Oral atorvastatin therapy increases nitric oxide-dependent cutaneous vasodilation in humans by decreasing ascorbate-sensitive oxidants

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Holowatz LA, Kenney WL. Oral atorvastatin therapy increases nitric oxide-dependent cutaneous vasodilation in humans by decreasing ascorbate-sensitive oxidants. Am J Physiol Regul Integr Comp Physiol 301: R763–R768, 2011. First published June 29, 2011; doi:10.1152/ajpregu.00220.2011.—Elevated low-density lipoproteins (LDL) are associated with cutaneous microvascular dysfunction partially mediated by increased arginase activity, which is decreased following a systemic atorvastatin therapy. We hypothesized that increased ascorbate-sensitive oxidant stress, partially mediated through uncoupled nitric oxide synthase (NOS) induced by upregulated arginase, contributes to cutaneous microvascular dysfunction in hypercholesterolemic (HC) humans. Four microdialysis fibers were placed in the skin of nine HC (LDL = 177 ± 6 mg/dl) men and women before and after 3 mo of a systemic atorvastatin intervention and at baseline in nine normocholesterolemic (NC) (LDL = 95 ± 4 mg/dl) subjects. Sites served as control, NOS inhibited, L-ascorbate, and arginase-inhibited + L-ascorbate. Skin blood flow was measured while local skin heating (42°C) induced NO-dependent vasodilation. After the established plateau in all sites, 20 mM 1001 1002 1003 1004 was infused to quantify NO-dependent vasodilation. Data were normalized to maximum cutaneous vascular conductance (CVC) (sodium nitroprusside + 43°C). The plateau in vasodilation during local heating (HC: 78 ± 4 vs. NC: 96 ± 2% CVCmax, P < 0.01) and NO-dependent vasodilation (HC: 40 ± 4 vs. NC: 54 ± 4% CVCmax, P < 0.01) was reduced in the HC group. Acute L-ascorbate alone (91 ± 5% CVCmax, P < 0.001) or combined with arginase inhibition (96 ± 3% CVCmax, P < 0.001) augmented the plateau in vasodilation in the HC group but not the NC group (ascorbate: 96 ± 2; combo: 93 ± 4% CVCmax both P > 0.05). After the atorvastatin intervention NO-dependent vasodilation was augmented in the HC group (HC postatorvastatin: 64 ± 4% CVCmax, P < 0.01), and there was no further effect of ascorbate alone (58 ± 4% CVCmax, P > 0.05) or combined with arginase inhibition (67 ± 4% CVCmax, P > 0.05). Increased ascorbate-sensitive oxidants contribute to hypercholesteromic associated cutaneous microvascular dysfunction which is partially reversed with atorvastatin therapy. 

CHOLESTEROLEMIA WITH elevated oxidized low-density lipoprotein (oxLDL) is a major risk factor for the development of atherosclerosis (21, 38, 40). One early indicator of preatherosclerotic vascular disease is a decrease in endothelial-derived nitric oxide (NO) and an increase in oxidant stress characterized by increased production of oxidants and decreased antioxidant clearing mechanisms (3, 6, 32–34). However, the precise characterization of these mechanisms in humans and how they contribute the hypercholesterolemia-induced microvascular dysfunction remains unclear.

The human cutaneous circulation is an accessible, representative regional circulation for investigating mechanisms of microvascular dysfunction (1, 5, 7, 13). Microvascular dysfunction occurring in the cutaneous circulation parallels changes that occur in the conduit arteries (7) and renal vascular beds (4). We have recently demonstrated that the reduction in cutaneous NO-dependent vasodilation with hypercholesterolemia is, in part, mediated by an increase in arginase activity (16). Increased arginase activity reduces substrate availability for NO synthase (NOS) and induces uncoupling of the enzyme into its monomeric form resulting in decreased functional NO synthesis and increased oxidant stress through augmented superoxide production (25). Furthermore, 3 mo of atorvastatin intervention decreases arginase activity, possibly through one of the pleiotropic effects [i.e., the overall benefits observed with statins appear to be greater than what might be expected from changes in lipid concentrations alone (27)] of the statin (3-hydroxy-3-methyl-glutaryl-CoA reductase-CoA reductase inhibitors). The roles of increased global oxidant stress alone, and more specifically from uncoupled NOS induced by increased arginase activity resulting in increased superoxide production, have not been explored.

Acute administration of the antioxidant ascorbate in superphysiological concentrations is a commonly utilized method for exploring oxidant stress mechanisms in humans (8, 9, 14, 17, 26, 31). While there are limitations due to the nonspecific oxidant-quenching capabilities of ascorbate, it improves NOS function across the vascular tree in a variety of disease states; however, these oxidant stress mechanisms and how they relate to upregulated arginase have not been systematically explored in hypercholesterolemic human skin. Therefore, the aim of this study was to determine the effects of acute localized ascorbate treatment alone and in combination with arginase inhibition in attenuated cutaneous NO-dependent vasodilation in hypercholesterolemic humans before and after 3 mo of a systemic atorvastatin intervention. We hypothesized that acute ascorbate treatment would augment NO-dependent vasodilation induced by a standardized local heating protocol (24, 29), and this would be amplified when arginase was inhibited. We further hypothesized that a short-term oral atorvastatin intervention would augment NO-dependent vasodilation through a decrease in ascorbate-sensitive oxidant mechanisms.

MATERIALS AND METHODS

Subjects. Experimental protocols were approved by the Institutional Review Board at The Pennsylvania State University and conformed to the guidelines set forth by the Declaration of Helsinki. Verbal and written consent was voluntarily obtained from all subjects prior to participation. This study was part of a larger series of studies utilizing the same participants; therefore the subject characteristics are the same as those that have been previously published (16). The order of these experiments was randomized. Nine healthy normocholesterolemic and nine hypercholesterolemic men and women (Table 1)
participated in this study consisting of functional in vivo assessment of cutaneous NO-dependent vasodilation during local skin warming. The hypercholesterolemic subjects were tested before and after a 3-mo atorvastatin intervention (10 mg daily). The normocholesterolemic control group was only tested once at enrollment. The subjects’ ages ranged from 40 to 62 yr and the groups (hypercholesterolemic and normocholesterol- olemic) were age-matched to account for any possible age-related changes in the local heating response (30). Furthermore, the subjects were nonobese, nonsmokers, nondiabetic, normally active (neither sedentary nor highly exercise trained), and not currently taking statins or other medications, including aspirin, vitamins, or antioxidants.

**Blood analysis.** Serum and plasma samples were obtained at enrollment and after the atorvastatin intervention and stored at −80°C for batched analysis of asymmetrical dimethyl L-arginine (Alpco Diagnostics, Salem, NH) and oxLDL (Mercodia, Uppsala, Sweden).

**In vivo vasoreactive studies.** All protocols were performed in a thermoneural laboratory with the subject semisupine and the experimental arm at heart level. Four intradermal microdialysis probes were inserted into the ventral forearm skin for localized delivery of pharmacological agents as previously described (15, 18). Microdialysis sites were perfused with: 1) 20 mM N\(^{4}\)-nitro-L-arginine methyl ester (L-NAME) to inhibit NO production by NOS throughout the duration of the local heating protocol; 2) 10 mM L-ascorbate to locally supplement supraphysiological concentrations of the antioxidant (14); or 3) a combination of 5.0 mM (S)-2-boronoethyl-L-cysteine-HCl (BEC) and 5.0 mM N\(^{6}\)-hydroxy-nor-L-arginine (nor-NOHA) to inhibit arginase (Calbiochem, San Diego, CA) and 10 mM ascorbate (FDA investigational drug number 78,954) (15); 4) lactated Ringer solution to serve as control. All pharmacological solutions were mixed immediately prior to usage, dissolved in lactated Ringer solution, and sterilized, using syringe microfilters (Acrodisc; Pall, Ann Arbor, MI). Solutions were also wrapped in foil to inhibit photodegradation of the agents. The concentrations of the pharmacological agents used in this study have been shown to be efficacious in younger, middle-aged, and older age groups by using the same intradermal microdialysis technique (13, 15, 18). Furthermore, the concentrations of the arginase inhibitor cocktail far exceed the KC for the inhibitors (BEC KC at pH 7.5 = 0.31 μM; nor-NOHA KC at pH 7.5 = 1.6 μM) (2).

An index of skin blood flow was measured using integrated laser-Doppler flowmetry probes, and local temperature was controlled with a local heater (MoorLAB Temperature Monitor SH02; Moor Instruments, Devon, UK) placed directly above each microdialysis membrane. This multipoint probe monitored blood flow from the area covered by the local heater directly over each microdialysis fiber. Arterial blood pressure was measured every 5 min using an automated brachial cuff (Cardiocap), which was verified with brachial auscultation. Cutaneous vascular conductance (CVC) was calculated as laser-Doppler flux divided by mean arterial pressure.

**Local heating protocol.** After the resolution of the initial insertion trauma with local skin temperature clamped at 33°C, a standardized local skin-warming protocol was performed to induce NO-dependent vasodilation (29). Local heating induces cutaneous vasodilation via two independent phases. The first phase of the local heating response includes an initial peak and nadir that are primarily mediated by sensory and adrenergic sympathetic nerve mechanisms (11, 12, 20) with a small NO contribution; the secondary phase is a slow rise to a predominantly NO-dependent plateau. This NO-dependent vasodilation at the plateau can be quantified by inducing the non-isof orm-specific NOS inhibitor L-NAME (29). The local heater temperature was increased from 33°C to 42°C at a rate of 0.1°C every second and then clamped at 42°C for the duration of the heating protocol. After skin blood flow reached an established plateau (30–40 min), 20 mM L-NAME was perfused to quantify NO-dependent vasodilation at all sites. Following a new post-L-NAME stabilization in skin blood flow, local temperature was increased to 43°C, and 28 mM sodium nitroprusside (SNP) was perfused to induce maximal cutaneous vasodilatation (CVC\(_{\text{max}}\)) (19, 23). In our previous work and in pilot work, this combination of heat and high concentration of SNP has been shown to induce maximal vasodilation. Higher temperatures (44°C) or increasing concentrations of SNP (50 mM) did not produce a further increase in absolute CVC (19).

**Data and statistical analysis.** Data were collected continuously and digitized at 40 Hz and stored for offline analysis with signal-processing software (Windaq; DATAQ Instruments). Skin blood flow data were normalized to a percent of maximal CVC (%CVC\(_{\text{max}}\)), and CVC data were averaged for a stable 5 min of baseline, plateau, post-L-NAME plateau, and maximal vasodilatation. Due to the transient nature of the local warming response, the initial peak and nadir CVC were visually identified as the highest and lowest values and averaged over 10 s. The L-NAME sensitive portion of local heating-induced vasodilation was calculated from the difference between the plateau and the post-L-NAME plateau. Because the late plateau phase of the local heating response is primarily dependent on NOS function, whereas the early phase has contributions from both sensory nerves and NOS (24, 29, 42), analysis and further discussion focus on the later phase of the cutaneous vasodilatory response.

Student’s unpaired t-tests were used to determine significant differences between the groups, and Student’s paired t-tests were used to determine the effects of the statin intervention on blood characteristics. A mixed-models, three-way, repeated-measures ANOVA was conducted to detect differences in %CVC\(_{\text{max}}\) between subject groups and for the statin intervention at the pharmacological treatment sites for the different phases of the local warming response (version 9.1; SAS). Specific planned comparisons with Bonferroni correction were performed when appropriate to determine where differences between groups, statin intervention, and localized drug treatments occurred. The level of significance was set at \(\alpha = 0.05\). Values are presented as means ± SE.

<table>
<thead>
<tr>
<th>Table 1. Subject characteristics</th>
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<tr>
<td>Subjects, men/women</td>
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<tr>
<td>Age, yr</td>
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<tr>
<td>Total cholesterol, mg/dl</td>
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<tr>
<td>HDL, mg/dl</td>
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<td>LDL, mg/dl</td>
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<tr>
<td>Triglyceride, mg/dl</td>
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<tr>
<td>Glucose, mg/dl</td>
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<tr>
<td>ADMA, μmol/l</td>
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<td>Oxidized LDL, U/l</td>
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Values are means ± SE. HDL, high-density lipoprotein; LDL, low-density lipoprotein; ADMA, asymmetrical dimethyl L-arginine. *P < 0.001 difference from the normocholesterol group; \(\text{tP} < 0.001\) difference due to the atorvastatin intervention.
RESULTS

Subject characteristics are presented in Table 1. The subjects were well matched for blood pressure, fasting blood glucose, and anthropometric characteristics. By design the hypercholesterolemic subjects had significantly higher total cholesterol, LDL cholesterol, and oxLDL (all $P < 0.05$). Serum triglycerides were also higher in the hypercholesterolemic group ($P < 0.05$). The 3-mo atorvastatin intervention decreased total cholesterol, LDL cholesterol, and oxLDL cholesterol ($P < 0.001$). However, oxLDL remained increased relative to the normocholesterolemic group ($P < 0.001$). There were no differences in plasma asymmetrical dimethyl l-arginine (an endogenous NOS inhibitor) between groups or with the atorvastatin intervention.

There was no difference in baseline, initial peak, and nadir \%CVC_{max} values for both groups across the localized treatment sites and with the atorvastatin intervention ($P > 0.05$). Similar to our previous findings, the plateau in local heating response was attenuated in the hypercholesterolemic group compared with the normocholesterolemic group, which is illustrated in Fig. 1A ($P < 0.001$). However, there was no difference in the plateau after NOS inhibition with l-NAME between the groups. After the atorvastatin intervention, the plateau was augmented such that there was no difference compared with the normocholesterolemic group ($P = 0.11$ vs. normocholesterolemic group). The plateau after NOS inhibition with l-NAME was decreased after the atorvastatin intervention, indicating that NO-dependent vasodilation increased with the atorvastatin intervention. The reduction in vasodilation at the plateau sensitive to l-NAME is shown in Fig. 2A. The hypercholesterolemic subjects had significantly higher total cholesterol, LDL cholesterol, and oxLDL (all $P < 0.05$). Serum triglycerides were also higher in the hypercholesterolemic group ($P < 0.05$). The 3-mo atorvastatin intervention decreased total cholesterol, LDL cholesterol, and oxLDL cholesterol ($P < 0.001$).

Figure 1, B and C shows the effects of localized ascorbate treatment alone and in combination with arginase inhibition on the plateau and the plateau after NOS inhibition with l-NAME. In the hypercholesterolemic group, these localized treatments augmented the plateau ($P < 0.01$) and reduced the plateau after NOS inhibition, compared with the control site leading to overall increase in the amount of vasodilation sensitive to NOS-inhibition (Fig. 2, B and C; $P < 0.01$). However, there was no difference in the amount of vasodilation sensitive to arginase inhibition. Interestingly, these localized treatments did not alter the plateau or the plateau after NOS inhibition in the normocholesterolemic group ($P > 0.05$).

Figure 1D illustrates the plateau in sites where NOS was inhibited with l-NAME throughout the duration of the local heating protocol. There was no difference between groups or due to the atorvastatin intervention (all $P > 0.05$).

Finally, absolute CVC_{max} (flux/mmHg) are presented in Table 2. There were no differences between groups or due to either localized microdialysis treatment or with the atorvastatin intervention (all $P > 0.05$).

DISCUSSION

The major new findings of this study are that acute administration of superphysiological concentrations of ascorbate aug-

![Fig. 1. Mean skin blood flow. Percentage of maximal cutaneous vascular conductance (\%CVC_{max}) at the plateau (black bars) in skin blood flow during local warming and after nitric oxide synthase (NOS) inhibition with l-NAME (grey bars) in normocholesterolemic control subjects, hypercholesterolemic subjects, and after the oral atorvastatin intervention in the control site (A), the ascorbate site (B), the ascorbate + arginase-inhibited site (C), and plateau in the  \textit{N}^\textit{G}-nitro-l-arginine methyl ester (l-NAME; D) continuously throughout local heating site. The arrow in A depicts the difference between the plateau and the post-l-NAME plateau to describe the amount of vasodilation due to functional nitric oxide production illustrated in Fig. 2. *$P < 0.001$ difference from the normocholesterolemic group; †$P < 0.01$ difference compared with the control site due to the localized microdialysis drug treatment; ‡$P < 0.001$ difference due to the atorvastatin intervention.

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ments cutaneous NO-dependent vasodilation induced by local heating in hypercholesterolemic humans. Contrary to our hypothesis, there was not an additional effect of concurrent arginase inhibition in combination with ascorbate alone. Ascorbate treatment and, as previously observed, arginase inhibition alone (16) increased the plateau phase of the local heating response and the vasodilation sensitive to NOS inhibition. Mechanistically, this may be related to an already maximized NO pathway with the localized treatments or to a ceiling effect as the %CVC_{max} values were near maximum. Three months of oral atorvastatin intervention decreased total, LDL and oxLDL cholesterol and normalized the plateau phase in the local heating response comparable to the normocholesterolemic control group. In addition, the atorvastatin intervention resulted in a decrease in the post-L-NAME plateau, suggesting that there was an overall increase in functional NO-mediated vasodilation. Together these findings suggest that there was an increase in arginase activity, thus increasing hydrogen peroxide and, in turn, superoxide-mediated attenuation in the cutaneous NO-dependent vasodilation induced microvascular dysfunction. However, after the intervention, acute ascorbate treatment no longer had an effect on the skin’s local heating response, i.e., functional NO bioavailability was maximized. In addition to atorvastatin’s effects on arginase/NOS uncoupling mechanisms, the observed decrease in ascorbate-sensitive oxidants may have been partially mediated by decreased NADPH oxidase activity and by altering cellular antioxidant capacity. Recently, NADPH oxidases and xanthine oxidases have been implicated in the reactive oxygen species-mediated attenuation in the cutaneous local heating response with elevated angiotensin II (28). Atorvastatin has been shown to alter NADPH oxidase function by decreasing the translocation of rac1 from the cytosol to the cellular membrane, which is necessary for NADPH activation, and it decreases expression of two key NADPH subunits, including p22phox and nox1 (41). Atorvastatin also increases antioxidant capacity by increasing catalase expression and activity, thus increasing hydrogen peroxide and, in turn, superoxide clearance (41). In total, there are several potential mechanisms by which the atorvastatin intervention may have decreases the intracellular oxidant load.

Table 2. Absolute maximal cutaneous vascular conductance

<table>
<thead>
<tr>
<th>Condition</th>
<th>Normocholesterolemic</th>
<th>Hypercholesterolemic</th>
<th>Atorvastatin</th>
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<tr>
<td>Control site</td>
<td>2.49 ± 0.29</td>
<td>1.85 ± 0.26</td>
<td>2.38 ± 0.36</td>
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<tr>
<td>Ascorbate</td>
<td>1.92 ± 0.25</td>
<td>1.89 ± 0.14</td>
<td>2.52 ± 0.30</td>
</tr>
<tr>
<td>Ascorbate + arginase-inhibited site</td>
<td>1.99 ± 0.39</td>
<td>1.82 ± 0.23</td>
<td>1.73 ± 0.30</td>
</tr>
<tr>
<td>l-NAME site</td>
<td>2.57 ± 0.65</td>
<td>2.24 ± 0.43</td>
<td>2.24 ± 0.30</td>
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Values are means ± SE in flux/mmHg. l-NAME, N^2-nitro-l-arginine methyl ester.

In this study, we utilized acute ascorbate treatment to examine in vivo oxidant stress mechanisms and to functionally evaluate NO metabolism in a human model. This highly potent but nonspecific antioxidant is commonly utilized in human vascular studies because it is readily soluble, easily cleared by the kidneys, is effective at quenching a number of oxidant species, and is the first step in confirming the involvement of oxidant stress mechanisms in vascular dysfunction. However, ascorbate has many limitations in terms of being able to mechanistically interpret the data as it is impossible to delin-

Fig. 2. Reduction in CVC with NOS inhibition (difference between the plateau and the post-L-NAME plateau) in normocholesterolemic control subjects, in hypercholesterolemic subjects, and after the oral atorvastatin intervention in the control site (A), the ascorbate site (B), and the ascorbate + arginase-inhibited site (C). *P < 0.001 difference from the normocholesterolemic group; †P < 0.01 difference compared with the control site due to the localized microdialysis drug treatment; ‡P < 0.001 difference due to the atorvastatin intervention.
eate the precise oxidant-producing enzyme and even identity of the oxidant that is contributing to the elevated reactive oxygen species environment. Furthermore, ascorbate also has many indirect actions that may be affecting the arginase/NOS uncoupling mechanisms of interest, including the ability to inhibit arginase (through inhibiting S-nitrosylation) and the stabilizing and resynthesizing actions on the essential NOS cofactor tetrahydrobiopterin. In the present study, ascorbate alone or in combination with arginase inhibition produced the same functional effect. Either there was a ceiling effect in the response and thus it was maximized with one of the localized treatments alone or the ascorbate and the arginase inhibitors are working through redundant pathways. Although these are all reasonable possibilities, we can only conclude that ascorbate-sensitive oxidants contribute to microvascular dysfunction with hypercholesterolemia, but the precise sources remain unknown and further investigations into these mechanisms are warranted.

One interesting finding that l-NAME treatment throughout the local heating protocol (Fig. 1D) resulted in the same magnitude of vasodilation (~40% CVCmax) across groups and is somewhat incongruent with the data evaluating NO-dependent vasodilation within each treatment site by perfusing l-NAME at the plateau. It would be logical to hypothesize that the magnitude of the vasodilation should be increased in the hypercholesterolemic group and decreased with atorvastatin therapy. In our work with primary human aging we also find that the non-NO-dependent plateau is ~40% CVCmax and not altered by aging (30). At the present time, we can only speculate that non-NO-dependent vasodilation is mediated by redundant neurotransmitter mechanisms and results in a highly reproducible robust vasodilation across populations.

Limitations. We set out to examine the role of oxidant stress mechanisms in hypercholesterolemia-induced cutaneous microvascular dysfunction and the effects of a systemic atorvastatin intervention. The present data is strengthened by the fact that the same subject pool underwent testing to examine in vivo and in vitro arginase mechanisms reported elsewhere (16). As part of that study, skin biopsy samples were obtained to examine arginase activity, expression, and NOS3 expression. In the present data set, it would have been ideal to examine oxidant stress markers in the biopsy samples and other indicators of NADPH oxidase and xanthine oxidase activity. However, we were limited in the amount of skin tissue we could reasonably obtain from the participants. Furthermore, we only measured one plasma oxidant stress indicator. Plasma oxLDL did decrease with the statin intervention, but this is only a general indicator of oxidant stress and may not be reflective of the intracellular oxidant environment.

We and others commonly use acute supraphysiological concentrations of ascorbate in human in vivo models as a pharmacological tool to examine oxidant stress mechanisms as they pertain to vascular dysfunction. At physiological concentration (22) or when supraphysiological concentrations are administered chronically (9), there is little evidence for ascorbate’s functional effectiveness for increasing NO production or improving vascular function.

Due to the reported pleiotropic effects of statins on vascular function it would have been ideal to have the normcholesterolemic subjects undergo the intervention phase of the study. Because we did not observe a decrement in NO-dependent vasodilation in the normcholesterolemic study group (i.e., their responses were defined as normal), it is unlikely that we would have observed a functional improvement in vascular function with the intervention.

Summary. In summary, we found that ascorbate-sensitive oxidants contribute to hypercholesterolemic-induced attenuated cutaneous NO-dependent vasodilation, which is reversed with a 3-mo atorvastatin intervention. The possible mechanisms mediating this statin-induced increase in functional NO bioavailability include a reduction in NOS uncoupling due to a decrease in arginase activity and/or through NADPH oxidase production and clearance mechanisms.

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