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Review

Hopes, disillusions and more hopes from vitamin C

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Abstract. In the current view of most biochemists and physiologists, the role of L-ascorbic acid (AA) in cell metabolism would be more or less confined to the scavenging of reactive oxygen species. Nevertheless, many data have been collected in our and other laboratories concerning the involvement of AA in many different aspects of cell metabolism. At the present time the molecular sites of action of AA have not been completely elucidated, but recent findings on the specific requirement of AA for the activity of several 2-oxoacid-dependent dioxygenases involved in cell signalling and the activation of transcription factors open new fascinating perspectives for further research.

Key words. Ascorbic acid; dehydroascorbic acid; redox regulation; 2-oxoglutarate-dependent dioxygenases; cell proliferation; hypoxia-inducible factor 1-α.

Introduction

'Once upon a time, the crew of a ship, very ill after a long permanence on the seas, eventually landed in an unknown island. A native sorcerer cured the sailors suffering evident symptoms of scurvy with leaves and fruits'. Many stories like this happened between the 15th and 18th centuries, in the period of long voyages of discovery, but the mysterious remedy present in fruit and vegetables could be recognized only much later, in the early thirties of the last century, when Szent-Györgyi identified ascorbic acid (AA) as the antiscorbutic factor [1]. High hopes were raised by this compound, which, in spite of not being an amine, received the name of vitamin C as an extension of the term 'vital amine' introduced by Casimir Funk. Since then, it was inferred that vitamin C can protect humans from almost any disease, from the common cold to cancer [2]. This originated the convinction – strongly supported by Nobel laureate Linus Pauling – that ingestion of high amounts of vitamin C is necessary to keep in good health. It was the time of megadoses, when hopes for vitamin C became higher and higher. Then followed doubt and disillusion, when several clinical trials questioned the beneficial effect of vitamin C [3].

A new boost of confidence in the properties of AA came out in parallel with establishment of a new theory stating that ageing and many diseases originate from the accumulation of free radicals [4]. As many free radicals are reactive oxygen species (ROSs), free-radical scavengers are often referred to as antioxidants. It was immediately clear that AA, being one of the most effective and widespread antioxidants in biological systems [5, 6], could have an important and decisive protecting role. The antioxidant function of AA has been largely discussed in recent reviews [7–8].

There is no doubt that AA can scavenge ROSs and organic radicals, yet it is necessary to remark that this action, which occurs mainly by means of nonspecific and nonenzymatic reactions, is definitely not the only function of vitamin C in cell metabolism. Moreover, correct evaluation of antioxidant function would require at least information on AA distribution and concentration, pres-

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ence of transition metals and pH values in different cell compartments. Unfortunately, we still lack a clear picture of these parameters, and consequently it is quite difficult to understand the specific role of AA in this context. In other words, overstressing the antioxidant function of AA could be tricky and lead again to disillusion. On the other hand, new fascinating perspectives could result from analysis of the role of vitamin C in many enzyme-mediated functions [9].

Understanding the role of AA in cell metabolism (in both animals and plants) seems at first an impossible task, since vitamin C is apparently involved in so many distinct physiological processes. But recent data make it possible to unravel, at least in part, this tangled skein and to identify the possible mechanisms underlying the pleiotropic action of vitamin C. To do so, some preliminary consideration is necessary to reduce the confusion often accompanying vitamin C.

Cell AA pool and the relationship between AA, AFR and DHA

AA is utilized in cell metabolism as an electron donor. Although donation of two reducing equivalents is also possible, AA normally functions as a one-electron donor, and the first oxidation compound generated is a radical known as ascorbic acid free radical (AFR), ascorbyl radical or monodehydroascorbate radical [10].

AFR generation occurs in all cell districts, since both enzyme-mediated and nonenzymatic AA-oxidizing reactions occur everywhere in the cell. In fact, several AA-dependent dioxygenases (see below) operate in different compartments, and in some of them AA is also oxidized to AFR by organic peroxides, hydrogen peroxide (in plants this reaction is catalyzed by AA peroxidase) and other ROSs. Therefore, AFR is continuously generated at high rates in the cell.

Is AFR involved in biological processes? The answer to this question seems to be yes. Navas et al. reported that AFR generated at the plasma membrane-extracellular matrix interface of plant cells induces hyperpolarization of the plasma membrane, leading to increased uptake of different nutrients, which in turn results in expansion and cell division [11]. AFR induces growth also in the human promyelocytic leukaemia cell line HL-60. At the concentration of about 10^{-8} M, stimulation of growth was 50% higher than that induced by AA itself [12]. Sakagami et al. [13] reported a tight relationship between AFR intensity and apoptosis-inducing activity. High levels of AFR occur in ultraviolet (UV)-B-irradiated tissues, and AFR formation is considered a hallmark of oxidative stress [14]. The increment in AFR content in the cells is partly due to increased AA oxidation and partly to lower efficiency in AFR reduction.

All this seems to suggest that the cell needs mechanisms controlling the amount of AFR present in the different cell compartments, under different environmental and developmental conditions.

AFR is an atypical free radical that is relatively stable due to highly delocalized unpaired electrons and reacts preferentially with itself (disproportionation), thus avoiding a free-radical chain reaction involving other substrates. Spontaneous disproportionation (i.e. intermolecular redox reaction) of two AFRs yields one AA and one dehydroascorbic acid (DHA) [10]. Remarkably, virtually all DHA present in the cell derives from the disproportionation of AFR.

Cells cannot avoid AFR generation, as this is part of the molecular logic of AA oxidation, but they can control its disproportionation. AFR disproportionation is strongly affected by H^+ concentration. The rate of AFR disproportionation is 1×10^5 M^{-1} s^{-1} at pH 7 but rises up to 1×10^8 M^{-1} s^{-1} at acidic pH values [15]. Therefore, the pH of a specific compartment can regulate the rate of AFR disproportionation.

As mentioned before, AFR is a radical with a half-life sufficiently long to make it a substrate for enzyme activity. The enzyme AFR reductase catalyzes a reaction that efficiently reconverts AFR to AA:

$$2\text{AFR} + \text{NAD(P)H} \xrightarrow{H^+} 2\text{AA} + \text{NAD(P)}^+$$

The K_m values of the enzyme for AFR range between 0.8 µM and 7 µM [16, 17]. AFR reductase has been observed in different cell compartments, including mitochondria, peroxisomes, endoplasmic reticulum and plasma membrane [16]. In plant cells, the enzyme is also present in the chloroplasts and the cytosol [18, 19].

It is evident that the more AFR reductase is present, the more AFR is reconverted to AA and the less AFR disproportionates. Since the products of AFR disproportionation are AA and DHA, we should ask ourselves if the importance of AFR reductase in the cell resides in the regeneration of AA or in its capability to limit AFR disproportionation and consequent DHA formation.

Both functions are important, and in some cases AA recycling prevails. In chloroplasts, where dioxygenic photosynthesis takes place, hydrogen peroxide formed in the Mehler reaction has been estimated to be 120 µmol s^{-1}, a concentration that could inhibit CO_2 fixation [17]. Therefore H_2O_2 must be removed by AA peroxidase activity, with net consumption of AA and generation of AFR. Although AA concentration in chloroplasts is very high (about 10 mM), in the absence of AFR and DHA recycling, all AA would be consumed in less than 2 min [17], thus indicating that AA transport from mitochondria (the site of AA synthesis in plants) to chloroplasts cannot keep pace with AA utilization. More often, AFR reductase is

required to control AFR disproportionation, thus preventing DHA formation and the potential damages deriving from the presence of large DHA amounts in different compartments. This is the case, e.g., of plant meristematic cells, which synthesize and consume AA at high rates and do not have vacuoles in which DHA can be segregated [20].

DHA is a harsh compound to the cell. Its administration induces a marked increase in intracellular AA content [21–23], but this is usually accompanied by negative metabolic consequences [21, 24]. DHA inhibits the activity of key enzymes such as hexokinase from erythrocytes [25] and malate dehydrogenase from spinach chloroplasts [26]; induces the oxidation of glutathione (and several thiol proteins) [21, 24] and progressively slows down the rate of cell division in onion root meristems [21]. These negative effects of DHA accumulation explain why the cells tend to keep DHA content as low as possible.

DHA can be reconverted to AA using glutathione (GSH) either in noncatalyzed or in enzyme-mediated reactions [26, 27]. Some DHA reductases use the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) as the electron donor [28], whereas an array of unrelated proteins sharing a dicysteinyl motif (C-X-X-C) can also catalyze DHA reduction in connection with the thioredoxin system [29]. Catabolism is probably another way contributing to keep DHA under control. DHA is converted (seemingly nonenzymatically) to diketogulonic acid (DKG), which is not reconvertible to AA and is further catabolized, although the intermediates of this pathway are not completely known [9, 30].

Since DHA can be reconverted to AA, the sum of AA + DHA is often defined as 'total vitamin C content' or 'total ascorbate'. This could be misleading, since, from a physiological point of view, AA and DHA are very different. As previously mentioned, DHA administration raises AA content, but leads to growth inhibition and other anomalies. The effects of direct administration of AA or (in plants) its physiological precursor L-galactono-γ-lactone (GalL) are exactly the opposite of those seen with DHA: increased thymidine incorporation, and an increase in the rate of cell division and in the redox state of thiol proteins [21, 31, 32]. These effects confirm that AA and DHA are not equivalent, nor is the way in which AA is obtained by the cell (either as AA itself or by uptake of DHA and its consequent reduction to AA) [21]. Interestingly, DHA reduction to AA occurs by simultaneous transfer of two electrons, and no AFR is formed in the reaction [33].

Uptake of AA and DHA

The uptake of AA and DHA is a peculiarity of eukaryotic cells, as AA cannot enter all microorganisms studied, and DHA entry in bacteria is less than could be accounted for by simple diffusion [34]. The mechanism of entrance of AA and DHA and reduction of the latter to AA in the eukaryotic cell are not completely clear. In most cases, such as astrocytes, adrenal and kidney cells, AA is the preferential absorbed form; conversely, in human leukocytes, cells of leukocytic origin, guinea pigs and kitten erythrocytes DHA is preferentially absorbed [35 and references therein]. AA and DHA enter the cell in different ways: DHA is taken up by means of glucose transporters (GLUTs), while AA enters through a Na^+-dependent transporter. In THP-1 cells, a monocyte-type human leukaemia cell line, DHA uptake occurs at high rate in the first 10 min and virtually stops thereafter [23]; AA uptake is linear and much slower than that of DHA when both are tested at the same concentration [23]. Similar results were obtained in plants [21]. However, AA concentration is usually much higher than that of DHA in both plant and animal cells. This means that in physiological conditions, cells accumulate AA by absorbing the reduced form of vitamin C. In oxidative stress conditions and whenever large AA oxidation to DHA occurs (e.g. in the presence of bacteria [34]), cells can provide themselves with AA by absorbing DHA and reducing it to AA. As previously mentioned, DHA is never accumulated, because beside DHA reductase proper, the majority of cells contain, a large number of proteins capable of reducing DHA to AA using either GSH or the thioredoxin system as a source of electrons. This keeps a DHA concentration gradient favouring continuous DHA uptake from the external environment [23].

It is helpful to remark that if the amount of DHA entering the cells is limited, glutathione disulfide generated by GSH oxidation is simply reconverted to GSH by glutathione reductase, thus ensuring cellular homeostasis; vice versa, if high amounts of DHA are transported, the amount of GSH necessary to reduce DHA exceeds the capacity of glutathione reductase, and new GSH biosynthesis can also be activated [21]. Nonetheless, overloading the system with DHA results in generalized oxidation of thiol-containing proteins, with negative consequences for cell metabolism [21, 24]. For example, the absorbance of large quantities of DHA induces acute injury to neurons, since these cells have relatively low capability to reduce DHA to AA. In the presence of astrocytes, which actively reduce DHA to AA, neuronal damage due to DHA treatment is much lower [36, 37].

Long-distance transport and intracellular delivery of vitamin C

As far as we know, there is no indication of any eukaryote capable of surviving without AA or erythroascorbic acid (the 5-carbon analogue of AA detected in yeast),

whereas AA is apparently not present in bacteria (with the exception of photosynthetic cyanobacteria, which are likely to have a small AA supply [9]).

It is well known that all plants and a large majority of animals can synthesize AA. Only humans, guinea pigs and few other species cannot operate de novo AA synthesis from sugars [9]. In plants, virtually all cells can synthesize AA, whereas in animals biosynthetic capability is unexpectedly restricted to liver (in most cases) or, in some species, to kidneys. Since AA is necessary for all cells, both AA-synthesizing and nonsynthesizing animals have the problem of ensuring proper delivery from the place of synthesis to the rest of the organism. This makes necessary the development of long-distance transport mechanisms via blood circulation, in which vitamin C must be protected from inappropriate AA oxidation processes.

The main risks that AA could face during long-distance transport and intracellular delivery are its interaction with transition metal ions such as copper, iron, molybdenum and cobalt. The central role of these metals in biology relies on their ability to exist in multiple oxidation states. However, the redox property that makes copper and iron essential elements of biological systems also contributes to their inherent toxicity. Redox cycling between Cu^{2+} and Cu^{+} or Fe^{3+} and Fe^{2+} can catalyse the production of ROSs, with consequent damage to lipids, proteins, DNA and other biomolecules [38]. AA, so largely present both in circulating blood and inside the cells, is possibly the main electron donor and therefore the major cause of damage deriving from redox reactions of transition metals. Copper and iron are kept under control by means of preventive mechanisms based on copper and iron routing by specific Cu and Fe chaperones. It is well known that copper enters the cell through a specific copper transporter and is then distributed by other specific protein chaperones that target various molecules for copper incorporation [39]. Although we have no direct evidence of the existence of AA chaperones in animals and plants, it is conceivable that AA routing might also require a protective system in vivo. It has been observed that although rapid AA breakdown occurs in buffer solution at pH 7.0–7.5 at 37 °C, under the same conditions, the molecule is very stable in circulating blood [40]. The view that 'close encounters' are possibly avoided by presently unknown mechanisms is also supported by the fact that AA and metal ions have a prooxidant effect in vitro that causes severe damage to lipids and DNA [37, 41, 42], whereas simultaneous iron and AA overload affects neither lipid oxidation [43] nor DNA damage [44] in vivo. Interestingly, earlier reports showed that AA can form ionic bonds with different proteins and peptides [45]. Due to its important implications, this aspect will surely be developed in the near future.

AA system and redox control of cell metabolism

AA is often seen as a system that, with its redox couples AA/DHA and AA/AFR, is involved in the regulation of different metabolic events. Although it is true that AA participates in a number of redox reactions, some theoretical considerations suggest caution in considering vitamin C essentially as a redox regulator.

In a cell, many metabolic processes require redox reactions, and therefore a comparably large number of biological responses have been reported to be redox controlled. Historically, the term 'redox state' refers to the ratio of the interconvertible oxidized and reduced forms of a specific redox couple. This was first applied to the $NAD(P)^{+}$/NAD(P)H couple, as described by Sir Hans Krebs [46]. Redox changes can involve simple redox couples or a series of redox pairs, as in the mitochondrial inner membrane and in the chloroplast. In these chains, the electron flow occurs according to a sequence of redox systems from more negative to more positive $E^{\circ 1}$ values. It is clear that changes in the redox state of a couple can influence the redox state of all other couples, and this can be very important not only for regulation of electron transfer in the chain, but also for control of important metabolic processes occurring in those organelles. This is the case of RNA synthesis in the mitochondrion, which is regulated by the redox state of the Rieske Fe-S protein (Rsp) [47], and in the chloroplast, where the process is apparently dictated by the redox poise of the cytochrome (cyt) b_6f complex [48]. Although the signalling molecule(s) downstream of the redox state of Rsp and cyt b_6f have not yet been identified, it can be concluded that RNA synthesis in mitochondria and chloroplasts is redox regulated.

After the discovery of the importance of ROSs and reactive nitrogen species (RNSs) as signal molecules activating many important cellular responses [49], the term 'redox state' has been also used to describe the general redox environment of a cell and the complex balance of oxidants (ROSs and RNSs) and antioxidants (including AA). In addition, it is often claimed that AA and other antioxidants are involved in the redox regulation of different processes. Unfortunately, this general use of the terms 'redox state' and 'redox regulation' is not well defined and, more important, lacks a quantitative characterization [50]. In most cases, the identification of a redox regulated process is based on studies in which a cell culture is challenged with ROSs/RNSs in the absence or presence of various antioxidants. Although these studies are certainly useful to understand basic aspects of cell regulation, the complexity of cell responses cannot be understood just in terms of AA redox changes. The AA system in the cell is much more than a simple redox couple (fig. 1): AA is present in almost all cell compartments; it is not only subjected to redox reactions, but AA and DHA are also con-

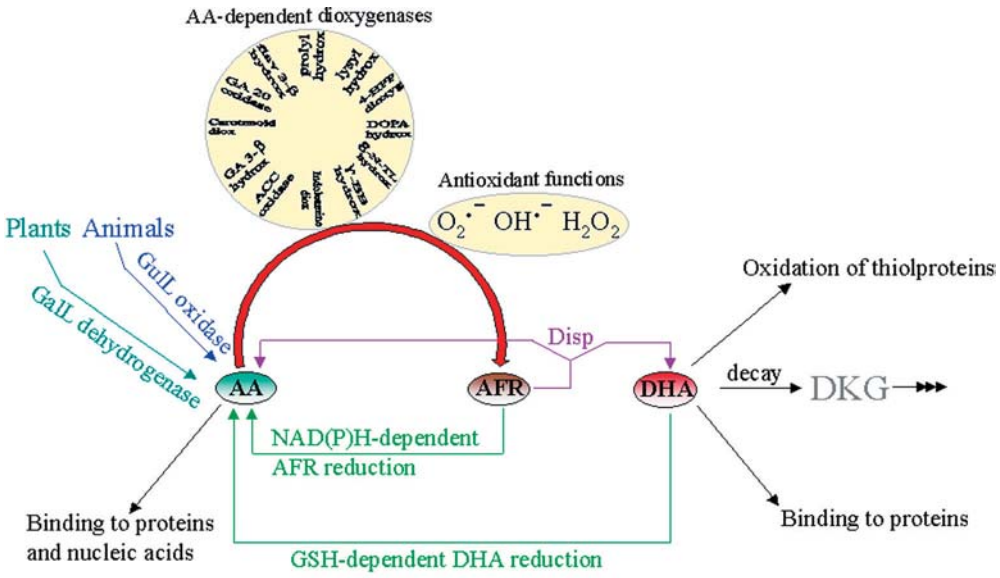

Figure 1. The ascorbic acid (AA) system. AA is synthesized in plants and animals via L-galactono-γ-lactone (GalL) and L-gulono-γ-lactone (GulL), respectively. AA is oxidized to ascorbic acid free radical (AFR) either in the reactions catalyzed by AA-dependent dioxygenases, or by reacting with reactive oxygen species (antioxidant function). AFR can either disproportionate, yielding AA and dehydroascorbic acid (DHA), or undergo reduction catalyzed by NAD(P)H-dependent AFR reductase. In turn, DHA can be reduced back to AA by GSH-dependent DHA reductases, react with thiol-containing proteins or undergo spontaneous decay to diketogulonic acid (DKG). Both AA and DHA can bind proteins, and AA can also bind nucleic acids. Only a few AA-dependent dioxygenases are listed. 4-HPP dioxyg, hydroxyphenylpyruvate dioxygenase; ε-*N*-TL-hydrox, ε-*N*-trimethyl-L-lysine hydroxylase; γ-BB hydrox, γ-butyrobetaine hydroxylase; ACC oxidase, 1-aminocyclopropane-1-carboxylate (ACC) oxidase; GA, gibberellic acid; flav, flavanone.

tinuously degraded into compounds that are not reconvertible to AA; eventually, AA and DHA form more or less stable complexes with proteins and nucleic acids [45, 51]. Explaining AA involvement in many important physiological phenomena simply on the basis of its antioxidant properties and its participation in hypothetical redox-regulated mechanisms, in the absence of quantitative parameters, will hardly help us in understanding those processes. Only a few studies tried to overcome the limits and uncertainties of simple qualitative analysis of redox regulation mechanisms. For instance, mathematical models have been proposed, defining the antioxidant role of AA in ozone detoxification in plant cell walls [52]. However, it should be considered that analysis of the AA system in this peculiar plant cell compartment is made simpler by relatively low concentrations of AA and absence of the enzymes responsible for its de novo synthesis and regeneration. Oxidation of AA to DHA in cell walls has been suggested to activate a signalling cascade [53], but the physiological significance of this system is still vague.

The biochemical role of vitamin C in cell metabolism

The special biochemistry of AA makes it an essential support for the activity of many enzymes. It has been demonstrated that AA stimulates endothelial nitric oxide synthesis by stabilizing the nitric oxide synthase cofactor tetrahydrobiopterin [54]. In addition, Wells et al. observed that AA induced a twofold increase in the activity of mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) and played an essential role in glucose-induced insulin release [55].

However, the main function of AA in biological systems seems to reside in its participation as a cosubstrate in reactions catalyzed by a number of 2-oxoacid-dependent dioxygenases (2-ODDs) that operate in the cells of all organisms [9].

2-ODDs catalyze the incorporation of O_2 into an organic substrate. In general, these enzymes share a catalytic mechanism specifically requiring Fe^{2+}, 2-oxoglutarate and AA [56]. In some cases, however, differences in cosubstrate requirements may occur. For example, 1-aminocyclopropane carboxylate oxidase (ACCO), the enzyme that catalyzes the last step in the synthesis of the plant hormone ethylene, does not require oxoglutarate, but is strictly dependent upon AA [57].

Different 2-ODDs catalyze reactions of hydroxylation, desaturation, oxidative ring closure and expansion. Such reactions, leading to the incorporation of oxygen in a given substrate, are critical steps in a surprisingly large number of biochemical pathways. The best-known reaction catalyzed by a member of 2-ODDs is posttranslational hydroxylation at carbon 4 of proline residues incorporated into polypeptide chains. The mechanism of

peptidyl-prolyl-4-hydroxylase (P4H), the enzyme responsible for this reaction, has been studied in detail. Molecular oxygen is split by the enzyme and used for both oxidative decarboxylation of 2-oxoglutarate and substrate oxidation. The latter is obtained via the generation of an iron-oxygen-atom complex, the ferryl ion, which hydroxylates an appropriate proline residue [58, 59]. Generation of the ferryl ion can proceed without subsequent hydroxylation in so-called uncoupled reaction cycles [60]. AA is utilized as a specific alternative acceptor of the ferryl oxygen in these reaction cycles. In the absence of AA, P4H is rapidly inactivated by self-oxidation [60, 61]. From a chemical point of view, this is a very difficult reaction since it requires 'handling' of the highly reactive ferryl ion. As mentioned above, this reaction mechanism, requiring specific AA oxidation to AFR, is widely used by enzymes involved in the synthesis of many important biologically active molecules, including proteins, hormones and neurotransmitters. The above-mentioned reaction mechanism of ACCO is different from that of P4H, in that O_2 is used simultaneously to oxidize both substrates ACC and AA without the involvement of oxoglutarate. In the absence of AA, ACCO also is quickly deactivated [62].

The central role of AA in the function of dioxygenases is the most convincing molecular explanation accounting for AA involvement in so many different and apparently non-related phenomena. This is supported by the analysis of the consequences of AA deficiency in animals and plants.

Organisms that are not capable of producing AA can be considered as knockout mutants impaired in AA biosynthesis. The analysis of the consequences of an AA-free diet in nonsynthesizing organisms (e.g. humans and guinea pigs) is the most straightforward strategy for understanding actual AA functions. It is well known that dietary deprivation of AA in humans and guinea pigs results in scurvy. The variety of symptoms occurring in scurvy can be simply explained by inactivation of a range of key AA-dependent 2-ODDs [63]. These include the already mentioned P4H (required for synthesis of functional collagen), tyrosine hydroxylase and dopamine-β-hydroxylase (involved in the synthesis of the neurotransmitter noradrenalin), ε-trimethyl-lysine dioxygenase and γ-butyrobetaine dioxygenase (necessary for the synthesis of carnitine), indoleamine 2,3-dioxygenase (involved in triptophan metabolism and kynurenine synthesis) and many more [63]. As will be discussed below, additional important roles have been identified for prolyl hydroxylation in other proteins, not related to collagen, that are involved in oxygen sensing and protein degradation under unfavorable environmental conditions.

Plants are a little bit more difficult since all species studied so far can synthesize AA, and no mutant completely devoid of AA has been described, clearly indicating that plants unable to synthesize AA are not viable. Plants with low AA content show altered growth and development, changes in the activity of enzymes of the AA system [32, 64] and low efficiency of the xanthophyll cycle [65]. Impaired activity of AA-dependent 2-ODDs can explain the effects of low AA content.

A number of AA-dependent 2-ODDs have been described in plants [66]. In addition to the already mentioned function of ACCO in the synthesis of ethylene, the list includes enzymes involved in the synthesis and/or catabolism of plant hormones gibberellins (GA 3 β hydroxylase; GA 20 oxidase) and abscisic acid (9′-*cis* epoxycarotenoid dioxygenases, also known as NCEDs). Correct hydroxylation of proline residues in the large class of plant hydroxyproline-containing proteins, catalyzed by P4H, has a paramount role in cell organization and in general plant development [67].

Detailed characterization of the *Arabidopsis* mutant *vitamin C 1* (*vtc*1) [68], which is impaired in an early step of AA biosynthesis and has about 70% less AA than the wild type, showed that also in plants, AA deficiency has much to do with impaired activity of 2-ODDs. Expression profiling of *vtc*1 plants, which are smaller than the wild type and show delayed flowering, demonstrated that lower vitamin C content significantly upregulates the expression of genes coding for NCEDs and two 2-ODD (At4g10500 and At2g36690), which may be involved in gibberellin synthesis. Increased expression of genes coding for AA-dependent enzymes in the AA-deficient *vtc*1 mutant is likely to be due to a compensation mechanism that counteracts the inadequate supply of AA itself. The balance between ABA and gibberellin content, a key issue in the control of plant growth and responses to environmental stresses, could be therefore regulated by AA availability.

An important consequence of AA-dependent regulation of the different metabolic pathways involving the activity of dioxygenases is that, upon decrease in AA availability, AA-dependent enzymes cannot work below a threshold value of AA concentration. This threshold is likely to be different for different dioxygenases, depending on their K_m for the cosubstrate AA. In principle, the order of inactivation of the different 2-ODDs will depend on their affinity for AA: the ones with lower affinity (higher K_m) are supposed to sense AA deficiency first (fig. 2).

Quantitative consumption of AA occurs as a consequence of the activity of different 2-ODDs. In plant tissues, inhibition of P4H activity induced a significant increase in AA content [67, 69], suggesting that in physiological conditions, P4H consumes AA at high rates. In animals, collagen is known to be the most abundant protein, so its hydroxylation necessarily requires large amounts of AA [61]. Little information is available concerning the amount of AA actually consumed by other 2-ODDs, although it is conceivable that they also contribute quantitatively to AA utilization.

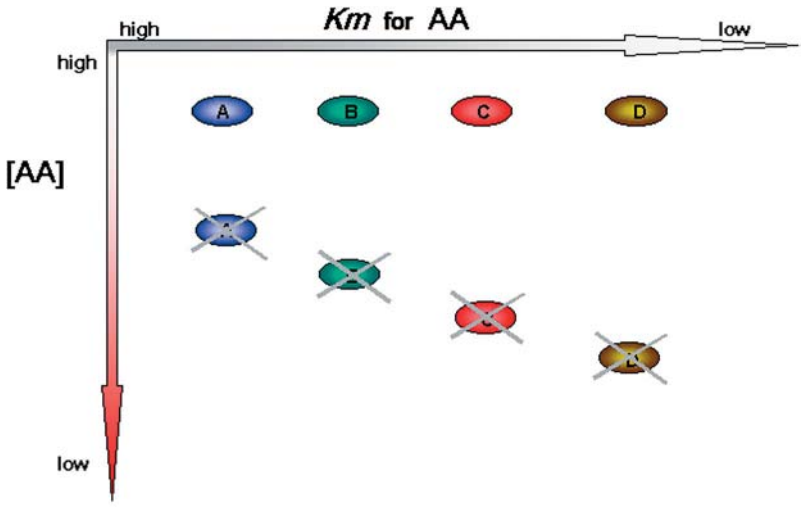

Figure 2. Scheme of the progressive drop in the activity of AA-dependent dioxygenases in AA deficiency. In conditions of normal AA availability, all AA-dependent dioxygenases (for clarity, only four hypothetical enzymes are represented in the scheme) can properly work. As far as AA concentration decreases, dioxygenases with higher K_m (lower affinity) for AA are inactivated first. Progressive inactivation of AA-dependent dioxygenases causes dramatic drawbacks to cell metabolism (scurvy).

The possibility of controlling the activities of different dioxygenases by modulating AA availability could have significant implications in many important issues, such as the control of cell proliferation.

AA is required for progression of the cell cycle

Cell division is a basic characteristic of all living organisms. In eukaryotes, the DNA replication phase (the S phase) is separated from cell division (the M phase) by a resting phase (G_0) and two gap phases (G_1 and G_2). Progression or block of cell division is regulated at the level of a number of different checkpoints [70]. This control mechanism operates at the G_1–S transition, and is mainly regulated by cyclin-dependent kinases. Experimental data indicate that AA is also implicated in the control of the G_1–S transition.

In plant cells, when the AA content of actively proliferating cells is experimentally lowered with the alkaloid lycorine [71, 72], cell division is blocked in G_1 but restored by supplying exogenous AA [31]. When AA content is experimentally increased, quiescent G_1 cells are induced to enter the S phase [73, 74]. This demonstrates that AA exerts a regulatory role in cell proliferation, and it has been suggested that cells with low AA content could have a longer G_1 and those with high AA content could be fast cycling [73, 75]. In other words, AA is a factor that, modulating the G_1 phase, regulates the duration of the cell cycle. Available evidence indicates that AA is involved in cell proliferation in enabling already competent cells to progress through the cell cycle, but not in inducing non-competent cells to overcome proliferation arrest [31, 76].

AA also stimulates cell cycle progression in animal cells, where it apparently stimulates the entry of quiescent 3T3 cells into S phase [12]. Moreover, AA influences the proliferation of human leukaemic and preleukaemic cells [77]. Both AA and AFR stimulate the proliferation of HL-60 cells in serum-limiting media by shortening the overall length of the cell cycle [78]. Lycorine treatment induced a decrease in AA content in rats, in parallel with a significant decrease in the number of polymorphonuclear leukocytes (PMNs) in both peripheral blood and in peritoneal exudates. Upon interruption of lycorine treatment, a new increase in the PMN number occurred (fig. 3) [79]. What is the molecular mechanism underlying AA involvement in cell division? A redox regulation mechanism has been suggested [80], but, as discussed previously, this hypothesis has not yet been fully substantiated. On the other hand, an increasing number of data point at a specific role of AA-dependent dioxygenases in the control of cell division at the G_1–S transition.

Inhibitors of the activities of AA-dependent dioxygenases, and namely of P4H, also result in inhibition of cell cycle progression [67, 81–82]. Conversely, exogenous AA stimulates both P4H and cell proliferation [83–85]. Also, the activity of dehydrohypusine hydroxylase, a dioxygenase that catalyzes the posttranslational modification of the eukaryotic initiation factor 5A (eIF-5A), is involved in the control of cell proliferation [86], although AA dependency of the hydroxylation reaction has not been demonstrated [87].

The connection between the posttranslational hydroxylation of proline residues by P4H and the cell cycle has not yet been fully understood. However, recent data showing that hydroxylation of specific proline residues in the tran-

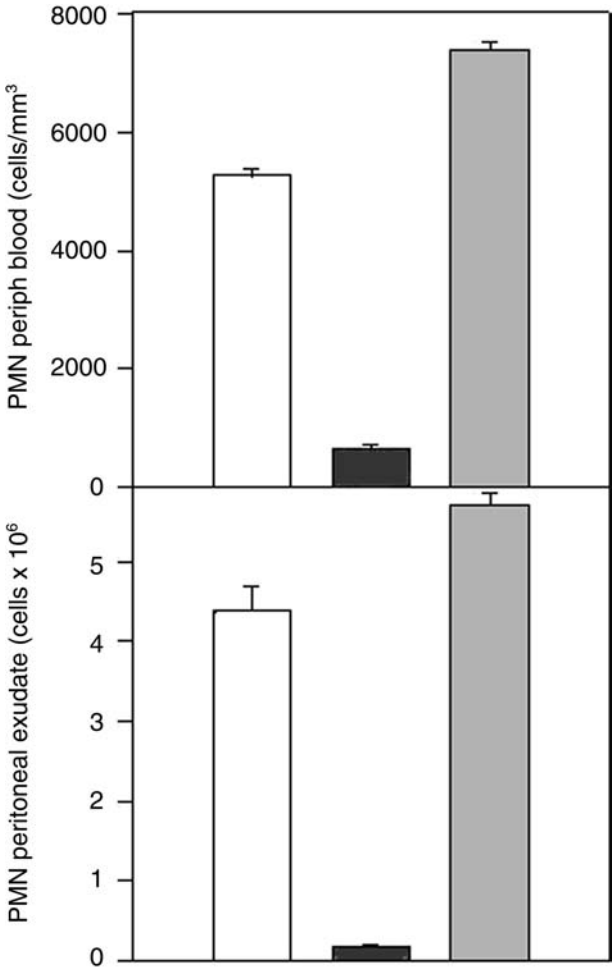

Figure 3. Effect of AA deficiency on rat polymorphonuclear leukocytes (PMN). The number of PMNs from peripheral blood (upper panel) and peritoneal exudates (lower panel) was analyzed in rats inoculated daily with the AA biosynthesis inhibitor lycorine (10 µg/g). Open bars: untreated controls; closed bars, rats treated 5 days with lycorine; grey bars, rats 1 week after interruption of lycorine treatment. Redrawn from [79].

scription factor hypoxia-inducible factor 1-α (HIF-1α) is necessary for its targeting to ubiquitination and degradation, demonstrate that P4H (and other AA-dependent 2-ODDs) can have a fundamental role in cell signalling [88, 89]. When P4H is not active, due to lack of any of its co-substrates, unhydroxylated HIF-1α is stable and activates a number of target genes [49, 90]. Recently, Knowles et al. [91] demonstrated that AA at physiological concentrations reduces the levels of HIF-1α by influencing the activity of P4H. Notably, although HIF-1α is also included in the long list of redox-regulated proteins [49], it has been clearly demonstrated that AA-dependent regulation of the stability of this transcription factor depends exclusively on AA involvement in the P4H reaction, since inhibition of P4H by an oxoglutarate homolog circumvents the effect of AA [91].

There is mounting evidence that the activity of P4H could be part of a general dynamic system controlling protein stability and turnover, and it is conceivable that in the near future, more and more targets will be discovered. For instance, it has been reported that ubiquitination and degradation of the large subunit of RNA polymerase II in response to UV radiation follows a similar mechanism [92]. Notably, the activities of other AA-dependent 2-ODDs besides P4H are apparently involved in important signal transduction pathways: this is the case of tyrosine hydroxylase [93] and aspartyl hydroxylase [94].

It is important to underline that HIF-1α is involved in angiogenesis, a process known to have a key role in cancer development [95]. This brings to mind the long and scathing debate on the function of AA in cancer treatment. Over the years, contrasting results have been obtained on this important issue [3, 96], and no reasonable conclusion could be drawn [97]. Once more, the antioxidant/prooxidant function of AA is invoked by the supporters of AA utilization in cancer therapies. However, studies on a presumptive selective cytotoxicity of AA against some cancer cell lines have been hampered by the observation that depending on the culture medium used, AA can contribute to the generation of H_2O_2 [98], thus triggering apoptotic responses. On the other hand, it has also been demonstrated that cancer cells are apparently more efficient than normal cells in accumulating AA, suggesting that their increased rate of cell proliferation could also be related to higher AA availability [99].

Could a basket of oranges be beneficial to cancer patients? We have no definitive response yet, but most likely it will not be very helpful. Perhaps now is the time to attempt an interpretation of these puzzling results based on our increased understanding of the role of AA in the activity of AA-dependent dioxygenases involved in the control of cell signalling. The above-mentioned HIF-1α is involved in angiogenesis [90] and constitutively highly expressed in cancer cells [100]. AA can suppress HIF-1α protein levels and transcriptional targets [91].

AA is also required for the hydroxylation, by aspartyl-β-hydroxylase (AAH), of aspartyl and asparaginyl residues in epidermal growth factor (EGF)-like domains [101] present in different signalling proteins involved in protein-protein interactions [102]. Some of these target proteins (Notch and Notch homologs) have roles in cell migration [103]. Interestingly, antisense suppression of AAH resulted in lower migration of cholangiocarcinoma cells [94]. In this case, increased expression and AA-dependent activity of AAH would result in increased infiltrative growth patterns of carcinoma cells.

These hypothetical contrasting actions of AA open the way to a completely new interpretation of its biochemical role in cancer and to strategies to be used in the near future.

Perhaps the main problem with the therapeutical use of AA in cancer is that AA delivery can be alternatively use-

ful or noxious, depending on the physiological state of the target cells. In some cases, it seems that our cells can live neither with AA, nor without it. The identification of specific AA transporters and/or chaperones that are likely to be present in biological systems will possibly allow us to target AA to the right place in the right quantity, thus overcoming possible problems resulting from inappropriate delivery.

Much research on AA has been done so far. All this experimental work seems to proceed following cycles of hope and deception. Maybe it is time to end the cycle characterized by excessive attention to antioxidant function and start a fascinating new one, based on careful investigation of AA involvement in cell signalling mechanisms.

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