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Role of Carbonic Anhydrases in Ferroptosis-resistance

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Abstract

Iron is essential for all the lives on earth but may trigger a switch toward ferroptosis, a novel form of regulated necrosis. Carbonic anhydrases (CAs) are ubiquitous enzymes from microbes to humans. The primary function of CAs is to regulate cellular pH by hydrating carbon dioxide (CO_2) to protons (H^+) and bicarbonate ions (HCO_3^-). Furthermore, CAs play roles in biosynthetic reactions, such as gluconeogenesis, lipogenesis, ureagenesis and are also associated with tumor metabolism, suggesting that CAs may be a potential target for the treatment of cancers. We have recently revealed a novel function of CA IX in ferroptosis-resistance by using human malignant mesothelioma cells. Herein, we aim to review the potential molecular association between ferroptosis and CAs, from the viewpoint of iron-metabolism, lipogenesis and signaling pathways both under physiological and pathological contexts.

Introduction

Carbonic anhydrase (CA; EC4.2.1.1) catalyzes the hydration of CO_2 to H^+ and HCO_3^- ions, which was first identified in human blood in 1933 [1]. Thus far, seven classes (α, β, γ, δ, ζ, η, θ) of CAs have been discovered and four of them (α, β, γ, and δ) have been profoundly investigated [2] by comparing the amino acid sequences in a variety of species. The biochemical traits of most CAs are characterized as zinc-dependent metalloenzymes which contain zinc ions (Zn^{2+}) in their active sites for catalysis [3]. α-CAs are present in vertebrates, bacteria, algae and cytoplasm of green plants, where they play a variety of roles in physiological reactions and vital metabolisms [4]; β-CAs has been identified predominantly in bacteria and algae, where it is indispensable for photosynthesis by modulating CO_2 uptake [5]; γ-CAs have been isolated from bacteria and archaea and reside especially in diatoms [6, 7]. In mammals, sixteen isozymes of α-CA have been identified with distinct roles, intracellular distributions and tissue-specific expression patterns [8]. Thus far, the best-known roles of α-CAs are pH regulation and CO_2 homeostasis. These functions are important for various physiological and pathological processes, including electrolyte secretion, biosynthetic reactions (*e.g.* gluconeogenesis, lipogenesis and ureagenesis), bone resorption and tumorigenesis as well [9-13].

Iron is the most abundant metal in humans and plays an essential role in all the life kingdoms [14-16]. An adult human accommodates 2.5~4 g of iron, of which 60% is present in red blood cells as hemoglobin. However, iron acts as a double-edged sword because it not only contributes to momentous biological events, encompassing oxygen transport (hemoglobin), ATP generation (cytochrome oxidases) and DNA synthesis (ribonucleotide reductases) [17] but also to oxidative stress in its excess via Fenton reaction [18], resulting in DNA damage, cell cycle arrest and even cell death (apoptosis and ferroptosis) [15, 19, 20]. Therefore,

maintenance of an appropriate local concentration of iron is essential for cell viability.

Mammals have conserved a complex but sophisticated system during the evolutionary processes, comprising iron absorption, storage and export with tight regulation. In humans, a fine balance between iron uptake and loss, based on the semi-closed system, maintains iron homeostasis. Approximately 1 mg of dietary iron intake is required to replenish the iron discharged through cell sloughing and excrement each day. Divalent metal transporter 1 (DMT1; SLC11A2) is present at the brush border membrane of duodenal luminal mucosa, transporting Fe(II) supplied from foods to cytoplasm of duodenal epithelial cells [21]. Then, cellular Fe(II) is transported across the basolateral membrane to the bloodstream of portal system via ferroportin (SLC40A1), the only known cellular iron exporter which is regulated by hepcidin [22]. Fe(III) is almost insoluble at neutral pH, stable and safer for the cells [18]. Transferrin (Tf), mostly produced by hepatocytes, is a Fe(III) carrier in the serum. Virtually all types of cells receive iron via transferrin receptor 1 (TfR1; CD71) and clathrin-mediated endocytosis of Tf-TfR1 binding complex [23] whereas some non-transferrin-bound transporters are recently reported [24]. The absorbed iron is thereafter released in the acidic environment of endosome where the Fe(III) will be reduced to Fe(II) by the ferrireductase STEAP3 [25]. Fe(II) is exported from endosome to cytosol by DMT1. In the cytosol, iron enters the labile iron pool transiently and participates in a variety of physiological processes, including heme synthesis and DNA replication. Surplus iron is stored, as Fe(III) in ferritin, a protein complex, consisting of heavy chain (FTH) and light chain (FTL).

Ferroptosis, as the name implies, is associated with Fe(II), leading to cell lethality [19, 26, 27]. Ferroptosis is characterized by dysregulation of iron metabolism, accompanied by lipid peroxidation. Cells undergoing ferroptosis are

biologically and morphologically distinct from other types of cell death, which exhibit a unique abnormality in mitochondrial morphology, displaying shrinkage and increased membrane density. Recent studies indicate that mitochondria play a pivotal role in cysteine deprivation-induced ferroptosis, leading to a series of the alterations in TCA cycle, electron transport chain and glutaminolysis [28, 29]. Ferroptosis appears to be determined by the cytosolic size of labile iron pool, which subsequently generates substantial amounts of lipid peroxides, resulting in rupture of biomembranes [30]. Ferroptosis can be specifically rescued by redox-inactive iron chelators and glutathione peroxidase 4 (GPX4) [31], a selenoenzyme that reduces phospholipid peroxides in membrane, using glutathione (GSH). GSH level is controlled by cytoplasmic cysteine level, which is under the tight regulation by cystine-glutamate antiporter (xCT) [28].

Currently, emerging evidence suggests that CA proteins play a crucial role in ferroptosis [32], due not only to the involvement in pH regulation, impacting on iron solubility and iron transporter activities [33], but also to functioning in biosynthetic reactions, including gluconeogenesis and lipogenesis with protective effects on lipid peroxidation [34]. Furthermore, our recent study indicated that inhibition of CA IX, the tumor-specific CA isoform, led to a mixture of cell death, including apoptosis and ferroptosis, accompanied by a significant increase in catalytic Fe(II) [32]. In this review, we aim to outline the potential molecular events in ferroptosis, surrounding the iron-metabolism, lipogenesis and associated signaling pathways, from the viewpoint of CAs-mediated physiological and pathological processes.

CA IX dictates tumor microenvironment

Hypoxia is a hallmark of advanced cancer and endows tumor cells with abundant

merits for proliferation and metastasis, based on aberrant activation of a heterodimeric transcription factor, hypoxia inducible factor-1 (HIF-1) [35]. *CA9*, encoding membrane-associated α-CA, is an inductively expressed gene in response to hypoxia in cancers. CA IX catalyzes the hydration of CO_2 into H^+ and HCO_3^-, which is required for tumor cells to maintain the optimal intracellular pH (pH*i*) to combat the deleterious condition caused by hypoxia [13, 36].

In addition to pH*i* regulation, extracellular acidity promoted by CA IX provides multiple merits for tumor cells. The extracellular acidosis facilitates extracellular matrix remodeling through overexpression and activation of reorganizational proteases, such as matrix metalloproteinases [37], and further may aid in evasion of apoptosis by adopting the pro-survival expressional pattern of *Bcl-2* family [38]. CA IX indeed contributes to the migration and invasion of tumor cells through enhancing MMP14-mediated collagen degradation in breast cancer cells [39]. *CA9* overexpression can increase metastatic potential through Rho-GTPase-associated epithelial-mesenchymal transition in a cervical cancer cell line [40, 41].

CA IX regulates iron metabolism and ferroptosis in tumor cells

Iron excess in tumor cells is tightly regulated in response to acidic extracellular pH (pH_e). High levels of catalytic Fe(II) in tumor cells are efficiently used for multiple cellular activities to ensure their rapid division and proliferation [42, 43]. DMT1 activity is precisely regulated by pH variation. An enhanced activity of DMT1 has been reported at an acidic pH_e in *Xenopus* oocytes [44]. The substantial H^+ coupling in DMT1 serves to increase an affinity to Fe(II) binding and promotes their simultaneous translocation. However, DMT1 does not behave like a typical ion-coupled transporter at higher pH_e. Furthermore, the Fe(II) transport appears not

to be related to H^+ influx at pH_e 7.4. Tandy S *et al.* have concluded that uptake of Fe(II) ascorbate across the apical membrane by Caco-2 human colon cancer cell line was significantly improved at both apical pH 6.5 and 5.5 in comparison to pH 7.5. Interestingly, when pH_e reached 6.5, Fe(II) ascorbate induced significant intracellular acidification in comparison to pH_e at 7.5. This response was abolished upon iron removal [45]. On the other hand, the association between pH and TfR1 activity appears weak because the affinity of TfR1 to di-ferric transferrin is lower in the acidic pH_e [46].

Our recent study revealed that CA IX plays an important role in iron metabolism in ACC-Meso-1 human malignant mesothelioma (MM) cells under hypoxia. Pharmacological inhibition (S4 and U104) or RNA*i*-mediated knockdown of *CA9* significantly decreased viability and migration of MM cells [32]. In addition, we found an increase in catalytic Fe(II) and lipid peroxidation, which were accompanied by overexpression of TfR1 and IRP1/2 and continuously diminishing ferritin in MM cells after CA IX inhibition. S4-induced cell death was partially rescued not only by apoptosis inhibitor (Z-VAD-FMK, 50 μM) but also by a redox-inactive iron chelator, deferoxamine (DFO; 0.5 μM) and a ferroptosis inhibitor, Fer-1 (3 μM). Intriguingly, the expressional patterns of TfR1 and ferritin were similar to that of erastin-induced ferroptosis [30] in the same MM cells. Indeed, ferroptotic cells displayed higher level of TfR1 and downregulation of ferritin, which was consistent with the elevated level of IRP1/2. In our previous study, CA IX expression showed an inverse correlation with TfR1 in MM cells within 48 h in hypoxic MM cells *in vitro* whereas a dramatically increased level of TfR1 occurred in MM cells cultured in hypoxia, in response to CA IX blocking [32].

Although the underlying mechanism and connection between CA IX and TfR1 remain to be elucidated, emerging evidence suggests that CA IX may present a

chaperone-like function as heat shock proteins (HSPs) in renal carcinoma cells, predicting prognosis [47]. The inhibitory effect of HSPs on endocytosis and recycling of Tf has accompanied a decrease in iron concentration [48, 49]. It has been proposed that the inhibition by HSPs of TfR1-mediated Tf endocytosis is associated with enhancement of actin polymerization and stabilization of the cortical actin cytoskeleton, which are characterized in CA9-enriched tumor cells for migration (**Figure 1**) [39, 50]. This scenario is also supported by other group, which has concluded that inhibition of HSPB1 is able to sensitize erastin-induced ferroptosis whereas overexpression of HSPB1 confers resistance to this lethality [51]. Of note, CA IX has a similar property to HSPs in cytoskeletal networks of tumor cells by regulating cytosolic filaments [39]. CA IX-associated alkalization of pH*i* is also able to produce the free-barbed actin ends, supporting actin polymerization and invadopodia elongation [52]. Moreover, CA IX has been shown to co-localize with F-actin, cofilin and α2/β1 integrin, resembling lamellipodia. Taken together, we here hypothesize that CA IX holds capability of suppressing endocytic vesicles and Tf/TfR1 mediated iron-uptake by maintaining a firm actin cytoskeleton (**Figure 1**). Interestingly, a shedding form of CA9 ectodomain has been reported, which originated from the surface of renal and non-renal tumors during progression [52]. In particular, this released form of CA9 ectodomain has been also found enriched in endocytic vesicles [53], suggesting that it may affect internalized Tf/TfR1 mediated iron influx.

Apart from the effects on TfR1, CA IX blocking showed a significant decrease in ferritin level [32]. The loss of ferritin mediated by NCOA4-associated autophagy in ferroptotic cells was discovered, which was coined as ferritinophagy [54]. This unsafe iron storage enlarges the size of labile iron pool and triggers lipid peroxidation. We observed ferritin overexpression in iron-rich MM cells,

accompanied by *CA9* overexpression in hypoxia. Here, either CA IX inhibition either by selective inhibitors or *si*RNA-mediated knockdown induced a dramatic decrease in ferritin level. In addition to ferritinophagy which indeed occurred in *CA9*-repressed MM cells, there was an increased level of lipidated LC3B and LAMP1 [32]. We speculated that intracellular acidosis is another determinant involved in ferritin degradation. It should be noted that the structures of ferritin and apoferritin are pH-dependent. Ferritin and apoferritin nanoparticles adsorbed on a mica substrate displayed a variation in their size when the pH of the surrounding medium is altered [55, 56]. Decreasing pH in solution resulted in smaller size of the nanoparticles whereas a successive increase in pH again enlarged the particle size. The pH-dependent alteration in size of these nanoparticles may be related to the dis- and re-assembling of the protein shell of ferritin. These results and other evidence suggest that pH*i* neutralization mediated by CA IX has a crucial role in stabilization of ferritin in hypoxic MM cells to avoid the toxicity resulting from labile iron. Of note, the role of mitochondrial ferritin (FTMT) is mostly unknown, especially whether it is engaged in the mitophagy/autophagy, induced by CA IX inhibition. Further studies are necessary on FTMT because its overexpression confers resistance to erastin-induced ferroptosis in HT-1080 cells [57].

CA IX has a capacity to maintain an appropriate neutral pH*i* in tumor cells, which depend on glycolysis for assimilating energy and simultaneously producing a large amount of proton and lactate [58]. Blocking CA IX activity will induce intracellular acidosis and reduce cell viability. Indeed, a shift of cell death mode, from apoptosis to necrosis, has been observed in colorectal cancer cells, exposed to low pH microenvironment [59]. This necrosis is characterized by mitochondrial depolarization, ATP depletion and superoxide accumulation, suggesting that

ferroptosis might be involved. In addition to the common features of mitochondrial morphology, as shrinkage and increased membrane density, we also observed the mitochondrial fragmentation in response to CA IX inhibition in MM cells [32]. These findings are consistent with the observation from other groups that ferroptotic cells display significant mitochondrial fission with overexpression of *dynamin-related protein-1* (*DRP1*) [32, 60]. Therefore, neutral pH*i* regulated by CA IX plays an important role in maintaining mitochondrial activity and integrity, which preserve tumor cells against ferroptosis.

It is noteworthy that acidic condition inside stomach and duodenum is also involved in solubilizing Fe(III) and reducing Fe(III) to the Fe(II) in the presence of ascorbic acid which acts as an enhancer of iron-uptake [61, 62]. The effect of ascorbic acid on iron solubility shows a pH-dependence. Notably, although CA IX has been considered as a hypoxic marker in solid malignancies, it is also expressed in the healthy gastrointestinal mucosa [63, 64] overwhelming the other normal tissues, suggesting that CA IX-mediated pH regulation is required for the iron uptake in the duodenal mucosa.

In addition to CA IX, CA XII has been also regarded as an important drug target because it has been observed overexpressed in a variety of cancers [65, 66]. Several groups have revealed that a combined silencing of *CA9* and *CA12* generates a synergistic effect on decreasing cell viability, relative to the single knockdown in tumor cells. Moreover, *CA12* expression is upregulated in response to *CA9* knockdown in 3D-cultured HT29 colorectal cancer cells [67]. The evidence suggests that CA XII may be crucial to certain types of tumor cells and may act as an emergency backup of CA IX. It remains to be seen whether CA XII can play a substitution to CA IX, engaged in the regulation of iron metabolism.

CAs antagonize lipid peroxidation via lipogenesis

Lipid peroxidation is a predominant characteristic of ferroptosis, initiated by a reaction between hydroxyl radicals and polyunsaturated fatty acids [68, 69]. Consequently, the generated lipid peroxides disrupt plasma membrane and other cellular membranes, eventually inducing cell death [70]. A role of CAs in lipogenesis was studied first by using locusts (*Schistocerca gregaria*) in the 1990s [71]. Later, a significant inhibitory effect on lipogenesis has been revealed by treating rat hepatocytes with acetazolamide, a pan-CA inhibitor, leading to a constrained HCO_3^- supply. The HCO_3^- supply is indispensable as a substrate of pyruvate carboxylase (EC6.4.1.1), which carboxylates pyruvate into oxaloacetate in the mitochondria. CA V_A and CA V_B are the only CA isoforms, existing in mitochondria and are essentially involved in metabolic pathways, including ureagenesis and lipogenesis [72, 73]. The produced HCO_3^- by mitochondrial CA $V_{A/B}$ is necessary for oxaloacetate synthesis, which is subsequently used for citrate formation **(Figure 2)**. Then, citrate will be translocated into the cytosol through tricarboxylic acid transporter and reversely converted to acetyl-CoA for fatty acid synthesis. In the cytosol, CA II-mediated production of HCO_3^- provides an adequate substrate for acetyl-CoA carboxylase, which links the central energy metabolism to lipid biosynthesis and is rate-limiting for the *de novo* synthesis of lipids [72]. Therefore, both CA II and CA $V_{A/B}$ appear to be involved in the vital processes of lipogenesis.

CA III is a cytosolic protein and is dominantly expressed in tissues characterized by a high rate of oxygen consumption (*Ex*. liver, adipose tissue) [74]. The role of CA III in lipogenesis is unclear, but *Ca(r)3* is primarily expressed in liver, brown and white adipose tissue, and stores lipids in mice. CA III appears to be nutritionally regulated and several evidences suggest that CA III participates in lipogenesis [75]. Both *Car3* mRNA and protein levels are proportionally associated

with insulin concentration in the adipose tissue because *de novo* lipogenesis would occur during the nutrient-rich states when insulin levels increase. However, this will also increase expression of NADPH oxidases, which subsequently elevate the levels of superoxide and H_2O_2 [76, 77].

Furthermore, researches have revealed that CA III may act as a radical scavenger and protect cells against oxidative stress [78, 79]. *CA3* overexpressing NIH/3T3 cells reveal faster proliferation and more resistance to oxidative stress by H_2O_2. On the contrary, CA III-depleted rat fibroblast cells displayed susceptibility to H_2O_2-induced apoptosis indicated by caspase-3 activation [80]. Indeed, CA III contains a higher number (five) of cysteine residues with respect to other cytosolic CAs having only a single cysteine. Among the five cysteine residues, two of them are highly reactive, termed Cys183 and Cys188, which locate on the molecular surface of the protein and undergo *S*-glutathionylation in the crystal structure to avoid the irreversible oxidation [74]. These evidences suggest that CA III may act as a radical scavenger (superoxide and H_2O_2) to protect cells against oxidative damage.

Of note, all the CA II, CA III and CA V are enriched in the tumor tissues in general, which supports the substantial lipogenesis for proliferation. This may give rise to an enhancement of lipid storage, principally attributable to CA II and CA V in tumor cells. Meanwhile, increased CA III may work to protect tumor cells against lipid peroxidation and ferroptosis.

Additionally, CAs have been regarded as a primary driver of hepatic gluconeogenesis [81-83]. HCO_3^- generated by CAs is utilized as a substrate for the first reaction of hepatic gluconeogenesis, which has been reportedly promoted in type 2 diabetes [83]. An increase in CAs activity enhances the availability of these substrates, resulting in higher hepatic glucose production [84]. A higher

level of glucose will cause a higher risk of lipid peroxidation in human erythrocytes and rat glomeruli [85]. In contrast, a decrease in glucose synthesis has been found in guinea pig hepatocytes upon inhibition of mitochondrial CA V, which is associated with reduction of pyruvate carboxylation rate [86]. It is still vague which CA isoform dominantly promotes glucose production in the normal and pathological conditions. Further studies are necessary to clarify the details.

CAs modulate ferroptosis via signaling pathway

Iron overload is closely associated with the aberrant activation of the RAS-MAPK pathway [87, 88], which can be triggered by intracellular acidosis **(Figure 3)** upon CAs inhibition [89]. Previous studies have indicated RAS-MAPK pathway is involved in expansion of labile iron pool in engineered BJ-fibroblast-derived cells by up-regulating of *TfR1* [90]. Recently, Ye *et al.* has reported that high mobility group box 1 (HMGB1) plays a crucial role in erastin-induced ferroptosis by up-regulating TfR-1 through RAS-JNK/p38 signaling pathway in HL-60 cells which harbored a *NRAS* mutation ($NRAS^{Q61L}$) [91]. Either RAS or JNK/p38 suppression by pharmacological inhibitors dramatically reduced TfR-1 level. An earlier study had uncovered the physiological binding of RAS protein with TfR-1 in human colon carcinoma cells [92]. Furthermore, iron overload-induced cell death was also dependent on RAS-MAPK pathway in ovarian cancer cells, which was associated with *heme oxygenase-1* overexpression. Treatment with MAPK inhibitor, U0126, remarkably reversed this cell death [88].

Interestingly, RAS-MAPK activity appears to be required for cancer cells to maintain an appropriate CA IX level that is associated with a cooperation between transcription factor, specific protein 1 (Sp1) and transcriptional co-activator P300 during the early development in multiple cancer cells **(Figure 3)**. Indeed, Milanini-

Mongiat *et al.* has identified two phosphorylation sites (threonine 453 and 739) in Sp1 which is directly phosphorylated by ERK1/2 kinases [93]. However, an inverse association has been revealed between CA IX overexpression and ERK activation at full confluence *in vitro*, suggesting CA IX may have a role as negative feedback effector that represses RAS-MAPK pathway [94]. Notably, ERK kinases-dependent ferroptosis has also been reported in embryonal U57810 and myoblast C2C12 cell lines [95]. These evidences suggest that aberrant activation of RAS-MAPK pathway may enhance ferroptosis primarily by increasing TfR1 expression. However, this conclusion appears controversial and the relationship between *RAS* mutation status and ferroptosis has not been verified well [31]. We hypothesize that *RAS* mutations sensitize cancer cells of certain genotype to erastin-induced ferroptosis. However, it may vary in distinct genetic backgrounds and cell types in cancers. Further studies are necessary to identify the decisive pathways involved in ferroptosis of tumor cells.

Mitochondrion has been demonstrated to play a crucial role in cysteine-deprivation-induced ferroptosis, accompanied by alterations of mitochondrial membrane potential and TCA cycles which are closely associated with mitochondrial iron metabolism [30]. However, the mechanisms of iron transport across mitochondrial membrane remained unclear thus far. Sheftel suggested that transferrin-containing endosomes come into contact with mitochondria, termed transient "kiss and run" **(Figure 4)**, which subsequently results in an increase in mitochondrial iron [96]. Indeed, CA IX-suppressed MM cells showed an enhanced endocytic activity, accompanied by increased new syntheses of both endosomes and lysosomes [32].

Emerging evidence further revealed that an appropriate communication between the endoplasmic reticulum (ER) and mitochondria is required for cellular

iron homeostasis. The ER–mitochondria encounter structure (ERMES) is a protein complex which functions not only in lipid and protein exchange but also iron homeostasis between the ER and mitochondria [97]. Loss of ERMES function induces an iron deficiency response. Of note, an increase in catalytic Fe (II) has been found in both ER and mitochondria during ferroptosis [32, 98]. Moreover, iron overload induced by high-fat diet can diminish mitochondrial antioxidant enzyme MnSOD, which is associated with ER stress pathway [99]. These evidences suggest that ER-mitochondria junction plays an important role in iron transport. Therefore, excessive iron accumulated in ER may lead to mitochondrial iron-overload.

We recognized a significant event of mitochondrial fission occurred in MM cells after CA IX inhibition accompanied by overexpression of *DRP1*, a mitochondrial fission gene [32]. Simultaneously, we observed that the cell death was associated with autophagy-driven ferritin degradation (ferritinophagy). To date, the mechanism of ferritinophagy induction is poorly understood in ferroptotic cells. We hypothesize that the mitochondrial fission resulting from iron overload plays a role as an executioner of ferritinophagy in CA IX-suppressed MM cells **(Figure 4)**. This scenario is supported by the previous study that ferric ammonium citrate-induced mitochondrial fission is positively regulated by DRP1 both in human marrow stromal and mouse neuronal cells [100]. Ikeda Y *et al.* has indicated that *Drp1* knockout mice showed downregulated autophagosome formation and autophagic flux in cardiomyocytes [101]. Basit F *et al.* has demonstrated that *DRP1* knockdown attenuated BAY 87-2243-induced ferroptosis in melanoma cells [60].

The function of autophagy in survival remains controversial in tumor cells. Moderate and chronic autophagy appears to be utilized by tumor cells to adapt

themselves to the harsh microenvironment during tumor progression [102, 103]. Hence, enhanced autophagy by CA IX inhibition in MM cells may be a protective response to the rapid collapse of pH*i* homeostasis. However, such protective response can be lethal to the MM cells, resulting in a vicious cycle, because iron-enriched proteins (ferritin) and organelles (fragmented mitochondria) will be degraded through a lysosome-dependent pathway, releasing abundant catalytic Fe(II) to the cytosol and promoting iron-mediated lipid peroxidation and ferroptosis.

Conclusion

CAs are involved in many physiological and pathological processes, including gluconeogenesis, lipogenesis and iron metabolism. Suppression or dysregulation of the expressional levels and activities of CAs can increase cytoplasmic labile iron pool concomitant with excessive catalytic Fe(II), which leads to intracellular radical generation and lipid peroxidation, culminating in ferroptosis and apoptosis. A clearer understanding of the association among CA isozymes, lipogenesis and iron/lipid-metabolism would be a beneficial strategy for the treatment of cancers and other various disorders.

Competing interests

The authors have no conflicts of interests to disclose.

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Figure legends

Figure 1. Roles of carbonic anhydrase IX in hypoxic tumor cells.

Carbonic anhydrase IX (CA IX) participates in the regulation of iron metabolism of malignant mesothelioma (MM) cells under hypoxia through (1) suppressing transferrin (Tf)/transferrin receptor (TfR1) endocytosis via enhancement of actin cytoskeleton and (2) stabilization of ferritin. CA IX-mediated intracellular neutral pH supports actin polymerization and invadopodia formation. The endocytic extracellular domain (ECD) of CA IX may affect iron release by suppressing internalized Tf in clathrin-coated vesicles/endosomes. NBC1: sodium bicarbonate cotransporter 1 (SLC4A4). Refer to text for details.

Figure 2. Collaborative role of CA II, CA III and CA V in lipogenesis.

Mitochondrial CA isoform (CA V) mediated HCO_3^- production is indispensable to oxaloacetate synthesis in the presence of pyruvate carboxylase (EC 6.4.1.1). The newly synthesized oxaloacetate reacts with acetyl-CoA, which is formed by oxidative decarboxylation of pyruvate from glycolysis in mitochondrial matrix, to yield citrate. Citrate will be transported to cytosol through the tricarboxylate anion carrier system (SLC25A1) and converted to acetyl-CoA again by ATP-citrate lyase (EC 2.3.3.8) in association with coenzyme A (CoA) and ATP. Subsequently, malonyl-CoA will be formed by a catalyzing reaction among ATP, acetyl-CoA and HCO_3^- which is produced by CA II. Eventually, malonyl-CoA is utilized for fatty-acid biosynthesis. During this process, CA III protects cells from elevated oxidative stress induced by H_2O_2 generated by NADPH oxidases-mediated superoxide production. CA, carbonic anhydrase.

Figure 3. Molecular association of CA IX in ferroptosis.

CA IX under hypoxia controls iron acquisition by suppressing acidosis-induced RAS/JNK/p38 pathway which subsequently enhances TfR-1 expression and induces ferroptosis. Further, aberrant activation of ERK1/2 initiated by RAS/RAF/MEK pathway has been suggested to play a role in triggering ferroptosis. Alternatively, activation of ERK1/2 is involved in stabilizing both HIF-1α and CA IX via transcription factor Sp1 and transcriptional co-activator P300, respectively. These pathways and factors generate a negative feedback loop to protect cancer cells from acidosis-induced iron-overload and ferroptosis. CA, carbonic anhydrase.

Figure 4. Mitochondrial iron overload induced by CA IX suppression plays a central role in ferroptosis and apoptosis.

CA IX inhibition results in mitochondrial iron overload through physical contact between endosome/ER and mitochondrion. IRP1/2 activation through CA IX inhibition may further enlarge labile iron pool (LIP). Dysfunctional mitochondria caused by excessive iron subsequently activates ferroptosis and apoptosis pathways via mitochondrial fission-associated ferritinophagy and opening of mitochondrial permeability transition pore (mPTP), which triggers the caspase cascade via activating Apaf-1 in tumor cells. CA, carbonic anhydrase. See text for more details.

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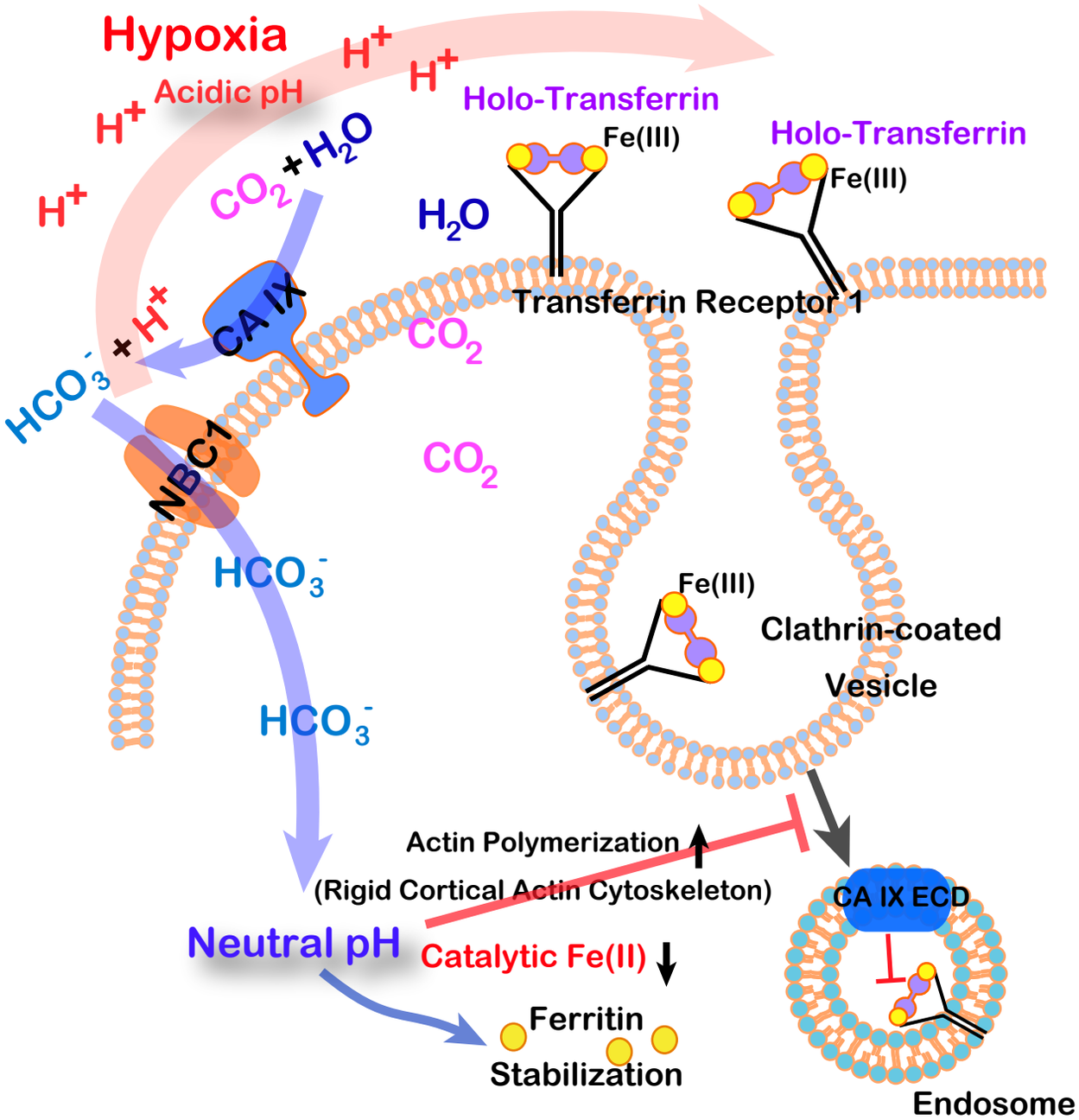
719


Hypoxia
Acidic pH
H+
H+
H+
H+
CO2 + H2O
H2O
Holo-Transferrin
Fe(III)
Holo-Transferrin
Fe(III)
Transferrin Receptor 1
CA IX
HCO3- + H+
CO2
CO2
NBC1
HCO3-
HCO3-
Fe(III)
Clathrin-coated
Vesicle
Actin Polymerization
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CA IX ECD
Neutral pH
Catalytic Fe(II)
Ferritin
Stabilization
Endosome

Figure 1

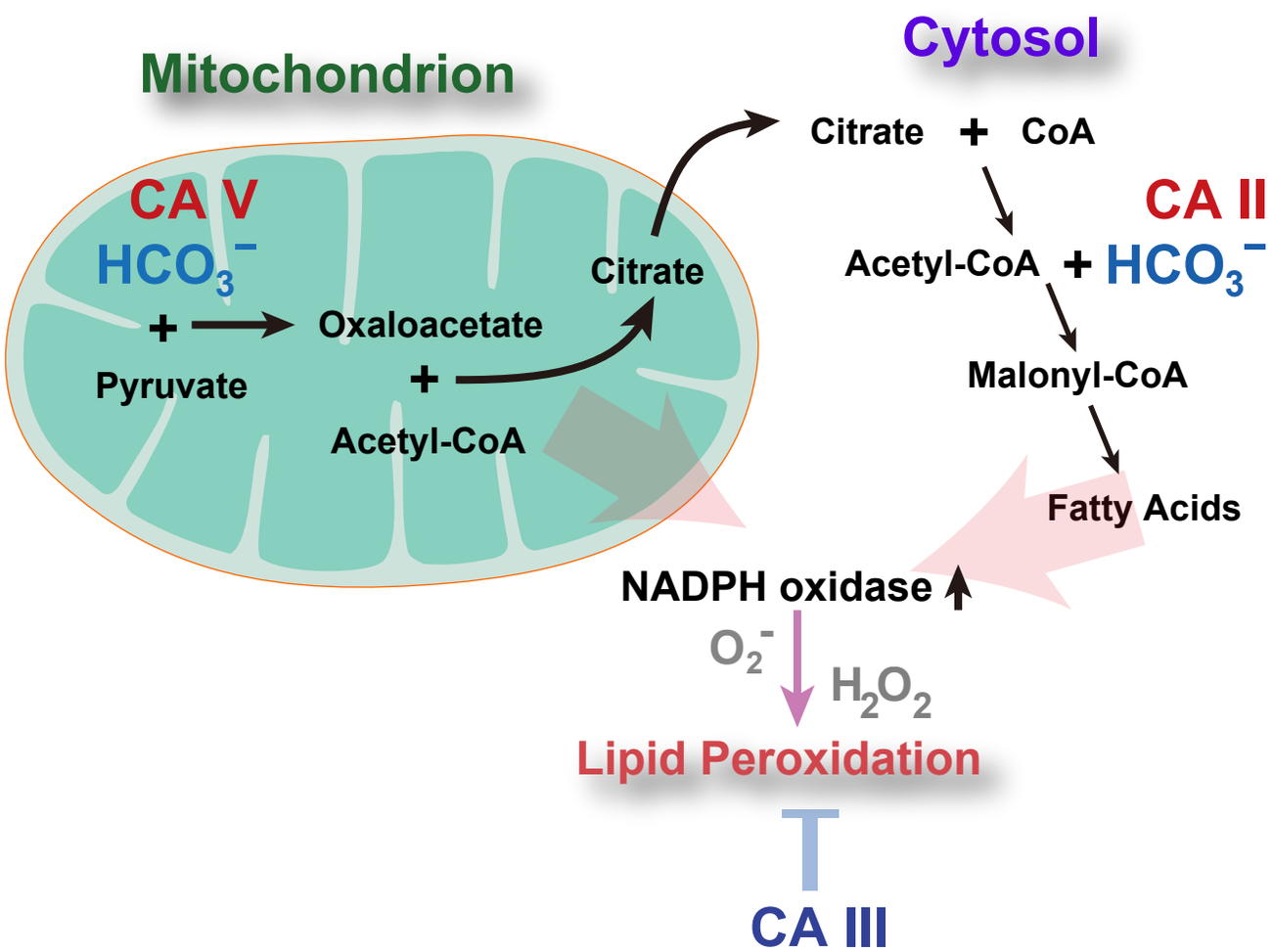
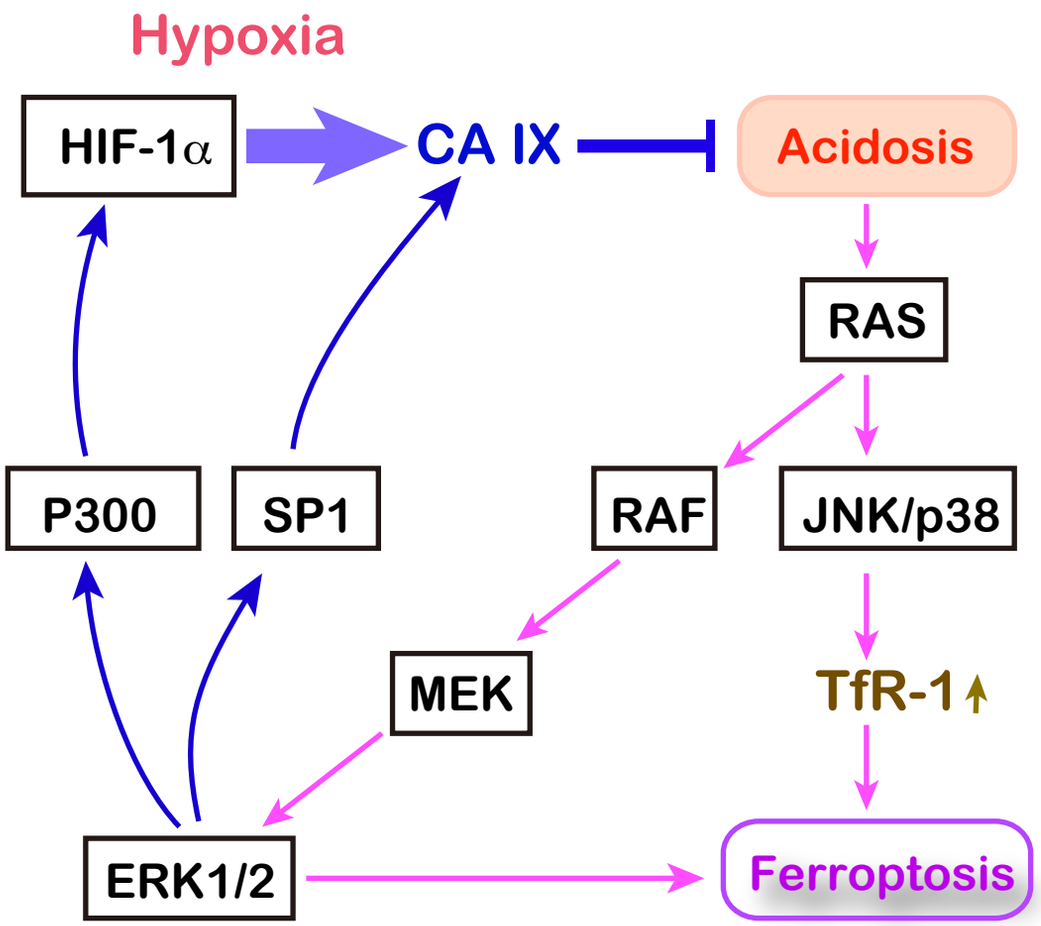

Mitochondrion
Cytosol
CA V
HCO_3^-
+
Pyruvate
Oxaloacetate
+
Acetyl-CoA
Citrate
Citrate + CoA
CA II
Acetyl-CoA + HCO_3^-
Malonyl-CoA
Fatty Acids
NADPH oxidase
O_2^-
H_2O_2
Lipid Peroxidation
CA III

Figure 2


Hypoxia
HIF-1α
CA IX
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RAS
P300
SP1
RAF
JNK/p38
MEK
TfR-1
ERK1/2
Ferroptosis

Figure 3

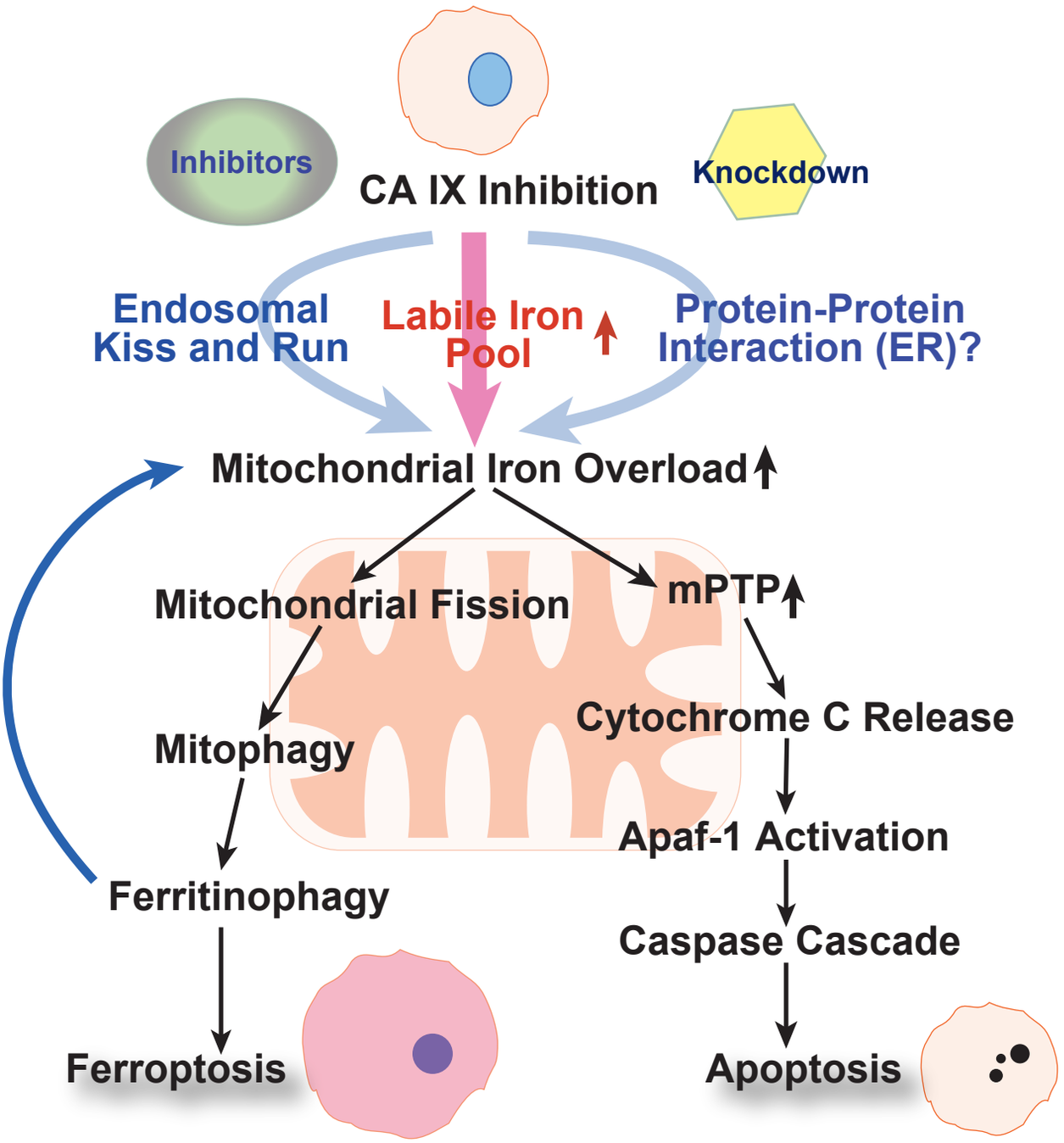
Inhibitors
CA IX Inhibition
Knockdown
Endosomal Kiss and Run
Labile Iron Pool
Protein-Protein Interaction (ER)?
Mitochondrial Iron Overload
Mitochondrial Fission
mPTP
Mitophagy
Cytochrome C Release
Apaf-1 Activation
Ferritinophagy
Caspase Cascade
Ferroptosis
Apoptosis

Figure 4