Mammalian stanniocalcin-1 activates mitochondrial antioxidant pathways: new paradigms for regulation of macrophages and endothelium

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Sheikh-Hamad D. Mammalian stanniocalcin-1 activates mitochondrial antioxidant pathways: new paradigms for regulation of macrophages and endothelium. Am J Physiol Renal Physiol 298: F248–F254, 2010. First published August 5, 2009; doi:10.1152/ajprenal.00260.2009.—The mammalian homolog of the fish calcium regulatory hormone stanniocalcin-1 (STC1) is ubiquitously expressed and likely functions in an autocrine/paracrine fashion. Mammalian STC1 does not appear to exert significant effects on serum calcium, and its physiological role remains to be determined. In macrophages, STC1 decreases intracellular calcium and cell mobility; attenuates the response to chemoattractants; and diminishes superoxide generation through induction of uncoupling protein-2 (UCP2). In cytokine-treated endothelial cells, STC1 attenuates superoxide generation and the activation of inflammatory pathways [c-Jun NH2-terminal kinase (JNK) and NF-kB]; maintains the expression of tight junction proteins, preserving the endothelial monolayer seal; and decreases transendothelial migration of leukocytes. Combined, the effects of STC1 on endothelial cells and macrophages predict potent anti-inflammatory action. Indeed, application of the anti-glomerular basement membrane (GBM) glomerulonephritis model to STC1 transgenic mice that display increased expression of STC1 transgene in endothelial cells and macrophages yields renal protection. Our data suggest that STC1 activates antioxidant pathways in endothelial cells and macrophages and displays cytoprotective and anti-inflammatory actions.

What is Stanniocalcin-1?

STC1 is an important calcium regulatory hormone in fish, in which elevation of serum calcium triggers the release of STC1 from the corpuscles of Stannius (75) (organs associated with the kidneys) to inhibit calcium influx through the gill and intestine (48, 77) and maintain stable calcium concentrations in the blood. Fish and mammals express two stanniocalcin genes, STC1 and STC2 (81). Mammalian stanniocalcin genes are similarly structured; both have four exons and display conserved exon-intron boundaries (38). The fish STC1 gene contains five exons (57); exons 3 and 4 correspond to exon 3 in mammalian STC1 and STC2, suggesting that mammalian STC1 and STC2 are derived from a common ancestral gene (53). Mammalian STC1 and STC2 contain N-glycation motifs, PKC/PKA consensus sequences, a signal peptide sequence of ~24 amino acids, and a prosequence of ~15 amino acids that are subsequently cleaved to yield the mature proteins; consistently, mammalian STC1 and STC2 are secreted phosphoglycoproteins (60). STC1 and STC2 have moderately conserved primary amino acid sequences, especially at their NH2-terminal domains with spatial conservation of cysteine residues, suggesting that they might have similar biological functions, although recombinant STC2 protein does not displace STC1 from its putative receptor (83).

In humans, STC1 is a protein of 247 amino acids that is 90% similar to salmon STC1 over the first 200 NH2-terminal amino acids (64). Mammalian STC1 mRNA is ubiquitously expressed, and the highest level of expression is found in the ovary, kidney, prostate, and thyroid (12, 64, 81). It was previously suggested that STC1 protein does not circulate in the blood of mammals (19) except during pregnancy and lactation (22); however, recent reports suggest that mammalian STC1 is blood borne (35, 40), likely attached to a soluble protein (40). The distributions of STC1 mRNA and protein are not always parallel. For example, in situ hybridization studies in the kidney reveal restricted expression of STC1 mRNA in the cortical and medullary collecting ducts, yet the protein is detected along the entire nephron (18, 87). Similarly, the expression of STC1 mRNA does not parallel the expression of the protein in cells of the ovaries (81) and uterus (76). The ubiquitous distribution of STC1 mRNA in mammalian organs, the wider tissue distribution of STC1 protein compared with the mRNA, and the fact that STC1 is a secreted protein suggest that STC1 functions in an autocrine/paracrine manner. The function of mammalian STC1 is yet to be defined, but the diverse schemes regulating its expression (32, 34, 41, 65, 73, 89, 90) and its dysregulation in malignancies (28, 86) suggest that STC1 plays important roles in the normal physiology of many organs.

Like STC1, mammalian STC2 is ubiquitously expressed; in humans, the primary site of STC2 production is the pancreas...
(60), where the protein localizes to a subpopulation of islet cells, suggesting involvement of STC2 in glucose and energy metabolism (60). Consistent with this, deletion of STC2 produces overweight mice (11). Abnormalities in the expression of STC2 have also been associated with various malignancies (5, 13).

**Stanniocalcin-1 Receptors**

There are results that suggest the existence of STC1-binding protein (receptor). This conclusion is based on experiments using stanniocalcin-alkaline phosphatase fusion protein, which was used in binding assays yielding results consistent with the presence of high-affinity (0.25–0.8 nM), saturable, and displaceable binding sites for STC1 in cells of the kidney, liver, breast, and ovaries (56, 58, 66, 70). Analysis of membrane fractions suggested that 90% of the binding sites are mitochondrial, while ~10% are located at the plasma membrane (56). It was suggested that STC is produced by one cell type, secreted, and targeted to neighboring cells, where it is bound to cell surface receptors, trafficked, and sequestered in the mitochondria (56). Membrane and mitochondrial receptors have similar binding affinities, suggesting that they are identical (56). As to targeting of STC1 to the mitochondria, two models have been proposed by Sazonova et al. (70): one model envisions the membrane receptor acting first as a signal transducer and second as a chaperone to facilitate translocation of STC1; alternatively, the membrane-bound receptor may serve as a signal transducer, which then passes STC1 to a unique mitochondrial receptor for internalization. Consistent with these models, we have recently shown (85) internalization of recombinant STC1 from the medium and subsequent sequestration of the protein in the mitochondria of freshly isolated murine peritoneal macrophages within 10 min.

**Phenotype of Transgenic Stanniocalcin-1 Mice**

To date, two transgenic STC1-overexpressing mice have been generated (27, 80). Muscle-specific overexpression of STC1, driven by the myosin light chain-2 promoter (27), produced dwarf mice that displayed normal blood pressure and normal reproductive capacity when transgenic males were mated with wild-type (WT) females. Serum phosphate levels were normal, but serum calcium levels were elevated; the latter finding was attributed to stimulated osteoclast activity (27). Notably, compared with WT littermates, transgenic mice were hyperphagic (32% higher food intake per g body wt), consumed more oxygen (14% more per g body wt), and had leaner fat pads and faster clearance of glucose (27), yet transgenic mice displayed normal serum free fatty acids, IGF-binding protein-1, thyroxine (T₄), and growth hormone (GH) (27). These mice were noted to have mitochondrial swelling (27), suggesting that STC1 affects mitochondrial function and/or structure.

In the second transgenic mouse line(s), STC1 expression is driven by the metallothionein I minimal promoter (80) and displays strong preferential expression of the transgene in the liver, heart, brain, endothelial cells, and macrophages (35, 80); serum calcium levels are normal, while serum phosphate levels are slightly higher. Here, too, transgenic mice are smaller compared with WT littermates yet display no abnormalities in serum GH and IGF-1 or steady-state mRNA levels of pituitary TSH β-subunit, FSH β-subunit, LH β-subunit, or α-glycoprotein subunit (80).

In light of observations by others and data from our lab, I would like to propose a plausible explanation for the dwarf phenotype in both transgenic mice. A report by James and coworkers (25) suggested uncoupling of the mitochondria by STC1. In agreement with this observation, our data suggest that STC1 upregulates the expression of mitochondrial uncoupling protein-2 (UCP2) in macrophages (85) and UCP3 in cardiomyocytes (52a). And it is highly likely that STC1 upregulates uncoupling proteins in other tissues. This is interesting because transgenic overexpression of UCP3 in skeletal muscle produces hyperphagic mice that are smaller in size compared with WT littermates and display leaner fat pads and faster clearance of glucose (17), a phenotype similar to that of STC1 transgenic mice (27). Since both transgenic mouse lines display high serum levels of STC1 (27, 80), it is highly likely that the dwarf phenotype of these transgenic mice results from STC1-mediated upregulation of uncoupling proteins in different tissues; as a consequence, the mice are hyperphagic and consume more oxygen—to compensate for the hypermetabolic state caused by uncoupling of the mitochondria (see below).

A STC1 knockout (KO) mouse line has been generated and displays normal phenotype (10), yet this mouse line has not been challenged (e.g., applying a disease model) to determine the impact of STC1 deletion on disease states.

**STC1 Suppresses Superoxide Generation in Macrophages Through Induction of Mitochondrial Uncoupling Proteins: A New Paradigm for Regulation of Macrophage Function**

We have shown that STC1 inhibits macrophages through several interrelated pathways: STC1 attenuates intracellular second messenger signals through calcium and decreases macropage mobility (42); in addition, it inhibits macrophage response to chemoattractants (42) and induces UCP2 expression [in freshly isolated mouse peritoneal macrophages and cultured murine macrophages (Raw 264.7 cells)]—decreasing mitochondrial membrane potential and superoxide generation (85). Superoxides are important for macrophage function and viability. As discussed below, UCP2 plays a critical role in the regulation of superoxide generation in macrophages; hence the recognition that STC1 decreases superoxide generation in macrophages (through induction of UCP2) places it in the forefront of immunity and cytoprotection.

As shown in Fig. 1, transfer of electrons (e⁻) from high-energy molecules (NADH and FADH₂; products of the Krebs cycle in the mitochondrial matrix) along the respiratory chain (complexes I–IV) generates a higher H⁺ ion concentration in the mitochondrial intermembrane space compared with the matrix, increasing mitochondrial membrane potential; concomitantly, superoxide production (formed by electron reduction of oxygen during electron transport) is also increased. H⁺ ions travel back to the matrix through the ATP synthase complex, facilitated by the favorable electrochemical gradient, generating ATP in the process (39, 71, 82). Alternatively, H⁺ ions may leak back to the matrix through uncoupling proteins (localized at the inner mitochondrial membrane and serving as H⁺ channels), bypassing ATP synthase—dissipating the H⁺ gradient. While oxygen consumption and the utilization of NADH and FADH₂ continue, ATP generation diminishes,
on superoxide is independent of NADPH oxidase, as suppression of superoxide generation by STC1 is observed in gp91phox-null macrophages (85). Conventional wisdom has it that the production of physiologically relevant ROS in macrophages occurs exclusively via the NADPH oxidase system. Thus the identification of a physiologically relevant and STC1/UCP2-regulatable ROS-generating system in macrophage mitochondria introduces a new paradigm for the regulation of immunity and inflammation.

Moreover, recent data suggest that lipopolysaccharide (LPS) increases mitochondrial superoxide generation through down-regulation of UCP2 (26). Since STC1 upregulates, while LPS downregulates, UCP2 in macrophages (26) we hypothesized that STC1 acts as a naturally occurring LPS antagonist. Indeed, STC1 blunts the rise in superoxide generation in LPS-treated peritoneal macrophages (85). This finding is critically important, as it suggests a role for STC1 in regulating innate immunity. Finally, superoxides and other ROS damage proteins and nucleic acids and have been implicated in aging and neurodegenerative disease (6, 59); thus STC1 may potentially delay aging-related organ dysfunction.

As mentioned above, uncoupled mitochondria are less efficient in ATP generation (7, 45, 68). Indeed, we find lower ATP levels in STC1-treated macrophages (85). The decline in cellular ATP levels can lead to cell death (69). On the other hand, the reduction in ROS generation can reduce apoptosis (46, 54). Hence, induction of UCP2 by STC1 could potentially decrease cell viability (due to the decrease in ATP) or increase it (due to suppression of superoxide generation). Of interest, we find increased cell viability in STC1-treated macrophages; however, STC1 induces cell cycle arrest at the G1 phase (85). Consistent with this, a recent report suggested that lower cellular ATP levels may promote cell survival by inducing cell cycle arrest (55). Thus, despite the reduction in ATP levels in STC1-treated macrophages, cell survival is enhanced, suggesting cytoprotection likely due to the combined reduction in ATP levels and superoxide generation. Our findings are in agreement with data from Zhang et al. (92) showing that transfection of Paju cells with STC1-expressing plasmid increased cell resistance to ischemic challenge, or elevated intracellular free calcium, induced by treatment with thapsigargin (92). Similarly, in multipotent stem cells, STC1 reduces apoptosis of lung cancer epithelial cells under conditions of low pH and hypoxia (4). In contrast, Law et al. (52) suggested that STC1 may promote apoptosis in human colon adenoma cells, while Nguyen et al. (62) suggested that STC1 reduces proliferation of mouse embryonic fibroblasts following oxidative injury, likely through suppression of MEK/ERK. The response to STC1 may be cell specific. In addition, cancer cells have escaped cell cycle regulatory signaling, and hence one should carefully interpret their response to stimuli that affect cell survival. Thus the response of colon cancer cells to STC1 does not negate a cytoprotective role for STC1 as we and others have reported (4, 92).

**STC1 Opposes Action of Cytokines on Endothelium: A New Paradigm for Regulation of Transendothelial Permeability**

It is well established that cytokines increase endothelial permeability, enhancing transmigration of inflammatory cells (21, 84). Attachment between neighboring cells is facilitated
by tight junctions and adherent junctions (20). Tight junctions contain the transmembrane protein occludin, which is bound to zonula occludens-1 (ZO-1) (29). Adherent junctions are formed by clusters of transmembrane proteins that belong to the cadherin family—linked intracellularly to catenins, which promote anchorage to the actin cytoskeleton (30, 43). Through cytoskeletal association, the tight junctions and adherent junctions form the apical junction complex, which plays an important role in regulating leukocyte transmigration (24).

Stimulation of endothelial cells by cytokines, frequently referred to as “activation,” involves the convergence of signaling mediators that include ROS, stress-activated protein kinases [c-Jun NH₂-terminal kinase (JNK)], and NF-κB (16, 31, 47). This “activation” is associated with increased expression of adhesion molecules (ICAM-1, VCAM-1, and E-selectin) and increased permeability—facilitating transendothelial migration of leukocytes (1, 67, 74).

We previously showed expression of STC1 mRNA in endothelial cells in vivo (72) and localization of the protein at the apical surface (8). In cultured endothelial cells STC1 regulates gene expression (8), and in TNF-α-treated endothelial cells STC1 stabilizes permeability through a number of interrelated mechanisms (14): 1) it attenuates the generation of ROS and the activation of signaling pathways that promote inflammation (JNK and NF-κB); 2) it opposes the effects of cytokines on the expression of tight junction proteins (ZO-1, occludin, claudin-1) (14); and 3) importantly, it inhibits cytokine-induced but fails to affect VEGF-induced endothelial permeability (14). A proposed scheme for the action of STC1 in endothelial cells is shown in Fig. 2. Is the effect of STC1 on the endothelium relevant to endothelial biology? The answer is yes, as STC1 dose-dependently diminishes the migration of macrophages and T cells across cytokine (IL-1β)-treated endothelial monolayer (8).

Transgenic Overexpression of STC1 Affects Energy Metabolism and Superoxide Generation

As discussed above, STC1 suppresses macrophage mobility and function (42), inhibits cytokine-induced rise in endothelial permeability (14), and diminishes transendothelial migration of inflammatory cells (8): through these combined and unique actions, STC1 is predicted to possess potent anti-inflammatory action. To test this hypothesis, we applied the anti-GBM disease model to STC1 transgenic mice that display preferential expression of STC1 transgene in endothelial cells and macrophages (35, 80). Experimental anti-GBM glomerulonephritis (GN) is an inflammatory disease characterized by proteinuria, macrophage and T-cell infiltration, glomerular crescent formation, and Th1 antibody and cytokine responses; it leads to progressive renal failure over days to weeks (37, 49, 50). The expression of proinflammatory cytokines (such as IL-1β) is markedly elevated in anti-GBM GN (51, 61, 88, 91); hence, increased endothelial permeability is expected. Our data show striking protection from anti-GBM-induced kidney injury in STC1 transgenic mice (35). Compared with WT mice, STC1 transgenic mice exhibited 1) diminished infiltration of inflammatory macrophages into the glomeruli; 2) marked reduction in crescent formation and sclerotic glomeruli (75% and 90%, respectively); 3) decreased interstitial fibrosis (80%); 4) preservation of kidney function and lower blood pressure; and 5) diminished C3 deposition in the glomeruli and reduced expression of macrophage inflammatory protein-2 (MIP-2) and transforming growth factor-β2 (TGF-β2) in the kidney. Moreover, compared with baseline, WT mice, but not STC1 transgenic mice, had more proteinuria and marked reduction in urine output. STC1 had no effect on the expression of cytokines characteristic of either Th1 or Th2 activation. Additionally, STC1 overexpression does not appear to affect antibody response or complement activation in the context of anti-GBM GN. Recent reports suggest that macrophages are common effectors for both CD4 and CD8 T cell-dependent injury in anti-GBM (36) and that macrophage depletion diminishes the recruitment of T cells to the kidney and provides renal protection (23, 33). Thus inhibition of macrophage infiltration into the glomeruli as we found in STC1 transgenic mice appears to be necessary and sufficient to ameliorate anti-GBM disease. Our data are consistent with these reports and support our hypothesis that through direct inhibition of macrophages and attenuation of transendothelial migration of inflammatory cells, STC1 provides potent anti-inflammatory action. Indeed, this is what we find.

Collectively, our observations identify STC1 as a key regulator of macrophage and endothelial function and may potentially lead to the development of new therapeutic targets to modulate immunity/inflammation.

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DISCLOSURES

No conflicts of interest are declared by the author.
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