Molecular interactions of serotonin (5-HT) and endothelin-1 in vascular smooth muscle cells: in vitro and ex vivo analyses

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Bhaskaran S, Zaluski J, Banes-Berceli A. Molecular interactions of serotonin (5-HT) and endothelin-1 in vascular smooth muscle cells: in vitro and ex vivo analyses. Am J Physiol Cell Physiol 306: C143–C151, 2014. First published November 6, 2013; doi:10.1152/ajpcell.00247.2013.—Elevated levels of serotonin (5-HT) and endothelin-1 (ET-1) may be involved in cardiovascular complications of diabetes mellitus. Data suggest supraphysiological concentrations of 5-HT (10\(^{-6}\) M) potentiate the ability of ET-1 to stimulate DNA synthesis and vascular smooth muscle cell (VSMC) proliferation in vitro via activation of mitogen-activated protein kinase (p42/44 MAPK) and Janus kinase 2 (JAK2) pathways. Additionally, 5-HT enhances agonist-induced contractions via p42/44 MAPK and an unknown tyrosine kinase. However, the exact mechanisms of the 5-HT/ET-1 interactions and whether these effects occur at physiological levels (10\(^{-9}\) M) are unknown. Therefore, we hypothesized that interactions between 5-HT and ET-1 at physiological concentrations in VSMC enhanced activation of both p42/44 MAPK and JAK2 pathways contributing to vascular growth and contractile responses. With the use of rat VSMC and Western blot analysis, our data suggest no effect of acute (30 min) preincubation with 5-HT (10\(^{-9}\) M) and/or ET-1 (10\(^{-9}\) M) on the activation of either pathway in normal or high glucose conditions. To determine if there was altered vascular reactivity in intact vessels we tested the effects of 5-HT and ET-1 interaction using myographs to measure isometric contractions of rat thoracic aortic rings. 5-HT (10\(^{-9}\) M) and ET-1 (10\(^{-12}\) M) stimulate enhanced contractile responses to each other that were inhibited by JAK2 and p42/44 MAPK antagonists. Our findings demonstrate that both 5-HT and ET-1 at physiological concentrations could interact with each other and activate p42/44 MAPK and JAK2 signaling pathways to cause an increase in smooth muscle contraction that could lead to altered vascular function.

Diabetes; serotonin (5-HT); endothelin-1 (ET-1); vascular smooth muscle cell (VSMC); p42/44 mitogen-activated protein kinase (p42/44 MAPK); Janus kinase 2 (JAK2)

Diabetes mellitus is a leading cause of renal disease and an independent risk factor for cardiovascular disease complications in the United States. Accumulating evidence suggests that serotonin [5-hydroxytryptamine (5-HT)], a well-known mediator and a potent vasoconstrictor, may also stimulate pathological processes in diabetes (16). 5-HT has been associated with cardiovascular diseases such as atherosclerosis (10) and pulmonary hypertension (14), but it has been overlooked in some diseases because of its low circulating physiological levels (estimated to be in the 10\(^{-9}\) M range; Ref. 28) and a lack of understanding of the roles of 5-HT in the peripheral systems. One way by which physiological levels of 5-HT may have an effect on vascular tone is through the interaction with other vasoactive substances including ET-1, which could exacerbate the pathophysiological mechanisms.

Evidence suggests that in the diabetic condition, there are elevated levels of 5-HT and ET-1, and they can both enhance each other’s actions in vitro (17, 19, 28), but the physiological effect of this interaction, underlying molecular mechanisms, as well as their role in vascular remodeling in diabetes and vascular contraction are not yet completely elucidated. It is important to understand 5-HT/ET-1 interaction mechanisms that may accelerate the development cardiovascular complications such as hypertension and atherosclerosis, which commonly occur at an increased rate in diabetic patients compared with their age-matched controls. Additionally, renovascular complications also occur during diabetes. 5-HT involvement in renal damage in diabetes is supported by studies which demonstrated that the administration of sarpogrelate, a 5-HT\(_{2A}\) receptor antagonist, reduced albuminuria in type 2 diabetic patients (22). In addition, an increased correlation between plasma 5-hydroxyindole-3-acetic acid (5-HIAA—a derivative metabolite of 5-HT) and urinary albumin excretion in men with type 2 diabetes was also reported by Fukui et al. (9) suggesting a possible role for the use of 5-HT levels and its metabolites as a biomarker.

Our previous studies in VSMC have shown that 5-HT stimulates JAK2 and STAT1 via the 5-HT\(_{2A}\) receptors under both normal and high glucose conditions but does not depend on reactive-oxygen species (ROS; Ref. 4). 5-HT via the 5-HT\(_{2A}\) receptor activates the p42/44 MAPK pathway in VSMCs but does not activate the JNK and p38 pathways (3). It is known that U0126, a MEK inhibitor, attenuated 5-HT-induced VSMC contraction ex vivo, suggesting the involvement of p42/44 MAPK pathway (23, 29). Watts (28) demonstrated that 5-HT (10\(^{-9}\) M) stimulated potentiation of vascular contraction in response to norepinephrine (NE) (10\(^{-9}\) M) and ET-1(10\(^{-9}\) M), which utilizes the p42/44 MAPK pathway and an undetermined tyrosine kinase. The mechanisms by which the p42/44 MAPK pathway participates in VSMC contraction were either due to de-activation of caldesmon (a calmodulin-binding protein, which activates actin-myosin ATPase; Ref. 1) or by phosphorylating MLCK (12). The unidentified tyrosine kinase involved in the enhanced contraction we speculate may be JAK2. JAK2 has recently been shown to be critical to the ability of angiotensin II (ANG II) to cause contraction (6).

Furthermore, elevated levels of renal ET-1 are associated with an expansion of mesangial cells and collagen deposition in the glomeruli of diabetic mice (21). ET-1-mediated activation of the ETA receptor stimulated the renal TGF-β production and inflammation in diabetic rats (25). ET-1 through the ETA receptor in VSMCs acts as signaling molecules to activate JAK2 and stimulate the NAD(P)H

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oxidase-induced production of ROS (5). In bovine aortic endothelial cell cultures, high glucose stimulates the release of ET-1 locally, which may participate in the development of atherosclerosis (31). Experimental data demonstrated that in the human mammary artery, threshold concentrations of ET-1 (10⁻¹⁰ M and 10⁻⁹ M) potentiated the vasoconstrictor effects of NE (10⁻⁸ M) and 5-HT (10⁻⁸ M) in a calcium-dependent manner (32). It was previously shown that ET-1 alone treatment induced contraction of the rabbit basilar artery utilizes p42/44 MAPK and JAK2 pathways (33); however, the molecular mechanisms of how JAK2 is involved in ET-1-induced VSMC contraction are not yet entirely elucidated.

Therefore, we tested the hypothesis that the interaction between 5-HT and ET-1 in VSMC at physiological concentrations would cause enhanced activation of both p42/44 MAPK and JAK2 signaling pathways in vitro for acute preincubations of the cultured VSMCs with 5-HT and/or ET-1 that could contribute to altered cellular functions especially in hyperglycemic conditions. We also hypothesized that the physiological levels of 5-HT and ET-1 interact to stimulate an increase in the contractile response in arteries ex vivo that may lead to altered vascular functions that may contribute to early development of cardiovascular injury as a consequence of diabetes.

Fig. 1. A: concentration-response curve of serotonin (5-HT): activation of p44 MAPK pathway in normal glucose cultured vascular smooth muscle cells (VSMCs). VSMCs were treated with V (Vehicle: water) and increasing concentrations of 5-HT for 10 min. Top: densitometric analysis of Western blots utilizing the cultured VSMCs. *Statistical significance compared with the control. Bottom: representative Western blots. Densitometry data are shown as means ± SE; n = 4. B: concentration-response curve of 5-HT: activation of p44 MAPK pathway in high glucose cultured VSMC. VSMCs were treated with V and increasing concentrations of 5-HT for 10 min. Top: densitometric analysis of Western blots utilizing the cultured VSMCs. *Statistical significance compared with the control. Bottom: representative Western blots. Densitometry data are shown as means ± SE; n = 4.

Fig. 2. A: concentration-response curve of endothelin-1 (ET-1): activation of p42 MAPK pathway in normal glucose cultured VSMC. VSMCs were treated with V (Vehicle: water) and increasing concentrations of ET-1 for 10 min. Top: densitometric analysis of Western blots utilizing the cultured VSMCs. *Statistical significance compared with the control. Bottom: representative Western blots. Densitometry data are shown as means ± SE; n = 4. B: concentration-response curve of ET-1: activation of p42 MAPK pathway in normal glucose cultured VSMC. VSMCs were treated with V (Vehicle: water) and increasing concentrations of ET-1 for 10 min. Top: densitometric analysis of Western blots utilizing the cultured VSMCs. *Statistical significance compared with the control. Bottom: representative Western blots. Densitometry data are shown as means ± SE; n = 4.
**METHODS**

**Cell culture.** VSMC were derived from aortas of euthanized male Sprague-Dawley rats and placed in phosphate-buffered saline (PBS) with penicillin and streptomycin (P/S). Samples were dissected in sterile conditions by cutting into helical strips and removing the endothelium with a moist cotton swab. Strips were cut into 2-3-mm pieces and incubated in 5 ml of 45% fetal bovine serum (FBS) in DMEM with P/S in p60 culture dishes. Cells were incubated at 37°C in humidified 5% CO₂-95% O₂. Normal glucose (NG) media were changed every 2 days until cells were confluent. Confluent plates were split by adding trypsin to detach the cells and then were grown in 10% FBS in DMEM with P/S in the plastic tissue culture flasks. Subsequently, cells were subcultured into six-well plates at a 1:6 split ratio, and the medium was changed every 2 days. Cells in the six-well plates were grown to ~50% confluence, and either NG media (5.5 mM) or high glucose media (HG: 25 mM) were added as needed. Media (NG or HG) were changed every 2 days until cells were confluent in the six-well plates. Once 90% confluent, media were changed to fresh serum-free media containing NG or HG with P/S 24 h before experiments to ensure quiescence. Cells from the six-well plates treated either with 5-HT or ET-1 or both for 10 min for concentration-response studies and 30 min with either 5-HT or ET-1 for acute preincubation studies (9). After experiments were carried out, the cells were washed with an ice-cold PBS solution. The cells were scraped and lysed with 250 μl of lysis buffer [Bill’s buffer (40 mM Tris·HCl, 0.1 mM EDTA, and 0.1 mM EGTA pH 7.5), Phosphatase Inhibitor Cocktail 1, Phosphatase Inhibitor Cocktail 2, Protease Inhibitor Cocktail 3, 1 mM Na₃VO₄, 1 mM PMSF, 1 M deoxycholate (sodium deoxycholic acid), 0.01% NaPO₄, and 0.1% SDS]. The lysates were then sonicated and centrifuged at 13,000 rpm, 4°C for 15 min, supernatant total protein was measured (Bio-Rad, Hercules, CA) and then frozen at ~80°C until used.

**Western blotting.** The VSMC lysates were boiled for 5 min and separated on SDS-polyacrylamide gels (SDS-PAGE; 7.5% polyacrylamide) with 2-mercaptoethanol as the reducing agent and bromophenol blue as the tracking dye and then transferred onto nitrocellulose membranes. Membranes were blocked for 1 h in a 5% BSA-TBS-T solution and then washed thrice (twice with TBS-T rinse, 5 min TBS). Incubation was done overnight at 4°C using either phospho-specific for p42/44 MAPK or JAK2 (1:1,000 concentration in 10 ml BSA/TBS) as the primary antibody. Blots were washed once with TBS-T for 20 min and twice with TBS for 10 min. Membranes were

![Fig. 3. A](http://ajpcell.physiology.org/)

**A:** levels of active p42 MAPK in normal glucose cultured VSMC for acute 5-HT preincubation. VSMCs were treated for 10 min with 6 treatment groups: V (Vehicle: water); ●, 5-HT -9 (5-HT 10⁻⁹ M); ■, ET-1 -9 (ET-1 10⁻⁹ M); ●, ET-1 -8 (ET-1 10⁻⁸ M); ●, 5-HT -9 ET-1 -9 (ET-1 10⁻⁹ M added after a 30-min preincubation of VSMC with 5-HT 10⁻⁹ M); and ■, 5-HT -9 ET-1 -8 (ET-1 10⁻⁸ M added after a 30-min preincubation of VSMC with 5-HT 10⁻⁹ M) in the 6-well VSMC culture plate, respectively. All samples were derived at the same time and processed serially. *Top:* densitometric analysis of Western blots utilizing the cultured VSMCs. *Statistical significance compared with the control. Bottom:* representative Western blots. Densitometry data are shown as means ± SE; n = 5.

**B:** levels of active p42 MAPK in high glucose cultured VSMC for acute 5-HT preincubation. VSMCs were treated for 10 min with 6 treatment groups: V (Vehicle: water); ●, 5-HT -9 (5-HT 10⁻⁹ M); ■, ET-1 -9 (ET-1 10⁻⁹ M); ●, ET-1 -8 (ET-1 10⁻⁸ M); ●, 5-HT -9 ET-1 -9 (ET-1 10⁻⁹ M added after a 30-min preincubation of VSMC with 5-HT 10⁻⁹ M); and ■, 5-HT -9 ET-1 -8 (ET-1 10⁻⁸ M added after a 30-min preincubation of VSMC with 5-HT 10⁻⁹ M) in the 6-well VSMC culture plate, respectively. All samples were derived at the same time and processed serially. *Top:* densitometric analysis of Western blots utilizing the cultured VSMCs. *Statistical significance compared with the control. Bottom:* representative Western blots. Densitometry data are shown as means ± SE; n = 5.
Isolated vessel function studies. Rat aortas from male Sprague-Dawley rats were cleaned of debris and connective tissue but with endothelial intact and cut into 3- to 4-mm rings and suspended in a Danish Myo Technology (DMT) myograph. The endothelium was left intact knowing that it has receptors for 5-HT and ET-1 and that would mimic actual physiological conditions. Myograph chambers contained 5 ml of a physiological salt solution (PSS; containing 130 mM NaCl, 4.69 mM KCl, 1.18 mM KH2PO4, 1.18 mM MgSO4·7H2O, 14.8 mM NaHCO3, 5.55 mM dextrose, and 26.3 μM EDTA) with 5% CO2-95% O2 bubbled at a constant rate. Aortic rings were placed under 30 mN of passive tension and equilibrated for 30 min. Viability was determined by measuring a contraction obtained by adding a maximal concentration of potassium chloride (KCl). After peak contraction was achieved, the baths were washed by removing the PSS and exchanging it for fresh until baseline tension was reached and then the vessels were left to rest for 30 min.

Animal studies. All animal procedures were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. All animal procedures were approved and followed in accordance with the institutional guidelines of the Institutional Animal Use and Care Committee of Oakland University. Male Sprague-Dawley rats (325–350 g) were purchased from Harlan (Madison, WI). Rats used for our experiments were euthanized using CO2 gas, aortas were removed and stored in freshly prepared PBS, and experiments were conducted immediately.

Data analysis and statistics. Data are presented as means ± SE for the number of experiments in parentheses. Statistical analysis for the Western protein blot data was carried out with the Graph Pad Prism program (GraphPad Software, San Diego, CA). When comparing two groups, the appropriate Student’s t-test was used. One-way ANOVA followed by a Student-Newman-Keuls post hoc test was performed when comparing three or more groups to determine significance. Band density was quantified using the program National Institutes of Health Image.

Chemicals. Molecular weight standards, acrylamide, sodium dodecyl sulfate (SDS), N'-methylene-bisacrylamide, protein assay reagents, and nitrocellulose membranes were purchased from Bio-Rad Laboratories. Monoclonal antibody to phosphorylated tyrosine/threonine-specific p42/44 MAPK was purchased from Abcam (Cambridge, MA), and nonphosphorylated tyrosine/threonine-specific p42/44 MAPK, anti-phosphotyrosine-specific JAK2, and nonphosphotyrosine-specific JAK2 antibodies were purchased from Cell Signaling Technology (Beverly, MA). The JAK2 inhibitor was purchased from Calbiochem-EMD Millipore (Billericia, MA). Goat anti-mouse IgG and anti-rabbit IgG were acquired from Amersham (Princeton, NJ). 5-HT, ET-1, AG490, Tween-20, and all other chemicals were purchased from the Sigma Chemical (St. Louis, MO).

![Graph](https://example.com/graph.png)

**Fig. 4.** A: levels of active JAK2 in normal glucose cultured VSMC for acute 5-HT preincubation. VSMCs were treated for 10 min with 6 treatment groups: V (Vehicle: water); 5-HT-9 (5-HT 10⁻⁹ M); ET-1-9 (ET-1 10⁻⁹ M); ET-1-8 (ET-1 10⁻⁸ M); ET-1-10⁻⁸ (ET-1 10⁻⁹ M); ET-1-9 (ET-1 10⁻⁹ M) added after a 30-min preincubation of VSMC with 5-HT 10⁻⁹ M; and ET-1-9 (ET-1 10⁻⁹ M) added after a 30-min preincubation of VSMC with 5-HT 10⁻⁹ M in the 6-well VSMC culture plate, respectively. All samples were derived at the same time and processed serially. Top: densitometric analysis of Western blots utilizing the cultured VSMCs. *Statistical significance compared with the control. Bottom: representative Western blots. Densitometry data are shown as means ± SE; n = 4. B: levels of active JAK2 in high glucose cultured VSMC for acute 5-HT preincubation. VSMCs were treated for 10 min with 6 treatment groups: V (Vehicle: water); 5-HT-9 (5-HT 10⁻⁹ M); ET-1-9 (ET-1 10⁻⁹ M); ET-1-8 (ET-1 10⁻⁸ M); ET-1-9 (ET-1 10⁻⁹ M) added after a 30-min preincubation of VSMC with 5-HT 10⁻⁹ M; and ET-1-9 (ET-1 10⁻⁹ M) added after a 30-min preincubation of VSMC with 5-HT 10⁻⁹ M in the 6-well VSMC culture plate, respectively. All samples were derived at the same time and processed serially. Top: densitometric analysis of Western blots utilizing the cultured VSMCs. *Statistical significance compared with the control. Bottom: representative Western blots. Densitometry data are shown as means ± SE; n = 4.

![Graph](https://example.com/graph.png)

**Fig. 5.** Isometric contraction in endothelium intact rat thoracic aorta with ET-1 and AG490. Effect of vehicle (DMSO, ●) and AG490 (AG490 10⁻⁶ M, ●) on the contraction of isolated rat thoracic aorta to ET-1. *Statistical significance and decrease in the contraction in the presence of AG490 compared with Vehicle at ET-1 >10⁻⁹ M. Values are shown as means ± SE; n = 9.
RESULTS

Concentration-response curves of 5-HT and ET-1 in cultured VSMC: activation of p42/44 MAPK proteins. It is known that in VSMC, 5-HT $10^{-6}$ M, and ET-1 $10^{-7}$ M maximally activate JAK2 under normal glucose conditions (4, 21). To determine the activation pattern of the p42/44 MAPK by 5-HT and ET-1 in cultured VSMC under normal and high glucose conditions, concentration-response curves were performed. Our results indicate an increase in the activation of p44 MAPK proteins with increasing concentrations of 5-HT under normal and high glucose conditions respectively (Fig. 1, A and B). We also demonstrate an increase in the activation of p42 MAPK proteins at increasing concentrations of ET-1 under normal and high glucose conditions respectively (Fig. 2, A and B). The concentration-response curves indicate that high glucose alone can increase the basal activation of p42/44 MAPK proteins at low concentrations of 5-HT and ET-1. Therefore, we chose 5-HT $10^{-9}$ M for our combination experiments.

Acute 5-HT $10^{-9}$ M preincubation and the effects on the activation of p42 MAPK proteins and JAK2 proteins in VSMC under normal or high glucose conditions. Previous in vitro data have suggested that 5-HT, at supraphysiological concentrations (5-HT $10^{-6}$ M), can potentiate the ability of ET-1 to stimulate VSMC proliferation (27). Wanatabe et al. (27) suggest that the mechanisms for the enhanced effect involve both the p42/44 MAPK and JAK2, so we tested the ability of 5-HT $10^{-9}$ M to interact with ET-1 to cause enhanced activation of both JAK2 and p42/44 MAPK, specifically in the high glucose conditions. Our data revealed that acute 5-HT $10^{-9}$ M preincubation for 30 min was not sufficient to enhance the ET-1 stimulated activation of either p42/44 MAPK (Fig. 3, A and B) and JAK2 proteins (Fig. 4, A and B) even under high glucose conditions. We also observed that acute preincubation with ET-1 at $10^{-9}$ M and $10^{-8}$ M concentrations and simultaneous addition of 5-HT $10^{-9}$ M and ET-1 $10^{-9}$ M did not enhance the activation of p42/44 MAPK and JAK2 proteins in VSMC even under high glucose conditions (data not shown).

Thus our in vitro data confirm that physiological concentrations of both 5-HT and ET-1 are not sufficient to augment the effects of each other in activating the p42/44 MAPK and JAK2 proteins in cultured VSMC. However, whether this is true in other cultured cells such as glomerular mesangial cells and tubular epithelial cells that could potentially lead to extracellular matrix accumulation in the kidney glomeruli, especially under high glucose conditions still needs to be further investigated.

Potential involvement of JAK2 in ET-1- and 5-HT-induced contractions. As our next step, we performed contractile studies of rat thoracic aortic rings to test whether 5-HT and ET-1 utilizes p42/44 MAPK and JAK2 proteins to cause smooth
muscle contraction that may lead to accelerated vascular injury such as hypertension. Previous studies show that both 5-HT and ET-1 utilize p42/44 MAPK to cause smooth muscle contraction (2, 8, 33), but whether they also activated JAK2 pathway was not investigated. The contractile studies of the rat thoracic aortic rings were performed using myograph technology. Chambers were covered to minimize light and preincubated for 30 min with the JAK2 inhibitor AG490 (10^{-6} \text{M}). Evidence suggests that AG490 exhibits an inhibitory effect of the JAK2 activity (13). AG490 was dissolved in dimethylsulfoxide (DMSO), and thus DMSO was used as the vehicle. Our results indicate that JAK2 is utilized by ET-1 to mediate smooth muscle contraction (Fig. 5), but 5-HT did not activate JAK2 to participate in the smooth muscle contractile mechanism since either adding AG490 or another JAK2 inhibitor did not statistically attenuate the 5-HT-induced smooth muscle contraction (Fig. 6).

5-HT and ET-1 interaction in smooth muscle contraction. It has previously been shown that the p42/44 MAPK is involved in the 5-HT/ET-1 interaction mechanism in the rat tail artery (4). However, the involvement of JAK2 in the 5-HT/ET-1 interaction mechanism has never been investigated in VSMC. Therefore, we used the JAK2 inhibitor AG490 to determine the involvement of JAK2. At first, we tested the effect of ET-1-induced contractility of rat thoracic aortic rings preincubated with supraphysiological concentration of 5-HT (10^{-5} \text{M}) and also tested the contractile response by preincubating the tissues with physiological concentration of 5-HT (10^{-9} \text{M}). In the absence of AG490, we found a significant increase in the maximal contraction in the tissues preincubated with 5-HT 10^{-6} \text{M} (Fig. 7A) and preincubated with 5-HT 10^{-9} \text{M} (Fig. 7B). In the presence of AG490 the contraction is attenuated, suggesting that the 5-HT/ET-1 interaction mechanism may involve the JAK2 pathway in VSMC. However, the attenuation response for tissues preincubated with 5-HT 10^{-9} \text{M} was more pronounced compared with the response from tissues preincubated with 5-HT 10^{-9} \text{M}, suggesting that elevated levels of 5-HT in diabetes could enhance the contractile response and contribute to vascular injury. Additionally, we observed that enhanced ET-1-induced smooth muscle contraction for both 5-HT 10^{-6} \text{M} and 5-HT 10^{-9} \text{M} preincubation involved the activation of Rho-kinase pathway as assessed by using Rho-kinase inhibitor 10^{-6} \text{M}.

Since, our in vitro data indicated that under normal glucose conditions, ET-1 10^{-8} \text{M} stimulated significant activation of the p42/44 MAPK protein, we wanted to test whether ET-1 concentrations <10^{-8} \text{M} could possibly enhance the effects of 5-HT induced VSMC contraction. It was observed that preincubation of ET-1 at concentrations 10^{-11} \text{M} (Fig. 8A) and at 10^{-12} \text{M} (Fig. 8B) enhanced 5-HT induced VSMC contraction respectively. Furthermore, we also observed that the ET-1 10^{-12} \text{M} potentiated the 5-HT-induced contraction involved the activation of p42/44 MAPK, JAK2, and Rho-kinase pathway as assessed by utilizing p42/44 MAPK inhibitor (U0126 10^{-6} \text{M}), JAK2 inhibitor (AG490 10^{-6} \text{M}), and Rho-kinase inhibitor 10^{-9} \text{M} respectively (Fig. 9).

DISCUSSION

These data suggest that physiological concentrations of 5-HT and ET-1 interact with each other to enhance the smooth muscle contraction in tissues obtained from nondiabetic rats. Further testing in tissues from diabetic rats, especially with elevated levels of 5-HT and ET-1 chronically, may indicate altered vascular function. We speculate that this may be due to enhanced vascular effects due to the 5-HT/ET-1 interaction mechanisms.

To date, diabetes remains a serious public health problem. The aging of the population, together with increasing obesity and declining physical activity, suggests that the number of diabetic individuals will continue to increase. Early treatment for high blood pressure and strict glycemic control may forestall the development of diabetes. Even though there has been significant improvement in the regulation of diabetes, the underlying molecular mechanisms to understand the pathophysiology that contributes to the progression of diabetic complications still remain largely unknown and warrants further investigation.

Several studies suggest an interaction between 5-HT and ET-1 that could contribute to the vascular remodeling in pathological conditions; this is the first in vitro study that is looking into the molecular mechanisms of the 5-HT/ET-1 interaction in diabetes. In vitro experimental results in VSMC...

Fig. 8. A: isometric contraction in endothelium intact rat thoracic aorta with ET-1 10^{-11} \text{M} preincubation and 5-HT curves. Effect of vehicle (preincubation of the tissue with water for 30 min, ●) and ET-1 10^{-11} \text{M} (preincubation of the tissue with ET-1 10^{-11} \text{M} for 30 min, ▲) on the contraction of isolated rat thoracic aorta to 5-HT. *Statistical significance and increase in contraction compared with vehicle-incubation tissue. Values are shown as means ± SE; n = 6. B: isometric contraction in endothelium intact rat thoracic aorta with ET-1 10^{-12} \text{M} preincubation and 5-HT curves. Effect of vehicle (preincubation of the tissue with water for 30 min, ●) and ET-1 10^{-12} \text{M} (preincubation of the tissue with ET-1 10^{-12} \text{M} for 30 min, ▲) on the contraction of isolated rat thoracic aorta to 5-HT. *Statistical significance and increase in contraction compared with vehicle-incubation tissue. Values are shown as means ± SE; n = 6.
suggest that high glucose conditions alone could significantly activate p42/44 MAPK and JAK2 pathways at lower concentrations of 5-HT and ET-1 to augment both vascular function and growth responses that are involved in the development of diabetic complications. Previous studies suggested an enhanced 5-HT/ET-1 interaction in cultured VSMCs at supra-physiological concentrations of 5-HT and ET-1 (27); our in vitro Western blot data in VSMC suggest the absence of the priming effect of 5-HT on ET-1 and vice versa at physiological concentrations of 5-HT and ET-1, to activate p42/44 MAPK and JAK2 proteins, even under high glucose conditions for acute preincubations. However, the effect of 5-HT/ET-1 interaction mechanisms in vivo at physiological concentrations needs to be further investigated.

Previous studies show that 5-HT utilizes p42/44 MAPK pathway to cause smooth muscle contraction (2). Our ex vivo contractility studies show for the first time that 5-HT does not require JAK2 to cause VSMC contraction although JAK2 is involved in the ET-1-induced VSMC contraction. The experimental data obtained suggest that both 5-HT and ET-1 each potentiate a contraction in VSMC in response to the other, at near physiological levels of 5-HT and ET-1 found in vivo (7, 9, 18). Our results also showed that the p42/44 MAPK, JAK2, and Rho-kinase pathways may participate in the 5-HT/ET-1-induced enhanced contractile response of VSMC. These data might suggest that overactivation of these pathways that involve 5-HT and ET-1 may contribute to the development of vascular complications in chronic conditions like diabetes.

Figure 10 represents a potential mechanism for the enhanced contraction observed with 5-HT preincubation. We speculate that low concentrations of 5-HT when added will result in increases in intracellular calcium levels below those needed to elicit a contraction (step 1 in Fig. 10) but that could act as a “primer” for the response when stimulated with ET-1 (step 2 in Fig. 10). This may also happen in a similar manner when ET-1 is the preincubation agonist. Additionally, 5-HT may also allow for the inactivation of the regulatory phosphatase, Src homology 2 domain phosphatase-1 (SHP-1). This would allow enhanced activation of the JAK2 pathway by ET-1 and a more enhanced contraction. 5-HT may generate ROS [such as hydrogen peroxide (H2O2)], which can also inhibit the ability of SHP-1 to regulate the activity of JAK2. Since levels of 5-HT are elevated in pathological conditions such as diabetes (17, 19), it is possible that the priming effect of 5-HT would be more pronounced for 5-HT preincubation as our myograph data indicates that higher concentrations of 5-HT could sig-

![Fig. 9. Isometric contraction in endothelium intact rat thoracic aorta with ET-1 10^{-12} M preincubation and 5-HT curves. Effect of vehicle/ET-1 (DMSO with ET-1 10^{-12} M for 30 min, ●), AG490/ET-1 (AG490 10^{-6} M with ET-1 10^{-12} M for 30 min, ▲), Rho-kinase inhibitor/ET-1 (Rho-kinase inhibitor 10^{-6} M with ET-1 10^{-12} M for 30 min, ✡), and vehicle/vehicle (water with DMSO for 30 min, ●) on the contraction of isolated rat thoracic aorta to 5-HT. +Statistical significance and increase in contraction of the tissues with ET-1 10^{-12} M preincubation (vehicle/ET-1) compared with others. *Statistical significance and decrease in contraction in the presence of AG490, UO126, and Rho-kinase inhibitors. Values are shown as means ± SE; n = 4–7.](http://ajpcell.physiology.org/)

![Fig. 10. Proposed signaling mechanism of 5-HT preincubation before the addition of ET-1 to stimulate VSMC contraction. Ca^{2+}, calcium ions; SR, sarcoplasmic reticulum; PLC, phospho-lipase C; PIP_{2}, phosphatidylinositol 4,5-bisphosphate; IP_{3}, inositol trisphosphate; DAG, diacylglycerol; PKC, protein kinase C; NAD(P)H oxidase, nicotinamide adenine dinucleotide phosphate-oxidase; O_{2}\cdot^{-}, superoxide ions; H_{2}O_{2}, hydrogen peroxide; SHP-1, Src homology 2 domain phosphatase-1.](http://ajpcell.physiology.org/)
significantly enhance ET-1-induced smooth muscle contraction (Fig. 7A). Also, the ability of ET-1 to activate JAK2 in VSMCs has been shown to be dependent on ROS production (30), so this is also an avenue that warrants further investigation.

Current treatment options have been shown to slow the progression of the disease but also increase the risk of vascular injury in diabetic patients (29). Studies show that patients with diabetes are at markedly higher risk for developing coronary, cerebral, and peripheral atherosclerosis (15). Thus research in understanding the diabetes pathophysiological mechanisms at the molecular level may provide additional adjunctive therapeutic treatment options that could significantly reduce the associated vascular complications. Furthermore, insulin can stimulate the suppressor of cytokine signaling-3 expression that could inhibit the insulin signal transduction through JAK/STAT pathway and the insulin receptor substrates (26). JAK2 can also activate other signaling cascade pathway members including the p42/44 MAPK pathway (11). Also, alterations in SHP-1 signaling pathway not only activate JAK2 (24) but also increases inflammatory response (24). Thus the cross-talk existing between the various intracellular signaling pathways emphasizes the intricate relationship between them that needs to be thoroughly investigated especially in diabetic conditions.

Conclusions. Our in vitro studies in the rat VSMC are the first to report the inability of 5-HT and ET-1 at physiological concentrations to enhance the activation of p42/44 MAPK and JAK2 pathways. On the contrary, we provide our first evidence using myograph experiments that 5-HT/ET-1 interaction at physiological concentrations increases smooth muscle contraction that could lead to the early development of vascular complications. Even though our results were generated using low-resistance arteries, the effects of 5-HT/ET-1 interactions in high-resistance arteries that might further contribute to increase in total peripheral resistance warrants further investigation. Hence, it is imperative to understand the potential molecular mechanisms of 5-HT/ET-1 interaction, because targeted inhibition of 5-HT/ET-1 effects in vivo may reduce the risk of vascular abnormality that is critical in the treatment of diabetic chronic patients.

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DISCLOSURES
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AUTHOR CONTRIBUTIONS

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