Maternal Hypercholesterolemia in Pregnancy Associates With Umbilical Vein Endothelial Dysfunction
Role of Endothelial Nitric Oxide Synthase and Arginase II

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Objective—Human pregnancy that courses with supraphysiological hypercholesterolemia (MSPH) correlates with atherosclerotic lesions in fetal arteries. It is known that hypercholesterolemia associates with endothelial dysfunction in adults, a phenomenon where nitric oxide (NO) and arginase are involved. However, nothing is reported on potential alterations in the fetoplacental endothelial function in MSPH. The aim of this study was to determine whether MSPH alters fetal vascular reactivity via endothelial arginase/urea and l-arginine transport/NO signaling pathways.

Approach and Results—Total cholesterol <280 mg/dL was considered as maternal physiological hypercholesterolemia (n=46 women) and ≥280 mg/dL as MSPH (n=28 women). Maternal but not fetal total cholesterol and low-density lipoprotein-cholesterol levels were elevated in MSPH. Umbilical veins were used for vascular reactivity assays (wire myography), and primary cultures of umbilical vein endothelial cells to determine arginase, endothelial NO synthase (eNOS), and human cationic amino acid transporter 1 and human cationic amino acid transporter 2A/B expression and activity. MSPH reduced calcitonine gene–related peptide-umbilical vein relaxation and increased intrima/media ratio (histochemistry), as well as reduced eNOS activity (l-citrulline synthesis from l-arginine, eNOS phosphorylation/dephosphorylation), but increased arginase activity and arginase II protein abundance. Arginase inhibition increased eNOS activity and l-arginine transport capacity without altering human cationic amino acid transporter 1 or human cationic amino acid transporter 2A/B protein abundance in maternal physiological hypercholesterolemia and MSPH.

Conclusions—MSPH is a pathophysiological condition altering umbilical vein reactivity because of fetal endothelial dysfunction associated with arginase and eNOS signaling imbalance. We speculate that elevated maternal circulating cholesterol is a factor leading to fetal endothelial dysfunction, which could have serious consequences to the growing fetus. (Arterioscler Thromb Vasc Biol. 2013;33:2444-2453.)

Key Words: arginase ■ cholesterol ■ endothelium ■ nitric oxide ■ pregnancy
NO synthases. Because NO synthesis depends on l-arginine uptake in human placenta endothelium,10–17 MSPH could result in altered l-arginine transport and NO synthesis, that is, the l-arginine/NO pathway, in fetal endothelium. l-Arginine uptake occurs predominantly via the human cat-ionic amino acid transporters (hCATs) family.13 hCATs family includes ≥5 members, that is, hCAT-1, hCAT-2A, hCAT-2B, hCAT-3, and hCAT-4,12,13 of which the high-affinity, low-capacity hCAT-1 is the main isoform expressed in human umbilical vein endothelial cells (HUVEC).15,16 a cell type exposed to oxygen- and nutrient-enriched blood (ie, arteriolar-like blood, reaching fetal circulation).18 Interestingly, altered hCAT-1 activity could result in abnormal NO synthesis in HUVEC.10,11,15,17 Hypercholesterolemia also associates with reduced endothelial NO synthase (eNOS) activity paralleled by higher arginases expression and activity.19,20 l-Arginine is the substrate for arginase activity, thus competing with eNOS for this amino acid.21 Furthermore, patients with hypercholesterolemia exhibit increased activity of endothelial arginase I and arginase II (ARGII) forms.19,22 However, the potential effect of MSPH on arginases activity and expression and its consequences in modulation of fetal endothelial function is unknown.4,6 Because endothelial dysfunction precedes hypercholesterolemia-associated atherosclerosis even without structural vascular abnormalities,23 the hypothesis of this study was that MSPH will associate with fetal endothelial dysfunction and umbilical vein reactivity caused by fetal endothelial dysfunction where arginases will play crucial roles.

The results show that pregnant women with MSPH exhibit reduced umbilical vein dilation and eNOS activity, but higher hCAT-1 mediates l-arginine transport without changes in eNOS or hCAT-1 expression in HUVEC. Parallel assays show higher arginase activity and ARGII expression in MSPH. In addition, umbilical veins exhibit a higher intima/media ratio in MSPH. MSPH was associated with significantly higher TCh levels in the maternal plasma, compared with MPH, but was not associated with elevated TCh in the umbilical cord vein (ie, the fetal circulation). Thus, we suggest that MSPH could be a maternal pathological condition that associates with altered umbilical vein reactivity caused by fetal endothelial dysfunction. This could be an emerging mechanism accounting for the onset of the atherogenic process in the fetus from mothers with MSPH increasing the risk for an adverse fetal outcome and for the development of adulthood diseases.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Study Groups
With the exception of maternal weight, none of the other maternal or newborn variables changed during the third trimester compared with before pregnancy or first trimester of pregnancy (Table 1).24–30

Maternal and Newborn Plasma Lipids
Maternal blood TCh, high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), or very-low-density lipoprotein-cholesterol and triglycerides

## Table 1. Clinical Variables of Pregnant Women and Newborns

<table>
<thead>
<tr>
<th>Variables</th>
<th>MPH (n=46)</th>
<th>MSPH (n=28)</th>
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<tr>
<td><strong>Maternal</strong></td>
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<tr>
<td>Age, y</td>
<td>31.5±0.6 (24–40)</td>
<td>32.4±0.7 (25–38)</td>
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<tr>
<td>Height, cm</td>
<td>165±2 (154–174)</td>
<td>161±3 (149–175)</td>
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<tr>
<td>Weight, kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before pregnancy</td>
<td>66.5±1.1 (52–85)</td>
<td>62.6±1.6 (46–79)</td>
</tr>
<tr>
<td>First trimester</td>
<td>65.6±1.3 (52–84)</td>
<td>62.0±1.4 (48–80)</td>
</tr>
<tr>
<td>Second trimester</td>
<td>66.7±1.2 (53–81)</td>
<td>65.9±1.6 (52–81)</td>
</tr>
<tr>
<td>Third trimester</td>
<td>72.3±1.0 (61–85)*</td>
<td>72.0±1.6 (57–92)*</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
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<tr>
<td>Before pregnancy</td>
<td>24±0.7 (22–27)</td>
<td>24±0.9 (21–26)</td>
</tr>
<tr>
<td>First trimester</td>
<td>24±0.6 (22–25)</td>
<td>24±0.7 (22–25)</td>
</tr>
<tr>
<td>Second trimester</td>
<td>24±0.8 (20–27)</td>
<td>25±0.8 (23–29)</td>
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<tr>
<td>Third trimester</td>
<td>26±0.7 (22–31)</td>
<td>27±1.2 (21–34)</td>
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<td>Systolic blood pressure, mmHg</td>
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<tr>
<td>Before pregnancy</td>
<td>110±1 (90–121)</td>
<td>110±1 (89–123)</td>
</tr>
<tr>
<td>First trimester</td>
<td>111±1 (90–130)</td>
<td>110±2 (90–120)</td>
</tr>
<tr>
<td>Second trimester</td>
<td>110±1 (100–120)</td>
<td>110±1 (100–130)</td>
</tr>
<tr>
<td>Third trimester</td>
<td>114±1 (100–130)</td>
<td>114±2 (100–130)</td>
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<tr>
<td>Diastolic blood pressure, mmHg</td>
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<tr>
<td>Before pregnancy</td>
<td>66±1 (61–79)</td>
<td>69±1 (58–78)</td>
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<tr>
<td>First trimester</td>
<td>67±1 (60–76)</td>
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<td>66±1 (60–75)</td>
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<td>73±2 (60–100)</td>
</tr>
<tr>
<td>Mean arterial pressure, mmHg</td>
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<td></td>
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<tr>
<td>Before pregnancy</td>
<td>82±1 (71–92)</td>
<td>81±1 (72–89)</td>
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<td>First trimester</td>
<td>82±1 (70–90)</td>
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<tr>
<td>Second trimester</td>
<td>80±1 (73–87)</td>
<td>82±1 (73–97)</td>
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<tr>
<td>Third trimester</td>
<td>85±1 (73–97)</td>
<td>85±2 (67–97)</td>
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<td>Glycemia, mg/dL</td>
<td>81.4±2.2 (73–94)</td>
<td>80.8±1.1 (73–90)</td>
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<td>OGTT, mg/dL</td>
<td>78.0±1.0 (68–90)</td>
<td>78.5±1.2 (70–87)</td>
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<td>Glycemia 2 h after glucose</td>
<td>104.6±3.8 (70–143)</td>
<td>109.4±4.3 (64–143)</td>
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<td>Sex, female/male</td>
<td>30/16</td>
<td>17/11</td>
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<td>Gestational age, wk</td>
<td>39.1±0.2 (37–41)</td>
<td>39.2±0.2 (38–41)</td>
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<tr>
<td>Birth weight, g</td>
<td>3324±76 (2550–3370)</td>
<td>3277±83 (2580–3410)</td>
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<tr>
<td>Height, cm</td>
<td>50.0±0.4 (47–53)</td>
<td>49.2±0.3 (47–55)</td>
</tr>
<tr>
<td>Ponderal index, g/cm²×100</td>
<td>2.58±0.03 (2.35–2.89)</td>
<td>2.68±0.03 (2.5–2.87)</td>
</tr>
</tbody>
</table>

Women with maternal physiological (MPH, <280 mg/dL TCh) or supraphysiological hypercholesterolemia (MSPH, ≥280 mg/dL TCh) in pregnancy were included (see Material and Methods). Weight, BMI, and blood pressure were determined before pregnancy and at first (0–14 wk of gestation [wg]), second (14–28 wg) or third (28–40 wg) trimesters of pregnancy. Data are mean±SD (range). BMI indicates body mass index; MPH, maternal physiological hypercholesterolemia; MSPH, maternal supraphysiological hypercholesterolemia; OGTT, oral glucose tolerance test; and TCh, total cholesterol. *P<0.02 vs corresponding values before of pregnancy or at first trimester. before pregnancy were similar in MPH (Figure 1A) and MSPH (Figure 1B). Maternal TCh was higher in the second and third trimester compared with first trimester of pregnancy or values
before pregnancy in both conditions. HDL-C, LDL-C, and very-low-density lipoprotein-cholesterol levels were unaltered along pregnancy in MPH; however, LDL-C was higher in the third trimester compared with before pregnancy or first trimester in MSPH. The increase in maternal TCh in the second and third trimester from values in first trimester in MSPH was higher than changes in MPH. LDL-C level at third trimester was higher than before pregnancy and first trimester in MSPH, and the increase in MSPH was higher than that in MPH. Maternal triglyceride blood level in the third trimester was higher than values before pregnancy or at first trimester in MPH or in MSPH (Figure 1C). However, increase in maternal triglyceride was similar in MPH and in MSPH. TCh, HDL-C, and triglycerides levels in newborn umbilical blood were unaltered by MSPH (Figure 1D).

**Umbilical Vein Morphology**

Intima layer thickness was higher, but media thickness was unaltered in umbilical veins from MSPH compared with MPH (Figure 2). Moreover, higher amplitude of projections of the internal elastic layer into the media from the intima is seen in MSPH compared with MPH. In none of the samples studied, CD68+ cells were found (not shown).

**Umbilical Vein Reactivity**

Calcitonine gene–related peptide (CGRP) caused a maximal dilation ($D_{max}$) of umbilical vein rings from MPH ($D_{max}=28\pm7\%$; Figure 3A) that was higher than in vein rings from MSPH ($D_{max}=69\pm3\%$; Figure 3B). The CGRP $D_{max}$ in MPH was unaltered by the arginases inhibitor S-(2-boronoethyl)-L-cysteine (BEC). However, the presence of BEC-increased CGRP dilation ($D_{max}=13\pm5\%$) in vein rings from MSPH. CGRP EC$_{50}$ was higher in MSPH compared with MPH (Table 2). Similar results were obtained in umbilical vein rings preconstricted with serotonin (not shown). BEC reduced the CGRP EC$_{50}$ in MPH and in MSPH, with MSPH EC$_{50}$ being around 1 order of magnitude lower compared with MPH. Inhibition of NOS activity with L$N^\text{G}$-nitro-L-arginine methyl ester (L-NAME) caused a reduction in vein rings dilation in the absence of BEC; however, CGRP dilation was abolished in the presence of L-NAME+BEC in MPH and in MSPH. The NO donor sodium nitroprusside caused comparable dilation in veins from MPH and MSPH (Figure 3C).

**Arginases and eNOS Protein Abundance**

ARGII protein abundance was higher (1.6±0.1-fold) in MSPH compared with MPH; however, arginase I protein abundance was barely detected (Figure 3D). Arginase I and total eNOS (Figure 3E) protein abundance were unaltered by MSPH.

**Arginase Activity**

Cells from MSPH exhibit higher $v_o$ for overall arginase activity compared with MPH, and BEC blocked arginase activity.
NOS Activity

L-Citrulline formation in cells from MSPH was lower than MPH and inhibited by L-NAME (Figure 5A). BEC increased L-citrulline formation reaching similar values in MPH and in MSPH. L-NAME-inhibited L-citrulline formation was higher (1.6±0.2-fold) in MPH compared with MSPH (Figure 5B). NOS activity was higher in the presence of BEC reaching similar maximal values in both cell types (Figure 4A; Table 3). BEC-inhibited overall arginase activity was also higher in MSPH compared with MPH (Figure 4B) and was semisaturable in both conditions (Figure 4C). Eadie-Hofstee plot of this data were biphasic (Figure 4D). Saturable BEC-inhibited arginase activity exhibited its higher V_max without altering the apparent K_m in MSPH compared with MPH (Figure 4E; Table 3) and Eadie-Hofstee plot was linear (Figure 4F).

1-Arginine Transport

1-arginine uptake v_i was higher (2.8±0.1-fold) in MSPH compared with MPH and increased by BEC reaching similar values in both conditions (Figure 6A). Overall 1-arginine transport was semisaturable in MPH but saturable in MSPH in the absence of BEC (Figure 6B). BEC increased overall transport reaching comparable maximal transport values in MPH and in MSPH. Eadie-Hofstee plot of overall transport was biphasic in MPH but linear in MSPH in the absence of BEC; however, it was linear in MPH or MSPH in the presence of BEC (Figure 6C). Saturable 1-arginine transport exhibited higher V_max and V_max/K_m with no significant changes in the apparent K_m, in MSPH compared with MPH (Figure 6D; Table 3). Saturable transport data were linear in an Eadie-Hofstee plot (Figure 6E). Parallel assays showed that neither hCAT-1 nor hCAT-2A/B protein abundance was altered in cells from MSPH compared with MPH (Figure 6F).

Discussion

This study shows that MSPH associates with umbilical vein endothelial dysfunction and altered umbilical vein reactivity compared with MPH. A higher maternal LDL-C, without changes in HDL-C or very low-density lipoprotein-cholesterol, or triglycerides in MSPH was seen. However, newborns from MSPH exhibit normal levels of TCH, HDL-C, and triglycerides. MSPH reduced endothelium-derived NO-dependent dilation of umbilical vein rings and associated with higher intima/media ratio in umbilical veins compared with MPH. Arginase activity inhibition reversed MSPH effect on vein ring dilation. ARGII protein abundance and activity were increased, but eNOS activity was reduced in HUVEC from MSPH. MSPH and arginase inhibition also increased the V_max/K_m for 1-arginine transport in MSPH, without changing hCAT-1 or hCAT2A/B protein abundance. Thus, MSPH results in human umbilical vein endothelial dysfunction likely because of an imbalance between eNOS and ARGII activity and expression. Furthermore, we identified a cut-off point at which risk of human umbilical vein endothelial dysfunction increases in pregnant women with MSPH. These findings suggest a potential mechanism that could be accounting for the reported atherogenic process in the fetus from mothers with MSPH perhaps reflecting an in utero fetal programming of this disease.

MSPH Cut-Off Point

In 28 of 74 pregnant women studied, we observed reduced reactivity of umbilical vein rings, increased umbilical vein intima/media ratio, as well as altered eNOS and ARGII activity, and 1-arginine transport kinetics in HUVEC. All of these women had a TCH>280 mg/dL. We, therefore, defined MSPH as TCH≥280 mg/dL, that is, the level associated with vascular pathologies, and MPH as TCH<280 mg/dL. This level of maternal plasma TCH was established as the cut-off point for this study. This cut-off point is similar to TCH level in pregnant women showing increased oxidative stress in maternal and fetal blood, and homogenized placenta, and exhibit reduced maternal omental artery reactivity and altered composition of the fetal blood fatty acids. Furthermore, increased early atherosclerosis markers, such as fatty streaks and lipid peroxidation, in human fetal aorta or in 7- to 14-year-old children born from mothers with TCH level over this cut-off point is reported. Because TCH, HDL-C, and triglyceride plasma...
levels were unaltered in newborns from women with MSPH, confirming previous observations.33,34 We speculate on the possibility that MSPH is a pathological condition of the mother coursing with altered human fetoplacental vasculature. A role for LDL-C is suggested in this phenomenon because its maternal plasma concentration was higher in MSPH compared with MPH and because LDL-C is transferred across the placenta.18

Vascular Reactivity and ARGII
MSPH reduced umbilical vein dilation in response to the endothelium-dependent vasodilator CGRP.28,35 Because dilation caused by sodium nitroprusside (NO donor) was unaltered by MSPH, the umbilical vein endothelium rather than smooth muscle is likely altered. Thus, reduced CGRP sensitivity or reactivity of umbilical vein endothelium from MSPH is suggested. Interestingly, hypercholesterolemia associates with increased intima/media thickness in human carotid artery, which correlates with reduced brachial artery flow mediated dilation.35,36 Our results also show increased umbilical vein intima/media ratio in MSPH; thus, a well-preserved endothelium/smooth muscle structure is required for proper reactivity of these vessels. We did not identify CD68+ cells in these preparations suggesting that vascular dysfunction caused by MSPH is not dependent on the intimal recruitment of monocyte/macrophages and may, therefore, represent an initial step of atherogenic programming in fetal arteries.

CGRP dilation in umbilical vein rings from MSPH was increased by BEC (arginase inhibitor), suggesting that arginase activity could limit CGRP dilation response in these vessels. However, because BEC did not restores CGRP dilation in vessel rings from MSPH to values in MPH, an alternative mechanism(s) than arginase activity in response to CGRP is likely in MSPH, including eNOS uncoupling or reduced bioavailability of the eNOS cofactor tetrahydrobiopterin. Because ARGII protein abundance in MSPH is ≈1.6-fold than in MPH, arginase activity (likely ARGII) will predominate over the effect of a change in ARGII expression in CGRP dilation in umbilical veins. The latter is feasible because BEC was assayed for 30 minutes in our study, and the reported endothelial ARGII protein half-life is ≈4 hours.37 Thus, ARGII could play a role in umbilical vein reactivity in MSPH.

Figure 3. Human umbilical vein reactivity, arginases, and endothelial nitric oxide synthase (eNOS) expression. Dilation in response to calcitonine gene–related peptide (CGRP; 5 minutes) in human umbilical vein rings at birth from pregnancies where the mother exhibited (A) maternal physiological hypercholesterolemia (MPH) or (B) maternal supraphysiological hypercholesterolemia (MSPH). Vein rings were preincubated in the absence (○) or in the presence of 20 µmol/L S-(2-boronoethyl)-l-cysteine (BEC; 10 minutes; ●), 100 µmol/L N\(^{\text{\textendash}}\)-nitro-l-arginine methyl ester (L-NAME; 30 minutes; □) or BEC+L-NAME (▲). C, Dilation in response to 10 µmol/L sodium nitroprusside (5 minutes) in umbilical vein rings in MPH and MSPH. D, Western blots (representative blot of other 9 different umbilical veins) for arginase I (ARGI) or arginase II (ARGII) in human umbilical vein endothelial cell (HUVEC; β-actin: internal control) from MPH or MSPH. Bottom, ARGI or ARGII/β-actin ratio densitometries normalized to 1 in MPH. E, Western blots for eNOS in HUVEC from MPH or MSPH as in D. Bottom, eNOS/β-actin ratio densitometries normalized to 1 in MPH. *P<0.05 vs all other values. Values are mean±SEM (n=9–18).

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ARGII protein abundance in MSPH compared with MPH is feasible. In addition, Eadie-Hofstee plot of arginase saturable activity was linear, suggesting that mainly, if not only, ARGII activity could account for urea generation in HUVEC.

Vascular Reactivity and the l-Arginine/NO Pathway

L-NAME (NOS inhibitor) reduced CGRP dilation in the absence of BEC in MPH or MSPH. Thus, NOS activity was required for CGRP dilation in umbilical veins. Because NO-dependent CGRP dilation in MPH was ≈5.5-fold compared with MSPH, NOS activity was probably reduced to a greater extent in MSPH compared with MPH. Interestingly, CGRP dilation was abolished by L-NAME+BEC, suggesting that CGRP effect is potentiated after arginases inhibition in vessels from either conditions. Because HUVEC from MSPH exhibit unaltered eNOS protein abundance compared with MPH, lower CGRP dilation in MSPH may result from reduced NOS activity. The reduced eNOS activity in MSPH could result from larger reduction of eNOS activity compared with MPH, NOS activity was probably reduced to a greater extent in MSPH compared with MPH. Interestingly, CGRP dilation was abolished by L-NAME+BEC, suggesting that CGRP effect is potentiated after arginases inhibition in vessels from either conditions. Because HUVEC from MSPH exhibit unaltered eNOS protein abundance compared with MPH, lower CGRP dilation in MSPH may result from reduced NOS activity. The reduced eNOS activity in MSPH could result from larger reduction of eNOS activation-associated Ser1177 phosphorylation, compared with a less pronounced reduction of eNOS inactivation-associated Thr495 phosphorylation. Furthermore, a larger eNOS inhibition should be expected in MSPH because increased eNOS activity caused by reduced inhibitory Thr495 phosphorylation could be a phenomenon that will counteract eNOS inactivation as a result of a lower Ser1177 phosphorylation–dependent stimulation of this enzyme. It is known that eNOS activity is reduced by the semiessential neutral amino acid l-glutamine likely because of l-citrulline depletion in HUVEC. Thus, an alternative mechanism for eNOS inhibition could be increased fetal blood l-glutamine level in MSPH. However, young adult female pigs selected genetically for low- or high-plasma TCh exhibit similar umbilical vein blood l-glutamine levels. Thus, because MSPH associates with reduced eNOS activity in HUVEC and because TCh, HDL-C, and triglycerides in human umbilical vein blood were unaltered by MSPH, it is possible that reduced eNOS activity was not caused by a

Table 2. CGRP Dilation in Human Umbilical Vein Rings

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MPH</th>
<th>MSPH</th>
<th>MSPH/MPH</th>
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<tr>
<td>–BEC</td>
<td></td>
<td></td>
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<tr>
<td>–L-NAME</td>
<td>0.15±0.001</td>
<td>0.50±0.001*</td>
<td>3.30±0.001</td>
</tr>
<tr>
<td>+L-NAME</td>
<td>1.69±0.041*</td>
<td>1.06±0.032†</td>
<td>0.63±0.017†</td>
</tr>
<tr>
<td>+BEC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>–L-NAME</td>
<td>0.06±0.001*‡</td>
<td>0.004±0.001*†‡</td>
<td>0.07±0.01†‡</td>
</tr>
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<td>+L-NAME</td>
<td>n.m.</td>
<td>n.m.</td>
<td>…</td>
</tr>
<tr>
<td>–BEC/+BEC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>–L-NAME</td>
<td>2.70±0.001</td>
<td>114.3±0.001§</td>
<td>42.3±0.04</td>
</tr>
<tr>
<td>+L-NAME</td>
<td>n.m.</td>
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<td>…</td>
</tr>
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Umbilical vein rings were obtained from term pregnancies where women exhibited maternal physiological (MPH, <280 mg/dL TCh) or supraphysiological hypercholesterolemia (MSPH, ≥280 mg/dL TCh) in pregnancy (see Materials and Methods). Vein rings were incubated in the absence (–) or in the presence (+) of 20 μmol/L BEC (10 min) and 100 μmol/L-NAME (30 min) and the response to CGRP (0.1–1000 nmol/L; 5 min) was assayed. CGRP half-maximal effective concentration (EC50) was calculated (see Materials and Methods). Values are means±SEM (n=12); BEC indicates S-(2-boronoethyl)-L-cysteine; CGRP, calcitonin gene-related peptide; L-NAME, L-N-g-nitro-l-arginine methyl ester; MPH, maternal physiological hypercholesterolemia; MSPH, maternal supraphysiological hypercholesterolemia; n.m., not measurable; and TCh, total cholesterol.

*P<0.05 vs –BEC–L-NAME in MPH.
†P<0.05 vs –BEC+L-NAME in MSPH.
‡P<0.05 vs corresponding values in –BEC/+L-NAME.
§P<0.05 vs corresponding value in MPH.

Figure 4. Kinetics of arginase activity. A, Urea formation in human umbilical vein endothelial cell (HUVEC) from pregnancies where the mother exhibited maternal physiological hypercholesterolemia (MPH; ○, ●) or maternal supraphysiological hypercholesterolemia (MSPH; □, ◼) in the absence (○, □) or in the presence (●, ◼) of 20 μmol/L S-(2-boronoethyl)-l-cysteine (BEC; 10 minutes). B, Arginase activity in MPH (○) or MSPH (●) from data in A. C, Overall arginase activity at initial velocity (80 minutes; 37°C) in whole extracts from cells exposed to different concentrations of l-arginine as in B. D, Eadie-Hofstee plot of data in C. E, Saturable arginase activity from data in C (see Methods). F, Eadie-Hofstee plot of data in E. Values are means±SEM (n= 11–13).
Human umbilical vein endothelial cells were isolated from term pregnancies where women exhibited maternal physiological (MPH, <280 mg/dL TCh) or supraphysiological hypercholesterolemia (MSPH, ≥280 mg/dL TCh) in pregnancy (see Materials and Methods). Arginase activity corresponds to urea formation inhibited by 20 μmol/L BEC (10 min). Saturable l-arginine transport was derived from the overall l-arginine transport (see Methods) in cells incubated in the absence (–) or in the presence (+) of 20 μmol/L BEC (10 min). \( V_{\text{max}}^{\text{F}} \) is the relative effect of MSPH vs MPH on the maximal arginase activity capacity \( (V_{\text{max}}/K_{\text{m}}^{\text{F}}) \) in the absence or in the presence of BEC, respectively. Values are mean±SEM (n=12). BEC indicates S-(2-boronoethyl)-L-cysteine; MPH, maternal physiological hypercholesterolemia; MSPH, maternal supraphysiological hypercholesterolemia; TCh, total cholesterol; \( v \), initial velocity; and \( V_{\text{max}}^{\text{F}} \), maximal velocity.

\(* P<0.05 \) vs corresponding values in MPH.

Table 3. Kinetic Parameters for Arginase Activity and l-Arginine Transport

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MPH</th>
<th>MSPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>( v_r ), pmol/μg protein per minute</td>
<td>0.100±0.001</td>
<td>0.152±0.002*</td>
</tr>
<tr>
<td>( V_{\text{max}}^{\text{F}} ), pmol/μg protein per minute</td>
<td>1.99±0.07</td>
<td>2.98±0.08*</td>
</tr>
<tr>
<td>( K_{\text{v}} ), μmol/L</td>
<td>0.31±0.09</td>
<td>0.31±0.07</td>
</tr>
<tr>
<td>( V_{\text{max}}^{\text{F}}/K_{\text{v}} ), pmol/μg protein/min/(μmol/L)</td>
<td>6.42±1.03</td>
<td>9.61±2.30*</td>
</tr>
<tr>
<td>( K_{\text{m}}^{\text{F}} ), pmol/μg protein/min/(μmol/L)</td>
<td>0.015±0.004</td>
<td>0.010±0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.50</td>
</tr>
</tbody>
</table>

Saturable l-arginine transport

\(~\)BEC

| \( v_r \), pmol/μg protein per second       | 0.050±0.003                | 0.141±0.004*                |
| \( V_{\text{max}}^{\text{F}} \), pmol/μg protein per minute | 3.7±0.5                    | 12.9±0.8*                   |
| \( K_{\text{v}} \), μmol/L                 | 73±41                      | 172±35                      |
| \( V_{\text{max}}^{\text{F}}/K_{\text{v}} \), pmol/μg protein/min/(μmol/L) | 0.051±0.017               | 0.075±0.01*                 |
| \( K_{\text{m}}^{\text{F}} \), pmol/μg protein/min/(μmol/L) | 0.0031±0.0018             | <10⁻¹⁰                      |
|                                            |                            | 1.47                         |

+BEC

| \( v_r \), pmol/μg protein per second       | 0.226±0.003                | 0.199±0.003                 |
| \( V_{\text{max}}^{\text{F}} \), pmol/μg protein per minute | 16.6±1.6                   | 19.5±1.9                    |
| \( K_{\text{v}} \), μmol/L                 | 126±43                     | 131±44                      |
| \( V_{\text{max}}^{\text{F}}/K_{\text{v}} \), pmol/μg protein/min/(μmol/L) | 0.132±0.03                | 0.149±0.06                  |
| \( K_{\text{m}}^{\text{F}} \), pmol/μg protein/min/(μmol/L) | 0.0035±0.0018             | <10⁻¹⁰                      |
|                                            |                            | 1.13                         |

\( l \)-arginine transport compared with MPH, a reduced eNOS activity caused by other than reduced \( l \)-arginine transport mechanism is likely. BEC caused an increase of \( l \)-arginine transport and NOS activity in cells from MPH or MSPH thus \( l \)-arginine was apparently preferentially metabolized via arginases rather than eNOS. This is likely because the \( V_{\text{max}}/K_{\text{m}}^{\text{F}} \) of arginase activity in HUVEC from MPH or MSPH was only \( \approx 1.9 \) fold that in MPH. Thus, a metabolically less efficient eNOS caused by a higher arginase activity in HUVEC from MPH compared with MPH is likely. ARGII metabolizes \( l \)-arginine, the substrate for eNOS in HUVEC and other cell types, suggesting that a lower \( l \)-arginine bioavailability could result in reduced eNOS activity in MSPH. Because \( l \)-arginine uptake is required for eNOS activity in HUVEC, a reduced uptake and further delivery of this amino acid to eNOS could occur in MSPH. Because HUVEC from MSPH exhibit higher \( V_{\text{max}} \) and \( V_{\text{max}}^{\text{F}}/K_{\text{m}}^{\text{F}} \) for potential detrimental effect of circulating \( l \)-glutamine in the human fetal endothelium.

Inhibition of arginase activity was associated with activation of eNOS in HUVEC from MPH or MSPH. In MSPH, it could result from a dual phenomenon involving higher Ser¹⁷⁷ phosphorylation–associated activation in addition to lower Thr⁴⁹⁵ phosphorylation–associated inactivation of eNOS. The same could happen in MPH but at lower magnitude compared with MSPH because BEC-increased NOS activity in MSPH was only \( \approx 1.9 \) fold that in MPH. Thus, a metabolically less efficient eNOS caused by a higher arginase activity in HUVEC from MPH compared with MPH is likely. ARGII metabolizes \( l \)-arginine, the substrate for eNOS in HUVEC and other cell types, suggesting that a lower \( l \)-arginine bioavailability could result in reduced eNOS activity in MSPH. Because \( l \)-arginine uptake is required for eNOS activity in HUVEC, a reduced uptake and further delivery of this amino acid to eNOS could occur in MSPH. Because HUVEC from MSPH exhibit higher \( V_{\text{max}} \) and \( V_{\text{max}}^{\text{F}}/K_{\text{m}}^{\text{F}} \) for
in HUVEC. Furthermore, MSPH-associated alterations in endothelial function could result from changes in l-arginine transport activity rather than altered umbilical vein blood or maternal blood l-arginine concentration. This is supported by reports showing that altered maternal or fetal blood TCh does not alter umbilical vein and maternal blood l-arginine concentration.46,47

In summary, MSPH is a pathological condition of the mother that associates with reduced human umbilical vein dilation compared with pregnant women coursing with a physiological increase in plasma cholesterol. We established a cut-off point for MSPH on the basis of umbilical vein alterations described in this study. HUVEC from MSPH exhibit reduced endothelial l-arginine/NO (likely eNOS derived) but...
increased arginases (likely ARGII)/urea signaling pathways compared with MPH. Arginases inhibition restored eNOS activity and drove increased L-arginine transport (likely via hCAT-1) in cells from MPH and MSPH. The results show that HUVEC dysfunction in MSPH has consequences on umbilical vein reactivity, a phenomenon that could be a major complication for the growing fetus. Although fetal cholesterol levels were normal at term birth, maternal hypercholesterolemia may influence fetal cholesterol levels at early stages of pregnancy. It is, therefore, possible that the altered endothelial function and vascular responses observed at birth reflect earlier pathogenic programming by MSPH. Alternatively, pathogenic effects may result from metabolic products other than TCHs that are increased in MSPH and are capable of crossing the placental barrier. Considering that MSPH treatment or prevention studies are necessary to establish a causal effect, the results here reported highlight the possibility that alterations of umbilical vein function associated with increased maternal cholesterol levels will likely begins in prenatal life.

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We thank the personnel at the Hospital Clínico Pontificia Universidad Católica de Chile labor ward for the support in placentas supply.

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Disclosures

E. Guzmán-Gutiérrez and T. Sáez hold Comisión Nacional de Investigación en Ciencia y Tecnología (CONICYT)-PhD (Chile) fellowships. The other authors report no conflicts.

References


**Significance**

An increase in the maternal plasma cholesterol during pregnancy is a physiological phenomenon resulting from requirements of the growing fetus. Increased maternal plasma cholesterol over the physiological requirements, that is, supraphysiological, associates with vascular dysfunction and atherosclerotic lesions in human fetal arteries. Our findings show that a supraphysiological increase in the maternal plasma cholesterol in pregnancy constitutes a pathological condition causing alterations in the human umbilical vein endothelial function. Thus, even when high cholesterol-associated atherosclerosis occurs in arteries, we demonstrate that over a certain level of maternal total cholesterol in pregnancy (cut-off >280 mg/dL total cholesterol in this study), the umbilical veins will exhibit changes in the physiology of the endothelium with deleterious consequences on umbilical vein reactivity. The latter could have as a consequence a restricted placenta-to-fetus blood flow resulting in major complications for the growing fetus.
Maternal Hypercholesterolemia in Pregnancy Associates With Umbilical Vein Endothelial Dysfunction: Role of Endothelial Nitric Oxide Synthase and Arginase II
Andrea Leiva, Camila Díez de Medina, Rocío Salsoso, Tamara Sáez, Sebastián San Martín, Fernando Abarzúa, Marcelo Farías, Enrique Guzmán-Gutiérrez, Fabián Pardo and Luis Sobrevia

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SUPPLEMENTAL MATERIAL

Expanded Materials and Methods

Study groups

Human placentas were collected from 74 full-term pregnancies from women undergoing vaginal delivery with labor (31 women) and women with cesarean delivery without labor or without preterm rupture of membranes (43 women). Pregnant women invited to participate in this study were selected based on the following exclusion criteria: maternal overweight or obesity (pre-pregnancy body mass index (BMI) >25), multiple pregnancy, fetal malformations, hypertensive syndrome, preeclampsia (PE), intrauterine growth restriction (IUGR), pre-gestational and/or gestational diabetes mellitus (GDM). All pregnant women were nonsmoking, did not consume alcohol or drugs, and were without intrauterine infection or any other medical or obstetrical complications. Women that during pregnancy developed overweight or obesity in pregnancy, PE, IUGR and/or GDM, were excluded of the study groups. The investigation conforms to the principles outlined in the Declaration of Helsinki. Ethics Committee approval from the Faculty of Medicine of the Pontificia Universidad Católica de Chile and patient informed consent were obtained.

Blood cholesterol and triglyceride measurement

All pregnant women admitted to the study were screened for total blood cholesterol (TCh), high-density lipoprotein (HDL-), low density lipoprotein (LDL-) or very-low density lipoprotein (vLDL-) cholesterol and triglycerides. The measurements were done in maternal whole brachial venous blood taken between 0-14 (first trimester), 14-28 (second trimester) and 28-40 (third trimester) weeks of gestation. At birth, whole umbilical blood was collected and assayed for TCh, HDL-cholesterol and triglycerides. TCh, HDL-cholesterol and triglycerides were determined via standard enzymatic-colorimetric assays (Cobas Integra Cholesterol (CHOLL), Cobas Integra HDL-Cholesterol (HDL_C), Cobas Integra Triglycerides (TRICL) kits) (Roche Diagnostic Corporation, Indianapolis, IN, USA) in a Cobas® 8000 modular analyser series (Roche Diagnostic Corporation) at the Clinical Laboratory of the Universidad Católica Clinical Hospital (Chile). LDL- and vLDL-cholesterol were calculated from TCh, HDL-cholesterol and triglyceride levels by applying the Friedewald’s equation.1,2

Women with <280 mg/dL TCh at term were considered as MPH and those ≥280 mg/dL TCh corresponded to MSPH in this study. The cut-off point established for MSPH for this study was defined based on (a) our results show that all assays performed in samples from pregnant women with ≥280 mg/dL TCh exhibited significant changes compared with samples from pregnant women with <280 mg/dL TCh, (b) patients with a TCh level close to this cut-off point are reported to associate with fetal fatty streaks (~281 mg/dL, 3 ~280 mg/dL 4) and (c) this value is higher than the mean of TCh level (~247 mg/dL, range 184-315 mg/dL) considered as normal in pregnancy (i.e., MPH) by different groups.5

Tissue preparation, histochemistry and immunohistochemistry

Sections (~6 cm³) of umbilical cords were fixed (12 hours) in 10% neutral buffered formalin (3.7% (v/v) formaldehyde, 29 mmol/L NaH₂PO₄·H₂O, 46 mmol/L Na₂HPO₄ (pH 7.2, 4°C)).6 Samples were then dehydrated with graded ethanol solutions (70% for 30
minutes, 96% for 30 minutes (2 cycles) and 100% for 60 minutes (2 cycles)) followed by exposure to xylol (60 minutes (2 cycles)). Samples were then included in paraffin to generate the tissue blocks by a sequential exposure to xylol:Paraplast plus = 1:1 v/v (30 minutes, 60ºC) (McCormick Scientific, St. Louis, MO, USA) and Paraplast (60 minutes, 60ºC (3 cycles)). Slides of 5 µm in thickness of tissue blocks were obtained in a 1212 model Leitz microtome (Wetzlar, Germany), adhered to 0.1% poly-L-lysine (Sigma-Aldrich, St Louis, MO, USA)-coated glass slides and dried (1 hour, 25ºC).

Prior to histochemical assays a group of tissue slices were stained with haematoxylin and 0.5% eosin for morphological analysis as described. Histochemistry was done in deparaffinized and rehydrated tissue slices incubated (15 minutes, 25ºC) in a modified Verhoeff-hematoxylin solution following standard procedures. Tissue slices were then washed (3 cycles) in distilled water and then differentiated by incubation in 2% ferric chloride (30 seconds, 25ºC) (Sigma-Aldrich) and 5% sodium thiosulphate (1 minute, 25ºC) (Merck, Darmstadt, Germany). The nuclear contrast was performed by staining the tissues (3 minutes, 25ºC) with 0.2% Green Light (Merck).

For immunohistochemistry assays deparaffinized and rehydrated tissue slides were subjected to heat-induced antigen retrieval using citrate buffer ((mmol/L) 10 citric acid, 10 sodium citrate) (pH 6.0, 18ºC) (Merck Millipore, Darmstadt, Germany) in a steam cooker for 15 minutes at 95ºC. Samples were then treated with 3% H2O2 in phosphate buffered solution (PBS) ((mmol/L) 130 NaCl, 2.7 KCl, 0.8 Na2HPO4, 1.4 KH2PO4 (pH 7.4, 18ºC)) for 30 minutes to quench endogenous peroxidase activity. After rinsing in PBS (pH 7.4, 18ºC) for 5 minutes, all tissue slides were incubated for 1 hour with protein blocking solution (Cas-Block, Zymed Laboratories, South SanFrancisco, CA, USA). A monoclonal mouse anti-CD68 primary (1:200, 12 hours, 4ºC) (Thermo Scientific, West Palm Beach, FL, USA) and policlonal rabbit anti-mouse secondary (1:1000, 1 hour, 25ºC) (Invitrogen Corporation, Camarillo, CA, USA) antibodies were used. Immunostaining was performed using horseradish peroxidase-conjugated secondary antibody was determined with NovaRED kit (Vector, Burlingame, CA, USA), treating for 2 minutes. Tissue slides were counterstained with Harris hematoxylin. Specificity of the staining was determined by incubation of sections in the absence of the primary antibody. Positive control for CD68+ cells was performed in human lymph node following the same above described protocol.

Tissue slices were then dehydrated (as described above), cleared in xylol and mounted with the hydrophobic synthetic resin, fast drying mounting HI-MO medium (Bio-Optica, Milano, Italy). To estimate the distances between the endotelium and intima and between the intima and media in umbilical veins, tissue samples were mounted under a CX21-Olympus microscope (Olympus, Tokyo, Japan) and images were captured (6 fields, 40X magnification) using an Olympus DP-71 digital camera (Olympus) and the Olympus DP-BSW software 03.01 (Olympus). The distances between the media and intima and the intima and endothelium from umbilical veins (20 determinations per tissue slice) were measured in each captured field using the Image-Pro Plus software 3.0 (Media Cybernetics, Silver Spring, MD, USA).

**Human umbilical vein reactivity**

Ring segments of 2-4 mm in length were dissected from human umbilical veins in ice-cold PBS (pH 7.4, 4ºC). Vein rings were mounted in a myograph (610M Multiwire Myograph System, Danish Myo Technology A/S, Denmark) for isometric force measurements in a Krebs physiological solution ((mmol/L) 118.5 NaCl, 4.7 KCl, 25
NaHCO₃, 1.2 MgSO₄, 1.2 KH₂PO₄, 2.5 CaCl₂, 5.5 D-glucose, 0.3 L-arginine (37°C, pH 7.4)). Vein rings were constantly bubbled with a mixture of 95% O₂/5% CO₂. The optimal diameter for each vessel was adjusted through the determination of the maximal active response evoked by 65 mmol/L KCl. Endothelium-dependent relaxation was evaluated as the concentration-dependent response to calcitonine gene related peptide (CGRP, 0.1-1000 nmol/L, 5 minutes) (Sigma-Aldrich) in KCl-preconstricted vessels. Experiments were performed in umbilical vein rings in the absence or presence of 20 µmol/L S-(2-boronoethyl)-L-cysteine, HCl (BEC, ARGs inhibitor, 30 minutes) (Calbiochem, San Diego, CA, USA), 100 µmol/L N⁶-nitro-L-arginine methyl ester (L-NAME, NOS inhibitor, 30 minutes) or 10 µmol/L sodium nitroprusside (SNP, NO donor, 5 minutes) (Sigma-Aldrich). Changes in isometric tension were recorded using the software LabChart (LabChart 7 for Windows, ADInstruments, Australia) coupled to a PowerLab (PowerLab 8/30 Data Acquisition System, ADInstruments, Australia).

The tissue responses are given as a percentage of the maximal contraction caused by 32.5 mmol/L KCl. The concentration of CGRP that causes 50% of the maximal vasodilation (i.e., half-maximal effective CGRP concentration (EC₅₀)) was calculated by the three parametric logistic equation (Hill slope = 1):

\[ Y = D_c + \frac{(D_{CGRP} - D_c)}{1 + 10^{(X - \log EC_{50})}} \]

where \( X \) is the logarithm of CGRP concentration and \( Y \) is vasodilation caused by CGRP, \( D_c \) is maximal contraction caused by 32.5 mmol/L KCl in the absence of CGRP, \( D_{CGRP} \) is vasodilatation in the presence of CGRP, and \( \log EC_{50} \) corresponds to the \( EC_{50} \).

**Cell culture**

Human umbilical vein endothelial cells (HUVEC) were isolated by collagenase (0.25 mg/mL, Collagenase Type II from Clostridium histolyticum (Boehringer, Mannheim, FRG)) digestion from umbilical cords obtained at birth from pregnancies with MPH (n = 46) or MSPH (n = 28) and cultured (37°C, 5% CO₂) in 1% gelatin-coated petri dishes (100 mm diameter) up to passage 2 in medium 199 (M199; Gibco Life Technologies, Carlsbad, CA, USA) containing 5 mmol/L D-glucose, 10% new born calf serum (NBCS), 10% fetal calf serum (FCS) (Gibco), 3.2 mmol/L L-glutamine and 100 U/mL penicillin-streptomycin (primary culture medium, PCM) (Gibco). Sera included in the PCM contained 4-8 mg/dL total cholesterol with <1 mg/dL HDL-cholesterol. Twenty-four hours prior experiment incubation medium was changed to 2% NBCS/FCS containing M199. Experiments were performed in the absence or presence (30 minutes) of 10 µmol/L BEC or 100 µmol/L L-NAME.

**ARGs activity**

Total urea production from L-arginine was measured as described. In brief, confluent HUVEC cultures were washed (x2) with cold PBS (4°C, pH 7.4) and incubated (5 minutes, on ice) with lysis buffer (1 µmol/L pepstatine A, 1 µmol/L leupeptine, 200 µmol/L phenylmethylsulfonyl fluoride, 50 mmol/L Tris-HCl (pH 7.5), 0.2% Triton X-100). Cell lysate was sonicated (3 cycles of 20 pulses at 150 Watts) and total protein content was determined by Bradford method (BioRad, CA, USA). Aliquotes (70 µg) were
preincubated (10 minutes, 55°C) with 100 mmol/L MnCl₂ in 50 mmol/L Tris-HCl (pH 7.5) in the absence or presence of 20 μmol/L BEC, and then mixed with L-arginine (0-50 mmol/L, 60 minutes, 37°C, pH 7.4). The reaction was stopped by addition (400 μL) of an acid mix (H₂SO₄:H₃PO₄:H₂O = 1:3:7 v/v) and incubated (45 minutes, 100°C) in 9% a-isonitrosopropiophenone (25 μL) for colorimetric determination of urea. Aliquots of 200 μL were then transferred into a 96-well plate and absorbance at 540 nm was measured in a microplate reader (Thermo Labsystems, Waltham, MA, USA).

Urea formation rate was derived from the slopes of lineal phases of urea formation from 50 mmol/L L-arginine. ARGs activity values were adjusted to the one phase exponential association equation considering the least squares fit (equation 1):

\[ v_i = V_m \cdot (1 - e^{-kt}) \]

where \( v_i \) is initial velocity, \( V_m \) is mayor velocity of ARGs activity at a given time (0, 30, 60 or 120 minutes) and L-arginine concentration (1-50 mmol/L), \( t \) is time, and \( e \) and \( k \) are constants.

Overall ARGs activity at initial rates was adjusted to the Michaelis-Menten hyperbola plus a non-saturable, lineal component. The saturable maximal velocity (\( V_{\text{max}} \)) and apparent Michaelis-Menten constant (\( K_m \)) for ARGs activity were calculated by the single Michaelis-Menten asymptotic hyperbola equation (equation 2):

\[ \frac{v}{V_{\text{max}}} = \frac{[\text{Arg}]}{K_m + [\text{Arg}]} \]

where \( v \) is the initial reaction velocity relative to the \( V_{\text{max}} \) at a given L-arginine concentration ([Arg]). Each assay was done in duplicate and activity was expressed as pmol urea/μg protein/minute.

The relative effect of MSPH compared with MPH on saturable ARGs activity kinetic parameters was estimated from the maximal transport capacity (\( V_{\text{max}}/K_m \)) values in the absence (control) or presence of BEC by (equation 3):

\[ \frac{1}{x/cF} = \frac{x \cdot K_m \cdot V_{\text{max}}}{x \cdot V_{\text{max}} \cdot C \cdot K_m} \]

where \( x \) and \( C \) correspond to \( V_{\text{max}} \) and \( K_m \) for saturable ARGs activity in MSPH and MPH, respectively, in the absence or presence of BEC.

**L-Citrulline formation assay**

NOS activity was determined by incubation of cells with 100 μmol/L L-arginine and 9 μCi/mL L-[³H]arginine (30 minutes, 37°C) in the absence or presence of 100 μmol/L L-NAME) and/or 20 μmol/L BEC in HEPES buffer solution ((mmol/L) 50 HEPES, 100 NaCl, 5 KCl, 2.5 CaCl₂, 1 MgCl₂ (pH 7.4)). The fraction of L-[³H]citrulline formation from L-[³H]arginine inhibited by L-NAME was considered NOS activity.Digested cells (95% formic acid) were passed through an activated cation ion-exchange resin Dowex-50W
(50X8-200) and L-[3H]citrulline was determined in H2O eluate as described.14 NOS activity was calculated as the fraction of L-[3H]citrulline formation blocked by L-NAME.

**L-Arginine transport**

Overall L-arginine transport (0-1000 μmol/L L-arginine, 3 μCi/mL L-[3H]arginine (Perkin-Elmer, Boston, MA, USA), 1 minute, 37°C) was measured in cells pre-incubated (overnight) in PCM containing 2% sera (NBCS/FCS) as described.8 Overall and saturable L-arginine transport kinetic parameters were calculated as for ARGs activity (equations 1-3). The relative effect of MSPH compared with MPH or the relative contribution of ARGs to saturable L-arginine transport kinetic parameters was estimated as above for ARGs activity (equation 3) with X and C corresponding to $V_{max}$ and $K_m$ for saturable L-arginine transport in MSPH and MPH, respectively, in the absence or presence of BEC.

**Western blotting**

Total protein was obtained from confluent cells washed twice with ice-cold PBS and harvested in 100 μL of lysis buffer (63.7 mmol/L Tris/HCl (pH 6.8), 10% glycerol, 2% sodium dodecyl sulphate, 1 mmol/L sodium orthovanadate, 50 mg/mL leupeptin, 5% 2-mercaptoethanol) as described.8 Cells were sonicated (6 cycles, 5 seconds, 100 Watts, 4°C), and total protein was separated by centrifugation (14000 g, 15 minutes, 4°C). Proteins (50 μg) were separated by polyacrylamide gel (10%) electrophoresis and transferred to Immobilon-P polyvinylidene difluoride membranes (BioRad Laboratories, Hertfordshire, UK). The proteins were then probed with primary polyclonal goat anti-hCAT-1 (1:200 dilution, 8 hours, 4°C) or anti-hCAT-2A/B (1:200 dilution, 8 hours, 4°C; against isoforms A (hCAT-2A) and B (hCAT-2B) of hCAT-2), rabbit anti-ARG1 (1:200 dilution, 12 hours, 4°C), anti-ARGII (1:200 dilution, 2 hours, room temperature) and anti-total eNOS (i.e., phosphorylated + non-phosphorylated eNOS) (1:200 dilution, 2 hours, room temperature) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal mouse anti-eNOS phosphorylated in Serine1177 (Ser1177) (1:1000 dilution, 1 hour, room temperature), anti-eNOS phosphorylated in Threonine495 (Thr495) (1:1000 dilution, 1 hour, room temperature) (BD Transduction Laboratories, San Jose, CA, USA) and anti-β-actin (1:5000, 1 hour, room temperature) (Sigma-Aldrich) antibodies. After a first blotting for phosphorylated Ser1177-eNOS (P-Ser1177-eNOS) the membranes were stripped to be then subjected to a further blotting now for P-Thr495-eNOS. This membrane was then stripped for a second time to blot against total eNOS. For detection of total and phosphorylated Ser1177- or Thr495-eNOS protein isolation was done from cells in the absence or presence of 20 μmol/L BEC. Membranes were rinsed in Tris buffer saline Tween 20 and incubated (1 hour) in TBS-T/0.2% BSA containing secondary horseradish peroxidase-conjugated rabbit anti-goat, goat anti-rabbit or anti-mouse antibodies (Santa Cruz Biotechnology). Proteins were detected by enhanced chemiluminescence and quantified by densitometry as described.8

**Statistical analysis**

Values for TCh, HDL-, LDL- and vLDL-cholesterol, and triglycerides are given as mean ± S.D. For assays in vitro the values are mean ± S.E.M., where n indicates the number of different cell cultures (3-4 replicates). The normality of the data was determined with Kolmogorov-Smirnov test. Comparisons between two groups were performed by
means of Student’s unpaired *t*-test or Mann-Whitney test for parametric or non-parametric data, respectively. The difference between more than two groups were performed by analysis of variance (ANOVA) or Friedman test for parametric or non-parametric data, respectively. If ANOVA or Friedman test demonstrated a significant interaction between variables, post hoc analyses were performed by the multiple-comparison Bonferroni or Dunns correction test, respectively. The statistical software GraphPad Instat 3.0b and Graphpad Prism 6.0a (GraphPad Software Inc., San Diego, CA, USA) were used for data analysis. *P*<0.05 was considered statistically significant.
Supplemental References


