A Peculiar Result and a Fanciful Hypothesis Regarding l-Arginine

To the Editor:

In the January 2003 issue of this Journal, Chen et al observed that l-arginine supplementation did not affect lesion formation in the hypercholesterolemic apolipoprotein E knockout mice, and it negated the protective effect that iNOS gene deficiency has in this model. Their findings are discordant with those observed in most other hypercholesterolemic models in which the administration of supplemental l-arginine improves vasodilation, increases endothelial synthesis of NO, reduces the generation of superoxide anion, suppresses the activation of oxidant sensitive transcriptional proteins, and reduces monocyte adhesion, infiltration, and lesion formation.1-7 In most hypercholesterolemic models, l-arginine appears to be rate-limiting for the synthesis of NO. This may be due to increases in plasma asymmetric dimethylarginine (ADMA, an endogenous NOS inhibitor), expression of tissue arginase, or NO synthase uncoupling.8-10

Attempting to explain the results of Chen et al, in his editorial Loscalzo11 proposes the fanciful hypothesis that supplemental l-arginine could increase plasma levels of homocyst(e)ine, a substance known to induce endothelial dysfunction and atherosclerosis. Unfortunately, this hypothesis lacks any mechanistic basis or experimental support. Loscalzo notes correctly that l-arginine is a precursor for guanidinoacetate (GAA), a reaction catalyzed by l-arginine:glycine amidinotransferase (AGAT). The methylation of GAA by S-adenosylmethionine yields creatine and S-adenosylhomocysteine. Loscalzo speculates that supplemental arginine could increase homocysteine by this pathway. However, the article he cites to support his speculation revealed that GAA, not l-arginine, increased homocysteine levels in Sprague-Dawley rats.12 Notably, GAA markedly suppressed the activity of AGAT. The negative regulation of AGAT activity by GAA would inhibit the conversion of l-arginine to GAA. Thus, the cited article does not provide evidence that l-arginine supplementation would increase homocysteine levels.

Not only is there absence of evidence, there is evidence of absence. l-Arginine administration does not increase plasma homocysteine levels in humans. In patients with peripheral arterial disease and hyperhomocyst(e)inemia, oral l-arginine supplementation (24g/d for 8 weeks) did not affect homocyst(e)ine levels.13 In another study, intravenous infusion of l-arginine (3 g) in diabetic patients actually reduced plasma homocyst(e)ine.14 Notably, we have shown that homocysteine inhibits NO synthesis by increasing the elaboration of ADMA.15 Homocysteine impairs the activity of DDAH (dimethylarginine dimethylaminohydrolase), the enzyme that degrades ADMA. Because it has a critical sulfhydryl in its active site, DDAH is vulnerable to oxidative attack.16 Homocysteine forms a mixed disulfide with DDAH, and thereby reduces the degradation of ADMA.18 This mechanism accounts for our recent observation that experimental hyperhomocysteinemia in humans induces an endothelial vasodilator dysfunction that is associated with an increase in plasma ADMA, and that is reversed by intravenous infusion of l-arginine (Stuhlinger MC, Oka RK, Graf EE, Schmolzer I, Upson BM, Kapoor O, Szuba A, Malinow MR, Wascher TC, Pachinger O, Cooke JP, unpublished observations, 2003).

To conclude, l-arginine does not increase plasma homocysteine levels in most hypercholesterolemic and atherosclerotic humans, the majority of investigators report that l-arginine supplementation improves vascular function. Supplemental l-arginine may exert this beneficial effect by opposing pathological increases in plasma levels of ADMA, tissue arginase, or NOS uncoupling.

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In Response:

Our original intent was to test the hypothesis that l-arginine supplementation would reduce atherosclerosis in apolipoprotein E (apoE) knockout mice by providing substrate for eNOS.1 In light of recent data that the iNOS isoform is potentially proatherogenic,2,3 we were concerned that l-arginine might also increase iNOS-derived NO, confounding our results. Thus, we studied both apoE knockout mice and apoE/iNOS double-knockout mice, reasoning that l-arginine could not serve as substrate for iNOS in the double

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knockout mice. We were surprised to find that L-arginine supplementation did not reduce atherosclerotic burden in the apoE knockout mice, a result in agreement with other reports of the apoE knocked out mouse model of atherogenesis. Furthermore, we found that L-arginine supplementation eliminated the protective effect of iNOS deficiency in the apoE/iNOS double-knockout mice. To examine potential mechanisms for these effects, we measured oxidized and reduced biotinier levels, as well as MDA-TBA reactive material as markers of lipid oxidation.

In his accompanying editorial, Loscalzo pointed out another potential mechanism: that L-arginine may increase plasma levels of homocysteine, because creatine synthesis accounts for the major fraction of total body arginine utilization and results in a significant methylation demand. Loscalzo’s analysis of the literature and our unexpected results was, we believe, extremely logical and cogent. Thus, the hypothesis certainly merits experimental testing.

We have three comments regarding Cooke’s and Sydow’s letter. First, they do not acknowledge that, while some studies suggest a beneficial effect of L-arginine on atherosclerosis and endothelial function, other studies fail to show such effects. A review of the literature suggests that this issue is far from clear. Thus, it becomes all the more important to establish under what conditions L-arginine may be beneficial and may in fact be contraindicated.

Second, their letter misstates that Loscalzo cites the article by Stead et al to provide evidence that L-arginine supplementation increases homocysteine levels. Rather, if one reads the editorial carefully, Loscalzo cites numerous references that show that creatine synthesis represents the major fraction of total body arginine utilization and results in a significant methylation demand. Loscalzo’s analysis of the literature and our unexpected results was, we believe, extremely logical and cogent. Thus, the hypothesis certainly merits experimental testing.

Third, we agree that hyperhomocysteinemia impairs endothelial function. However, these effects do not negate the possibility that L-arginine supplementation may, under certain conditions, increase homocysteine levels.

We believe the results of our study and the issues raised by Loscalzo in his editorial are best resolved by experimental study, so that we can determine when and if L-arginine supplementation is beneficial and, equally importantly, when it might not be.

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Paul Huang
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Looking only at the group means, the results might seem as there is no difference between measurements from serum and diluted plasma. However, if we look at the individual data (Figure) it can be seen that although there is good agreement between the duplicates of serum samples (an average 5.9% difference from the mean), the variation is large between the diluted plasma samples of the same individuals, and in 3 of the 5 samples, zero activity was measured in one or both of the duplicates.

In agreement with recent reports\(^5\)\(^6\) and the product information of the ELISA kit,\(^2\) our results also show that measuring sCD40L from EDTA-treated plasma samples is unreliable. Therefore, we also suggest measuring sCD40L activities from serum and not from plasma samples.

### In Response:

We share the concern of Dr Bereczki and colleagues regarding the importance of using appropriate specimens in the currently available sCD40L ELISAs (Bender MedSystem and R&D) and have referred to this important issue in a recent publication (Schonbeck et al\(^1\)). Several issues are pertinent to the concerns of Dr Bereczki et al:

1. First and foremost, it is of utmost importance to avoid activation of platelets during the post-harvesting sample procedure, because sCD40L can be released within minutes in high concentrations from this cell type. Indeed, platelets are commonly considered the predominant source of sCD40L in the bloodstream. Thus, the sCD40L concentrations obtained in serum, as generated by clotting of whole blood, should be interpreted with care. Indeed, in a previous report, Garlichs et al\(^2\) used serum to measure sCD40L levels and reported an average of 25 ng/mL sCD40L, even in healthy individuals (typically thought to have sCD40 levels <2 ng/mL). Such concentrations might be considered the "absolute blood level of sCD40L," combining freely circulating sCD40L and CD40L expressed in circulating cells, namely platelets, T cells, and mononuclear phagocytes. Therefore, the authors (and other groups with whom the authors have communicated) consider the use of serum specimens to determine circulating sCD40L levels to be questionable.

2. In light of the above, it is commonly considered crucial to remove platelets from blood preparations without activation if one desires to determine the levels of freely circulating sCD40L. Indeed, the authors have performed extensive studies comparing levels of sCD40L in serum, platelet-rich plasma, platelet-poor plasma and plasma centrifuged at 2000g (the technique the authors used). Platelet counts and sCD40L levels detected in these healthy subjects are shown in the Table, demonstrating that, in our hands, removal of platelets is crucial for appropriate analysis.

3. The authors have informed Bender MedSystems about these circumstances, suggesting respective modifications in their protocol.

4. Using plasma, the authors have furthermore compared Heparin, Citrate, and EDTA as anticoagulants. Although Citrate provided the highest reproducibility, EDTA also yielded reliable and reproducible read-outs (variation coefficient<10%). These data were verified in "spiking experiments," in which recombinant sCD40L was added to plasma samples of healthy or atherosclerotic subjects and recovery rates determined.

5. Finally, the data provided by Bereczki et al appear somewhat unclear to the authors. Their Figure indicates that serum and diluted plasma samples (stated to have been prepared in a
fashion similar to that used by the authors) indeed provide comparable data (with cases 1, 3, and 5, demonstrating rather marginal standard variations). Also, it remains to be determined why the high standard deviations were observed in only 2 of 5 samples; if EDTA comprises a more generic problem for the ELISA, values would be expected “all over the place.” Unfortunately however, Bereczki et al do not provide any information regarding the platelet contamination in their preparations, information that might be crucial to understand their findings better.

The authors hope that the above discussion reveals the rationale of avoiding the use serum in our studies on sCD40L and, furthermore, affirms the reliability of the data provided in our original manuscript.

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<tr>
<th>G force</th>
<th>Platelets, $\times 10^3$/mL</th>
<th>sCD40L, ng/mL</th>
</tr>
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<tbody>
<tr>
<td>PRP 200g</td>
<td>5480</td>
<td>20.37</td>
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<tr>
<td>PPP 800g from cellular fraction after collecting PRP</td>
<td>100</td>
<td>0.81</td>
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<tr>
<td>Plasma 2000g</td>
<td>Nondetectable</td>
<td>0.27</td>
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<tr>
<td>Plasma 10 000g</td>
<td>Nondetectable</td>
<td>1.02</td>
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<tr>
<td>Serum</td>
<td>21</td>
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Platelet Counts and sCD40L Levels

PRP indicates platelet-rich plasma; PPP, platelet-poor plasma.


Should Soluble CD40 Ligand Be Measured From Serum or Plasma Samples?
Dániel Bereczki, Emőke Nagy, András Pál, Mária T. Magyar and József Balla

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