



CO-mediated cytoprotection is dependent on cell metabolism modulation

Cláudia Figueiredo-Pereira^{a,1}, Daniela Dias-Pedroso^{a,b,1}, Nuno L. Soares^{a,b},
Helena L.A. Vieira^{a,b,c,*}

^a CEDOC, Faculdade de Ciências Médicas/NOVA Medical School, Universidade Nova de Lisboa, 1169-056, Lisboa, Portugal

^b UCIBIO, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Portugal

^c Instituto de Biologia Experimental e Tecnológica (iBET), Apartado 12, 2781-901, Oeiras, Portugal

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ABSTRACT

Carbon monoxide (CO) is a gasotransmitter endogenously produced by the activity of heme oxygenase, which is a stress-response enzyme. Endogenous CO or low concentrations of exogenous CO have been described to present several cytoprotective functions: anti-apoptosis, anti-inflammatory, vasomodulation, maintenance of homeostasis, stimulation of preconditioning and modulation of cell differentiation. The present review revises and discusses how CO regulates cell metabolism and how it is involved in the distinct cytoprotective roles of CO. The first found metabolic effect of CO was its increase on cellular ATP production, and since then much data have been generated. Mitochondria are the most described and studied cellular targets of CO. Mitochondria exposure to this gasotransmitter leads several consequences: ROS generation, stimulation of mitochondrial biogenesis, increased oxidative phosphorylation or mild uncoupling effect. Likewise, CO negatively regulates glycolysis and improves pentose phosphate pathway. More recently, CO has also been disclosed as a regulating molecule for metabolic diseases, such as obesity and diabetes with promising results.

1. Introduction

Carbon monoxide (CO) was first described to be endogenously produced in 1949, when it was found in exhaled human breath [1]. Only 20 years later, the enzyme responsible for CO production was identified. Tenhunen and colleagues have discovered that heme degradation by microsomal heme oxygenase (HO) originates CO, iron (Fe²⁺) and biliverdin [2]. In fact, HO catalyzes the first and limiting rate step of heme oxidative degradation by opening Fe-protoporphyrin IX ring into free Fe²⁺, CO and biliverdin, whose reaction consumes O₂ and NADPH as electrons donor [3]. HO activity is cytoprotective for two main reasons: (i) it eliminates free heme, which is potentially very toxic, and (ii) its products present beneficial properties. First, biliverdin is rapidly converted by biliverdin reductase into bilirubin, which is a potent anti-oxidant [4]. Second, iron binds to iron regulatory proteins (IRP) increasing ferritin expression, which promotes cytoprotection [5]. Finally, CO emerges as a gasotransmitter with several beneficial biological functions revised in Refs. [6–10], namely maintenance of tissue homeostasis, anti-inflammation, cytoprotection, cardioprotection,

promotion of preconditioning and tolerance state, regulation of cell differentiation/proliferation and modulation of cell metabolism. The present review tackles how endogenous CO or low concentrations of exogenous CO modulate cell metabolism in different cells and tissues, and discusses CO's potential impact on human health.

2. Carbon monoxide releasing molecules (CORM)

The development of carbon monoxide releasing molecules (CORM) emerges due to the potential application of this gasotransmitter as a therapeutic agent in Medicine. The use of CO gas presents intrinsic limitations because of its great affinity to hemoglobin, forming carboxyhemoglobin and hampering optimal oxygen delivery. Therefore, the development of strategies to deliver CO in a safer mode is needed. CORMs are metal or transition metal carbonyl complexes that function as a prodrug to deliver CO under temporal and spatial controlled conditions [11,12]. In fact, CORM design must take into account water solubility, biocompatibility, toxicity, absorption, distribution, metabolism and excretion properties [11]. Herein four different types of

Abbreviations: CNS, central nervous system; CO, carbon monoxide; CORM, Carbon monoxide-releasing molecule; DMSO, dimethyl sulfoxide; GSH, reduced glutathione; GSSG, oxidized glutathione; HO-1, Heme-oxygenase 1; Nrf, nuclear respiratory factor; PGC-1 α , peroxisome proliferator-activated receptor gamma co-activator-1 α ; PPP, Pentose phosphate pathway; ROS, reactive oxygen species; TCA, Tricarboxylic acid cycle; TFAM, mitochondrial transcription factor

* Corresponding author. UCIBIO Departamento de Química Faculdade de Ciências e Tecnologia Universidade Nova de Lisboa, 2829-516, Caparica, Portugal.

E-mail address: hl.vieira@fct.unl.pt (H.L.A. Vieira).

¹ equally contributed.

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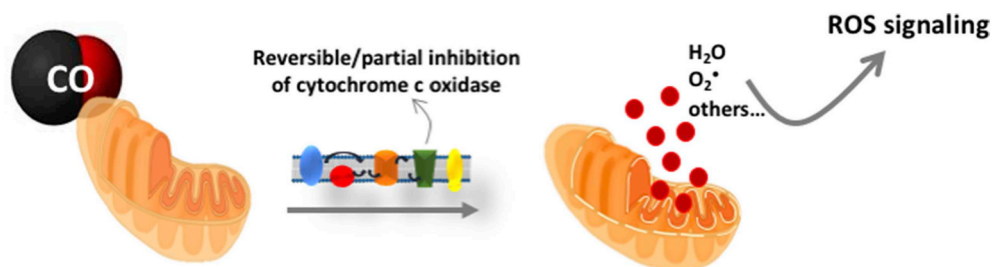


Fig. 1. CO promotes mitochondrial ROS generation. CO can bind and inhibit cytochrome *c* oxidase, which accumulates electrons at the mitochondrial electron transport chain. Accumulation of electrons enhances the probability of O_2 reduction into anion superoxide, which is quickly converted into other ROS, namely hydrogen peroxide. Because low amounts of CO partially and transiently binds to cytochrome *c* oxidase low levels of ROS are generated that act as cell signaling molecules.

CORMs are discussed as modulators of cell metabolism. CORM-2 and CORM-3 contain a ruthenium center and release CO with $t_{1/2}$ of 1 min [13,14]. While CORM-2 is soluble in DMSO, CORM-3 is water soluble and only slightly increases carboxyhemoglobin levels when injected *in vivo*, which is a great advantage [15]. CORM-A1 has boron in its center and releases CO in a pH- and temperature-dependent manner with $t_{1/2}$ of 21 min, being a slow releaser [16]. Of note, CORM biological effect might be also dependent on CO release kinetics. Finally, CORM-401 presents a manganese center, is water soluble and provides at least 3 mol of CO *per* mole of compound [17]. Moreover, CORM-401 is relatively stable in PBS buffer, and 1 mM of CORM-401 releases 0.33 mol equivalent of CO gas in 4 h [17]. In biological systems, CORM-3 and CORM-A1 are the most studied with well-established functions of vasodilation, anti-inflammation and cytoprotection.

3. ATP assessment following CO treatment

The first clues about the CO's potential modulation of cell metabolism emerged in 2004 when Lavitrano and colleagues have found an improved cardiac metabolic status in CO-treated pigs before ischemia and reperfusion injury [18]. In fact, pre-treatment with CO gas (250 ppm) increased heart levels of ATP and phosphocreatine, which is a high-energy phosphate cellular reserve. Likewise, in hepatocytes, endogenous CO derived from heme-oxygenase activity or exogenous CO exposure increases ATP production that, in turn, activates p38 MAPK signaling [19]. *In vivo*, CO gas exposure also increased ATP levels in liver and in *in vitro* primary cultures of hepatocytes. These higher concentrations of ATP are associated with higher viability of hepatocytes and mice in response to TNF- α treatment and fulminant hepatitis, respectively [20]. The CO improvement of cellular energy metabolism is dependent on soluble guanylyl cyclase [20]. Nevertheless, in both models (heart and liver), CO-induced higher ATP levels can also be a consequence of increased cell viability rather than an improvement of cellular metabolism. Only later, more accurate studies properly revealed the role of CO in cell metabolism regulation, which is discussed below.

4. CO acts *via* mitochondrial ROS signaling

Several studies have demonstrated that many CO's biological beneficial effects are dependent on mitochondrial ROS generation, reviewed in Refs. [10,21,22]. In fact, CO-induced low levels of mitochondrial ROS act as signaling molecules, as a preconditioning-like effect, which in turn, promote different biological responses: cytoprotection, anti-inflammatory, modulation of cell metabolism or cellular differentiation. In this section, the molecular mechanisms of mitochondrial ROS generation are discussed. It is worth of note that for ROS to signal and to exert beneficial properties, they must be at low concentrations.

Despite all the described biological functions of CO, its pathways and in particular, its molecular targets in biological systems are still a matter of great debate. CO is a quite chemically inert molecule and it binds to transition metals present in proteins [23,24]. In biological

systems, the typical target of CO are heme-containing proteins, namely soluble guanylate cyclase, cytochromes, hemoglobin and myoglobin. Notably, CO can only bind to reduced Fe^{2+} , limiting the potential target proteins, in contrast to NO that donates electrons to Fe^{3+} and accepts electrons from Fe^{2+} [24].

High levels of CO are toxic and lead to tissue hypoxia at systemic level and to mitochondrial damage at cellular level. In fact, cytotoxic effects of exogenous CO involve cytochrome *c* oxidase inhibition and defects on mitochondrial metabolism and energy production. This has been demonstrated in isolated mitochondria from human muscle by the direct analysis of the activity of respiratory chain complexes [25]. Likewise in HEK293 cells, it was also demonstrated that HO-1 overexpression and endogenous CO production also moderately decreased cellular respiration along with partial inhibition of cytochrome *c* oxidase [26]. In fact, CO binds to cytochrome *c* oxidase and slows down the rate of electron transport, leading to electrons accumulation, in particular at complex III. Thus, the lifetime of the ubisemiquinone state of coenzyme Q is prolonged, increasing the propensity to reduce O_2 into superoxide (O_2^-), which is enzymatically converted to other ROS, in particular hydrogen peroxide (H_2O_2) that can then act as signaling molecules [21,27]. Likewise, CO-mediated inhibition of cytochrome *c* oxidase can be partial or transitory, which enables low levels of ROS production without further damaging mitochondria [28] (Fig. 1).

Zuckerbraun and colleagues have demonstrated that CO generates mitochondrial ROS and promotes anti-inflammatory effects in macrophages [27]. Low concentrations of exogenous CO partially inhibited cytochrome *c* activity, generating mitochondrial ROS, which in turn limited LPS-induced inflammation by promoting p38 MAPK phosphorylation and decreasing TNF- α levels. The presence of antimycin A or in respiration deficiency ρ^0 cells CO's anti-inflammatory effect is reverted, indicating its dependency on mitochondrial ROS signaling [27]. Likewise, CO is anti-inflammatory in macrophages *via* PPAR β/γ SUMOlation and p38 activation, whose upstream effectors are mitochondrial ROS. CO-induced generation of mitochondrial ROS depends on a mild uncoupling effect *via* targeting the uncoupling protein 2 (UCP2), since whenever Ucp2 gene is knocked out, CO anti-inflammatory capacity is lost along with a decrease on mitochondrial ROS production [29]. In airway smooth muscle cells (ASMC), CO modulates cell proliferation by generating mitochondrial ROS [30]. Finally, in primary culture of cerebellar neurons, CO gas prevented apoptosis mediated by excitotoxicity and by oxidative stress in a ROS dependent manner [31]. Co-treatment with the anti-oxidant butyl-hydroxytoluene and CO reverted the anti-apoptotic role of this gasotransmitter [31]. In summary, low concentrations of exogenous CO or endogenous CO target mitochondria, generating low amounts of ROS, which act as signaling molecules (Fig. 1). Nevertheless, one cannot disregard that high CO levels inhibit mitochondrial respiratory chain and generate toxic amounts of ROS and oxidative stress. Subcellular localization of CO can also regulate ROS production, for instance Taillé and colleagues have demonstrated that CORM-2 treatment inhibited the activity of NAD(P)H oxidase cytochrome b_{558} , which decreases plasmatic membrane ROS production, while there is an increase on mitochondrial ROS generation by targeting cytochrome *c* oxidase [30]. Therefore, exogenous CO

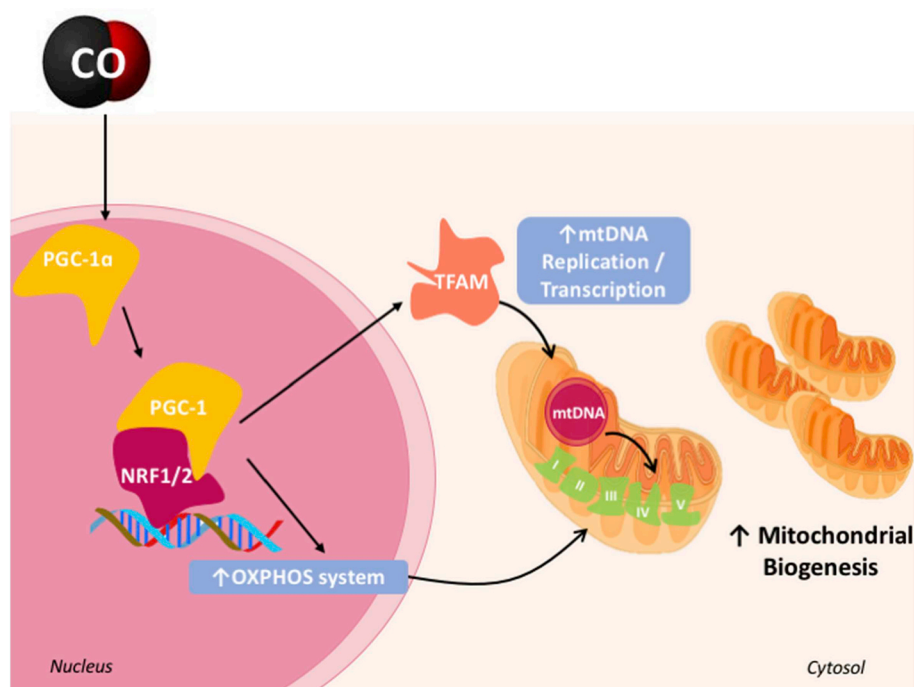


Fig. 2. CO stimulates mitochondrial biogenesis. CO exposure upregulates the expression of peroxisome proliferator-activated receptor gamma co-activator-1 α (PGC-1 α) and the nuclear respiratory factor-1 and 2 (Nrf-1/2), which regulate the expression of nuclear encoding mitochondrial proteins. PGC-1 α interaction with Nrf-1/2 also coordinates the upregulation of the mitochondrial transcription factor (TFAM) that modulates transcription of mitochondrial DNA. These factors are all involved in CO-induced mitochondrial biogenesis.

(delivered by CORMs) and endogenously produced CO may present different subcellular localizations, with potential different effects on ROS generation and signaling.

5. CO stimulates mitochondrial biogenesis

The consequence of CO targeting mitochondria is not limited to ROS production, since this gasotransmitter also activates mitochondrial biogenesis (Fig. 2). CO has been demonstrated to promote cytoprotection *via* activation of mitochondrial biogenesis in several different cellular models with distinct damage triggering factors. In 2007, it was found by Suliman and colleagues that CO stimulates mitochondrial biogenesis in cardiomyocytes using *in vivo* and cell culture models [32,33]. Transient elevations of intracellular CO levels increased number of mitochondrial DNA and the protein content of complexes I–V. Likewise, CO upregulated the expression of nuclear respiratory factor-1 and -2 (Nrf-1, Nrf-2), peroxisome proliferator-activated receptor gamma co-activator-1 α (PGC-1 α) and mitochondrial transcription factor (TFAM), which are key transcription factors controlling mitochondrial biogenesis. Moreover, the CO-induced mitochondrial biogenesis was dependent on hydrogen peroxide generation and independent on nitric oxide synthase activation [32]. Still, CO stimulation of mitochondrial biogenesis was cardioprotective by limiting doxorubicin-induced apoptosis [33]. Likewise, in a mice sepsis model induced by peritonitis, CORM-3 treatment improved cardiac mitochondrial function *via* stimulation of mitochondrial biogenesis in a PGC-1 α and ROS dependent manner [34]. Interestingly, HO-1 overexpression and CO production activated the mitochondrial biogenesis Nrf2 transcription factor, along with upregulation of the anti-inflammatory proteins IL-10 and the IL-1 receptor antagonist (IL-1Ra) [35]. These data couples mitochondrial biogenesis with the CO anti-inflammatory role in LPS-induced inflammation cellular model and in mice challenged with *Escherichia coli* sepsis [35]. Furthermore, activation of mitochondrial biogenesis by HO-1 rescued mice from lethal *Staphylococcus aureus* sepsis, *via* redox-regulated NF-E2-related factor-2 signaling [36]. In another model, HO-1 activity, along with CO production, protects macrophages against endoplasmic reticulum stress *via* mitochondrial biogenesis and in a protein kinase RNA-like endoplasmic reticulum kinase (PERK)-dependent manner [37]. The naturally

occurring flavonoid Quercetin presents several beneficial biological effects including promotion of mitochondrial biogenesis in a HO-1/CO axis dependent manner in HepG2 cells [38]. Finally, CO gas protected astrocytes against apoptosis by increasing mitochondrial population in a Bcl-2 dependent manner [28]. Likewise, Kim and colleagues demonstrated that CORM-2 activation of L-type Ca²⁺ channel mediates PGC-1 α /ERR α axis, which leads to mitochondrial biogenesis and maintenance of astrocyte function in astrocytes [39]. The same authors have recently demonstrated that CORM-2 increases HIF-1 α stabilization, which is necessary for ERR α -mediated mitochondrial biogenesis [40]. This data can be controversial since HIF-1 α stabilization is usually associated with an increase of glucose uptake and glycolytic metabolism [41]. Semenza and colleagues have demonstrated in renal carcinoma cells that HIF-1 negatively regulates mitochondrial biogenesis by preventing C-MYC activity, which in turn reduces PGC-1 β expression and mitochondrial biogenesis [42]. This controversy can be partially explained by the different PGC-1 family members: PGC-1 α and PGC-1 β [41]. Moreover, the same researchers also shown that under hypoxia, HIF-1 modulates the expression of cytochrome *c* oxidase subunit 4 (COX4), by decreasing COX4-1 and increasing COX4-2, which promotes COX activity, oxygen consumption and ATP production [43]. Thus the same group demonstrated two different effects of HIF on glycolysis and mitochondrial metabolism. Of note, Semenza group work has been developed in cancer cell lines, while Kim's research has been done in primary cultures of astrocytes, which may also influence the potential different responses to HIF stabilization. Taken all together, in different cell types and models, CO-induced mitochondrial biogenesis is essential for its cytoprotective and anti-inflammatory roles (Fig. 2). Data are summarized in Table 1.

6. CO and mitochondrial quality control

CO-mediated mitochondrial function also implicates modulation of mitochondrial quality control. Mitochondrial quality control can be defined as the balance between mitochondrial biogenesis and elimination of dysfunctional mitochondria *via* autophagy (mitophagy) [44,45].

In hepatocytes, CO activates protein kinase R (PKR)-like endoplasmic reticulum (ER) kinase (PERK), which in turn promotes nuclear translocation of transcription factor EB (TFEB) [46]. TFEB

Table 1
CO and mitochondrial function.

Biological function: mitochondrial biogenesis			
Experimental Model	Assessment of mitochondrial biogenesis	Biological outcome of CO	Ref.
Cardiomyocytes (<i>in vivo</i> and cell culture)	Increased content of mtDNA, mitochondrial proteins (complexes I-IV) Upregulation of Nrf1, Nrf2, TFAM and PGC-1 α	Mitochondrial biogenesis in a ROS dependent manner	[32]
<i>In vivo</i> mouse model for doxorubicin-induced heart failure	Increased content of mtDNA, mitochondrial proteins Upregulation of TFAM	Cardioprotection against doxorubicin-induced apoptosis <i>via</i> mitochondrial biogenesis	[33]
In mice peritonitis-induced sepsis model	Increased content of mtDNA Upregulation of PGC-1 α	Cardioprotection in sepsis context <i>via</i> mitochondrial biogenesis Reduces mortality	[34]
In mice <i>Escherichia coli</i> -induced sepsis	Upregulation of Nrf2	Anti-inflammatory effect Upregulation of IL-10 and IL-1Ra	[35]
In mice <i>Staphylococcus aureus</i> -induced sepsis	Increased content of mtDNA, mitochondrial protein (citrate synthase) Upregulation of Nrf1, Nrf2, TFAM and PGC-1 α	Reduction of mortality Anti-inflammatory effect <i>via</i> upregulation of NF-E2-related factor-2	[36]
Macrophages	Increased content of mtDNA Upregulation of Nrf1, TFAM and PGC-1 α	Cytoprotection against thapsigargin-induced endoplasmic reticulum stress	[37]
Hepatocytes (HepG2 cell line)	Increased content of mtDNA, mitochondrial proteins (complex IV) Upregulation of Nrf1, TFAM and PGC-1 α	Mitochondrial biogenesis promoted by Quercetin <i>via</i> CO production	[38]
Primary culture of mouse astrocytes	Increased content of mtDNA	Cytoprotection against oxidative stress-induced apoptosis	[28]
Primary culture of mouse astrocytes	Increased content of mitochondrial proteins (cytochrome c and cytochrome c oxidase) Upregulation of PGC-1 α	Cytoprotection/maintenance of astrocytic function	[39]
Mouse ischemic brain injury model	Upregulation of PGC-1 α	Improvement of astrocytic mitochondrial function	[40]
Biological function: mitochondrial metabolism			
Experimental Model	Effects and Mechanisms of CO	Biological outcome of CO	Ref.
Diabetic kidney	Upregulation of carnitine, citrate, deoxynucleotide, dicarboxylate and ADP/ATP carriers Increase of cytochrome c oxidase activity	Improvement of mitochondrial metabolism Reversion of diabetes-induced lower mitochondrial transport carriers	[49]
Isolated mouse liver mitochondria	Inhibition of mitochondria membrane depolarization induced by oxidative stress Increased activity of cytochrome c oxidase	Anti-apoptotic effect	[50]
Primary culture of astrocytes Isolated mouse cortex non-synaptic mitochondria	Increased specific activity of cytochrome c oxidase and O ₂ consumption Increased content of mtDNA Bcl-2 dependent mechanism	Oxidative metabolism improvement Increased ATP production	[28]
Primary culture of astrocytes Isolated rat cortex non-synaptic mitochondria	Inhibition of mitochondria membrane depolarization induced by oxidative stress ROS dependent mechanism Protein glutathionylation signaling	Anti-apoptotic effect	[51]
Primary culture of astrocytes and neurons	Increased ATP production Purinergic signaling	Astrocytic-neuronal communication for preventing neuronal cell death induced by oxidative stress	[53]
Microglia challenged with LPS	Increased ATP production Decreased glycolytic metabolism Maintenance of ATP production	Anti-neuroinflammatory effect	[54]
Human teratocarcinoma NT2 cell line	Enhanced mitochondrial population Higher expression of pyruvate and lactate dehydrogenase Higher levels of tricarboxylic acid cycle metabolites	Improvement of neuronal differentiation	[58]
Embryonic stem cell differentiation into cardiomyocytes	Improvement of mitochondrial function Increased mtDNA	Differentiation and maturation of cardiomyocytes	[59]
Human prostate cancer cells	Shifts from glycolytic into oxidative metabolism	Anti-Warburg effect Sensitizes cancer cells to respond to chemotherapy by cell metabolism regulation Promotes apoptosis	[60]
Biological function: mitochondrial uncoupling & CO			
Experimental Model	Effects and Mechanisms of CO	Biological outcome of CO	Ref.
Isolated rat renal mitochondria (<i>in vivo</i> treatment)	Decreased levels of state 3 respiration Decreased respiratory control index	Uncoupling mitochondrial respiration	[65]
Isolated rat heart mitochondria	Increased levels of state 2 respiration Modulation by adenine nucleotide translocator (ANT) and uncoupling proteins (UCP)	Uncoupling mitochondrial respiration	[66]
Isolated rat heart mitochondria	Decreased hydrogen peroxide production at complex I level Dependent on phosphate carrier and dicarboxylate carrier	Uncoupling mitochondrial respiration	[67]
Human endothelial EA.hy926 cells	Increased oxygen consumption Increased proton leak Increased state 2 respiration	Uncoupling mitochondrial respiration	[68]

(continued on next page)

Table 1 (continued)

Microglia challenged with LPS	Higher levels of oxygen consumption and ATP generation Dependent on mild uncoupling and ROS production	Mild uncoupling effect Anti-neuroinflammatory effect	[54]
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translocation is involved in lysosomal and mitochondrial biogenesis. In TFEB deficient cells, CO failed to promote Parkin translocation into mitochondria and to induce mitophagy. Likewise, knockdown expression of TFEB also decrease expression of lysosomal genes (Lamp1, cathepsin B and TPP1), along with a decrease on mitochondrial DNA, mitochondrial biogenesis markers (PGC-1 α , Nrf1 and TFAM) and mitochondrial proteins, such as COX II, COX IV and cytochrome c [46]. In summary, TFEB emerges as a key regulator of CO-dependent cytoprotection in hepatocytes, being associated with modulation of mitophagy and mitochondrial biogenesis [46].

The term mitochondrial quality control can be also applied for the maintenance of mitochondrial function *via* regulation of fusion/fission processes, which promote dynamic changes in mitochondrial morphology in response to environment, nutritional conditions and potential stress. In fact, CO modulation of mitochondrial quality control also involves fusion and fission processes. Actually, increased expression of HO-1, caused by hemin exposure, protects astroglial C6 cell line against manganese (Mn²⁺) induced oxidative stress and cell death *via* regulation of mitochondrial quality control [47]. Hemin treatment limited mitochondrial fragmentation and mitophagy and improved mitochondrial interconnectivity, along with a decrease on hydrogen peroxide and anion superoxide levels [47]. Nevertheless, it is not clear which are the downstream pathways related to mitochondrial fusion/fission and mitophagy, and which are the involved HO-1 products. In addition, Hull and colleagues have described how mice overexpressing HO-1 are protected against DOX-induced cardiotoxicity *via* modulation of mitochondrial quality control [48]. Overexpression of HO-1 led to mitochondrial biogenesis activation assessed by increased expression of TFAM, PGC-1 α and Nrf1, and presence of mitochondrial DNA. Moreover, HO-1 overexpression also decreased fission and increased fusion levels, measured by morphology and expression of mitochondrial fission 1 (Fis1) and mitofusin 1 and 2 (Mfn1 and Mfn2). Finally, HO-1 improved basal levels of mitophagy immediately after DOX treatment, while at 14 days later HO-1 prevented mitophagy progression since it is much higher at WT animals [48].

In summary, a tight-controlled crosstalk between the mitochondrial processes: biogenesis, fusion, fission and mitophagy is necessary to promote cellular homeostasis. The exact role of CO in this tight control needs further clarification, in particular how CO regulates mitophagy, which are the molecular players and its crosstalk to biogenesis and fission/fusion processes.

7. CO modulation of mitochondrial metabolism

The direct effect of CO on mitochondrial metabolism has also been described, namely the activity of mitochondrial respiratory chain complexes, oxygen consumption or mitochondrial membrane permeabilization.

It is well established that in diabetes, the kidney presents lower levels of mitochondrial transport carriers. This effect is reverted by CO exposure. Overexpression of HO-1 or its up-regulation by treatment with cobalt protoporphyrin (CoPP) led to a robust increase in carnitine, citrate, deoxynucleotide, dicarboxylate and ADP/ATP carriers, along with an increase on cytochrome c oxidase activity [49], suggesting an improvement on mitochondrial metabolism. In isolated liver mitochondria, low concentrations of CO gas (CO-saturated PBS solutions) improved ADP/ATP translocase activity and inhibited mitochondrial membrane permeabilization (MMP), which is a key step in apoptosis control. CO protection against MMP was assessed by

inhibition of mitochondrial swelling, mitochondrial depolarization, inner membrane permeabilization and cytochrome c release, as well as by modulation of cytochrome c oxidase activity [50]. CO treatment limited MMP in a ROS dependent manner, since whenever ROS are scavenged by β -carotene CO did not inhibit MMP. In addition, CO transiently decreased specific activity of cytochrome c oxidase during the first 10 min of exposition and at 30 min there was an increase of its activity [50]. This CO-induced two-step response in cytochrome c activity was also observed in non-synaptic mitochondria isolated from rat cortex [28]. In this study, CO prevented astrocytic cell death by reinforcing mitochondrial metabolism, namely CO increased ATP production and oxygen consumption. CO pushed the glycolysis/oxidative phosphorylation (OXPHOS) balance towards OXPHOS and increased specific activity of cytochrome c oxidase, along with an enhancement of mitochondrial population [28]. Likewise, in astrocytic primary culture and enriched non-synaptic mitochondria from rat cortex, low concentrations of CO gas also prevented MMP. In fact, CO improved ADP/ATP mitochondrial inner membrane translocation and maintained mitochondrial metabolism in a ROS dependent manner *via* mitochondrial protein glutathionylation signaling [51]. Therefore, one may speculate that CO generates small amounts of ROS by partially and transitorily inhibiting cytochrome c oxidase. These ROS act as signaling molecules that, in turn, improve mitochondrial metabolism and function [28,50,51]; including cytochrome c oxidase activity [28,50]. Nevertheless, the molecular mechanisms underlying this dual effect of CO on cytochrome c oxidase activity is not yet clearly disclosed. The hypothesis about CO modulation of mitochondrial metabolism in a ROS dependent manner is deeper discussed in Refs. [10,22,52]. Finally, in the brain context, CO-increased astrocytic production of ATP and purinergic signaling is also important for glia cell-neuron communication and the maintenance of neuronal function and survival in response to oxidative stress [53].

Inflammatory response in macrophages and microglia is accompanied by cellular metabolic changes, favoring glycolysis over oxidative phosphorylation. In microglia, CO presents anti-neuroinflammatory properties, which are associated with CO-induced cell metabolism modulation [54]. Actually, low concentrations of CORM-401 decreased glycolytic metabolism, maintaining ATP production and mitochondrial respiration in microglia challenged with lipopolysaccharide (LPS) [54]. The CO-increased respiration levels is associated with an uncoupling activity. While higher concentrations of CORM-401 or prolonged periods of CORM-401 exposure also inhibited mitochondrial respiration and decreased ATP production, suggesting a biphasic effect of CO on microglial metabolism.

During neuronal differentiation there is a metabolic switch: from mainly glycolytic metabolism (cell proliferation phase) into oxidative metabolism and increased mitochondrial population (cell differentiation phase) [55–57]. Likewise, CORM-A1 supplementation of differentiation medium containing retinoic acid improved neuronal differentiation of human embryonic teratocarcinoma NT2 cell line by favoring mitochondrial metabolism [58]. CORM-A1 supplementation increased neuronal yield along with enhanced mitochondrial population, higher expression of pyruvate and lactate dehydrogenase and increased the percentage of ¹³C incorporation from ¹³C-glucose into the tricarboxylic acid cycle metabolites. Whenever NT2 cells were differentiated under 5% of oxygen levels, which promotes glycolytic metabolism, there was a reversion on CO-improved neuronal differentiation [58]. Likewise, in another cell type, HO-1/CO axis is essential for stem cell differentiation into mature and functional cardiomyocytes in a

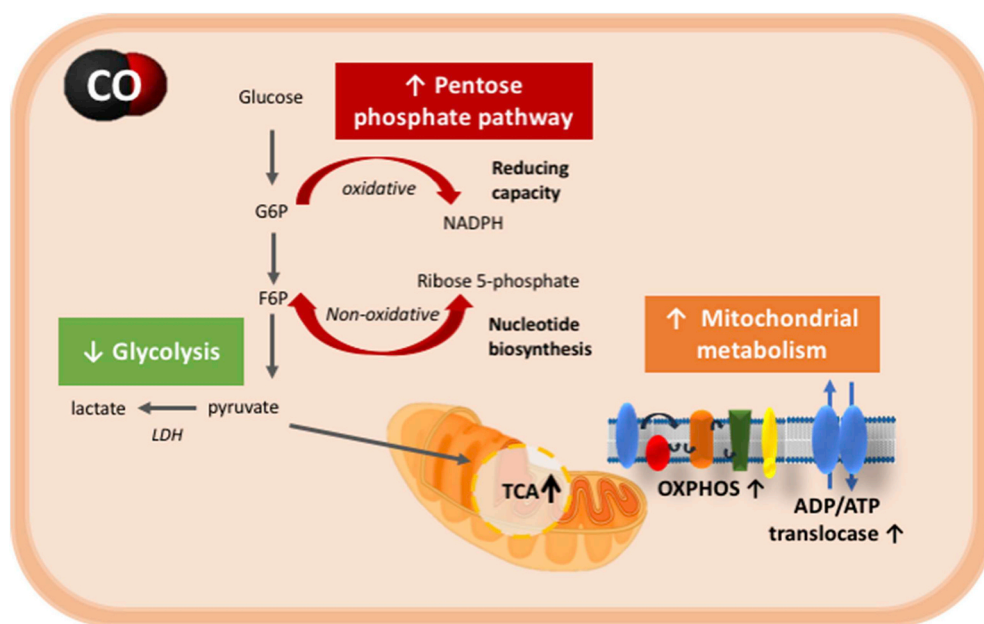


Fig. 3. Main cellular metabolic pathways modulated by CO: mitochondrial metabolism, glycolysis and pentose phosphate pathway. Low levels of CO enhance TCA, improve mitochondrial OXPHOS, increase oxygen consumption and production of ATP. Accordingly, in most of the tested models (cell type and pathophysiological conditions) CO decreases glycolysis. Finally CO modulates redox processes: ROS appear as signaling molecules for CO mode of action and one of the cytoprotective properties of CO is anti-oxidant. Thus it is not surprising that CO also stimulates PPP, a critical pathway responsible for maintaining the reducing capacity of the cell, as well as the cellular nucleotides pool.

manner that is dependent on mitochondrial biogenesis [59].

Cancer cells are known to present Warburg effect, which consists in having mainly glycolytic metabolism, even in the presence of normal levels of oxygen. In human prostate cancer cells, HO-1 is mainly present in the nucleus with low enzymatic activity and correlates with worse clinical outcomes. Overexpression of HO-1 or exogenous administration of CO improved prostate cancer cell response to chemotherapy increasing apoptosis levels and mitotic catastrophe. Likewise, CO limited growth of human prostate cancer xenografts. This sensitization is consequence of an increased mitochondrial metabolism induced by CO. CO increased oxygen consumption and pushed cell metabolism towards mitochondria, leading to cell metabolic exhaustion [60] and an anti-Warburg effect mode of action. Furthermore, CO act in a ROS dependent manner, since whenever ROS are depleted using a cocktail of pegylated catalase and superoxide dismutase, CO loses its effect on mitochondrial metabolism [60].

In summary, CO emerges as a molecule able to reinforce oxidative mitochondrial metabolism in several different models, kidney brain, heart, cancer cells leading to homeostasis, cell survival, cell differentiation and anti-tumorigenesis effects (Fig. 3, Table 1).

8. CO decreases glycolysis

Along and in accordance with CO-mediated effect on oxidative metabolism, this gasotransmitter also decreases glycolysis in three distinct models (Fig. 3). In a model of ischemic myocardium, CO gas inhalation during 200 min and reaching 5% of COHb levels, improved metabolic conditions by decreasing glycolysis. Levels of lactate in the myocardium were 50% lower in pig pre-conditioned with CO gas than in control pigs. Accordingly, CO-treated animals presented higher glucose levels in myocardium than control animals [61]. In a research work about breast cancer cell adhesion and transmigration across lung endothelial cells, CORM-401 was found to decrease transmigration by cell metabolism modulation [62]. CORM-401 differently controls cell metabolism: in lung endothelial cells CO decreased glycolytic metabolism (assessed by extracellular acidification rate) and activated mitochondrial respiration (assessed via oxygen consumption rate). While in breast cancer cells CO decreased both mitochondrial respiration and glycolysis [62]. Cell adhesion and transmigration are highly bioenergetic demanding cellular processes, being cell metabolism a promising target for therapy. Finally, in EA.hy926 endothelial cell line CORM-401 treatment also shifts metabolism from glycolysis to oxidative

phosphorylation for ATP production. This effect is accompanied by a slight mitochondrial depolarization and mild uncoupling effect; which in turn activates mitochondrial respiration via mitochondrial Ca^{2+} signaling [63]. Moreover, CORM-401 accelerates mitochondrial NADH consumption and increases mitochondrial Ca^{2+} content, which is in accordance with the fact that Ca^{2+} activates pyruvate dehydrogenase (and other hydrogenases) to feed NADH into electron transport chain, in particular complexes I and II [63]. Nevertheless, in obese mice fed with high fat diet, oral administration of CORM-401 reduces body weight and accelerates glucose metabolism in adipocytes by improving glycolysis [64]. Therefore CO action on metabolism might depend on tissue type and on pathophysiological conditions. One can speculate that CO pushes the biological system to an homeostatic status. For instance, in tumor context and cancer cell lines, CO accelerates oxidative phosphorylation pushing glycolysis down [62,63], in obesity models CO improves glucose homeostasis [64] or in ischemic conditions CO optimizes the use of oxygen [61]. Data and references are summarized in Table 2. Finally, it can also be considered that CO-induced mitochondrial uncoupling might increase mitochondrial respiration as a compensatory effect, and this is further discussed and explored in the next section.

9. CO promotes a mild uncoupling effect

In apparent controversy, hemin-activation of HO-1 or treatment with CORMs (CORM-2, CORM-3 and CORM-A1) decreased state 3 oxygen consumption in isolated mitochondria [65]. State 3 represents oxygen consumption for oxidative phosphorylation that is associated with ATP production. Other studies using isolated mitochondria also point to a mild uncoupling effect of CO. In isolated mitochondria from rat hearts, low concentrations of CORM-3 promoted an augmentation of state 2 respiration (oxygen consumption in the absence of ADP, meaning an increase on oxygen consumption uncoupled of mitochondrial potential and ATP production [66]), while CORM-3 decreases state 3 respiration. This CO-induced mild uncoupling effect is mediated by adenine nucleotide translocator (ANT) and uncoupling proteins (UCP), since their pharmacological inhibition attenuated the CO effect on state 2 respiration [66]. Guanosine 5'-diphosphate (GDP) is general inhibitor for all UCP family proteins, nevertheless UCP2 and UCP3 are both expressed in cardiac mitochondria. Furthermore, CORM-3-mediated uncoupling effect is also associated with modulation of mitochondrial ROS generation since CORM-3 decreases hydrogen peroxidase production at

Table 2
CO and glycolysis.

Biological function: Glycolysis			
Experimental Model	Effects and Mechanisms of CO	Biological outcome of CO	Ref.
<i>In vivo</i> pig model of ischemic myocardium	Lower levels of lactate and higher levels of glucose in response to ischemia	Metabolic cardioprotection in ischemic context	[61]
Lung endothelial cells Breast cancer cells	Decreased glycolytic metabolism and increased mitochondrial respiration in endothelial cells	Decreased cancer cell transmigration	[62]
EA.hy926 endothelial cell line	Decreased glycolytic and mitochondrial metabolism in cancer cells Acceleration of NADH consumption Increased mitochondrial calcium levels, activating mitochondrial respiration	Modulation of cell metabolism in cancer and endothelial cells Metabolic shift from glycolytic to oxidative	[63]
<i>In vivo</i> obesity mouse model High fat diet Adipocytes	Dependent on mild mitochondrial uncoupling effect Decreased mitochondrial ATP production Increased glycolysis Dependent on mitochondrial uncoupling effect	Metabolic shift from oxidative to glycolytic	[64]

complex I level. In addition, this uncoupling effect of CORM-3 in isolated mitochondria was also found to be dependent on phosphate carrier [67]. Inhibition of phosphate carrier with N-ethylmaleimide reverted CORM-3 effect on reducing mitochondrial potential. Likewise, higher levels of mitochondrial phosphate were found following treatment with CORM-3, along with mitochondrial swelling [67]. One could speculate that since these effects were found in isolated mitochondria, a cell-free system, could not be physiologically representative. Nevertheless, in intact in human endothelial EA.hy926 cells, CORM-401 increased oxygen consumption rate simultaneously with an increase of proton leak and decrease of mitochondrial reserve capacity, indicating an uncoupling effect. This effect is dependent on the activation of mitochondrial large-conductance calcium-regulated potassium channel (mitoBK_{Ca}) [68]. Furthermore, low concentrations of CORM-401 are anti-inflammatory *via* cell metabolism modulation in microglia [54]. In this system, LPS-triggered inflammation promoted lower levels of oxygen consumption, mitochondrial function and cellular ATP generation, while CORM-401 reverted all these effects. Still, CORM-401 promoted a mild uncoupling outcome without affecting cellular ATP concentration, but decreasing mitochondrial membrane potential and generation of ROS [54]. In conclusion, one can speculate that CO-mediated mild and transitory uncoupling (Fig. 4) promotes mitochondrial homeostasis and anti-oxidant effect, which may in turn maintain mitochondrial function and cellular metabolic status. In summary, CO-induced mild uncoupling effect improves mitochondrial function *via* a compensatory mechanism and ROS signaling, these data are summarized in Table 1.

10. CO stimulates pentose phosphate pathway (PPP)

Mitochondria are the main source of cellular ROS generation under physiological and pathological conditions. Moreover, reduced glutathione (GSH) is the major anti-oxidant component of the cell, and its

recycling depends on NADPH as source of electrons. NADPH is mostly generated at the pentose phosphate pathway (PPP). Thus, it is not surprising that CO may also direct or indirectly modulate PPP. In fact, the first evidence of CO regulation of PPP was demonstrated in 2014 by two different groups and in different biological systems. CO-treatment of whole blood increased levels of reduced GSH in the cytosol of red blood cells. CO promoted stimulation of PPP, and whenever PPP was inhibited by 2-deoxyglucose, reduced GSH levels are partially decreased [69]. Nevertheless, other mechanisms in red blood cells were also found to be involved in CO-increased GSH levels. CO promoted deglutathionylation of hemoglobin at Cys93 and Cys112, which is a significant source of reduced GSH. Finally, CO also increased glutathione reductase activity without promoting glycolysis [69]. The axis HO-1/CO protects cancer cells against oxidative stress, in particular in leukemia. Yamamoto and colleagues have found that CO promotes a shift from glycolysis to PPP, supplying NADPH to reduce glutathione and limit oxidative stress [70]. CO inhibited the heme containing enzyme cystathionine β-synthase (CBS) that, in turn, reduced methylation of PFKFB3, which decreases its activity and fructose 2,6-biphosphate (F-2,6-BP) production. Since F-2,6-BP is an allosteric activator of the rate limiting enzyme of glycolysis, phosphofructokinase-1, there was an acceleration of PPP with the consequent increased cellular resistance to oxidative damage by presenting higher levels of reduced glutathione [70].

In the human endothelial cell line EA.hy926, CORM-401 promoted NO production and triggered Ca²⁺ release from the endoplasmic reticulum, increasing Ca²⁺ intracellular levels. Inhibition of NO synthase by L-NAME limited NO production and prevents Ca²⁺ signaling, indicating that CO increase of Ca²⁺ is dependent on NO generation. Furthermore, CO-induced NO production was prevented by 6-aminonicotinamide, which is an inhibitor of PPP. PPP is the major enzymatic source of NADPH, a molecule required for NO synthase [71]. Therefore, reinforcement of PPP by CORM-401 is also important for NADPH

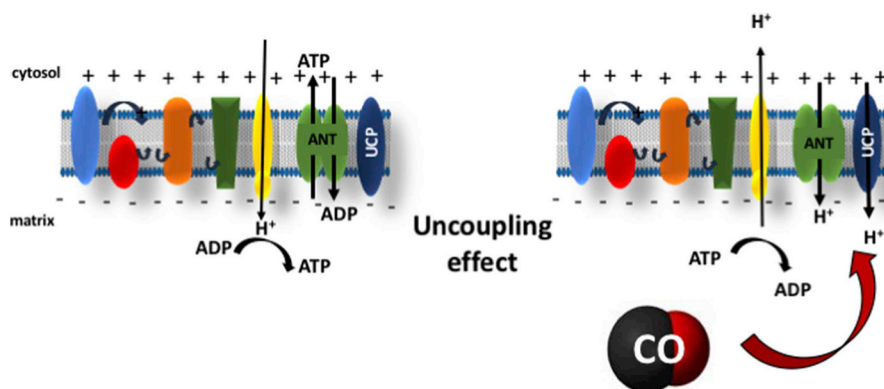


Fig. 4. CO promotes mild uncoupling effect in mitochondria. CO-induced mild uncoupling is dependent on ANT and UCP and this event is associated with anti-inflammatory role of CO. It is speculated that a small leakage of protons into mitochondrial matrix may generate ROS and accelerate respiratory chain for maintaining mitochondrial membrane potential, both events shall improve mitochondrial function and metabolism.

Table 3
CO and pentose phosphate pathway.

Biological function: Pentose Phosphate Pathway			
Experimental Model	Effects and Mechanisms of CO	Biological outcome of CO	Ref.
Red blood cells	Stimulation of pentose phosphate pathway (PPP) 2-deoxyglucose reverts PPP stimulation	Increased reduced glutathione levels	[69]
Leukemia cancer cells	Limitation of glycolysis favoring PPP Dependent on inhibition of cystathionine β -synthase and on inhibition of phosphofructokinase-1 (rate limiting glycolytic enzyme)	Increased cancer cell resistance against oxidative stress	[70]
EA.hy926 endothelial cell line	Promotion of Ca^{2+} release from the endoplasmic reticulum Acceleration of PPP and in consequence NADPH production	Higher reduced glutathione levels Increased NO production	[71]
Human neuroblastoma SH-SY5Y cell line	Increased expression and activity of glucose 6-phosphate dehydrogenase Higher levels of reduced glutathione	Improvement of neuronal differentiation	[74]

production, required in nitric oxide (NO) synthesis in endothelial cells [71]. It is well established that during neuronal differentiation, there is a switch from glycolytic to oxidative mitochondrial metabolism [72,73]. Thus, neurons are mostly oxidative and must present an effective anti-oxidant defense, being GSH the major cellular non-enzymatic anti-oxidant factor. In a recent paper, Almeida and colleagues used human neuroblastoma SH-SY5Y cells as models for neuronal differentiation and exposed them to CORM-A1. CORM-A1 improved neuronal differentiation by increasing the final yield of neurons, by reinforcing PPP activity and increasing cellular GSH/GSSG ratio [74]. CO treatment enhanced protein expression and activity of glucose 6-phosphate dehydrogenase (G6PD), which is the rate-limiting enzyme of PPP. Whenever G6PD was knocked down there was a reversion of CO-induced improvement of neuronal differentiation, while pharmacological inhibition of GSH synthesis did not change neuronal differentiation. Likewise, CO increased the reduced and oxidized glutathione (GSH/GSSG) without changing total pool of GSH, which is in accordance with CO-induced activation of PPP [74]. In conclusion, CO emerges as a modulator of PPP, but much data is still needed for deeply and precisely describe the underlying molecular mechanisms (Fig. 3, Table 3).

11. CO, obesity and diabetes diseases

Modulation of metabolism by CO is not limited to cellular metabolism, but this gasotransmitter also plays a role in metabolic diseases (diabetes and obesity), which is discussed herein.

In intact mouse islet, exogenous CO gas improved glucose-stimulated insulin secretion in a cGMP signaling dependent manner [75]. Likewise, CO stimulated the activities of the lysosomal/vacuolar enzymes: acid glucan-1,4- α -glucosidase and acid α -glucosidase (acid α -glucoside hydrolases), which are involved in glycogen hydrolyzation [75].

In an obesity mouse model based on high fat diet, chronic and preventive intraperitoneal (IP) administration of CORM-A1 reverted obesity by decreasing body and epididymal fat weight and reduced fasted blood glucose and insulin in a food intake independent manner [76]. Furthermore, in epididymal fat tissue, CORM-A1 treatment increased expression levels of uncoupling protein-1 (UCP-1), PGC-1 α and Nrf-1, which are considered markers of mitochondrial heat production and mitochondrial biogenesis. These facts are in accordance with the higher levels of oxygen consumption and heat production of CORM-A1 treated mice [77]. Moreover, chronic treatment with CORM-A1 can reverse established dietary-induced obesity, hyperglycemia, and insulin resistance [77]. Oral administration of CORM-401 also decreased body weight and improved insulin sensitivity and glucose tolerance in high fat diet mouse model for diabetes [64]. CORM-401 reverted the increase on cellular size of epididymal white adipocytes following high fat diet and reduced macrophage infiltration in white fat tissue, decreasing the levels of pro-inflammatory factors (IL-6, IL-1 β and HO-1). In contrast to the previous works, the described underlying molecular

mechanism is based on reinforcement of glycolytic metabolism for increasing ATP intracellular levels in white adipocytes [64]. In fact, CORM-401 did not revert the reduction of Pgc-1 α mRNA expression in white adipocyte tissue. Nevertheless, in brown adipocyte tissue, CO restored the levels of Pgc-1 α and Ucp-1 mRNA [64], which might indicate an increase on mitochondrial population and/or function. In summary the effects of CO described by Braud's and Hosick's teams are similar but the underlying metabolic mechanisms in white adipocytes are different.

In the same high fat diet model of diabetes, fibroblast growth factor 21 (FGF-21) is necessary for CO gas to reduce obesity, glucose tolerance and improve insulin sensitivity following high fat diet [78]. The CO-induced increased expression of FGF-21 in liver and primary hepatocytes is dependent on mitochondrial ROS generation. In hepatocytes, CO increased protein levels of complex III and IV, as well as of Nrf-1, TFAM and PGC-1 α , which are associated with increased mitochondrial biogenesis, and accordingly enhances oxygen consumption. Likewise, CO also increased mitochondrial population in white fat tissue, which converts into beige tissue by switching cell metabolism. All these mitochondrial events are dependent on FGF-21 presence [78]. In summary, CO improves mitochondrial metabolism in hepatocytes and converts white adipocytes into beige ones, in order to prevent obesity and diabetes type 2.

In a model of metabolic syndrome, based on mice fed with high fat diet, CO (CORM-3 administration) improved cardiac function by modulation of mitochondrial population quality [79]. High fat diet for 12 weeks decreased intrinsic contractile function in left ventricle. This cardiac dysfunction was accompanied by an alteration in mitochondrial morphology and function, including: (i) an increase of mitochondrial volume and number, along with higher expression levels of the mitochondrial fusion genes Mfn2 and OPA1 and mitochondrial biogenesis gene PGC-1 α ; and (ii) a decrease of oxygen consumption and mitochondrial membrane potential [79]. These mitochondrial events were reverted by two CORM-3 IP injections. Furthermore, high fat diet does not modulate autophagy, while CORM-3 treatment with high fat diet promoted autophagy assessed by LC3I/II ratio [79]. In an apparent contradiction, Lancel and colleagues showed that CO reverted the high fat diet-induced mitochondrial biogenesis and fusion, while most of data in the literature claim the cardioprotection ability of CO is dependent on mitochondrial biogenesis.

The regulatory role of CO in obesity and diabetes is an emerging research field and more data are necessary to disclose its mechanisms and the targeted cells and tissues.

12. Conclusions

The fact that CO targets mitochondria, in particular cytochrome c oxidase, originates several consequences: (i) generation of ROS for downstream signaling, (ii) alterations in oxygen consumption implying uncoupling effects and/or improvement of mitochondrial respiration and ATP production, (iii) regulation of mitochondrial quality control,

including mitochondrial biogenesis, mitophagy and fusion/fission. Likewise, related to cell metabolism and redox response, CO also appears to modulate glycolysis and pentose phosphate pathway. Because these are vital cellular processes, endogenous CO production or exogenous CO treatment have great impact on cellular function at distinct cells and tissues, namely: liver, heart, brain, and also in metabolic diseases, such as diabetes and obesity.

Declaration of competing interest

The authors state there is no conflict of interest in the manuscript entitled “CO-mediated cytoprotection is dependent on cell metabolism modulation”.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2020.101470>.

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