^+ content in mice livers fed with increasing amounts of the compound (0, 5, 25 or 50 mg/kg bw), resulting in Sirt1 stimulation.[84] Although the precise mechanisms remain unclear, data from this study indicate that the administration of proanthocyanidins increases the content of several precursors for NAD biosynthesis, including nicotinamide, tryptophan and nicotinic acid, and decreases transcript levels for two important NAD^+-consuming enzymes, namely Parp1 and Cd38.[84] Importantly, while Sirt1 activity is clearly increased in response to EPI, little data is available for other sirtuins, particularly those located in the mitochondria, which are known to modulate the activity of the Krebs cycle and respiratory chain enzymes.

Activation of cell surface receptors

In addition to the above-mentioned mechanisms, a limited number of studies have focused on the potential contribution of selected cell surface receptors in mediating the beneficial effects of EPI. Studies in C2C12 myotubes have shown that pharmacological inhibition and small interfering RNA (siRNA)-mediated knockdown of G-protein coupled oestrogen receptors, which are expressed in multiple tissues including cardiac and skeletal muscle,[85,86] blunted the increase in mitochondrial content and the upregulation of PGC1α-NRF-TFAM signalling observed in response to EPI (3-10 μM).[32] Currently, there is no evidence to indicate that EPI can bind to classical oestrogen receptors (i.e., α and β),[32] which suggests that the effect of EPI is not mechanistically dependent on oestrogen receptor signalling *per se*, but acts on mitochondrial biogenesis signals that are regulated by oestrogens. Oestrogen receptors located in the nucleus are important targets of PGC1α and can directly regulate the expression of Krebs cycle and β-oxidation genes.[72] Furthermore, binding of oestrogen stimulates the

synthesis of cAMP and cGMP as well as the release of calcium, which in turn stimulates several pathways that can enhance mitochondrial biogenesis.[87]

Previous studies have also suggested that ∂-opioid receptors may underlie some of the beneficial effects of EPI on mitochondrial functions.[43] In this study, supplementing mice with EPI for 10 days increased in state 3 respiration in isolated cardiac mitochondria along with changes in membrane fluidity. These effects were abolished when mice were co-treated with the ∂-opioid receptor antagonist naltrindole. However, it remains unclear how the ∂-opioid receptor can signal to mitochondria.

6. Therapeutic potential:

With a favourable safety profile, pharmacokinetics and bioavailability, as well as ~~and~~ proven benefits on mitochondrial content and function, EPI has emerged as an interesting therapeutic to prevent or mitigate mitochondrial dysfunction. Evidence suggests that EPI could provide benefits in the context of diseases with broadly different aetiologies. For instance, in type 2 diabetes, skeletal muscle mitochondrial abnormalities are believed to contribute to insulin resistance and muscle fatiguability.[88–90] Using a high-fat diet model of insulin resistance in mice, Ramirez-Sanchez et al.[6] reported that EPI supplementation can restore mitochondrial content and improve mitochondrial function in the heart. Moreover, mitochondrial dysfunction is increasingly associated with an array of neurodegenerative diseases, including Alzheimer's and Huntington disease.[91] For most of these disorders, a common pathogenic mechanism, involving a complex interplay between reduced oxidative capacity, impaired mitochondrial network dynamics, oxidative stress, loss of proteostasis and proinflammatory signalling, is believed to be involved.[91,92] Although this area has been relatively underexplored, a recent study suggested that EPI could have beneficial neuroprotective effects. For instance, epigallocatechin gallate reportedly has neuroprotective effects in a mouse model of MPTP-induced neurotoxicity disease, which is well known to mimic mitochondrial alterations observed in dopaminergic neurons from Parkinson's patients.[93] Oral supplementation with epigallocatechin gallate (25 mg/kg for 7 days) resulted in increased dopamine levels, and lowered oxidative stress in the *substantia nigra*, which was associated with improved motor function.[93]

Fragmentation of the mitochondrial network has been implicated in many neurodegenerative and metabolic diseases because it impairs bioenergetic efficiency, increases ROS production and augments susceptibility to mPTP opening.[94 95] Interestingly, PGC1α positively regulates mitofusin-2 (MFN2) expression at the transcriptional level,[96] and conversely, proper regulation of mitochondrial activity by PGC1α depends on correct MFN2

expression.[97] Therefore, EPI supplementation could contribute to alleviate mitochondrial network fragmentation, an effect that could be beneficial in pathological states associated with mitochondrial fragmentation, such as type 2 diabetes, obesity or neurodegenerative diseases.[94]

7. Conclusion

A number of conclusions can be derived from the available literature on the effects of epicatechin on mitochondria. Most importantly, it appears clear that supplementation with EPI over several (4-12) weeks is effective at improving mitochondrial content and function in muscle, and potentially other tissues. EPI could thus represent an interesting adjuvant therapy for the management of diseases associated with mitochondrial dysfunction. With regard to the underlying mechanisms, the available data indicate that EPI is effective at promoting mitochondrial biogenesis at physiological concentrations, which can account at least in part for the increase in tissue mitochondrial content. This effect is mediated mainly by the stimulation of NO- and SIRT1-dependent signalling, converging on PGC1α and major nuclear transcriptional complexes. In addition, EPI appears to have direct acute effects on mitochondria *in vitro*, a phenomenon that impacts membrane fluidity, respiratory capacity and ROS production. However, the underlying mechanisms and their physiological significance remain to be established. Some evidence suggests that the benefits of EPI are most readily observed in sedentary subjects or diseased patients that have lower oxidative capacities. The impact of this type of supplementation in active individuals is less obvious. Importantly, bioavailability studies indicate that orally administered EPI is extensively metabolised in the gut, with a number of secondary metabolites appearing in the blood at concentrations that are equivalent to or in excess of EPI. The impact of these secondary metabolites on mitochondrial function is completely unknown and should be investigated, considering the fact that the types of secondary metabolites generated in the gut vary extensively across species.

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Figure legend

Figure 1: Chemical structure of flavanol stereoisomers (−)-epicatechin, (+)-epicatechin, (−)-catechin, (+)-catechin.

Figure ~~1~~2: Effect of (-)-epicatechin supplementation on metabolic pathways involved in mitochondrial biogenesis.

EPI can stimulate mitochondrial biogenesis through a combination of overlapping and redundant pathways. EPI modulates the activity of some kinases and cellular receptors involved in NO production to increase its cellular content. The regulatory role of NO on mitochondrial biogenesis involves the activation of NOS and guanylate cyclase to generate cyclic GMP, which in turn activates protein kinase A (PKA). PKA phosphorylates CREB and thus allowing its nuclear translocation and activation of the PGC-1 gene, a co-activator for NRF-1, a transcription factor for mitochondrial biogenesis. Moreover, NO production promotes the dissociation of NRF2 and Kelch-like ECH-associated protein 1 to promote the nuclear translocation of NRF2 and its interaction with antioxidant response element to promote mitochondrial biogenesis. EPI, through modulation of nicotinamide adenine dinucleotide metabolism, activates sirtuins that deacetylate PGC1a and enhance its transcription activity. PGC1a induces mitochondrial biogenesis by activating transcription factors, including NRF1 and NRF2, which activates TFAM that drives transcription and replication of mtDNA.

Abbreviations: EPI, (-)-epicatechin; GPER, G-protein coupled estrogen receptor; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; cGMP, cyclic guanosine monophosphate; PKA, protein kinase A; CREB, cAMP-response-element-binding protein; NRF1, nuclear respiratory factor 1; NRF2, nuclear factor erythroid-2-related factor 2; AMPK, AMP-activated protein kinase; PGC1a, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; SIRT1, sirtuin-1; SIRT3, sirtuin-3; SOD2, superoxide dismutase 2; TFAM, mitochondrial transcription factor A; mtDNA, mitochondrial DNA; NuGEMPs, nuclear gene encoding mitochondrial proteins

Table 1: Effect of (-)-epicatechin supplementation on mitochondrial structure.

Reference	Species	Muscles or cells	Protocol	Results
Bitner et al. (2018)[5]	Cell culture	832/13 ß cells	Overnight culture with **10 µM EPI**	↑ CI, CII, CIII, CIV and CV content
Chidambaram et al.(2018)[33]	Cell culture	Neuroblastoma cells (SH-SY5Y)	3 or 10 µg/mL of cocoa 12 h prior to MPP intoxication	↑ CIV content in intoxicated cells ∅ effect in control cells
Moreno-Ulloa et al. (2018)[32]	Cell culture	C2/C12 myotubes	3 or **10 µM EPI** during 48 h	↑ Porin content and mitochondrial inner mass
Ramirez-Sanchez et al. (2018)[4]	Cell culture	BCAEC	1 µM during 48 h	↑ Mitofilin, CII, CIV and CV content
Schwarz et al. (2018)[45]	Human (recreationally men and women, n=20)	Vastus lateralis	Oral capsule: 2 x 100 mg/day during 4 weeks associated or not with endurance training program. No diet control.	∅ CII, CIV and CS content with EPI
Rowley et al. (2017)[30]	Cell culture	INS-1-derived 832/13 rat insulinoma cell	25 µg/mL of monomeric catechins (~2.5 µM of EPI) for 24 h	↑ CIII, CIV and CV content

Lee et al. (2016)[46]	Mice (C57BL/6N)	Soleus Gastrocnemius	Hindlimb suspension during 14 days with oral gavage of EPI: 2 x 1 mg/kg bw/day	Restore CIV content
Ramirez-Sanchez et al. (2016)[6]	Cell culture	HCAEC	100 nM EPI for 48 h	↑ Mitofilin, CI, CII, CIII and CV content
Ramirez-Sanchez et al. (2016) [6]	Mice (C57BL/6)	Heart	High fat diet fed mice Oral gavage: 2 x 1 mg/kg bw/day for 15 days	↑ Mitofilin and porin content
Moreno-Ulloa et al. (2015)[39]	Senile mice (C57BL/6)	Heart Quadriceps	Oral gavage: 2 x 1 mg/kg bw/day for 15 days	↑ Mitofilin, CI and CV content
Ramirez-Sanchez et al. (2014)[42]	Mice (B6.129Sgcdtm Mcn/J)	Heart Quadriceps	Oral gavage 1 mg/kg bw/day for 14 days	↑ Mitofilin, porin and CV content
Watanabe et al. (2014)[41]	Mice (C57BL/6)	Gastrocnemius, Soleus	Oral gavage of cacao powder containing 6.43% (w/w) of epicatechin 50 mg/kg bw/ day for 14 days	↑ mtDNA/nDNA and CPT-2
Hüttemann et al. (2013)[51]	LRC Rat	Plantaris	Oral gavage: 2 x 1 mg/kg bw/day for 30 days	↑ Mitochondrial volume, cristae abundance, CIV subunit II content
Moreno-Ulloa et al. (2013)[47]	Cell culture	BCAEC	2 nM EPI for 48 h	↑ Mitofilin, CI and CII content

Panneerselvam et al. (2013)[43]	Mice (C57BL/6)	Heart	Oral gavage: 1 mg/kg bw/day for 10 days	↑ Rigidity/fluidity ratio
Hütteman et al. (2012)[44]	Mice (C57BL/6)	Quadriceps	Oral gavage: 2 x 1 mg/kg bw/day for 14 days detraining period following a 5-week training period	↑ CI, CIII, CIV and CV content
Nogueira et al. (2011)[40]	Mice (C57BL/6N)	Heart Quadriceps Plantaris	Oral gavage: 2 x 1 mg/kg bw/day for 15 days	Heart: ↑ Mitofilin, porin and CII content, cristae membrane surface area, mitochondrial volume density Quadriceps: ↑ CI, CII, CIII and CV content, mitofilin, porin Plantaris: ↑ Cristae membrane area, mitochondrial volume density
Taub et al. (2012)[49]	Human (HF and DT2, n = 5)	Vastus lateralis	Chocolate square and cocoa beverages containing 50 mg of EPI 2x/day for 3 months. No diet control; no lifestyle control.	↑ Mitofilin, porin, CI, CV content and cristae perimeter

mtDNA, mitochondrial DNA; nDNA, nuclear DNA; CPT-2, carnitine palmitoyltransferase 2; CI, respiratory chain complex I; CII, respiratory chain complex II; CIII, respiratory chain complex III; CIV, respiratory chain complex IV; CV, respiratory chain complex V or ATP synthase; CS, Citrate synthase; HF, heart failure patients; DT2, type II diabetes patients; HCAEC, human coronary artery endothelial cells; EPI, (-)-epicatechin; BCAEC,

5 bovine coronary artery endothelial cells; LRC, low running capacity. ↑, increase; ∅, unchanged. Bold type: supraphysiological dose of EPI
6 (>10μM).

Table 2: Effect of (-)-epicatechin supplementation on mitochondrial function

Author	Species or model	Muscles or cells	Protocol	Results
Energy production				
Bitner et al. (2018)[5]	Cell culture	832/13 ß cells	24 h with 5 or **10µM EPI**	↑ basal, maximal and glucose stimulated respiration
Bitner et al. (2018)[5]	Cell culture	C2C12 cells	4-h pretreatment of vehicle, **5 or 10 µM EPI** before a 4-h exposure to 500 µM of H_2O_2	∅ effect with 5µM 10µM of EPI partially blunt maximal respiration decrease
Kener et al. (2018)[62]	Cell culture	INS-1 derived 832/13 ß cells	24-h incubation with 0.1 µM EPI	Mitochondrial respiration in permeabilised cells: ↑ coupled and uncoupled respiration with glucose as substrate
Moreno-Ulloa et al. (2018)[32]	Cell culture	C2/C12 myotubes	3 or **10 µM EPI** for 48 h	↑ CS activity
Ramirez-Sanchez et al. (2018)[4]	Cell culture	BCAEC	1 µM EPI for 48 h	↑ CS activity
Rowley et al. (2017)[30]	Cell culture	INS-1-derived 832/13 rat insulinoma cells	25 µg/mL of monomeric catechins (~2.5 µM of EPI) for 24 h	Mitochondrial respiration in permeabilised cells: ↑ CI and CII-driven respiration (state 3) ↑ CI-driven respiration (state 4)

				↑ ATP production
Santos et al. (2017)[64]	Cell culture	MRC-5	30 min exposure to **10 µM EPI** followed by an 1 h exposure with AMD	↑ CI activity and ATP biosynthesis
Ramirez-Sanchez et al. (2016)[6]	Cell culture	HCAEC	100 nM EPI for 48 h	↑ CS activity
Ramirez-Sanchez et al. (2016) [6]	Mice (C57BL/6)	Heart	High fat diet fed mice Oral gavage: 2 x 1 mg/kg bw/day for 15 days	↑ CS activity
Taub et al. (2016)[56]	Human (sedentary subjects, n=10)	Vastus lateralis	Chocolate square containing 26 mg EPI, 1x/day for 3 months. No diet control.	↑ CS activity
Kopustinskiene et al. (2015)[60]	Rat (Wistar)	Heart	Mitochondrial respiration on isolated mitochondria. EPI supplementation from 0.3 to 2.4 µM	↑ State 2 respiration in a dose-dependent manner ↑ State 3 respiration at low EPI concentration with PM, S and PC as substrate ↓ State 3 respiration at high EPI concentration ↓ Cytochrome c release in presence of EPI

Moreno-Ulloa et al. (2015)[39]	Senile mice (C57BL/6)	Heart Quadriceps	Oral gavage: 2 x 1 mg/kg bw/day for 15 days	↑ CS activity
Nichols et al. (2015)[75]	Cell culture	Mouse primary cortical neurons	0.1 or 0.3µM EPI for 24 h	↑ uncoupled respiration following oxygen-glucose deprivation Ø on basal and maximal coupled respiration
Elbaz et al. (2014)[52]	Cell culture	Human fibroblasts, pancreatic cells	Mitochondrial respiration with 0, **20, 50, 100 or 200 µM EPI**	↑ CIV respiration with 100 and 200 µM EPI in pancreatic cells Ø in human fibroblast
Ramirez-Sanchez et al. (2014)[42]	Mice (B6.129Sgcdtm Mcn/J)	Heart Quadriceps	Oral gavage 1 mg/kg bw/day for 14 days	↑ CS activity
Moreno-Ulloa et al. (2013)[47]	Cell culture	BCAEC	2 nM EPI for 48 h	↑ CS activity
Panneerselvam et al. (2013)[43]	Mice (C57BL/6)	Heart	Oral gavage: 1 mg/kg bw/day for 10 days	Mitochondrial respiration in isolated mitochondria: ↑ CI-driven respiration (state 3) Ø, CI-driven respiration (state 4) and CII-driven respiration (state 3 and 4)
Hütteman et al. (2012)[44]	Mice (C57BL/6)	Quadriceps	Oral gavage:	↑ CIV activity

			2 x 1 mg/kg bw/day for 14 days detraining period following a 5-week training period	
ROS metabolism				
Chidambaram et al. (2018)[33]	Cell culture	Neuroblastoma cells (SH-SY5Y)	**8.4 or 28µM** 12 h prior to MPP intoxication	↑ SOD activity in intoxicated cells Ø SOD activity in control cells
Rowley et al. (2017)[30]	Cell culture	INS-1-derived 832/13 rat insulinoma cell	25 µg/mL of monomeric catechins (~2.5 µM of EPI) for 24 h	↑ GSH/GSSG, GSH content, cell viability following a ROS-induced apoptosis
Santos et al. (2017)[64]	Cell culture	MRC-5	30-min exposure to **10 µM** EPI followed by 1-h exposure with AMD for SOD and catalase assay 30-min exposure with **10, 100 or 500 µM** EPI followed by a 24-h exposure with AMD for oxidative damage to proteins and lipids	↑ Catalase and SOD activities ↓ Protein carbonyl levels, TBARS
Shaki et al. (2017)[65]	Rats (Wistar)	Whole brain	Oral gavage: 50 mg/kg bw/day for 10 days Intra hippocampal injection of homocysteine (0.5 µmol/µL) 5 days prior to sacrifice	↓ Mitochondrial peroxidation, ROS production induced by homocysteine ↑ GSH content following homocysteine injection

Ramirez-Sanchez et al. (2016)[6]	Mice (C57BL/6)	Quadriceps	Oral gavage: 1 mg/kg bw/day for 15 days	↑ SOD2 and catalase activity
Taub et al. (2016)[56]	Human (sedentary subjects, n=10)	Vastus lateralis	Chocolate square containing 26 mg EPI, 1x/day for 3 months. No diet control.	↑ GSH/GSSG ↓ Protein carbonyl levels
Moreno-Ulloa et al. (2015)[39]	Mice (C57BL/6)	Heart Quadriceps	Oral gavage: 2 x 1 mg/kg bw/day for 15 days	↑ SIRT3, SOD2, catalase, TRX protein content ↑ catalase activity
Ramirez-Sanchez et al. (2014)	Mice (B6.129Sgcdt mMcn/J)	Heart Quadriceps	Oral gavage 1 mg/kg bw/day for 14 days	↑ SIRT3, SOD2, TRX protein content ↑ SOD2 and catalase activity
Panneerselvam et al. (2013)[43]	Mice (C57BL/6)	Heart	Oral gavage: 1 mg/kg bw/day for 10 days	↑ ROS production in isolated mitochondria during state 3 respiration
Ramirez-Sanchez et al. (2013)[66]	Human (DT2 and HF patients, n=5)	Vastus lateralis	Chocolate square and cocoa beverages containing 50 mg EPI. Regular diet.	↑ SIRT3, SOD and catalase protein content ↑ SOD2 and catalase activity
Calcium handling				
Panneerselvam et al. (2013)[43]	Mice (C57BL/6)	Heart	Oral gavage: 1 mg/kg bw/day for 10 days	↑ Calcium-induced mitochondrial swelling capacity

CI, respiratory chain complex I; CII, respiratory chain complex II; CIV, respiratory chain complex IV; CS, citrate synthase; HF, heart failure patients; DT2, type II diabetes patients; HCAEC, human coronary artery endothelial cells; EPI, (-)-epicatechin; BCAEC, bovine coronary artery endothelial cells; MRC-5, human lung fibroblasts; AMD, amiodarone; state 4 respiration represent oxygen consumption in absence of ADP (mitochondria not

making ATP), state 3 represent oxygen consumption in presence of ADP (mitochondria actively making ATP); PM, pyruvate + malate; S, succinate; PC, palmitoyl-L-carnitine; SOD, super oxide dismutase; GSH, reduced glutathione; GSSG, oxidised glutathione; ROS, reactive oxygen species; SIRT3, sirtuin-3; TRX, thioredoxin. ↑, increase; ↓, decrease; ∅, unchanged. Bold type: supraphysiological dose of EPI (>10μM).

15 **Table 3: Effect of (-)-epicatechin supplementation on mitochondrial biogenesis pathways.**

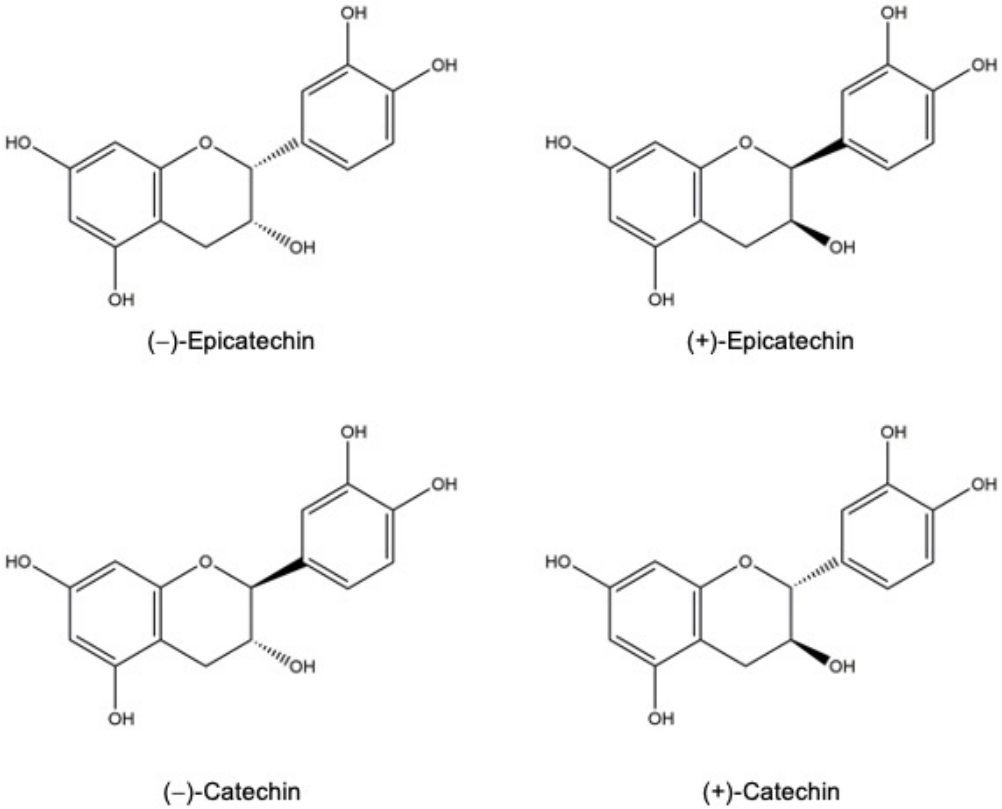
Author	Species	Muscles or cells	Protocol	Results
Huang et al. (2019)[73]	Rat (Sprague-Dowley)	Liver	Oral gavage: 2 x 40 mg/kg bw EPI with 24 h delay	↑ NRF2 protein translocation into the nucleus Ø NRF2 mRNA and protein
Chidambaram et al. (2018)[33]	Cell culture	Neuroblastoma cells (SH-SY5Y)	**8.4 or 28µM** 12 h prior to MPP intoxication	↑ PPARγ, PGC1α, NRF2 and TFAM content in intoxicated cells Ø PPARγ, PGC1α, NRF2 and TFAM content in control cells
Moreno-Ulloa et al. (2018)[48]	Cell culture	C2/C12 myotubes	3 or **10 µM EPI** for 48 h	↑ TFAM, NRF2, Porin content
Ramirez-Sanchez et al. (2018)[4]	Cell culture	BCAEC	1 µM EPI for 48 h	↑ p-SIRT1/SIRT1 and TFAM
Rowley et al. (2017)[30]	Cell culture	INS-1-derived 832/13 rat insulinoma cells	25 µg/mL of monomeric catechins (~2.5 µM of EPI) for 24 h	Ø cytoplasmic NRF2 protein content ↑ NRF1 mRNA, NRF2 mRNA and NRF2 protein translocation into the nucleus
Aragones et al. (2016)[84]	Rat (Wistar)	liver	Oral gavage: 5, 25 or 50 mg/kg bw/day proanthocyanidins containing 52 µmol/g EPI for 21 days	↑ NAM, NAD^+ protein content in a dose dependent manner ↑ SIRT1, SIRT2, SIRT3, SOD2 mRNA levels in a dose dependent manner

				↑ Sirt1 activity in a dose dependent manner
Lee et al. (2016)[46]	Mice (C57BL/6N)	Soleus Gastrocnemius	Hindlimb suspension for 14 days with oral gavage of EPI: 2 x 1 mg/kg bw/day	↑ PPARß and TFAM protein content ∅ PGC1α protein content
Ramirez-Sanchez et al. (2016)[6]	Cell culture	HCAEC	100 nM EPI for 48 h	↑ PGC1α, SIRT1 and TFAM protein content
Ramirez-Sanchez et al. (2016) [6]	Mice (C57BL/6)	Heart	High fat diet fed mice Oral gavage: 2 x 1 mg/kg bw/day for 15 days	↑ PGC1α, SIRT1 and TFAM protein content
Taub et al. (2016)[56]	Human (sedentary subjects, n=10)	Vastus lateralis	Chocolate square containing 26 mg EPI, 1x/day for 3 months. No diet control.	↑ PGC1α, pLKB1 pAMPK protein content
Moreno-Ulloa et al. (2015)[39]	Mice (C57BL/6)	Heart Quadriceps	Oral gavage: 2 x 1 mg/kg bw/day for 15 days	↑ PGC1α, SIRT1, TFAM and NRF2 protein content
Nichols et al. (2015)[75]	Cell culture	Mouse primary cortical neurons	0.1 or 0.3 µM EPI for 24 h	↑ pAkt/Akt, pCREB/CREB
Ramirez-Sanchez et al. (2014)[42]	Mice (B6.129SgcdtmMcn/J)	Heart Quadriceps	Oral gavage 1 mg/kg bw/day for 10 days	↑ PGC1α and TFAM protein content
Ramirez-Sanchez et al. (2013)[66]	Human (DT2 and HF patients, n=5)	Vastus lateralis	Chocolate square and cocoa beverages containing 50 mg epicatechin. Regular diet.	↑ pSIRT1 protein content ↓ acetylated PGC1α

Hüttemann et al. (2013)[51]	LRC Rat	Plantaris	Oral gavage: 2 x 1 mg/kg bw/day for 30 days	↑ PGC1α, PGC1ß, pMKK3/6, pp38 MAPK, MEF2A and TFAM protein content
Moreno-Ulloa et al. (2013)[47]	Cell culture	BCAEC	2 nM EPI for 48 h	↑ TFAM protein content
Nogueira et al. (2011)[40]	Mice (C57BL/6N)	Quadriceps	Oral gavage: 2 x 1 mg/kg bw/day for 15 days	↑ TFAM protein content
Taub et al. (2012)[49]	Human (HF and DT2, n=5)	Vastus lateralis	Chocolate square and cocoa beverages containing 50mg of epicatechin 2x/day for 3 months. No diet control; no life style control.	↑ SIRT1, PGC1α and TFAM protein content ↓ acetylated PGC1α

MCT, monocrotaline; NRF1, nuclear respiratory factor 1; NRF2, nuclear factor erythroid-2-related factor 2; BCAEC, bovine coronary artery endothelial cells; EPI, (-)-epicatechin; SIRT1, sirtuin-1; pSIRT1, phosphorylated-sirtuin-1; SIRT2, sirtuin-2; SIRT3, sirtuin-3; TFAM, mitochondrial transcription factor A; PPARγ, peroxisome proliferator-activated receptor gamma; PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PPARß, peroxisome proliferator-activated receptor beta; NAD^+, nicotinamide adenine dinucleotide; NAM, nicotinamide; SOD2, super oxide dismutase 2; pLKB1, phosphorylated liver kinase B1; pAMPK, phosphorylated AMP-activated protein kinase; pMKK3/6, phosphorylated MAPK kinase 3/6; pp38 MAPK, phosphorylated p38 mitogen-activated protein kinase; MEF2A, myocyte enhancer factor 2A; pAKT: phosphorylated protein kinase B; pCREB: phosphorylated cAMP response element-binding protein. ↑, increase; ↓, decrease; ∅, unchanged. Bold type: supraphysiological dose of EPI (>10µM).

25 Figure 1:



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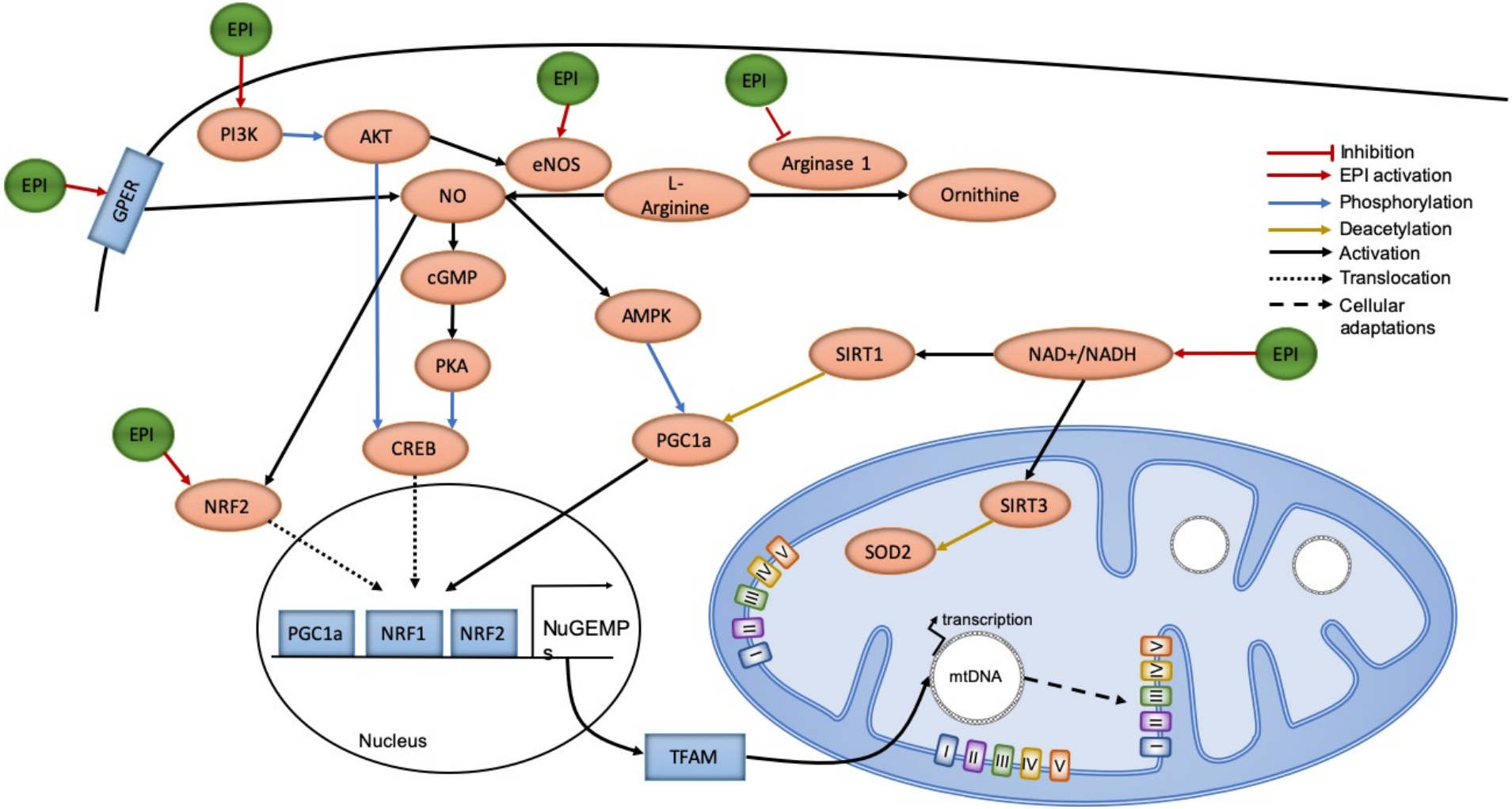
28 Figure 2:



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