

# Role of S-Equol, Indoxyl Sulfate, and Trimethylamine N-Oxide on Vascular Function

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Gut microbiota have been emerging as important contributors to the regulation of host homeostasis. Accordingly, several substances converted by gut microbiota can have beneficial or adverse effects on human health. Among them, S-equol, which is produced from the isoflavone daidzein in the human and animal gut by certain microbiota, exerts estrogenic and antioxidant activities. Indoxyl sulfate, which is metabolized in the liver from indole converted from dietary tryptophan by bacterial tryptophanases in the colon, is known as a protein-bound uremic toxin. Trimethylamine N-oxide, which is generated via the oxidization of gut microbiota-derived trimethylamine by hepatic flavin monooxygenases, is known as an accelerator of atherosclerosis. The aforementioned gut-derived substances could be potential regulators of systematic tissue/organ function, including the vascular system. Macro- and microvascular complications of cardiovascular

and metabolic diseases, including atherosclerosis, hypertension, and diabetes, occur systemically and represent the principal cause of morbidity and mortality. Vascular endothelial and smooth muscle dysfunction play pivotal roles in the development and progression of vasculopathies. We herein review the link between the aforementioned gut-derived substances and endothelial and vascular smooth muscle cell function. This information will provide a conceptual framework that would allow the development of novel preventive and/or therapeutic approaches against vasculopathies.

**Keywords:** blood pressure; endothelium; hypertension; indoxyl sulfate; S-equol; TMAO; vascular smooth muscle

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Vascular dysfunction is undoubtedly associated with the onset and maintenance of hypertension, as well as with the initiation and development of vascular complications related to several chronic diseases, including diabetes, hypertension, and atherosclerosis.<sup>1–6</sup> Blood vessels contain two primary cell types, endothelial cells (ECs) and vascular smooth muscle cells (VSMCs), both of which exert an essential functions in sustaining vascular homeostasis.<sup>7</sup>

ECs constantly generate a number of vasoactive and trophic substances that regulate inflammation, VSMC growth, platelet function, plasmatic coagulation, and vasomotion under normal conditions.<sup>8,9</sup> ECs play a pivotal role in vascular tone regulation by generating and releasing several factors, including endothelium-derived relaxing factors (EDRFs) and contracting factors.<sup>9–12</sup> Among them, three EDRFs, including nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF), and prostacyclin, and several endothelium-derived contracting factors, including angiotensin II, endothelin-1, vasoconstrictor prostanoids, and uridine adenosine tetraphosphate, have been well known.<sup>9–13</sup> During aging and/or in several disease states, including hypertension, diabetes,

hypercholesterolemia, and atherosclerosis, an imbalance between EDRFs and endothelium-derived contracting factors levels has been observed in different vasculatures.<sup>8–10</sup> Therefore, manipulating the balance between endothelium-derived factors is an important strategy for preventing the initiation and development of vascular dysfunction and complications.

VSMCs are another major cell type that forms the blood vessels. Distinct from other mature cell types throughout the body, VSMCs do not terminally differentiate but maintain a remarkable plasticity.<sup>4–6,14</sup> Fully differentiated medial VSMCs of mature blood vessels retain quiescence and express various genes and proteins related to important components that regulate contraction and relaxation, allowing them to regulate systemic and local blood pressure via vascular tone control.<sup>4,6,14</sup> In response to vascular injury or changes in local environmental cues, differentiated/contractile VSMCs are capable of switching to a dedifferentiated phenotype characterized by increased proliferation, migration, and extracellular matrix synthesis consistent with the reduced expression of contractile markers.<sup>4–6,14</sup> Given the key role of VSMC dysfunction in the remodeling process during the

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development of vascular diseases,<sup>4-6,14,15</sup> determining causative factors and molecular mechanisms underlying abnormal proliferation, migration, apoptosis, senescence, and calcification in VSMCs is critical for a comprehensive understanding on the initiation and development of vascular dysfunction and for the establishment of therapies and preventive strategies against vascular diseases.

A growing body of evidence has suggested a relationship between gut microbiota and several cardiovascular and metabolic diseases.<sup>16-24</sup> A number of substances derived from the gut microbiome, microbial metabolites, and bacterial structural components have been found to affect host homeostasis. Given the adverse or beneficial effects of such substances on many physiological functions, controlling gut dysbiosis, defined as deleterious changes to the composition or number of gut bacteria, has been an important strategy against the development and/or progression of numerous diet-related diseases, including cardiovascular diseases.<sup>16-24</sup>

The present review summarizes some of the experimental and clinical evidence indicating that gut-derived substances can affect vascular function, especially focusing on the relationship between cellular function and three substances, S-equol, indoxyl sulfate, and trimethylamine *N*-oxide (TMAO), in ECs and VSMCs.

## S-EQUOL

Equol [7-hydroxyl-3-(4-hydroxyphenyl)chroman] is produced from soy isoflavone daidzein in human and animal gut by certain bacterial biotypes that can cross individuals.<sup>25,26</sup> A number of studies have suggested that S-equol is responsible for the metabolic and cardiovascular benefits of soy.<sup>27-29</sup> Considerable evidence has suggested that S-equol can affect several phenomena in not only ECs but also VSMCs (Figure 1).

In VSMCs, S-equol inhibited the proliferation, collagen, and total protein syntheses, migration, and mitogen-activated protein kinase activity of human aortic smooth muscle cells (HASMCs) in a concentration-dependent manner,<sup>30</sup> suggesting that S-equol may confer protective effects on the vascular system by inhibiting vascular remodeling and neointima formation.

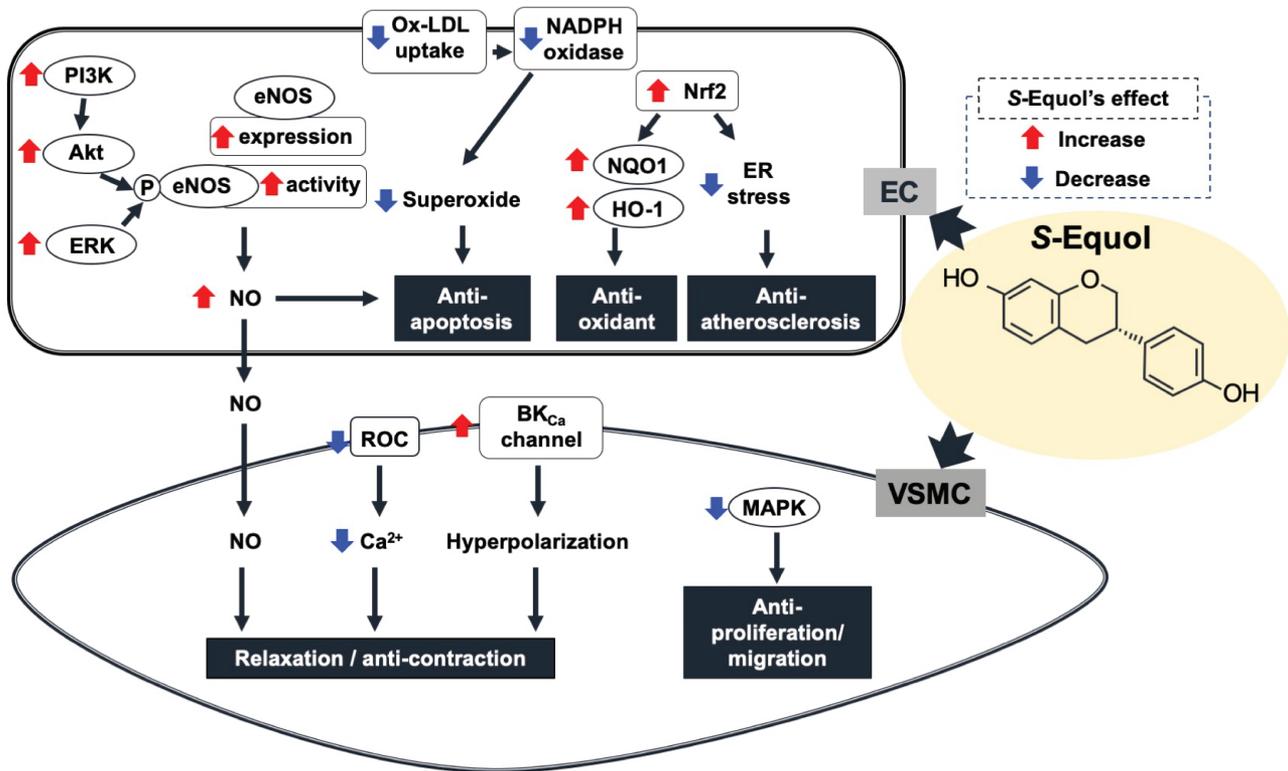
In ECs, S-equol suppresses oxidized low-density lipoprotein-induced apoptosis via decreased superoxide production by nicotinamide adenine dinucleotide phosphate oxidase and increased NO production in human umbilical vein ECs (HUVECs)<sup>31</sup> and inhibits H<sub>2</sub>O<sub>2</sub>-induced apoptosis by reducing intracellular reactive oxygen species (ROS) generation and increasing the expression of phosphorylated-p38 mitogen-activated protein kinase and Bcl-2 in bovine aortic ECs.<sup>32</sup> These findings suggest that S-equol exerts antiapoptotic effects in ECs. Another study showed that S-equol acutely activates endothelial NO release at basal cytosolic Ca<sup>2+</sup> levels by activating extracellular signal-regulated kinase (ERK) 1/2 and Akt independent of classic estrogen receptor (ER) signaling.<sup>33</sup> Inhibiting mitochondrial ROS abolished S-equol-mediated activation of Akt, ERK1/2, endothelial NO synthase (eNOS) phosphorylation, and NO production, as well as the relationship

between S-equol-stimulated mitochondrial ROS generation and epidermal growth factor receptor kinase transactivation and F-actin cytoskeleton reorganization.<sup>34</sup> Hence, identifying novel actions of S-equol may provide valuable insights into therapeutic strategies that improve endothelial function in cardiovascular disease.

With regard to vascular tone regulation, S-equol can promote relaxation in a variety of arteries, including the carotid arteries, cerebral arteries,<sup>35</sup> aorta,<sup>33,36,37</sup> and basilar arteries.<sup>38</sup> In rat carotid arteries, S-equol-induced relaxation via endothelium-, nitric oxide synthase (NOS)-, and K<sup>+</sup> channel-independent pathways, which was preserved during angiotensin II-induced hypertension.<sup>35</sup> Another study showed that S-equol-induced relaxation of rat thoracic aorta was NO dependent.<sup>33</sup> In human uterine arteries, S-equol-induced relaxation was mediated by its calcium antagonistic action through its antagonism of receptor-dependent but not voltage-dependent Ca<sup>2+</sup> channels.<sup>39</sup> S-Equol increased regional cerebral blood flow in rats and promoted concentration-dependent but endothelium-independent relaxation of rat cerebral basilar arteries, which was mediated by the large-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> (BK<sub>Ca</sub>) channel.<sup>38</sup> In fact, in stably expressed multiple K<sup>+</sup> channels in HEK293 cells, S-equol inhibited several cardiac K<sup>+</sup> currents at relatively high concentrations but increased BK<sub>Ca</sub> current at very low concentrations, suggesting that S-equol was safe for cerebral vascular disorders.<sup>40</sup> These evidences suggest that S-equol could cause endothelium-dependent and/or -independent relaxation depending on the artery.

Elevated insulin level, an important pathophysiological condition for type 2 diabetes, results from insulin-resistant states. Prolonged elevation of circulating insulin levels can promote several types of systematic dysfunctions, including vascular diseases.<sup>3</sup> We very recently demonstrated that S-equol can prevent the augmentation of serotonin-induced contraction in carotid arteries receiving prolonged treatment with increased insulin levels, which may have been due to increased BK<sub>Ca</sub> channel activity in carotid artery smooth muscle cells.<sup>41</sup> Therefore, S-equol may possibly prevent the development of vascular dysfunction in high insulin conditions and type 2 diabetes.

In *in vivo* studies, a dietary soy protein-rich diet for 12–16 months can better modulate blood pressure *in vivo*, antioxidant and eNOS gene expression, and intracellular glutathione levels compared with a soy protein-deficient diet in male rats.<sup>42</sup> S-Equol treatment (low-dose, 10 mg/kg and high-dose, 20 mg/kg orally for 4 weeks) could dose-dependently decrease systolic blood pressure in deoxycorticosterone acetate-salt hypertensive rats by inhibiting angiotensin-converting enzyme activity and increasing the NO production.<sup>43</sup> Removal of dietary soy isoflavones reduced endothelium-derived NO levels in ovariectomized rats, while S-equol supplementation (200 µg/day subcutaneously for 4 weeks starting at the 16th week after receiving an isoflavone-deficient diet) partially improved NO-mediated endothelial function.<sup>36</sup> In addition, S-equol treatment (0.05% and 0.1% for 12–14 weeks) displayed antiatherosclerotic properties in apolipoprotein E knockout mice fed a high-fat



**Figure 1.** Effects of *S*-equal on vascular endothelial and smooth muscle cells. *S*-equal has several beneficial effects, including anti-apoptosis, anti-oxidation, and anti-atherosclerosis; production of nitric oxide in endothelial cells; anti-proliferation and/or migration; and promotion of vascular smooth muscle cell relaxation. Details are provided in the text. Abbreviations: BK<sub>Ca</sub>, large-conductance calcium-activated potassium channel; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; HO-1, heme oxygenase-1; MAPK, mitogen-activated protein kinase; NO, nitric oxide; Nrf2, NF-E2-related factor 2; NQO1, NAD(P)H:quinone oxidoreductase 1; Ox-LDL, oxidized low-density lipoprotein; PI3K, phosphoinositide 3-kinase; ROC, receptor-operated calcium channel; VSMC, vascular smooth muscle cell.

diet by inhibiting endoplasmic reticulum stress through the activation of nuclear factor-erythroid 2-related factor 2 in ECs.<sup>44</sup> The aforementioned *in vivo* studies imply that *S*-equal has potential benefits against vascular dysfunction among not only postmenopausal women but also those with hypertension and metabolic disorder-associated atherosclerosis.

Taking these *in vitro* and *in vivo* evidences together, *S*-equal appears to be a clinically safer alternative to feminizing estrogens for the prevention of cardiovascular diseases among both men and women.

Nonetheless, further investigations are required to elucidate the underlying mechanisms. For instance, *S*-equal exerts several biological effects by binding to ERs, with its binding affinity being stronger to ER $\beta$  than ER $\alpha$ .<sup>45,46</sup> Given that ERs are not equally distributed among different tissues/cells, *S*-equal might have different effects depending on the ratio of ER $\alpha$  and ER $\beta$  isoforms present. Furthermore, *S*-equal may exert its effects by independently binding to ERs.<sup>33</sup> Considering that the primary target(s) of *S*-equal in ECs and VSMCs related to the aforementioned effects remain unclear, future investigations will be required.

The production of *S*-equal has attracted considerable attention, with several excellent reviews having been written on *S*-equal-producing phenotype, *S*-equal-producing microorganisms, and *S*-equal-producing populations in

the human gut.<sup>25,26</sup> *S*-Equal is produced by the action of gut bacteria in some individuals called *S*-equal-producers. The prevalence of *S*-equal-producers in Asian countries has been reported to be 50%–60%, with Western having a much lower prevalence (25%–30%) than Asian countries.<sup>47</sup> Observational studies have suggested that *S*-equal production was associated with decreased risk of certain diseases or conditions, including obesity<sup>48</sup>, hypertension, and vascular dysfunction.<sup>48–51</sup> Clinical trials have reported that the beneficial effect of soy on cardiovascular health, particularly on the normalization of lipids profiles,<sup>52,53</sup> inflammatory markers,<sup>54</sup> vascular function,<sup>54,55</sup> and blood pressure,<sup>53,55</sup> was only present or more pronounced in equal-producers than nonproducers, although others did not.<sup>56,57</sup> However, most of these data were from nonprespecified subgroup analysis of equal-producers who were not randomized accordingly. Recently, Ahuja *et al.*<sup>58</sup> investigated the cross-sectional association between dietary isoflavones and equal-producer status and coronary artery calcification, a biomarker of coronary atherosclerosis, among Japanese men. Their results subsequently showed that equal-producers had lower coronary artery calcification than equal nonproducers independent of cardiovascular risk factors. The absence of an inverse association between coronary arterial calcification and dietary isoflavones therefore indicated that equal may

be an important factor for the atheroprotective properties of dietary isoflavones.<sup>58</sup> Further prospective studies and clinical trials are warranted to expand on such observations.

Despite the growing body of evidence suggesting a relationship between microbiota profile and cardiovascular diseases, some questions about *S*-equol currently remain unresolved. For instance, the extent of differences in *S*-equol production under physiological and pathophysiological states have yet to be determined. Considering that the actual microorganisms involved in *S*-equol production currently remain unknown and that the production phenotype and *S*-equol production itself may be modified by dietary habits, drug consumption, and disease duration, aging, or sex, understanding the association between *S*-equol levels, microbiota population, and lifestyle among humans with and without cardiovascular diseases should be encouraged to elucidate the preventive or therapeutic effects of *S*-equol as a nutraceutical or pharmaceutical agent against cardiovascular diseases.

## INDOXYL SULFATE

Indoxyl sulfate is a protein-bound uremic toxin that has deleterious effects on the vascular system. Dietary protein-derived tryptophan is metabolized to indole by tryptophanase, which is produced by intestinal bacteria, such as *Escherichia coli*. Indole is then absorbed into the blood from the intestine, metabolized to indoxyl sulfate in the liver, and normally excreted into the urine.<sup>59</sup> During uremia, however, the reduced renal clearance of indoxyl sulfate results in elevated circulating levels, as observed in patients with chronic kidney disease (CKD).<sup>21–24,60</sup>

Organic anion transporters (OATs) are involved in the cellular uptake of indoxyl sulfate and play a role in the impairment of endothelial and smooth muscle functions.<sup>61</sup> Among the OATs related to indoxyl sulfate uptake, OAT1, and OAT3 are expressed in ECs<sup>61</sup> and VSMCs.<sup>62–65</sup> Studies have shown that the aryl hydrocarbon receptor (AhR) is an intracellular receptor for indoxyl sulfate.<sup>66,67</sup> The AhR is a ligand-activated transcriptional factor that mediates adaptive and toxic responses in cells.<sup>67,68</sup> Indoxyl sulfate induces a number of inflammation-related substances in ECs and VSMCs via OATs and AhR. Using the small interfering RNA technique, indoxyl sulfate-induced interleukin-6 (IL-6) expression in both HUVECs and HASMCs, which were suppressed by OAT3 small interfering RNA, AhR small interfering RNA, and nuclear factor- $\kappa$ B (NF- $\kappa$ B) subunit p65 small interfering RNA. This suggests that indoxyl sulfate induces IL-6 expression in both ECs and VSMCs via the OAT3/AhR/NF- $\kappa$ B pathway.<sup>62</sup> Given that IL-6 plays an important role in the initiation and amplification of inflammation, OAT3/AhR/NF- $\kappa$ B pathway suppression can be effective in preventing indoxyl sulfate-induced inflammation. Indoxyl sulfate not only induced the expression inflammatory substances, including cytokines, but also amplified cytokine-induced responses in ECs and VSMCs. Using EC-specific AhR knockout mouse, Ito *et al.*<sup>68</sup> demonstrated that indoxyl sulfate could enhance tumor necrosis factor (TNF)- $\alpha$ -induced leukocyte–endothelial interactions

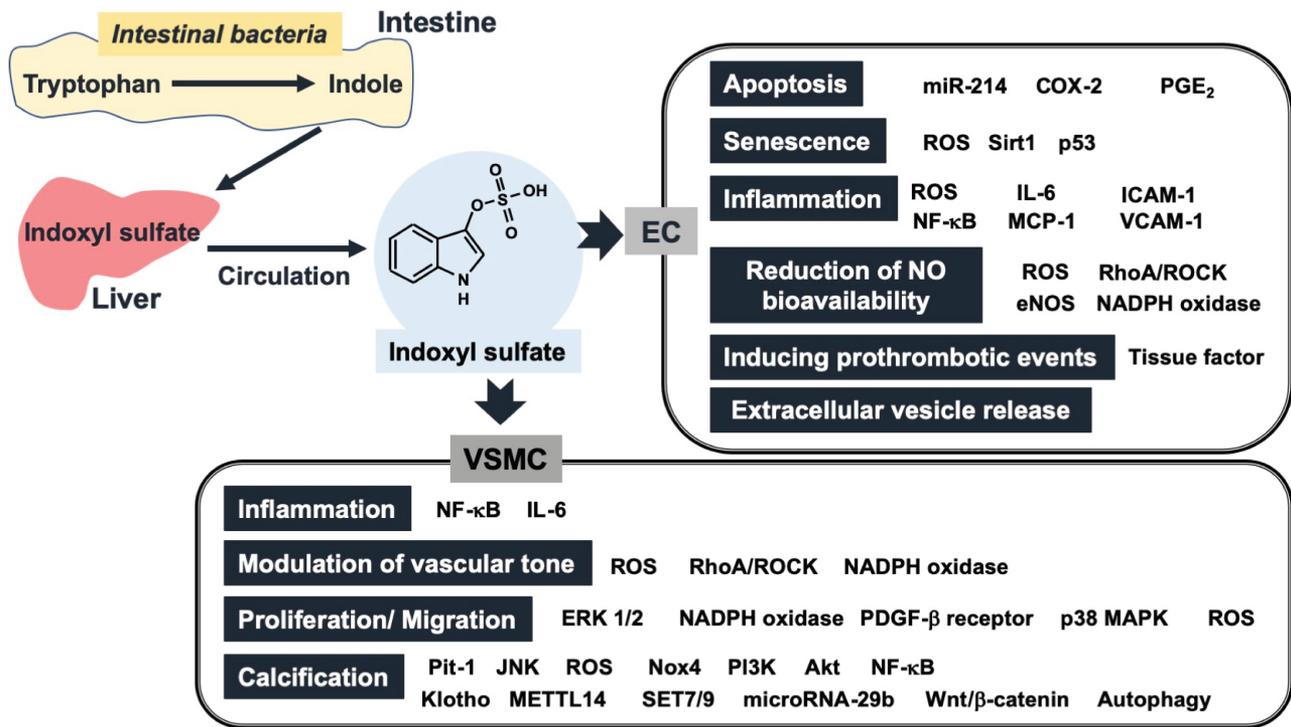
due to activator protein-1 transcriptional activity through AhR. Morita's laboratory found that indoxyl sulfate activates AhR and increases oxidative stress in HUVECs via Nox4 (a component of nicotinamide adenine dinucleotide phosphate oxidase), resulting in enhanced monocyte chemoattractant protein-1 expression.<sup>69</sup> In addition, indoxyl sulfate-induced monocyte chemoattractant protein-1 expression was related to indoxyl sulfate uptake via OATs in human ECs.<sup>61</sup> These evidences strongly suggest that indoxyl sulfate plays a key role in the development of atherosclerosis and that manipulation of indoxyl sulfate-related molecules (e.g., OATs and AhR) may be a potential approach against atherosclerosis.

Tissue factor is the primary initiator of blood coagulation *in vivo* and has been implicated in the pathogenesis of cardiovascular disorders and development of atherosclerotic diseases. Studies have shown that indoxyl sulfate increased tissue factor production in ECs and peripheral blood mononuclear cells, which are two cellular sources of tissue factor in the blood, via AhR activation.<sup>70</sup> This increase in tissue factor expression was associated with increased procoagulant activity.<sup>70</sup> Thus, given that indoxyl sulfate may be an initiation factor that increases thrombotic events, suppression of indoxyl sulfate-mediated signaling may prevent thrombotic complications associated with atherosclerosis.

Several reports have suggested an association between indoxyl sulfate and cellular senescence in ECs. Indoxyl sulfate suppresses Sirt1 activity in association with a reduction in intracellular nicotinamide phosphoribosyltransferase activity and NAD<sup>+</sup> content, leading to the acceleration of cellular senescence due to oxidative stress, with cellular senescence in HUVECs being mediated by AhR.<sup>71</sup> In addition, indoxyl sulfate promoted cellular senescence in HUVECs by increasing ROS production and p53 activity.<sup>72</sup> Considering the involvement of vascular senescence in the development of cardiovascular diseases, these evidences suggest that suppressing the indoxyl sulfate–AhR signaling pathway to regulate cellular senescence may be a novel approach against cardiovascular diseases.

EC apoptosis has been an important pathological feature in the development of vascular disease. Indoxyl sulfate downregulated microRNA-214 (miR-214) consistent with enhanced apoptosis in mouse aortic ECs, while cyclooxygenase-2 (COX-2) had been determined to be a target gene of miR-214, the inhibition of which reduced indoxyl sulfate-induced ECs apoptosis along with the suppression of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) secretion.<sup>73</sup> Thus, miR-214 plays a protective role against indoxyl sulfate-induced EC apoptosis by direct downregulation of cyclooxygenase-2/prostaglandin E<sub>2</sub> signaling, making it a potential target for indoxyl sulfate-induced EC injury.

Extracellular vesicles released by different cells, including ECs, have been closely associated with vascular dysfunction.<sup>74</sup> Indoxyl sulfate increases the release of extracellular vesicles from ECs, which promotes VSMC proliferation by inducing transforming growth factor- $\beta$  production.<sup>75,76</sup> Although mechanisms underlying indoxyl sulfate-induced release of extracellular vesicles remain unclear, the aforementioned



**Figure 2.** Effects of indoxyl sulfate on vascular endothelial and smooth muscle cells. Indoxyl sulfate induces apoptosis, senescence, prothrombotic events, reduction of nitric oxide bioavailability, and release of extracellular vesicle in endothelial cells and inflammation, proliferation and/or migration, calcification, and modulation of vascular tone in vascular smooth muscle cells. Details are provided in the text. Abbreviations: COX-2, cyclooxygenase-2; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; ICAM-1, intercellular adhesion molecule-1; IL-6, interleukin-6; JNK, c-Jun *N*-terminal kinase; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; METTL14, methyltransferase-like 14; miR-214, microRNA-214; NF-κB, nuclear factor-κB; PDGF, platelet-derived growth factor; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; PI3K, phosphoinositide 3-kinase; Pit-1, phosphate transporter 1; ROCK, Rho-associated protein kinase; ROS, reactive oxygen species; SET7/9, lysine methyltransferase 7/9; VCAM-1, vascular cell adhesion molecule-1.

data suggest that indoxyl sulfate may be a causative factor in the pathogenesis of vascular access stenosis.

Indoxyl sulfate affects many cellular functions in not only ECs but also VSMCs<sup>77,78</sup> (Figure 2) and induces the proliferation and migration of VSMCs through platelet-derived growth factor-β receptors and ROS generation.<sup>79</sup>

Vascular calcification is an independent risk factor for the development of cardiovascular diseases and a prognostic indicator of end-stage renal disease.<sup>78,80</sup> Phosphorus (Pi) is an important regulator of vascular calcification, with Pi transport via type-III sodium (Na)-Pi cotransporters, PiT1, and PiT-2, being a crucial step in calcification.<sup>81</sup> Indoxyl sulfate promoted Pit-1 expression in part by activating the c-Jun *N*-terminal kinase pathway related to the mechanism of indoxyl sulfate-induced osteoblastic differentiation and matrix mineralization.<sup>82</sup> Indoxyl sulfate-induced ROS generation via Nox4 upregulation and the expression of osteoblast-specific proteins, including core binding factor 1, alkaline phosphatase, and osteopontin in HASMCs.<sup>83</sup> The activation of the phosphoinositide-3-kinase/Akt/NF-κB pathway was also related to VSMC calcification induced by indoxyl sulfate.<sup>84</sup> Several reports have suggested an association between indoxyl sulfate, calcification, and epigenetic regulators in VSMCs. Indoxyl sulfate increased CpG hypermethylation of the *Klotho* gene, decreased *Klotho* expression in HASMCs, and potentiated calcification in HASMCs.<sup>85</sup>

Methyltransferase-like 14-dependent m6A methylome in VSMCs is also related to the development of indoxyl sulfate-induced calcification.<sup>86</sup> Indoxyl sulfate promoted osteoblastic differentiation and calcification of VSMCs and reduced the expression of lysine methyltransferase 7/9, one of the important histone methyltransferases.<sup>87</sup> In addition, indoxyl sulfate was able to activate autophagy, with the inhibition of autophagy partly suppressing the stimulating effect of indoxyl sulfate on the expression of both runt-related transcription factor 2 and calcium deposition.<sup>87</sup> In HASMCs, indoxyl sulfate accelerates calcification through miRNA-29b-dependent regulation of Wnt/β-catenin signaling.<sup>88</sup>

Studies have shown that acutely exposing indoxyl sulfate to normal control vessels decreases ACh-induced endothelium-dependent relaxation in the thoracic aorta of mice<sup>89</sup> and rats<sup>90</sup>, and reduces in the superior mesenteric artery of rats.<sup>91</sup> Such acute effects of indoxyl sulfate on endothelium-dependent relaxation may be attributed to decreased NO bioavailability. ACh-induced endothelium-dependent relaxation is largely mediated by NO in the thoracic aorta, with EDHF also contributing to superior mesenteric artery relaxation in addition to NO.<sup>92</sup> We demonstrated that the indoxyl sulfate-mediated reduction in ACh-induced relaxation within the rat superior mesenteric artery was still preserved following COX inhibition by indomethacin or COX plus EDHF signaling inhibition by

indomethacin plus small ( $SK_{Ca}$ )- and intermediate ( $IK_{Ca}$ )-conductance calcium-activated  $K^+$  channels.<sup>91</sup> However, the difference in ACh-induced relaxation between vehicle and indoxyl sulfate treated groups was eliminated by NOS inhibition or NOS/COX inhibition.<sup>91</sup> Therefore, acute exposure of rat superior mesenteric artery to indoxyl sulfate impaired ACh-induced endothelium-dependent relaxation due to the reduction in NO signaling rather than alterations in other EDRFs, such as EDHF and prostacyclin. In addition to acute treatment with indoxyl sulfate, prolonged *in vitro* treatment with indoxyl sulfate impaired ACh-induced relaxation in the aorta of female wild-type mice whereas AST-120 (an oral charcoal adsorbent, described below) improved relaxation and prevented indoxyl sulfate-induced EC loss (assess using CD31 expression) and intercellular adhesion molecule-1/vascular cell adhesion molecule-1 upregulation.<sup>89</sup> Furthermore, *in vivo* AST-120 treatment in a mice model of CKD (i) improved ACh-induced aortic relaxation, (ii) reduced aortic expressions of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1, (iii) decreased aorta systolic expansion rate, and (iv) prevented the increase in pulse wave velocity.<sup>89</sup> Moreover, treatment with indoxyl sulfate impaired vascular responses, such as increased phenylephrine-induced contraction and decreased ACh-induced relaxation of the aorta, in 5/6 nephrectomized rats, all of which were improved by ROS scavengers or RhoA/Rho kinase (ROCK) pathway blockade.<sup>93</sup>

Despite the presence of complicating mechanisms underlying indoxyl sulfate-induced EC and VSMC dysfunction (Figure 2), the manipulation of the aforementioned signalings may constitute an effective strategy. AST-120 adsorption of indoxyl sulfate can also be an effective strategy for blocking the deleterious effects of indoxyl sulfate. Indeed, AST-120 treatment has been shown to reduce oxidative stress<sup>94,95</sup> and improve flow-mediated vasodilation in patients with CKD<sup>94</sup> and endothelium-dependent relaxation in uremic rat arteries<sup>95</sup> while exerting protective effects against the progression of atherosclerosis.<sup>96</sup> Considering the aforementioned evidence suggesting that indoxyl sulfate is undoubtedly a causative factor for the development of vascular dysfunction, decreasing its levels (e.g., through AST-120) may be an effective and novel therapeutic strategy for the treatment of cardiovascular diseases.

Indoxyl sulfate is excreted from the circulating blood into the urine by healthy kidneys.<sup>97</sup> Generally, indoxyl sulfate is metabolized by dietary tryptophan.<sup>69,97</sup> Thus, a high-protein diet and gut microflora influence the increase in circulating indoxyl sulfate levels among patients with mild renal dysfunction or without CKD.<sup>98,99</sup> However, indoxyl sulfate can easily accumulate in patients with renal dysfunction, especially those with impaired renal tubular excretory function.<sup>97</sup> Patients with advanced CKD have higher levels of circulating indoxyl sulfate than those without CKD. In addition, the increased accumulation of indoxyl sulfate has been associated with future risk.<sup>100,101</sup> On the other hand, an increased plasma indoxyl sulfate levels had been associated with increased carotid intima-media thickness among patients with chronic coronary artery disease with preserved renal function.<sup>102</sup> Also, a recent study using a comprehensive metabolomic profiling of plasma in patients

with type 2 diabetes to explore metabolites associated with atherosclerosis found that plasma levels of inositol and indoxyl sulfate were associated with carotid maximal intima-media thickness and/or flow-mediated vasodilation.<sup>103</sup> Moreover, subjects with coronary artery disease had significantly higher plasma levels of inositol and indoxyl sulfate than those without an apparent history of cardiovascular disease.<sup>103</sup> These findings suggest that increased plasma indoxyl sulfate levels can accelerate atherosclerosis not only in patients with severe renal dysfunction but also in those with early nephropathy or normal renal function. Thus, impaired renal function is not the sole reason for high concentrations of circulating indoxyl sulfate. Given that diabetes profoundly alters the gut microenvironment and is associated with a distinct gut microbial composition and metabolism,<sup>104,105</sup> it could be also related to elevated circulating indoxyl sulfate levels. Nonetheless, further investigations on the balance between generation and excretion of indoxyl sulfate in each cardiovascular disease will be required.

### TRIMETHYLAMINE N-OXIDE

Dietary betaine, choline, L-carnitine, and other choline-containing compounds, which are the principal nutrient precursors of TMAO, are metabolized to trimethylamine (TMA) by gut microbiota and several enzymes. TMAO is a compound generated by the liver via a flavin-monooxygenase 3 oxidation of gut microbiota-derived TMA, which is absorbed in the intestines and delivered to the liver via the portal circulation.<sup>16,18,106</sup> Dietary choline intake has been mechanistically linked to atherosclerotic plaque formation by increasing circulating TMAO levels derived from gut microbiome metabolism of choline to TMA.<sup>21,107</sup>

A growing body of evidence has suggested that TMAO has various adverse effects at the EC level.<sup>106</sup> In HUVEC and aortas from *ApoE* knockout mouse, TMAO exerted proinflammatory effects through nucleotide-binding oligomerization domain-like receptor family, pyrin domain-containing 3 (NLRP3) inflammasome activation partly due to the inhibition of the sirtuin 3-superoxide dismutase 2-mitochondrial ROS signaling pathway.<sup>108</sup> TMAO was able to induce the expression of inflammatory markers in both primary human aortic ECs (i.e., cyclooxygenase-2, IL-6, E-selectin, and intercellular adhesion molecule-1) and VSMCs (cyclooxygenase-2, IL-6, tumor necrosis factor- $\alpha$ , and intercellular adhesion molecule-1), increase leukocyte adhesion to ECs, and activate mitogen-activated protein kinases (p38 mitogen-activated protein kinase and ERK1/2) and NF- $\kappa$ B.<sup>109</sup> These evidences suggest that TMAO has proinflammatory abilities in both ECs and VSMCs. Decreased self-repair capacity in ECs may play a role in the initiation of atherosclerosis. TMAO impaired the self-repair capacity of HUVECs and increased monocyte adhesion partly due to the activation of the protein kinase C/NF- $\kappa$ B/vascular cell adhesion molecule-1 pathway.<sup>110</sup> Another study showed that TMAO increased senescence in HUVECs through suppression of SIRT1 expression, increased oxidative stress, and p53/p21/Rb pathway activation.<sup>111</sup> Activation of the ROS-thioredoxin interacting

protein-NLRP3 inflammasome contributed to TMAO-induced inflammation (i.e., increased production of IL-1 $\beta$  and IL-18) and endothelial dysfunction (i.e., reduced eNOS expression and NO production).<sup>112</sup> These evidences indicate that TMAO is a novel positive regulator of endothelial dysfunction. Although the receptors for TMAO have not yet to be identified, the aforementioned signaling molecules may provide information on suppressing TMAO-related endothelial dysfunction and atherogenesis.

Several reports have investigated the relationship between TMAO and vascular tone regulation (Table 1). Accordingly, an association between elevated circulating TMAO levels and endothelial dysfunction, including decreased eNOS-derived NO bioavailability in the aorta, had been observed in Fischer-344 rats.<sup>113</sup> In a reduced uterine perfusion pressure rat model of preeclampsia, increased levels of circulating TMAO, increased superoxide production, and proinflammatory cytokines in the aorta, reduced aortic relaxation, and hypertension had been observed, all of which were normalized following TMAO inhibitor treatment (3,3-dimethyl-1-butanol).<sup>114</sup> In a rat model of CKD, 3,3-dimethyl-1-butanol treatment normalized ACh-induced endothelium-dependent relaxation and eNOS activity and reduced superoxide production and proinflammatory cytokine (i.e., tumor necrosis factor- $\alpha$  and IL-6) expressions in the aorta.<sup>115</sup> These evidences suggest that TMAO is a causative factor for the development of endothelial dysfunction in aging, preeclampsia, and CKD and that targeting TMAO may be a novel strategy for the prevention and treatment of patients with cardiovascular disease.

However, only a few direct evidences have been available regarding the relationship between TMAO and vascular function (Table 1). Intraluminal exposure to TMAO (1  $\mu$ mol/l for 4 hours) had no effect on ACh-induced relaxation in adipose arterioles from healthy volunteers.<sup>116</sup> In our recent study, acute exposure to TMAO (300  $\mu$ mol/l

for 1 hour) specifically impaired EDHF-type relaxation in rat femoral arteries but not superior mesenteric arteries.<sup>117</sup> In that study, we found that ACh-induced femoral arterial relaxation was similar between the control and TMAO-exposed groups, whereas ACh-induced femoral arterial relaxation observed in the presence of *N*<sup>w</sup>-nitro-L-arginine (L-NNA, a NOS inhibitor) plus indomethacin (a COX inhibitor) was greatly impaired in the TMAO-treated group. In addition, under indomethacin treatment (i.e., preserved NO and EDHF components), ACh-induced femoral arterial relaxation was slightly weaker in the TMAO-exposed group than in the control group. We also found that ACh-induced NO-mediated femoral artery relaxation was similar between the control and TMAO-exposed groups under treatment with indomethacin and 1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole (TRAM-34, *I*K<sub>Ca</sub> inhibitor) and apamin (SK<sub>Ca</sub> inhibitor) (two *K*<sub>Ca</sub> channel inhibitors related to the source of EDHF). Furthermore, ACh-induced femoral arterial relaxation was considerably small and similar between both two groups under three EDRFs blockades established via treatment with L-NNA, indomethacin, TRAM-34, and apamin. Finally, we showed that ACh-induced relaxation was similar between the control and TMAO-exposed groups under (i) intact, (ii) L-NNA plus indomethacin, and (iii) L-NNA, indomethacin, TRAM-34, and apamin conditions in the rat superior mesenteric arteries. Therefore, the aforementioned findings suggest that TMAO could specifically affect endothelial function with variations among different vessels.

A growing body of evidence has suggested that TMAO affects the cardiovascular system, exerting both harmful or beneficial effects.<sup>118,119</sup> These discrepancies may have resulted from the limited number of studies investigating the effects of TMAO at concentrations close to physiological levels in mammals. In fact, chronic, low-dose, oral TMAO treatment could reduce diastolic dysfunction in the pressure-overloaded heart of hypertensive rats.<sup>120</sup> On

**Table 1.** Evidence of the relationship between vascular tone and TMAO

Conditions	Responses	References
Direct exposure of TMAO to vessels		
Adipose arterioles from healthy volunteers [1 $\mu$ mol/l for 4 h]	No effect on ACh-induced relaxation	Malik <i>et al.</i> <sup>116</sup>
Superior mesenteric artery of rat [300 $\mu$ mol/l for 1 h]	No effect on ACh-induced relaxation	Matsumoto <i>et al.</i> <sup>117</sup>
Femoral artery of rat [300 $\mu$ mol/l for 1 h]	Impaired ACh-induced EDHF-type relaxation	Matsumoto <i>et al.</i> <sup>117</sup>
Treatment with DMB, an inhibitor of trimethylamine formation to reduce TMAO levels		
Aorta of aged rat [male Fisher-344 rat (~22 months old), with vs. without DMB]	Improvement of ACh-induced relaxation No effect on SNP-induced relaxation	Li <i>et al.</i> <sup>113</sup>
Aorta of CKD model rat [5/6 nephrectomy rat, with vs. without DMB]	Improvement of ACh-induced relaxation No effect on SNP-induced relaxation	Li <i>et al.</i> <sup>115</sup>
Aorta of RUPP model rat [with vs. without DMB]	Improvement of ACh-induced relaxation No effect on SNP-induced relaxation	Chen <i>et al.</i> <sup>114</sup>

Abbreviations: ACh, acetylcholine; CKD, chronic kidney disease; DMB, 3,3-dimethyl-1-butanol (an inhibitor of trimethylamine formation); EDHF, endothelium-derived hyperpolarizing factor; RUPP, reduced uterine perfusion pressure; SNP, sodium nitroprusside; TMAO, trimethylamine *N*-oxide.

the other hand, Jaworska *et al.*<sup>121</sup> observed that older rats presented higher levels of plasma TMA, which is associated with alterations in gut bacteria composition, structural, and functional changes in the colon, and increased penetration of TMA from the colon to portal blood. The same authors found that close to physiological concentrations of TMA reduced proliferation and viability of human VSMCs, and that TMAO did not exert cytotoxic effects at concentrations exceeding its physiological levels by 1,000-fold.<sup>121</sup> Thus, understanding the role of not only TMAO but also its precursor TMA in the initiation and development of vascular dysfunction is necessary.

A comprehensive understanding of the direct effects of TMAO on vascular function and their molecular mechanisms could provide a potentially novel therapeutic target for the treatment of vascular diseases.

Evidence from experimental and clinical investigations has confirmed the role of the gut microbiota in TMAO metabolism, with recent seminal reviews focusing on the relationship between gut microbiota and TMAO in cardiovascular diseases.<sup>106,122</sup> For example, the association between gut microbiota dysbiosis and increased circulating TMAO levels had been observed in several pathophysiological states, such as atherosclerosis,<sup>123</sup> preeclampsia,<sup>124</sup> and CKD.<sup>125</sup> Given that microbiota-derived TMA is an important precursor of TMAO generation, the regulation of TMA production, including gut microbiota remodeling (e.g., antibiotics, synbiotics, probiotic functional products, and some natural molecules), and blocking of microbiota TMA lyases (e.g., 3,3-dimethyl-1-butanol<sup>126</sup>) can be strategies in the regulation of circulating TMAO. In addition, inhibiting hepatic flavin-monooxygenase 3 activity (e.g., trigonelline, a compound from *Trigonella foenum-graecum*<sup>127</sup> and guggulsterone, a nuclear factor farnesoid X receptor antagonist<sup>128</sup>) to inhibit the conversion from TMA to TMAO can also be a beneficial strategy. Thus, a theoretical basis for controlling the gut microbiota to regulate TMAO levels will be beneficial for preventing and/or treating cardiovascular diseases.<sup>106,107,129</sup> However, one should note that reducing TMAO may also have adverse effects. Therefore, we believe that a new TMAO-targeting therapeutic approach against cardiovascular diseases will be established in the near future.

The number of publications on gut-derived substances and host homeostasis has been growing rapidly. This review mainly focused on the effects of S-equal, indoxyl sulfate, and TMAO on vascular functions. Notably, other substances, such as bile acids and short-chain fatty acids, also play a role in the regulation of vascular functions.<sup>16–18,20,21</sup> Alterations in vascular tone regulation, including the generation of several endogenous vasoactive substances and responsiveness thereto, and their signaling pathways by gut-derived substances may depend on vessel type, exposure duration (e.g., acute or chronic), and host status (sex, age, or disease). Despite the ongoing questions as mentioned previously, we believe that further knowledge on the manipulation of gut-derived substances

will lead to new approaches in the prevention and treatment of vascular diseases.

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## DISCLOSURE

The authors declared no conflict of interest.

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