



## PAPER

# Sibutramine metabolites increase glucose transport by cultured rat muscle cells

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**BACKGROUND:** The anti-obesity agent sibutramine, a serotonin and noradrenaline reuptake inhibitor (SNRI), has been shown to reduce insulin resistance and improve glycaemic control in obese-diabetic *ob/ob* mice and overweight type 2 diabetic patients.

**OBJECTIVE:** To investigate whether sibutramine or its metabolites act directly on muscle cells to improve glucose uptake and insulin action.

**DESIGN:** Uptake of the non-metabolized glucose analogue 2-deoxyglucose was measured in cultured L6 rat muscle cells after incubation with sibutramine, its two pharmacologically active metabolites and related agents.

**RESULTS:** Sibutramine itself ( $10^{-8}$ – $10^{-6}$  M) did not significantly affect 2-deoxyglucose uptake during incubations up to 72 h. The primary amine metabolite M2 ( $10^{-7}$  and  $10^{-6}$  M) increased basal and insulin-stimulated 2-deoxyglucose uptake (by 12% and 34%) after 24 h incubation. These effects of M2 were lost by 72 h incubation. However, the secondary amine metabolite M1 ( $10^{-6}$  M) increased basal and insulin-stimulated 2-deoxyglucose uptake (by 50%) after 72 h incubation, although M1 was ineffective after 24 h. M2 stimulated 2-deoxyglucose uptake in the presence of LY-294,002 (an inhibitor of phosphatidylinositol 3-kinase) but the effect of M2 was inhibited by cytochalasin B, which acutely blocks glucose transporters. Incubations with serotonergic, noradrenergic and dopaminergic agents, or agents known to stimulate release or inhibit reuptake of these substances in nervous tissues indicated that the sibutramine metabolites were not affecting 2-deoxyglucose uptake via mechanisms associated with their SNRI properties.

**CONCLUSIONS:** Sibutramine metabolites can improve insulin-sensitive 2-deoxyglucose uptake by cultured muscle cells independently of SNRI effects.

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**Keywords:** sibutramine; glucose transport; cultured L6 muscle cells; insulin action

## Introduction

The anti-obesity agent sibutramine is a serotonin and noradrenaline reuptake inhibitor (SNRI) which induces satiety, stimulates thermogenesis and reduces weight gain in rodents.<sup>1–6</sup> In clinical studies sibutramine promoted weight loss in obesity,<sup>6–8</sup> and preliminary evidence indicates that sibutramine improves glycaemic control in overweight type 2 diabetic patients.<sup>9,10</sup>

Sibutramine reduced insulin resistance and weight gain in obese-diabetic *ob/ob* mice.<sup>5</sup> An improved insulin action could be due to the weight loss itself or to the potential for sibutramine or its active metabolites (M1 and M2) to

improve insulin action, at least in part, independently of the weight-reducing effect. To investigate this possibility, sibutramine, its metabolites and various structurally and pharmacologically related agents were exposed to cultured L6 muscle cells. Glucose uptake by the cells was assessed in the absence and presence of added insulin using the non-metabolized analogue 2-deoxyglucose.

## Materials and methods

### Materials

Cell culture reagents were from Gibco (Paisley, Scotland), plastic were from Sarstedt (Leicester) and Hi-safe II scintillant was from Fisons (Loughborough). 2-Deoxy-D-[<sup>3</sup>H]-glucose (15.0 Ci/mmol) was from Amersham International (Amersham); sibutramine hydrochloride, its metabolites and nordexfenfluramine were from Knoll Pharmaceuticals (Nottingham), and nisoxetine hydrochlor-

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ide was from Research Biochemicals International (St Albans). Other chemicals were of analytical grade from Sigma (Poole) and BDH (Poole).

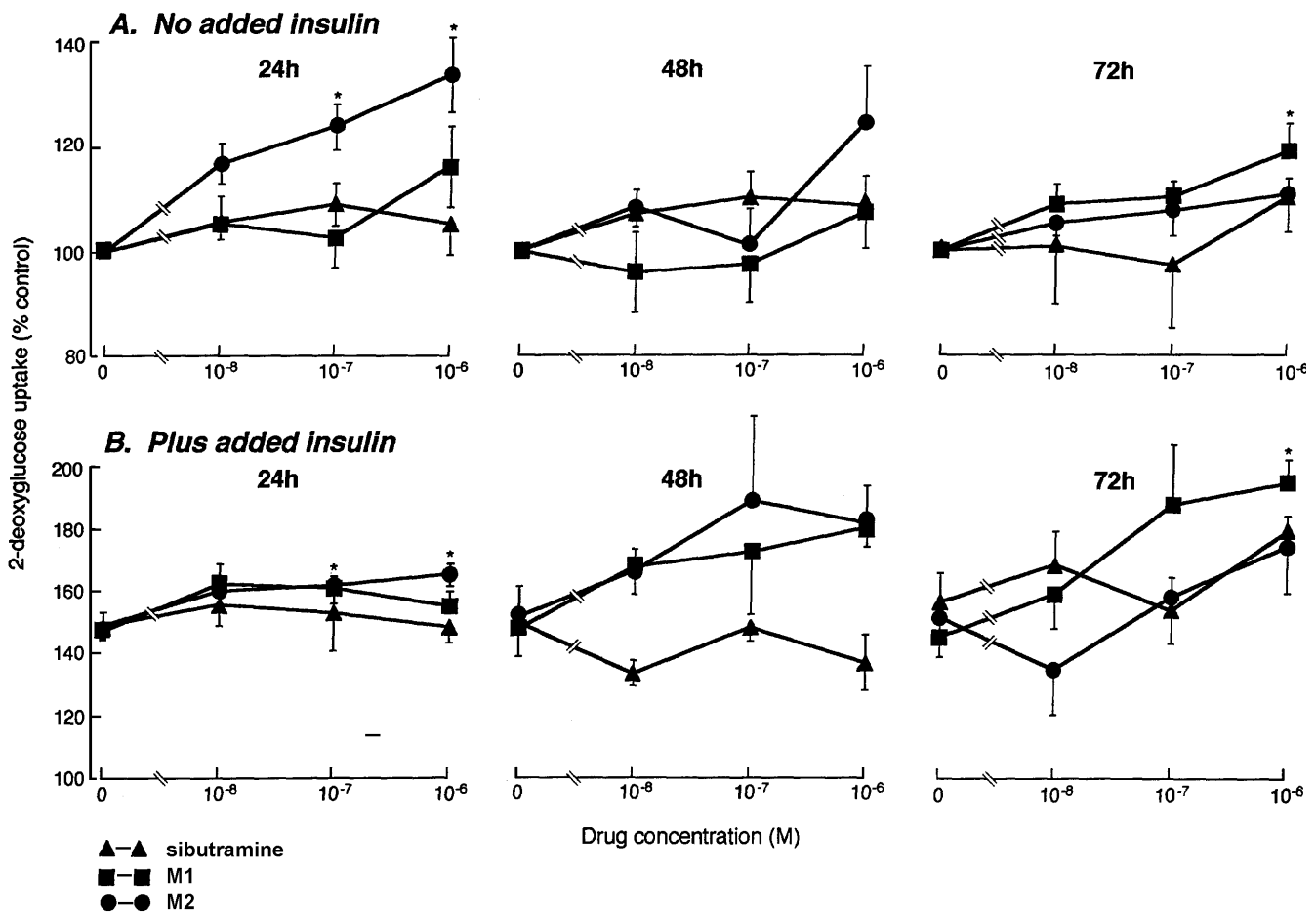
### Cell culture

Monolayers of rat L6 muscle cells (European Culture Collection, Porton Down) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 1 mM glutamate, 1 mM pyruvate and 25 mM glucose.<sup>11</sup> The medium was supplemented with 5% foetal calf serum (FCS), 100 U/ml penicillin G, 100 µg/ml streptomycin and 25 µg/ml amphotericin B. Cells were maintained at 37°C with humidified 95% air and 5% CO<sub>2</sub>. Experiments were undertaken in 24-well plates seeded from preconfluent flasks with 5 × 10<sup>5</sup> cells in 1 ml. The cells (passages 8–18) were grown to confluence and the medium was changed to DMEM containing 0.5% FCS and 2.5 mM glucose for 24 h to induce differentiation

and fusion of myoblasts into myotubes. Myotubes were then incubated with test compounds for 24–72 h.

### Deoxyglucose uptake

Cell monolayers were washed with glucose-free Krebs Ringer bicarbonate (KRB) buffer at 22°C, then incubated in 1 ml of this buffer supplemented with 0.1 mM 2-deoxy-D-glucose and 2-deoxy-D-[<sup>3</sup>H]-glucose (0.2 µCi/ml) for 10 min at 22°C. Buffer was then aspirated and cells were washed twice with ice-cold KRB buffer, lysed with 0.5 ml 1 M NaOH, and radioactivity was counted in 5 ml Hi-safe II scintillant using a Packard 1900TR liquid scintillation counter. Experiments were undertaken in multiples of six wells on at least two occasions. Six control wells and six wells containing a test concentration of insulin were included in each 24-well plate. Uptake of 2-deoxyglucose was expressed as the percentage change compared with control (100%). Control (basal) uptake of 2-deoxyglucose was about 9 pmol/10<sup>5</sup>



**Figure 1** Effect of sibutramine and its metabolites M1 and M2 on 2-deoxyglucose uptake by L6 muscle cells after incubation for 24, 48 and 72 h with or without added insulin (10<sup>-8</sup> M) for the last 24 h of the incubations. Values are mean ± s.e.m., n = 12. \*P < 0.05 vs control (no drug).

cells/min in the present studies. Data are presented as mean ± sem and compared using Student's unpaired *t*-test with Bonferroni's correction. Probability values of *P* < 0.05 were considered to be significant.

## Results

### Sibutramine and its metabolites

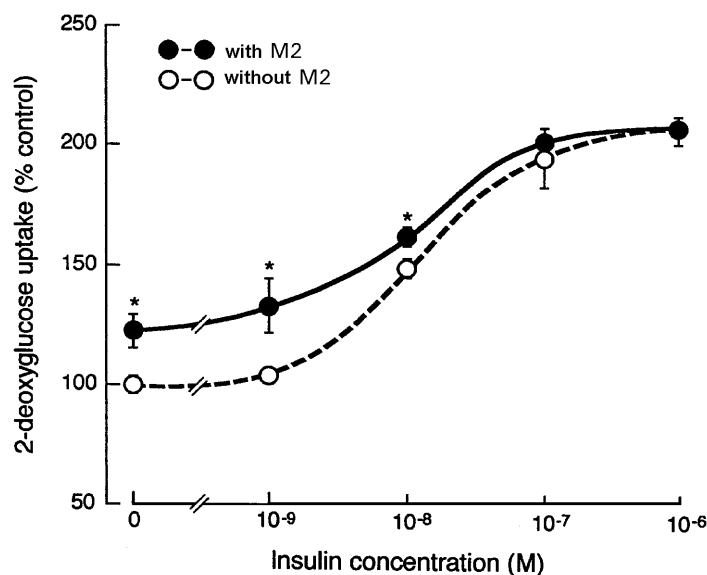
L6 muscle cells were incubated with 10<sup>-8</sup>–10<sup>-6</sup> M sibutramine or each of its metabolites (M1 and M2) for 24, 48 and 72 h prior to determination of 2-deoxyglucose uptake. In the absence of added insulin, incubation with sibutramine did not significantly alter 2-deoxyglucose uptake (Figure 1A). However M2 (10<sup>-7</sup> and 10<sup>-6</sup> M) produced a small concentration-related increase in 2-deoxyglucose uptake (by 23% and 34% respectively; *P* < 0.05) after 24 h, but did not produce a significant effect after longer incubation times. In contrast M1 (10<sup>-6</sup> M) significantly increased 2-deoxyglucose uptake (by 18%, *P* < 0.05) only after 72 h incubation.

Addition of insulin (10<sup>-8</sup> M for 24 h) to the control incubation medium increased 2-deoxyglucose uptake by about 40% (*P* < 0.01; Figure 1B). Addition of insulin (10<sup>-8</sup> M) for the last 24 h of incubations with sibutramine (24–72 h) had no further effect on 2-deoxyglucose uptake. However, in the presence of added insulin, M2 (10<sup>-7</sup> and 10<sup>-6</sup> M) produced a small further increase (by 12% and 16%, respectively; *P* < 0.05) in 2-deoxyglucose uptake after 24 h, but not after longer incubation times. M1 (10<sup>-6</sup> M) increased insulin-stimulated 2-deoxyglucose uptake by a further 50% (*P* < 0.05 compared with insulin only) after incubation for 72 h.

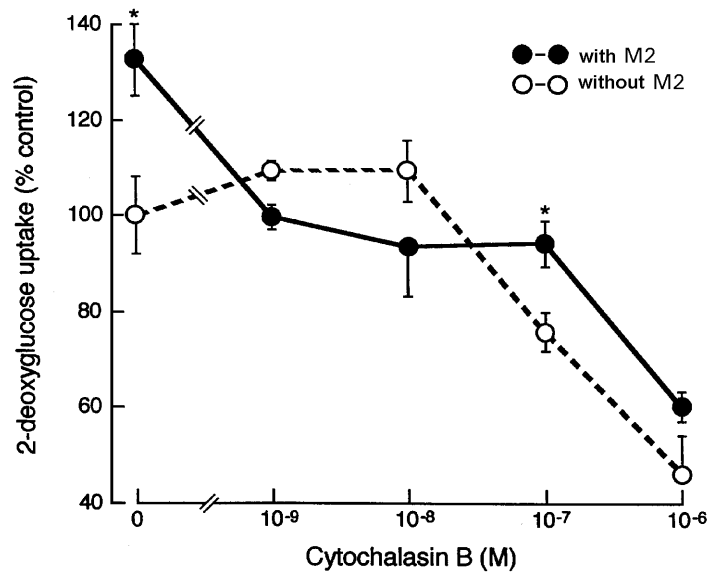
To investigate the mechanism of action of M2 on 2-deoxyglucose uptake, L6 cells were incubated for 24 h with his metabolite at 10<sup>-6</sup> M. M2 increased insulin-stimulated 2-deoxyglucose uptake at low insulin concentrations (10<sup>-9</sup> and 10<sup>-8</sup> M), but there was no additional effect of M2 on 2-deoxyglucose uptake at higher insulin concentrations (10<sup>-7</sup> and 10<sup>-6</sup> M); Figure 2). Inhibition of glucose transporters by incubation for 24 h with cytochalasin B (10<sup>-9</sup>–10<sup>-6</sup> M) decreased basal and M2-stimulated 2-deoxyglucose uptake in a concentration-dependent manner (Figure 3). Incubation for 24 h with LY-294,002 (10<sup>-9</sup>–10<sup>-5</sup> M), an inhibitor of the insulin-signalling intermediate phosphatidylinositol 3-kinase (P13-K), decreased basal uptake of 2-deoxyglucose in a concentration-related manner, but did not affect M2-stimulated 2-deoxyglucose uptake (Figure 4). Incubation for 24 h with an inhibitor of protein synthesis, cycloheximide, produced a concentration-related reduction of basal and M2-stimulated 2-deoxyglucose uptake (Figure 5).

### Other agents

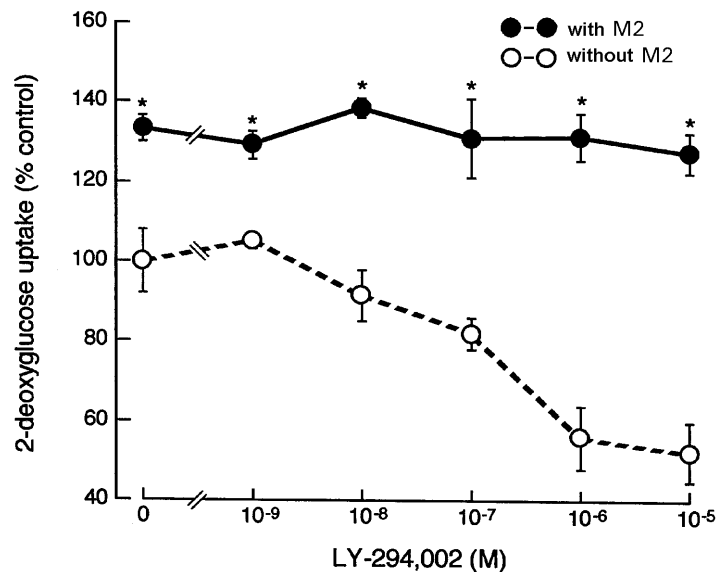
Fluoxetine and nisoxetine (10<sup>-8</sup>–10<sup>-6</sup> M), which selectively inhibit the reuptake of serotonin and noradrenaline respectively,<sup>12,13</sup> did not significantly alter 2-deoxyglucose uptake after 24 h incubations with and without added insulin (10<sup>-8</sup> M) (Figure 6). At 10<sup>-6</sup> M, serotonin and dopamine reduced insulin-stimulated 2-deoxyglucose uptake (by 17% and 23%, respectively, *P* < 0.05), and dopamine (10<sup>-6</sup> M) reduced 2-deoxyglucose uptake (by 17%, *P* < 0.05) after 24 h incubation without added insulin (Figure 7). Lower concentrations of serotonin and dopamine, and the range



**Figure 2** Effect of insulin on 2-deoxyglucose uptake by L6 muscle cells after 24 h incubation with and without M2 (10<sup>-6</sup> M). Values are mean ± s.e.m., *n* = 12. \**P* < 0.05 vs insulin only.



**Figure 3** Effect of cytochalasin B on 2-deoxyglucose uptake by L6 muscle cells after incubation for 24 h with  $10^{-6}$  M M2 and without M2. Values are mean  $\pm$  s.e.m.,  $n = 6$ . \* $P < 0.05$  vs without M2.



**Figure 4** Effect of LY-294,002 on 2-deoxyglucose uptake by L6 muscle cells after incubation for 24 h with  $10^{-6}$  M M2 and without M2. Values are mean  $\pm$  s.e.m.,  $n = 6$ . \* $P < 0.05$  vs without M2.

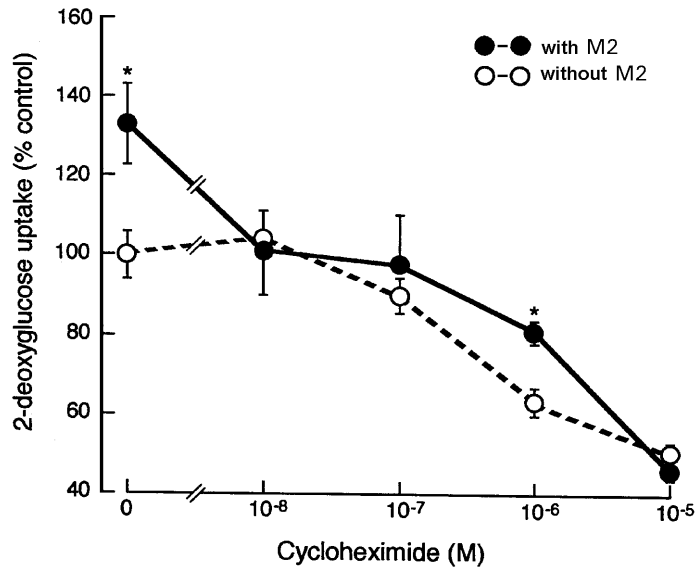
of noradrenaline concentrations tested ( $10^{-8}$ – $10^{-6}$  M) did not significantly alter 2-deoxyglucose uptake.

The serotonin releaser fenfluramine and its active metabolite nordexfenfluramine ( $10^{-6}$ – $10^{-8}$  M) did not significantly alter 2-deoxyglucose uptake after 24 h incubation without added insulin (Figure 8). However insulin-stimulated 2-deoxyglucose uptake was increased by  $10^{-8}$  and  $10^{-7}$  M nordexfenfluramine (each by 17%,  $P < 0.05$ ). The

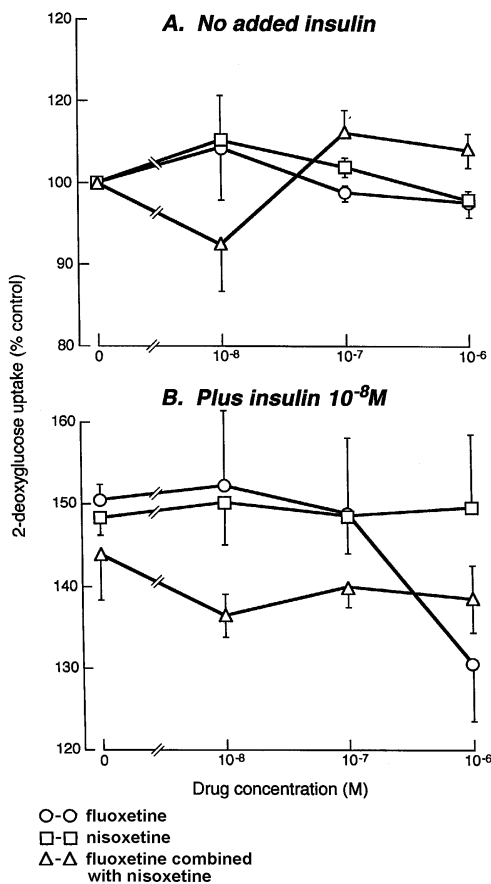
noradrenaline releaser phentermine ( $10^{-6}$ – $10^{-8}$  M) did not affect 2-deoxyglucose uptake after 24 h incubations with and without added insulin.

## Discussion

The results show that sibutramine metabolites can increase 2-deoxyglucose uptake by cultured L6 muscle cells. Sibutramine



**Figure 5** Effect of cycloheximide on 2-deoxyglucose uptake by L6 muscle cells after incubation for 24 h with  $10^{-6}$  M M2 and without M2. Values are mean  $\pm$  s.e.m.,  $n = 6$ . \* $P < 0.05$  vs without M2.

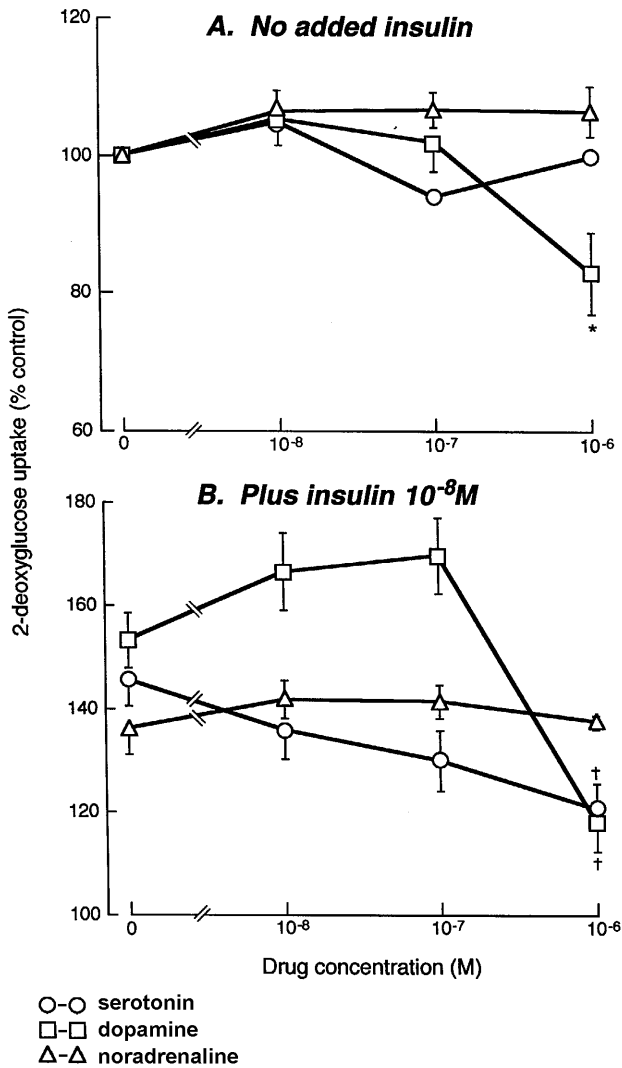


**Figure 6** Effect of fluoxetine, nisoxetine, and fluoxetine combined with nisoxetine on 2-deoxyglucose uptake by L6 muscle cells after incubation for 24 h with and without added insulin ( $10^{-8}$  M). Values are mean  $\pm$  s.e.m.,  $n = 6$ .

is metabolized *in vivo* by the liver to the secondary amine metabolite M1, and then to the primary amine metabolite M2.<sup>14</sup> M2 increased 2-deoxyglucose at 24 h whereas M1 was not effective at 24 h but became effective by 72 h. This raises the possibility that gradual conversion of M1 to M2 might at least partly account for the effectiveness of M1 after M2 in L6 muscle cells. It has recently been noted that M2 can stimulate 2-deoxyglucose uptake in isolated mouse soleus muscle (Liu and Stock, personal communication).

Although M2 increased 2-deoxyglucose uptake by muscle cells incubated without added insulin (basal uptake), this does not exclude a need for the presence of at least some insulin, since the cells were previously exposed to insulin in the FCS. However M2 produced a greater enhancement of 2-deoxyglucose uptake without added insulin and at low (submaximally stimulating) insulin concentrations. Since there was no increase produced by M2 at a maximally stimulating insulin concentration (Figure 2), this suggests that M2 does not increase maximal insulin responsiveness, as defined previously.<sup>15</sup> This is consistent with an effect of M2 to increase glucose uptake mediated via the insulin-signalling cascade.

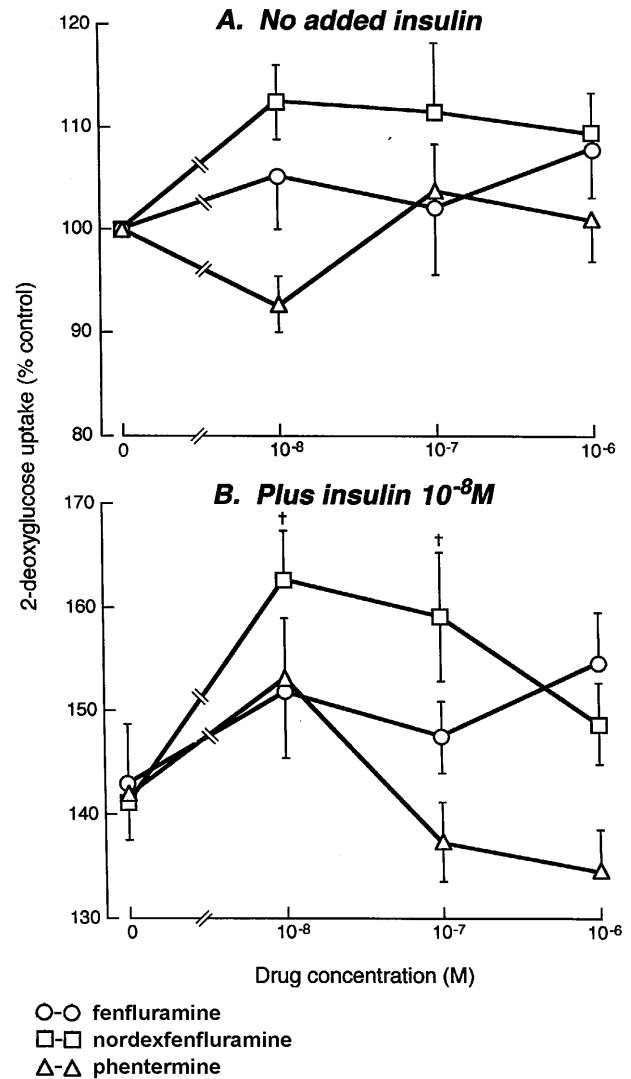
The insulin-signalling pathway(s) controlling glucose uptake involve PI3-K as an early component.<sup>16</sup> This step is inhibited by LY-294,002,<sup>17</sup> and a reduction in 2-deoxyglucose uptake was observed using this inhibitor (Figure 4). Since LY-294,002 did not inhibit M2-stimulated 2-deoxyglucose uptake it is likely that M2 acts distally to PI3-K. The fungal alkaloid, cytochalasin B acutely blocks sodium-independent glucose transporters.<sup>18</sup> Reduced uptake of 2-deoxyglucose by M2 in the presence of cytochalasin indicates that M2 promotes glucose transport via a facilitated sodium-independent transporter such as GLUT1 and GLUT4 found



**Figure 7** Effect of serotonin, dopamine and noradrenaline on 2-deoxyglucose uptake by L6 muscle cells after incubation for 24 h with and without added insulin ( $10^{-8}$  M). Values are mean  $\pm$  s.e.m.  $n=6$ . \* $P < 0.05$  vs control (no insulin); † $P < 0.05$  vs insulin only.

in muscle cells.<sup>19</sup> Reduced uptake of 2-deoxyglucose with cycloheximide shows that the transport process makes partial use of newly synthesized protein during the 24 h period of incubation.<sup>20,21</sup> This occurred to a similar extent with M2, supporting the view that M2 increases 2-deoxyglucose uptake through the normal glucose transport processes, possibly involving increased translocation of sodium-independent glucose transporters into the plasma membrane.

Thus M2 might activate or enhance signalling pathways and biological effects that mediate insulin action, rather than cellular mechanisms that are entirely independent of insulin. Improved insulin sensitivity by M2 in muscle cells is consistent with the amelioration of insulin resistance during sibutramine treatment in *ob/ob* mice,<sup>5</sup> and the improved glycaemic control during sibutramine treatment in obese



**Figure 8** Effect of fenfluramine, nordexfenfluramine and phentermine on 2-deoxyglucose uptake by L6 muscle cells after incubation for 24 h with and without added insulin ( $10^{-8}$  M). Values are mean  $\pm$  s.e.m.,  $n=6$ . † $P < 0.05$  vs insulin only.

type 2 diabetic patients.<sup>9,10</sup> Although decreased adiposity itself would be expected to reduce insulin resistance,<sup>22,23</sup> the present study suggest that M2 also acts directly on muscle to improve insulin action. Skeletal muscle is quantitatively the major site of insulin-stimulated glucose disposal, and an important focus of insulin resistance in obese type 2 diabetic patients.<sup>24,25</sup>

Sibutramine acts as an SNRI to induce satiety in rodents, indirectly enhancing activation of 5HT<sub>2A/2C</sub> receptors and  $\alpha_1$  and  $\beta_1$  adrenoceptors.<sup>4,14,26-28</sup> To investigate whether these mechanisms could account for increased 2-deoxyglucose uptake in muscle cells, experiments were undertaken with serotonin, noradrenaline, fluoxetine (serotonin reuptake inhibitor)<sup>13</sup> and nisoxetine (noradrenaline reuptake inhibitor).<sup>12</sup> None of these agents increased 2-deoxyglucose

uptake, suggesting that M2 is not acting on the muscle cells via serotonin or noradrenaline mediated mechanisms. Although it is unlikely that sibutramine could indirectly enhance dopamine receptor function,<sup>14</sup> there was no evidence that dopamine could stimulate 2-deoxyglucose uptake in muscle. Indeed, high concentrations ( $10^{-6}$  M) of serotonin and dopamine decreased 2-deoxyglucose uptake.

The serotonin-releasing anorectic agent fenfluramine has been reported to increase insulin action *in vivo* and *in vitro*, independently of its weight-reducing effect.<sup>29–31</sup> Although fenfluramine itself did not significantly affect 2-deoxyglucose uptake into the muscle cells, its active metabolite nordexfenfluramine increased insulin-stimulated 2-deoxyglucose uptake, presumably via a mechanism that is independent of serotonin.<sup>32</sup> The lack of effect of the anorectic agent phentermine (a noradrenaline and dopamine releaser)<sup>33</sup> on 2-deoxyglucose uptake by the muscle cells substantiates that an improvement of insulin sensitivity is not a general feature of amphetamine-based anorectic agents.<sup>34</sup>

Thus, sibutramine acts via its metabolites to increase insulin-sensitive glucose uptake by cultured L6 muscle cells. This is independent of the SNRI properties of sibutramine, and provides a means of reducing insulin resistance which is separate from that associated with decreased adiposity.

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