massive amounts of SIRT1 and FoxO3a were expressed (Fig. 4). The synergistic action of insulin and HG on the up-regulation of MHC was clearly demonstrated by a quantitative densitometric analysis (Fig. 5B).

Glucose-dependent opposing effects of insulin on SIRT1 and FoxO3a are achieved through PI3-kinase and mTOR activities

The myogenic action of insulin/IGFs has been shown to be mediated through the activation of PI 3-kinase and Akt signaling pathways (7, 23, 25, 53). In addition, several lines of evidence have demonstrated mTOR involvement in myogenesis (39, 47). To test whether the activities of PI 3-kinase and mTOR are involved in the regulation of SIRT1 and FoxO3a contents in response to insulin under LG or HG conditions, we used LY294002 and rapamycin, potent inhibitors of PI 3-kinase and mTOR, respectively. Consistent with previous reports (8, 26, 41), the myogenic action of insulin as assessed by MHC expression, clearly detectable only in the presence of HG, was completely abolished by these compounds (Fig. 6A and 6C, *lower panels, lanes 4-6*). Importantly, these compounds also inhibited the insulin-mediated suppression of SIRT1, while increasing its cellular content during 24 h HG exposure (Fig. 6A and 6C, *upper panels, lanes 4-6*). Interestingly, these compounds were also effective under LG conditions, and completely abolished insulin's action (Fig. 6A and 6C, *middle panels, lanes 1-3*), although insulin exerted completely opposing effects on the regulation of SIRT1 and FoxO3a abundances (Fig. 6A and 6B, *upper and middle panels, lane 2 vs. lane 5*). We also monitored insulin-dependent phosphorylations of Akt and S6 in the presence of LY294002 and rapamycin (Fig. 6B and 6D). As expected, LY294002 abolished insulin-dependent phosphorylation of Akt (S473 and T308); on the other hand,

rapamycin tended to increase insulin-dependent Akt phosphorylation (Figure 6B and 6D, *first and second panels*) as previously reported (52). Phosphorylation of S6 was high under basal conditions but was slightly induced by insulin, and either LY294002 or rapamycin had a negative effect on this phosphorylation (Fig. 6B and 6D, *fourth panel*).

Reduction of SIRT1 activity is not sufficient to restore C2C12 differentiation status in the presence of insulin

Finally, we attempted to restore the poor differentiation seen under LG conditions by exposure to sirtinol, an inhibitor of SIRT1 (20, 32). Consistent with a previous study (14), 24 h exposure to sirtinol under LG conditions restored MHC expression to levels comparable to those observed in HG-exposed C2C12 myotubes (Fig. 6E, *third panel, lanes 1-4*). However, sirtinol failed to restore MHC expression (Fig. 6E, *third panel, lanes 5~8*) in the presence of insulin, a condition in which FoxO3a is highly expressed (*second panel, lanes 5-8*).

Changes in extracellular glucose levels during the course of overnight incubation

To precisely document the importance of ambient glucose levels in the phenomena described above, extracellular glucose concentrations were monitored during the course of overnight incubation of differentiated C2C12 myotubes. As shown in Fig. 7, extracellular glucose levels gradually decreased but remained high levels (approximately 13 mM) even after an 18 h incubation when differentiated C2C12 myotubes were cultured in HG-DMEM (*closed circles*), while glucose in the medium was almost completely exhausted when the cells were cultured in LG-DMEM for 18 h (*open circles*).

DISCUSSION

In the present study, we demonstrated that low serum-induced differentiation of C2C12 cells is significantly influenced by extracellular glucose levels (Fig. 1), concurrently with glucose-dependent alterations in the amounts and subcellular localizations of SIRT1 and FoxO3a (Fig. 2 and 3) both of which have recently been implicated as negative regulators of myogenesis (14) (21). Consistent with many previous reports studying myogenesis by using mostly IGFs (6, 7, 12), insulin also exerts a myogenic action in a manner dependent upon PI 3-kinase and mTOR activities, as assessed by MHC expression (Fig. 6); however, we cannot rule out the possibility that insulin activates IGF-I receptors since a relatively high concentration of insulin was required to stimulate MHC expression within 24 hours (Fig. 5). Surprisingly, however, we found the potency of insulin's myogenic action to also be remarkably affected by extracellular glucose levels, and that insulin exerts its potent myogenic effect only in the presence of relatively high levels of glucose, while its potency is significantly compromised in the absence of sufficient glucose (Fig. 5), perhaps due to massive increases in SIRT1 and FoxO3a, serving as negative regulators of this process, which are induced by insulin treatment under LG conditions (Fig. 4 and 6). Taken together, these results reveal an important interplay between glucose availability and insulin in the regulation of myogenesis, which is achieved at least partially through alterations in the cellular contents and nuclear abundances of SIRT1 and FoxO3a. Our data also document opposing effects of insulin, depending upon glucose availability, and thereby on the regulation of SIRT1 and FoxO3a amounts in differentiating C2C12 myotubes (Fig. 6A). Since insulin/IGFs often display opposite biological effects, *e.g.* proliferation and differentiation, depending upon conditions and circumstances (8, 11, 28, 30), our

data presented herein provide a conceptual framework for understanding the mode of insulin actions (and presumably those of IGFs) by providing evidence that insulin's myogenic action is profoundly influenced by glucose availability mediated through regulation of SIRT1 and FoxO3a, both of which have been shown to be directly involved in the determination of cellular fates including proliferation, differentiation and senescence in various cell types (1, 4, 5, 44, 50, 51).

Effects of ambient glucose levels on the regulations of SIRT1 and FoxOs during myogenesis

A recent report revealed an important SIRT1 role in myogenesis by showing that overexpression of SIRT1 inhibited myogenesis, whereas either siRNA-mediated suppression of SIRT1 or sirtinol enhanced it by altering the acetylation states of MyoD and the histone acetylase p300/CBP (14). Although the SIRT1 expression level was shown to be slightly decreased upon differentiation of C2C12, the authors focused primarily on the importance of the regulation of its enzymatic activity rather than the expression level of SIRT1. In the present study, we found that SIRT1 expression is influenced by glucose levels and that SIRT1 abundance is significantly reduced in C2C12 myotubes differentiated under HG conditions, a culture condition obviously potentiating myogenesis (Fig. 1). In addition, 24 h exposure of differentiating C2C12 myotubes to HG (*days 5-6*) was also sufficient to decrease SIRT1 abundance (Fig. 2C), which apparently contributed to the potentiation of myogenesis as assessed by MHC expression levels (Fig. 5). Although acute redistribution was not observed with either glucose or insulin in the present study (Fig. 2B), recent reports revealed the existence of a nucleocytoplasmic shuttling mechanism for SIRT1 (24, 50). Thus, our data strongly

suggest that SIRT1 abundance and its localization being sensitively influenced by ambient glucose levels are also directly involved in the regulation of myogenesis as a prerequisite for regulating its enzymatic activity at suitable site(s). Consistent with this idea, a recent report demonstrated SIRT1 transcription to be regulated by metabolic states *via* the HIC1:CtBP corepressor complex (55). These changes in SIRT1 abundance in skeletal muscle cells may contribute not only to myogenesis but also metabolic adaptations during glucose deprivation, such as regulation of mitochondrial gene expression and fatty acid utilization (17, 29).

Similar to what was observed in SIRT1 regulation, we found decreases in both the cellular and the nuclear abundance of FoxO3a to also be coupled to the potentiation of myogenesis, which can be induced under HG conditions (Fig. 1, 3). These findings are consistent with a previous report showing that siRNA-mediated suppression of FoxOs enhanced myogenesis, while its overexpression inhibited differentiation (21). Although the importance of nucleocytoplasmic shuttling of FoxOs by posttranslational modifications such as the interplay between the Akt-mediated phosphorylation and the SIRT1-mediated deacetylation in response to growth factors and oxidative stress is well established (3, 4), acute redistribution of FoxO3a was not detected with changes in the ambient glucose level (Fig. 3B), while its nuclear exclusion was rapidly induced by insulin as previously reported (49). Since C2C12 myocytes also express other FoxO family transcription factors including FoxO1 and FoxO4, in addition to FoxO3a (21), our data at this stage cannot address the magnitude of the FoxO3a contribution to myogenic inhibitory action. However, our present data support previous reports showing that both SIRT1 and FoxO3a serve as negative myogenic regulators (14, 21), and also provide evidence supporting the important participation of these proteins in the

process of myogenesis achieved through the regulation of their amounts and subcellular localizations. As discussed below, the physiological importance of glucose-dependent alterations in cellular SIRT1 and FoxO3a abundance in myogenesis further underscores our striking finding that insulin exerts distinct effects regulating the abundances of these proteins depending upon glucose availability, which is tightly coupled with myogenic differentiation status (Fig. 4 and Fig. 5).

Interplay between ambient glucose and insulin in the regulation of SIRT1 and FoxO3a and its involvement in myogenesis

In the present study, we found that the effects of ambient glucose levels can be exerted within 24 h, according to the cellular abundances of both SIRT1 and FoxO3a (Fig. 2 and Fig. 3), in differentiating C2C12 myotubes. Similarly, the drastic changes in SIRT1 and FoxOs abundances in various tissues including skeletal muscle have been reported for *in vivo* experiments showing that starvation increases SIRT1 and FoxOs, which are re-suppressed by re-feeding within 24 h (15, 16, 38, 45). In this regard, we observed that the extracellular glucose concentration falls to less than 0.5 mM after a 24 h incubation when differentiating C2C12 cells are cultured in LG-DMEM (Fig. 7), even though DMEM containing 5 mM glucose (LG) is a conventional medium routinely utilized to maintain various cell types. This is probably because myotubes are post-mitotic multi-nuclear cells that consume vast amounts of glucose as an energy source. Consequently, the cells cultured under LG conditions were perhaps experiencing an environment similar to the condition of glucose starvation, of varying degrees, during the 24 h incubation, even though the LG media were replaced daily, while when cells were cultured under HG conditions they were continually exposed to

pathophysiologically high levels of glucose for 24 h (Fig. 7). Hence, our observations indicate that these gross culture environments including the consequences of glucose consumption and/or exhaustion, not just the initial glucose concentration, apparently contribute to regulating SIRT1 and FoxO3a, which is in turn responsible for the modulation of myogenesis during the 24 h incubation.

One of the most intriguing observations is that insulin remarkably increases the cellular contents of both SIRT1 and FoxO3a under only LG conditions (Fig. 4A, *lanes 1~6*), while insulin completely fails to increase FoxO3a, instead decreasing the SIRT1 amount in the presence of HG (Fig. 4A, *lanes 7~12*). Moreover, our most striking finding is that insulin is unable to exert its myogenic action under LG conditions (Fig. 5A, *lanes 1~5*), while its potency is maximized under HG conditions, as assessed by MHC expression levels (Fig. 5A, *lanes 6-10*). Although it is well established that insulin and IGFs serve as potent myogenic stimulators (7, 12, 21), our present data reveal insulin's myogenic action to be significantly influenced by glucose availability. In addition, our data strongly suggest that the insulin-induced massive accumulations of these negative myogenic transcriptional regulators, SIRT1 and FoxO3a, provoked under LG conditions in differentiating C2C12 myotubes counteract the myogenic stimulatory potency that insulin intrinsically possesses.

The important functional interrelationships between SIRT1 and FoxOs have been established in various organisms (18), and SIRT1 and FoxOs including FoxO3a have been shown to physically interact with each other to regulate their functions in a wide array of cell types (4, 10, 36). Thus, although either SIRT1 or FoxO3a alone is reportedly able to interfere with the process of myogenesis (21), it is likely that SIRT1 and FoxO3a are cooperatively involved in this interference, properly responding to

alterations of culture circumstances such as glucose availability and the presence of insulin. In an attempt to evaluate the contribution of the myogenic inhibitory actions of these proteins, we utilized sirtinol to eliminate the deacetylase activity of SIRT1, and found that although the poor differentiation state under LG conditions is significantly restored within 24 h by sirtinol (Fig. 6C, *lower panel, lanes 1~4*) as previously reported (14), the sirtinol-dependent restoration of increased MHC expression is completely abolished in the presence of insulin (*lanes 5~8*). These data not only further confirm the opposing actions of insulin, i.e. that insulin can serve as a negative, rather than a positive, myogenic factor when glucose availability is low, but also suggest that the reduction of SIRT1 enzymatic activity alone might be insufficient to overcome the poor differentiation status in the presence of insulin, a condition under which FoxO3a is remarkably increased (Fig. 6C, *middle panel, lanes 5~8*). Thus, the considerably augmented FoxO3a may still be functional to some extent even in the presence of insulin during a 24 h incubation, which perhaps contributes to interference with the promotion of myogenesis. Moreover, since both FoxO1 and FoxO3a have been shown to increase the expressions of the ubiquitin ligases MAFbx and MuRF1, responsible for muscle atrophy *via* increased protein degradation (46, 49), the increased FoxO3a may also participate in the stimulation of protein degradation governed by these FoxO-inducible ubiquitin ligases. In any case, together with previous studies showing that overexpression of FoxOs results in reduced muscle mass in transgenic mice (27) and also retards myogenesis (21), our present data suggest that the massively increased FoxO3a plays a pivotal role in exerting the inhibitory actions of insulin at least under these experimental conditions.

Another interesting observation presented in this study is that the opposing

actions of insulin depending upon ambient glucose levels were both completely abolished by LY294002 (Fig. 6A) or rapamycin (Fig. 6B). These results indicate crucial involvements of the PI 3-kinase and mTOR activities stimulated by insulin in exerting insulin actions on SIRT1 and FoxO3a depending upon ambient glucose environments (Fig. 6), although the insulin-induced decrease in FoxO3a under HG conditions is not apparent due to its undetectable expression (Fig. 6AB, *middle panels, lanes 4~6*), as was the case with that observed in SIRT1 (*upper panels, lanes 4~6*). Recently, Southgate *et al.* showed that the elevation of FoxO1 protein levels induced the non-phosphorylated form of 4EBP1, followed by reductions in Raptor and mTOR protein amounts (48). Together with our finding that the opposing actions of insulin on FoxO3a protein levels which depend upon ambient glucose concentrations are both abolished by rapamycin (Figure 6), it is reasonable to speculate that insulin stimulates either negative or positive feedback loops on the mTOR-FoxO axis, depending on ambient glucose levels. Future work should be directed toward increasing our understanding of the mechanisms underlying the differential effects of insulin on SIRT1 and FoxOs depending upon glucose availability in order to solve the mystery of how insulin/IGFs exert diverse, and in some instances opposing, biological actions.

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Figure legends

Fig.1. Low-serum induced C2C12 differentiation was affected by extracellular glucose levels

(A, B) C2C12 myoblasts were cultured in LG-DMEM (5 mM glucose) + 10% FBS, then switched to differentiation media (DMEM + 2% calf serum; D-medium) containing 5 mM (LG) or 22.5 mM (HG) glucose (*Day 0*). The cells were continuously cultured with D-medium changes every 24 h. (A) At *Day 4* of differentiation under LG (*panel a*) or HG (*panel b*) conditions, the myotube formations were observed using a microscope. (B) Relative numbers of myotubes defined as multinuclear myotubes which contained more than 5 nuclei, were determined. Statistical analysis was performed using the paired *t*-test. $^{\#}P<0.05$ (n=5). (C) On the indicated day, cell lysates were prepared, and the same amounts of proteins were subjected to western blotting using anti-myosin, anti-myogenin and anti-β–actin antibodies. Each experiment was repeated three times and representative results are shown.

Fig.2. Cellular abundance of SIRT1 and its localization are controlled by extracellular glucose levels during myogenesis

(A) C2C12 myoblasts were differentiated into myotubes under LG conditions (5 mM glucose in DMEM + 2% CS, low glucose; LG) or HG conditions (22.5 mM glucose in DMEM + 2% CS, high glucose; HG) for 6 days. The myotubes were then fixed and immunostained using anti-SIRT1 antibody (*a, c*) or anti-IgG as a negative control (*e*). DAPI staining was performed at the same time to confirm the position of the nucleus (*b, d, f*). (B) C2C12 myoblasts were differentiated into myotubes under LG conditions for 6

days. The myotubes were then incubated under LG or HG conditions for 2 h. Cells were fixed, and immunostained using anti-SIRT1 antibody and DAPI. (C) C2C12 myoblasts were differentiated into myotubes under LG or HG conditions for the indicated days. The cell lysates were prepared as described in *Materials and Methods* and the same protein samples were subjected to Western blotting using anti-SIRT1 antibody. (D) C2C12 myoblasts were differentiated into myotubes under LG conditions for 6 days. Next, the media were switched to LG or HG conditions and the myotubes were then further incubated for the indicated times. The cell lysates were prepared as described in *Materials and Methods* and the same protein amounts were subjected to Western blotting using anti-SIRT1 antibody. (E) Densitometric analysis of (D). Statistical analysis was performed using one-way ANOVA as described in *Materials and Methods* ($^{\#}P<0.05$ compared to control (0 h), n=3). (F, G) C2C12 myoblasts were differentiated into myotubes under HG conditions for 6 days. Next, the media were switched to LG conditions and the myotubes were then further incubated for the indicated time. (F) The cell lysates were subjected to Western blotting using anti-SIRT1 antibody. (G) Total RNAs were purified and subjected to Real Time PCR analysis to measure SIRT1 mRNA amounts as described in *Materials and Methods*. Statistical analysis was performed using the paired *t*-test. $^{\#}P<0.05$ (n=3). All experiments were performed at least three times and similar results were obtained.

Fig.3. Cellular abundance of FoxO3a and its localization are controlled by extracellular glucose levels during myogenesis

(A) C2C12 myoblasts were differentiated into myotubes under LG conditions (5 mM glucose in DMEM + 2% CS, low glucose; LG) or HG conditions (22.5 mM glucose in

DMEM + 2% CS, high glucose; HG) for 6 days. The myotubes were then fixed and immunostained using anti-FoxO3a antibody (*a, c*) or anti-IgG as a negative control (*e*). DAPI staining was performed at the same time to confirm the position of the nucleus (*b, d, f*). (B) C2C12 myoblasts were differentiated into myotubes under LG conditions for 6 days. The myotubes were then incubated under LG or HG conditions for 2 h. Cells were fixed and immunostained using anti-FoxO3a antibody and DAPI. (C) C2C12 myoblasts were differentiated into myotubes under LG or HG conditions for the indicated days. The cell lysates were prepared as described in *Materials and Methods* and the same protein amounts were subjected to Western blotting analysis using anti-FoxO3a antibody. (D) C2C12 myoblasts were differentiated into myotubes under LG conditions for 6 days. Next, the media were switched to LG or HG conditions and the myotubes were then further incubated for the indicated time. The cell lysates were subjected to Western blotting analysis using anti-FoxO3a antibody. (E) Densitometric analysis of (D). Statistical analysis was performed using one-way ANOVA followed by Tukey's posttest ($^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ (n=3) compared to control (0 h, HG)). (F, G) C2C12 myoblasts were differentiated into myotubes under HG conditions for 6 days. Next, the media were switched to LG conditions and the myotubes were then further incubated for the indicated time. (F) The cell lysates were subjected to Western blotting using anti-FoxO3a antibody. (G) Total RNA was purified and subjected to Real Time PCR analysis to measure FoxO3a mRNA amounts as described in *Materials and Methods*. Statistical analysis was performed using the paired *t*-test. $^{\#\#\#}P<0.001$ (n=3). All experiments were performed at least three times and similar results were obtained.

Fig.4. Effects of insulin on SIRT1 and FoxO3a abundances depend upon

extracellular glucose levels

(A) C2C12 myoblasts were differentiated into myotubes under LG conditions for 6 days. Then, the media were switched to HG (22.5 mM glucose in DMEM + 2% CS) or control LG (5 mM glucose in DMEM + 2% CS) in the presence or absence of the indicated concentration of insulin for 24 h. The amounts of SIRT1, FoxO3a and β-actin were monitored by Western Blotting (B) Densitometric analysis of SIRT1 abundance during the time course experiments. Statistical analysis was performed using one way ANOVA followed by Tukey's posttest ($^{*}P<0.05$, $^{**}P<0.01$ (n=3) compared to LG control (LG, 0 nM insulin); $^{\#}P<0.05$, $^{\#\#}P<0.01$ (n=3) compared to HG control (HG, 0 nM insulin)) (C) Densitometric analysis of FoxO3a abundance during the time course experiments. Statistical analysis was performed using one way ANOVA followed by Tukey's posttest (LG; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ (n=3) compared to LG control (LG, 0 nM insulin))

Fig.5. Insulin myogenic potency is altered by extracellular glucose levels

(A) C2C12 myoblasts were differentiated into myotubes under LG conditions for 6 days. Then, the media were switched to HG (22.5 mM glucose in DMEM + 2% CS) or control LG (5 mM glucose in DMEM + 2% CS) in the presence or absence of the indicated concentration of insulin for 24 h. The amounts of MHC were monitored by Western Blotting. (B) Densitometric analysis of (A). Statistical analysis was performed using one way ANOVA followed by Tukey's posttest (HG; $^{\#}P<0.05$ (n=3) compared to control (HG, 0 nM insulin)).

Fig.6. Dissecting the interplay between glucose and insulin effects on SIRT1 and

FoxO3a expressions and their involvements in myogenesis

(A, C) C2C12 myoblasts were differentiated into myotubes under LG conditions for 6 days. The media were then switched to HG or LG with or without 100 nM insulin in the presence or absence of 10 μM LY294002 (A) or 50 nM rapamycin (C) for 24 hours. The amounts of MHC, SIRT1 and FoxO3a were monitored by Western Blotting. (B, D) C2C12 myoblasts were differentiated into myotubes under LG conditions for 6 days. Then, the myotubes were treated with 100 nM insulin for 5 min in the presence or absence of 10 μM LY294002 (B) or 50 nM rapamycin (D) under LG or HG conditions. The phosphorylations of Akt (S473 and T308) and S6 as well as total Akt and β-actin were analyzed by Western Blotting. (E) C2C12 myoblasts were differentiated into myotubes under LG conditions for 6 days. The cells were then cultured for 24 h in the presence of the indicated concentrations of sirtinol under LG conditions (*lanes 1~4*) or LG plus 100 nM insulin (*lanes 5~8*). The amounts of MHC, SIRT1 and FoxO3a were monitored by Western Blotting. All experiments were repeated at least three times and representative results are shown.

Fig. 7. Glucose consumption by C2C12 myotubes

C2C12 myoblasts were differentiated into myotubes for 6 days. The media were then switched to fresh medium containing LG (*closed circles*) or HG (*open circles*) and glucose concentrations were measured at the indicated time.