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Long-term high glucose concentration influences Akt, ERK1/2, and PTP1B protein expression in human aortic smooth muscle cells

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ABSTRACT

Hyperglycemia stimulates a plethora of intracellular signaling pathways within the cells of the vascular wall resulting in dysfunction-associated pathologies. Most of the studies reported so far explored the effect of rather short-time exposure of smooth muscle cells to high glucose concentrations. To mimic situation in Type 2 diabetes in which vascular wall is constantly exposed to circulating hyperglycemia, we report here the long-term (7 days) effect of high glucose concentration on human media artery smooth muscle cells. This consists in up-regulation of PTP1B protein expression, down-regulation of basal Akt phosphorylation, and elevation of basal ERK1/2 activation. Acute stimulation of cells in high glucose with insulin down-regulated PTP1B expression, slightly decreased ERK1/2 activity, and activated Akt, whereas oxidative stress up-regulated Akt and ERK1/2 phosphorylation. In conclusion, long-term high glucose and acute oxidative stress and insulin stimulation imbalance the expression of activated kinases Akt and ERK1/2 and of dephosphorylating PTP1B in the insulin signaling pathway.

Hyperglycemia and insulin resistance are major hallmarks of Diabetes mellitus associated vascular complications. The circulating high glucose concentration alters both the vascular endothelial cells (ECs) and the underneath smooth muscle cells (SMCs). The latter come in direct contact with blood glucose after injury—associated ECs removal, and in indirect contact while encountering the glucose permeating or transported through ECs layer. Recent data bring evidence on subtle changes in intracellular signaling mechanisms induced by hyperglycemia in vascular SMCs, such as decreased levels of inhibitory guanine nucleotide regulatory protein (G_i)α and of ANG(1–7), stimulation of TNF-α ectodomain shedding, up-regulation of connective tissue growth factor expression followed by accumulation of fibronectin and type I collagen [1–4]. In the abnormal release of collagen type I and vascular fibrosis participates the extracellular signal-regulated kinase (ERK) 1/2, $\alpha v\beta_3$-integrin, and TGF-β1 [5]. In vitro, high (25 mM) glucose caused increased production of integrin-associated protein by vascular SMCs [6]. Moreover, signaling via growth arrest gene 6 (Gas6)-receptor tyrosine kinase Ax1 showed glucose-concentration dependent effects, increasing vascular SMCs survival in low, 5.5 mM glucose, and cells migration in high, 27.5 mM glucose [7].

In hyperglycemia, arterial SMCs confront also a pro-oxidant environment enriched in reactive oxygen species (ROS) generated by glucose autoxidation and nonenzymatic glycation or as a result of cytosolic NADPH-oxidase activation and uncoupling of the mitochondrial respiratory chain [8]. Reportedly, fission-mediated fragmentation of mitochondria resulted in elevated production of ROS and in high glucose induced cell death [9]; others demonstrated that exacerbated oxidative stress causes a series of intracellular signaling events that culminate with SMCs apoptosis [1]. Oxidative stress promotes also vascular SMCs calcification associated with an increased expression and transactivity of Runx2, a key transcription factor for osteogenic differentiation [10].

Reportedly, insulin acts as a mitogenic stimulus that up-regulates vascular SMCs proliferation via Akt and ERK1/2 signaling pathways [11], and hyperinsulinemia promotes vascular remodeling and increased movement of SMCs from media to neointima causing an accelerated rate of restenosis [12,13]; in cultured vascular SMCs, chronic insulin treatment induced time-dependent upregulation of PI3-kinase-δ and Akt, and suppression of the expression and function of protein tyrosine phosphatase 1Beta (PTP1B) [13]; the latter process lead to enhanced PDGFR signaling and neointima hyperplasia [14]. Insulin-like Growth Factor-1 Receptor (IGF-1R) supports aortic SMCs growth, migration, and extracellular matrix formation [1], and stimulates SMCs proliferation [15]. Taken together, the Type 2 diabetes insults such as hyperglycemia, oxidative stress and insulin modify the intracellular signaling in vascular SMCs, trigger dedifferentiation of their normal contractile phenotype, and therefore alter the vasomotor tone. The defective insulin-induced vasodilation of diabetic vasculature can be restored by overexpression of active Akt [16].

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In this study, we tried to identify the effect of prolonged (7 days) effect of high glucose concentration on SMCs from the media layer of human aorta in an attempt to mimic situation in Type 2 diabetes in which vascular wall is constantly exposed to circulating hyperglycemia. Other reports show the effect of rather short-time exposure of vascular SMCs to high glucose concentrations, such as 5 min [5], 24 h [17], 48 h [5,18], and 72 h [2]. We questioned further on expression of phosphorylating kinases (Akt and ERK1/2) and of dephosphorylating PTP1B, and their adjustment under oxidative stress and insulin stimulation. We present evidence that long-term high glucose imbalance the expression of these molecules down-regulating Akt activation and up-regulating mitogenic ERK1/2 and non-receptor PTP1B in the insulin signaling pathway.

Materials and methods

Cells culture. Human aortic SMCs from media explants of fetal thoracic aorta [19], at passage 5 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with essential and non-essential amino acids, sodium selenite, ascorbic acid, 10% fetal calf serum (vol/vol), antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml neomycin), and 5.5 mM D-glucose, in a 5% CO_2/95% O_2 incubator, at 37 °C. After 1 week, the culture medium was replaced: half of the dishes were exposed to fresh DMEM containing 5.5 mM D-glucose (low glucose condition), while the other half were transferred in DMEM supplemented with 22.5 D-glucose (high glucose condition). Cells were analyzed after the next 7 days, when confluent and with the characteristic hills and valley pattern.

Cells stimulation and immunoblotting. Confluent SMCs were washed with phosphate buffer saline (PBS) solution, pH 7.4, and exposed for 10 min to either 1 mM H_2O_2 in PBS or 1 μM insulin in PBS (after 17 h in serum-free medium). Subsequently, cells were washed with PBS and solubilized in 10 mM Tris–HCl buffer containing 5 mM EDTA, 1% Triton X-100, 1 μM PMSF, 1 μM benzamidin, and 10 μg pepstatin, pH 7.4. Lysates were clarified by centrifugation, and protein concentration was assayed by Ammidoblack staining. Protein extracts (40 μg/lane) were resolved by 8% SDS–PAGE, transferred onto nitrocellulose membranes (Bio-Rad Laboratories, CA, USA), and blots blocked with 3% bovine serum albumin (BSA) in 10 mM PBS containing 0.05% Tween 20. The primary antibodies used were: rabbit polyclonal IgG corresponding to phosphorylated Ser 473 of Akt1 of human origin and rabbit polyclonal IgG corresponding to amino acids 345–480 of Akt1 of human origin (both from Santa Cruz Biotechnology, Inc.), monoclonal antibody to ERK1 + ERK2 and rabbit polyclonal to β-actin (both from Abcam, Cambridge, UK), affinity-purified rabbit anti-human PTP1B IgG (Calbiochem, Germany), and affinity purified anti-phospho-ERK1/ERK2 (T202/Y204) (R&D Systems, www.RnDSystems.com). The secondary antibody was anti-rabbit IgG-HRP (R&D Systems). Immunoblots were visualized using enhanced chemiluminescence reagents (Pierce Biotechnology, Rockfod, IL) and Kodak X-ray films (Eastman Kodak Co., Rochester, New York). Densitometry of the bands was carried out with Total Lab (TL 100, v2006) nonlinear dynamics program, and the value for each band was normalized to the level of β-actin in the sample load, or expressed as ratio of phosphorylated protein per total protein.

Immunofluorescence staining. SMCs were cultured to confluence on glass coverslips in medium supplemented with 5.5 mM or 22.5 mM D-glucose and stimulated (as above). Cells were rinsed with PBS, fixed in 2% *p*-formaldehyde, washed with PBS, and permeabilized with 0.1% Triton X-100 in PBS (3 min). Cells were rinsed again with PBS, and the unspecific binding sites were blocked in 1% BSA in PBS supplemented with 10% normal goat serum (45 min). The primary antibodies used (5 μg/ml) were mouse monoclonal to ERK1 + ERK2 (Abcam, Cambridge, UK) and affinity-purified rabbit anti-human PTP1B IgG (Calbiochem, Germany); after 17 h at 4 °C cells were washed with PBS, and incubated with goat anti-mouse IgG (whole molecule)-FITC (Sigma, dilution 1:64) in 1% BSA in PBS supplemented with 10% normal goat serum (2 h). A final PBS wash was performed, and cells nuclei were localized by counterstaining with DAPI (4 μg/50 ml)(1 min). The coverslips were mounted in 90% glycerol, and cells were examined with the Nikon, Eclipse TE 300 fluorescence microscope (Japan); images were captured with Digital Net camera DN100 (Nikon, Japan).

Experimental diabetes. Golden Syrian hamsters (5 weeks old, 97–106 g body weight) were i.p. injected with streptozotocin (STZ), as in [20]. The age-matched control hamsters were injected with an equal volume of saline solution. After 4 weeks since the start of the experiment, animals were euthanized, the aorta dissected out and processed for immunoblotting, as for cultured SMCs. The experiments were according to "Guide for the care and use of laboratory animals" published by US National Institutes of Health (NIH publication No. 83-25, revised 1996), and have been approved by the Ethic Committee of ICBP "N. Simionescu".

Data analysis. The results are presented as means ± standard error of the mean from five independent cell culture experiments. Statistical significance was determined by one-way ANOVA. Differences were considered significant at $p < 0.05$.

Results and discussion

Cells characterization

The human aortic SMCs used in this study exhibit an elongated spindle- or ribbon-shaped morphology devoid of any contaminants, grow as multilayer with the characteristic hills and valley pattern (as assessed by phase-contrast microscopy), show the presence of bundles of myofilaments in the cytoplasm and of numerous caveolae at the cells periphery (as demonstrated by electron microscopy), have positive reaction for smooth muscle α-actin and negative reaction for von Willebrand factor (as shown by immunofluorescence) [19], have positive reaction for vinculin (in immunoblotting and immunohistochemistry experiments)(Fig. 1 in Supplementary data, and [21]), and display functional store-operated channels responsive for capacitative calcium entry [22].

Selection of high glucose concentration

Reportedly, vascular SMCs have been exposed in vitro to various over-normal glucose concentrations such as 20 mM [23], 22 mM [5], 23.1 mM [2], 25 mM [6,15,18,24,25], 27.5 mM [7], and 30 mM [8]. Experiments reported here were performed on human aortic SMCs grown in medium supplemented with 22.5 mM D-glucose (high glucose); cells in medium with 5.5 mM D-glucose (low glucose) were used for comparison.

Akt phosphorylation is down-regulated by prolonged culture in high glucose and is improved by acute oxidative stress and insulin stimulation

In an attempt to simulate situation in Type 2 diabetes, we checked next the response of molecules from the insulin signaling pathway (Akt, ERK1/2, and PTP1B) to the long time insult of high glucose concentration, and their regulation by acute oxidative stress and insulin.

Densitometric measurements showed that 7 days culture in medium containing 22.5 mM D-glucose down-regulated by ~3.5-fold protein expression of Ser 473 phosphorylated-Akt, compared to the level in cells grown in low glucose condition (Fig. 1A). The glucose concentration-dependent down-regulation of Akt activa-

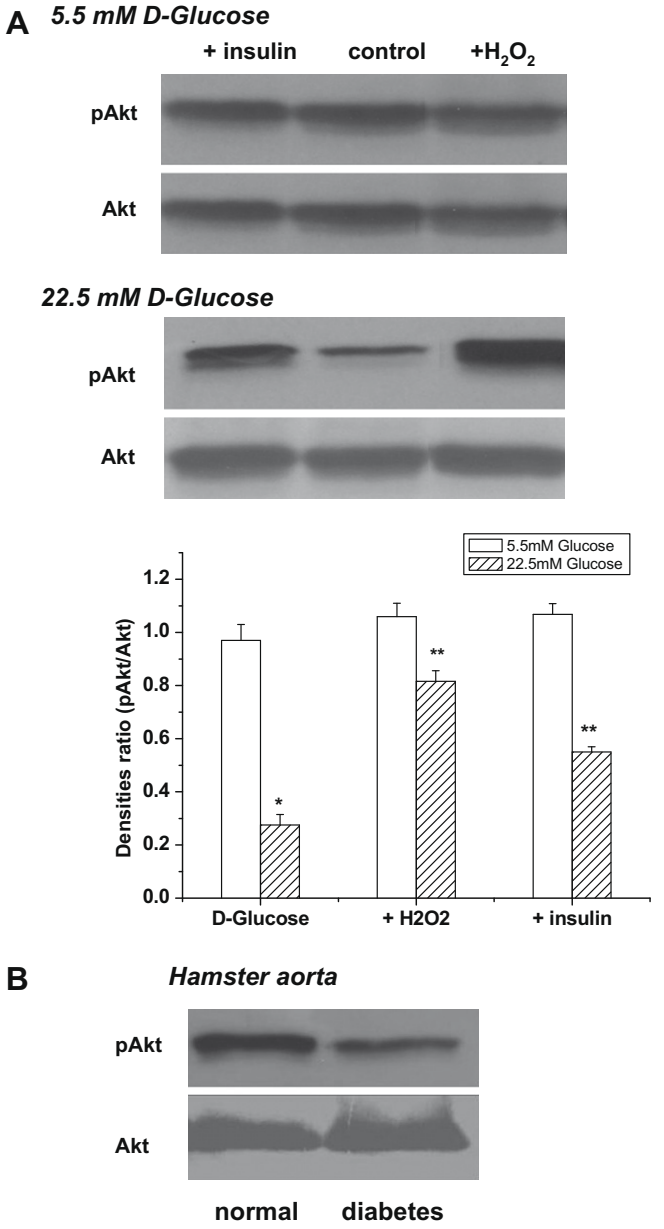

Fig. 1. Akt phosphorylation. (A) Immunoblot and densitometric evaluation of activated Akt in human aortic SMCs (normalized to total Akt). $^{*}p < 0.05$ compared with cells grown in 5.5 mM D-glucose; $^{**}p < 0.05$ compared with cells grown in 22.5 mM D-glucose, not stimulated with H_2O_2 or insulin; Akt bands from at least five individual immunotransfers were used for densitometry. (B) Immunoblot of Akt in aortic lysates of normal and 4 weeks diabetic hamsters (No. of animals = 4).

tion was validated in the aortic lysates of diabetic hamsters (blood glucose ~ 20 mM) that displayed ~1.5-fold reduced Akt phosphorylation, as compared to aorta of age-matched normal animals (Fig. 1B). This effect was recorded also in SMCs from another vascular bed, i.e. the mesenteric arteries of WKY and SHRSP rats grown in medium containing 25 mM glucose [25]. Moreover, other laboratories reported greater activation of Akt and mTOR in low glucose, compared to high glucose condition [7]. In cells cultured for 7 days in 22.5 mM D-glucose, both oxidant and insulin stimulation (for 10 min) up-regulated Akt phosphorylation by ~3- and 2-fold, respectively (versus the level in non-stimulated cells grown in high glucose condition). Although the activating effect of H_2O_2 and insulin on vascular SMCs Akt is known [11,13,26], we demonstrate here that it is produced also in SMCs affected by the long-term maintenance in high glucose.

ERK1/2 phosphorylation is up-regulated by prolonged culture in high glucose and acute oxidative stress stimulation

To examine ERK1/2 (T202/Y204) phosphorylation after 7 days culture of aortic SMCs in medium with 22.5 mM D-glucose, immunoblotting and immunohistochemical approaches were used. Densitometric measurements of the blots showed that prolonged high glucose up-regulated by ~2-fold ERK1/2 phosphorylation, compared to cells in medium with 5.5 mM D-glucose (Fig. 2A); in line with this result is the significantly enhanced staining of the mole-

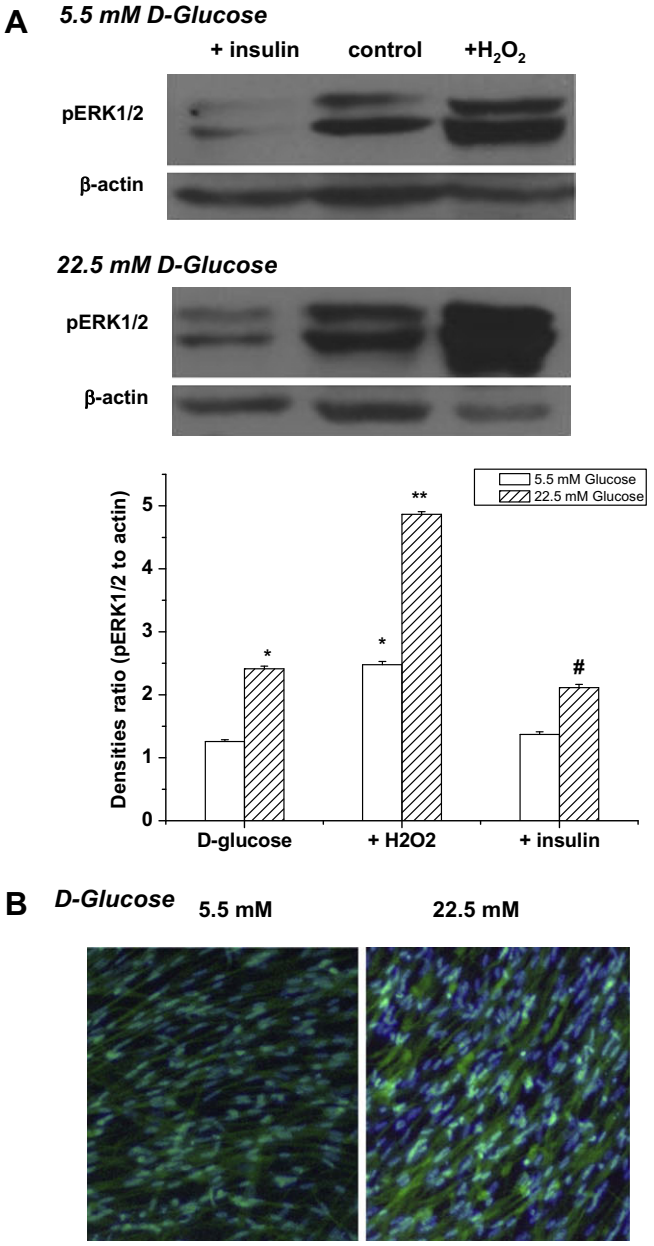

Fig. 2. ERK1/2 phosphorylation. (A) Immunoblot and densitometric evaluation of activated ERK1/2 in human aortic SMCs (normalized to β-actin). $^{*}p < 0.05$ compared to in cells grown in 5.5 mM D-glucose condition; $^{**}p < 0.05$ compared with in cells grown in 22.5 mM D-glucose; $^{\#}p < 0.05$ compared with cells grown in 5.5 mM D-glucose and stimulated with 1 μM insulin; $n = 7$. (B) Immunofluorescence localization of ERK1/2 in human aortic SMCs cultured in medium supplemented with 5.5 mM D-glucose and 22.5 mM D-glucose. In these merged images, ERK1/2 appears in green within the cells cytoplasm, and the cells nuclei in blue (DAPI staining). Note the evident enhanced ERK1/2 protein in cells in high glucose, compared to low glucose condition. Magnification: 10×. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

cule in cells grown in 22.5 mM D-glucose versus 5.5 mM D-glucose (Fig. 2B). The reaction is absent in cells reacted only with the secondary antibody (Fig. 2 in Supplementary data). In another SMCs culture originating from mesenteric resistance arteries from streptozotocin-injected or hypertensive mice, activation of mitogenic pathways was reported to occur very fast, at even 5 min high glucose (22 mM) stimulation [5]. We show here that in cells cultured for 7 days in 22.5 mM D-glucose, acute stimulation with 1 mM H_2O_2 doubled ERK1/2 phosphorylation, while 1 μM insulin treatment was apparently ineffective (~11% diminishment compared to the level in untreated cells) (Fig. 2A). Thus, the SMCs affected by the long-term maintenance in high glucose were still susceptible to acute oxidant stimulation, while not responsive to insulin.

PTP1B expression is up-regulated by long-term maintenance in high glucose concentration and is down-regulated by acute insulin stimulation

In the context of insulin resistance, PTP1B has a major role, since this enzyme dephosphorylates the autophosphorylated insulin receptor, disrupts the ability of the receptor to respond to insulin binding, decreases insulin signaling, and diminishes cellular glucose uptake. Despite these important roles, there is a paucity of information on protein tyrosine phosphatases regulation in SMCs [27]. Here we questioned on PTP1B response to 7 days maintenance of SMCs in 22.5 mM D-glucose. Densitometric evaluation of the immunoblots showed that 22.5 mM D-glucose up-regulated PTP1B protein level by ~2-fold (compared to 5.5 mM D-glucose condition). This result is in line with PTP1B cell associated immunofluorescence staining that appears more intense in VSMCs in 22.5 mM versus 5.5 mM D-glucose (Fig. 3C). The prolonged exposure of the cells to an even higher glucose concentration (i.e. 30 mM) conduct to the occurrence of an additional band reactive with the PTP1B antibody, of apparent molecular mass slightly higher than 51 kDa (Fig. 3A). One may infer that the latter band represents an irreversibly oxidized form of the enzyme, since chronic exposure to hyperglycemia is an optimal environment for oxidative-stress development. Glucose concentration-dependent PTP1B up-regulation was observed also in the aortic lysates of streptozotocin-injected diabetic hamsters (comparative with aortic lysates of normal hamsters) (Fig. 3B). Reportedly, PTP 1B expression was up-regulated also in vascular SMCs migrating from

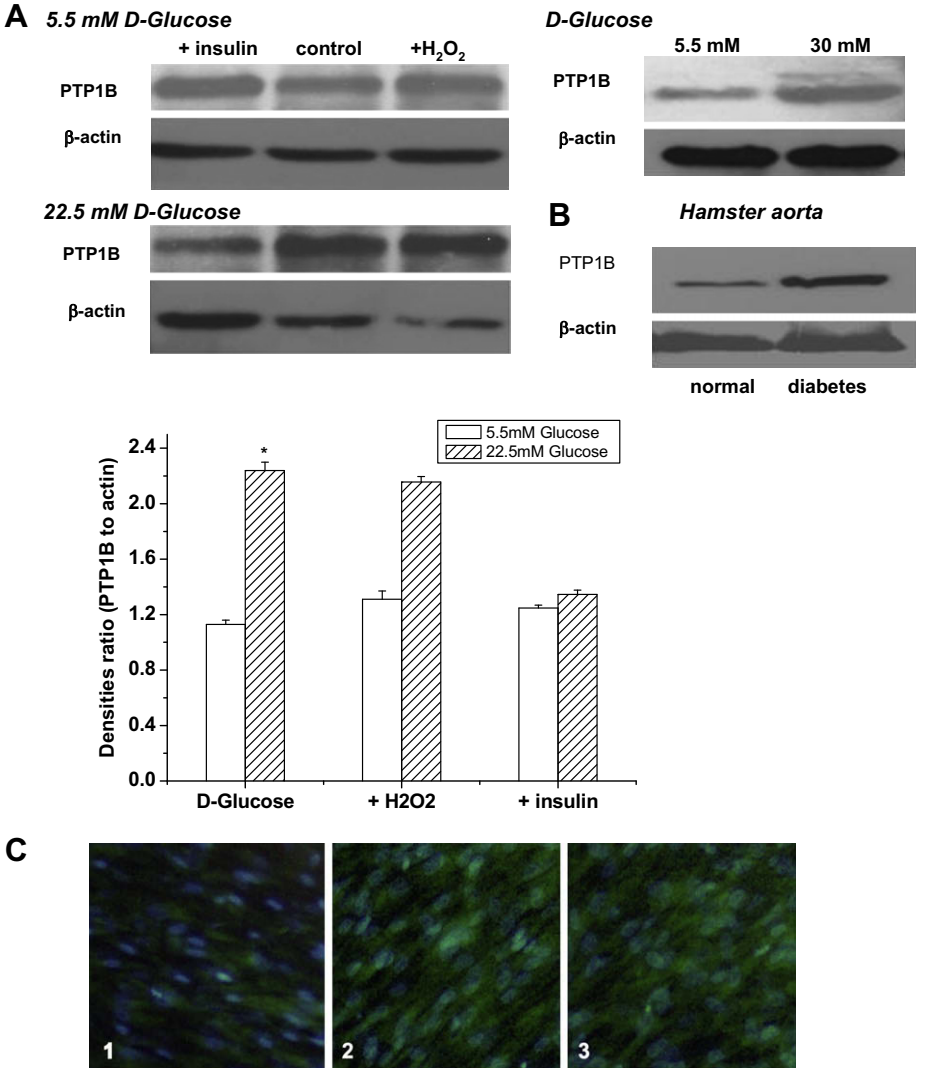

Fig. 3. PTP1B expression. (A) Immunoblot and densitometric evaluation of PTP1B in human aortic SMCs (normalized to β-actin). $^{*}p < 0.05$ compared with cells grown in 5.5 mM D-glucose; $n = 6$. An immunoblot for PTP1B expression in cells maintained for 7 days in 30 mM D-glucose is also included here. (B) A representative example of PTP1B protein expression in aortic lysates from normal and 4 weeks diabetic hamsters (No. of animals = 4). (C) Immunofluorescence localization of PTP1B in human aortic SMCs. (1) Cells cultured in medium with 5.5 mM D-glucose; (2) cells in medium supplemented with 22.5 mM D-glucose; (3) cells in medium with 22.5 mM D-glucose and stimulated with 1 mM H_2O_2 for 10 min. In these merged images, PTP1B was stained in green within the cells cytoplasm, while the cells nuclei in blue (DAPI staining). Magnification: 20×. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

tunica media of the artery to the intima (subsequently to acute injury), or to the neointima suggesting participation of the enzyme in vessel wall remodeling and neointima formation during postangioplasty restenosis [12,13].

We show here that acute stimulation with 1 mM H_2O_2 (10 min) did not influence PTP1B protein expression in high glucose condition (Fig. 3A), a result in line with the comparable immunostaining recorded in SMCs preparations in 22.5 mM D-glucose stimulated or not by the oxidant (Fig. 3C). The reaction is absent in cells reacted only with the secondary antibody (Fig. 2 in Supplementary data). The acute treatment with 1 μM insulin in long-term 22.5 mM D-glucose condition reduced by ~40% PTP1B expression (Fig. 3A). Others reported that in differentiated cultured primary rat aortic SMCs insulin amplified PDGF-induced cell motility by suppressing the expression and function of PTP1B [13].

Thus, in an experimental set up that mimics situation in Type 2 diabetes in which vascular wall is constantly exposed to circulating hyperglycemia, we report here the long-term (7 days) effects of high glucose concentration on human media artery SMCs. The results show down-regulation of Akt activation and up-regulation of mitogenic ERK1/2 and of dephosphorylating PTP1B. The imbalance in protein expression of these molecules from insulin signaling pathway is further altered by acute oxidative stress and insulin stimulation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.07.141.

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