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Tissue Transglutaminase Promotes Serotonin-Induced AKT Signaling and Mitogenesis in Pulmonary Vascular Smooth Muscle Cells

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Abstract

Tissue transglutaminase 2 (TG2) is a multifunctional enzyme that cross-links proteins with monoamines such as serotonin (5-hydroxytryptamine, 5-HT) via a transglutamidation reaction, and is associated with pathophysiologic vascular responses. 5-HT is a mitogen for pulmonary artery smooth muscle cells (PASMC) that has been linked to the pulmonary vascular remodeling underlying pulmonary hypertension development. We previously reported that 5-HT-induced PASMC proliferation is inhibited by the TG2 inhibitor monodansylcadaverine (MDC); however, the mechanisms are poorly understood. In the present study we hypothesized that TG2 contributes to 5-HT-induced signaling pathways of PASMC. Pre-treatment of bovine distal PASMC with varying concentrations of the inhibitor MDC led to differential inhibition of 5-HT-stimulated AKT and ROCK activation, while p-P38 was unaffected. Concentration response studies showed significant inhibition of AKT activation at 50 μM MDC, along with inhibition of the AKT downstream targets mTOR, p-S6 Kinase and p-S6. Furthermore, TG2 depletion by siRNA led to reduced 5-HT-induced AKT activation. Immunoprecipitation studies showed that 5-HT treatment led to increased levels of serotonylated AKT and increased TG2-AKT complex formations which were inhibited by MDC. Overexpression of TG2 point mutant cDNAs in PASMC showed that the TG2 C277V transamidation mutant blunted 5-HT-induced AKT activation and 5-HT-induced PASMC mitogenesis. Finally, 5-HT-induced AKT activation was blunted in SERT genetic knock-out rat cells, but not in their wild-type counterpart. The SERT inhibitor imipramine similarly blocked AKT activation. These results indicate that TG2 contributes to 5-HT-induced distal PASMC proliferation via promotion of AKT signaling, likely via its serotonylation. Taken together, these results provide new insight into how TG2 may participate in vascular smooth muscle remodeling.

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Keywords

Transglutaminase 2; AKT; S6K; S6; serotonin; SERT

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a well-recognized neurotransmitter and vasoconstrictor [1]; additionally, it stimulates the proliferation and migration of a variety of cell types including lung, kidney, prostate, and mast cells [2, 3]. At near-physiologic concentrations, 5-HT also acts as a mitogen for pulmonary arterial smooth muscle cells (PASMC) in culture [4]. Extensive clinical and experimental studies have implicated 5-HT in the pathologic pulmonary vascular smooth muscle remodeling that underlies idiopathic pulmonary arterial hypertension (PAH) [5, 6], a progressive disease that is ultimately fatal despite available therapies.

Tissue transglutaminase (TG2) is a ubiquitous multifunctional protein that catalyzes the post-translational modification of proteins via a calcium-dependent transglutamidation reaction [7]. It may also modify proteins with monoamines; when the amine is 5-HT the process is referred to as serotonylation [8]. It has become increasingly recognized that TG2-mediated changes modulate a variety of cellular responses such as cytoskeletal reorganization, cell adhesion, cell signaling and survival [9–11], and subsequently impact various pathophysiologic responses involved in inflammation and cancer [12]. For example, TG2 is an essential participant in the epidermal growth factor-stimulated signaling pathway leading to cancer cell migration and invasion [13, 14]. Furthermore, TG2 has been linked to vascular remodeling. Guilluy et al. reported TGase-dependent Rho activation and depletion by 5-HT in vascular SMCs [15], and also elevation of serotonylated RhoA in platelets and vascular tissues of PAH patients [16]. Our own studies have shown elevated serotonylated fibronectin in the serum of PAH patients and in animal models of pulmonary hypertension that we have linked to TG2 [17]. Moreover, the TG2 inhibitor ERW1041E mitigates pulmonary hypertension in a rodent model [18]. Furthermore, our recent findings indicate that TG2 contributes to hypoxia-induced pulmonary vascular smooth muscle cell mitogenesis (Penumatsa et al. in press).

PASMC express not only 5-HT receptors (5-HTRs), but also the 5-HT transporter (SERT) [5], and the mitogenic effect of 5-HT on SMCs requires the combinatorial actions of SERT and 5-HT receptors [19]. SERT belongs to a monoamine transporter family that is known to actively move 5-HT intracellularly, and earlier we reported that 5-HT is actively transported intracellularly in pulmonary vascular cells [20]. Furthermore, there is clinical evidence to support a role for SERT in PAH development [21]. Thus, we propose a pathway by which SERT-mediated intracellular 5-HT participates in SMC responses via TG2; however, the mechanisms for this are only partly understood.

Our lab has shown that 5-HT-induced PASMC mitogenesis requires p42/44 ERK MAP kinase [22], RhoA/ROCK [19], and AKT pathways [23]. Guilluy et al. reported TGase-dependent RhoA activation and depletion by 5-HT in proximal vascular smooth muscle cells using supra-physiologic 5-HT concentrations of 10 μM [15]. In contrast, our cell model

system is based on the use of near-physiologic 1–2 μM levels of 5-HT. Moreover, in contrast to previous studies, in the present study we have used a modified cell system with primary pulmonary vascular smooth muscle cells isolated from distal bovine vessels, rather than the proximal arteries, since this is thought to more closely resemble the human PAH pathology that predominantly affects the distal pulmonary vasculature [24]. Using this modified cell system, in the present study we have investigated the potential role of TG2 in signaling pathways in 5-HT-induced bovine (B) PASMC mitogenesis. We initially confirmed that the TG2 inhibitor MDC mitigates 5-HT-induced BPASMC mitogenesis in the modified culture model. Next we investigated the effect of MDC on a number of signaling pathways and found that in addition to the previously reported inhibitory effect on ROCK, 5-HT-induced AKT activation is inhibited by MDC. This inhibition extends to the downstream targets of AKT activation including pS6 and S6K. Furthermore, these effects on the AKT pathway were confirmed by studies using TG2 knock-down. To examine the mechanism, we utilized TG2 cDNA expression to show that 5-HT-induced AKT activation is inhibited by a TG2 mutant that is transamidation defective. Immunoprecipitation studies were used to investigate possible serotonylation of AKT, and binding of TG2 with AKT. Finally SERT knock-out cells were examined for their levels of AKT activation compared to control cells. These findings provide evidence that the AKT pathway is a novel TG2 target in the serotonin system.

2. Materials and methods

2.1. Cell Culture

Primary bovine (calf) pulmonary arterial cells isolated from lung distal pulmonary vessels (measuring approximately 0.5 mm - 1.5 × 105 mm in diameter) were cultured by a modification of the method of Ross as described [24] and maintained in DMEM medium (Life Technologies, Grand Island, NY) with 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA), 100 units/ml penicillin and 100μg/ml streptomycin, and used between passages 3 to 6. Smooth muscle cell morphology was confirmed by morphological appearance under phase-contrast microscopy and by immunofluorescence staining for smooth muscle α-actin. SERT−/− gene knock-out PASMCs were isolated from SERT knock-out rats generated by Dr. Edwin Cuppen and colleagues using N-ethyl-N-nitrosourea-driven target-selected mutagenesis [25]. The corresponding rat wild type (WT) PASMCs were isolated from a colony of animals licensed from GenOway to Dr. Stephanie Watts; SERT−/− and WT cells were isolated and cultured as above. Cells were maintained in a humidified, 5% CO_2 incubator at 37°C.

2.2. Plasmids and siRNA

pcDNA:TG wt, pcDNA:TG C277V and pcDNA:TG R580L plasmids were gifts (M. Antonyak and R. Cerione; Cornell University) [26]. 19mer ready-to-use custom duplex siRNA oligonucleotides for bovine TG2 were purchased as Silencer Custom Designed siRNA from Life Technologies and used at 50 nM final concentration. Nontargeting siRNA control was purchased as Silencer Negative Control siRNA from Life Technologies.

2.3. Antibodies

Phosphorylation of AKT, ERK, MYPT1, p-38, p70 S6 Kinase 1, and S6 were analyzed using phospho-specific antibodies. Antibodies to phospho-p42/44 ERK MAP kinase (Thr202/Tyr204), phospho-Akt (Ser473), phospho-AKT (Thr308), AKT, phospho-S6K1 (Thr421/Ser424), S6K1, phospho-mTOR (Ser2481), mTOR, phospho-S6 ribosomal protein (Ser235/Ser236) and S-6 were from Cell Signaling Technology (Beverly, MA). Anti-phospho-myosin phosphatase targeting subunit (MYPT1 (Thr696) rabbit polyclonal antibody was from Upstate Group, Inc. (Lake Placid, NY). Antibodies against MYPT-1, p42/44 ERK, TG2 and GAPDH were from Santa Cruz Biotechnology (San Diego, CA). The serotonin antibody was from Sigma (St. Louis, MO). HRP-conjugated mouse and rabbit secondary antibodies were from Cell Signaling Technology (MA).

2.4. Reagents

Serotonin (5-HT) was purchased from Sigma and dissolved in water. Monodansylcadaverine (MDC) was purchased from Sigma and dissolved in methanol (10 mg/ml). Equal volumes of methanol were used as vehicle such that all groups contained the same final amount of methanol. Imipramine was purchased from Sigma and dissolved in water. 5-biotinamidopentylamine (5-BP) was purchased from Thermo Scientific (Rockford, IL) and dissolved in water.

2.5. Cell transfection

Cells at 70–90% confluence were transfected with plasmid DNA using Lipofectamine 2000 (Life Technologies) for 5 hr as recommended. For siRNA, cells were transfected at 40–50% confluence using Lipofectamine 2000 (Life Technologies).

2.6. 3H-thymidine incorporation

Smooth muscle cells were grown to 80% confluence in 96 well dishes, transfected, and growth-arrested in 0.1% FBS for 48 hr. Each group contained 6 wells. Cells were then incubated with 1 μM 5-HT or vehicle for 20 hr, followed by labeling with methyl-3H-thymidine (20 mCi/ml; PerkinElmer Life Sciences, Boston, MA) for 4 hr. In some experiments, inhibitors were added 30 min before the 5-HT. After labeling, media from wells was removed and the cells were harvested into Unifilter-96-well microplates using a Packard harvester. Incorporation of 3H-thymidine was measured by a Trilux liquid scintillation and luminescence counter (PerkinElmer Life Sciences).

2.7. Immunoblotting

Cells were washed with ice-cold phosphate buffer saline (PBS), and incubated for 15 min on ice in 1% Triton-X 100 lysis buffer containing protease and phosphatase inhibitor cocktails. Lysates were centrifuged at 14,000 × g for 10 min to collect supernatants, and boiled for 10 mins in reducing SDS Sample Buffer (Boston Bioproducts, Ashland, MA). Cellular lysates were resolved using Novex 4–20% Tris-Glycine gels (Life Technologies), and immunoblotted as described in [27]. Immunoreactive bands were visualized using the ECL Chemiluminescent Western Blotting Detection kit (Thermo Scientific) by the Protein Simple FluorChem E Digital Darkroom gel imaging system. Quantification of bands was performed

by densitometry with Un-Scan-It Version 5.1 gel analysis software (Silk Scientific, Orem, UT), and protein phosphorylation was normalized to total protein band densitometry individually.

2.8. Immunoprecipitation

Immunoprecipitation was carried out as described [27]. Briefly, Pierce Protein A/G Plus Agarose beads (Thermo Scientific, IL) were pre-blocked with 1 mg/ml bovine serum albumin (BSA; Sigma) for 1 hr at 4°C, followed by washing. Beads were then coupled to antibody by incubating together for 2 hrs at room temperature. After washing, antibody-conjugated beads were incubated overnight at 4°C with cell lysates that had been lysed in Pierce IP Lysis Buffer (Thermo Scientific). Following washing in IP Washing Buffer (Thermo Scientific), beads were collected and boiled in reducing SDS Sample Buffer (Boston Bioproducts).

2.9. Immunocytochemical analysis of TG2 activity

For measurement of intracellular TG2 activity, 5-BP incorporation was visualized using fluorochrome-conjugated streptavidin HRP. Briefly, PASMCs grown on cover slips were serum-starved overnight and incubated with 5-BP (400 μM). Cells were then pretreated with MDC (200 μM) or vehicle for 30 minutes followed by 5-HT (1 μM) treatment for 1 hour. For negative control, 5-BP incubation was omitted. After a brief wash with PBS, cells were fixed with 4% formaldehyde (Tousimis, Rockville, MD) and permeabilized with 0.4% Triton X-100 in PBS. Permeabilized cells were then blocked with 5% BSA in PBS and incubated with Streptavidin AlexaFluor 555 HRP conjugate (Life Technologies) for 1 hour in blocking buffer. TG2 localization in permeabilized cells was visualized by incubating with anti-TG2 antibody and AlexaFluor® 488 conjugated secondary antibody (Life Technologies). Cover slips were mounted on to slides using Vectashield mounting medium with DAPI (Vector Labs, Burlingame, CA) and sealed. The stained cells were imaged under Axio light microscope (CarlZeiss AG) using Volocity software (PerkinElmer). TG2 activity was quantitated by measuring the 5-BP staining intensity per cell using ImageJ analysis software.

3.0. Statistical analysis

Data are shown as Means ± SD. Statistical analysis was performed using Student’s t-test and ANOVA; differences were considered as significant where $p<0.05$.

3. Results

3.1. MDC treatment of PASMC mitigates 5-HT-induced mitogenesis

Initially we investigated the role of TG2 on 5-HT-induced mitogenesis in our modified cell system. Distal BPASMC cells that had been serum-starved were treated with 5-HT overnight in the presence of increasing concentrations of the TG2 inhibitor MDC (2–200 μM) or vehicle, followed by thymidine incorporation assay. The concentration response study showed that 20 μM of MDC significantly inhibited 5-HT-induced mitogenesis of distal BPASMC (Figure 1). Inhibition of thymidine uptake by 20 μM MDC was also observed in the absence of 5-HT treatment.

3.2. MDC treatment of PASMC mitigates 5-HT-induced AKT and ROCK activation

We previously reported that 5-HT-induced mitogenesis of PASMC requires the coordinate signaling of the ERK MAPK, Rho/ROCK, and AKT pathways [19, 22, 23]. To investigate the role of TG2 on these pathways, serum-starved BPASMC were pretreated with increasing concentrations of MDC (50–200 μM) for 50 mins, followed by 10 mins 5-HT stimulation. Following lysis the lysates were immunoblotted for activation of key signaling molecules. MDC (50 μM) was found to significantly inhibit AKT (S473) activation down to background levels (Fig 2A). In addition, MYPT-1 (Thr 696) phosphorylation as a measure of ROCK activation was inhibited at 50 μM MDC. Inhibition of p-ERK activation required the higher concentrations of 100–200 μM MDC. In contrast p-38 activation was not affected by any of the MDC concentrations tested.

3.3. MDC inhibits activation of mTOR, S6 kinase and S6

Next the effect of MDC on downstream targets of AKT was similarly tested. MDC was found to significantly inhibit the activation of mTOR, S6 kinase and its downstream target S6 by 5-HT at the same concentration (50 μM) as for AKT (Figure 3).

3.4. TG2 siRNA knock-down reduces 5-HT-induced AKT activation

To independently examine the role of TG2 in 5-HT-induced signaling, TG2 knock-down was carried out by transfection of cells with either TG2 or control siRNAs, followed by 10 min 5-HT stimulation of cells 48hr later. Cells transfected with the TG2 siRNA, but not with control siRNA, showed ~ 50% reduction in 5-HT-induced AKT activation (Fig. 4A, B). The TG2 siRNA knock-down was validated by immunoblot (Fig. 4C), which showed a corresponding reduction of ~47% TG2 expression.

3.5. The TG2 C277V mutant cDNA blocks 5-HT-induced AKT activation and mitogenesis

Next TG2 mutant cDNAs were used in over-expression studies to study effects on 5-HT-induced responses of BPASMC. The TG2:C277V mutant is transamidation defective, while the TG2:R580L mutant is defective in GTP binding activity [26]. Expression of the myc-tagged cDNAs was validated by immunoblotting for myc (Fig. 5A top panel). The same lysates were then immunoblotted for AKT, and it was found that expression of the pcDNA:TG2:C277V mutant, but not the pcDNA:TG2:R580L mutant or of the pcDNA control plasmid, significantly reduced 5-HT-induced AKT activation (Figs. 5A and B). The effect of TG2 cDNA expression on BPASMC mitogenesis was next examined using thymidine incorporation, and TG2:C277V expression was found to significantly attenuate 5-HT-induced thymidine uptake, whereas the WT and R580L TG2 forms did not (Fig. 5C).

3.6. 5-HT-treated BPASMC have increased levels of serotonylated AKT and TG2 binding to AKT

To further investigate the mechanism by which TG2 may participate in 5-HT-induced AKT activation, the possibility of AKT modification by serotonylation was investigated. For this purpose, AKT was immunoprecipitated from untreated and 5-HT stimulated distal BPASMC, and the precipitated material immunoblotted with anti-5-HT antibody. 5-HT

treatment was found to result in increased detection of serotonylated AKT (s-AKT: Figure 6A), and this was reduced by MDC treatment.

To further examine the mechanism, potential complex formation between endogenous TG2 and AKT was tested by immunoprecipitation methods. Unstimulated and 5-HT-stimulated cells were lysed, and lysates immunoprecipitated with anti-TG2 antibody coupled to protein Sepharose beads. The precipitated material was then immunoblotted for both AKT and TG2, and it was found that 5-HT treatment results in increased binding of AKT with TG2 (Figure 6B). Furthermore, this association was reduced upon pre-treatment of cells with MDC.

3.7. 5-HT-induced AKT activation is reduced in SERT knock-out cells and by the SERT inhibitor imipramine

To investigate the upstream mechanisms of 5-HT-induced AKT activation, vascular smooth muscle cells isolated from SERT genetic knock-out rats and their wild type counterparts were examined for their responses. 5-HT stimulation of wild type cells showed substantial AKT activation, whereas the knock-out cells showed a blunted response (Fig. 7A and B). Similar blunted responses were observed for p-S6K and p-S6 in the SERT knock-out cells compared to the wild type counterparts (Fig. 7A and B). In addition, BPASMC were pre-treated with increasing concentrations of the SERT inhibitor imipramine. Following 5-HT stimulation, 25 μM imipramine substantially inhibited 5-HT-induced activation of p-AKT and its downstream targets p-S6K and p-S6, whereas there was no effect on control tubulin levels (Figure 7C).

3.8. 5-HT stimulates TG2 activity in distal BPASMC

To further investigate the link between 5-HT and TG2, TG2 activity was assayed by immunocytochemistry to measure 5-BP incorporation. Distal BPASMC were grown on coverslips and incubated with or without 5-BP, and then treated with vehicle, 5-HT or 5-HT + 200 μM MDC. Cells were then stained with TG2 antibody to detect TG2 localization, and with a streptavidin conjugate for detecting 5-BP incorporation, followed by processing for immunofluorescent examination. The TG2 staining demonstrated cytosolic TG2 expression in all groups (Fig. 8 Row A). The streptavidin staining (Fig. 8 Row B) for 5-BP incorporation showed increased cytosolic fluorescence in the 5-HT-treated group (middle panel) compared to the vehicle control group (left-hand panel), and this was inhibited by MDC treatment (right-hand panel). Overlay of the TG2 and 5-BP immunofluorescent images (Fig. 8 Row C) showed that the increased 5-BP fluorescence colocalized with TG2 (middle panel). The Fig. 8D graph shows the quantification of the relative 5-BP fluorescent intensities normalized to number of cells per image.

4. Discussion

An increasing number of disease-associated processes are being attributed to tissue transglutaminase such as cancer progression, inflammation, and vascular remodeling including pulmonary hypertension [7, 12]; however, the mechanisms are not well-understood. At the cellular level there is considerable data on the action of TG2 on matrix reorganization via modification of cytoskeletal and ECM proteins [11]. However, potential

effects of TG2 on signaling pathways are relatively understudied. Previous reports have shown effects of TG2 on serotonylation of the small GTPases RhoA and Rab [8]; while others have proposed modulatory effects of TG2 on EGF, PDGF, HIF and NFkB pathways [9, 10, 14, 28]. TG2 modulation of serotonin-mediated RhoA signaling in pulmonary vascular cells has been reported by another group, although this was in the context of supra-physiologic levels of serotonin [15]. In the present study we have postulated that TG2 may be involved in 5-HT related signaling pathways in the context of near-physiologic (1–2 μM) levels of serotonin, and in pulmonary vascular cells isolated from distal vessels.

Our initial finding of MDC inhibition of 5-HT-induced mitogenesis of distal PASMC (Figure 1) confirmed our earlier findings [27], and established that TG2 may be participating in our modified cell culture system. The inhibition of thymidine uptake by 20 μM MDC observed in the absence of 5-HT suggests background TG2 activity, and could be due to endogenous production of 5-HT in PASMCs, based on our pilot studies (K. Penumatsa et al, unpublished). Our analyses of the effects of MDC on 5-HT-induced signaling pathways showed that AKT activation was the most sensitive to MDC (Figure 2), showing substantial inhibition at 50 μM. In addition, we observed inhibition of 5-HT-induced ROCK activation, albeit at a higher MDC concentration. This confirms that 5-HT-induced ROCK activation involves TG2, even at 5-HT levels that are lower than originally reported [15]. In addition, ERK1/2 MAPK activation was inhibited by MDC, albeit at a higher concentration of 200 μM; whereas p38 was not affected, indicating specificity of effect and a differential action of TG2 on AKT, ROCK and ERK1/2 MAPK. On this basis we focused on the novel observed effect on the AKT pathway, and the finding that MDC also inhibited the activation of the AKT downstream targets mTOR, p70 S6 kinase, and S6 corroborates the observed effect on the AKT pathway (Figure 3). The reduction in 5-HT-induced AKT activation by TG2 knock-down (Figure 4) further supports the participation of TG2 in this pathway.

Our findings of TG2 binding with AKT, and the detection of increased serotonylated AKT following 5-HT treatment indicates a TG2-mediated post-translational modification of AKT (Figure 5). Our results showing mitigation of 5-HT-induced AKT activation and of 5-HT-induced PASMC mitogenesis by the TG2 transamidation-defective C277V mutant (Figure 6) support a mechanism involving a TG2-mediated cross-linking modification.

The upstream mechanisms of serotonin-stimulated AKT activation are not well-understood, and likely differ from the canonical pathway of AKT activation via receptor tyrosine kinases. Our present finding that 5-HT-induced AKT, S6K and S6 activation is attenuated in SERT KO cells compared to the control wild-type cells (Figure 7A) indicate a role for SERT-mediated pathways in AKT activation. Moreover, the observed mitigating effect of the SERT inhibitor imipramine on 5-HT-induced p-AKT is further consistent with a role for SERT. Thus the present findings are supportive of a TG2-dependent mechanism since the SERT transporter would provide the intracellular 5-HT necessary for the serotonylation modification of AKT (Figure 7B). A role for SERT in AKT activation in vivo in a rat model of PH has been noted by others [29], and our findings here would indicate a TG2-mediated mechanism for this. An earlier report showed that 5-HT-driven AKT activation in PASMC was sensitive to 5-HT receptor (5-HTR) inhibitors [23]; however this was based on the use of only pharmacologic inhibitors, whereas the present study utilizes both pharmacologic and

genetic inhibitions of SERT which support the concept that 5-HT-driven AKT also involves SERT. Indeed, these pathways would not be mutually exclusive since 5-HTR signaling is known to lead to Ca^{2+} release, which could in part provide a mechanism for TG2 activation since TG2 activity is calcium-dependent [7].

A recent report has shown an effect of TG2 overexpression on the AKT pathway via a novel mechanism involving conformational changes induced by protein-protein interaction, rather than by post-translational modification [13], and at present we cannot rule out that such a mechanism could occur in combination with a serotonylation reaction in our case, since we also found TG2 binding to AKT (Fig. 6). The intracellular TG2 activity assay indicated that near-physiologic levels of 5-HT stimulates TG2 activity, which independently supports our hypothesis of TG2 involvement in 5-HT-triggered AKT pathway (Fig. 8).

5. Conclusion

Taken together, these data suggest that TG2 is a novel intermediary that transduces 5-HT signals to AKT in the serotonin pathway in pulmonary vascular smooth muscle cells, and may be an interventional target in the development of vascular diseases such as PH.

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Abbreviations

MDC	monodansylcadaverine
PASMC	pulmonary arterial smooth muscle cells
5-HT	5-hydroxytryptamine (serotonin)
SERT	serotonin transporter
TG2	transglutaminase 2 aka tissue transglutaminase

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Highlights

- Serotonin-induced pulmonary vascular smooth muscle cell proliferation is inhibited by the TG2 inhibitor monodansylcadaverine (MDC).
- MDC inhibited 5-HT-stimulated AKT activation and its downstream targets mTOR, p-S6 Kinase and p-S6, but not pP38.
- TG2 siRNA led to reduced 5-HT-induced AKT activation.
- Serotonin led to increased levels of serotonylated AKT and increased TG2:AKT complex formation which were inhibited by MDC.
- Serotonin-induced AKT activation was blunted by TG2 C277V cross-linking mutant expression, and attenuated in SERT genetic knock-out cells and by the SERT inhibitor imipramine.

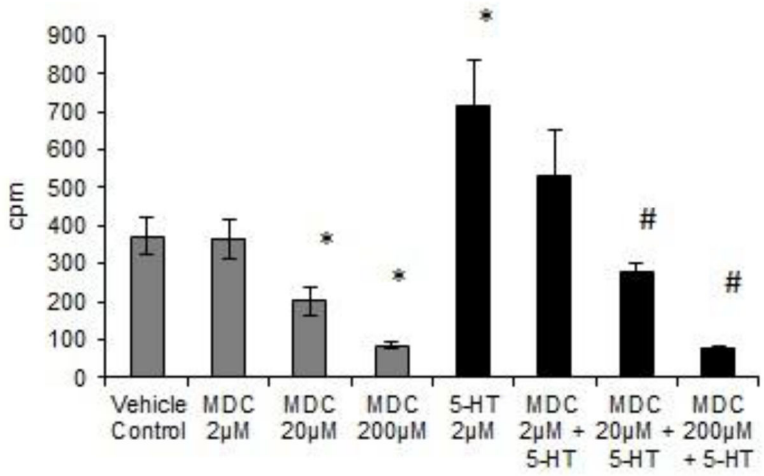

Figure 1. MDC ameliorates 5-HT-induced mitogenesis of distal BPASMC

Cells were serum-starved overnight, and following 30 mins. pre-treatment with increasing concentrations of MDC (2–200 μM), were stimulated with 2 μM 5-HT overnight (18 hrs), followed by incubation with 3H-thymidine. Cpm= counts per minute. Shown is a representative experiment with n=8 per group. *$p<0.05$ for groups vs. untreated group; #$p<0.05$ for groups vs 5-HT treated group.

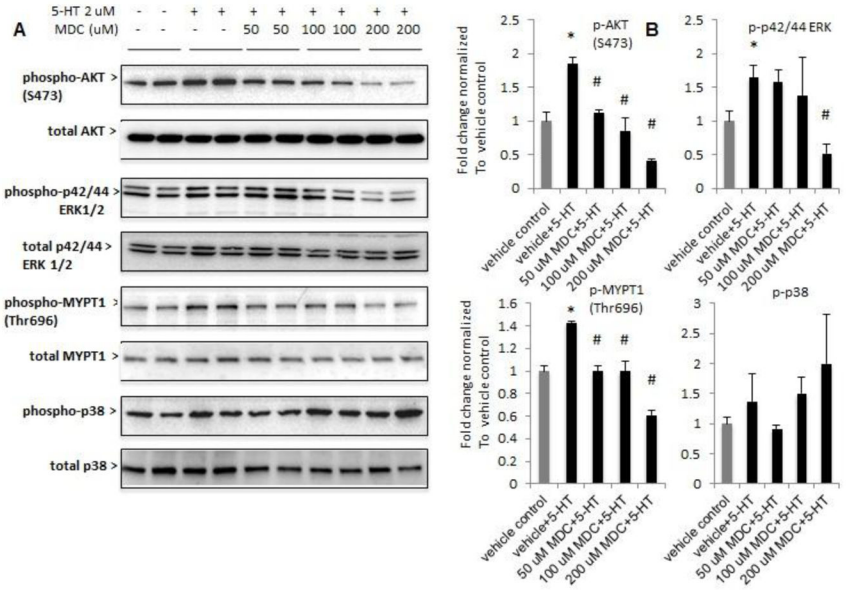

Figure 2. MDC mitigates 5-HT-induced AKT and ROCK activation

A) Serum-starved BPASMC were pretreated with increasing concentrations of MDC (50–200 μM) for 50 mins; then stimulated with 5-HT for 10 mins. Following lysis, lysates were immunoblotted with phospho-specific antibodies against AKT, MYPT-1, p-ERK and p-38, and with antibodies against total protein. Shown is a representative experiment with duplicates. B) Graphical depiction of the densitometric mean +SD values of phospho-proteins normalized to total protein values from 3 separate experiments shown as fold change compared to the vehicle control group that was assigned a value of 1; *$p<0.05$ for + 5-HT treated group vs. untreated group; #$p<0.05$ for groups vs + 5-HT treated group.

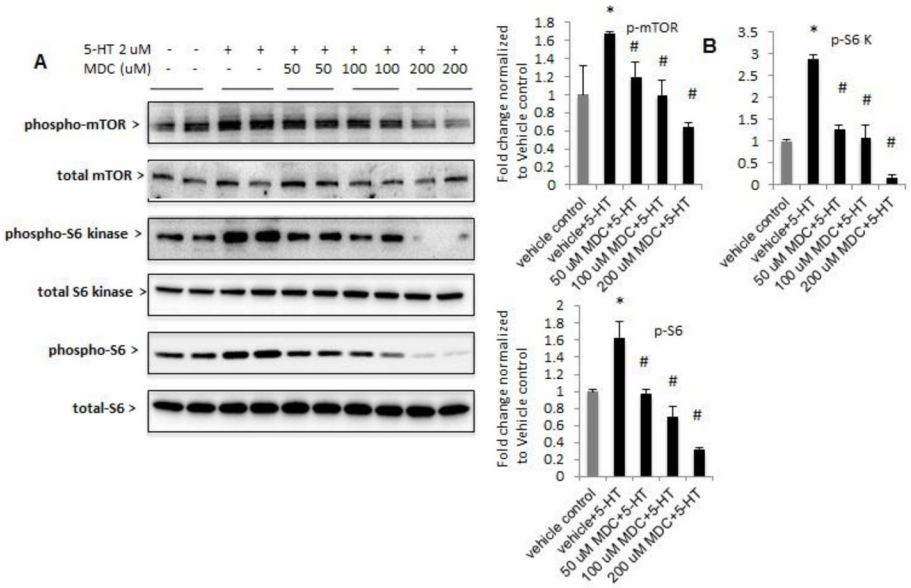

Figure 3. MDC mitigates 5-HT-induced activation of the AKT downstream targets mTOR, p70 S6 Kinase and p-S6

A) Serum-starved BPASMC were pretreated with increasing concentrations of MDC (50–200 μM) for 50 mins; then stimulated with 5-HT for 10 mins. Following lysis, lysates were immunoblotted with phospho-specific antibodies and with antibodies against total proteins. Shown is a representative experiment containing duplicates. B) Graphical depiction of the densitometric mean +SD values of phospho-proteins normalized to total protein values from 3 separate experiments shown as fold change compared to the vehicle control group that was assigned a value of 1; *p<0.05 for + 5-HT treated group vs. untreated group; #p<0.05 for groups vs + 5-HT treated group.

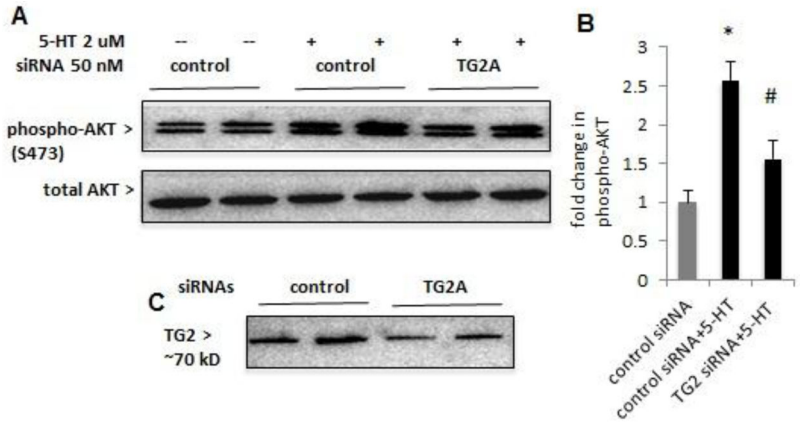

Figure 4. TG2 knock-down in distal BPASMC attenuates 5-HT-induced AKT activation

A) BPASMC were transfected with control or TG2 siRNAs at 50 nM, switched to 0.1% serum medium, and stimulated with 5-HT 48 hrs later. Lysates were immunoblotted with phospho-specific and total AKT antibody; shown is a representative experiment with duplicates. B) Graphical depiction of the normalized densitometric phospho-AKT band values as fold change compared to the unstimulated control group that was assigned a value of 1; *$p<0.05$ for + 5-HT treated group vs. untreated group; #$p<0.05$ for TG2 siRNA + 5-HT treated group vs control siRNA + 5-HT treated group. C) Immunoblot of siRNA-transfected cells showing reduction of TG2 expression levels by TG2A siRNA.

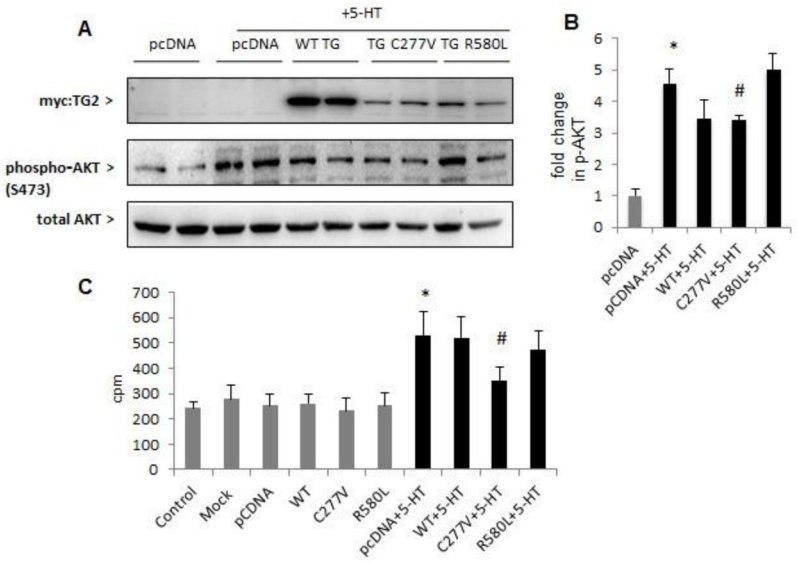

Figure 5. TG2 C277V mutant cDNA expression mitigates 5-HT-induced AKT activation and thymidine uptake

A) Distal BPASMC were transfected with pcDNA vector, wt TG2, TG2 C277V mutant, or TG2:R580L mutant cDNAs, and stimulated with 5-HT 48 hrs later. Lysates were immunoblotted with myc antibody to validate cDNA expression (top panel); with phospho-specific AKT antibody (middle panel); and with total AKT antibody (bottom panel). Shown is a representative experiment with duplicates. B) Graphical depiction of the densitometric values of transfected group bands normalized to total AKT values and shown as fold change compared to the pcDNA group that was assigned a value of 1; *p<0.05 for + 5-HT treated group vs. untreated group; #p<0.05 for C277V + 5-HT treated group vs. pcDNA+ 5-HT treated group. C) Cells transfected with plasmids were stimulated with 5-HT overnight and subjected to 3H-thymidine uptake assay. Cpm= counts per minute. Shown is a representative experiment with n=8 per group; *p<0.05 for + 5-HT treated group vs. untreated group; #p<0.05 for C277V + 5-HT treated group vs. pcDNA + 5-HT treated group.

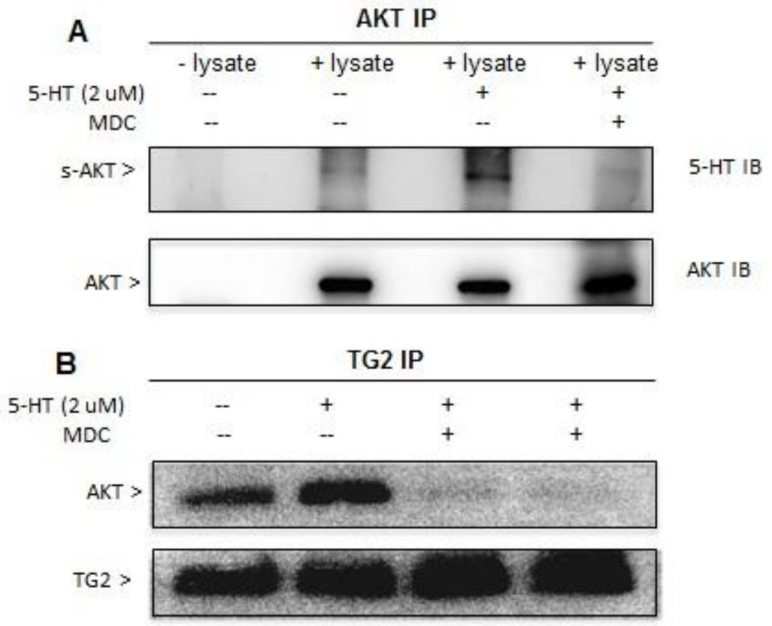

Figure 6. 5-HT stimulation of distal BPASMC increases AKT serotonylation and AKT binding to TG2

A) 5-HT stimulates AKT serotonylation. Serum-starved distal BPASMC were stimulated with 5-HT, washed 3X, then lysed and incubated with AKT antibody-coupled beads. The precipitated material was then immunoblotted with AKT and 5-HT antibodies, the latter to detect serotonylated AKT (s-AKT). B) 5-HT stimulation leads to increased AKT and TG2 association. Serum-starved distal BPASMC were stimulated with 5-HT in the absence or presence of MDC, washed 3X, then lysed and incubated with TG2 antibody-coupled beads. The precipitated material was immunoblotted with AKT and TG2 antibodies. Shown are representative experiments.

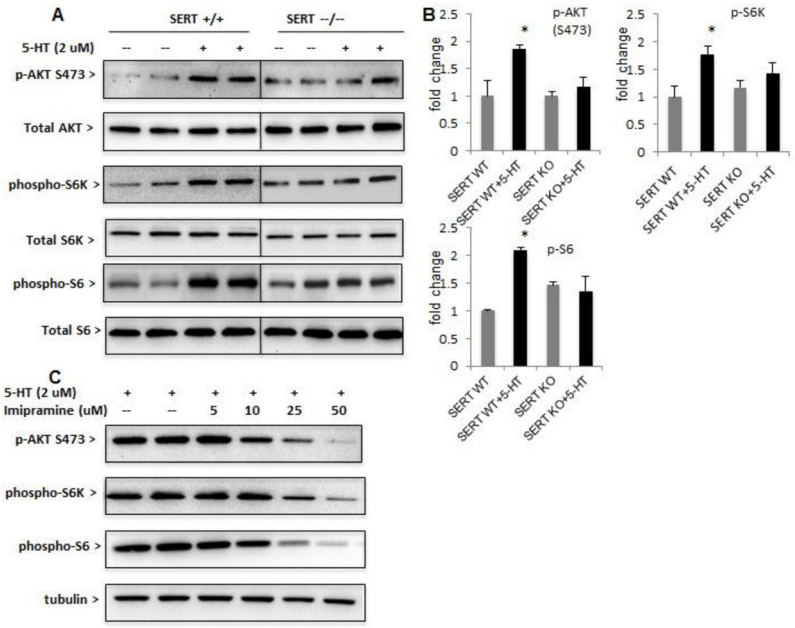

Figure 7. 5-HT-induced AKT activation is reduced in SERT knock-out cells and by the SERT inhibitor imipramine

A) Rat pulmonary arterial smooth muscle cells from SERT gene knock-out (SERT−/−) and wild type (SERT+/+) animals were stimulated with 5-HT, and immunoblotted for AKT, S6k and S6 activation. Shown is a representative experiment with duplicates. B) Graphical depiction of the densitometric band values of activated proteins normalized to total protein values and shown as fold change compared to the unstimulated SERT WT group that was assigned a value of 1; *p<0.05 for SERT WT + 5-HT treated group vs. untreated SERT WT. C) Serum-starved BPASMC were pre-treated with increasing concentrations of the 5-HT transporter imipramine (5–50 μM) for 50 mins, then stimulated with 5-HT for 10 mins. Lysates were immunoblotted with designated antibodies. Shown is a representative experiment.

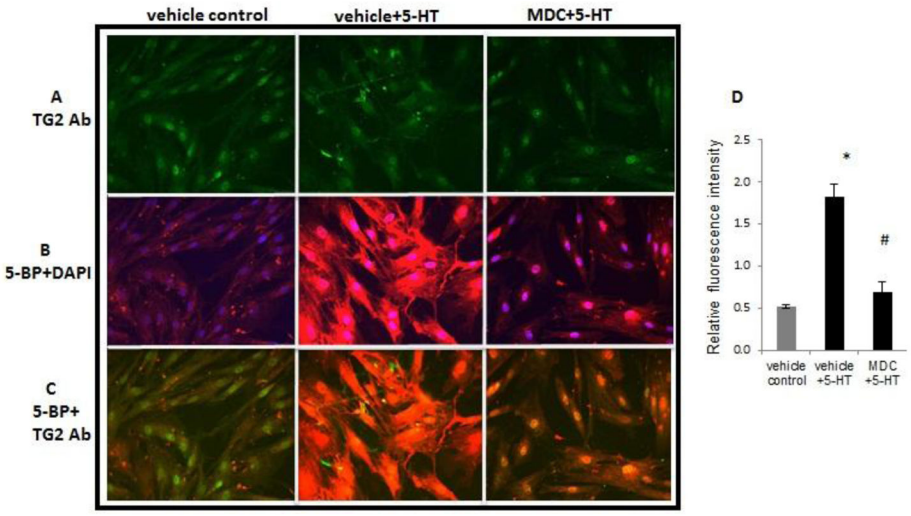

Figure 8. 5-HT stimulates TG2 activity in distal BPASMC

Cells were grown on coverslips and incubated with or without 5-BP overnight. Cells were then treated with vehicle, 5-HT, or 5-HT + 200 μM MDC, and stained with Row A) TG2 antibody; Row B) streptavidin conjugate for 5-BP incorporation, and DAPI. Row C) depicts the overlay of TG2 and 5-BP staining, showing the colocalization. Shown are representative fields for each group. D) Graph depicts relative fluorescence intensity of Row B fields. *$p<0.05$ for + 5-HT treated group vs. untreated group; #$p<0.05$ for groups vs vehicle + 5-HT treated group.