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SHORT COMMUNICATIONS

On the role of 5-hydroxytryptamine in the peripheral action of fenfluramine: studies with the isolated rat soleus muscle

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The anorectic effects of fenfluramine are mediated through interactions with the brain monoamine system which regulates energy balance [1]. Of particular importance is the serotoninergic system in which fenfluramine selectively releases 5-hydroxytryptamine(5-HT*) and inhibits its reuptake [2–4]. Several studies have convincingly demonstrated that fenfluramine improves glucose metabolism independent of weight changes, and that this action of the drug is mediated by augmented peripheral glucose utilization [5, 6]. It has been claimed that fenfluramine acts on the periphery by increasing muscle glucose uptake, usually in the presence of insulin [7–11]. *In vivo* studies in man [12, 13], dogs [14], rats [15] and mice [16] produced conflicting results on the possible effects of fenfluramine on skeletal muscle glucose metabolism and on the role of insulin in this interaction. Possible interactions between fenfluramine and 5-HT regarding modulation of muscle glucose uptake were first studied by Kirby and Turner [17] and Bichi *et al.* [18]. Their results suggested that the effect of fenfluramine was inhibited by the selective 5-HT antagonist methysergide, while 5-HT increased further the synergism between fenfluramine and insulin on glucose uptake; 5-HT showed also a stimulatory effect of its own. It was therefore concluded that fenfluramine might act as a partial agonist of 5-HT receptors in skeletal muscle, or that it might release 5-HT from local pools [19].

We have recently studied in detail the effects of *d*- and *l*-fenfluramine on glucose uptake into isolated rat soleus muscles and skeletal muscle cells in culture; in none of the experimental conditions used could we demonstrate a drug effect on either basal or insulin-stimulated hexose transport [20]. In the present work we extend these studies to include the possible involvement of 5-HT in mediating the effects of fenfluramine in the presence or absence of insulin.

Material and methods

Chemicals. The 2[1-2-^{3}H]deoxy-glucose (dGlc), 30.2 Ci/mmol, was purchased from Du Pont-New England Nuclear (Boston, MA). [U-^{14}C]Sucrose, 556 mCi/mmol, was from the Radiochemical Centre (Amersham, U.K.). Crystalline porcine insulin (26.8 units/mg) was a gift from Eli Lilly & Co. (Indianapolis, IN). Stock solutions (1 mg/mL) in 10^{-3}N HCl, pH 3.0, were kept at 4°; for further use, the solution was diluted in buffer containing 0.25% (w/v) bovine serum albumin (BSA). Hepes, BSA (fraction V), dGlc, cytochalasin B and 5-HT were purchased from the Sigma Chemical Co. (St Louis, MO). *d*(+)Fenfluramine and *l*(−)fenfluramine were a gift from Technologie Servier (Orleans, France). Dulbecco's modified Eagle's medium (DMEM) was from Gibco Laboratories (Grand Island, NY). All other chemicals were reagent grade.

The purity of the *d*- and *l*-enantiomers of fenfluramine was checked as previously described [20].

Animals. Young male albino rats (60–80 g) of the Sabra strain (Hebrew University, Jerusalem, Israel) received standard laboratory chow and water *ad lib.*

Measurement of dGlc uptake in isolated soleus muscles. Non-fasted rats were killed by cervical dislocation, the soleus muscles quickly removed and incubated in 20-mL glass vials sealed with a rubber stopper, continuously gassed with O_2/CO_2 (95:5, v/v) in a metabolic shaker (40 cycles/min) at 37°. The [^{3}H]dGlc uptake assay in the isolated soleus muscles has been previously described [20–22].

We [21, 22] and others [23–25] showed that an intracellular pool of free glucose in soleus muscles incubated at various glucose concentrations is unlikely to dilute the free intracellular [^{3}H]dGlc in the uptake assay. We have previously established that the standard washing procedure effectively eliminated free glucose from the extracellular space [20].

Glucose determination. Glucose concentration in plasma samples from rats and in aliquots from incubation media was determined by the glucose oxidase method using a glucose analyser.

Statistical analysis. Results are expressed as means ± SE. The non-parametric Kruskal-Wallis test [26] was used for mutliple comparisons.

Results and discussion

Ex vivo *hexose transport in soleus muscles isolated from fenfluramine-treated rats.* The *l*- or *d*-fenfluramine enantiomers were injected i.p. into the rats. After 180 min the animals were killed, the soleus muscles removed, washed and [^{3}H]dGlc uptake measured. Blood glucose at death was 6.5–8.5 mM and plasma insulin 4–14 μUnits/mL, with no significant difference between the control and treated animals. Table 1 shows that the *in vitro* basal dGlc uptake was not influenced by *in vivo* treatment with *l*- or *d*-fenfluramine (2.0 and 20.0 mg/kg) and that the stimulation by insulin was similar in muscles from control and fenfluramine-

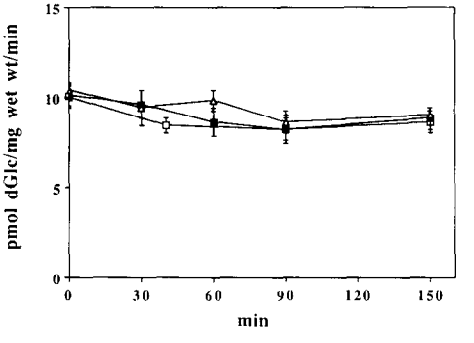

Fig. 1. Lack of effect of 5-HT on basal dGlc uptake in isolated rat soleus muscles. Soleus muscles were incubated in DMEM containing 9.0 mM glucose without (□) or with 10^{-8} (■) or 10^{-6} M (△) 5-HT at 37° under O_2:CO_2 (95:5). At the indicated times the muscles were taken for the standard [^{3}H]dGlc uptake assay. Points represent mean ± SE (N = 5).

* Abbreviations used: BSA, bovine serum albumin; dGlc, 2-deoxy-D-glucose; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid; 5-HT, 5-hydroxytryptamine; KRB, Krebs-Ringer bicarbonate buffer.

Table 1. dGlc uptake in soleus muscles isolated from fenfluramine-treated rats

Treatment	dGlc uptake (pmol dGlc/mg wet weight/min) Basal	+1.7 nM insulin	% Increase
Saline	9.7 ± 0.5	17.6 ± 0.7	+81
d-Fenfluramine			
2.0 μg/kg	9.3 ± 0.7	17.4 ± 1.1	+87
20.0 μg/kg	9.9 ± 0.8	18.5 ± 0.9	+86
l-Fenfluramine			
2.0 μg/kg	9.3 ± 0.6	19.2 ± 1.5	+106
20.0 μg/kg	8.8 ± 0.6	18.6 ± 1.0	+111

Rats weighing 60–80 g were injected i.p. with *d*- or *l*-fenfluramine (dissolved in normal saline). After 180 min the rats were killed, the soleus muscles removed, and incubated for 20 min (in a metabolic shaker at 37° under O_2:CO_2 atmosphere) in DMEM supplemented with 0.25% (w/v) BSA with or without 1.7 nM (0.25 mUnits/mL) insulin. The muscles were then taken for the standard [^{3}H]dGlc uptake assay. Basal dGlc uptake rate in freshly isolated soleus muscles was 8.8 ± 2.5 pmol/mg wet weight/min (N = 25). Differences between the control groups and the fenfluramine treatment groups were not significant (Kruskal–Wallis test). Values are means ± SE (N = 6–12).

treated rats. The plasma concentration of fenfluramine was found to be at near therapeutic level 3 hr after injecting 2 mg/kg fenfluramine [27]. In additional experiments, pretreatment with 0.2 and 10.0 mg/kg *l*- or *d*-fenfluramine had no effect on the basal or insulin-stimulated dGlc uptake (data not shown). Thus, a single dose of fenfluramine (whether at pharmacological or suprapharmacological level) was not a potent modulator of the basal or of the insulin-stimulated hexose transport in muscle.

In vitro *hexose transport rates in isolated soleus muscles treated with 5-HT, fenfluramine and insulin.* It has been postulated that fenfluramine acts on muscle via the serotoninergic system [17–19]. This may seem reasonable since fenfluramine augments the release and inhibits the reuptake of 5-HT both in the central nervous system and in thrombocytes [1–4, 28, 29]. Soleus muscles were incubated in the absence or presence of 10^{-8} M and 10^{-6} M 5-HT, and dGlc uptake rates measured at the indicated times (Fig. 1). At both concentrations 5-HT had no significant effect on the basal dGlc uptake. In this experiment the basal transport capacity remained constant throughout. Occasionally, we observed a time-dependent decrease in the basal hexose transport rate due to progressive deterioration of the isolated muscles. 5-HT did not reverse such decline of the uptake (data not shown).

Since Bichi *et al.* [18] tested the effect of 5-HT at higher concentrations (5×10^{-6}–5×10^{-4} M), similar experiments were performed. Table 2 shows that neither 5-HT at these high concentrations, nor *l*- or *d*-fenfluramine increased the rate of dGlc uptake in isolated soleus muscles whether insulin was present or not, with the exception of 10^{-4} M 5-HT. At this extremely high concentration, a modest but statistically significant stimulation of dGlc uptake above the insulin effect was noted in the presence of *l*-fenfluramine.

To exclude the possibility of deterioration of the isolated muscles during 90 min of incubation, short-term (20 min) experiments were performed. Again, no effect of 10^{-4} M 5HT alone or together with 3.7 μM *l*- or *d*-fenfluramine was observed. (The respective dGlc uptake rates were 8.8 ± 0.5, 8.6 ± 0.4 and 9.3 ± 0.5 in comparison with 9.1 ± 0.3 pmol dGlc/mg wet weight/min in the control group, N = 5.)

Since Kirby and Turner [17] and Bichi *et al.* [18] utilized rat hemidiaphragms we also studied the effect of fenfluramine on the hexose transport in this tissue. We could not demonstrate stimulatory effects of either *d*- or *l*-fenfluramine alone or together with insulin on hexose uptake (data not shown).

One conclusion of our study is that the *in vivo* peripheral effects of fenfluramine are neither the consequence of a direct drug effect on muscle cells, nor the result of interaction with serotoninergic systems, since 5-HT seems to be inert at the muscle glucose uptake level. In keeping with this view are the studies of Storlien *et al.* [30], Geelen [31, 32] and Mounie *et al.* [33] which show that the interaction of fenfluramine with the liver is of prime importance for the metabolic effect of the drug. Storlien *et al.* [30] have also suggested that *d*-fenfluramine reduces the hypothalamic noradrenergic tone which in turn reduces the

Table 2. Lack of effect of fenfluramine and 5-HT on insulin-stimulated dGlc uptake

Treatment	dGlc uptake (pmol/mg wet weight/min) + Saline	+ 1.7 nM insulin
Saline	6.2 ± 0.2	9.7 ± 0.7
10^{-6}M 5-HT	6.0 ± 0.3	8.8 ± 0.7
+3.7 μM *l*-fenfluramine	6.2 ± 0.3	9.1 ± 0.7
+3.7 μM *d*-fenfluramine	5.9 ± 0.2	8.2 ± 0.3
10^{-4}M 5-HT	5.5 ± 0.1	10.3 ± 1.0
+3.7 μM *l*-fenfluramine	5.9 ± 0.2	11.7 ± 0.3*
+3.7 μM *d*-fenfluramine	5.8 ± 0.2	8.7 ± 0.5

Soleus muscles were incubated in DMEM supplemented with 10 mM glucose, and the appropriate additions, for 90 min in a metabolic shaker at 37° under O_2:CO_2. At the end of the incubation the muscles were taken for the standard [^{3}H]dGlc uptake assay. Differences between the control groups (saline or insulin treated muscles) and treatment groups were not significant except for * $P < 0.01$ when compared with its respective control (Kruskal–Wallis test). Values are means ± SE (N = 5).

neural drive to hepatic glucose output and improves hepatic response to insulin. Also these authors failed to observe a significant improvement of glucose metabolism by fenfluramine in most skeletal muscles. Fenfluramine reduces the circulating level of triglycerides [30, 34, 35] and increases glucocorticoid levels via the hypothalamic–pituitary–adrenal axis [30]; these factors have pronounced effects on glucose metabolism [35–38] and could therefore mediate part of the peripheral effects of fenfluramine.

In summary, we found no direct effect of fenfluramine on skeletal muscle glucose uptake, whether the drug was given alone or in combination with insulin and/or 5-HT. Hepatic and CNS effects of the drug seem to be of major importance; both may lead to a secondary effect on peripheral glucose metabolism by altering the metabolic and hormonal milieu of tissues.

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Staurosporine inhibition of intracellular free Ca^{2+} transients in mitogen-stimulated Swiss 3T3 fibroblasts

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The microbial alkaloid staurosporine is a potent inhibitor of protein kinase C [1] and a weaker inhibitor of tyrosine protein kinase activity [2]. The biological effects of staurosporine are generally ascribed to its ability to inhibit protein kinase C [3–5]. Staurosporine is a potent inhibitor of fibroblast DNA synthesis and proliferation [6], although a number of studies have shown that protein kinase C is not essential for fibroblast proliferation [6–9]. This has led to the suggestion that staurosporine has effects in addition to inhibition of protein kinase C that account for its effect on cell proliferation [6]. A common event that occurs early in the action of a number of growth factors and mitogens is a transient increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$)* [10, 11]. This increase in $[Ca^{2+}]_i$ has been implicated in mediating the effects of these agents on cell proliferation [11–13]. We have examined the effects of staurosporine on the increase in $[Ca^{2+}]_i$ caused by growth factors and mitogens and on the release of Ca^{2+} from intracellular stores using Swiss 3T3 fibroblasts.

Materials and methods

Staurosporine and *myo*-inositol 1,4,5-trisphosphate [IP_3(1,4,5)] were purchased from Calbiochem (San Diego, CA). Arachidonic acid, vasopressin-^{8}Arg, bradykinin and phorbol 12-myristate 13-acetate (PMA) were purchased from the Sigma Chemical Co. (St Louis, MO). $^{45}CaCl_2$ (25 mCi/mg) was purchased from the Amersham Corp. (Arlington Heights, IL) and [^{32}P]orthophosphoric acid (9 Ci/μmol) from Dupont NEN (Boston, MA). Platelet-derived growth factor (PDGF) as the B chain homodimer was purchased from Bachem Inc. (Torrance, CA). Aequorin was provided by Dr John Blinks, Mayo Clinic. Swiss 3T3 fibroblasts were provided by Dr H. R. Herschmann, University of California, Los Angeles, CA. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and harvested at each passage with 0.5% trypsin and 0.5 mM EGTA before becoming confluent. Studies were conducted on cells between passages 37 and 58. Measurement of $[Ca^{2+}]_i$ employed Swiss 3T3 fibroblasts loaded with the Ca^{2+}-sensitive photoprotein aequorin by a low Ca^{2+} centrifugation technique as previously described [11]. The cells were plated in DMEM containing 10% fetal calf serum and allowed to attach to the surface of a culture dish for 18 hr, then they were exposed to DMEM without fetal calf serum for 2 hr. Growth factors and mitogens dissolved in 0.2 mL DMEM without fetal calf serum were added to the culture dish. Light emitted by the aequorin-loaded cells was measured as previously described [11] and converted to an estimate of $[Ca^{2+}]_i$ using the calibration method of Allen and Blinks [14]. Cells were exposed to staurosporine for 3 hr before and during the exposure to the growth factors and mitogens.

The ATP-dependent uptake and agonist-induced release of $^{45}Ca^{2+}$ by the endoplasmic reticulum of saponin-permeabilized Swiss 3T3 fibroblasts was measured by the method of Ghosh *et al.* [15]. Preliminary studies showed that $^{45}Ca^{2+}$ uptake by the permeabilized Swiss 3T3 cells had reached a plateau by 6 min. IP_3(1,4,5), 10 μM, and arachidonic acid, 50 μM, were added to the preparation at 6.25 min, and $^{45}Ca^{2+}$ remaining in the cells was measured at 7 min. Staurosporine was added to the preparation at 0 min.

For measurement of ligand-dependent PDGF receptor phosphorylation, Swiss mouse 3T3 cells were grown to confluency, in 35 mm tissue culture dishes, in DMEM containing 10% fetal calf serum. Forty-eight hours after the last medium change, the confluent cultures were washed in 37° serum-free labeling medium (phosphate-free DMEM containing 2% bovine serum albumin, 2 mM L-glutamine, buffered to pH 7.2 with 10 mM HEPES). The washed cultures were then incubated for 4 hr in serum-free labeling medium (4 mL/dish) containing 2 mCi [^{32}P]orthophosphate

* Abbreviations: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; PMA, phorbol 12-myristate 13-acetate; IP_3(1,4,5), *myo*-inositol 1,4,5-trisphosphate; PDGF, platelet-derived growth factor; DMEM, Dulbecco's modified Eagle's medium; EGTA, ethyleneglycolbis(aminoethylether)tetra-acetate; and HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.