Vascular Pharmacology 62 (2014) 113–119

Contents lists available at ScienceDirect

Vascular Pharmacology

journal homepage: www.elsevier.com/locate/vph

Reprint of "Alteration of endothelial proteoglycan and heparanase gene expression by high glucose, insulin and heparin"☆

J. Han [1], L.M. Hiebert *

Department of Veterinary Biomedical Sciences, University of Saskatchewan, Saskatoon, Saskatchewan, S7N 5B4, Canada

ARTICLE INFO

Article history:
Received 5 April 2013
Received in revised form 16 July 2013
Accepted 2 August 2013
Available online 16 May 2014

Keywords:
Diabetic cardiovascular complications
Endothelial cells
Syndecan
Heparanase
Heparin

ABSTRACT

Heparan sulfate proteoglycans (HSPGs) contain a core protein with glycosaminoglycans attached. Reduced glycosaminoglycan, in endothelial HSPGs syndecan and perlecan, is associated with diabetic cardiovascular complications but changes in core protein remain controversial. Since heparanase degrades heparan sulfate, we wished to determine if changes in endothelial heparanase mRNA, by high glucose (HG), correlate with changes in syndecan and perlecan core proteins, and to observe effects of heparin or insulin. RNA was isolated from cultured human aortic endothelial cells treated with HG (30 mM), insulin (0.01 units/mL), heparin (0.5 μg/mL), HG plus heparin and/or insulin for 24 h. Real time PCR revealed that HG alone significantly increased heparanase, decreased syndecan with no effect on perlecan mRNA. Heparin or insulin significantly prevented the increase in heparanase but decreased perlecan mRNA while heparin, but not insulin, prevented the decrease in syndecan mRNA in HG treated cells. HG plus heparin and insulin increased heparanase and syndecan mRNA compared to all other treatments and decreased perlecan mRNA compared to control and HG alone. Heparin may protect endothelium from HG injury by reducing heparanase and increasing syndecan while insulin inhibits heparanase expression. Effects with insulin plus heparin suggest interference in transcriptional regulation of heparanase and syndecan genes.

1. Introduction

Hyperglycemia is a risk factor for cardiovascular complications in diabetes mellitus. Injury of endothelial cells (ECs), that normally regulate vascular tone and permeability and help maintain hemostasis, contribute to diabetic cardiovascular complications. Mechanisms suggested to mediate EC damage in hyperglycemia include increased oxidative stress, increased production of advanced glycosylation end-products (AGEs), altered coagulation and fibrinolytic processes, and increased production of cytokines [5,9]. In addition, degradation of heparan sulfate proteoglycans (HSPGs) may be an important mechanism contributing to endothelial injury and could be due to release of bioactive factors associated with heparan sulfate (HS) chains [21].

HSPG, a macromolecule found in all mammalian tissues and cells, contains two to three glycosaminoglycan (GAG) HS chains [11]. HS is a prominent component of blood vessels and the most common GAG found on the EC surface and in the extracellular matrix (ECM). Typical HSPGs in blood vessels are syndecans and perlecan which are synthesized and secreted by ECs. Syndecans are mainly found on the cell surface and perlecan is present in the extracellular matrix (ECM) [20]. Evidence suggests that biosynthesis of HS and HS chain sulfation in HSPGs are decreased in the hyperglycemic state [8,18,22]. HS GAGs were decreased in arteries of diabetic patients [45]. HS GAGs but not core protein were decreased in cultured ECs and kidney cells treated with high glucose [41,44]. Studies of renal biopsies also showed a decrease in HS GAGs without changes in core protein [38]. The concentration of arterial HS was negatively correlated with plasma glucose concentration in diabetic monkeys [8]. However, it is unknown whether the decrease in GAGs in ECs, associated with hyperglycemia, is accompanied by a decrease in the gene expression of HSPG core proteins.

The endoglucuronidase, heparanase, induced by high glucose, could be responsible for the cleavage of HS chains but not core proteins of HSPGs. Heparanase mRNA and protein activity are expressed in ECs treated with high glucose [14]. Studies in brain melanoma cells have shown that HS in syndecan and perlecan were targets for heparanase degradation [33]. However, a correlation between decreased HSPG core proteins and increased heparanase in ECs under hyperglycemic conditions has been poorly studied.

In addition to its antithrombotic activities, the GAG heparin is a vasodilator [29] and lowers blood pressure [48]. Heparin and/or insulin

Abbreviations: Heparan sulfate proteoglycans, HSPGs; Heparan sulfate, HS; Endothelial cells, ECs; Glycosaminoglycans, GAGs; Extracellular matrix, ECM; Human aortic endothelial cells, HAECs; Calcium and magnesium-free Dulbecco's phosphate-buffered saline, CMF-DPBS; Threshold cycle, C_T; Porcine aortic endothelial cells, PAECs.

☆ This article is a reprint of a previously published article. The article is reprinted here for the reader's convenience. For citation purposes, please use the original publication details; Vascular Pharmacology, 59(3-4), pp. 112-118.

DOI of original article: http://dx.doi.org/10.1016/j.vph.2013.08.001.

* Corresponding author at: Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Drive, Saskatoon, Saskatchewan, S7N 5B4, Canada. Tel.: +1 306 966 7358.

E-mail address: linda.hiebert@usask.ca (L.M. Hiebert).

[1] Present address: Department of Cell Biology, Lerner Research Institute, Cleveland Clinic Foundation, 9500 Euclid Ave, Cleveland, OH 44195, USA.

1537-1891/$ – see front matter
http://dx.doi.org/10.1016/j.vph.2014.05.001

inhibited heparanase upregulation induced by high glucose in cultured ECs [14]. Although heparin and insulin affect heparanase expression and subsequent digestion of HS chains in ECs treated with high glucose, their influence on syndecan and perlecan core proteins is not well studied. In this study, high glucose treated cultured human aortic endothelial cells (HAECs) were used as a model for diabetic EC injury. Gene expressions of syndecan, perlecan and heparanase were determined following treatment with high glucose and/or insulin and/or heparin. The mechanism of transcriptional gene regulation was also studied.

2. Materials and methods

2.1. Human aortic endothelial cell (HAEC) culture

HAECs, basal medium (Medium 200) and cell growth supplement (LSGS kit) were purchased from Cascade Biologics (Portland, OR, USA). HAECs were transferred into 30 mL flasks from a vial of frozen cells and were cultured at 37 °C with 5% CO_2/95% air in a humidified environment until confluent. Confluent HAECs were then washed twice with calcium and magnesium free Dulbecco's phosphate-buffered saline (CMF-DPBS) and cells were detached by trypsin (0.025% with EDTA in CMF-DPBS) for 2 to 3 min at room temperature. Cells were resuspended in medium and transferred to 35 mm dishes for experiments.

2.2. Treatment of cultures

Confluent HAECs were incubated with control medium, glucose (30 mM), insulin (0.01 unit/mL), heparin (0.5 μg/mL), glucose plus insulin, glucose plus heparin, and glucose plus insulin plus heparin for 24 h (3 dishes/group). In a separate study, confluent HAECs were treated with control medium, glucose (30 mM), glucose plus actinomycin D (10 μg/mL), glucose plus insulin plus heparin, and glucose plus insulin plus heparin plus actinomycin D for 2, 4, 8 and 24 h (3 dishes/group).

2.3. Detection of gene expression by real time quantitative PCR

Total RNA was isolated from HAECs using the RNeasy® Mini Kit from QIAGEN (Mississauga, Ontario, CA). Briefly, medium was removed from dishes, and then cells were washed with cold CMF-DPBS (4 °C). Procedural steps from the lysis of cells to obtaining total RNAs were performed according to the manufacturer's instructions. RNA concentrations were determined by a Nanodrop™ 1000 Spectrophotometer (Thermo Scientific). A ratio of absorbance at 260/280 nm > or = 2.0 indicated RNA pure enough for cDNA synthesis.

cDNA was made from 1 μg of cell RNA from each sample in a 20 μL reaction using the AffinityScript™ QPCR cDNA Synthesis Kit from Stratagene (La Jolla, CA, USA) according to the methods described in the manufacturer's instructions. The Oligo (dT) primer (3 μL each reaction) was used. The reaction program was: primer annealing at 25 °C for 5 min, cDNA synthesis at 45 °C for 15 min and reaction termination at 95 °C for 5 min. Newly synthesized cDNAs were stored at −20 °C for later use.

Brilliant® II SYBR® Green QPCR Master Mix Kit (Stratagene) was used to determine gene expression from samples of synthesized cDNA. The cDNA samples were diluted 1:20 for the PCR reactions. The following primers were used: heparanase sense primer 5′-GGCAAGT ATTCTTTGGAGCA-3′ and antisense primer 5′-TGGATTGT CAGTGTTT GTGC-3′; perlecan sense primer 5′-TGCCTGAGGACATAGAGACC-3′ and antisense primer 5′-TCGGAATAAA CCATCTGGA-3′; syndecan-1 sense primer 5′-TG CAGGTGCTTTGCAAGATA-3′ and antisense primer 5′-TTCTGGAGACGTGGGAATA-3′; β-actin (internal control) sense primer 5′-GGCATCCTCACCCTGAAGTA-3′ and antisense primer 5′-GAAGG TCTCAAACATG ATCT-3′. Mixtures were assembled according to the manufacturer's instructions in a 25 μL mixture, with final concentrations of 200 nM for each primer and 5 μL for each template cDNA. The real time PCR reactions were performed in Mx3005P® QPCR Systems from Stratagene. The PCR cycling programs used were: 1 cycle at 95 °C for 10 min to activate the DNA polymerase and 40 cycles at 95 °C for 30 seconds, 58 °C for 1 min and 72 °C for 1 min to detect and report fluorescence during the annealing and extension step of each cycle.

2.4. Statistical analyses

Relative gene expression data were calculated by using the threshold cycle (C_T) value of real time quantitative PCR following the $2^{-\Delta\Delta CT}$ method described by Livak et al. [27] using the formula $\Delta\Delta C_T = (C_T - C_{TActin})_{Treatment} - (C_T - C_{TActin})_{Control}$. The data are presented as the fold change in gene expression normalized to the housekeeping reference gene β-actin and relative to the untreated control. For the untreated control sample, $\Delta\Delta C_T$ equals zero and 2^0 equals one, so that the fold change in gene expression, relative to the untreated control, equals one. For treated samples, the fold change in gene expression is relative to the untreated control. For the regulation of gene expression by actinomycin D, relative gene changes at different times compared to time 0 was also calculated by using the $2^{-\Delta CT}$ method, where $\Delta C_T = C_{T.time\ 0} - C_{T.time\ n}$.

All data are expressed as mean ± standard deviation (SD) from three culture dishes per group. A one-way ANOVA was used to determine significant differences between groups. A two-tailed *t*-test was used to test differences between the two groups. $P < 0.05$ was considered significant. Regression analysis was used to show relative mRNA inhibition by actinomycin D with time.

3. Results

3.1. Gene expression

To determine gene expression of syndecan, perlecan and heparanase under hyperglycemic conditions and the effect of insulin and/or heparin, total RNA was isolated from treated cultured cells and gene expression was detected by quantitative real time PCR. The heparanase, syndecan and perlecan genes were expressed in control ECs. High glucose significantly increased heparanase mRNA, while heparin or insulin alone had no effect on heparanase mRNA compared to control. In the presence of high glucose, insulin or heparin alone decreased heparanase mRNA compared to glucose alone with expression similar to that in control cells, while the combination of insulin and heparin enhanced heparanase mRNA compared to all other treated groups (Fig. 1).

High glucose alone inhibited syndecan mRNA but insulin or heparin alone had no effect on syndecan mRNA compared to control cells. In the presence of high glucose, heparin, but not insulin, maintained syndecan mRNA at control levels. Insulin plus heparin significantly increased syndecan mRNA compared to all other groups (Fig. 1).

High glucose alone had no effect on perlecan mRNA. Insulin or heparin alone significantly decreased perlecan mRNA compared to control. When high glucose was present, insulin, heparin and insulin plus heparin significantly decreased perlecan mRNA compared to both control and high glucose treatment alone (Fig. 1).

3.2. Mechanism of gene regulation

To help determine the mechanism of gene expression, the three experimental groups with the most amplified mRNAs, were treated with or without actinomycin D, a DNA transcription suppressor. These groups were, for heparanase mRNA, ECs treated with high glucose or high glucose plus insulin plus heparin; and for syndecan mRNA, ECs treated with high glucose plus insulin plus heparin.

Gene expression of heparanase relative to β-actin was increased with time in high glucose treated cells with or without actinomycin D and was significantly increased at 24 h compared to 0 h (Fig. 2A). The expression of heparanase mRNA was increased and β-actin mRNA did not change with time in cells without actinomycin D (Fig. 2B), while