Insulin sensitivity and nutrient utilisation in skeletal muscle

by

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Chapter 3: Development and characterisation of an *in vitro* system using adipose tissue-conditioned media to study the effect of adipose tissue on glucose uptake in skeletal muscle cells

3.1 Introduction

Skeletal muscle is a major determinant of whole body energy utilisation and glucose metabolism. *In vivo,* a strong inverse relationship between visceral fat mass and insulin-stimulated glucose disposal in skeletal muscle has been consistently demonstrated (Colberg et al. 1995; Virtanen et al. 2005). Both skeletal muscle mass and function as well as regional fat distribution determine insulin sensitivity and metabolic risk of (Atlantis et al. 2009). A low ratio of visceral fat to skeletal muscle is one of the key determinants of a favourable metabolic profile (Kim et al. 2004). Interactions between adipose tissue and skeletal muscle, via adipose tissue-derived secretory factors, have been proposed to modify muscle energy metabolism. As a consequence of the expansion of fat mass and increased infiltration and activation of macrophages, the altered secretory function of adipose tissue in obesity has been associated with insulin resistance which is central to the pathophysiology of obesity-related metabolic abnormalities (Reaven 2005).

In vitro co-culture of adipocytes and skeletal muscle cells has been used to elucidate the effect of adipose tissue on muscle insulin sensitivity, although with somewhat contradictory findings (Section 1.6.2). The limited reproducibility of the effect of adipokines on muscle insulin sensitivity and the discrepancy between findings from *in vitro* and *in vivo* studies raise questions about the nature of adipose tissue-skeletal muscle interactions of the *in vitro* co-culture systems used to study them. One of the major

limitations of these models is the use of isolated adipocytes. Although adipocytes are the primary source of some adipokines including leptin and adiponectin (Lafontan and Viguerie 2006), many cytokines are also released from the stromal-vascular fraction, in particular activated macrophages, of adipose tissue (de Luca and Olefsky 2008). Accordingly, the net effect of adipose tissue on muscle metabolism under physiological conditions reflects the actions of secretory factors from both the adipocytes and cells in the stromal component.

The use of fat explants facilitates the study of the interactions between adipose tissue and other tissue types under physiological conditions, or the regulation of metabolism within a particular fat depot. It has been shown for example that SC adipose tissue is highly responsive to insulin-induced up-regulation of the mRNA expression and activity of LPL, whereas LPL in omental fat is resistant to the anti-lipolytic effect of insulin (Fried et al. 1993). IAB fat explants from obese humans secreted more pro-inflammatory cytokines including IL-6 and PAI-1 as compared to SC fat (Fain et al. 2004). Moreover, 90% of the factors released from the adipose tissue were from cells other than adipocytes (Fain et al. 2004). I therefore aimed to develop a novel *in vitro* system using adipose tissue-conditioned media (CM) to study the net effect of secretory factors from SC and IAB fat on basal and insulin-stimulated glucose uptake in skeletal muscle cells.

3.2 Materials and methods

3.2.1 Adipose tissue-conditioned media-skeletal muscle myotube culture

Primary human skeletal muscle and L6 cells were cultured on 12-well plates as described in Section 2.2. CM was generated using SC and IAB fat explants obtained from three male obese patients (50.7 ± 4.4 yr; BMI 45.7 ± 1.8kg/m^2) undergoing gastric bypass

surgery for the treatment of morbid obesity. CM was generated over 168 h incubation (Section 2.3). Primary human or L6 myotubes were incubated with 0.5 – 1 ml of CM for 0.5 – 24 h in the presence of 0.5% or 1% BSA (w/v) in an atmosphere of 5% CO_2 at 37°C.

3.2.2 Cell viability of adipose tissue explants

The viability of adipose tissue explants was determined by measuring the LDH activity in CM and nuclear DNA fragmentation of tissue explants. LDH activity in CM collected every 24 h during the 168 h incubation was determined enzymatically using a commercially available colorimetric assay as described in Section 2.4.1. DNA fragmentation of adipose tissue explants, cultured for 0 h, 48 h and 240 h, was analysed by separation of fragmented DNA and visualisation of apoptotic DNA laddering (Section 2.4.2).

3.2.3 Myotube viability

Primary human and L6 myotubes were incubated with CM collected after 48 h of culture (optimal time for adipose tissue viability; Figure 3.2) of IAB adipose tissue explants. Based on pilot studies showing an effect of CM on modifying basal glucose uptake in myotubes (Figure 3.4), CM at a concentration of 1:2 was used in the present study. LDH activity was measured in the media and expressed relative to total protein content (Section 2.4.1). The effect of CM on myotube viability was determined by comparing the LDH activity released from untreated cells (low control) to that from myotubes after culturing with CM and that from cells lysed with 0.4% Triton X-100 (v/v) for maximal LDH release (high control) (Mathieson et al. 1993).

3.2.4 Glucose uptake

After culturing with CM (concentrations 1:2 – 1:128), myotubes were incubated with or without 100 – 500 nM insulin (Actrapid, Novo Nordisk, Bagsvæd, Denmark) for 20 – 60 min in an atmosphere of 5% CO_2 at 37°C. 2-deoxy-D-[^{3}H] glucose uptake was measured as described in Section 2.7.

3.2.5 Data analysis

Data are expressed as means ± SEM. Statistical analyses were performed by one-way or two-way ANOVA with Bonferroni post hoc tests to compare treatment difference using the GraphPad Prism Program. Significance was accepted at $P < 0.05$.

3.3 Results

3.3.1 LDH activity in the adipose tissue-conditioned medium

The overall profile of LDH activity in CM from IAB and SC fat was similar at each time point (Figures 3.1A and B). LDH activity in CM collected after 24 h of culture was 5 – 70 times higher than that in the CM collected at the rest of the time points ($P < 0.001$). There was a gradual, but not significant, increase in LDH activity from 72 and 96 h onwards in CM from SC and IAB fat respectively, suggesting that there may be an increased loss of membrane integrity in adipose tissue explants when they were kept in culture for 72 h or longer.

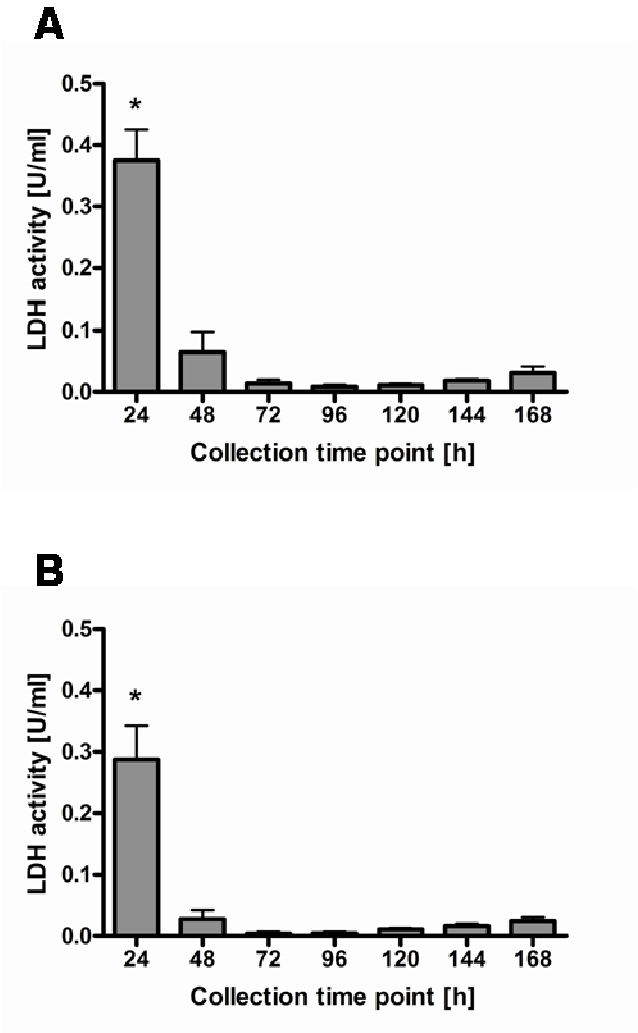

Figure 3.1. Lactate dehydrogenase (LDH) activity of adipose tissue-conditioned media generated from visceral (A) and subcutaneous (B) fat explants during a collection period of 168 h (n = 4). CM was collected and refreshed every 24 h for 168 h. LDH activity in the CM was measured using the Cytotoxicity Detection Kit^{PLUS} [LDH]. Data are shown as mean ± SEM. * $P < 0.001$ compared to all other time points.

3.3.2 Detection of DNA fragmentation in cultured adipose tissue explants

DNA fragmentation of adipose tissue explants cultured for 0 h, 48 h and 240 h were 3%, 13% and 35% respectively (Figure 3.2A). Culturing fat explants for 240 h resulted in a 12-fold increase ($P < 0.05$) in DNA fragmentation, while that in tissue cultured for 48 h was not different from non-cultured control (0 h). DNA ladder at multiples of ~180 base pairs has been considered as the hallmark of cell death (Elmore 2007). As shown in Figure 3.2B, no distinct bands of oligonucleosome-length electrophoresed DNA were detected in soluble fractions of DNA from all samples. These results confirmed a minimal degradation of DNA, and thus a low level of cell death, in tissue explants cultured for 48 h.

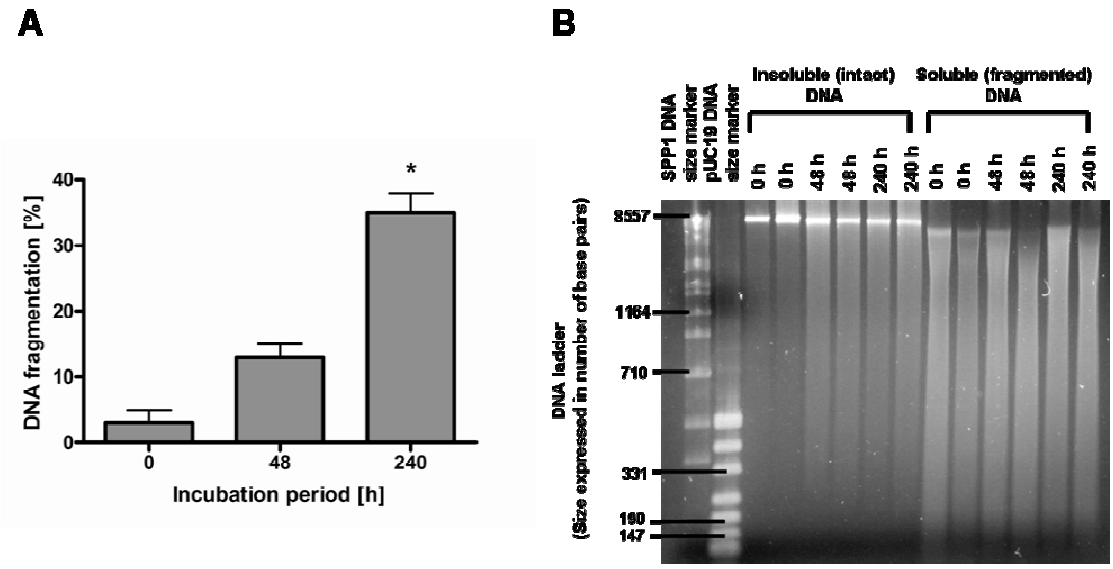

Figure 3.2. DNA fragmentation in adipose tissue explants. DNA fragmentation was assessed by measuring percentage of DNA fragmentation (A) and visualising SYBR Gold-stained agarose gel of electrophoresed DNA (B). After cultured for 0 h, 48 h and 240 h, insoluble (intact) and soluble (fragmented) DNA were extracted from visceral fat explants in duplicate using DNAzol and phenol/chloroform/isoamyl alcohol respectively. A: DNA concentration in both fractions was quantified using the Quant-iT^{TM} PicoGreen Assay®. Percentage of DNA fragmentation was calculated as the ratio of the concentration of soluble DNA to that of total DNA. B: 45 ng DNA was loaded onto a 2% agarose gel stained with SYBR® Gold Nucleic Acid Gel Stain and visualised under UV light. Data are shown as mean ± SEM. * $P < 0.05$ compared to non-cultured control (0 h).

3.3.3 Myotube viability

No significant changes in the confluence of either L6 or primary human myotubes were observed under light microscopy after 6 h incubation with CM. In each type of muscle cell culture, the released LDH activity from untreated cells (low control) was compared to that from cells lysed with 0.4% Triton X-100 (v/v) (high control) and from those cultured with CM. Compared to low control, LDH activity released from high control was 6-fold ($P < 0.001$; Figure 3.3A) and 5-fold ($P = 0.001$; Figure 3.3B) higher in L6 and primary human myotubes respectively. The released LDH activity from both types of myotubes after culturing with CM was not different from that of low control, suggesting a minimal effect of CM on myotube viability in either type of muscle cell culture.

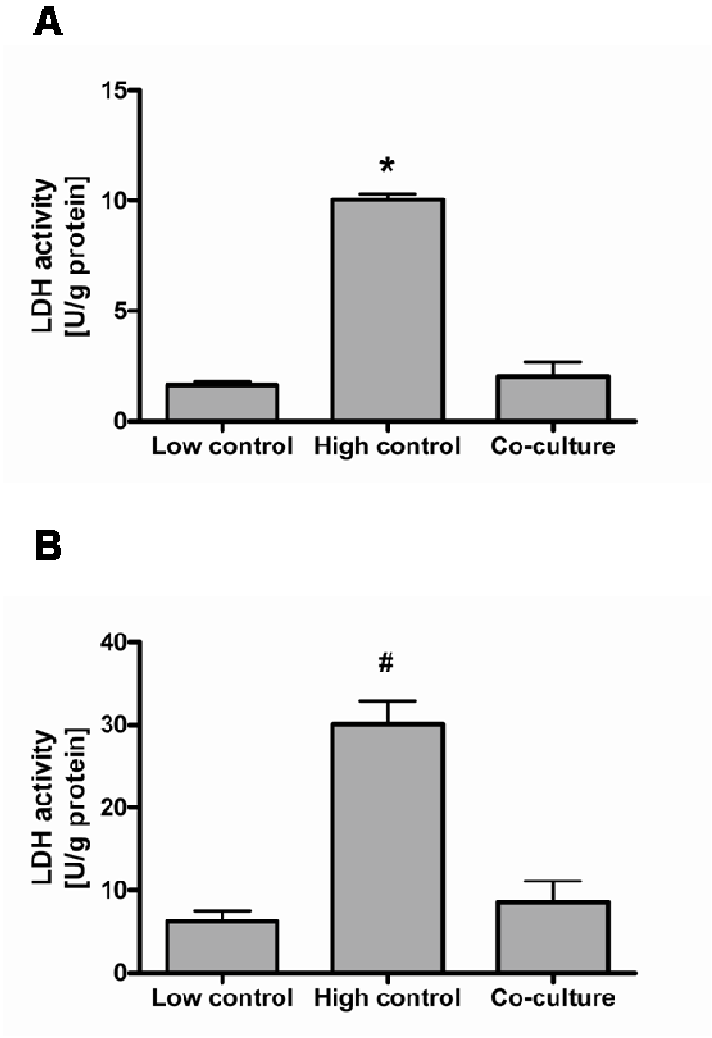

Figure 3.3. Lactate dehydrogenase (LDH) activity released by L6 (A) and primary human myotubes (B) after cultured with adipose tissue-conditioned media for 6 h (n = 3). Skeletal muscle cells were incubated with visceral fat-conditioned media (collected after 48 h of culture) at a concentration of 1:2 for 6 h. Treatments were completed in triplicate. The media was collected and LDH activity was measured using the Cytotoxicity Detection Kit^{PLUS} [LDH]. Data are shown as mean ± SEM. * $P < 0.001$ and # $P = 0.001$ compared to untreated cells (Low control).

3.3.4 Optimisation of the adipose tissue-conditioned media-myotube system

3.3.4.1 Concentration of CM

A pilot adipose tissue-conditioned media-myotube system was established by incubating primary human myotubes (derived from an obese individual) with CM generated from SC and IAB fat of the same donor. CM from each fat depot, collected after 24 h and 72 h incubation respectively, were cultured with myotubes at concentrations 1:2 – 1:16 (1% BSA; w/v) for 16 h prior to glucose uptake assay.

CM, generated from either fat depot, collected after 24 h of culture did not affect basal glucose uptake in human myotubes. At concentrations 1:2 and 1:4, CM from IAB fat cultured for 72 h increased basal glucose uptake in human myotubes by 52% ($P < 0.01$) and 46% ($P < 0.05$) respectively (Figure 3.4A). In contrast, CM from SC fat collected at the same time point had no effect on basal glucose uptake at all concentrations tested (Figure 3.4B). The data suggest that 1:2 and 1:4 may be the optimal concentrations to demonstrate depot-specific effects of adipose tissue on muscle glucose uptake and therefore were used in the subsequent optimisation trials.

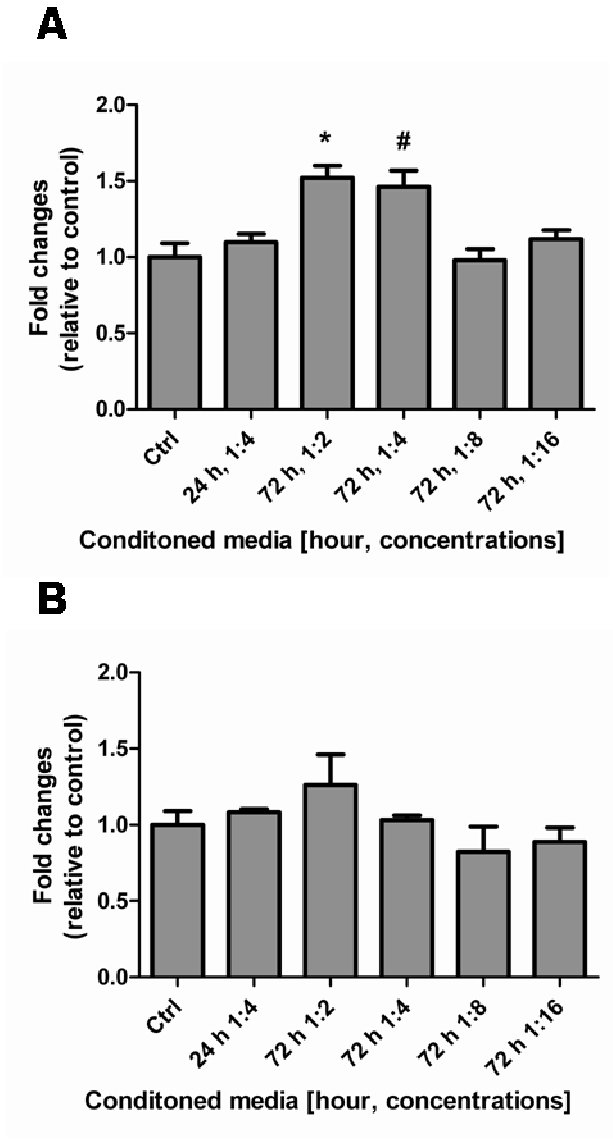

Figure 3.4. Dose-response effect of adipose tissue-conditioned media generated from visceral (A) and subcutaneous (B) fat on basal glucose uptake in primary human myotubes. Primary human myotubes derived from an obese individual were incubated with adipose tissue-conditioned media collected after 24 and 72 h of culture and at concentrations 1:2 – 1:16. The culture was kept for 16 h. Myotubes were then incubated in uptake buffer containing 2-deoxy-D-[^{3}H] glucose (1 μCi/well; 10 μM) for 15 min. Treatments were completed in triplicate. Data are shown as mean ± SEM and are expressed as fold changes from the control (Ctrl). * $P < 0.01$ and # $P < 0.05$ compared to Ctrl.

3.3.4.2 Volume of CM

On 12-well plates, cell cultures are usually maintained and treated with 1 ml of media (Ciaraldi et al. 1995; McIntyre et al. 2004). Limited access to adipose tissue samples, however, led me to explore the possibility of using a smaller volume of CM in the culture. Primary human myotubes from an obese individual were incubated with 0.5 ml or 1 ml of CM, collected after 72 h of culture and at concentrations 1:2 and 1:4 generated from an obese donor. The culture was kept for 16 h followed by glucose uptake assay.

Neither confluence nor morphology of myotubes was affected by the reduced volume of media during the culture. In general, there was no volume effect of CM on basal glucose uptake in myotubes except when culturing with CM from IAB fat at concentration 1:2, the reduction in volume resulted in a 55% increase in muscle basal glucose uptake ($P < 0.05$; Figure 3.5). The data suggest that the volume of media used in the culture, at least as demonstrated with 0.5 ml and 1 ml, may not be a critical factor in determining the effect of CM and thus 0.5 ml of CM was used in subsequent studies.

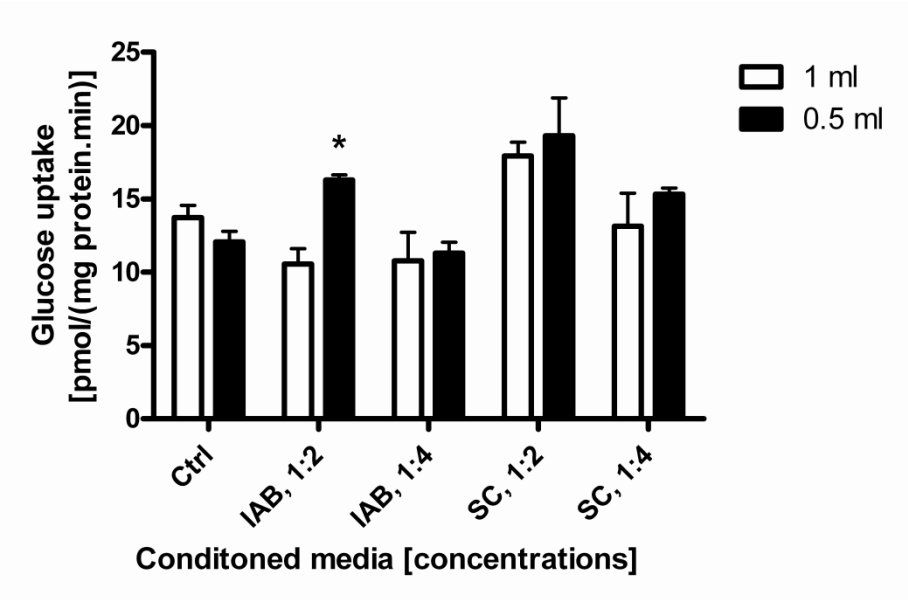

Figure 3.5. Volume effect of adipose tissue-conditioned media generated from visceral (IAB) and subcutaneous (SC) fat on basal glucose uptake in primary human myotubes. Primary human myotubes derived from an obese individual were cultured with adipose tissue-conditioned media, collected after 72 h of culture and at concentrations 1:2 and 1:4, for 16 h. Myotubes were then incubated in uptake buffer containing 2-deoxy-D-[^{3}H] glucose (1 μCi/well; 10 μM) for 15 min. Treatments were completed in triplicate. Data are shown as mean ± SEM and are expressed as fold changes from the control (Ctrl). * $P < 0.05$ compared to 1 ml of the corresponding treatment.

3.3.4.3 Collection time point of CM

In the initial trials CM collected after 24 h and 72 h of culture were used and the later showed a depot-specific effect on basal glucose uptake in myotubes. The effect of CM from other collection time points, however, has not been determined due to limited sample availability. CM (concentration 1:2) generated from three obese tissue donors, each collected at 48 h, 72 h and 96 h after culture, were incubated with primary human myotubes from an obese individual. CM increased basal glucose uptake in myotubes by up to 60% (P = 0.3; Figure 3.6A) with CM from IAB fat collected at 48 h and a similar increase (61%) with CM from SC fat collected at 72 h (P = 0.1; Figure 3.6B). The effect of CM on muscle glucose uptake, from either fat depot, collected at the three time points was not different from each other. Based on the results of the viability assays of cultured adipose tissue explants which showed minimal cell death when tissue samples were cultured for 48 h (Figure 3.2), CM collected at this time point was used in the subsequent studies.

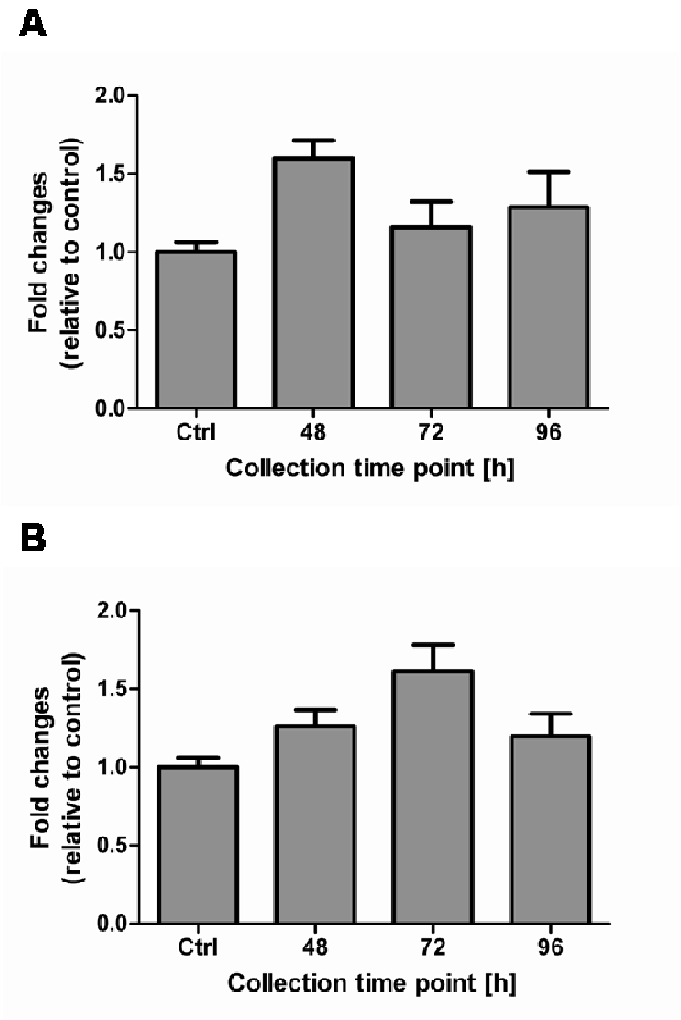

Figure 3.6. The effect of adipose tissue-conditioned media from visceral (A) and subcutaneous (B) fat, collected after 48, 72 and 96 h of culture, on basal glucose uptake in primary human myotubes (n = 3). Primary human myotubes derived from an obese individual were incubated with adipose tissue-conditioned media (concentration 1:2) for 16 h. Myotubes were then incubated in uptake buffer containing 2-deoxy-D-[^{3}H] glucose (1 μCi/well; 10 μM) for 15 min. Treatments were completed in triplicate. Data are shown as mean ± SEM and are expressed as fold changes from the control (Ctrl).

3.3.4.4 Concentration of BSA and duration of culture

CM was generated in the presence of BSA (1%; w/v) which was commonly used in *in vitro* adipocyte culture as a stabiliser in the medium (Schlesinger et al. 2006). Accordingly, the same BSA concentration has been kept in the culture.

The effect of BSA concentration on basal glucose uptake in skeletal muscle cells was determined. Primary human myotubes were incubated in basal cell culture medium (α-MEM) with 0.5% or 1% BSA (w/v) for 0.5 – 48 h. 1% BSA (w/v) significantly increased glucose uptake by 60% ($P < 0.05$; Figure 3.7A) when compared to 0.5% BSA (w/v) after 24 h incubation. No concentration effect of BSA has been observed in other culture durations. The data suggest that at concentrations 0.5 – 1% BSA (w/v) has minimal effect on muscle glucose uptake.

Primary human myotubes were incubated with CM, generated from IAB and SC fat after 48 h of culture and at concentration 1:2, from the same obese tissue donor for 0.5 – 48 h. BSA concentration in the culture was kept at 0.5% and 1% (w/v) in parallel studies. Increasing BSA concentration from 0.5% to 1% (w/v) resulted in a 17% ($P < 0.05$) and 31% ($P < 0.01$) decrease in muscle basal glucose uptake when cultured with CM from IAB fat for 6 h and 24 h respectively (Figure 3.7B). In contrast, BSA concentration did not affect basal glucose uptake when myotubes were cultured with CM from SC fat (Figure 3.7C). Culturing myotubes with CM from either fat depot in 0.5% BSA (w/v) for 6 h appeared to be the optimal conditions in respect to increasing basal glucose uptake in myotubes.

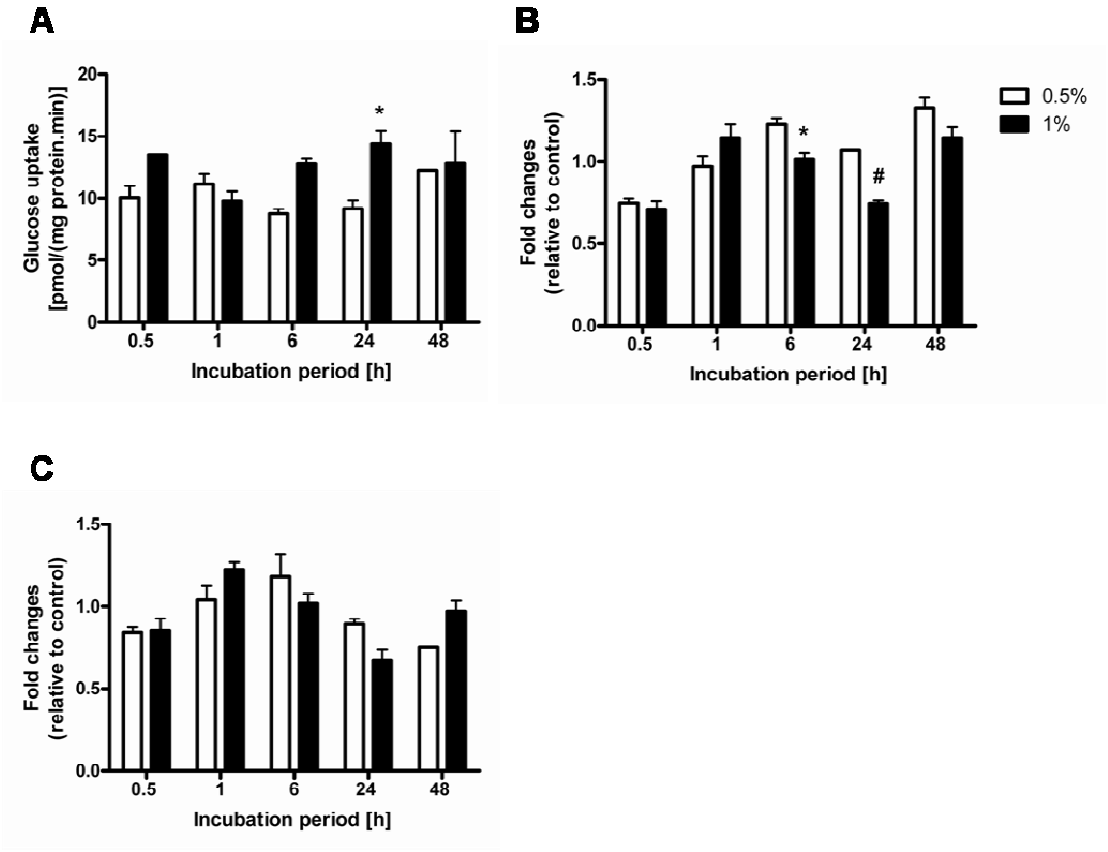

Figure 3.7. Concentration and duration effect of BSA exposure on basal glucose uptake in primary human myotubes. Primary human myotubes were cultured with basal cell culture medium (A), adipose tissue-conditioned media generated from visceral (B) and subcutaneous (C) fat, collected after 48 h of culture and at concentration 1:2. The culture was kept for 0.5 – 48 h and with BSA concentrations of 0.5% and 1% (w/v) in parallel studies. Myotubes were then incubated in uptake buffer containing 2-deoxy-D-[^{3}H] glucose (1 μCi/well; 10 μM) for 15 min. Treatments were completed in triplicate. Data are shown as mean ± SEM and are expressed as fold changes from the corresponding controls. # $P < 0.01$ and * $P < 0.05$ compared to 0.5% BSA (w/v).

3.3.5 Optimisation of conditions to study insulin-stimulated glucose uptake in skeletal muscle cells

The low degree of insulin responsiveness in cultured human skeletal muscle cells may limit its use in assessing insulin responsiveness and the mechanisms involved in the regulation of insulin sensitivity (Perriott et al. 2001). Optimisation of conditions to study the effect of insulin stimulation on glucose uptake in skeletal muscle cells was performed on L6 myotubes, a skeletal muscle cell line of rat origin with consistent sensitivity and responsiveness to insulin stimulation (Huang et al. 2002; Ceddia et al. 2005) (Section 1.6.3.1).

3.3.5.1 Dose-response and duration of insulin exposure

100 nM and 20 min insulin exposure were conditions commonly used to study the effect of insulin stimulation on glucose uptake in skeletal muscle cells of both rat and human origin (McIntyre et al. 2004; Ceddia et al. 2005). Perriott et al (2001) incubated primary human myotubes with 500 nM for 1 h and reported a consistent 2- to 3-fold increase in glucose uptake in response to insulin stimulation, a magnitude of effect similar to that observed in human forearm muscle balance studies *in vivo*. In the present study, L6 myotubes were serum-starved overnight followed by incubating with 100 nM and 500 nM of insulin for 20 or 60 min in parallel studies and the insulin was washed off prior to the glucose uptake assay.

Exposures to 20 min of 100 nM and 60 min of 500 nM insulin resulted in maximal increase in glucose uptake in L6 myotubes of 20% ($P = 0.5$; Figure 3.8A) and 29% ($P = 0.1$; Figure 3.8B) respectively. The insulin response, however, was not as robust as reported in the literature (Ceddia et al. 2005; Palanivel et al. 2006).

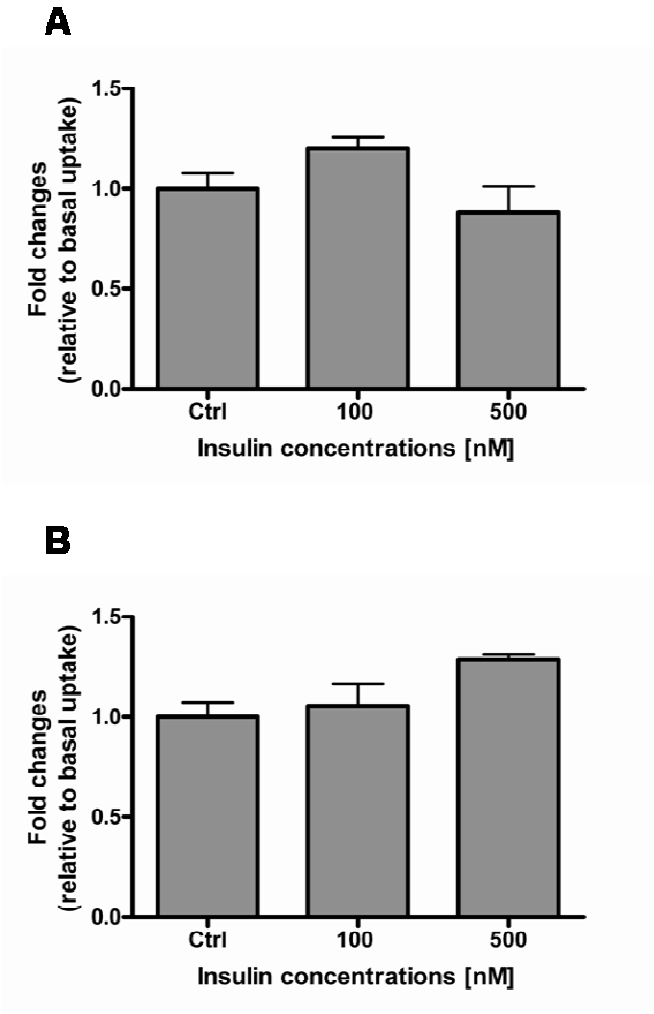

Figure 3.8. Insulin-stimulated glucose uptake in L6 myotubes. L6 myotubes were incubated with 100 nM and 500 nM insulin for 20 min (A) or 60 min (B) in parallel studies. Insulin was washed off prior to the glucose uptake assay. Myotubes were then incubated in uptake buffer containing 2-deoxy-D-[^{3}H] glucose (1 μCi/well; 10 μM) for 15 min. Treatments were completed in triplicate. Data are shown as mean ± SEM and are expressed as fold changes from the basal uptake.

3.3.5.2 Cytochalasin B-inhibitable glucose uptake

Although glucose uptake in skeletal muscle takes place primarily via carrier-facilitated transport (Section 1.3.2.1), non-specific uptake, i.e., passive diffusion, also contributes to a small amount of glucose uptake and may thus mask the effect of insulin stimulation. Cytochalasin B is an inhibitor of GLUT1- and GLUT4-mediated glucose transport and is commonly used to account for non-specific glucose uptake in *in vitro* models studying glucose transport system in skeletal muscle (Sarabia et al. 1992; Ryder et al. 1999).

A stock solution of 0.01 M cytochalasin B (Sigma-Aldrich # C6762) was prepared in DMSO and stored at 4°C until use. L6 myotubes were incubated 6 h with CM (collected after 48 h of culture and at concentration 1:2) from IAB and SC fat of an obese individual. The myotubes were rinsed with PBS followed by incubating with 100 nM insulin for 20 min. Non-carrier-mediated glucose uptake was measured in parallel studies with the presence of 10 μM cytochalasin B (Ceddia et al. 2005) during the glucose uptake assay, which was then subtracted from the total to determine specific uptake, i.e., uptake inhibitable by cytochalasin B.

Under control conditions, cytochalasin B inhibited basal and insulin-stimulated glucose uptake by 54% ($P < 0.001$) and 63% ($P < 0.001$) respectively (Figure 3.9A). Insulin induced a 34% ($P < 0.001$) increase in absolute glucose uptake whereas non-specific uptake was not affected by insulin. When non-cytochalasin B-inhibitable uptake was subtracted from the corresponding experimental values, carrier-mediated uptake accounted for 53 – 70% of total uptake (Figure 3.9A). When only the carrier-mediated uptake was taken into consideration, the effect of insulin on increasing glucose uptake increased from 34% to 52% (Figures 3.9B and C).

In cultures with CM, the effect of insulin on increasing glucose uptake was similar when glucose uptake was expressed in both absolute and cytochalasin B-inhibitable uptake. Insulin had no effect when L6 myotubes were cultured with CM from IAB fat (Figures 3.9B and C). In the presence of CM from SC fat, insulin induced a ~ 50% ($P < 0.05$) increase in glucose uptake.

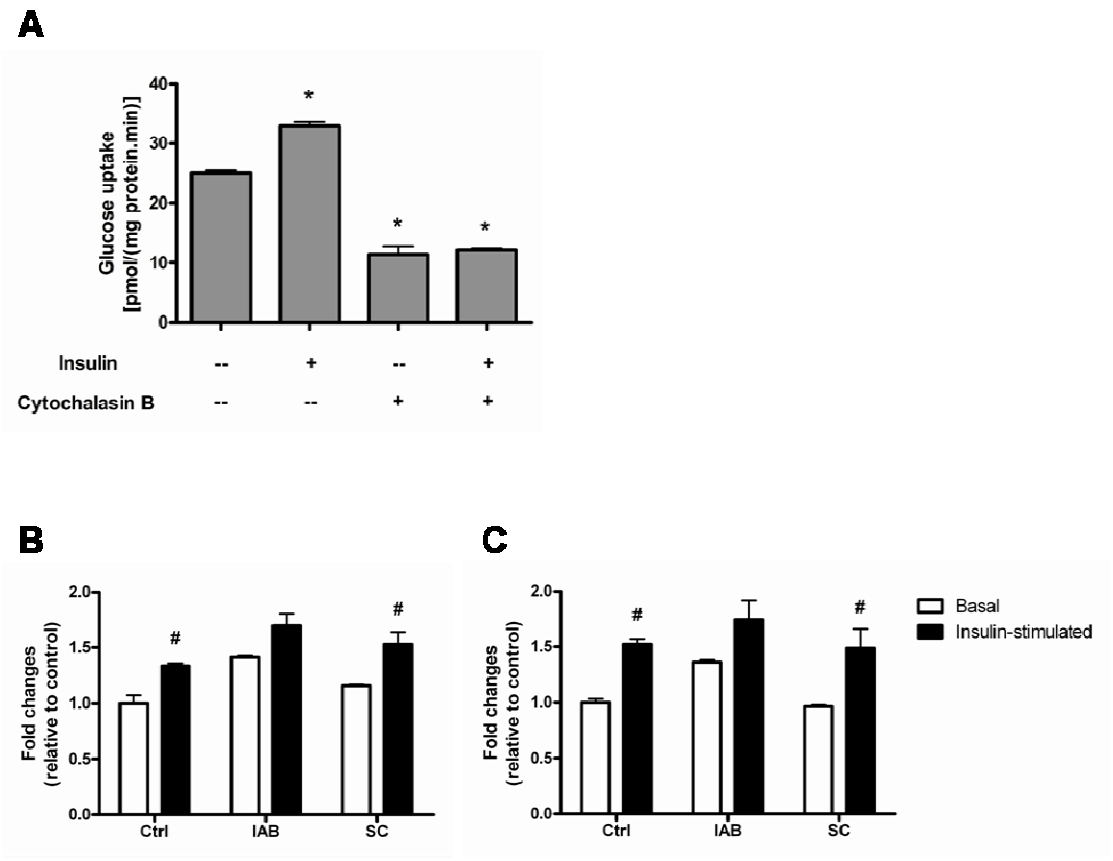

Figure 3.9. The effect of insulin stimulation on specific glucose uptake in L6 myotubes. L6 myotubes were incubated with adipose tissue-conditioned media (concentration 1:2) generated from visceral (IAB) and subcutaneous (SC) fat for 6 h, followed by an exposure to 100 nM insulin for 20 min. Myotubes were then incubated in uptake buffer containing 2-deoxy-D-[^{3}H] glucose (1 μCi/well; 10 μM) for 15 min. Non-specific uptake was determined with the presence of 10 μM cytochalasin B during the uptake assay (A). Basal and insulin-stimulated glucose uptake was expressed as fold changes relative to controls (Ctrl) reported in absolute (B) and cytochalasin B-inhibitable uptake (C). Treatments were completed in triplicate. Data are shown as mean ± SEM. * $P < 0.001$ compared to uptake in the absence of insulin and cytochalasin B. # $P < 0.05$ compared to the basal uptake of the corresponding treatment.

In the previous study, cytochalasin B inhibited glucose uptake by 53 – 70%. Klip et al (1992) reported that non-specific uptake should account for < 20% of the total uptake. Currently cytochalasin B was only added during the glucose uptake assay. A longer exposure to cytochalasin B may maximise its effect on inhibiting carrier-mediated glucose transport. L6 myotubes were incubated in α-MEM with 1% BSA (w/v) for 6 h (same as the duration of the culture with CM), followed by 20 min insulin stimulation (100 nM) and 15 min glucose uptake assay. 10 μM cytochalasin B was added at various time points: 1) before the 6 h incubation, 2) before insulin stimulation and 3) before glucose uptake assay in parallel studies and the inhibitor was retained until the end of the uptake assay in all cases.

Incubating L6 myotubes with cytochalasin B for 15, 35 and 395 min inhibited 83 – 90% of total glucose uptake. The inhibitory effect of cytochalasin B was not different between exposure durations and was not affected by insulin stimulation (Figure 3.10A). When the corresponding non-specific uptake was subtracted from total uptake, insulin induced a small (13 – 14%; $P < 0.01$; Figure 3.10B) and yet significant increase in carrier-mediated glucose uptake with no difference between the three cytochalasin B exposure durations. Extended exposure to cytochalasin B may affect myotube viability, as suggested by the great variations in non-specific uptake and the low protein content of the cell culture (data not shown). The presence of cytochalasin B during insulin exposure and glucose uptake assay was therefore a preferred condition for insulin stimulation studies.

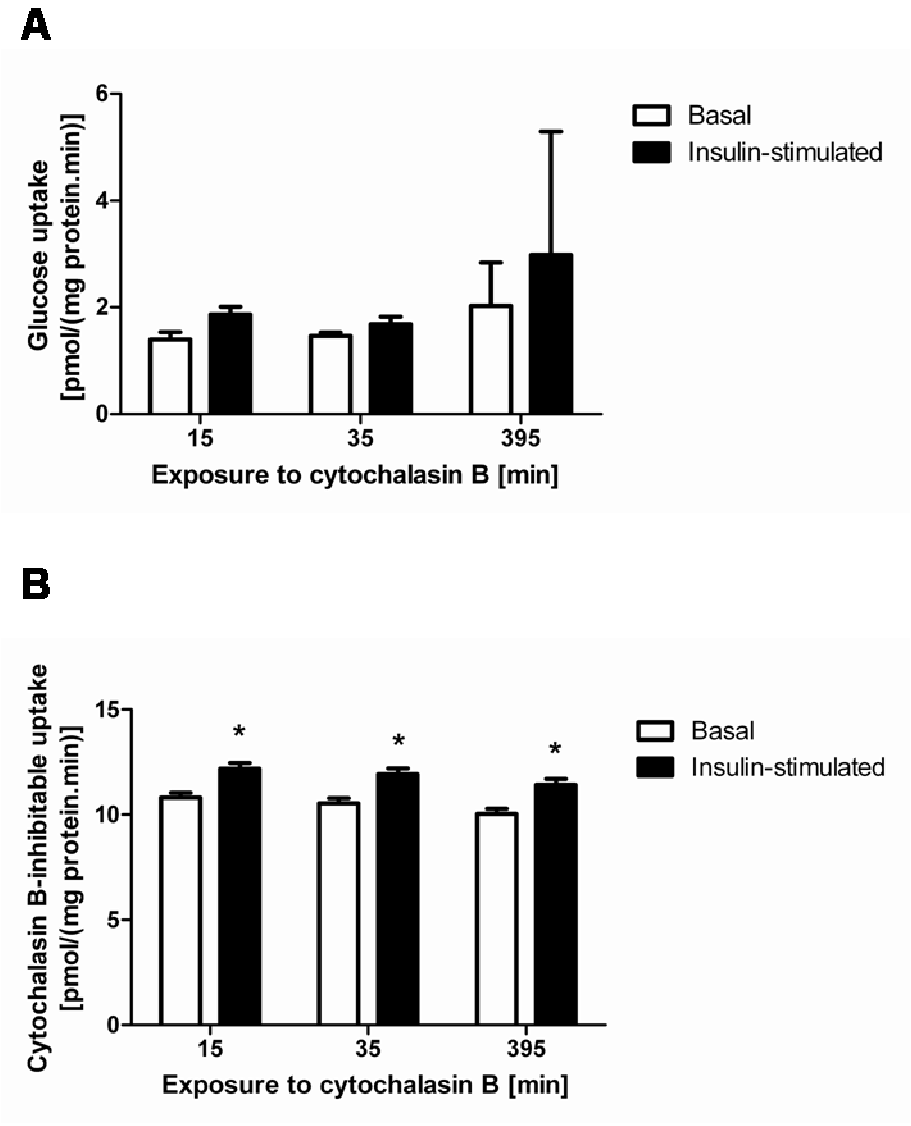

Figure 3.10. The effect of cytochalasin B exposure on inhibiting carrier-mediated glucose uptake in L6 myotubes. 10 μM cytochalasin B was added to L6 myotubes during glucose uptake assay (15 min), insulin stimulation (35 min) and 6 h incubation with α-MEM and 1% BSA (w/v) (395 min) and was retained until the end of uptake assay. Myotubes were incubated in uptake buffer containing 2-deoxy-D-[^{3}H] glucose (1 μCi/well; 10 μM) for 15 min. Non-specific uptake (A) and carrier-mediated uptake (B) was determined. Treatments were completed in triplicate. Data are shown as mean ± SEM. * $P < 0.01$ compared to the corresponding basal uptake.

3.3.6 Effect of subcutaneous fat on basal and insulin-stimulated glucose uptake in primary human myotubes

The optimised conditions were used to determine the effect of secretory factors from SC fat on basal and insulin-stimulated glucose uptake in skeletal muscle cells. Primary human myotubes established from three healthy lean female (48.3 ± 1.8 yr; BMI 22.6 ± 0.9 kg/m^2) and three obese female (48 ± 3 yr; BMI 49.4 ± 4.2 kg/m^2) were cultured with CM (concentrations 1:4 – 1:128) for 6 h. The myotubes were then incubated with or without 100 nM insulin for 20 min prior to glucose uptake assay. Non-carrier-mediated glucose uptake was determined by parallel studies in the presence of 10 μM cytochalasin B and was subtracted from all experimental values.

In the absence of CM (control), insulin had no effect on glucose uptake in myotubes from both healthy lean and obese subjects (Figure 3.11A). In the three cell lines established from healthy lean individuals, only one showed an increase in glucose uptake in response to insulin and none of the difference between basal and insulin-stimulated glucose uptake was significant (P = 0.72; Figure 3.11B). Similarly, all cell lines established from obese subjects were non-responsive to insulin stimulation (Figure 3.11C) although none of the muscle donors were diagnosed as insulin-resistant. Since no significant effect of insulin stimulation was shown under the control conditions, the effect of CM from SC fat on insulin sensitivity could not be determined.

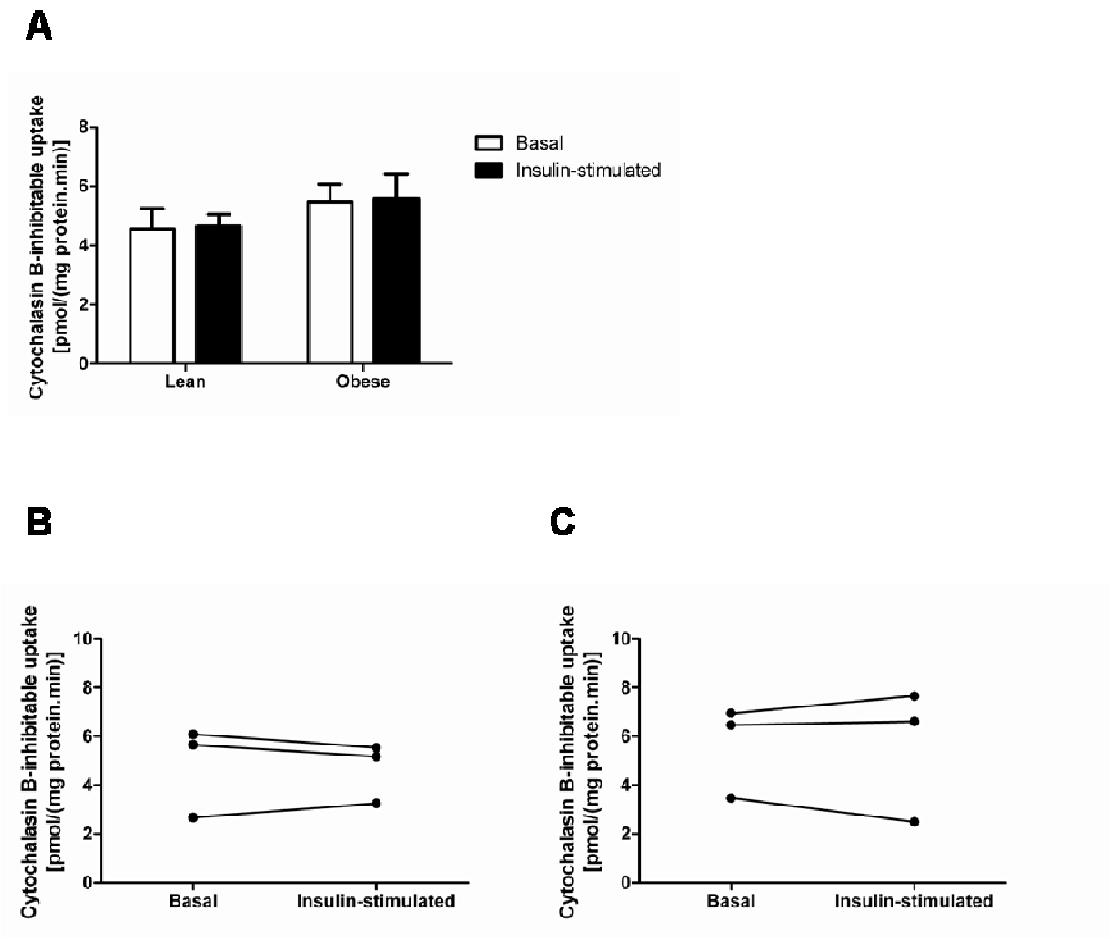

Figure 3.11. Insulin sensitivity of primary human myotubes. Primary human myotubes established from healthy lean (n = 3) and obese (n = 3) subjects were incubated with or without 100 nM insulin for 20 min. Myotubes were then incubated in uptake buffer containing 2-deoxy-D-[^{3}H] glucose (1 μCi/well; 10 μM) for 15 min. Non-specific uptake was determined in the presence of 10 μM cytochalasin B. Basal and insulin-stimulated cytochalasin B-inhibitable uptake in healthy lean and obese myotubes (A). Insulin sensitivity of individual cell lines established from healthy lean (B) and obese (C) individuals. Treatments were completed in triplicate. Data are shown as mean ± SEM.

In the presence of CM, basal glucose uptake of myotubes from lean and obese subjects was not different at all concentrations tested (1:4 – 1:128; Figure 3.12). The dose-response effect of CM on basal glucose uptake was similar in both types of myotubes, in which CM at concentrations 1:4 – 1:32 increased basal glucose uptake with the maximal effect (21 – 28%) at 1:8. The effect of CM, however, was only significant in myotubes from obese donors, in which CM at concentrations 1:4 and 1:8 increased glucose uptake by 25% and 28% respectively ($P < 0.05$).

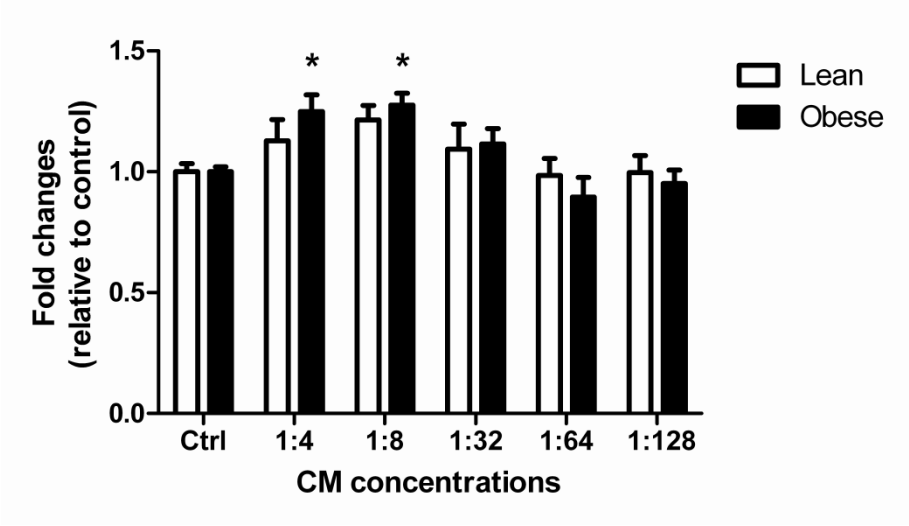

Figure 3.12. The effect of secretory factors from subcutaneous fat on basal glucose uptake in primary human myotubes. Primary human myotubes established from healthy lean (n = 3) and obese (n = 3) subjects were cultured with subcutaneous fat-conditioned media for 6 h. Myotubes were then incubated in uptake buffer containing 2-deoxy-D-[^{3}H] glucose (1 μCi/well; 10 μM) for 15 min. Non-specific uptake was determined in the presence of 10 μM cytochalasin B. Treatments were completed in triplicate. Data are shown as mean ± SEM. * $P < 0.05$ compared to the corresponding control (Ctrl).

3.4 Discussion

The present study demonstrates the feasibility of using an adipose tissue-conditioned media-myotube system to determine the effect of fat depots on glucose uptake in skeletal muscle cells. The viability of adipose tissue explants and skeletal muscle cells was confirmed by the measurement of LDH activity in the CM. In addition, nuclear DNA fragmentation was used to validate the integrity of the tissue samples (Gullicksen et al. 2004; Lappas et al. 2004). The optimal culture duration of 48 h prior to the collection of CM is consistent with that previously found to be optimal to study adipokine secretion from adipose tissue explants (Casabiell et al. 1998; Fain et al. 2006). The culture conditions were optimised and the system was established by incubating myotubes with 0.5 ml CM with a final concentration of 0.5% BSA (w/v) in an atmosphere of 5% CO_2 at 37°C for 6 h, followed by insulin stimulation and/or glucose uptake assay. Basal glucose uptake in primary human myotubes, from both healthy lean and obese individuals, was modified by the net effect of secretory factors from SC fat.

The novelty of the present system was culturing skeletal muscle cells with conditioned media generated from adipose tissue explants, instead of isolated adipocytes, which demonstrated the net effect of secretory factors from the whole tissue on muscle metabolism in an *in vitro* model. Adipose tissue produces > 100 secretory factors contributed by both mature adipocytes and cells in the stromal-vascular component, in particular activated macrophages (Hauner 2004). While some adipokines, including leptin (Section 1.4.3.1), adiponectin (Section 1.4.3.2) and TNF-α (Section 1.4.3.3), are known to have significant physiological functions in energy metabolism and inflammatory responses, the function and clinical significance of most secretory factors remain to be fully elucidated. This *in vitro* model facilitates the study of the involvement

of individual cytokines, as well as their interactions and the mechanisms by which they occur, in functional characteristics of adipose tissue.

The culture system was optimised to ensure the viability and functional characteristics of both the adipose tissue explants and skeletal muscle cell culture were not compromised in the experiments. In order to minimise the effect of serum on the adipose tissue explants prior to the collection of CM, tissue explants were incubated in serum-free basal cell culture medium (α-MEM with 1% BSA (w/v)). This is in contrast to other models in which adipocytes were cultured in skeletal muscle cell differentiation medium (2% FBS; v/v) (Dietze et al. 2002; Sell et al. 2008). In the presence of the undefined variety of growth factors and hormones in FBS, the secretory function of adipocytes may be affected (Isozaki et al. 1999). The effect of CM on myotube viability was also minimised by reducing the concentration of BSA in the culture system. CM is currently generated in the presence of BSA, which is commonly used in adipocyte/adipose tissue cultures as stabiliser in the medium (Schlesinger et al. 2006). The reduction of BSA concentration in the system increased the responsiveness of primary human myotubes to the effect of secretory factors from adipose tissue on glucose uptake. Taken together, the characteristics of the culture system confirmed the utility of the model. Accordingly, the system may be used to determine the effect of adipose tissue-skeletal muscle interactions on other aspects of skeletal muscle energy metabolism.

In contrast to the conservation of metabolic characteristics in myotubes developed from individuals of various phenotypes documented previously (Ukropcova et al. 2005), insulin had no effect on glucose uptake in primary human myotubes irrespective of insulin sensitivity of the muscle donors *in vivo*. In the present study, the conditions to

study insulin-stimulated glucose uptake were optimised in L6 myotubes, a rat skeletal muscle-derived cell line commonly used to investigate insulin-stimulated glucose metabolism *in vitro* due to their high sensitivity and responsiveness to insulin stimulation (Huang et al. 2002; Ceddia et al. 2005) (Section 1.6.3.1). A significant 1.1- to 1.5-fold increase in glucose uptake has been shown when exposing L6 myotubes to 100 nM insulin for 20 min. Although primary muscle cell cultures are considered to be the best to reflect muscle metabolism *in vitro*, experiment-to-experiment variability, as a result of source and processing of biopsies, may compromise the reproducibility of studies (Bonavaud et al. 1997). The known discrepancy of myogenic cell lines and primary cells may also suggest the need to optimise study conditions specific to the type of cell culture used (Bonavaud et al. 1997).

In summary, I describe here an adipose tissue-conditioned media-skeletal muscle myotube culture system which preserves the physiological *in vivo* crosstalk between adipose tissue and skeletal muscle in an *in vitro* system to study glucose uptake and potentially other aspects of energy metabolism in skeletal muscle. The culture system has been optimised to study insulin-stimulated glucose uptake in L6 myotubes, while conditions to demonstrate the effect of insulin stimulation in primary human skeletal muscle cells are yet to be determined.