Two Faces of Vitamin C: AA vs. DHA

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Historically, vitamin C has been associated with many regulatory processes that involve specific signaling pathways. Among the most studied signaling pathways are those involved in the regulation of aging, differentiation, neurotransmission, proliferation, and cell death processes in cancer. This wide variety of regulatory effects is due to the fact that vitamin C has a dual mechanism of action. The reduced form of vitamin C (ascorbic acid, AA) is an essential micronutrient of small size; it is soluble in water and has two dissociable protons with pKa values of 4.2 and 11.8. At physiological pH, its reduced form predominates as the monovalent ascorbate anion (AA); when it loses the second proton, it is oxidized to dehydroascorbic acid (DHA).

vitamin C signal transduction cancer cell death necroptosis

1. Introduction

The reduced form of vitamin C (ascorbic acid, AA) is an essential micronutrient of small size; it is soluble in water and has two dissociable protons with pKa values of 4.2 and 11.8. At physiological pH, its reduced form predominates as the monovalent ascorbate anion (AA); when it loses the second proton, it is oxidized to dehydroascorbic acid (DHA) [1][2][3]. Most mammals can synthesize vitamin C from D-glucose in the liver, except guinea pigs, bats, and higher primates, including humans, due to the absence of the enzyme L-gulonolactone oxidase, which catalyzes the last step of the biosynthesis of vitamin C [4]. Therefore, to meet the body's requirements, vitamin C must be incorporated into the diet [1]. The best-known function of vitamin C is as an antioxidant agent that can act as a cofactor of enzymatic reactions involved in the synthesis of catecholamines, carnitine, cholesterol, amino acids, and some hormonal peptides, as well as in the maintenance of brain function and the protection of central nervous system (CNS) structures [1][3][5][6][7].

AA uptake in different cells is performed by the sodium-ascorbate cotransporters SVCT1 and SVCT2, which stereospecifically transport the reduced form of vitamin C, L-ascorbate [8][9][10][11]. Vitamin C can also be transported in its oxidized form, DHA, through the facilitative glucose transporters GLUT1, GLUT2, GLUT3, GLUT4, and GLUT8 [12][13][14][15][16]. However, for a long time, it has been postulated that the contribution of DHA to the accumulation of vitamin C in tissues is relatively low [3][17][18][19].

2. Molecular Pathways Regulated by Vitamin C

One of the first targets for vitamin C was discovered via its relationship to the NF–κB pathway. In this pathway, vitamin C has an inhibitory function; in studies carried out in endothelial cells, millimolar doses of AA inhibited NF-

κB and IL-8 activation in response to tumor necrosis factor (TNF) [20]. In this entry, the authors also evaluated the toxicity generated by high doses of vitamin C supplementation and did not detect cell damage or lipid peroxidation [20][21]. Furthermore, they were able to determine that the inhibition of the NF-κB pathway was not due to the antioxidant activity of vitamin C, but rather to the direct inhibition of IκB kinase α/β (IKKα/β) [20][21][22][23]. In line with this notion, IKKα/β is a kinase responsible for the phosphorylation of IκBα protein that maintains NF-κB-p65 in the cytoplasm [24]. IκBα phosphorylation is a signal for proteasomal degradation of this protein, allowing NF-κB-p65 nuclear translocation (**Figure 1**A), triggering the activation of specific genes [24][25][26]. In line with these findings, it was postulated that AA is a regulator of IKKα/β activity; however, subsequent studies determined that AA has no action on IKKα/β [22][27]. Interestingly, it was shown that DHA was a regulator of IKKα/β mediated by directly binding to this kinase, inhibiting it, and finally controlling the activity of NF-κB [27]. This function of DHA was determined through immunoprecipitation experiments using p-IκBα-GST where derivatives of vitamin C, AA, DHA, oxalic acid, and threonic acid were used. Only treatment with DHA inhibited IκBα-phosphorylation, and this inhibition was mediated by DHA directly blocking the activity of IKKα/β and p38, likely competing for the binding of ATP to the active site of IKKβ [22][27]. Given this evidence, it was concluded that vitamin C has a dual action against reactive oxygen species (ROS). Intracellularly, AA would fulfill its antioxidant function by neutralizing ROS, generating DHA. Thus, the intracellular accumulation of DHA would block the activation of NF-κB, involving vitamin C in signaling processes that control inflammatory responses and cell death among others (**Figure 1**A).

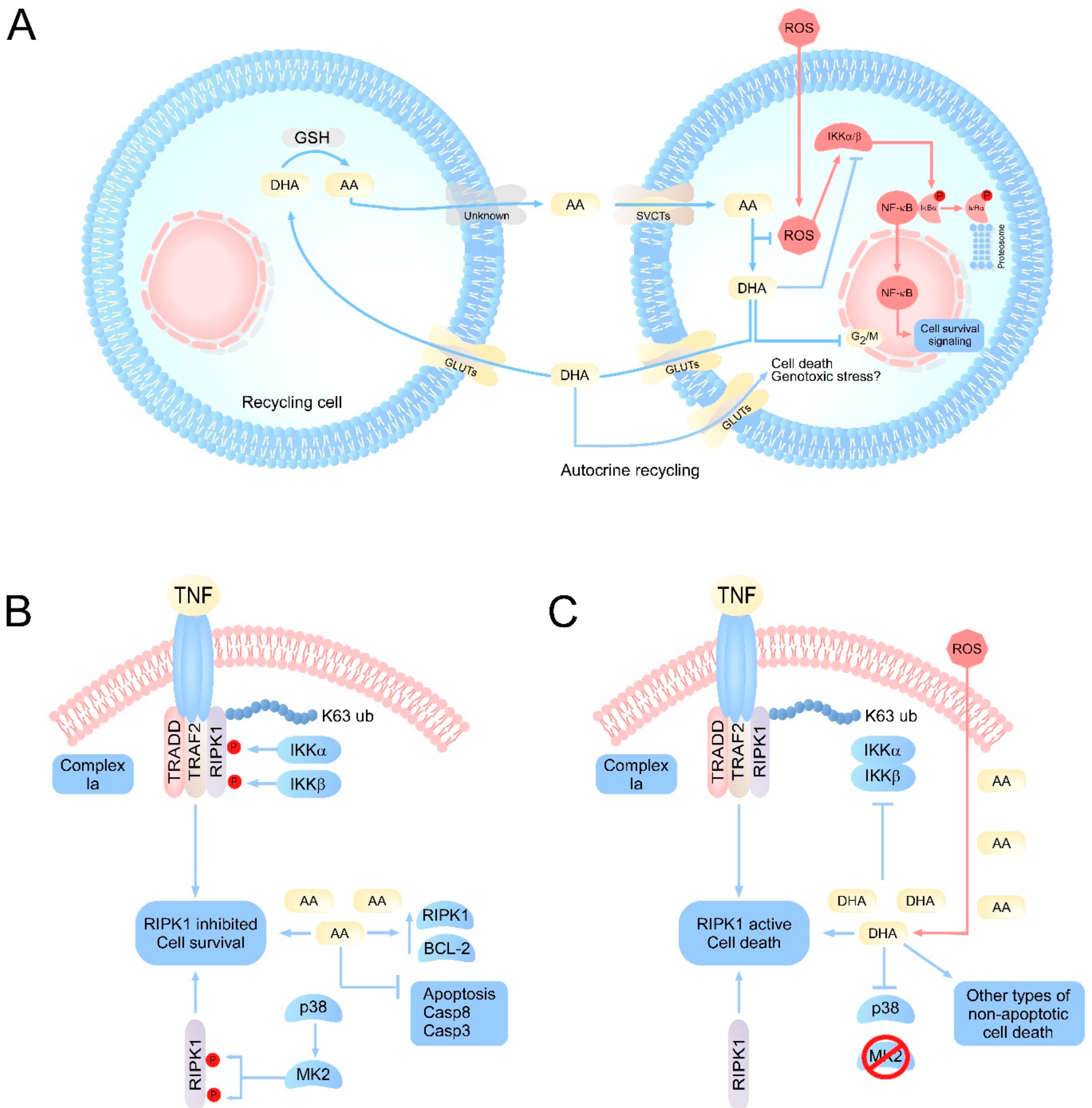

Figure 1. Integrative vision of the principal molecular pathways regulated by vitamin C. (**A**) Scheme of vitamin C recycling in normal cells or cells with oxidative stress. Under normal conditions, AA concentrations remain homeostatically stable due to the efficient recycling of DHA by specialized cells. However, under conditions of oxidative stress or inefficient recycling, an accumulation of intracellular DHA can occur. DHA would target the inhibition of IKK α/β, metabolic enzymes such as GAPDH, as well as the production of genotoxic stress, resulting in the induction of cell death. (**B**) Intracellular effects of vitamin C on signaling pathways associated with cell death. The physiological levels of AA would have a protective function intracellularly, favoring the inhibition of apoptosis by inducing overexpression of antiapoptotic genes, as well as caspases. At the same time, AA could maintain RIPK1

in its inhibited state, which favors cell survival. (**C**) Under pathophysiological or acute oxidative stress conditions, ROS overload induces a massive oxidation of AA to DHA, intracellularly. The accumulation of DHA results in the inhibition of IKK α/β, and p38, which can trigger the activation of RIPK1 and cell death due to necroptosis, in cells that accumulate high concentrations of vitamin C, such as neurons. AA: ascorbic acid; DHA: dehydroascorbic acid; SVCT2s: sodium-dependent vitamin C transporter; GLUTs: glucose transporters; RIPK1: receptor-interacting serine/threonine-protein kinase 1; MK2: p38MAPK-activated protein kinase 2; TRADD: TNFR1-associated death domain protein; TRAF2: TNF receptor associated factor 2.

Another kinase-dependent pathway that is regulated by vitamin C is that of the mitogen-activated protein kinases (MAPK), which involves three other MAPK-dependent pathways, extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 kinase, which are involved in proliferation, differentiation, and apoptosis, respectively [10][28][29]. The first studies examining the relationship between vitamin C and MAPK concluded that vitamin C was involved in in vitro cell death processes, but the mechanism of action was unknown. Thus, the possible regulation of MAPK-ERK mediated by vitamin C was analyzed. For this, leukemia cell lines were treated with AA (0-500 μM) for 1 to 3 h in order to analyze ERK activation by in vitro phosphorylation assays. In cells treated with concentrations as low as 100 μM AA, phosphorylation of ERK and therefore activation was induced [10][30]. Thus, it was proposed that the regulation of ERK mediated by vitamin C would be associated with eventual apoptotic processes that are observed in certain tumor lines when treated with vitamin C because ERK activation is associated with proliferative processes and cell death. However, to date, it has been shown that the use of pharmacological doses of AA induces tumor death from conventional necrosis due to the extracellular generation of H_2O_2 [31][32], as discussed in detail later. At the same time, it has also been shown that AA can antagonize apoptosis in cancer cells induced by classical mechanisms, such as treatment with doxorubidicin, TRAIL, or FAS [33][34][35]. In line with this notion, treatment with physiological doses of AA in neuronal cultures induces overexpression of antiapoptotic genes, such as Bcl-2, and decreases the expression of proapoptotic genes, such as Bax and caspase 8 [19]. Thus, the current evidence suggests that physiological doses of AA could inhibit apoptosis rather than activate this death pathway. Furthermore, AA-mediated ERK activation could be associated with neuronal arborization mechanisms, which would be an indicator of neuronal "good health" [10].

3. Vitamin C as a Cell Cycle Regulator

The cell cycle is regulated by interactions between cyclins and cyclin-dependent kinases (cdk). The cyclin–cdk complex is up- or downregulated by phosphorylation. When DNA damage occurs, cells can be arrested at the G1/S, S, or G2/M cell cycle checkpoints for DNA repair or to enter cell death processes [36][37]. In addition, AA has the ability to regulate the cell cycle directly. During periods of oxidative stress, AA can trigger cell cycle arrest at the S-phase checkpoint [38]. In line with this notion, a recent study in primary human fibroblasts showed that AA treatment decreased the expression of 31 genes [39]. Interestingly, of these 31 genes, 12 corresponded to tRNA synthetases and translation initiation factor subunits, which are required for cell cycle progression [39]. Strikingly, the effects on the cell cycle generated by vitamin C are frequently observed when it is used in combination with pro-oxidant molecules [40][41][42][43]. When AA was used in combination with agents that induce oxidative stress,

growth was inhibited, and the cell cycle arrested at the G2/M checkpoint [44]. Co-incubation of AA together with pro-oxidant molecules triggered cell death, possibly due to necrosis [41]. This suggests that the effects observed and attributed to AA could possibly be triggered by DHA because, when AA is co-incubated with pro-oxidant molecules, it must neutralize ROS and oxidize to DHA. In addition, the treatments were generally for long periods of time and involved a single high-concentration supplementation, which would favor the oxidation of AA. Currently, there is evidence that supports the hypothesis that DHA could be the trigger for cell cycle arrest; when primary hepatocyte cultures were treated with AA, DNA synthesis and cell proliferation were observed [45]. However, when cells were treated with DHA, some proliferation was induced, but it was not sustained [45]. Thus, it is again unclear whether the impact of vitamin C on cell proliferation is due to AA or DHA. Thus, determining which form of vitamin C controls the regulatory functions of proliferation is essential before possible pharmacological use, such as its use as an antineoplastic.

4. Vitamin C as an Enzymatic Cofactor

Epigenetic modifications are reversible changes that affect the genomic structure of DNA, which dictates the accessibility of transcriptional machinery to its sequence, thus regulating gene expression [46]. In particular, chromatin modifications include DNA methylation/demethylation and histone modification, which are introduced by the action of different enzymes. In this context, the influence of various metabolites on enzymatic activity has been widely described [47]. For example, different intermediaries of glycolysis and the citric acid cycle can introduce modifications, such as acetylation or methylation [47]. In the same way, the action of various vitamins in the generation of epigenetic modifications has also been reported [48], indicating that these molecules would also play a role in enzymatic function. Particularly, vitamin C can act as a cofactor of the Fe^{2+} and 2-oxoglutarate (2-OG) family of dioxygenase enzymes [49], which includes important epigenetic regulators, such as Jumonji-C domain-containing histone demethylases (JHDMs) and TET hydroxylases, the latter associated with the conversion of 5-methylcytosine (5mC) into 5hydroxymethylcytosine (5hmC) in DNA [50].

Vitamin C deficiency generates scurvy, a condition in which collagen synthesis is mainly affected; it acts as a cofactor for proline hydroxylase and lysine hydroxylase [51], which are part of the Fe^{2+} and 2-OG-dependent dioxygenase family. Although they have various substrates, they share a conserved mechanism of action: a Fe^{2+} ion is coordinated at the enzyme’s active site, which binds to 2-OG, permitting entry of the substrate and binding of an oxygen molecule [49]. Subsequently, the oxidative decarboxylation of 2-OG and the generation of ROS will oxidize the substrate and release secondary products [52]. In this context, vitamin C would be essential for maximum enzymatic activity, and it has been reported that the need for vitamin C in these reactions arises from its function as an electron donor to maintain an iron pool in its oxidation state +2 [53][54]. However, previous reports show that the effect of vitamin C is not reproduced in the presence of other antioxidant agents, and its direct interaction with the catalytic domain of TET enzymes has been described, which would indicate a specific role of vitamin C in the dioxygenase family [55]. Nonetheless, it should be noted that vitamin C has been associated with iron metabolism, increasing its uptake into the intracellular environment (and thus increasing the labile iron pool of Fe^{2+}) [56]; therefore, vitamin C may be necessary for the maintenance of adequate levels of Fe^{2+}.

Regarding the role of vitamin C in the action of epigenetic enzymes, one of the first analyses was performed on methylated oligonucleosomes in vitro, which were subjected to a histone demethylation reaction in the presence of a nuclear pellet obtained from HeLa cells. In these cells, the production of formaldehyde decreased by ~50% in the absence of vitamin C, indicating that it would be necessary for histone demethylase activity [57]. On the other hand, in vitro DNA demethylation assays in the presence of the catalytic domain of TET2 and different concentrations of vitamin C showed a progressive increase in 5hmC levels in relation to control conditions, an effect that was observed in a time-dependent manner, suggesting that vitamin C accelerates the hydroxymethylation reaction [58]. Additionally, the incubation of vitamin C with the catalytic domain of TET2 showed a progressive extinction of the latter's intrinsic fluorescence (determined by the presence of tyrosine residues that intrinsically fluoresce) in a concentration-dependent manner, thus suggesting a direct interaction between these two molecules [58].

5. Regulation of Necroptosis by Vitamin C: AA as Apoptosis Inhibitor and DHA as RIPK1 Activator

Necroptosis is a pathway that has recently been characterized, and its scope determined at a physiological and pathophysiological level. After determining that receptor-interacting serine/threonine-protein kinase 1 (RIPK1) regulated cell death due to necroptosis, three independent research groups reported and characterized another protein that was necessary for the execution of this pathway, RIPK3 [59][60][61]. Knockout animals and genome-wide siRNA screening, showed that RIPK3 triggers a metabolic alteration that changed the response to TNF from apoptosis to necroptosis [60][61] that required Caspase-8 (Casp8) to be inhibited or absent [62]. Furthermore, in response to TNF, an interaction occurred between RIPK1-RIPK3-Casp8 and the death adapters TNFR1-associated death domain protein (TRADD) and Fas-associated protein with death domain (FADD) [62]. This set of proteins (RIPK1/RIPK3/Casp8/TRADD/FADD) was called complex IIb, to differentiate it from the canonical activation pathway of apoptosis in response to TNF, composed of casp-8/FADD/TRADD, called complex IIa [63][64]. However, the existence of the necroptosis executor protein, mixed lineage kinase domain-like (MLKL), which is activated by RIPK3-dependent phosphorylation, was subsequently determined [65][66]. The discovery of MLKL showed that complex IIb was not necessary for the execution of necroptosis due to the existence of another complex called the "necrosome," composed only of RIPK1/RIPK3/MLKL [63]. Activation of MLKL by phosphorylation induces formation of tetramers and their translocation to the plasma membrane [66][67], resulting in the exposure of phosphatidylserine and the formation of pores that trigger stress osmotic and death by cell explosion. Cell death by the MLKL-dependent necroptotic pathway induces the release of damage-associated molecular pattern (DAMP), which induces inflammation, greater activation of necroptosis, and eventually other parallel death pathways [68].

Current studies suggest that RIPK1 is the critical protein that regulates cell death mechanisms of apoptosis and necroptosis [69][70][71]. The regulation of RIPK1 depends mainly on two factors: ubiquitylation and phosphorylation [72][73][74]. The ubiquitination of RIPK1 depends on the activity of cIAP1/2 and LUBAC that act as E3 ubiquitin ligases [75]. With regard to the regulatory mechanisms of RIPK1 phosphorylation, these are only beginning to be elucidated. It is known that RIPK1 can also be inhibited through phosphorylation by IKKα/β [76]. The activity of IKKα/β on RIPK1 is independent of the activation of NF-κB. Under physiological conditions (absence of death

stimuli), IKKα/β directly phosphorylates RIPK1 in an inhibitory manner, maintaining it in the plasma membrane and preventing the formation of complex IIa, IIb or the necrosome [76]. Alternatively, when IKKα/β complex is inhibited, an inhibitory hypophosphorylation of RIPK1 occurs, which induces the activation of RIPK1 mediated by the autophosphorylation of serine 166 and finally triggers necroptosis or uncontrolled apoptosis [76]. RIPK1 can also be inhibited by MAPK-activated protein kinase 2 (MK2) in the cytosol [77] by direct phosphorylation of serines 321 and 336, independent of IKKα/β activity [77][78]. MK2-mediated phosphorylation of RIPK1 prevents complex IIb and necrosome formation in the cytosol independent of death ligands (intrinsic control of necroptosis) because it prevents RIPK1 from being able to bind Casp-8 or FADD [78], thus preventing the activation of apoptosis or necroptosis (depending on the stimulus) (**Figure 1**B,C). The signaling pathways that regulate apoptosis and necroptosis are closely related. Generally, both routes act as negative feedback for the other. Thus, when death ligands, such as TNF or FasL, bind their receptor, they activate complex IIa, activating Casp-8 that cleaves RIPK1 at aspartate 324, favoring apoptosis [79]. Conversely, when there are death stimuli and pro-apoptotic proteins, such as Casp-8 and BAX, are inhibited or expressed at low levels and/or antiapoptotic proteins, such as those of the Bcl-2 family, are overexpressed, necroptosis is activated via complex IIb or the necrosome [80].

Interestingly, the reduced form of vitamin C, AA, has been widely reported as an inhibitor of apoptosis because it decreases the activity of Casp-3 and Casp-8, increases the levels of Bcl-2, prevents the release of cytochrome C from the mitochondria, and decreases the expression of BAX [19][34][35][81][82][83]. Thus, under physiological conditions, AA would be a potent inhibitor of apoptosis (**Figure 1**B). Conversely, vitamin C can induce death in tumor cells, largely due to necrosis [31][32][84][85]. However, current literature indicates that there is a signal transduction mechanism specifically regulated by the oxidized form of vitamin C, DHA. The first studies that postulated vitamin C as a regulator of signal transduction pathways were activating NF-κB with TNF and determined that vitamin C was a potent inhibitor of this pathway [20][21]. Subsequently, DHA inhibition of NF-κB activity via inhibiting the kinase activity of IKKα/β and p38 in response to TNF was shown [22][27]. In this scenario, DHA would be a potent inhibitor of survival signals by suppressing the activation of NF-κB (**Figure 1**C). As a consequence of the production and/or accumulation of DHA in the cell, cell death is finally triggered [18][86]. In line with this notion, and as described above, there is a close relationship between the effects of DHA and the regulation of necroptosis through RIPK1. Under physiological conditions, vitamin C is found as AA; therefore, IKKα/β is active, keeping RIPK1 phosphorylated in an inhibitory way [76]. At the same time, under physiological conditions, p38 activates MK2 by phosphorylation, which inhibits RIPK1 phosphorylation in the cytoplasm [77][78]. Interestingly, DHA specifically inhibits IKKα/β and p38 [22][27].

Accumulating evidence suggests that DHA primarily targets the activation of RIPK1 to prevent its inhibitory phosphorylation. At the same time, AA would precondition cells to necroptosis as it is a potent inhibitor of apoptosis [34][35]. Thus, under pathophysiological conditions where the oxidation of AA to DHA is favored, DHA could stimulate the induction of cell death mainly by necroptosis (**Figure 1**B,C). To meet this requirement, it is necessary for cells to accumulate a sufficient amount of vitamin C to trigger intracellular death stimuli. Thus, the pro-necroptotic effect of DHA could be limited only to cells that accumulate high concentrations of vitamin C, which have a limited capacity to keep AA reduced, are highly sensitive to oxidative stress, and are also refractory to apoptosis. Unfortunately, the only cells that meet these requirements are neurons, which accumulate up to 10 mM AA

intracellularly [6], rapidly oxidize AA to DHA [17], and, in the adult brain, are refractory to apoptosis, expressing very low levels of Casp-8 and -3 and overexpressing antiapoptotic proteins [87][88].

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