Serotonin (5-hydroxytryptamine, 5-HT) is an established mitogen and vasoconstrictor. 5-HT has been reported to potentiate the proliferative effects of both angiotensin II (ANG II) and endothelin-1 in vascular smooth muscle cells (VSMCs) in vitro (24, 25). These are two vasoactive peptides that have recognized roles in diabetes (6, 17). However, in diabetes, the plasma levels of 5-HT are reported to be elevated (7, 15). The mechanisms for this elevation are still unclear. However, treatment with sarpogrelate, a 5-HT2A receptor antagonist, can reduce albuminuria in patients with diabetes who have early-stage diabetic nephropathy (20). Treatment with sarpogrelate also prevents the increase in blood glucose levels caused by 5-HT in Otsuka Long-Evans Tokushima fatty rats (21). In addition, in rats with streptozotocin (STZ)-induced diabetes, sarpogrelate also significantly lowered fasting blood glucose levels, with a corresponding increase in insulin levels, and prevented STZ-induced polydipsia, hyperphagia, bradycardia, and hypertension (22). Therefore, the role for 5-HT as a causative factor in diabetic complications warrants further inquiry.

Investigators at our laboratory (2) previously reported that inhibition of ANG II-induced JAK2 activation reduced proteinuria in the STZ-induced diabetes rat model. Because 5-HT is a known mitogen and because inhibition of the 5-HT2A receptor in vivo demonstrates some protective effects in diabetes, we examined the ability of 5-HT to activate the JAK/STAT pathway. While studies have shown the presence of 5-HT2A, 5-HT2B, and 5-HT1B receptors in vascular smooth muscle cells (VSMCs), there are currently no data regarding 5-HT2B and 5-HT1B receptor activation of the JAK/STAT pathway in VSMCs and resultant potential alterations in 5-HT signaling in diabetes. Therefore, we tested the hypothesis that 5-HT differentially activates the JAK/STAT pathway in VSMCs and endothelium-denuded thoracic aorta in vivo. 5-HT (10−6 M) treatment resulted in increased cell proliferation and increased DNA synthesis, which were inhibited by the JAK2 inhibitor AG490. Further studies with apocynin, diphenyleneiodonium chloride, catalase, and virally transfected superoxide dismutase had no effect at either glucose concentration on activation of the JAK/STAT pathway by 5-HT. Therefore, we conclude that 5-HT activates JAK2, JAK1, and STAT1 via the 5-HT2A receptors in a reactive oxygen species-independent manner under both normal and high glucose conditions.

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either normal (5 mM) or high glucose (25 mM) concentration. After the experiments were conducted, the cells were washed with an ice-cold phosphate-buffered saline (PBS) solution with sodium orthovanadate (1 mM). Modified RIPA lysis buffer (500 μl) was added to each plate and allowed to remain on ice for 18 h before the addition of antagonists. All antagonists were incubated for 5 min for the JAK2 and JAK1 studies and 10 min for activation in the STAT studies.

STZ-induced diabetes. All studies were conducted with the approval of the Medical College of Georgia Animal Care Committee and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (225–250 g), purchased from Harlan Laboratories (Indianapolis, IN), were rendered diabetic by administration of a single intravenous injection of STZ (60 mg/kg) in fresh 0.1 M citrate buffer, pH 4.5. Age-matched control rats were administered buffer only. The diabetic state was confirmed 48 h later by measurement of tail blood glucose level using the Accu-Chek glucometer (Roche Diagnostics, Indianapolis, IN). All rats administered STZ had a blood glucose concentration exceeding 15 mM and thus were considered diabetic. All diabetic rats were treated with up to 2 U/day insulin via insulin pellets (LinShin Canada, Scarborough, ON, Canada) to prevent ketoacidosis. Insulin treatment did not result in normalization of blood glucose. All animals were fed standard Purina rat chow (Ralston Purina, Richmond, IN), had free access to tap water ad libitum, and were maintained on a 12:12-h light-dark cycle.

Isolation of rat thoracic aorta. Sham tissues or tissues obtained from rats treated with STZ and STZ with ketanserin were quick frozen with liquid nitrogen, pulverized with a liquid nitrogen-cooled mortar and pestle, and solubilized in lysis buffer (0.5 mol/l Tris-HCl (pH 6.8), 10% sodium dodecyl sulfate (SDS), 10% glycerol) with protease inhibitors (0.5 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and a tyrosine phosphatase inhibitor (1 mM sodium orthovanadate). Homogenates were sonicated for 1 min at setting 7 in a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) and then centrifuged (11,000 g for 10 min at 4°C) and the protein concentration for each sample was assessed using a modification of Bradford’s method (27).

Immunoblotting protocol. VSMC and tissue lysates were resolved by performing 7.5% SDS-PAGE, transferred to nitrocellulose membranes, blocked for 60 min at 22°C in Tris-buffered saline with 0.05% Tween 20 (TTBS), pH 7.4, with 5% skimmed milk powder. Membranes were incubated overnight at 4°C with affinity-purified anti-phosphotyrosine-specific and total JAK and STAT antibodies (JAK1, JAK2, STAT1, and STAT3). Membranes were washed with TTBS and then incubated with the appropriate secondary antibodies, either a goat anti-rabbit IgG or a goat anti-mouse horseradish peroxidase conjugate (60 min, 22°C). After being washed with TTBS, the bands were visualized using SuperSignal substrate chemiluminescence (Pierce Biotechnology, Rockford, IL) and Kodak Biomax film. Membranes were also incubated with smooth muscle α-antin antibody (Oncogene, Boston, MA) and the appropriate secondary antibody to ensure equal loading of total protein. Molecular mass markers assessed specificity of the bands.

Adenoviral infection of cells. The recombinant adenoviruses expressing control green fluorescent protein and Cu/Zn-SOD were obtained from D. Fulton (Medical College of Georgia). Monolayers were incubated with recombinant adenovirus at a multiplicity of infection of 100. After infection, normal 10% FBS supplemented DMEM was added for the cell recovery period, followed by serum starvation in serum-free normal and high glucose DMEM. Experiments were performed as described previously.

DHE staining. Aortas were placed into freezing mold with Tissue-Tek OCT (Sakura Finetek, Torrance, CA) and snap frozen with liquid nitrogen. Samples were then sectioned on a cryostat, place on slides and in situ superoxide generation was evaluated in the cryosections with the oxidative fluorescent dye dihydroethidium (DHE). Cryosections (12 μm) were incubated with DHE (2 μmol/l) in PBS, with or without polyethylene glycol (PEG)-SOD (150 U/ml), which was added 30 min before staining. Fluorescence images were obtained with a Bio-Rad MRC 1024 scanning confocal microscope. For each slide, at least five images from different sections of the slide were captured and average staining intensity was calculated with MetaMorph software (Universal Imaging, Downingtown, PA).

Cell proliferation assay and Coulter counting. Proliferation was measured using the Cell Titer 96 aqueous nonradioactive cell proliferation assay (Promega, Madison, WI) as described previously (16). Briefly, VSMCs were grown to confluence in a 75-mm2 flask and detached with trypsin-EDTA (0.05% trypsin, 0.53 M EDTA; Life Technologies, Carlsbad, CA). Cells (n = 20,000) were plated into 96-well plates and allowed to settle for 4 h in DMEM supplemented with 10% FBS. Before experiments, cells were growth arrested in serum-deprived DMEM for 24 h (time 0). Cells were then stimulated with 10–6 M 5-HT. After timed 5-HT exposure, the phenazine methosulfate/MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenoxy)-2-(4-sulfophenyl)]-2H-tetrazolium salt] mix was added to each well (final volume of 20 μl/100 μl of medium) and then incubated for an additional 60 min in 5% CO2 at 37°C. A 10% SDS solution was then added to stop the reaction, and the absorbance of formazan was measured at 490 nm. The JAK2 inhibitor AG490 (10 μM) was added 16 h before experimentation.

5′-Heymidine incorporation. VSMCs were plated in 96-well plates and maintained in DMEM supplemented with 10% FBS as described for the cell proliferation assay above. After (24 and 48 h) 5-HT exposure, cells were pulsed with 1 μCi/ml [3H]thymidine (New England Nuclear, Boston, MA) and then harvested into trichloroacetic acid-precipitable material. Cells were washed with PBS, incubated in 10% trichloroacetic acid at 4°C, dissolved at room temperature in 0.1 M tris(hydroxymethyl)aminomethane, and dried on filter paper. The filter paper was washed three times with PBS, and then samples were placed in scintillation liquid and counted with a scintillation counter (Beckman, Palo Alto, CA). Data were plotted as cpm/well. Each experimental data point represents duplicate wells from at least 10 different experiments. The JAK2 inhibitor AG490 (10 μM) was added 16 h before experimentation.

Data analysis and statistics. Quantitation of band density was performed using NIH Image software (Scion). Band density is reported in arbitrary densitometric units and was examined using two-way ANOVA and the Student-Newman-Keuls post hoc test. Samples were compared within normal and high glucose treatment groups and across all treatments to determine whether glucose alone had any effect. In vivo data were studied using one-way ANOVA and the Student-Newman-Keuls post hoc test. Data are reported as means ± SE for the number of animals and samples indicated. Values were considered significant at P < 0.05.

Chemicals. Molecular mass standards, acrylamide, SDS, N,N,N′,N′-methylene-bisacrylamide, N,N,N′,N′-tetramethylenediamine, protein assay reagents, and nitrocellulose membranes were purchased from Bio-Rad Laboratories. STZ, BW7123C86, RU24969, CGS12066A, ketanserin tartrate, and GR55562 were purchased from Tocris (Ballwin, MO). Monoclonal antibodies to IAK1 and JAK2 were procured from Transduction Laboratories (Lexington, KY). Anti-phosphotyrosine antibodies for STAT1 and STAT3 were purchased from Cell Signaling Technology (Beverly, MA). The anti-phosphotyrosine antibodies for JAK1 and JAK2 were obtained from Biosource International (Camarillo, CA). DHE was purchased from Molecular Probes (Eugene, OR). The SuperSignal substrate chemiluminescence detection kit was obtained from Pierce Biotechnology. Goat anti-mouse IgG and anti-rabbit IgG were acquired from Amersham Biosciences.

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5-HT activation of the JAK/STAT pathway in VSMCs. In these studies, we found that 5-HT (10^{-6} M, 5-min stimulation) increased the phosphorylation of JAK2 and that this was decreased by the presence of the 5-HT_{2A} receptor antagonist ketanserin (10 nM) (Fig. 1A). A 5-min stimulation period was chosen because it was determined to be the point of maximal activation of JAK2 by 5-HT (data not shown). Similar results were obtained for the activation of JAK1 (Fig. 1B). However, unlike the increased basal level of phosphorylation of JAK2 in high-glucose conditions, high glucose alone does not enhance basal activation of JAK1. There was no significant activation of TYK2 under any conditions by 5-HT (data not shown). However, unlike previous studies with ANG II demonstrating an enhanced activation of JAK2 by ANG II in the presence of high glucose (19), the activation of JAK2 by 5-HT did not show this enhanced effect.

Furthermore, we found that stimulation by 5-HT resulted in an increase in the phosphorylation of STAT1. A 5-min stimulation period was chosen because it was determined to be the point of maximal activation of JAK2 by 5-HT (data not shown). These findings are different from those in skeletal muscle, in which STAT3 has been shown to coprecipitate with the 5-HT_{2A} receptor. We did not observe any STAT5 protein in the cell lysates (data not shown) or any activation of either STAT protein in the presence of high glucose alone.

Involvement of 5-HT_{2B} and 5-HT_{1B} receptors in activating the JAK/STAT pathway. To determine whether the 5-HT_{2B} and 5-HT_{1B} receptors were also involved in the activation of the JAK/STAT pathway, we treated VSMCs with the 5-HT_{2B} receptor agonist BW723C86. Under conditions of normal and acute high glucose, we observed no activation of the JAK/STAT pathway by BW723C86 (data not shown). When the VSMCs were treated with the 5-HT_{1B} receptor agonists, we obtained mixed results. While we did observe a slight activation of JAK2 with the 5-HT_{1B} receptor agonist RU24969 (data not shown), we did not observe any change in the phosphorylation of JAK2 with the 5-HT_{1B} receptor agonist CGS12066A (data not shown). However, as expected, we did observe a basal increase in phosphorylated JAK2 in the high-glucose treatments (data not shown), but there was no enhancement of the 5-HT stimulation such as that observed with ANG II treatment (19). We observed no activation of JAK1 with BW723C86, CGS12066A, or RU24969 (data not shown). High-glucose treatment alone did not increase the basal JAK1 activation in VSMCs (data not shown). To determine whether the RU24969 was acting selectively, we further investigated the role of the 5-HT_{1B} receptor with the 5-HT_{1B} receptor antagonist GR55562 (100 nM). We observed that treatment with GR55562 had no effect on the ability of 5-HT to stimulate increased levels of JAK2 phosphorylation (data not shown). These data suggest that neither the 5-HT_{1B} nor the 5-HT_{2B} receptors are involved in activation of the JAK/STAT pathway in VSMCs.

Role of ROS in 5-HT-induced activation of the JAK/STAT pathway. To further investigate the mechanisms of 5-HT activation of the JAK/STAT pathway in VSMCs, we studied the potential role of ROS. Under the normal glucose conditions, we found that neither the NAD(P)H oxidase inhibitor apocynin (100 nM) nor the flavoprotein inhibitor DPI (1 μM) had any effect on the 5-HT-induced phosphorylation of JAK2 (Fig. 3). Under conditions of high glucose, we found that only DPI significantly inhibited 5-HT-induced activation of JAK2 (Fig. 3). We next treated VSMCs with PEG-conjugated catalase to inhibit the formation of H_{2}O_{2} and found that it had no effect on

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5-HT-induced activation of JAK2 under conditions of normal or high glucose (Fig. 3). Treating the cells with PEG-SOD also had no effect under the conditions of normal and high glucose (Fig. 3). To determine whether the lack of effect with PEG-SOD treatment was due to inadequate cell permeability, we transfected VSMCs with a Cu/Zn-SOD adenovirus. Overexpression of the SOD virus still had no effect on the 5-HT-induced activation of JAK2 (Fig. 4). The lack of effect was not due to an expression problem, because we observed significant expression of the SOD protein (Fig. 4). In the high-glucose treatment groups, we again observed the expected increase in basal JAK phosphorylation levels (Fig. 4). However, this increase was not affected by the expression of the SOD virus.

To determine whether our experimental conditions were not adequate to observe inhibition of 5-HT signaling, we also examined the ability of 5-HT to activate the MAPK pathway. Activation of this pathway has been shown to be ROS dependent (9). Treatment with both catalase and SOD resulted in a significant inhibition of pMAPK levels under the conditions of both normal and high glucose when stimulated with 5-HT (Fig. 5).

Effect of 5-HT-induced JAK2 activation in VSMCs. To determine the consequences of the activation of JAK2 by 5-HT in VSMCs, we examined both cell proliferation and DNA synthesis. We found that 5-HT stimulated VSMC proliferation and that this proliferation was inhibited by the presence of the JAK2 inhibitor AG490 (Fig. 6A). Furthermore, we found that 5-HT at both 24 and 48 h stimulated DNA synthesis and that this increase also was blocked by the presence of AG490 (Fig. 6B).

In vivo activation of JAK2 by 5-HT. To determine whether 5-HT was activating the JAK/STAT pathway via the 5-HT2A receptor in vivo under the conditions of diabetes mellitus type 1 in VSMCs, we used the established STZ-induced diabetes model in the rat and the 5-HT2A receptor antagonist ketanserin. Treatment with ketanserin (5 mg/kg/day administered via drinking water) did not affect the blood glucose levels of the rats with STZ-induced diabetes (443 ± 14.2 mg/dl and 440 ± 20.3 mg/dl STZ alone and STZ with ketanserin, respectively). Treatment with ketanserin also had no effect on final body mass (196 ± 9 g and 220 ± 9 g STZ with ketanserin and STZ alone, respectively) or on fluid consumption (149 ± 4.2 ml/day and 157 ± 13.2 ml/day in STZ alone and STZ with ketanserin groups, respectively). However, phosphorylation of JAK2 (Fig.
A) was significantly increased in the rats with STZ-induced diabetes compared with their sham-treated counterparts, and this increase was inhibited in the presence of ketanserin. We observed similar increases with STAT1 and STAT3 but observed no inhibition of STAT3 phosphorylation with ketanserin (Fig. 7, B and C). However, we did observe a significant decrease in STAT1 phosphorylation with ketanserin treatment (Fig. 7B).

Furthermore, upon examination of sections of these aortas stained with DHE, we observed that after 2 wk of STZ-induced diabetes, there was a significant increase in the level of O$_2$ as shown by red DHE staining in the STZ tissues compared with the nondiabetic sham-treated controls (Fig. 8). Ketanserin treatment in the rats with STZ-induced diabetes had no significant effect on the level of O$_2$ as measured by DHE staining compared with the untreated group with STZ-induced diabetes. These data further suggest that the 5-HT$_{2A}$ receptor did not use ROS to activate JAK2 in vivo.

**DISCUSSION**

In agreement with previously published findings (11), we have found in the present study that 5-HT acted via the 5-HT$_{2A}$ receptor in VSMCs to activate the JAK/STAT pathway. Our data demonstrate that the 5-HT$_{2A}$ receptor antagonist ketanserin reduced activation of JAK2, JAK1, and STAT1. However, STAT3 was not activated by stimulation with 5-HT. This finding is different from the findings in skeletal muscle, in which it was previously shown that the 5-HT$_{2A}$ receptor activated JAK2 and STAT3 (11). Therefore, the present data...
suggest that in VSMCs, 5-HT signaling is different from that observed in skeletal muscle. While this differential activation of the JAK/STAT pathway members is not surprising, this report is the first of its involving 5-HT.

In addition, previous work (19) showed an increase in the basal level of JAK2 phosphorylation in VSMCs under high-glucose conditions. We also observed this change but found it to be specific to JAK2, because we observed no increased basal phosphorylation levels of JAK1, STAT1, or STAT3. The increased basal phosphorylation of JAK2 was not observed in cells treated with inhibitors of ROS or NAD(P)H oxidase. High glucose alone in endothelial cells is known to activate PKC via the polyol pathway, and this leads to the generation of NAD(P)H-dependent ROS and enhanced basal JAK phosphorylation (19). Therefore, the observed lack of increased basal phosphorylation of JAK2 in VSMCs under high-glucose conditions with these inhibitors is not surprising.
Unlike the results obtained with ANG II (19), another G protein-coupled receptor agonist that uses similar signaling pathways, we did not observe an enhanced activation of JAK2 by 5-HT under high-glucose conditions. These findings suggest that while there are many similarities between ANG II and 5-HT signaling, they use different mechanisms to activate the JAK/STAT pathway.

Furthermore, stimulation with the 5-HT2B receptor agonist BW723C86 did not activate JAK2 in VSMCs under conditions of normal or acute high glucose. These data suggest that although the receptor is present in VSMCs (26), it is not involved in 5-HT activation of the JAK/STAT pathway. This finding is consistent with previously published reports that although this receptor is present in VSMCs, it does not appear capable of activation (20, 25). The reasons for this are as yet unclear, but to date no one has shown measurable activation of this receptor in VSMCs in vitro. In addition, treatment with the 5-HT1B receptor agonist CGS12066A did not activate JAK2 and treatment with the 5-HT1B receptor agonist RU24969 activated JAK2 only at the highest concentration. However, the 5-HT1B receptor antagonist GR55562 did not affect the ability of 5-HT to activate JAK2. The discrepancy between these data suggests that RU24969 at high concentrations is not acting as a specific 5-HT1B receptor agonist. While the mechanism for this nonselectivity is unclear, it was previously reported (3). These data suggest that the 5-HT1B receptor also is not involved in 5-HT-induced activation of JAK2 in VSMCs.

Unlike the data reported for pulmonary VSMCs, in which the 5-HT1B receptor caused vasoconstriction and acted through NAD(P)H oxidase to increase \( \Delta O_2^{•−} \) (13), activation of JAK2 in aortic VSMCs by 5-HT was not affected by treatment with catalase, SOD, and apocynin. This finding was not universal, because we observed significant inhibition of 5-HT-induced MAPK activation by both catalase and SOD. Previous work demonstrated that activation of the MAPK pathway in VSMCs occurs via the 5-HT2A receptor (1, 26). These findings suggest that 5-HT differentially uses ROS as a second messenger within the same cell and through the same receptor. While the mechanisms by which 5-HT activates the JAK/STAT pathway have yet to be elucidated, this finding is novel and very different from the mechanism used by ANG II. To date, no one has demonstrated that ANG II simultaneously activates ROS-dependent and -independent signaling mechanisms through the same receptor type.

These data also suggest that more investigation into peripheral 5-HT receptors and their functions is warranted. These future investigations should take into account the vascular bed and whether the arteries are in a diseased state, because other work has demonstrated that in hypertension, both the 5-HT2B and 5-HT1B receptors are upregulated and functional only in aorta and superior mesenteric arteries from DOCA-salt-hypertensive rats (4, 5). Changes in 5-HT receptor function in any other disease state have not been addressed to date, even though there are reports that the circulating level of this vasoactive substance are increased (14). Furthermore, the reduction in albuminuria in patients with diabetes using the 5-HT2A receptor antagonist sapogrelate (20), as well as the effects on insulin and blood glucose levels (22), suggests a role for 5-HT in diabetes.

In addition, we observed an increase in JAK2, STAT1 and STAT3 tyrosine phosphorylation levels in endothelium-denuded rat thoracic aorta in vivo. Furthermore, treatment of diabetic rats with ketanserin prevented the increase in pJAK2 and pSTAT1 levels observed in aortas from untreated diabetic rats. The present report is the first one involving diabetes in which STAT3 activation in VSMCs appears not to have occurred through 5-HT. These data correspond to recently published results showing that inhibition of JAK2 in STZ-induced
diabetes had a protective effect in the kidney (2). This suggests that further investigation of the involvement of 5-HT in diabetic complications, particularly in the kidney, should be pursued.

In rat aortic VSMCs, 5-HT uses only the 5-HT2A receptor under the conditions of normal and acute high glucose to activate JAK2 and STAT1 in vitro. Furthermore, the activation of JAK2 by 5-HT appears to be independent of ROS and NAD(P)H oxidase. This finding is novel because other activators of the JAK/STAT pathway, such as ANG II, which is also a G protein-coupled receptor, appear to use ROS to activate this pathway. Furthermore, in vivo treatment with ketanserin reduced the activation of JAK2 and STAT1, suggesting that this pathway. Furthermore, in vivo treatment with ketanserin reduced the activation of JAK2 and STAT1, suggesting that 5-HT via the 5-HT2A receptor maybe involved in complications of diabetes mellitus type 1.

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