

Research article

## Inability of Serotonin to Activate the c-Jun N-terminal Kinase and p38 Kinase Pathways in Rat Aortic Vascular Smooth Muscle Cells

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### Abstract

**Background:** Serotonin (5-HT, 5-hydroxytryptamine) activates the Extracellular Signal-Regulated Kinase (ERK)/ Mitogen-Activated Protein Kinase (MAPK) pathways, in vascular smooth muscle cells. Parallel MAPK pathways, the c-Jun N-terminal Kinase (JNK) and p38 pathway, are activated by stimulators of the ERK/MAPK pathway. We hypothesized that 5-HT would activate the JNK and p38 pathways in rat vascular smooth muscle cells.

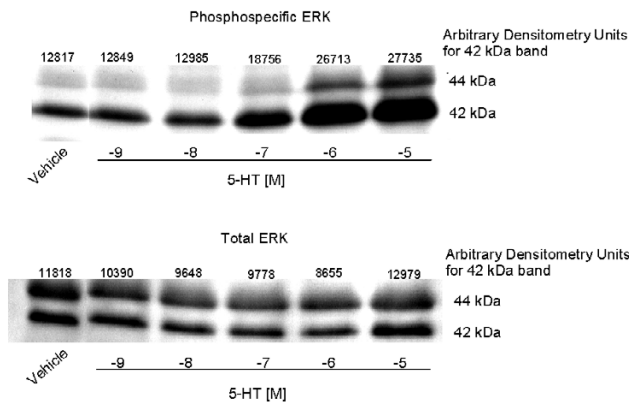
**Results:** Results were determined using standard Western analysis and phosphospecific JNK and p38 antibodies. No significant activation by 5-HT ( $10^{-9}$  –  $10^{-5}$  M; 30 min) of the JNK or p38 pathways, as measured by protein phosphorylation, was observed in any of these experiments. These experiments were repeated in the presence of the serine/threonine phosphatase inhibitor okadaic acid (1  $\mu$ M) and the tyrosine phosphatase inhibitor sodium orthovanadate (1  $\mu$ M) to maximize any observable signal. Even under these optimized conditions, no activation of the JNK or p38 pathways by 5-HT was observed. Time course experiments (5-HT  $10^{-5}$  M; 5 min, 15 min, 30 min and 60 min) showed no significant activation of JNK after incubation with 5-HT at any time point. However, we detected strong activation of JNK p54 and p46 (5- and 7 fold increases in bands p54 and p46, respectively over control levels) by anisomycin (500 ng/ml, 30 min). Similarly, a JNK activity assay failed to reveal activation of JNK by 5-HT, in contrast to the strong stimulation by anisomycin.

**Conclusion:** Collectively, these data support the conclusion that 5-HT does not activate the JNK or p38 pathways in rat vascular smooth muscle cells.

### Background

The mitogen-activated protein kinase (MAPK) family consists of three commonly recognized subgroups: the extracellular signal-regulated kinase (ERK), the c-jun-N-terminal kinase (JNK), also known as the stress activated

protein kinase (SAPK) and the p38 kinase. While many actions have been associated with activation of the ERK pathway, two particular functions of interest to our laboratory are mitogen-stimulated growth [1] and smooth muscle cell contraction [2]. Of the three MAPK path-



**Figure 1**  
Top: Blot of 5-HT-induced stimulation of the ERK/MAPK pathway in rat aortic vascular smooth muscle cells as measured by use of a phosphospecific ERK/MAPK antibody. Densitometry measurements are for the 42 kDa band. Blot is representative of four experiments. Bottom: Total ERK/MAPK antibody recognizes nonphosphorylated forms as well as phosphorylated protein. Densitometry measurements are for the 42 kDa band.

ways, activation of the ERK pathway and the intracellular signaling pathways associated with ERK activation are the best delineated. Known activators include reactive oxygen species [3,4], growth factors [5], and agonists of G-protein coupled receptors [6,7]. The two other MAPK pathways, the JNK and p38 pathways, have been implicated in a variety of similar cellular functions. Known activators of the JNK and p38 pathways in vascular smooth muscle cells include reactive oxygen species [8,9], mechanical strain [10,11], hypoxia [12] and a variety of cytokines and growth factors. The mechanisms of many cellular functions of the JNK and p38 pathways are not yet clearly defined. The JNK pathway is involved in apoptosis [13], arginine vasopressin-induced increases in smooth muscle  $\alpha$ -actin in vascular smooth muscle cells [14] as well as in phosphorylation of transcription factors c-jun, ATF-2 and ELK-1 and phosphorylation of Na-K-2Cl cotransporter [15]. The p38 pathway has been implicated in apoptosis [16], neointimal hyperplasia after vascular injury [17] as well as angiotensin II-induced contraction in vascular smooth muscle [18].

Recently, it has been noted that the JNK and p38 pathways can be activated by G-protein coupled receptor agonists, notably angiotensin II, in vascular smooth muscle cells [19,20]. In cardiac myocytes, both the p38 and JNK pathways have been activated by endothelin-1 (ET-1) and the  $\alpha_1$  adrenergic receptor agonist phenylephrine [21]. The ability of the JNK and p38 pathways to be activated by the same agonists of G-protein coupled receptors which activate the ERK pathway led to the investigation

of 5-HT as a possible activator of the JNK and p38 pathways. 5-HT, acting *via* the 5-HT<sub>2A</sub> receptor, is a known activator of the ERK pathway in vascular smooth muscle cells [2,22]. In these studies we tested the hypothesis that 5-HT would activate the JNK and p38 pathways in rat aortic vascular smooth muscle cells.

## Results

### The ERK pathway

In agreement with previously published studies from our lab, 5-HT caused a concentration-dependent activation of the ERK pathway (figure 1). This activation occurs maximally at five minutes of stimulation and returns to basal levels by thirty minutes [23]. This time course of activation is consistent with that of other G-protein coupled receptors.

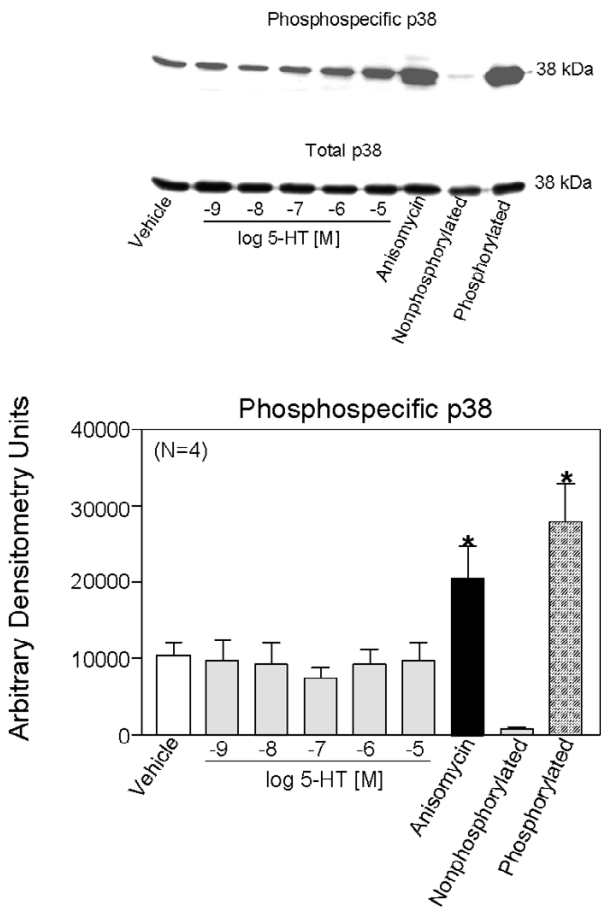
### The p38 pathway

The p38 pathway has demonstrated a different time course of activation *via* G-protein coupled receptors than the ERK pathway [24]. Using the time point (30 min) at which angiotensin II demonstrates maximal stimulation of the JNK pathway we performed a concentration response curve ( $10^{-9}$  –  $10^{-5}$  M) to 5-HT in vascular smooth muscle cells. Western analysis using phosphospecific antibodies was used to measure activation the p38 pathway. A total or non-phosphospecific antibody was used to ensure equal loading of p38 protein. After thirty-minutes of incubation with 5-HT ( $10^{-9}$  –  $10^{-5}$  M), there was no enhanced phosphorylation of p38 (figure 2, bottom). In contrast, incubation with anisomycin (500 ng/ml) induced significant phosphorylation of p38 (2-fold above basal). These data suggest that 5-HT does not activate the p38 pathway in rat vascular smooth muscle cells despite the ability of other agonists to activate the pathway in these cells.

### The JNK pathway

Figure 3 shows that thirty minutes of incubation with 5-HT ( $10^{-9}$  –  $10^{-5}$  M) resulted in a trend towards a modest increase in phosphorylation of the p46 JNK isoform. This trend, however, was not statistically significant. In contrast, anisomycin (500 ng/ml) induced significant phosphorylation of the p46 JNK isoform (5–7 fold above basal). Both the p54 and p46 bands were analyzed by densitometry (data for p54 not shown). However, incubation with 5-HT did not produce statistically significant activation in either band when compared to vehicle. These data suggest that 5-HT does not substantially activate the JNK pathway in vascular smooth muscle cells. However, the data obtained with anisomycin provides evidence that this pathway can be activated in these cells.

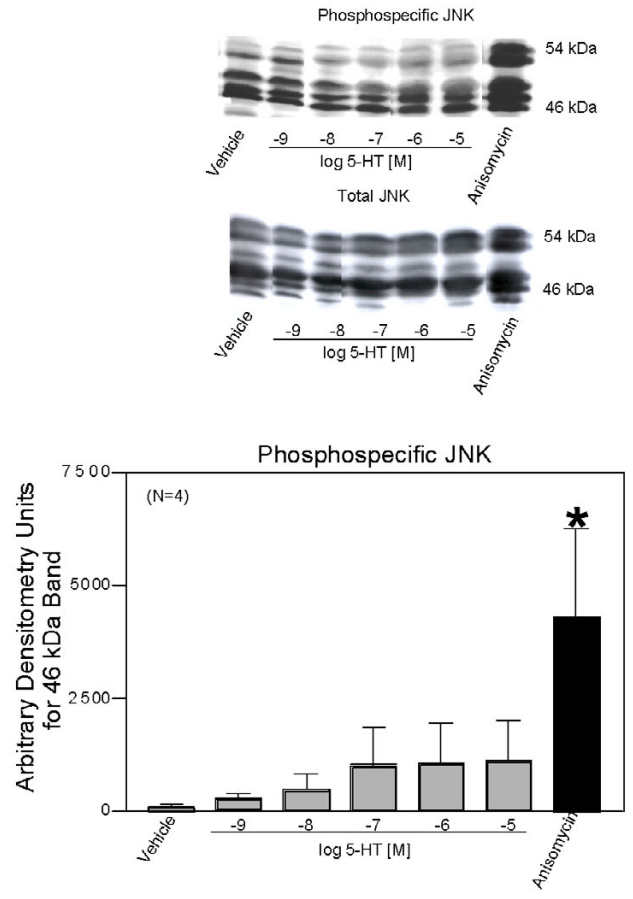
To confirm that thirty minutes was an optimal time point for observing 5-HT-induced phosphorylation of JNK, we



**Figure 2**  
 Top: Blot of 5-HT-induced stimulation of the p38 pathway in rat aortic vascular smooth muscle cells as measured by use of a phosphospecific p38 antibody. Blot is representative of four experiments. Total anti-p38 antibody recognizes nonphosphorylated forms as well as phosphorylated protein. Bottom: Densitometry measurements for p38 kDa. Bar graphs represent means and vertical bars the SEM for N = 4. \* = statistical difference for response in vehicle-incubated cell cultures.

next performed a time course of JNK phosphorylation after exposure to 5-HT in rat vascular smooth muscle cells. At no time point (5 min, 15 min, 30 min and 60 min) did 5-HT (10<sup>-5</sup> M) enhance JNK phosphorylation above basal levels (figure 4). These data fail to support a role for 5-HT-induced activation of the JNK pathway in vascular smooth muscle cells.

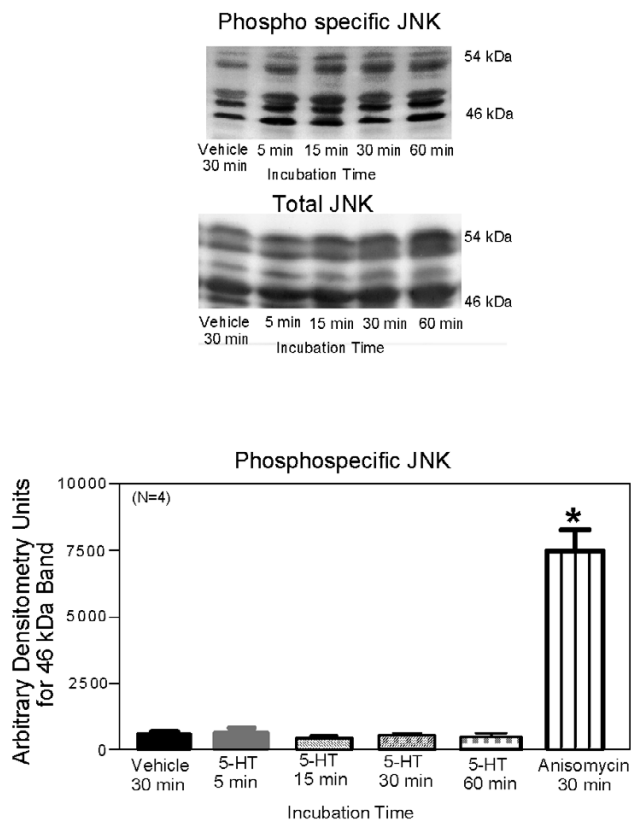
To demonstrate that a G-protein coupled receptor agonist could activate the JNK pathway in our vascular smooth muscle cells, we incubated the cells with angiotensin II (10<sup>-9</sup> – 10<sup>-5</sup> M) for thirty minutes. Angiotensin II (10<sup>-8</sup> – 10<sup>-5</sup> M) produced a significant increase in the level of JNK phosphorylation (figure 5). The authors



**Figure 3**  
 Top: Blot of 5-HT-induced (10<sup>-9</sup> – 10<sup>-5</sup> M) stimulation (30 minutes) of the JNK pathway in rat aortic vascular smooth muscle cells as measured by use of a phosphospecific JNK antibody. Blot is representative of four experiments. Middle: Total anti-JNK antibody recognizes nonphosphorylated forms as well as phosphorylated protein. Bottom: Densitometry measurements are for the 46 kDa band. Bar graphs represent means and vertical bars the SEM for N = 4. \* = statistical difference for response in vehicle-incubated cell cultures.

speculate that the differences in the level of phosphorylation of the p46 JNK isoform in the vehicle treatment group maybe due to differences in the JNK expression over time in cultured cells. The cells used in these experiments were of a later passage than those used for the 5-HT incubation studies. These data, in addition to that obtained with anisomycin, demonstrate that in these vascular smooth muscle cells the JNK pathway can be activated.

Due to the trend toward increased phosphorylation of both the p46 and p54 JNK isoforms by 5-HT, we next performed JNK activity assays to determine whether 5-



**Figure 4**

Top: Blot of 5-HT-induced ( $10^{-5}$  M) stimulation (5 min, 15 min, 30 min and 60 min) of the JNK pathway in rat aortic vascular smooth muscle cells as measured by use of a phospho-specific JNK antibody. Blot is representative of four experiments. Middle: Total anti-JNK antibody recognizes nonphosphorylated forms as well as phosphorylated protein. Bottom: Densitometry measurements are for the 46 kDa band. Bar graphs represent means and vertical bars the SEM for N = 4. \* = statistical difference for response in vehicle-incubated cell cultures.

HT had any significant effect on kinase activity. This assay was chosen to ensure that the western analyses used were sufficiently sensitive to detect changes in the activation of the JNK pathway. There was no significant difference in the levels of activity at any concentration of 5-HT ( $10^{-9}$  –  $10^{-5}$  M) after thirty minutes of incubation in vascular smooth muscle cells (figure 6). In contrast, anisomycin (500 ng/ml, 30 min) produced a significant increase in JNK activation (approximately 2-fold above basal). These data, in combination with the above findings, do not support a role for 5-HT in the activation of the JNK pathway in vascular smooth muscle cells.

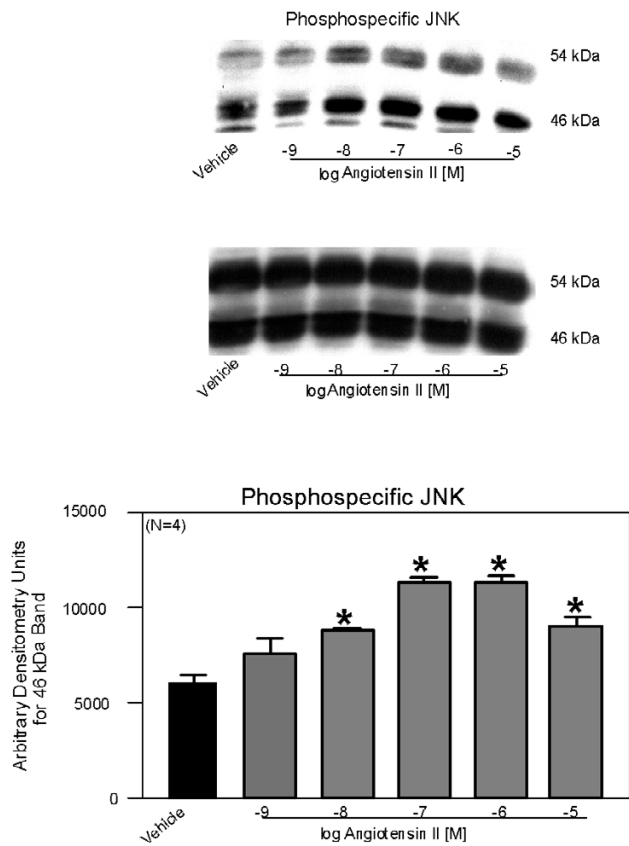
## Conclusions

5-HT is an activator of the ERK pathway in vascular smooth muscle cells. In contrast, 5-HT appears to be unable to activate the JNK and p38 pathways in vascular smooth muscle cells. No observable changes in activation, as measured by phosphorylation status, were seen at any of the 5-HT concentrations ( $10^{-9}$  –  $10^{-5}$  M) or time points (5 min, 30 min, 60 min and 2 hr) examined. The data from the kinase activity assay also demonstrated no activation of the JNK pathway by 5-HT.

The mitogen-activated protein kinase family is associated with many cellular functions. The ability of 5-HT to activate the ERK pathway is consistent with 5-HT's role as a mitogenic stimulus and vasoconstrictor. Several vasoactive G-protein coupled agonists, including angiotensin II and endothelin, have also been shown to activate the ERK pathway in vascular smooth muscle cells [6,21]. These same hormones also activate the JNK and p38 pathways. These agonists have different time course and concentration profiles for activation of the different MAPK pathways. ERK activation by angiotensin II and 5-HT occurs at relatively lower agonist concentrations and within five minutes of stimulation [19,23]. The activation of p38 and JNK pathways by angiotensin II and endothelin occurs at higher concentrations of agonist and requires a longer incubation with the stimulus [18,19,25]. There are data which also suggest that different signal transduction pathways are utilized for the different pathways [25–27]. This differential activation of MAPK pathways is most likely due to the role that each plays in the cell. The ERK pathway is associated with growth where as the p38 and JNK pathways are "stress response" pathways.

In light of data for the other G-protein coupled receptor agonists, the inability of 5-HT, also G-protein coupled receptor, was initially surprising. While 5-HT has never been shown to increase activation of either the p38 or JNK pathways, it has been linked to ERK activation. The 5-HT<sub>2A</sub> receptor is the primary serotonergic receptor which couples to activation the ERK pathway in vascular smooth muscle cells [28]. 5-HT's inability to activate the JNK and p38 pathways may be due to a lack of coupling of the 5-HT<sub>2A</sub> receptor signaling pathway to the components in the JNK and p38 pathways.

It has recently been suggested that G-protein coupled receptors with PDZ domains, SH2-containing domains and PTB domains participate in protein-protein interactions with partners other than G-proteins, such as Grb2 and JAK2, which may allow these receptors to bypass the G-proteins and utilize other signaling cascades (29). This ability of G-protein coupled receptors to modulate the signal cascade used, independent of G-proteins, suggests



**Figure 5**  
 Top: Blot of angiotensin II-induced ( $10^{-9}$  –  $10^{-5}$  M) stimulation (30 minutes) of the JNK pathway in rat aortic vascular smooth muscle cells as measured by use of a phosphospecific JNK antibody. Blot is representative of four experiments. Middle: Total anti-JNK antibody recognizes nonphosphorylated forms as well as phosphorylated protein. Bottom: Densitometry measurements are for the 46 kDa band. Bar graphs represent means and vertical bars the SEM for N = 4. \* = statistical difference for response in vehicle-incubated cell cultures.

a broader range of interaction with signaling components. This may provide one explanation for the 5-HT activation of the ERK and its lack of effect on the JNK and p38 pathways.

Another possible explanation for the differential activation of the MAPK pathways by 5-HT may relate to the varied roles 5-HT in different cell types and the multiple receptors which mediate these roles. When examining the physiological role of 5-HT in other cell types, in regards to apoptosis and cellular responses to stress, 5-HT is generally anti-apoptotic [30–32]. In particular, in neuronal cells the 5-HT<sub>1A</sub> receptor is upregulated under conditions of cellular stress and is anti-apoptotic [33,34]. The anti-apoptotic effects of 5-HT may be due to

scavenging of reactive oxygen species [35] as well as stimulation of the ERK pathway which results in inhibition of a caspase-3-like enzyme [32]. There are no currently published studies examining the role of 5-HT as an anti-apoptotic factor in vascular smooth muscle cells. It may be that under stressful conditions, such as a disease state or a loss of nutrients which occurs in a state of ischemia, the smooth muscle cells upregulate a new complement of 5-HT receptors [33–35]. These new receptors maybe involved in activation of the ERK, p38 and JNK pathways, but the studies presented here do not directly address this issue. They do, however, support the conclusion that the 5-HT<sub>2A</sub> receptor does not activate the p38 or JNK pathways in rat vascular smooth muscle cells.

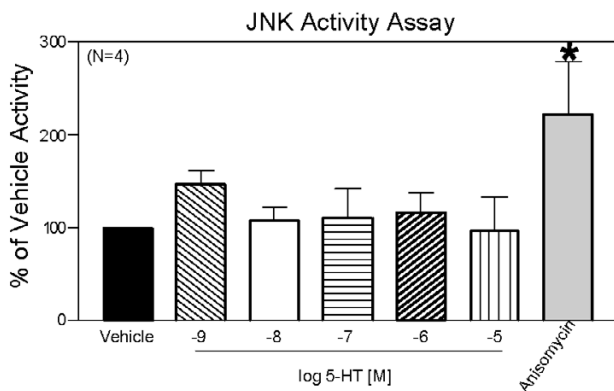
## Methods

### Aortic smooth muscle cell culture

Vascular smooth muscle cells were derived from the aorta of male Sprague-Dawley rats by an explant method previously described [36]. The smooth muscle cells were plated on to P-100's and grown to confluence. The cells were used between passages 2 and 9. The cells were positively stained for smooth muscle  $\alpha$ -actin (Oncogene Research Products, Boston, MA; Fluorescein labeled goat anti-mouse secondary antibody, Molecular Probes, Eugene, OR) with each new isolation.

### Aortic smooth muscle cells experiments

Cells (P-100 plates) were switched to physiological salt solution (4 ml) [consisting of (in mmol/L) NaCl, 103; KCl, 4.7; KH<sub>2</sub>PO<sub>4</sub>, 1.18; MgSO<sub>4</sub> · 7H<sub>2</sub>O, 1.17; CaCl<sub>2</sub> · 2H<sub>2</sub>O, 1.6; NaHCO<sub>3</sub>, 14.9; dextrose, 5.5; and CaNa<sub>2</sub>EDTA, 0.03] for one hour prior to addition of agonist. At this time, okadaic acid (1  $\mu$ M), sodium orthovanadate (1  $\mu$ M) and PD098059 (10  $\mu$ M) or vehicle (0.1–5 %DMSO) was added and allowed to equilibrate for one hour. PD098059, an inhibitor of MEK activation, was included in all experiments, except the ERK activation experiments, to increase the specificity of the phospho-JNK antibody which recognizes the 44 and 42 kDa ERK bands as well as bands at 54 and 46 kDa. In the presence of PD098059, the bands at 42 and 44 kDa were significantly reduced. Each dish was incubated with one agonist concentration [5-HT ( $10^{-9}$  –  $10^{-5}$  M), angiotensin II ( $10^{-9}$  –  $10^{-5}$  M) or anisomycin (500 ng/ml [37])] for thirty minutes. Plates were placed on ice and incubation buffer aspirated. Plates were washed with ice-cold phosphate-buffered saline containing sodium orthovanadate as a tyrosine phosphatase inhibitor (10 mM sodium phosphate, 150 mM NaCl, 1 mM Na<sub>3</sub>VO<sub>4</sub>, pH 7.0). Five hundred microliters of supplemented RIPA lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EGTA, 0.1% Triton X-100, 1 mM PMSF, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 1 mM sodium orthovanadate) were added to each dish and cells were harvested with a rub-



**Figure 6**

Bar graph of 5-HT-induced changes in JNK activity in rat aortic vascular smooth muscle cells. Cells were exposed to 5-HT ( $10^{-5}$  M) for thirty minutes before lysing. Bars represents the means and the vertical bars the SEM for N = 4. \* = statistical difference for response in vehicle-incubated cell cultures.

ber policeman. Lysates were centrifuged at 14,000 g for 10 minutes at 4°C. Protein concentrations of the resulting supernatant were measured using the BSA method (Bio-Rad, Hercules, CA). The gels were also stained with Gel Code Blue® (Pierce, Rockford, IL) to validate equal loading of protein.

#### Immunoblotting protocol

Supernatant (4:1 in denaturing loading buffer, boiled 5 minutes) was loaded, separated on 10% denaturing SDS-polyacrylamide gels, and transferred to Immobilon-P membranes. Membranes were blocked for 3–4 hours in Tris buffer saline + Tween-20 (0.1%; TBS-T) containing 4% chick egg ovalbumin and 0.025% sodium azide. Rabbit anti-phospho Erk MAPK (1:5000, Promega, Madison, WI), rabbit anti-phospho JNK MAPK (1:5000, Promega, Madison WI), rabbit anti-phospho p38 MAPK (1:1000, Cell Signaling, Beverly, MA), mouse anti-total Erk (1:5000, Zymed, San Francisco, CA) rabbit anti-total JNK MAPK (1:5000, Santa Cruz BioTechnologies, Santa Cruz, CA) or rabbit anti-total p38 MAPK (1:1000, Cell Signaling, Beverly, MA) were incubated with blots overnight (4°C). Following washes, secondary antibody linked to horseradish peroxidase [anti-rabbit (1:2000, Zymed Laboratories, S. San Francisco, CA) or anti-mouse (1:7500, Amersham, Arlington Heights IL)] was added for one hour and incubated with blots at 4°C. Enhanced chemiluminescence was performed using standard reagents (Amersham Laboratories, Arlington Heights, IL).

#### Kinase activity assay protocol

Primary vascular smooth muscle cell preparations were treated with agonists as described above. They were then lysed in a protein kinase lysis buffer [50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM sodium vanadate, 50 mM sodium fluoride, 20 mM beta-glycerophosphate, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, and protease inhibitors (1 mM PMSF, 1 ug/ml pepstatin A1, 1 ug/ml leupeptin, and 1 ug/ml aprotinin)]. Equal protein amounts of each cell lysate (usually 100 ug) were incubated for 3 h at 4°C in the presence of purified GST-c-Jun-(1–79) bound to glutathione-agarose beads (2.8 ug GST-c-Jun/ul beads) as previously described [38]. Beads were washed 2 times with HNTG buffer (HEPES 20 mM pH7.5, NaCl 150 mM, Triton X-100 0.10%, glycerol 10%) followed by an additional washing with HNTG buffer + 1% bovine serum albumin (BSA). Samples were then centrifuged at 13,000 rpm for 2 min and the supernatant was discarded. The pellets were washed 2 times with HNTG buffer and 2 times with JKAW buffer (HEPES 25 mM, glycerol 10%, MgCl<sub>2</sub> 20 mM, Na<sub>3</sub>VO<sub>4</sub> 0.1 mM, beta-glycerophosphate 12.5 mM, EGTA 0.5 mM, NaF 0.5 mM). The pellets were then resuspended in 50.5 µl JKAW reaction buffer containing 20 µCi [<sup>32</sup>P] ATP and 50 µM unlabeled ATP. After 30 min at 37°C, reactions were terminated by the addition of 8 ul SDS loading buffer, samples were boiled and separated by SDS-PAGE. Proteins were transferred to stabilized nitrocellulose membranes and the bands corresponding to phosphorylated c-Jun were counted by a phosphorimager (Storm model 860, Molecular Dynamics, Sunnyvale CA).

#### Data analysis

Cell experiments were performed three or four times with each repetition of the experiment being performed in cells from explants derived from different animals. Thus, experiments are representative of responses of 3 or 4 different animals. Unpaired Student's t tests were used where appropriate in comparing two group responses and a one way ANOVA test was used when comparing responses of three or more groups ( $p < 0.05$  considered statistically significant). Phosphorimager data was captured using Image Quant 5.1 software (Molecular Dynamics, SunnyVale, CA). Quantitation of all band densities was performed using the public domain NIH Image (v.1.62).

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