Vitamin C Deficiency Activates the Purine Nucleotide Cycle in Zebrafish*§

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Background: We investigated the effects of vitamin C status on the metabolome of adult zebrafish.
Results: Levels of inosine monophosphate (IMP) and AMP deaminase (AMPD) activity were enhanced in vitamin C-deficient zebrafish.
Conclusion: Vitamin C deficiency activates the purine nucleotide cycle in zebrafish.
Significance: The link between vitamin C deficiency and elevated AMPD activity is relevant to metabolic diseases.

Vitamin C (ascorbic acid, AA) is a cofactor for many important enzymatic reactions and a powerful antioxidant. AA provides protection against oxidative stress by acting as a scavenger of reactive oxygen species, either directly or indirectly by recycling of the lipid-soluble antioxidant, α-tocopherol (vitamin E). Only a few species, including humans, guinea pigs, and zebrafish, cannot synthesize AA. Using an untargeted metabolomics approach, we examined the effects of α-tocopherol and AA deficiency on the metabolic profiles of adult zebrafish. We found that AA deficiency, compared with subsequent AA repletion, led to oxidative stress (using malondialdehyde production as an index) and to major increases in the metabolites of the purine nucleotide cycle (PNC): IMP, adenylosuccinate, and AMP. The PNC acts as a temporary purine nucleotide reservoir to keep AMP levels low during times of high ATP utilization or impaired oxidative phosphorylation. The PNC promotes ATP regeneration by converting excess AMP into IMP, thereby driving forward the myokinase reaction (2ADP → AMP + ATP). On the basis of this finding, we investigated the activity of AMP deaminase, the enzyme that irreversibly deaminates AMP to form IMP. We found a 47% increase in AMP deaminase activity in the AA-deficient zebrafish, complementary to the 44-fold increase in IMP concentration. These results suggest that vitamin C is crucial for the maintenance of cellular energy metabolism.

Since the discovery of vitamin C (ascorbic acid, AA) and its function as an antiscorbutic micronutrient in humans, it has become increasingly evident that AA plays significant roles in disease prevention beyond scurvy (1). AA is involved in neurotransmitter biosynthesis and nitric oxide bioavailability (2), in gene transcription mediated by hypoxia-induced factor (HIF) (3–5), in maintenance of proper redox status by acting as an antioxidant (6–8) and facilitating the recycling of vitamin E (α-tocopherol, α-T) (9, 10). One of the difficulties with investigating the functions of AA in vivo is that animals commonly used for laboratory research, unlike humans, are able to synthesize AA. Humans depend on dietary intake of AA because they lack the functional gene for gulonolactone oxidase, the enzyme responsible for conversion of gulonolactone into AA (11). Gulonolactone oxidase knockout (Gulo−/−) mice have been used to study the effects of AA (12, 13). The zebrafish (Danio rerio) is a unique and novel model for studies of AA function. We chose zebrafish because this and other teleost species, like humans, lack a functional gulonolactone oxidase gene (14). Other beneficial attributes include its small size (< 1 g body weight) and short life span (15–17), as well as enabling assessment of integrative, whole animal effects. We hypothesized that specific metabolic pathways are at risk if AA and/or α-T are not sufficiently available. Our approach was to let our biological system respond to the insult (AA or α-T deficiency) and then pursue the underlying mechanism by exploiting the advantages of the zebrafish model. The objective of this study was to determine how and to what extent vitamin C and vitamin E deficiency affect metabolism in zebrafish. We quantified the impact of vitamin C and E status on the zebrafish metabolome by high-resolution tandem mass spectrometry using an untargeted metabolomics approach. Here we report the changes in metabolic profiles in relation to vitamin status and show that AA deficiency elicits stress responses in zebrafish that resemble oxidative stress responses in laboratory rodents and in humans. Our findings suggest that zebrafish are an appropriate model for studying the effects of vitamin C on metabolism.

MATERIALS AND METHODS

Chemicals—MS-grade methanol was purchased from J.T. Baker (Mansfield, MA). Water and 98% formic acid were purchased from EMD Chemicals (Gibbstown, NJ). Synthetic standards were purchased from Sigma-Aldrich (St. Louis, MO)
and TCI America (Portland, OR). Negative and positive ion calibration solutions were supplied by AB SCIEX (Foster City, CA).

Fish Husbandry—Tropical 5D strain zebrafish (D. rerio) were housed in the Sinnhuber Aquatic Research Laboratory at Oregon State University and studied in accordance with protocols approved by the Institutional Animal Care and Use Committee. Adult zebrafish were kept at standard laboratory conditions as described previously (18).

Feeding Experiment—Beginning at 42 days of age, zebrafish were fed defined experimental diets as described previously (19), with the following changes. Defined diets were prepared in 300-g batches with high (E+, 178 μmol RRR-α-tocopherol/kg diet) or low (E-, 22 μmol RRR-α-tocopherol/kg diet) levels of α-T. Both E+ and E– diets were prepared with low AA (C–, 50 mg AA/kg diet, added as Stay-C®, DSM Nutritional Products, Parsippany, NJ). At 98 days of age, the dietary ascorbic acid was increased to higher levels in both E+ and E– diets (C+, 350 mg AA/kg diet, added as Stay-C®). Thus, at 42 days of age, zebrafish were fed diets that were low in AA with or without α-T (C-E+ or C-E–) for 56 days. Subsequently, zebrafish were fed the high-vitamin C diet with or without E (C+E+ or C+E–) for an additional 21 days (supplemental Fig. S1). The feeding protocol was used to examine the consequences of AA supplementation to fish that had been AA-deficient. By sampling fish after the period of AA deficiency and after AA supplementation, this experiment resulted in four different zebrafish diet groups: C–E–, C–E+, C+E–, and C+E+.

Measurement of α-Tocopherol—Fish were euthanized by an overdose of tricaine (Sigma-Aldrich), flash-frozen in liquid nitrogen, and stored at −80 °C until analyzed. Individual, whole fish α-T concentrations were determined by HPLC with electrochemical detection as previously described (20).

Measurement of AA, Uric Acid, and Malondialdehyde (MDA)—Fish were euthanized by an overdose of tricaine, weighed, and homogenized in buffer (5% trichloroacetic acid (Sigma-Aldrich), 0.08% diethylenetriaminepentaacetic acid (Acros Organics, Morris Plains, NJ), 250 mM perchloric acid (Fisher Scientific, Fair Lawn, NJ), 0.4 mM dithioerythritol (Sigma-Aldrich)). Homogenates were then centrifuged, the supernatants collected, divided into two aliquots (one for MDA analysis and one for AA analysis), flash-frozen in liquid nitrogen, and stored at −80 °C until analysis. Individual, whole zebrafish AA and uric acid concentrations were determined by HPLC with electrochemical detection as described (21).

MDA concentrations were determined using a modified method of Hong et al. (22). Briefly, the supernatants (200 μl) were mixed with 0.2% butylated hydroxytoluene (20 μl) and 10 N NaOH (100 μl), incubated for 30 min at 60 °C, cooled to room temperature, mixed with 8.5% H₃PO₄ (1 ml), and cooled on ice for 5 min. An aliquot (250 μl) was then transferred to a fresh tube, mixed with 98% thiobarbituric acid (TBA, 150 μl), incubated for 30 min at 95 °C, cooled on ice, mixed with butanol (300 μl), and centrifuged at 16,000 × g (Eppendorf, Hauppauge, NY) at 10 °C for 5 min. The supernatant was then transferred to injection vials. 1,1,3,3-tetraethoxypropane standards were prepared as described previously (23). MDA separation was performed using a Shimadzu HPLC (Kyoto, Japan) with a Phenomenex Luna C18 column (250 × 4.6 mm, 5 μm, Torrance, CA) and a mobile phase consisting of 50% (v/v) methanol and 50% phosphate buffer (25 mM KH₂PO₄). Detection of MDA-TBA adducts was performed using a Shimadzu RF-10A spectrophotofluorometric detector with a xenon short-arc lamp (Ushio Inc., Tokyo, Japan) at the following wavelengths: excitation 532 nm, emission 553 nm. Zebrafish MDA concentrations were calculated by comparisons to the standard curve and then expressed per gram body weight.

Metabolite Extraction—Whole, frozen zebrafish (n = 5/group, n = 20 total) were ground in liquid nitrogen using a mortar and pestle, and the fish powder was weighed. A mixture of 80:20 methanol:water at −80 °C was used as the extraction solvent (61.2 μl/100 mg ground weight). Samples were mixed vigorously, allowed to rest 15 min at 4 °C, and then the supernatant clarified by centrifugation at 13,000 × g for 10 min, removed, and kept cold (−20 °C) while the pellet was re-extracted. The resulting supernatant was pooled with the first and stored at −80 °C until LC-MS/MS analysis.

LC-MS/MS—High-pressure liquid chromatography was performed on a Shimadzu Nexera system (Shimadzu, Columbia, MD) coupled to a hybrid quadrupole-time-of-flight mass spectrometer (TripleTOF™ 5600, AB SCIEX). Chromatographic separations were carried out on an InertSill phenyl-3 column (150 × 4.6 mm, 5 μm, MetaChem Technologies, Torrance, CA) for positive ion analysis and a Scherzo SMC18 column (150 × 2.3 mm, 3 μm, Imtakt, Philadelphia, PA) for negative ion analysis. For the phenyl-3 column, the flow rate was 0.4 ml/min, and mobile phases consisted of water (A) and methanol (B), both with 0.1% formic acid. The elution gradient was as follows: 0 min, 5% B; 1 min, 5% B; 11 min, 30% B; 23 min, 100% B; 35 min, 100% B; 37 min, 5% B; and 47 min, 5% B. Column temperature was held at 70 °C, and the injection volume was 10 μl. For the SMC18 column, the flow rate was 0.18 ml/min, and mobile phases consisted of water (A) and acetonitrile (B), both with 0.1% formic acid. Elution gradient was as follows: 0 min, 5% B; 5 min, 5% B; 32 min, 100% B; 35 min, 100% B; 37 min, 5% B; and 47 min, 5%. The column temperature was held at 50 °C, and the injection volume was 10 μl.

Mass spectrometry was performed on an AB SCIEX TripleTOF™ 5600 equipped with an electrospray ionization source. The instrument was operated in the information-dependent MS/MS acquisition mode with the collision energy set at 35 V and with a collision energy spread of 15 V. TOF MS acquisition time was 0.25 s, and MS/MS acquisition time was 0.17 s. The scan range was 70–1250 m/z for TOF MS and 50–1250 m/z for MS/MS. Ion source gas 1 and 2 and curtain gas (all nitrogen) were set at 50, 40, and 25, respectively. The source temperature was set at 500 °C and IonSpray voltage at 5.5/-5.5 kV. For quality control and to determine the degree of system variance relative to biological variance, an equal mixture of all zebrafish samples (n = 20) was injected periodically throughout the batch. Two-minute autocalibrations were performed every three samples.

Data Processing and Statistical Analyses—Statistical analysis of antioxidant and MDA concentrations were performed using GraphPad Prism software (GraphPad, La Jolla, CA). Significance (p < 0.05) was determined by a one-way analysis of vari-
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RESULTS AND DISCUSSION

Zebrafish ∝-T, AA, Uric Acid, and MDA Concentrations—The levels of ∝-T, AA, uric acid, and MDA were quantified in individual zebrafish (n = 10 per diet group) from each of the diet groups (Fig. 1). The ∝-T-deficient diets relative to the ∝-T adequate diets markedly decreased whole body ∝-T concentrations (Fig. 1A). AA supplementation increased ∝-T concentrations in the ∝-T-adequate fish. The ability of AA to recycle ∝-T may be responsible for this increase.

Both dietary ∝-T and dietary AA altered zebrafish AA concentrations. During the initial inadequate dietary AA period, zebrafish AA concentrations were low. However, during the high dietary AA period, the C+E+ zebrafish contained nearly double the AA concentrations than the C+E- fish, but these differences were not sufficient to reach statistical significance (Fig. 1B). These data suggest, however, that the lack of ∝-T caused increased oxidative stress leading to increased AA depletion.

Uric acid is a powerful antioxidant, and, like glutathione, has been hypothesized to increase in the absence of AA as a compensatory antioxidant mechanism (26). Zebrafish uric acid concentrations varied with dietary antioxidants. As would be predicted, the highest uric acid concentrations were found in the C-E- zebrafish. These concentrations were significantly higher than those in zebrafish fed the C+E- diet, whereas fish fed diets containing ∝-T had intermediate uric acid concentrations (Fig. 1C). Further study is required to explore the mechanism behind this phenomenon.
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MDA was measured in the same homogenate from each of the fish as were the AA and uric acid concentrations. Both dietary AA and α-T influenced zebrafish MDA concentrations, which were highest in C-E- fish and lowest in C+E+ fish, whereas omission of either antioxidant led to intermediate MDA concentrations (Fig. 1D).

Taken together, these findings demonstrate that low levels of dietary AA have greater influence on both MDA and uric acid concentrations than α-T. AA levels also had a major impact on glutathione concentrations (see below), whereas α-T did not.

**Discovery of Differentiating Metabolites and Identification**—PCA-DA analysis of the zebrafish dataset revealed significant differences in the polar metabolic profiles of the AA deficient and sufficient zebrafish (Fig. 2). The low and high E diet groups did not separate demonstrating that α-tocopherol deficiency had little or no effect on the metabolome of these zebrafish (Supplementary Fig. S2). Because no significant changes were observed between low and high E diet groups, the insufficient E groups were eliminated from further analysis, leaving only the C+E− and C-E+ diet groups. The PCA-DA plot also shows relatively tighter grouping from repeat injections of the pooled quality control sample, demonstrating instrument and system variance to be much lower than the biological variance in these zebrafish samples (Supplemental Fig. S2). To further highlight metabolite differences between AA-deficient and AA-sufficient zebrafish, p values from a Student’s t test were plotted against fold change, log 10 (C-E− versus C+E+) (Fig. 3). The metabolites with the smallest p values and greatest fold changes were selected for identification.

Metabolite identification was conducted with high-resolution MS, MS/MS fragmentation, and isotopic distribution, and, when available, synthetic standards were used to confirm retention time. Of the 81 metabolites identified, 64 were confirmed with synthetic standards, and the remaining 17 were identified by database MS/MS spectra comparison (Supplemental Table S1). An example of the comparison between a synthetic standard and a metabolite detected in a zebrafish sample in regards to retention time and MS/MS fragmentation is shown for glycero-phosphocholine (GlyPhCh) and IMP (Fig. 4). The feature at m/z 515.2120 eluting at 5.1 min was initially selected for identification because of a significant 15-fold change between zebrafish diet groups. After a database search, no metabolites were found matching the experimental m/z value and fragmentation pattern of the 515.2120 feature. After close examination of the MS/MS spectra, it was determined that the m/z 515.2120 ion at 5.1 min was a dimer of GlyPhCh that had an experimental m/z of 258.1106 and retention time of 5.1 min. The identity was further confirmed with a synthetic standard. The m/z 258.1106 [M+H]+ ion at the same retention time was quantified and pooled with the response of the m/z [2M+H]+ 515.2120 ion, which resulted in a much smaller (2.3-fold), nonetheless significant (p < 0.05), change between vitamin C diet groups.

Following the identification of significantly differentiating metabolites, a targeted data analysis approach was used to uncover concerted changes specific to a metabolic pathway or class of metabolites. The Human Metabolome Database (HMDB) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases were used to determine which metabolites were relevant to those identified previously.

**Purine Metabolism**—By far the most dramatic and coherent change seen in AA-deficient zebrafish was an increase in the levels of metabolites from the PNC. Specifically, IMP, AMP, and adenylosuccinate, which were increased 44-fold, 3-fold, and 2.4-fold, respectively (Fig. 5). In addition, the level of GMP was 9.3 times higher in AA-deficient zebrafish compared with the AA-supplemented fish. Because GTP is required to convert IMP to adenylosuccinate, the rise in GMP may be due to GTP depletion caused by activation of the PNC. The PNC is activated when ATP levels become compromised during times of cellular stress, such as intense exercise, oxygen depletion, or...
oxidative stress (27, 28). When cellular ATP levels decrease, the concentration of AMP increases in an exponential manner (29, 30). As such, AMP is able to serve as a highly sensitive regulatory molecule controlling multiple branch points in energy metabolism. The regulation of several enzymes greatly depends on the ratios of ATP to AMP and ADP, also known as the adenylate energy charge. One such enzyme is AMP-activated protein kinase (AMPK), which regulates physiological and cellular energy balance in response to energy needs (31). Acute activation of AMPK is thought to affect a multitude of anabolic and catabolic processes, including glucose uptake, fatty acid oxidation, and triacylglycerol synthesis (29). With such a broad range of downstream targets, a small change in AMPK activity can have a profound effect on whole-cell energy metabolism.

In chick cartilage cells, AA fortification prevented a decline in the adenylate energy charge over a 20-day period (32). Our

![Figure 2: PCA-DA scores plot of zebrafish fed the AA-deficient (n = 10) and AA-sufficient diet (n = 10) (DA1 × DA2, 70% of variance). Analysis is on the basis of the polar features detected in positive ion mode.](image)

![Figure 3: Volcano plot of polar metabolites detected in positive ion mode in zebrafish fed an AA-deficient and AA-sufficient diet with α-T supplementation (C+E+ versus C-E+). Log 10 (fold change) values are on the basis of average (n = 5/group) responses calculated by MarkerView software.](image)
metabolomics data suggest that zebrafish have a similar response to AA fortification. In another study, activation of AMPK in mouse cartilage cells promoted the development of an autophagic state by decreasing the activity of mammalian target of rapamycin. Moreover, the presence of AMPK was necessary to trigger this autophagic response (33). Additionally, activation of HIF1, a stimulator of cartilage cell autophagy and regulator of many genes associated with cancer and energy metabolism, was necessary for the activation of AMPK (33). AA decreases the active levels of HIF1 by acting as a cofactor for enzymatic hydroxylation of HIF1 by prolyl hydroxylase, resulting in targeted degradation (3–5). Given that the presence of reactive oxygen species can increase HIF1 activity (4, 5), vitamin C deficiency may induce onset of the autophagic process by three mechanisms: 1) by lowering the cellular adenylate charge, which results in AMPK activation and inhibition of mammalian target of rapamycin; 2) by decreasing HIF1 hydroxylation and its subsequent degradation; and 3) by allowing high levels of reactive oxygen species, leading to increased HIF1 activity, thereby promoting autophagy independent of AMPK.

Two reactions acting in concert are thought to be responsible for replenishing ATP levels and maintaining the ratios of ATP to AMP and ATP to ADP during times of high ATP utilization or impaired oxidative phosphorylation. First, the deamination of AMP to form IMP decreases AMP levels, which then, by mass action, pushes the myokinase reaction ($2\text{ADP} \rightarrow \text{AMP} + \text{ATP}$) toward ATP production (27). AMPD is responsible for irreversibly deaminating AMP to form IMP and is the main source of ammonia production in working muscle, especially fish muscle (27, 34). The production of ammonia from this reaction has been hypothesized to independently regulate energy metabolism by preventing the inhibition of phosphofructokinase by citrate, stimulating glycolytic flux (27). As an integral part of the PNC, AMPD is an energy-generating pathway operative in many animal tissues (35). In goldfish muscle, AMPD exists in the free or bound forms (24). Under resting

![FIGURE 4. Identification of metabolites using the untargeted metabolomics approach. A, LC-MS chromatogram and MS/MS spectra for a GlyPhCh synthetic standard and GlyPhCh found in zebrafish samples. XIC, extracted ion chromatogram. B, LC-MS chromatogram and MS/MS spectra for an IMP synthetic standard and IMP found in zebrafish samples.](image-url)
conditions, 35.6% of muscle AMPD in this fish species was found in the bound fraction in association with subcellular structural material such as myosin. High oxygen concentration, which is known to induce oxidative stress in goldfish tissues, alters the enzymatic activity and spatial distribution (free versus bound) of the enzyme. The stimulation of physical activity in the skeletal muscle of both rats (36) and trout (34) has also been shown to affect the distribution of AMPD between free and bound forms. In human erythrocytes, Tavazzi et al. (37) demonstrated that reactive oxygen species are directly responsible for activation of AMPD through sulfhydryl group modification.

On the basis of the increase in PNC metabolites in our metabolomics study, we hypothesized that AMPD activity would also be increased in AA-deficient zebrafish. To test this idea, we determined the activity of free and matrix bound AMPD in whole zebrafish on the basis of the rate of formation of ammonia upon addition of AMP. The assay for bound AMPD activity of the zebrafish fractions was initially optimized for linearity of reaction in terms of time of incubation (supplemental Fig. S3), protein concentration (supplemental Fig. S4), and substrate concentration (supplemental Fig. S5).

Bound AMPD activity of zebrafish ranged from 18.4 ± 1.90 to 29.3 ± 4.31 nmol/min/mg protein, whereas free AMPD activity of this fish species ranged from 21.0 ± 1.40 to 26.3 ± 4.87 nmol/min/mg protein (Fig. 6). These values are comparable with those measured in fresh whole rat brain (27 and 25 nmol/min/mg protein) and to the activities of control soluble and membrane-associated forms of AMPD from human cerebellum, which were 33 and 28 nmol/min/mg protein, respectively (25). These activities are similar to those reported in sheep, rat, and fetal human brain (25).

Feeding zebrafish a vitamin C-deficient diet produced a significant (p < 0.05) 47% increase in bound AMPD activity, but no change in free AMPD activity was observed (Fig. 6). This result suggests that the increased IMP levels in vitamin C-deficient zebrafish might be due to increased activity of AMPD. However, further work is needed to determine the mechanism...
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**FIGURE 6.** Bound and free AMPD activity in zebrafish fed an AA-deficient diet and in fish supplemented with AA, both groups with adequate dietary α-T (C+E+ versus C-E+). To account for unequal variance in the C+ and C- groups, a Welch’s correction was used for Student’s t test comparisons (n = 10/group). Data represent mean ± S.E.

behind the increase in activity in the vitamin C-deficient zebrafish.

Although it has been shown previously that AA deficiency can reduce the adenylate energy charge (32), the mechanism is unknown. Impaired oxidative phosphorylation, thought to be the cause of the major changes seen in this study, could potentially arise from impaired fatty acid oxidation. The decreased acylcarnitine levels seen in this study in the absence of AA support this view and are discussed below.

**Carnitine Metabolism**—The role of AA in carnitine synthesis is well established (38). AA acts as a cofactor aiding the hydroxylation of the carnitine precursors, trimethyl-lysine and butyrobetaine. Interestingly, we found no changes in the levels of free carnitine or its immediate biosynthetic precursor, butyrobetaine (Fig. 5). These observations may be due to the highly efficient reabsorption of the already present carnitine in combination with sufficient dietary intake. We do report, however, a global decrease in long chain acylcarnitines in the absence of AA. Although these changes in zebrafish may directly relate to inhibited synthesis of free carnitine without AA, altered lipolytic and lipogenic enzymatic activity may also play a role. When the black sea bream (*Acanthopagrus schlegelii*), was supplemented with AA, decreases in the activities of the lipogenic enzymes NADP-isocitrate dehydrogenase and glucose-6-phosphate dehydrogenase were observed, along with an increase in the activity of the lipolytic enzyme carnitine palmitoyltransferase (39). Although the increase in carnitine palmitoyltransferase activity with AA fortification was not statistically significant, a trend was apparent. The global increase in acylcarnitines seen in this study after AA supplementation may partially be due to the ability of AA to activate carnitine palmitoyltransferase, promoting the exchange of coenzyme A for acyl groups.

**Glycerophospholipid Metabolism**—The inhibitory effect of AA on GlyPhCh choline phosphodiesterase, an enzyme that converts GlyPhCh into glycerol and phosphocholine, has been explored previously in vitro (40). A recent study of AA deficiency in the Gulo−/− mice revealed differentiating levels of the downstream products of GlyPhCh choline phosphodiesterase, glycerol, phosphocholine, and choline (41). Here we report 2.3-fold lower levels of GlyPhCh in the AA-deficient fish (Fig. 5), supporting an inhibitory effect on GlyPhCh choline phosphodiesterase by AA. Interestingly, no change was seen for the downstream metabolites, phosphocholine and choline. This may be due to the ability of the organism to obtain sufficient quantities of choline from the diet or by methylation of phosphatidylethanolamine to form phosphatidylcholine.

Glyceric acid, a branch point between glycerolipid and carbohydrate metabolism, was decreased 2.5-fold in AA-deficient zebrafish. Because cellular energy conditions are stressed in AA-deficient cells because, at least partially, of the lack of fatty acid β-oxidation, glyceric acid produced from glycerol may be used as an alternative source of energy for oxidative phosphorylation. This is not far-reaching, given the high energy value of glyceric acid which, when converted to 3-phosphoglycerate, can directly enter glycolysis. The decrease in glycerol seen in previous AA deficiency studies also supports this notion (41). Although the idea that glyceric acid levels are affected by vitamin C has not been explored recently, several studies in the 1940s and 1950s showed a relationship between the two acids in cress seedlings and mice, both of which are able to synthesize AA. In cress seedlings, Isherwood (42) found that when AA was being actively synthesized and levels were increasing, glyceric acid pools also increased. The same authors also determined that glyceric acid is not a biosynthetic precursor to AA. Though humans and zebrafish lost the ability to synthesize AA endogenously, these previous results, taken together with our data, suggest that the regulatory mechanism by which AA increases glyceric acid levels may have been retained.

**Glutathione Metabolism**—Two separate studies on AA deficiency in the Gulo−/− mice reported an increase in total glutathione levels as well as changes in several precursors supportive of an increase in GSH synthesis (12, 41). In our zebrafish model of AA deficiency, we report similar congruent changes supporting an increase in GSH synthesis as well as an increase in total GSH levels (Fig. 5). Both GSH and GSSG increased in AA-deficient zebrafish as well as the direct GSH biosynthetic precursor, glycine, whereas serine, a precursor to glycine, decreased. Another direct precursor of GSH biosynthesis is cysteine, which can be formed from methionine. As described by Duggan et al. (41), cystathionine-β-synthase catalyzes the irreversible production of cystathionine, which can then be converted to cysteine. The activity of cystathionine-β-synthase depends greatly on the oxidation state of its heme group, a potential target of modulation by AA. Although cysteine was not detected in this study, the decrease in methionine levels in the absence of AA is suggestive of a shunt toward cysteine production and increased GSH synthesis.

Not only do our results highlight the unique ability of zebrafish to act as a model of AA deficiency without genetic manipulation, we have shown that AA plays a crucial role in energy metabolism by affecting AMP levels and regulating the activity of the PNC, specifically AMPD activity. Any effect of AA on the activity of AMPK should be explored, as it may have a global impact on cellular energy metabolism and be responsible for many of the metabolic changes presented here.
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