High fructose-mediated attenuation of insulin receptor signaling does not affect PDGF-induced proliferative signaling in vascular smooth muscle cells

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Abstract

Insulin resistance is associated with accelerated atherosclerosis. Although high fructose is known to induce insulin resistance, it remains unclear as to how fructose regulates insulin receptor signaling and proliferative phenotype in vascular smooth muscle cells (VSMCs), which play a major role in atherosclerosis. Using human aortic VSMCs, we investigated the effects of high fructose treatment on insulin receptor substrate-1 (IRS-1) serine phosphorylation, insulin *versus* platelet-derived growth factor (PDGF)-induced phosphorylation of Akt, S6 ribosomal protein, and extracellular signal-regulated kinase (ERK), and cell cycle proteins. In comparison with PDGF (a potent mitogen), neither fructose nor insulin enhanced VSMC proliferation and cyclin D1 expression. D-[^{14}C(U)]fructose uptake studies revealed a progressive increase in fructose uptake in a time-dependent manner. Concentration-dependent studies with high fructose (5 to 25 mM) showed marked increases in IRS-1 serine phosphorylation, a key adapter protein in insulin receptor signaling. Accordingly, high fructose treatment led to significant diminutions in insulin-induced phosphorylation of downstream signaling components including Akt and S6. In addition, high fructose significantly diminished insulin-induced ERK phosphorylation. Nevertheless, high fructose did not affect PDGF-induced key proliferative signaling events including phosphorylation of Akt, S6, and ERK and expression of cyclin D1 protein. Together, high fructose dysregulates IRS-1 phosphorylation state and proximal insulin receptor signaling in VSMCs, but does not affect

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PDGF-induced proliferative signaling. These findings suggest that systemic insulin resistance rather than VSMC-specific dysregulation of insulin receptor signaling by high fructose may play a major role in enhancing atherosclerosis and neointimal hyperplasia.

Keywords

Fructose; Insulin receptor substrate; Insulin; PDGF; Vascular smooth muscle cells; Proliferation

1. Introduction

Insulin resistance, characterized by metabolic abnormalities including glucose intolerance and dyslipidemia, is a risk factor of accelerated atherosclerosis (Bornfeldt and Tabas, 2011; Semenkovich, 2006; Taegtmeyer, 1996). While altered metabolic milieu is known to promote proatherogenic phenotype (Semenkovich, 2006), the contribution of vascular wall-specific insulin resistance toward atherosclerotic lesion progression remains unclear (Bornfeldt and Tabas, 2011; Semenkovich, 2006). In particular, vascular smooth muscle cells (VSMCs) that play a major role in atherosclerosis have been shown to exhibit proliferative and proapoptotic phenotypes upon target-specific deletion of insulin receptor signaling components (Lightell et al., 2011; Martinez-Hervas et al., 2014). For instance, insulin receptor gene deficiency in VSMCs leads to a decrease in insulin-induced Akt phosphorylation and an increase in extracellular signal-regulated kinase (ERK) phosphorylation with an accompanying increase in cell proliferation (Lightell et al., 2011). In a different study, siRNA-mediated downregulation of insulin receptor substrate-2 (IRS-2) in VSMCs diminishes insulin-induced phosphorylation of Akt and ERK thereby inducing a proapoptotic phenotype (Martinez-Hervas et al., 2014). Thus, dysregulation of insulin signaling in VSMCs at the level of insulin receptor and IRS-2 may result in proliferative and proapoptotic phenotypes, which would enhance and exacerbate atherosclerosis, respectively. Although high fructose consumption is known to induce insulin resistance and promote atherosclerosis (D'Angelo et al., 2005; Lu et al., 2013; Ning et al., 2015), it is unknown as to how fructose uptake in VSMCs regulates insulin receptor signaling and proliferative phenotype.

Previous studies demonstrate that high fructose diet induces insulin resistance in a rat model, as revealed by hyperinsulinemic-euglycemic clamp technique that shows a significant reduction in glucose infusion rate to maintain euglycemia (D'Angelo et al., 2005). In addition, high fructose diet-induced insulin resistance results in exaggerated atherosclerosis and neointima formation with a significant increase in smooth muscle cell accumulation (Lu et al., 2013; Ning et al., 2015). Since platelet-derived growth factor (PDGF) is a potent mitogen released at the site of arterial injury (Barrett and Benditt, 1987; Heldin and Westermark, 1999; Rubin et al., 1988), it is likely that high fructose-induced increase in neointima formation may occur through enhanced PDGF receptor signaling. Previously, we have shown that PDGF not only increases VSMC proliferation but also attenuates insulin-induced insulin receptor substrate (IRS-1/IRS-2)-associated PI 3-kinase/Akt signaling (Zhao et al., 2011). In the present study, we tested the hypothesis that high fructose-mediated dysregulation of insulin receptor signaling is associated with enhanced VSMC proliferation.

Using human aortic VSMCs, we determined the effects of high fructose on: i) IRS-1 serine phosphorylation and IRS-1/IRS-2 expression; ii) insulin *versus* PDGF-induced changes in the phosphorylation of Akt, S6 ribosomal protein (a downstream target of mTOR/p70S6K signaling), and ERK; and iii) cell cycle proteins and proliferation.

2. Materials and methods

2.1. Materials

Recombinant human PDGF-BB was purchased from R&D Systems (Minneapolis, MN). Human insulin (Novolin R) was obtained from local pharmacy. D-[U-^{14}C]fructose (specific activity: 240-360 mCi/mmol) was purchased from Moravek Biochemicals (Brea, CA). D-fructose was purchased from Sigma Chemical (St. Louis, MO). L-fructose was purchased from Omicron Biochemicals, Inc. (South Bend, IN). The primary antibodies for phospho-IRS1$^{Ser636/639}$ (2388), IRS-1 (3407), IRS-2 (3089), phospho-44/42 MAPK (ERK1/2; 4695), 44/42 MAPK (ERK1/2; 9102), phospho-Akt^{Thr308} (2965), Akt (4691), phospho-S6 ribosomal protein$^{Ser235/236}$ (4857), S6 ribosomal protein (2217), phospho-PDGFRβ^{Tyr751} (3161), PDGFRβ (3169), p27^{Kip1} (3686), cyclin D1 (2922), phospho-Rb^{Ser795} (9301), and β-actin (8457) were purchased from Cell Signaling Technology (Danvers, MA). All other chemicals were from Fisher Scientific (Fair Lawn, NJ) or Sigma Chemical (St. Louis, MO).

2.2. Cell culture and treatments

Human aortic VSMCs, vascular cell basal medium and smooth muscle growth supplement (SMGS) were purchased from ATCC (Manassas, VA). SMGS constituents and their final concentrations after addition to vascular cell basal medium were as follows: 5% FBS (vol/vol), 5 ng/ml human basic fibroblast growth factor, 5 ng/ml human epidermal growth factor, 5 μg/ml insulin, 50 μg/mL ascorbic acid, 10 mM L-glutamine. VSMCs (passages 3–5) were maintained in vascular cell basal medium containing SMGS (complete medium), 5.5 mM D-glucose, and antibiotic/antimycotic solution in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. After the attainment of confluence (~6–7 days), VSMCs were trypsinized, centrifuged, and seeded onto petri dishes or multiwell plates. Subconfluent VSMCs were maintained under SMGS (serum)-deprived conditions for 48 h to achieve quiescence and then subjected to treatments as described in the legends to the respective figures. Equimolar concentrations of L-fructose were used as the vehicle controls for D-fructose in respective experiments.

2.3. Cell proliferation

Subconfluent VSMCs were serum-deprived for 48 h and then treated with D-fructose (5.5 or 25 mM), insulin (100 nM) or PDGF (30 ng/ml) for 96 h. Fresh serum-free media containing the respective treatments were replaced every 48 h. VSMCs were then trypsinized and the changes in cell number were determined using Countess Counter (Life Technologies, Carlsbad, CA), as described (Pyla et al., 2013).

2.4. Immunoblot analysis

Immunoblot analysis was performed as described (Osman and Segar, 2016). VSMC lysates (20 μg protein per lane) were subjected to electrophoresis using precast 4–12% NuPage

mini-gels (Life Technologies). The resolved proteins were then transferred to PVDF membranes (EMD Millipore, Billerica, MA). Subsequently, the membranes were blocked in 5% nonfat milk and probed with the respective primary antibodies. The immunoreactivity was detected using HRP-conjugated horse anti-mouse secondary antibody (7076; Cell Signaling) or goat anti-rabbit secondary antibody (7074; Cell Signaling) followed by enhanced chemiluminescence (ECL; Thermo Scientific, Wilmington, DE). The protein bands were quantified by densitometric analysis using Image J.

2.5. D-[U-^{14}C] fructose uptake studies

Subconfluent VSMCs (100,000 cells/well) were serum-deprived for 48 h and then washed twice with Krebs-Ringer-phosphate (KRP) buffer containing 130 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 10 mM Na_2HPO_4, 0.8 mM $CaCl_2$ (pH 7.4). The washing buffer was removed completely and replaced with 500 μl radioactive cocktail (0.5 mM D-fructose and 0.5 μCi D-[U-^{14}C]fructose in KRP buffer). After incubation for different time intervals (2-30 min) at 37°C, the cells were washed twice with ice-cold KRP buffer and then lysed with 500 μl lysis buffer (0.2 N NaOH and 1% SDS) with intermittent shaking for 10 min. The lysates were analyzed using liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA, Model LS-6500).

2.6. Statistical analysis

Results are expressed as the means ± S.E.M. of at least three separate experiments. Statistical analyses of the data were performed using one-way analysis of variance (ANOVA) followed by Bonferroni t-test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. PDGF, but not fructose or insulin, enhances VSMC proliferation

Isolated aortic VSMCs from high fructose-fed rats have been shown to exhibit enhanced proliferation in response to serum trophic factors (Miatello et al., 2001). However, a direct regulatory effect of high fructose on VSMC proliferation has not yet been examined. Insulin has been shown to enhance VSMC proliferation or maintain VSMC quiescence (Pfeifle and Ditschuneit, 1981; Wang et al., 2003). In addition, previous studies have shown that insulin treatment does not result in an increase in VSMC proliferation (Staiger et al., 2005). PDGF is a potent mitogen and is known to enhance VSMC proliferation (Owens et al., 2004). In the present study, we determined the effects of high fructose *versus* insulin or PDGF on VSMC proliferation and cell cycle proteins. As shown in **Fig. 1A**, fructose or insulin did not show significant effects on VSMC proliferation, whereas PDGF exposure led to an increase in VSMC proliferation by ~2 ± 0.5 fold. Co-incubation with insulin did not result in significant changes in PDGF-induced VSMC proliferation. In addition, fructose or insulin treatment was not associated with a decrease in the expression of p27[kip1] (a cell cycle inhibitor) or an increase in the expression of cyclin D1 (a cell cycle protein) (**Fig. 1B**). In parallel, PDGF treatment led to a significant decrease in the expression of p27[kip1] by ~56 ± 3% with an accompanying increase in cyclin D1 expression by ~6 ± 1-fold. PDGF-induced changes in p27[kip1] and cyclin D1 expression were not significantly altered upon co-

incubation with insulin. Furthermore, PDGF enhanced the phosphorylation of retinoblastoma (Rb) protein by ~3.8 ± 0.1 fold (data not shown). Thus, treatment of VSMCs with PDGF, but not fructose or insulin, resulted in the transition to proliferative phenotype.

3.2. Fructose uptake occurs in a time-dependent manner in VSMCs

GLUT5 (fructose transporter) expression and fructose uptake have been demonstrated in several cell types including adipocytes and skeletal muscle cells (Buchs et al., 1998; Fukuzawa et al., 2013; Hajduch et al., 1998; Hajduch et al., 2003). Although we and several investigators have reported the expression of GLUT5 in VSMCs (Liu et al., 2011; Pyla et al., 2013), the ability of VSMCs to transport fructose has not been examined. As shown in **Fig. 2**, the uptake of D-[U-^{14}C]fructose occurred in a time-dependent manner in VSMCs. A significant increase in fructose uptake was observed within 2 min followed by a progressive increase in fructose transport for up to 30 min.

3.3. High fructose treatment enhances IRS-1 serine phosphorylation in VSMCs

Previous studies with hepatic and skeletal muscle tissues have shown that high fructose induces IRS-1 serine phosphorylation, which is reflected by diminished insulin-induced IRS-1 tyrosine phosphorylation and PI 3-kinase activity (Bezerra et al., 2000; Wei et al., 2005). Hence, we examined the likely regulatory effects of D-fructose on IRS-1 serine phosphorylation state in VSMCs. As shown in **Fig. 3,** exposure of VSMCs to D-Fructose at 5.5, 11, and 25 mM concentrations led to a progressive increase in the phosphorylation of IRS-1 by ~2.3 ± 0.2-, 4.1 ± 0.5-, and 6.1 ± 1.2-fold, respectively. Under these conditions, there were no significant changes in the expression levels of IRS-1 and IRS-2 proteins. Thus, high fructose treatment in VSMCs has the potential to dysregulate the phosphorylation state of IRS-1, a key adapter protein in insulin receptor signaling.

3.4. High fructose attenuates insulin-induced phosphorylation of Akt, S6 ribosomal protein, and ERK in VSMCs

Previously, we have shown that PDGF-induced IRS-1 serine phosphorylation is associated with diminished insulin-induced PI 3-kinase/Akt signaling in VSMCs (Zhao et al., 2011). In addition, insulin resistance has been shown to suppress PI 3-kinase/Akt signaling with an accompanying activation of ERK signaling in aortic tissues (Jiang et al., 1999). To examine how fructose-induced IRS-1 serine phosphorylation impacts Akt and ERK signaling in VSMCs, we determined the effects of D-fructose pretreatment on acute insulin stimulation. As shown in **Fig. 4A**, D-fructose pretreatment at 5.5 mM, 11 mM, and 25 mM concentrations led to progressive decreases in insulin-induced phosphorylation of Akt, S6 ribosomal protein, and ERK1/2. In particular, D-fructose pretreatment at 25 mM concentration resulted in significant diminutions in insulin-induced phosphorylation of Akt, S6, and ERK1/2 by 69 ± 3%, 78 ± 3%, and 50 ± 4%, respectively.

3.5. High fructose does not affect PDGF-induced phosphorylation of PDGF receptor-β, Akt, S6 ribosomal protein, and ERK in VSMCs

To examine whether high fructose-mediated attenuation of agonist-induced signaling events is specific for insulin, we determined the effects of high fructose on PDGF receptor

signaling in parallel. As shown in **Fig. 4B**, D-fructose pretreatment at 25 mM concentration did not affect PDGF receptor-β expression, PDGF-induced PDGF receptor-β tyrosine phosphorylation, or PDGF-induced key proliferative signaling events including phosphorylation of Akt, S6, and ERK.

3.6. High fructose does not affect PDGF-induced VSMC proliferation or PDGF-induced changes in p27^{kip1} and cyclin D1 expression

To further confirm whether PDGF-induced proliferative signaling events are refractory to high fructose, we examined the changes in VSMC proliferation and expression levels of p27^{kip1} and cyclin D1. As shown in **Fig. 5A**, D-fructose pretreatment at 25 mM concentration did not result in significant changes in PDGF-induced VSMC proliferation. In addition, D-fructose pretreatment did not affect PDGF-mediated decrease in p27^{kip1} expression or increase in cyclin D1 expression (**Fig. 5B**).

4. Discussion

Previous studies have shown that in rodents fed a 20-40% fructose diet, circulating concentration of fructose increases at a range of 0.2 to 1 mM (Patel et al., 2015). Fructose is known to be transported passively into several tissues including skeletal muscle and adipocytes through plasma membrane-localized fructose transporter (glucose transporter-5, GLUT5) (Douard and Ferraris, 2008). The present study provides evidence for fructose uptake in VSMCs for the first time using radiolabeled ^{14}C-fructose. Furthermore, we and several other investigators have demonstrated the expression of GLUT5 mRNA in VSMCs and aortic tissues (Liu et al., 2011; Pyla et al., 2013). In addition to the contribution from GLUT5-mediated fructose uptake, elevation of intracellular fructose concentration can occur through *de novo* synthesis from high glucose. This is achieved through the polyol pathway that involves the activation of aldose reductase (a rate-limiting enzyme) and the intermediary formation of sorbitol (Lanaspa et al., 2013; Yasunari et al., 1995). In this regard, exposure of VSMCs to 25 mM glucose results in the elevation of polyol pathway metabolites including sorbitol and fructose (Liu et al., 2011). Importantly, 25 mM fructose treatment has been shown to enhance intracellular fructose to a similar level with an accompanying induction of GLUT5 mRNA in VSMCs (Liu et al., 2011). The present findings reveal that at 11 to 25 mM concentrations, fructose has the potential to enhance IRS-1serine phosphorylation in VSMCs, thereby attenuating insulin-induced phosphorylation of downstream signaling components such as Akt and ribosomal protein S6. In addition, high fructose treatment inhibits insulin-induced phosphorylation of ERK. Contrary to our hypothesis, high fructose-mediated disruption of insulin receptor signaling does not affect PDGF-induced VSMC proliferation or key proliferative signaling events as evidenced by sustenance in the phosphorylation state of Akt, S6, and ERK and the expression level of cyclin D1.

Our findings on fructose dysregulation of insulin receptor signaling in VSMCs are in conformity with previous studies, which demonstrate high fructose-induced insulin resistance in hepatic tissue and skeletal muscle (Bezerra et al., 2000; Wei et al., 2005). For instance, high-fructose diet in rats leads to significant decreases in insulin-induced IRS-1 tyrosine phosphorylation and IRS-1 association with PI 3-kinase in the liver and skeletal

muscle (Bezerra et al., 2000). In rat primary hepatocytes treated with high fructose, IRS-1 serine phosphorylation is increased with an accompanying decrease in insulin-induced IRS-1 tyrosine phosphorylation (Wei et al., 2005). From a mechanistic standpoint, fructose-mediated dysregulation of IRS-1 and the resultant suppression of downstream signaling events may be attributable to several factors including methylglyoxal accumulation and c-jun N-terminal kinase (JNK) activation (Dhar et al., 2008; Liu et al., 2011; Riboulet-Chavey et al., 2006; Wei et al., 2005). It is noteworthy that, in high-fructose diet-fed rats, a significant accumulation of methylglyoxal has been observed in aortic tissues (Liu et al., 2011). In addition, high fructose treatment (15 to 25 mM) under *in vitro* conditions has been shown to enhance methylglyoxal accumulation in aortic VSMCs (Liu et al., 2011; Wang et al., 2006). Together, these findings suggest that high fructose-mediated accumulation of methylglyoxal may promote IRS-1 serine phosphorylation, thereby disrupting insulin receptor signaling in VSMCs.

Previous studies have shown that in a rat model of obesity, there is a selective resistance to PI 3-kinase/Akt signaling but not ERK pathway in aortic tissues (Jiang et al., 1999). Furthermore, in VSMCs isolated from insulin receptor-deficient mice, a decrease in insulin-induced Akt phosphorylation is associated with an increase in ERK phosphorylation, which may decrease $p27^{Kip1}$ expression to enhance proliferation (Lightell et al., 2011). Such selective insulin resistance in vascular cells would augment the atherogenic potential in insulin-resistant states (Gogg et al., 2009; Jiang et al., 1999). However, high fructose treatment diminishes insulin-induced activation of Akt, S6 (a downstream target of mTOR), and ERK in VSMCs (present study). Notably, methylglyoxal, an intermediary metabolite of fructose, has been previously shown to inhibit not only IRS-1 tyrosine phosphorylation and Akt signaling but also insulin-induced activation of ERK (Riboulet-Chavey et al., 2006). Thus, high fructose-mediated disruption of insulin receptor signaling including ERK does not result in enhanced VSMC proliferation, as evidenced in the present study.

To further understand the relationship between high fructose exposure and VSMC proliferative phenotype, the present study has also examined whether dysregulated insulin receptor signaling is associated with altered PDGF receptor signaling. Previously, high glucose has been shown to increase aldose reductase activity and fructose formation thereby upregulating PDGF receptor-β expression (Campbell et al., 2003; Kasuya et al., 1999), which in turn results in enhanced PDGF-induced VSMC proliferation. The present findings reveal that in high fructose-treated VSMCs, PDGF receptor-β expression remains unchanged. In addition, high fructose does not affect PDGF-induced PDGF receptor-β tyrosine phosphorylation. Furthermore, PDGF-induced phosphorylation of key proliferative signaling events (e.g., Akt, S6, and ERK) and the expression of cell cycle protein (e.g., cyclin D1) remain essentially the same with our without high fructose treatment. These findings suggest that, under the conditions of VSMC-specific insulin resistance resulting from high fructose-induced IRS-1 serine phosphorylation, the growth-promoting effects of PDGF will be preserved in the vessel wall.

It is noteworthy that the exaggerated neointima formation observed in previous studies with IRS-1-deficient mice has been attributed to altered metabolic milieu in insulin-resistant state (Kubota et al., 2003). High fructose is known to induce metabolic changes in the liver

including enhanced *de novo* lipogenesis (Samuel, 2011). In addition, high fructose consumption may lead to hepatic insulin resistance and dysregulation of lipid/lipoprotein profile in the systemic circulation (Herman and Samuel, 2016; Stanhope et al., 2015). In conclusion, systemic insulin resistance rather than VSMC-specific dysregulation of insulin receptor signaling by high fructose may play a major role in enhancing atherosclerosis and neointimal hyperplasia.

Acknowledgements

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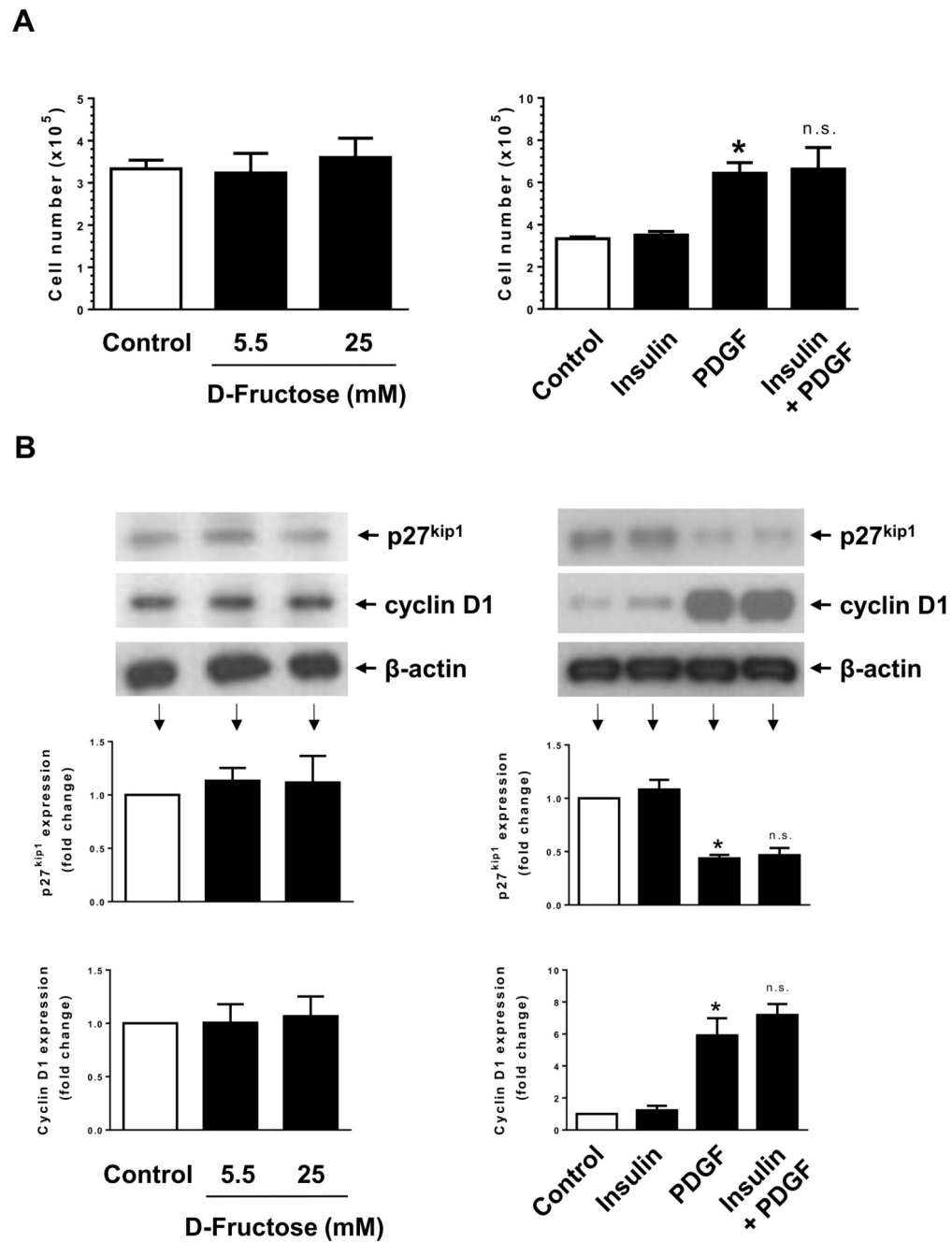
A

B

Fig. 1.
Effects of fructose, insulin, *versus* PDGF on VSMC proliferation. (**A**) Serum-deprived VSMCs were exposed to D-fructose (5.5 or 25 mM), insulin (100 nM), and/or PDGF (30 ng/ml) for 96 h to determine the changes in cell number using automated counter. (**B**) Serum-deprived VSMCs were exposed to similar concentrations of D-fructose (for 96 h) or insulin and/or PDGF (for 48 h) to determine the changes in p27^{kip1} and cyclin D1 expression by immunoblot analysis. *P < 0.05 compared with control; n.s. not significant compared with PDGF alone. n = 3.
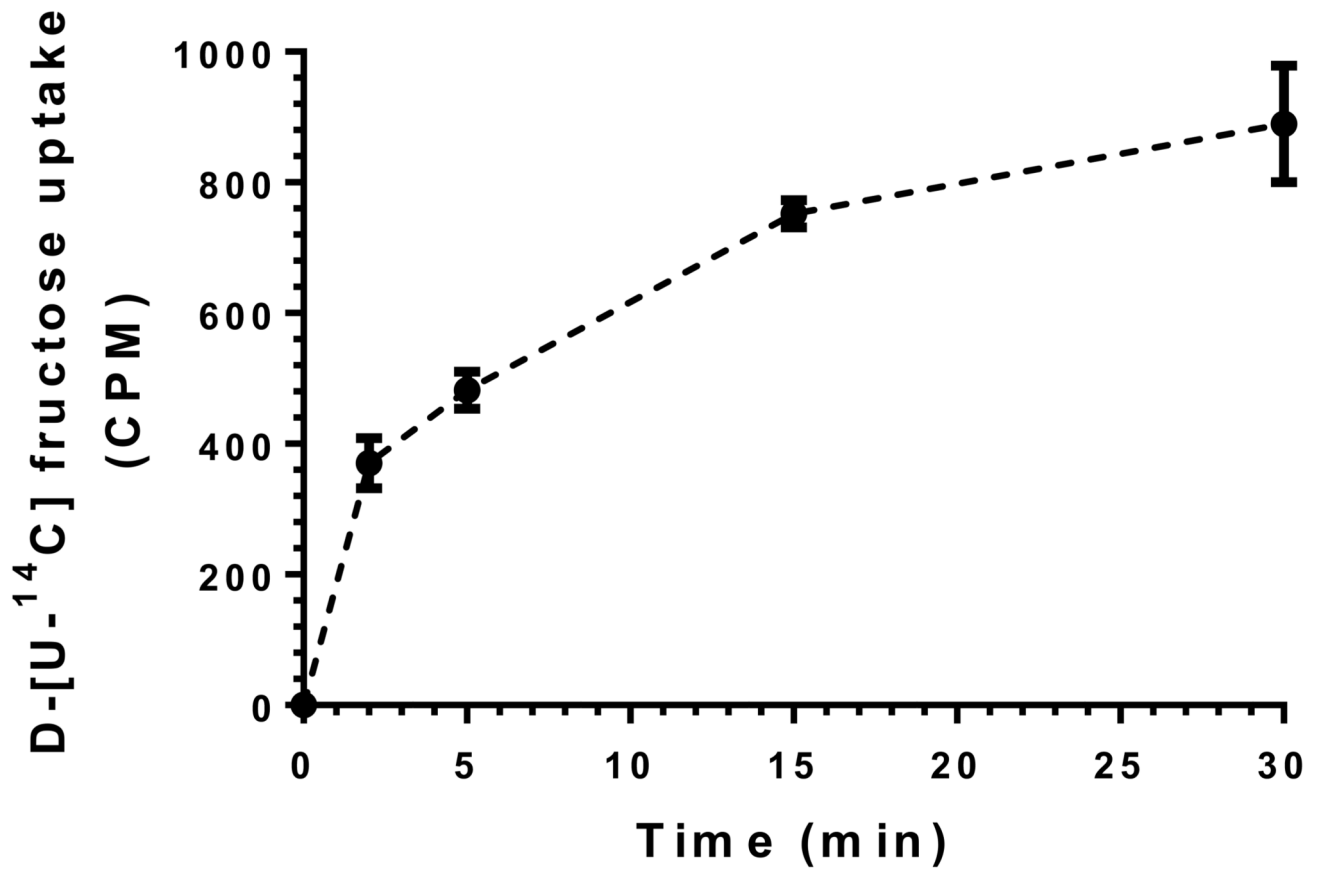
Fig. 2.
Time course of D-fructose uptake in VSMCs. Serum-deprived VSMCs were incubated in Krebs buffer containing 0.5 μCi D-[U-^{14}C] fructose for 2 to 30 min. Cellular uptake of radiolabeled fructose was then determined using liquid scintillation counter. n = 3.
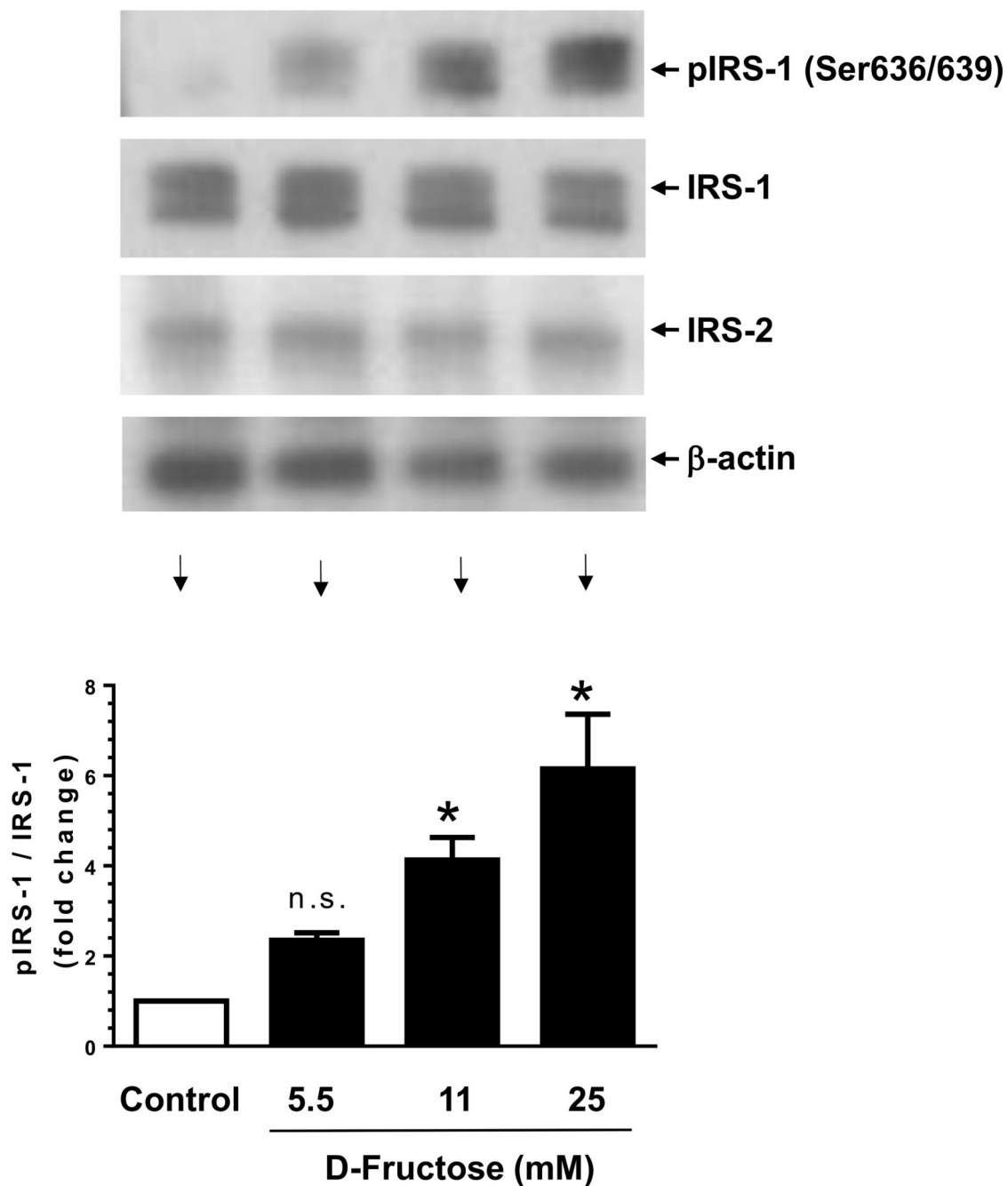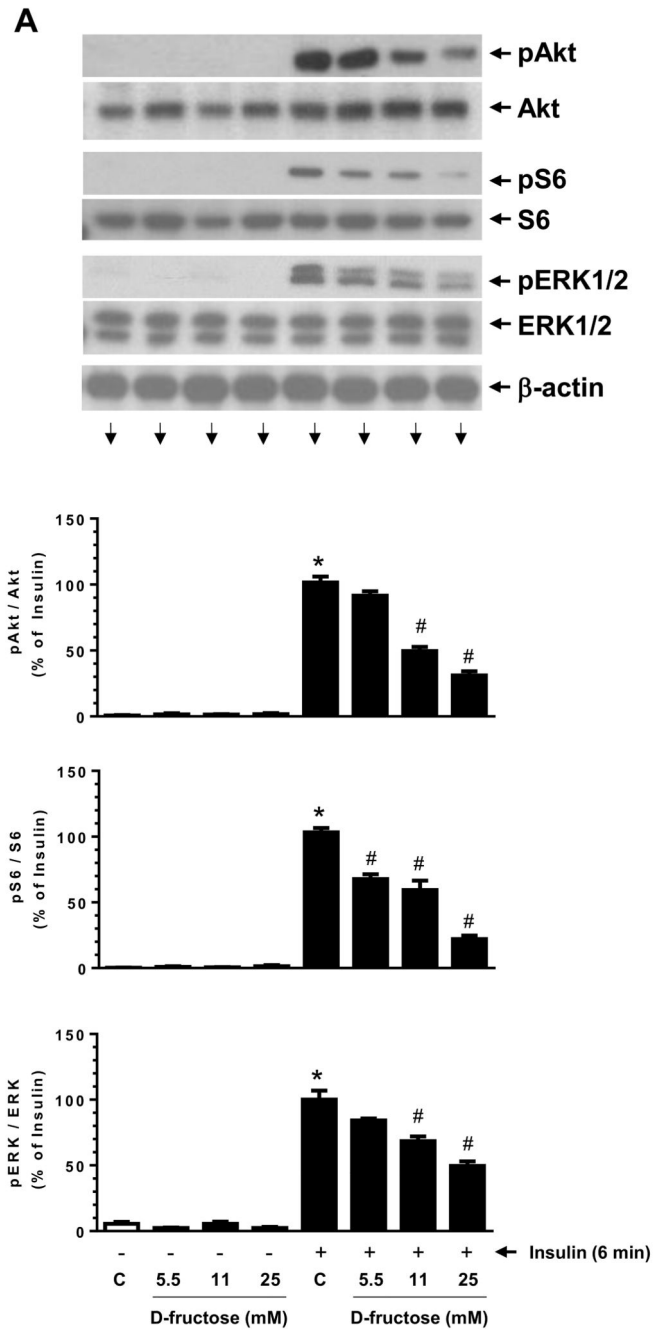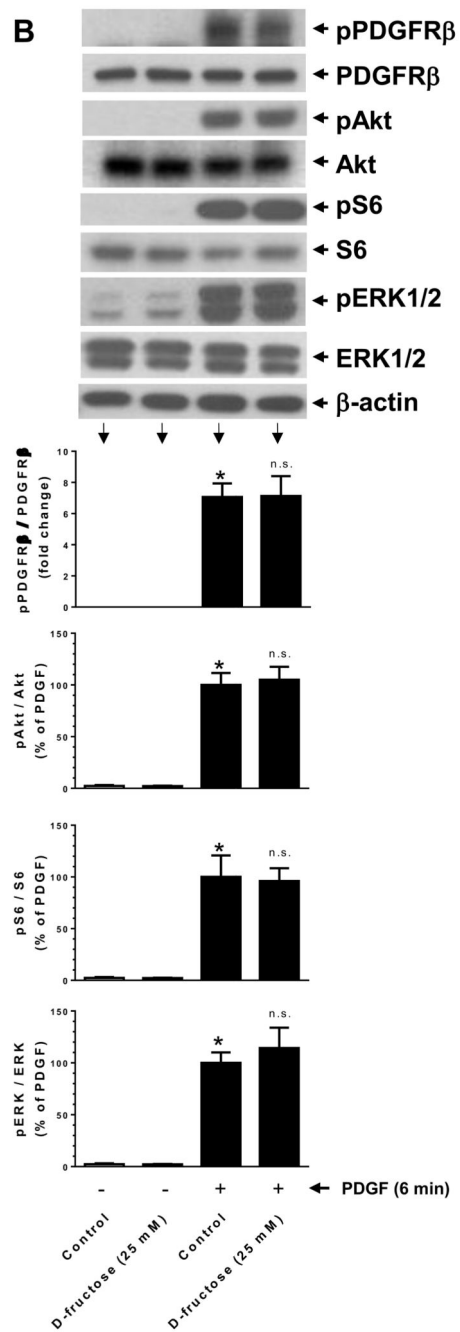
A

Fig. 4.
Effects of D-fructose pretreatment on insulin- *versus* PDGF-induced phosphorylation of
Akt, S6, and ERK in VSMCs. Serum-deprived VSMCs were pretreated with the indicated
concentrations of D-fructose for 48 h followed by acute stimulation with 100 nM insulin (**A**)
or 30 ng/ml PDGF (**B**) for 6 min. The cell lysates were then subjected to immunoblot
analysis using primary antibodies specific for pAkt, pS6, or pERK1/2. PDGF-treated cells
were also probed for pPDGFRβ and PDGFRβ expression. *P < 0.05 compared with

control; $^{\#}P < 0.05$ compared with insulin alone; n.s. not significant compared with PDGF alone. n = 3.
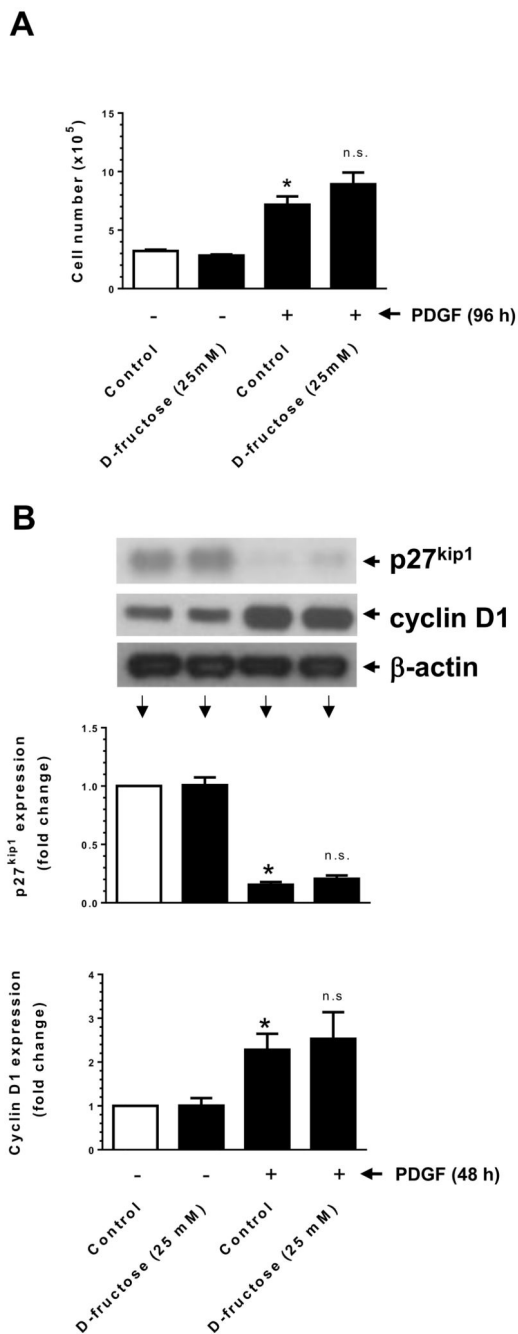
A

B

Fig. 5.
Effects of D-fructose pretreatment on PDGF-induced VSMC proliferation. Serum-deprived VSMCs were pretreated with 25 mM D-fructose for 48 h followed by exposure to 30 ng/ml PDGF for: (**A**) 96 h to determine the changes in cell number using automated counter; or (**B**) 48 h to determine the changes in p27^{kip1} and cyclin D1 expression by immunoblot analysis, as described. *P < 0.05 compared with control; n.s. not significant compared with PDGF alone. n = 3-6.