Nitric oxide regulates non-classical secretion of tissue transglutaminase

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Nitric oxide (NO) is an endogenous second messenger which acts as a potent vasodilator, anti-inflammatory, anti-thrombotic and pro-angiogenic agent in the vasculature. Recent studies revealed that the effects of NO on blood vessels are mediated in part by its ability to regulate protein trafficking machinery and vesicle-based exocytosis in vascular cells. Specifically, NO-dependent *S*-nitrosylation of *N*-ethylmaleimide sensitive factor (NSF), an ATPase that enables membrane fusion, was shown to inhibit exocytosis of vesicular secretory compartments such as endothelial Weibel-Palade bodies, platelet alpha granules and cytolytic granules from activated lymphocytes. Tissue transglutaminase (tTG or TG2) is a multifunctional protein synthesized and secreted by various cell types in the vasculature, which is involved in multiple vascular diseases, including atherosclerosis, vascular calcification and age-dependent aortic stiffening. Our recent findings indicate that tTG is delivered to the cell surface and the extracellular matrix (ECM) via a non-classical ER/Golgi-independent secretion pathway, which depends on the recycling endosomes and the NSF function. Here we report that NO attenuates the unconventional secretion of tTG in human aortic endothelial cells. NO-dependent downregulation of extracellular tTG levels via inhibition of its secretion might be a part of general physiological mechanism which limits externalization of adhesive, pro-inflammatory and thrombogenic proteins in the vasculature.

tTG is a ubiquitously expressed member of transglutaminase family which displays the transamidating/protein cross-linking function, but, unlike other transglutaminases, also possesses GTPase and disulfide isomerase activities, as well as additional non-enzymatic adhesive/signaling functions.[1,2] Among the latter, tTG is known to non-covalently bind and regulate signaling by integrins, syndecan-4 and platelet derived growth factor receptor (PDGFR).[3-5] The majority of tTG is localized in the cytoplasm, yet it is externalized from undamaged endothelial and smooth muscle cells, monocytes/macrophages, fibroblasts and osteoblasts, which all contain this protein on their surface and in the surrounding ECM.[1] Since tTG does not have leader peptide or hydrophobic domains typical for secreted proteins,[6] this dual topology appears surprising. While tTG is associated with cellular membranes, it is not localized to the ER/Golgi compartments involved in classical protein secretion.[1,2] Our latest studies showed that tTG is secreted via a constitutive non-classical pathway which includes phospholipid-mediated docking of cytoplasmic protein to perinuclear recycling endosomes, its delivery inside these transport vesicles, their anterograde movement and fusion with plasma membrane which releases intravesicular tTG onto the cell surface.[7] The functions of membrane fusion ATPase NSF, soluble NSF attachment receptor proteins (SNAREs) VAMP3 and SNAP23, and regulatory GTPase Rab11, appear all essential for tTG externalization.[7] While this secretion pathway is likely common for many cell types that express tTG, physiological mechanisms of its regulation remain unknown.

Studies by several laboratories began to unravel a complex role of tTG in vascular pathophysiology. The protein was found to contribute to pressure-induced inward remodeling in small arteries and both its enzymatic (protein cross-linking) and non-enzymatic functions are involved in this process.[8] tTG was also implicated in osteochondrogenic transdifferentiation of vascular smooth muscle cells in vitro and atherosclerotic calcification in human carotid arteries.[9,10] In addition, the cross-linking tTG activity was shown to promote macrophage infiltration into atherosclerotic lesions.[11] Finally, this activity of tTG was reported to contribute to age-dependent increases in vascular stiffness in rats, and is likely to decrease arterial compliance in aging humans.[12]

Nitric oxide is a key signaling molecule in the vasculature where it affects multiple aspects of blood vessel homeostasis, including vascular tone, leukocyte trafficking and platelet adhesion/aggregation.[13] Recent studies showed the ability of NO to regulate protein trafficking and secretion in vascular cells.[13,14] NO-mediated S-nitrosylation of Cys264 in NSF does not affect the ATPase activity of this molecular motor, but blocks its disassembly from complexes with SNAREs and inhibits membrane fusion.[14] This regulation occurs in endothelial cells where NO inhibits secretion of Weibel-Palade bodies,[15] in platelets where it prevents externalization of dense, lysosomal and alpha granules,[16] and in activated T lymphocytes where it impedes exocytosis of cytolytic granules.[17] In addition, NO S-nitrosylates dynamin, increasing its oligomerization and GTPase activity, and elevating the rate of receptor-mediated endocytosis.[18] These NO-mediated modifications of the key regulators of protein trafficking alter cell surface proteome by modulating extracellular levels and activities of adhesive, pro-inflammatory and thrombogenic proteins in vascular cells.[13]

Is tTG a target of NO-mediated regulation in the vasculature? Greenberg and colleagues showed that the cross-linking activity of tTG is inhibited by S-nitrosylation in vitro[19] and we found that endogenously produced NO suppresses tTG-mediated transamidation/cross-linking in human aortic endothelial

cells (HAECs) and in mouse aorta.[12] In agreement with diminished bioavailability of NO in aging blood vessels, we also observed a decrease in S-nitrosylation of tTG and an increase in tTG-mediated cross-linking in the aortas of old versus young rats and humans with little or no changes in the protein expression levels.[12] However, the regulation of tTG by NO does not stop here.

NO has recently been shown to alter tTG subcellular distribution and inhibit its deposition into the ECM of fibroblasts.[20] Given the abundance and prominent externalization of tTG in the vasculature[1,2,12] and the impact of NO on protein trafficking and secretion in blood vessels,[13,14] we sought to determine its effects on tTG localization and functions in vascular cells. Using cell surface biotinylation and immunofluorescence we observed that endogenous NO alters the subcellular distribution of tTG by increasing its levels on the surface and in the ECM of HAECs (data not shown). This was also the case with exogenous or endothelial-derived NO and localization of tTG in human aortic smooth muscle cells (HASMCs)(data not shown).Importantly, this NO-dependent regulation of tTG localization was also observed ex vivo when we found that reducing NO bioavailability by endothelial denudation or inhibition of nitric oxide synthase (NOS) and in vivo in the aortas of old versus young rats significantly increases the levels of ECM-associated tTG (data not shown).

To examine whether this regulation occurs on the level of tTG secretion to the cell surface, we defined the effects of NO on externalization of de novo synthesized protein in HAECs (**Fig. 1**). The cells were metabolically labeled, cell surface biotinylated and the amounts of de novo synthesized tTG in the fraction of biotinylated (cell surface) proteins were defined by immunoprecipitation. Analysis of time course of tTG externalization showed that NO donor S-nitrosoglutathione (GSNO) significantly inhibits the secretion of tTG, whereas NOS inhibitor L-nitroarginine methyl ester (L-NAME) increases the amounts of tTG on the surface of HAECs. Pretreatment of cells with endocytic inhibitor dynasore which blocks GTPase activity of dynamins,[21] did not alter the

levels of surface tTG at early time points of secretion, confirming that NO regulates the rate of tTG delivery to the cell surface (not shown). Therefore, we concluded that both exogenous and endogenously produced NO attenuates the process of tTG secretion in these cells (**Fig. 2**). This second layer of tTG regulation by NO in the vasculature appears important, because, in addition to inhibition of its cross-linking activity, it decreases extracellular levels of the protein, thereby diminishing its non-enzymatic impact on outside-in signaling by integrins, syndecan-4 and PDGFR.

How this regulation may occur? At this point, one can not exclude that S-nitrosylation of tTG itself by NO inhibits its externalization. Mapping and mutating the affected cysteine residues in tTG and analysis of secretion rates of the resulting mutants should address this possibility. However, more likely, NO-dependent modification of NSF, which inhibits its ability to disassemble SNARE complexes,[13,14] is pivotal for attenuation of tTG secretion. Specifically, the last step in tTG externalization, a fusion of tTG-bearing recycling endosomes with plasma membrane[7] might be impaired by NSF S-nitrosylation. While spatiotemporally controlled reversible NO-mediated regulation of protein trafficking machinery has been attested as a general pathophysiological mechanism in the vasculature, NSF is emerging as its principal redox sensor which impacts not only classical secretion pathways and fusion of Golgi-derived vesicles with plasma membrane, but also unconventional export of cytoplasmic secretory proteins such as tTG.

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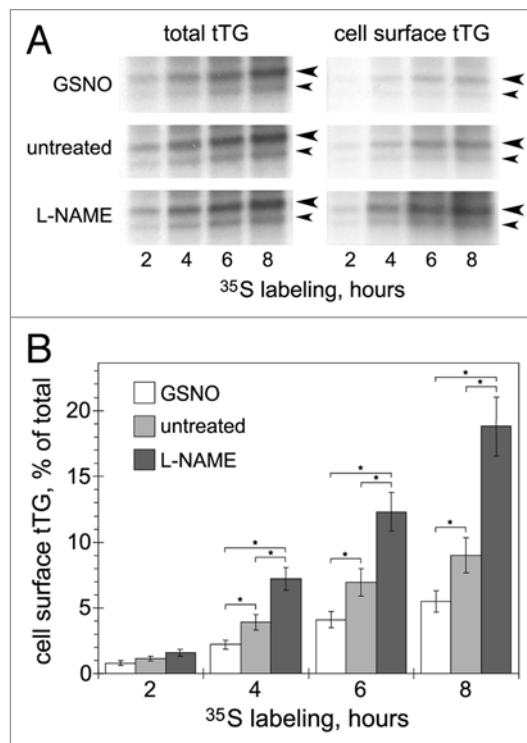
Figure 1. Effects of NO on externalization of endogenous tTG in HAECs. (A) Untreated cells and cells pretreated with NO donor GSNO (200 μM, 1 h) or NOS inhibitor L-NAME (100 μM, 18 h), were metabolically labeled with ^{35}S-Translabel for 0–8 h. At the end of labeling, cells were surface-biotinylated with Sulfo-NHS-LC-biotin and cell surface protein fractions were isolated on neutravidin-Agarose.[5,7] The de novo synthesized tTG was immunoprecipitated from total cell extracts (left parts) and cell surface protein fractions (right parts). The resulting tTG immune complexes were resolved by SDS-PAGE and visualized by fluorography. Large and small arrowheads mark the positions of tTG (~80 kDa) and its major proteolytic fragment (~68 kDa). (B) The relative amounts of de novo synthesized cell surface tTG were quantified by scintillation counting and compared to those of total tTG. Shown in (A) is a representative of four independent experiments. Bars in (B) depict means ± SEM. *p < 0.05.
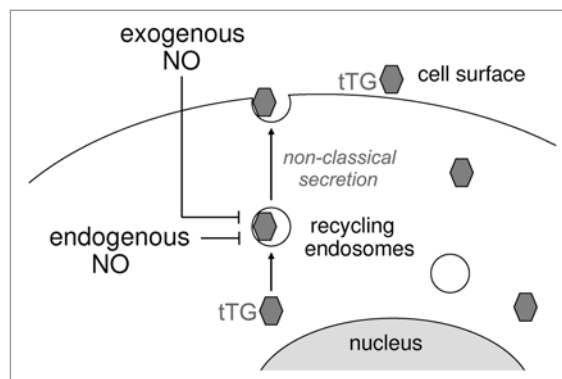

Figure 2. A scheme depicting the effects of exogenous and endogenously produced NO on non-classical secretion of cytoplasmic tTG.