R is for Arginine: Metabolism of Arginine Takes off Again, in New Directions

Running title: Michel; Homoarginine and cardiovascular disease

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First published in 1962, Ray Bradbury’s short story collection “R is for Rocket” provided a view of space travel with a prescience and sophistication that transformed the simpler notions of the time. And in a simpler era dating back only to the mid-1980s, L-arginine (abbreviated R in the single letter amino acid code) seemed to be just another amino acid, notable principally for its guanido group, which conferred arginine with a high pK and provided its distinction as one of the three basic amino acids. Abundant in many types of food, synthesized from simple precursors, or recycled in different tissues in the body, arginine is classified as a non-essential amino acid: except under extreme conditions, nutritional states of arginine deficiency are rare. Yet how much more complicated and interesting has arginine become in the past three decades!

The role of arginine as a humdrum amino acid was forever transformed by the discovery that this amino acid is a substrate for the formation of nitric oxide by all mammalian nitric oxide synthases1,2. The metabolic transformations of arginine and their roles in cardiovascular (patho) physiology have since been explored in many hundreds of papers2-7. The current issue of Circulation reports a new and intriguing aspect of arginine metabolism involving the compound homoarginine8, with even broader implications for our understanding of the roles of metabolic systems in vascular biology and disease.

Homoarginine looks a lot like arginine, except that it has an extra carbon (methylene group) in its side chain (Table 1). Because of the involvement of other arginine and arginine metabolites in vascular homeostasis2-7, this structural similarity has recently inspired numerous clinical studies exploring the relationship between plasma homoarginine levels and human disease states8-12. Unlike the methylated arginine metabolites, which are competitive inhibitors of NO synthases (6), homoarginine can serve as a NOS substrate (albeit not a very good one13-14). Since NO is generally viewed as a biologically salutary molecule, it seemed like a plausible
hypothesis that low plasma homoarginine levels would be associated with cardiovascular disease states, and higher homoarginine levels might be good for you. This hypothesis appears to be largely correct: except in one clinical study with somewhat contrary findings, low plasma homoarginine levels have been found to be associated with increased morbidity or mortality in patients with coronary artery disease, cerebrovascular disease, diabetes, chronic kidney disease, and other diseases. What is less clear is the molecular mechanism for these associations.

The study reported by Choe et al provides important new clues about homoarginine, while raising interesting new questions. Consistent with previous papers reporting an association between higher plasma homoarginine levels and reduced cardiovascular morbidity or mortality, Choe et al showed that higher homoarginine was independently associated with a reduction in mortality in stroke patients. The authors speculate that their data “suggest that homoarginine may positively influence the beneficial pathways of NO”, although no direct measurements of the activity of the NO pathway were reported in this study. This still seems like a plausible hypothesis, yet a direct effect of homoarginine on eNOS catalysis may be less likely: homoarginine appears to be a relatively poor NOS substrate in vivo and in vitro, and may even lead to NOS uncoupling. But the Cho et al paper goes well beyond prior clinical reports on homoarginine by performing genome-wide association studies (GWAS) analyzing single nucleotide polymorphisms in stroke patients stratified by plasma homoarginine levels. These analyses led to an intriguing and novel discovery that low plasma homoarginine levels are significantly associated with polymorphisms in the gene encoding arginine:glycine amidinotransferase (AGAT; Figure 1). AGAT encodes an enzyme known to be expressed in the kidney and mostly studied for its role in creatine metabolism- but more recently AGAT was shown to be involved in the synthesis of homoarginine. Another significant GWAS finding in
the Choe et al study\textsuperscript{8} was in the ornithine transcarbamoylase (OTC) gene, but this was a weaker association and was not followed up in detail; it is interesting to note that polymorphisms in OTC (a key enzyme in the urea cycle) have been previously implicated in GWAS studies of hypertension and stroke\textsuperscript{16}. Focusing in detail on the AGAT polymorphisms that they had discovered, the authors\textsuperscript{8} found that most of these polymorphisms were located in intronic regions of the AGAT gene, documenting just one mis-sense mutation (exchange of a glutamine residue for histidine). The effect of this amino acid change on AGAT enzyme activity was not explored in this study, nor were levels of AGAT protein measured. Yet the authors still provide several clear and compelling lines of evidence suggesting that AGAT has a key role in homoarginine metabolism. They showed that overexpression of AGAT increased homoarginine levels in a cell model, and also analyzed AGAT\textsuperscript{null} knockout mice. They found that AGAT\textsuperscript{null} knockout mice had low plasma homoarginine levels, and also showed that these mice had increased morbidity in an experimental stroke model that could be ameliorated by homoarginine feeding. Conversely, in a distinct knockout mouse model in which the gene for a different arginine-metabolizing enzyme called guanidinoacetate methyltransferase (GAMT) was deleted, the mice had higher homoarginine levels and smaller experimental strokes. The authors conclude: “These results provide evidence that higher homoarginine levels attenuate stroke severity and improve outcome in mice, and suggest that homoarginine is causally involved in the (patho) physiology of ischemic stroke.” This seems like a reasonable inference, yet the molecular mechanisms remain incompletely defined. Moreover, this study did not show that plasma homoarginine levels improved risk prediction over conventional risk factors. So before we start crop-dusting the population with homoarginine, let’s take a closer look at how homoarginine fits in to a complicated network that involves not only AGAT and GAMT, but also many other complexly-
modulated enzymes and pathways that regulate arginine and its metabolites.

Let’s start with arginine, which has been the most extensively studied of these compounds. Figure 1 presents a simplified (but still complicated!) scheme showing many of the intersecting cycles of arginine metabolism that appear to be most clearly related to cardiovascular (patho)physiology. Please also see Table 1 for molecular structures and for the references supporting the summary presented in the paragraphs below. L-arginine levels in the plasma are in the ~100 μM range, and intracellular arginine levels appear to be even higher. The NOS $K_m$ for L-arginine is much lower than its overall intracellular concentration, but it appears that arginine and arginine-metabolizing enzymes may have differential subcellular distributions, raising the possibility that arginine concentrations may be limiting for NOS in specific subcellular locales. Moreover, the presence of endogenous competitive inhibitors of NOS (discussed below) may raise the effective intracellular arginine concentration needed for full enzyme activity. In any event, the action of NOS on L-arginine yields NO plus L-citrulline, which can be recycled back to L-arginine by the sequential reactions catalyzed by arginosuccinate synthase (ASS) and arginosuccinate lyase (ASL). But it appears that most of the plasma L-arginine comes either from the diet or from recycling/proteolysis of cellular proteins, with a smaller fraction synthesized from other amino acids or recycled from L-arginine (at least in most cell types). The traffic of extracellular L-arginine (and other cationic amino acids, including L-lysine) into cells occurs via specific cell surface amino acid transporters, of which CAT-1 and related transporters are the best characterized. Arginine may also be catabolized by L-arginine:glycine amidinotransferase (AGAT), the enzyme recently implicated in homoarginine metabolism (15), as discussed above. But in most cell types, arginine catabolism is principally catalyzed by a family of arginase enzymes, which yield ornithine and urea; the ornithine that is...
formed by arginase can by metabolized back to L-arginine in multiple enzymatic steps.

Arginases are central to arginine flux in the cell because these enzymes may represent the quantitatively most important pathway for arginine catabolism- and arginase activity appears to be inhibited by homoarginine.

Let’s now move on to the methylated arginine compounds, which have been definitively linked to cardiovascular disease– and might represent another potential pathway whereby homoarginine may have a role. Mono- and asymmetrically $\text{N}^\text{O}$-dimethylated arginine (known as NMMA or ADMA, respectively) are potent NOS inhibitors, while symmetrically dimethylated arginine (SMA) is neither a NOS substrate or inhibitor. To form these arginine homologs, the terminal guanido nitrogens of arginine residues in proteins are methylated by a family of protein arginine methyltransferases (PRMT). Methylarginines play a broad role in cellular regulation well beyond their effects on NOS: these compounds are implicated in transcriptional regulation and in the metabolism of DNA and RNA$^7$. As is the case for arginine, methylated arginine compounds are released from cellular proteins by proteolysis, possibly in the context of cellular autophagy or apoptosis; increased levels of ADMA and NMMA are clearly implicated in vascular pathology and in the inhibition of NOS activity. The catabolism of ADMA and NMMA can be catalyzed either by arginases or by a family of dimethyl diaminoarginine hydrolase enzymes (DDAH), yielding citrulline, which can then be recycled back to arginine. It is possible that homoarginine might inhibit the actions of PRMT, thereby reducing levels of NMMA and ADMA and thus enhancing NOS activity. Arguing against this notion is the fact that plasma levels of ADMA and NMMA were unchanged in the AGATnull knockout mouse studied by Choe et al$^8$, although intracellular levels of these compounds were not measured.
Compared to the large body of clinical and biochemical data on the roles of arginine and methylated arginine compounds, much less is known about the metabolic transformations and biochemical roles of homoarginine (Figure 1 and Table 1). In mammals, homoarginine can come either from the diet or by synthesis from endogenous precursors; plasma homoarginine levels are about 100-fold lower than those of L-arginine. Homoarginine can serve as a NOS substrate, yielding NO plus homocitrulline as co-products. Yet the NOS Kₘ for homoarginine is about 20-fold higher than for arginine. Moreover, homoarginine is not a very effective substrate for NOS, and it’s not clear than higher homoarginine levels would necessarily lead directly to significant increases in NO synthesis. There are even some reports that homocitrulline— the enzymatic product of NOS action on homoarginine— might be associated with atherogenesis. Although homoarginine can serve as an arginase substrate with a low Kₘ, it’s not a very good arginase substrate, and it is plausible that homoarginine could actually function as an arginase inhibitor and thereby lead to an increase in intracellular arginine. But since intracellular arginine levels are already well in excess of the NOS Kₘ for its substrate, the direct consequences of homoarginine-dependent arginase inhibition on NOS activity are less obvious. Clinical studies have reported that chronic arginine supplementation may have deleterious effects, raising caveats about the possible long-term consequences of homoarginine supplementation- despite the clearly beneficial effects of short-term homoarginine supplementation in mouse stroke models, as reported by Choe et al. The AGATnull and GMATnull mouse knockout models that were carefully studied by Choe et al provide additional intriguing clues to possible mechanisms whereby homoarginine may modulate vascular (patho) physiology. Homoarginine can be formed from L-arginine and the amino acid L-lysine by the actions of our now-familiar arginine:glycine amidinotransferase...
enzyme (AGAT), as shown in Figure 1. AGAT is a mitochondrial enzyme that is expressed most abundantly in the kidney, and is critically involved in the synthesis of creatine, a vital metabolite in energy metabolism. Marked decreases in both homoarginine and in creatine were documented in plasma from AGATnull mice, associated with significant increases in the size of experimental stroke8. Yet previous studies of AGATnull mice documented multiple other metabolic changes in these knockout mice18. The metabolic phenotype of AGATnull mice is quite complex, and the link between homoarginine and stroke needs to be viewed in the context of numerous biochemical derangements in this knockout mouse model. Nonetheless, the fact that homoarginine but not creatine supplementation improved the stroke phenotype provides strong suggestive evidence for a central role for homoarginine in attenuating the severity of stroke in this mouse model. But despite the intriguing associations between homoarginine levels and stroke protection in these informative mouse models, the links between AGAT polymorphisms, homoarginine, and vascular disease in humans likely reflect a greater complexity.

In humans, AGAT expression appears to be highest in kidney and liver. Yet AGAT is also expressed in the myocardium, where levels of AGAT appear to be dynamically regulated in heart failure19. While arginine is clearly required for AGAT catalysis, the enzyme is somewhat promiscuous in its choice of co-substrates: AGAT generates homoarginine when lysine is used as the co-substrate, or instead promotes creatine synthesis when glycine is used (Figure 1). This enzymatic promiscuity may also depend on the specific tissue in which AGAT is expressed as well as the relative availability of lysine vs. glycine for catalysis. Since creatine synthesis can lead to the generation of the pro-atherogenic metabolite homocysteine20, subtle changes in the expression and activity of AGAT may have a marked effect on vascular pathobiology. Perhaps increased levels of plasma homoarginine are associated with decreased levels of homocysteine,
reflecting a change in the balance of AGAT activity between using lysine as a co-substrate (leading to homoarginine synthesis) instead of glycine (yielding creatine and homocysteine). Might lysine be the link? We don’t know, but it’s important to note that all but one of the AGAT polymorphisms identified in the Choe et al study are located in intronic regions, which might possibly lead to differences in AGAT expression or tissue distribution.

Clearly, more remains to be learned about the molecular mechanisms whereby differences in plasma homoarginine levels modulate stroke severity. It should now be clear that arginine metabolism can’t be viewed as a single pathway, or even as the confluence of multiple pathways. Rather, arginine metabolism must be viewed as a biochemical system, allowing for multiple points of connection and regulation within any particular cell type and for differing patterns of tissue-specific enzyme expression and modulation.

One of the best known short stories in Bradbury’s “R is for Rocket” is called “A Sound of Thunder”, which relates the tale of a time traveler who interferes with the thread of time by accidentally treading on a butterfly during his journey into the past. When the time traveler returns to the present after his mishap with the butterfly, he finds subtle but irrevocable changes in the world that he left behind to travel into the past. So it is for arginine metabolism: subtle changes in levels of one metabolite may lead to profound changes in the levels or biological activities of distantly-related metabolites. No matter what molecular mechanisms are ultimately identified in unraveling the association between homoarginine and disease, the Choe et al study serves as an exemplar of the need to view levels of specific metabolites as reflecting the products of interrelated and dynamic metabolic systems.

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### Table 1. Properties of selected arginine metabolites.

<table>
<thead>
<tr>
<th>Compound (key references)</th>
<th>Structure</th>
<th>eNOS $K_m$ or (IC$_{50}$)</th>
<th>Plasma/intracellular levels ($\mu$M)</th>
<th>Source or synthetic pathway</th>
<th>Major catabolic pathways</th>
<th>Relation of plasma levels to CV disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Arginine$^{12,5,13,14}$</td>
<td><img src="image" alt="Structure" /></td>
<td>$\sim3\mu$M</td>
<td>$\sim60$-$100/\gg200\mu$M</td>
<td>Proteolysis $\rightarrow$ diet, recycling via ASS/ASL, de novo synthesis</td>
<td>NOS, Arginases, AGAT</td>
<td>No clear relation. Acute infusion improves vascular function, but long-term supplementation may be harmful.</td>
</tr>
<tr>
<td>L-Homoarginine$^{8-14}$</td>
<td><img src="image" alt="Structure" /></td>
<td>$\sim70\mu$M</td>
<td>$\sim1\mu$M/?</td>
<td>Diet, recycling, OTC/ASS/ASL or via AGAT</td>
<td>NOS, Arginases?, others?</td>
<td>$\uparrow$ levels seen in stroke, CV disease; $\downarrow$ Levels are protective. Suppléments $\downarrow$ stroke in mice.</td>
</tr>
<tr>
<td>L-Citrulline$^{12}$</td>
<td><img src="image" alt="Structure" /></td>
<td>Not a NOS inhibitor/substrate</td>
<td>$\sim20$-$100/\sim20$-$30\mu$M</td>
<td>NOS product, product of DDAHs, diet</td>
<td>“citrulline cycle” via ASS/ASL</td>
<td>No clear relation to CV disease.</td>
</tr>
<tr>
<td>ADMA$^{2-7}$</td>
<td><img src="image" alt="Structure" /></td>
<td>$(-1\mu$M)</td>
<td>$0.4/\sim4\mu$M</td>
<td>PRMTs $\rightarrow$ proteolysis</td>
<td>DDAH-1 and DDAH-2, arginases</td>
<td>$\uparrow$ levels seen in CV disease</td>
</tr>
<tr>
<td>SMA$^{2-6}$</td>
<td><img src="image" alt="Structure" /></td>
<td>Not a NOS inhibitor/substrate</td>
<td>$\sim1\mu$M/?</td>
<td>PRMTs $\rightarrow$ proteolysis</td>
<td>Renal excretion</td>
<td>No clear relation to CV disease.</td>
</tr>
<tr>
<td>NMMA$^{2-6}$</td>
<td><img src="image" alt="Structure" /></td>
<td>$(-1\mu$M)</td>
<td>$0.4/\sim3\mu$M</td>
<td>PRMTs $\rightarrow$ proteolysis</td>
<td>DDAH-1 and DDAH-2, arginases</td>
<td>$\uparrow$ levels seen in CV disease</td>
</tr>
</tbody>
</table>

This table summarizes basic features of several major arginine metabolites relevant to cardiovascular disease states. The first column lists principal arginine metabolites and provides key references (mostly review articles) that support the information provided in this table and in the text; because of space limitations, the numerous relevant original research papers can’t be listed here. The structure of L-arginine (second column) differs from L-homoarginine by the additional methylene group present in the homoarginine side chain. The third column shows the concentration of the compound that yields half-maximal enzyme activity ($K_m$) for active substrates, or (in parentheses) the concentration that yields half-maximal suppression of enzyme activity for inhibitor compounds (IC$_{50}$). The concentrations of substrates supporting half-maximal eNOS enzyme activity ($K_m$ values) have been extensively validated for L-arginine in studies of the purified enzyme. In contrast, the eNOS $K_m$ value for L-homoarginine is based either on analyses of crude cell lysates or on studies of other NOS isoforms. The concentrations of NOS inhibitors yielding half-maximal enzyme inhibition (IC$_{50}$) are mostly determined in studies in which ADMA or NMMA are added to cultured endothelial cells and eNOS activity is estimated, and the IC$_{50}$ values for inhibitors depend on the extracellular arginine concentration. ADMA and NMMA appear to be competitive inhibitors of eNOS, while SMA serves as neither a substrate nor inhibitor of NOS isoforms. Plasma levels for these compounds have been reported in many studies, but the intracellular concentrations have been less extensively characterized. The relative roles of different synthetic and catabolic pathways are best known for arginine, and less so for the other arginine metabolites. Please see the text and Figure 1 for further discussion.
Figure Legend:

Figure 1. Principal pathways of arginine and homoarginine metabolism. Please see the text for details and for definition of abbreviations used in this figure.
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