Serotonin (5-Hydroxytryptamine), a Novel Regulator of Glucose Transport in Rat Skeletal Muscle*

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In this study we show that serotonin (5-hydroxytryptamine (5-HT)) causes a rapid stimulation in glucose uptake by ~50% in both L6 myotubes and isolated rat skeletal muscle. This activation is mediated via the 5-HTTA receptor, which is expressed in L6, rat, and human skeletal muscle. In L6 cells, expression of the 5-HTTA receptor is developmentally regulated based on the finding that receptor abundance increases by over 3-fold during differentiation from myoblasts to myotubes. Stimulation of the 5-HTTA receptor using methylserotonin (m-HT), a selective 5-HTTA agonist, increased muscle glucose uptake in a manner similar to that seen in response to 5-HT. The agonist-mediated stimulation in glucose uptake was attributable to an increase in the plasma membrane content of GLUT1, GLUT3, and GLUT4. The stimulatory effects of 5-HT and m-HT were suppressed in the presence of submicromolar concentrations of ketanserin (a selective 5-HTTA antagonist) providing further evidence that the increase in glucose uptake was specifically mediated via the 5-HTTA receptor. Treatment of L6 cells with insulin resulted in tyrosine phosphorylation of IRS1, increased cellular production of phosphatidylinositol 3,4,5-phosphate and a 41-fold activation of protein kinase B (PKB/Akt) activity. In contrast, m-HT did not modulate IRS1, phosphoinositide 3-kinase, or PKB activity. The present results indicate that rat and human skeletal muscle both express the 5-HTTA receptor and that 5-HT and specific 5-HTTA agonists can rapidly stimulate glucose uptake in skeletal muscle by a mechanism which does not depend upon components that participate in the insulin signaling pathway.

Serotonin, also known as 5-hydroxytryptamine (5-HT), is a neurotransmitter that has been implicated in the regulation of diverse physiological processes, including cellular growth and differentiation (1), neuronal development (2), and regulation of blood glucose concentration (3, 4). This functional diversity stems from the ability of the neurotransmitter to interact with multiple 5-HT receptors (currently classified as 5-HT1 through 5-HT7 with further subtypes within each receptor class) that can trigger and activate distinct intracellular signaling systems (5). For example, with the exception of the 5-HT3 receptor which operates as a ligand-gated ion channel, all known 5-HT receptors belong to the superfamily of G-protein coupled receptors which can, depending on receptor class and subtype, couple negatively or positively to adenyl cyclase (6), modulate ion channel activity (7), promote hydrolysis of phosphatidylinositol bisphosphate through activation of phospholipase C-β (8, 9), and stimulate the mitogen-activated protein kinase pathway (10).

Of major interest to us, however, has been the observation that administration of 5-HT, 5-HT precursors, or specific 5-HT receptor agonists and antagonists can modulate circulating levels of blood glucose in rodents. While some investigators have reported that 5-HT promotes hyperglycemia by a mechanism that may involve increased renal catecholamine release (11), there is a prevailing view that blood glucose is lowered by 5-HT and that this response can be suppressed by 5-HT receptor antagonists (3, 12–14). The precise nature by which 5-HT promotes hypoglycemia remains poorly understood, but it is unlikely to be related to changes in plasma insulin, since circulating levels of the hormone do not increase significantly following intraperitoneal administration of tryptophan, a 5-HT precursor (3, 14). This finding is consistent with the view that biogenic amines normally suppress, rather than enhance, pancreatic insulin release (14–17). An alternative possibility is that 5-HT may stimulate glucose transport by acting directly upon tissues such as skeletal muscle which, by virtue of its total body mass, could make a significant contribution toward a reduction in circulating levels of blood glucose. This proposition is based on recent work showing that rat fetal myoblasts express the 5-HTTA receptor (18) and that activation of this receptor enhances the expression of genes associated with myogenic differentiation and that of the fetal glucose transporter, GLUT3 (18). Increased expression of muscle glucose transporters may play a role in the hypoglycemic action of 5-HT, but changes in transporter expression are generally slow in onset (18), and previous work has shown that 5-HT precursors (e.g. 5-hydroxytryptophan) can reduce blood glucose in fed animals within 1 h of administration (3). In an attempt to assess whether 5-HT can stimulate skeletal muscle glucose transport, we have specifically addressed the following questions. (i) Do L6 muscle cells and skeletal muscle from rats and humans express the 5-HTTA receptor? (ii) Do 5-HT and specific 5-HTTA receptor agonists acutely regulate skeletal muscle glucose transport, and if so (iii) can the effects be explained on the basis of changes in the subcellular distribution of glucose transporters? (iv) Does the response involve cellular components (such as...
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IRIS1, phosphoinositide 3-kinase, and protein kinase B, which have been implicated in the insulin-induced activation of glucose transport in skeletal muscle?

**EXPERIMENTAL PROCEDURES**

**Materials**—L6 rat skeletal muscle cells were provided by Dr. Amira Klip (Toronto). d-Glucose and all other reagent grade chemicals for buffers were obtained from BDH (Poole, Dorset, UK). Sterile trypsin solution, 2-deoxy-d-glucose (2DG), cytochalasin B, and human insulin were obtained from Sigma (Poole, Dorset, UK). 2-(1,2-<sup>3</sup>H)deoxy-d-glucose was purchased from New England Nuclear (Dreieich, Germany).

**Cell Culture and Incubations**—Monolayers of L6 muscle cells were grown to the stage of myotubes as described previously (19) in a minimum essential medium containing 2% fetal calf serum and 1% antimotic/antibiotic solution (final concentration 100 units/ml penicillin, 100 μg/ml streptomycin, 250 mg/ml amphotericin B) at 37 °C in an atmosphere of 5% CO<sub>2</sub>, 95% air. Muscle cells were grown in six-well multidishes for transport measurements and in 15 cm culture dishes for subcellular fractionation studies.

**Rat and Human Skeletal Muscle Procurement**—Human soleus muscle was obtained from patients at Dundee Royal Infirmary while undergoing elective limb amputation surgery for peripheral vascular complications. Upon surgical excision, ~5 g of soleus muscle was rapidly frozen in liquid nitrogen and stored at −80 °C until required for study. For isolation of crude rat muscle membranes we used male Sprague-Dawley rats (200–250 g, Bantin & Kingman, Hull, UK) that were killed by cervical dislocation. Hindlimb skeletal muscle was excised and frozen in liquid nitrogen and stored at −80 °C until required. Rat and human skeletal muscles were homogenized and subjected to differential centrifugation for isolation of crude muscle membranes as described previously (20, 21). For glucose uptake studies in skeletal muscle, smaller rats were used as described below.

**Glucose Transport in L6 Muscle Cells and Isolated Rat Soleus Muscle**—L6 myotubes were exposed to insulin, 5-HT, or to a specific 5-HT<sub>2A</sub> receptor agonist (α-methylhydroxytryptamine (mHT), Tocris, Bristol, UK), antagonist (ketanserin tartrate, Tocris, Bristol, UK) or wortmannin (24) pretreatments and for periods indicated in the figure legends. Following the appropriate treatments 2DG uptake was assayed as described previously (22, 23). For glucose uptake in isolated rat muscle, male Sprague-Dawley rats (50 g, Bantin & Kingman) were killed by cervical dislocation and soleus muscle from both hindlimbs removed. Each isolated soleus muscle was cut into two strips, subsequently weighed (~15 mg), and pinned at the tendon ends onto an inert resin base of a six-well culture dish. Each well contained 3 ml of Krebs Henseleit buffer (KCl, 4.7 mM; CaCl<sub>2</sub>, 2.5 mM; K<sub>2</sub>PO<sub>4</sub>, 1.2 mM; MgSO<sub>4</sub>, 1.2 mM; NaHCO<sub>3</sub>, 25 mM; glucose, 25 mM; bovine serum albumin, 0.1% (w/v), pH 7.4) pregassed with O<sub>2</sub>/CO<sub>2</sub>(95%/5%) at 37 °C. Muscle strips were allowed to recover for 15 min at 37 °C with continuous oxygenation and gentle rotation on a platform shaker and then incubated for a further 30 min in the absence or presence of 1 millimolar insulin (Novo, Denmark) or 50 μM mHT. At the end of this period, muscle strips were rapidly washed three times with glucose-free KH buffer (at 37 °C) and then incubated for 10 min at 37 °C in uptake buffer (KH buffer containing 10 μM 2-[1,2-<sup>3</sup>H]deoxy-d-glucose (1 μCi/ml) and [<sup>14</sup>C]mannitol (0.2 μCi/ml), used as an extracellular reference marker). Following this incubation period muscle washes were processed three times with ice-cold saline and then maintained in cold saline for 40 min before blotting on filter paper and solubilization in 1 ml of 0.5 N NaOH at 60 °C for 45 min. Solubilized muscle extracts were then processed for liquid scintillation counting.

**Subcellular Fractionation of L6 Muscle Cells**—Total L6 cell membranes, plasma, and intracellular membranes were prepared from muscle cells as described previously (24). The protein content of each of the isolated membrane fractions was determined using the Bradford assay (25).

**SDS-PAGE and Immunoblotting**—Isolated membrane fractions from L6 cells and rat and human skeletal muscle were subjected to SDS-PAGE on 10% resolving gels and immunoblotted as previously reported (24). IRS1 immunoprecipitates were run on a 7% resolving gel. Nitrocellulose membranes were probed with antibodies against Thr<sub>308</sub> subunit of the Na,K-ATPase (Mck1, 1:100 generously provided by Dr. K. Seward, Harvard University (26)), GLUT1 (1:500, East Acres Biologicals, Southbridge, MA), GLUT1 (1:500, kindly provided by Dr. S. A. Baldwin, University of Leeds, Leeds, UK), mouse GLUT3 (1:500, kindly provided by Professor G. W. Gould, University of Glasgow), 5-HT<sub>2A</sub> receptor (1:700, PharMingen, San Diego, CA), anti-phospho PKB (1:100, Upstate Biotechnology Inc.), anti-PKB and anti-phospho PKB Ser<sup>737</sup> (New England Biolabs, Herts, UK). Primary antibody detection was performed using either horseradish peroxidase-conjugated anti-rabbit IgG (1:2000, SAPU, Scotland) or anti-mouse (1:2000, Scottish Antibody Production Unit) for 1 h and visualized using enhanced chemiluminescence (Amersham Life Sciences, Buckinghamshire, UK). Autoradiograms were quantitated using a Bio-Rad 670 densitometer.

**IRS1 Immunoprecipitation from L6 Lysates**—L6 cells were extracted on 10-cm plates in the lysis buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM sodium β-glycerophosphate, 50 mM NaF, 5 mM Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 1 μM microcystin-LR, 0.2 mM sodium fluoride, 1 mM benzamidine, 10 μM leupeptin, and 1% (v/v) 2-mercaptoethanol. 500 μg of cell lysate protein was centrifuged at 13,000 × g and IRS1 immunoprecipitated from lysates using a C-terminal IRS1 antibody (Upstate Biotechnology Inc.). Immunocomplexes were captured by incubation with protein A-arose beads and solubilized in Laemmli sample buffer prior to SDS-PAGE and immunoblotting as described above.

**Analysis of Phosphatidylinositol 3,4,5-Phosphate (PIP3) and PKB Activity in L6 Cells**—PIP3 was measured using a sensitive ligand binding displacement assay as reported previously (27). For analysis of PKB activity, L6 cells were extracted on 10-cm plates in lysis buffer (composition as described above). PKBs was immunoprecipitated from cell lysates using a PKB antibody (26) and kinase activity was assayed using a synthetic peptide substrate “crosstide” (GRPRTSS-FAEG) corresponding to the sequence in GSK3 surrounding the Ser residue phosphorylated by MAPKAPK1 and p70<sup>56</sup> as described previously (23, 28). One unit of activity was defined as that amount which catalyzed the phosphorylation of 1 nmol of substrate in 1 min.

**Statistical Analysis**—Statistical analysis was carried out using a two-tailed Student's t test. Data were considered statistically significant at p values ≤0.05.

**RESULTS AND DISCUSSION**

Previous work has shown that administration of 5-hydroxytryptophan and pargyline (a monoamine oxidase inhibitor that prevents breakdown of 5-HT) to rodents induces a profound lowering in blood glucose (3, 14). In these studies the resulting hypoglycemia could not be explained by an increase in insulin secretion, and the effect could be abolished when animals were pretreated with an inhibitor of aromatic amino acid decarboxylation, which prevents the conversion of 5-hydroxytryptophan to 5-HT (14). These observations collectively suggested that 5-HT was the active hypoglycemic agent. We entertained the possibility that one potential mechanism by which 5-HT may promote a lowering in blood glucose was by directly stimulating glucose uptake in skeletal muscle; a notion based on recent work showing that rat fetal myoblasts express the 5-HT<sub>2A</sub> receptor (18). To test this hypothesis we initially carried out SDS-PAGE and immunoblotting to determine whether the 5-HT<sub>2A</sub> receptor was expressed in total membranes prepared from L6 muscle cells and crude membranes from mature rat and human skeletal muscle. Rat brain and human liver microsomes were used as positive and negative controls, respectively. Using a monoclonal antibody that specifically recognizes the 5-HT<sub>2A</sub> receptor subtype, a single immunoreactive band of ~55 kDa was observed in all three muscle samples, which migrated alongside that seen in the rat brain sample (Fig. 1A). Quantitative analyses of immunoblot data from three separate experiments revealed that 5-HT<sub>2A</sub> receptor expression was higher by 3.2 ± 0.6-fold in fully differentiated L6 myotubes (day 8) compared with that in L6 myoblasts (day 3). The receptor difference in 5-HT<sub>2A</sub> receptor expression between myoblasts and myotubes could not be attributed to the aberrant loading of membrane protein on SDS gels, as no differences were observed in the abundance of the α1-Na,K-ATPase subunit in the same L6 membranes (Fig. 1B).

Having established that L6 cells and rat skeletal muscle express the 5-HT<sub>2A</sub> receptor, we investigated whether 5-HT...
acute regulated 2DG uptake in L6 myotubes and isolated rat soleus muscle. When muscle cells were exposed to increasing concentrations of 5-HT (between 1 nM and 100 μM), there was a dose-dependent increase in 2DG uptake (Fig. 2). Measurements of circulating 5-HT levels indicate that whole blood 5-HT is approximately 1 μg/ml (equating to ~5 μM), whereas the platelet-free plasma circulating concentration is ~10 nM (29). Thus, the observation that skeletal muscle expresses a 5-HT2A receptor and that glucose uptake can be stimulated within the physiological range of blood 5-HT implies that signaling via this receptor may represent a novel mechanism regulating muscle glucose uptake in vivo. In order to gain further insights into the mechanism by which 5-HT stimulates glucose uptake, all subsequent experiments were performed using maximally effective concentrations (50 μM) of either 5-HT or the 5-HT2A agonist methylserotonin (m-HT). Both agents increased 2DG uptake by ~50% (Fig. 3A), and identical results were obtained when using 50 μM quipazine (another 5-HT2 agonist, data not shown). The 5-HT- and m-HT-induced increase in 2DG uptake was lower than that seen in response to insulin (Fig. 3A), but neither 5-HT nor m-HT could elicit a significant additive stimulation when simultaneously presented to muscle cells with insulin (Fig. 3A). Very similar observations were made in isolated rat soleus strips in which insulin and m-HT caused a 2.7-fold and 50% increase in 2DG uptake, respectively (Fig. 3B). As with L6 cells, we found that exposing soleus strips simultaneously to insulin and m-HT did not result in any additive stimulation in glucose uptake (Fig. 3B). The finding that m-HT increases glucose uptake in both cultured muscle cells and isolated rat muscle in a manner similar to that seen in response to 5-HT is therefore consistent with the suggestion that the effects of the latter are also mediated via the 5-HT2A receptor. This view is further strengthened by the observation that the stimulatory effects of m-HT (and 5-HT, data not shown) were suppressed in the presence of submicromolar concentrations of the 5-HT2A antagonist, ketanserin (Fig. 3C). The observed antagonism takes place within the expected Kd range reported for the antagonist in the literature (i.e. <10 nM (6)) and is, moreover, in line with findings from other groups reporting that low nanomolar concentrations of ketanserin block 5-HT action via the 5-HT2A receptor subtype (30).

In order to determine whether the acute stimulation in 2DG uptake observed in the presence of m-HT was due to changes in the subcellular distribution of glucose transporters, we immunoblotted plasma (PM) and intracellular (IM) membrane fractions from L6 myotubes with antibodies against GLUT1, GLUT3, and GLUT4. Fig. 4 shows representative immunoblots from three separate experiments showing that m-HT induces an increase in the plasma membrane abundance of all three transporters by between 40 and 60%. The increase in surface GLUT content takes place as a result of their recruitment from the intracellular compartment, which showed a corresponding loss in each of the three GLUT proteins. The increase in plasma membrane GLUT content following exposure of L6 cells to m-HT was very rapid and highly reminiscent of that seen in response to treatment of muscle cells with insulin (23, 31). Nevertheless, the observed translocation of all three transporters seen in response to m-HT was less than that evoked by insulin (Fig. 4). Given that m-HT and insulin do not cause any additive increase in glucose uptake, it is conceivable that both stimuli signal via their respective membrane receptors onto the same intracellular pool of glucose transporters by either distinct or convergent signaling pathways.

To gain some insight into whether components of the insulin-signaling pathway may participate in 5-HT2A receptor signaling we first investigated the effects of the phosphoinositide 3-kinase (PI3K) inhibitor, wortmannin, on the agonist induced stimulation in glucose uptake. In line with previous work from our group, Fig. 5A shows that wortmannin induced a 50% reduction in basal glucose uptake and completely blocked insulin-stimulated glucose transport in L6 myotubes (22, 23, 31). However, exposure of muscle cells to 50 μM m-HT following pretreatment with 100 nM wortmannin resulted in a modest, but significant, increase in 2DG uptake by ~25% (similar results were also obtained when using the structurally unrelated PI3K inhibitor LY294002, data not shown). Since wortmannin is known to suppress the externalization of glucose transporters that recycle between the cell surface and endosomal compartment (32, 33), we believe that this is likely to contribute to the reduced activation in glucose uptake by m-HT. The finding that m-HT is still capable of causing a significant stimulation in glucose uptake in the presence of wortmannin, whereas insulin fails to elicit any increase, is consistent with the idea that PI3K participates in insulin signaling but not in 5-HT2A mediated signaling. In an attempt to resolve this issue further, we investigated whether 5-HT2A receptor stimulation modu-
lated the phosphorylation status of IRS1 and the activities of PI3K and protein kinase B.

Fig. 5B shows an anti-phosphotyrosine blot of IRS1 immunoprecipitates prepared from control and insulin- and m-HT-treated muscle cells. Insulin, but not m-HT, induced tyrosine phosphorylation of IRS1, suggesting that the latter was not a downstream target for the 5-HT2A receptor. Moreover, analyses of IRS1 precipitates with an antibody against the regulatory 85-kDa PI3K subunit revealed that p85 was only associated with IRS1 in insulin-treated cells. However, since 5-HT2A belongs to the family of G-protein-coupled receptors and heterotrimeric G-protein-regulated forms of PI3K have been identified (34), it is plausible that 5-HT may stimulate PI3K independently of IRS1. It is also noteworthy that G-protein-coupled receptors have been shown to activate PKB in human phagocytes and COS-7 cells ectopically expressing muscarinic acetylcholine receptors that couple to G_{i} and G_{o} (35, 36). Since PKB lies downstream of PI3K, and we and others have implicated it in the insulin-mediated translocation of GLUT4 (23, 37, 38), activation of PI3K by a G-protein-coupled receptor may represent one potential mechanism of stimulating PKB and hence glucose uptake in muscle. However, the data shown in

![Fig. 3. Effects of serotonin (5-HT), methylserotonin (m-HT), and insulin (Ins) on 2DG uptake in L6 muscle cells and incubated rat soleus muscle strips. A, L6 myotubes were incubated with insulin (100 nM) for 30 min, with 5-HT (50 μM) or m-HT (50 μM) for 10 min, or with insulin and m-HT (Ins, 30 min/m-HT, added during the last 10 min of insulin incubation). 2-[1,2-3H]deoxy-D-Glucose was assayed as described under “Experimental Procedures.” Values represent means ± S.E. from at least three separate experiments. B, rat soleus strips were incubated for 30 min with m-HT (50 μM), insulin (1 milliunit/ml), or with insulin and m-HT (Ins/m-HT). Results are means ± S.E. of seven different experiments. C, L6 myotubes were incubated with m-HT (50 μM) for 10 min in the absence or the presence of various concentrations (1 nM to 1 μM) of ketanserin tartarate (KT). Results are the means ± S.E. of three different experiments. The asterisk indicates a statistically significant change (p < 0.05) compared with the respective basal value, the annotation a indicates a significant difference compared with the 5-HT or m-HT values (p < 0.05).]

![Fig. 4. Effect of methylserotonin (m-HT) and insulin on the abundance of GLUT1, GLUT3, and GLUT4 in subcellular membrane fractions from L6 myotubes. L6 myotubes were incubated with 50 μM m-HT for 10 min or 100 nM insulin for 30 min prior to cell harvesting and subcellular fractionation as described under “Experimental Procedures.” 20 μg of plasma membrane (PM) or internal membrane (IM) protein was applied to polyacrylamide gels and analyzed by SDS-PAGE. Immunoblotting was performed using isoform-specific antibodies to the three glucose transporters as described under “Experimental Procedures.”]

![A L6 myotubes B Soleus C L6 myotubes PM IM PM IM a a a a a 5-HT m-HT Ins Ins/ m-HT 5-HT m-HT Ins Ins/ m-HT 5-HT m-HT KT (μM) - + + + + 0.001 0.01 0.1 1 PM PM IM IM - α1 Na,K-ATPase - GLUT1 - GLUT4 - GLUT3](image)
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In summary, our results show that the 5-HT$_{2A}$ receptor is expressed in rat and human skeletal muscle. Stimulation of this receptor with 5-HT or a specific 5-HT$_{2A}$ agonist causes a rapid stimulation in glucose transport that occurs as a result of the increased recruitment of glucose transporters from an intracellular pool to the cell surface. The post-receptor signaling events involved in eliciting this stimulation currently remain unknown, but they do not involve signaling molecules that participate in early events of insulin signaling (i.e. IRS1, PI3K, or PKB). The finding that the 5-HT$_{2A}$ receptor can modulate glucose transport is likely to be physiologically significant given that plasma 5-HT levels are known to increase during muscle exercise (40) and fall during diabetes (29); conditions during which utilization of glucose is significantly modulated in skeletal muscle. Understanding how the 5-HT$_{2A}$ receptor signals an increase in muscle glucose uptake may prove potentially valuable in developing new strategies aimed at improving glucose utilization in skeletal muscle during circumstances when this tissue may be profoundly resistant to insulin action.

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Table I

Table I: Effects of insulin, serotonin (5-HT), and methylseratonin (m-HT) on cellular PIP3 levels and protein kinase B activity in L6 muscle cells

| Condition       | PI3K activity | PKB activity |
|-----------------|---------------|--------------|
|                 | pmol/mg protein | milliunits/mg protein |
| Basal           | 2.4 ± 0.04     | 0.33 ± 0.05  |
| Insulin         | 5.4 ± 0.4*     | 13.7 ± 0.47  |
| 5-HT            | 2.3 ± 0.8      | 0.26 ± 0.06  |
| m-HT            | 2.6 ± 0.5      | 0.39 ± 0.14  |
| Wortmannin/insulin | 2.5 ± 0.04    | 0.23 ± 0.17  |

* Statistically significant change (p < 0.05) compared with the respective basal value.

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