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1. Introduction
1.1 Extracellular Matrix

The extracellular matrix (ECM) is the noncellular component present within all tissues and organs, and provides not only essential physical scaffolding for the cellular constituents but also triggers crucial biochemical and biomechanical cues that are required for tissue morphogenesis, differentiation and homeostasis.

Although, fundamentally, the ECM is composed of water, proteins and polysaccharides, each tissue has an ECM with a unique composition and topology that is generated during tissue development through a dynamic and reciprocal, biochemical and biophysical dialogue between the various cellular components (e.g. epithelial, fibroblast, adipocyte, endothelial elements) and the evolving cellular and protein microenvironment. Indeed, the physical, topological, and biochemical composition of the ECM is not only tissue-specific, but is also markedly heterogeneous. Cell adhesion to the ECM is mediated by ECM receptors, such as integrins, discoidin domain receptors and syndecans.

Moreover, the ECM is a highly dynamic structure that is constantly being remodeled, either enzymatically or non-enzymatically, and its molecular components are subjected to a myriad of post-translational modifications. Through these physical and biochemical characteristics the ECM generates the biochemical and mechanical properties of each organ, such as its tensile and compressive strength and elasticity, and also mediates protection by a buffering action that maintains extracellular homeostasis and water retention. In addition, the ECM directs essential morphological organization and physiological function by binding growth factors (GFs) and interacting with cell-surface receptors to elicit signal transduction and regulate gene transcription. The biochemical and biomechanical, protective and organizational properties of the ECM in a given tissue can vary tremendously from one tissue to another and even within one tissue, as well as from one physiological state to another. In fact, the ECM is involved in several physiologic processes of degradation and remodeling and moreover in regulating the behavior of the cells that contact it, influencing their development, function, shape, proliferation, and migration. This last
phenomenon holds a pivotal role during the development of inflammatory and pathologic status, such as in the atherosclerosis and formation of metastasis.

The ECM is composed of two main classes of polysaccharides: glycosaminoglycans (GAGs), which are usually found covalently linked to protein in the form of proteoglycans (PGs) and fibrous proteins (Schaefer and Schaefer, 2010). The main fibrous ECM proteins are collagens, elastins, fibronectins and laminins. PGs fill the majority of the extracellular interstitial space within the tissue in the form of a hydrated gel (Jarvelainen et al. 2009). PGs have a wide variety of functions that reflect their unique buffering, hydration, binding and force-resistance properties.

1.2 Glycosaminoglycans and Proteoglycans

Proteoglycans (PGs) are biological molecules composed of a specific core protein substituted with covalently linked glycosaminoglycan (GAG) chains. Hyaluronan (HA) is an exception to this definition, as it lacks a protein core. GAGs are linear, sulfated, negatively charged polysaccharides, which can be divided into two classes, namely sulfated GAGs comprising chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), heparin, and heparin sulfate (HS), and non-sulfated GAGs such as HA. GAG chains are made up of disaccharide repeating regions containing acetylated amino sugar moieties (N-acetylgalactosamine or N-acetylglucosamine) and mainly uronic acid (glucuronic acid or iduronic acid).

The biosynthesis of proteoglycans, in addition to the ordinary biosynthetic processes of O- and N-linked oligosaccharide components as in other glycoproteins, further requires biosynthesis of glycosaminoglycans in the Golgi apparatus, which can be considered to be a most complex biosynthetic process for complex carbohydrates. It requires a number of glycosyltransferases and sulfotransferases and involves multiple subregions of the Golgi apparatus, which poses a great challenge for researchers in elucidating the organization and regulatory mechanisms involved in proteoglycan biosynthesis.
PGs can be classified into three main groups according to their localization, extracellularly secreted, those associated with the cell surface and intracellular.

The wide molecular diversity of PGs derives from the multitude of possible combinations of protein cores, O-linked and N-linked oligosaccharides, and various types and numbers of GAG chains. The specific structural characteristics of GAG types provide some of the structural basis for the multitude of their biological functions. PGs exhibit numerous biological functions acting as structural components in tissue organization, and affect several cellular parameters, such as cell proliferation, adhesion, migration and differentiation. PGs interact with growth factors and cytokines, as well as with growth factor receptors, and are implicated in cell signaling.

### 1.3 Hyaluronan

Hyaluronan is considered the simplest of the GAGs because it consists of a regular repeating sequence of non sulfated disaccharide units and because it’s not bound to a core protein. It is an unusual polysaccharide that has a simple chemical structure but extraordinary properties. It is synthesized as a large, negatively charged, unbranched polymer that is composed of repeating disaccharides (from 2000 to 25000) of glucuronic acid and N-acetylglicosamine linked with β-1,3 and β-1,4 glycosidic bonds respectively (Figure 1).

![Figure 1 - Repeated disaccharidic structure of hyaluronan](image-url)
Although HA belongs to the family of glycosaminoglycans it differs from these in many ways. Other GAGs are made as PGs that are synthesized and assembled in the rough endoplasmic reticulum and Golgi apparatus, and are secreted in a similar way to other glycoproteins. Moreover, whereas other GAGs are synthesized inside the cell and released by exocytosis, HA is spun out directly from the cell surface by an enzyme complex that is embedded in the plasma membrane. Many of the functions of HA depend on specific hyaluronan-binding proteins and proteoglycans, some of which are constituents of the extracellular matrix, while others are integral components of the surface of cells.

HA has multiple functions, such as space filling, hydration, lubrication of joints, and provision of a matrix through which cells can migrate (Toole, 2004). HA is actively produced during tissue injury, tissue repair, and wound healing (Slevin et al. 2002). In addition to providing a framework for ingrowth of blood vessels and fibroblasts, HA also regulates many aspects of molecular mechanisms of tissue repair, such as activation of inflammatory cells to mount an immunological response and regulation of behavior of epithelial cells and fibroblasts. Elucidation of the role and mechanisms of HA is crucial in aiding the development of novel therapy for many diseases.

1.4 **Biosynthesis of Hyaluronan**

HA is synthesized as an unmodified polysaccharide by three different, but related, hyaluronic acid synthases (HASs). These are multipass transmembrane enzymes, the active sites of which protrude from the inner face of the plasma membrane. HA is extruded through porelike structures onto the cell surface or into the ECM during its polymerization. HA biosynthesis requires not only HASs participation but it needs also the synthesis of UDP-sugars throughout a complex pathway organized in several steps. The enzymes use UDP-β-N-acetyl-D-glucosamine and UDP-β-D-glucuronate as substrates. Two additional enzymes are necessary for the
glucuronic acid biosynthesis: the UDP-glucose pyrophosphorylase (UGPP) and the UDP-glucose dehydrogenase (UGDH). The first enzyme transfers glucose-1-phosphate to the UTP, producing UDP-glucose. This sugar is used as a substrate by the UGPP in an NAD+-dependent, 2-fold oxidative reaction to generate UDP-glucuronic acid. The conversion of glucose to UDP-GlcNAc is mediated by the hexosamine biosynthetic pathway (HBP). This pathway merges from glycolysis using fructose-6-phosphate (Fruc-6-P) to form glucosamine-6-P (GlucN-6-P) and this main reaction is catalyzed by the rate-limiting enzyme glutamine:fructose-6-phosphate-amidotransferase (GFAT). GlucN-6-P is rapidly acetylated, isomerized to N-Acetylg glucosamine-6-phosphate (GlucNAc-1-P) and activated to UDP-N-acetylg glucosamine (UDP-GlucNAc) that serves as common precursor for all amino sugars used for the synthesis of glycoproteins, glycolipids, and PGs (Figure 2).

**Figure 2- HA biosynthesis**
In mammals the synthesis of HA is guaranteed by three enzymes on the cell membrane (HAS1, 2 and 3); they differ in kinetic characteristics and product size. The existence of three different HAS isoforms with different characteristics implies that these enzymes have distinct biological and physiological roles. All the HAS isoforms are highly homologous in their amino acid sequences and have similar hydropathic features, suggesting that they are similarly organized within the membrane. (Itano et al. 1996).

In human the genes encoding for the three HAS’s are located on different autosome: HAS1 is located on Chr 19q13.4, HAS2 on Chr 8q24.12 and HAS3 on Chr 16q22.1. The expression profile of HAS genes during development are spatially and temporally regulated, suggesting that HA may play a different role during development in different tissues or cell types (Itano, 2008). Structurally, all HAS enzymes are integral membrane proteins composed of seven membrane-spanning regions with hydrophobic amino acid cluster and large cytoplasmatic loops (Figure 3). Unlike typical glycosyltransferases, this enzyme is localized in the plasma membrane.

To complete the rapid HA-turnover in physiological conditions, this GAG is cleaved by enzymes known as hyaluronidases. In human there are six different genes encoding for hyaluronidases with different properties and cellular locations (Stern, 2004).
1.5 The exosamine biosynthetic pathway

Uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc) is synthesized from glucose as the final product of the hexosamine biosynthetic pathway (HBP) (Figure 4).

Only a small percentage of the glucose entering in the cells, approximately 2–5% of intracellular glucose, depending on cell type, is routed to the HBP. Furthermore, flux through the pathway is thought to be regulated largely by the levels of glucose and the rate-limiting enzyme GFAT.
Glutamine:fructose-6-phosphate amidotransferase). In fact, after entering the cells, glucose is rapidly phosphorylated and converted to fructose-6-phosphate (F-6-P), which is mainly metabolized through the glycolytic pathway. However, under chronic hyperglycemic conditions of a diabetic state, a greater part of F-6-P is diverted by the action of GFAT and converted into glucosamine-6-phosphate (GlcN-6-P). GlcN-6-P is then metabolized into various hexosamine products, including the main product UDP-GlcNAc. This elevated UDP-GlcNAc has three effects on the pathway. At first, UDP-GlcNAc feedback inhibits GFAT, thus blunting synthesis; also provide glycosidic precursors for the synthesis of glycoproteins, glycolipids and proteoglycans. Furthermore, it can be used to covalently modify proteins and affect their functions, through a single O-linked glycosylation, referred to O-GlcNAcylation. These O-GlcNAc modifications play critical roles in regulation of wide spectrum of cellular functions. Since its first discovery, it has been suggested that elevated O-GlcNAc contributes to many deleterious effects of hyperglycemia and glucotoxicity including insulin resistance, diabetic cardiovascular damage associated with oxidative stress and inflammation (Masson et al. 2006).

### 1.6 **O-GlcNac modification**

The HBP connects a nutrient-sensing system to a signaling pathway by providing GlcN-6-P, the key precursor of UDP-GlcNAc, the substrate for O-GlcNac modification. Indeed, O-GlcNAcylation results from the addition of a single UDP-GlcNAc on the hydroxyl groups of Ser and/or Thr residues of target proteins. O-GlcNAcylation is a unique type of glycosylation that it is not elongated to more complex glycan structures and is not restricted to the cell surface and/or luminal face of secreted proteins. This nucleocytoplasmic dynamic post-translational modification is rapidly responsive to hormones, nutrients, and cellular stress. Enzymes that catalyze the addition and removal of O-GlcNAc have been cloned and characterized (Figure 5). Unlike protein phosphorylation, where ~650 genetically distinct enzymes regulate the addition and removal of
phosphate, just two catalytic polypeptides catalyze the turnover of O-GlcNAc; a uridine diphospho-N-acetylglucosamine: peptide β-N-acetylglucosaminyl transferase (OGT; EC 2.4.1) and a neutral β-N-acetylglucosaminidase (O-GlcNAcase; EC 3.2.1.52).

![Figure 5- O-GlcNAcylation cycles](image)

The sensitivity of O-GlcNAc levels to extracellular glucose concentrations supports a model in which O-GlcNAc plays a role as a nutritional sensor, regulating cell growth in response to the available nutrients by altering protein-protein interactions, intracellular localization, protein turnover and activity. Studies showing that altering UDP-GlcNAc levels, and increasing and decreasing levels of O-GlcNAc, disrupt cell cycle lend additional support to this model (Butkinaree et al. 2010).

Several studies have shown that such a crosstalk between GlcNAcylation and phosphorylation exists, as disturbing phosphorylation events affects GlcNAcylation levels and vice versa.

Site-mapping studies have shown that on some proteins O-GlcNAc and O-phosphate compete dynamically for the same serine or threonine hydroxyl moiety. This reciprocal occupancy seems to produce different activities or stability in the proteins (Figure 6).
In certain proteins, O-GlcNAc and O-phosphate can also occur next to each other. Adjacent occupancy by each modification reciprocally influences the functions or turnover of proteins, as has recently been reported for the tumour suppressor p53 (Yang et al. 2006).

Our group demonstrated that HAS2 could be phosphorylated by AMPK in the intracytoplasmic loop, which is important for HAS glycosyltransferase activity (Vigetti et al. 2011). Bioinformatic analysis have shown the presence of a putative AMPK phosphorylation consensus motif at treonine 110 (T110). Therefore, we mutated T110 to alanine (T110A) in order to prevent phosphorylation, and we found that the T110 residue can be target of AMPK and that the phosphorylation of HAS2 strongly reduces the HA synthetic activity. Because regulatory phosphorylation modulates the function of cellular proteins similar to that of O-GlcNAcylation, in the
present study we investigated whether HAS2 is subject to GlcNAc protein modification, and its consequence for the activity and the stability of HAS2.

1.7 O-GlcNAc transferase (OGT)

OGT catalyses the addition of $O$-GlcNAc to protein. OGT was identified in and first purified from rat liver, and has since been cloned in rats, humans, *C. elegans* and other organisms. In mammals, OGT gene is highly conserved and is present as a single X linked gene localized near the centromere where recombination rates are low. This chromosomal region is linked to several neurological diseases, including Parkinson’s disease (Nemeth et al. 1999). Although there is only one OGT gene present, mammalian has three different isoforms, all of which share an identical catalytic domain, but differ in the number of tetratricopeptide (TPR) repeat motifs found at the N-terminus. The longest form (116-kDa, ncOGT) is a nucleocytoplasmic isoform and the next longest isoform (103-kDa, mOGT) is mitochondrial. The shortest form of OGT (sOGT; 73-kDa) is also nuclear/cytoplasmic. In addition to the differential targeting of these isoforms, the level of expression varies depending on the target tissue. OGT is highly expressed in the pancreatic-β-cells and in the brain (Hanover et al. 1999). All OGT isoforms consist of two distinct domains: the N-terminal domain contains TPR motifs, which are common protein–protein interaction domains, while the C-terminal is the catalytic domain that is highly conserved. The main difference among OGT isoforms is the number of TPRs each isoform contains (Figure 7).

![Figure 7: Structure of OGT isoforms](image)
The mechanism by which OGT specifically modifies proteins are unclear, although it appears that UDP-GlcNAc concentration, protein–protein interactions, glycosylation, and phosphorylation may all play a role. The mRNA and protein expression levels of OGT are tissue-specific and are dependent upon specific cellular signaling, such as nutrient availability. Several proteins have been identified that interact with OGT (Yang et al, 2002), and while these interactions are not known to change the activity of OGT, they do act to anchor and/or target OGT to signaling and transcriptional complexes. These interactions may modulate the activity of OGT by affecting its localization, modulating the binding of substrate proteins, or targeting to complexes where it is specifically activated by signal transduction events. Notably, OGT is both O-GlcNAc modified and tyrosine phosphorylated (Lubas and Hanover, 2000). Finally, OGT activity is influenced by the levels of its donor substrate UDP-GlcNAc, suggesting that as levels of UDP-GlcNAc change within the cell, OGT will target a different population of substrates (Kreppel and Hart, 1999).

The coordinate regulation of substrate binding, substrate preference, association with target complexes, and post-translational modification may mediate OGT in such a way that it specifically modifies different proteins in response to diverse signals, appropriately regulating cellular function.

1.8 O-GlcNAcase

O-GlcNAcase is a soluble, cytosolic β-N-acetylglucosaminidase expressed in all tissues examined and predominantly in brain (Wells et al. 2002). O-GlcNAcase is well conserved in mammals, with 97.8% identity between the human and mouse gene, and 29% identity (and 43% homology) between the human and C. elegans gene (Gao et al. 2001). O-GlcNAcase co-purifies with a complex of proteins suggesting that, like OGT, it is regulated by its interactions with other proteins (Gao et al. 2001). In addition, it has been shown that O-GlcNAcase is phosphorylated, suggesting an additional mechanism of regulation. O-GlcNAcase is efficiently inhibited by PUGNAC (O-(2-acetamido-2-deoxy-D-glucopyranosyl)idene)amino-N-
phenylcarbamate; Haltiwanger et al. 1998). The gene encodes two alternatively spliced isoforms of OGA, the longest form of OGA has two distinct domains, an O-GlcNAcase domain at the N-terminus that shows high homology with bacterial hyaluronidases and a putative acetyltransferase domain at the C-terminus (Schultz and Pils, 2002). The acetyltransferase domain may have histone acetyltransferase (HAT) activity (Toleman et al. 2004) but its sequence is dissimilar to other, canonical HAT domains (Figure 8). Interestingly, OGA contains a caspase-3 cleavage site between these domains (Wells et al. 2002). Two variants of O-GlcNAcase, in which amino acids 250–345 and 250–398 (exons 6 and 7) are deleted, have no activity suggesting that the O-GlcNAcase active site is within the N-terminal half of this protein.

![O-GlcNAcase structure](image)

**1.9 O-GlcNAc and disease**

The HBP connects a nutrient-sensing system to a signaling pathway by providing the substrate for O-GlcNAc modification of cytosolic and nuclear proteins in all cell types, including those involved in the cardiovascular system. This dynamic post-translational modification is rapidly responsive to hormones, nutrients, and cellular stress. It is reciprocal with phosphorylation on some proteins and is thought to play an analogous role to this modification in cellular regulation. O-GlcNAc modification alters protein-protein interactions, intracellular localization, protein turnover and activity (Zachara and Hart, 2002). The list of proteins targeted by O-
GlcNAcylation is ever growing and includes key intracellular proteins such as transcription factors, cytoskeletal proteins, tumor suppressor and oncogenes, nuclear and cytosolic proteins and enzymes. Accumulating data suggest that global O-GlcNAc protein modification is regulated in a cell-cycle dependent manner and that any disruption of this cycling results in major cell-cycle defects. In this connection, a recent study by Slawson et al. showed that excessive O-GlcNAc modification alters cell-cycle progression while intact O-GlcNAcylation is nevertheless necessary for proper cell cycle function. Several studies indicate that O-GlcNAc might induce nuclear localization of some transcription factors and may affect their DNA binding activities. For instance, transcriptional activation of Sp1, a ubiquitous transcription factor, has been reported several times to be induced (Goldberg et al. 2005), or inhibited (Yang et al. 2001) by O-GlcNAcylation. Other transcription factors have also been shown to be modified by O-GlcNAc including cAMP response element-binding protein (CREB) (Lamarre-Vincent and Hsieh-Wilson, 2003) and pancreatic duodenal homeobox (PDX-1) (Gao Y et al. 2003). More recent findings have strengthened that increased levels of O-GlcNAc have been implicated as a pathogenic contributor to glucose toxicity and insulin resistance, which are major hallmarks of type 2 diabetes and diabetes-related cardiovascular complications. Marshall and colleague demonstrated that flux through the HBP is responsible for the development of insulin resistance and remarked that the development of insulin resistance requires glucose, insulin, and glutamine. The role of glutamine in the development of insulin resistance has been found to occur via the regulation of GFAT, the rate limiting enzyme in the HBP. Inhibition of GFAT with either azaserine or DON abrogated the effects of hyperglycemia on the development of insulin resistance (Rajapakse et al. 2009). Studies have shown that an increase in cellular UDP-GlcNAc and O-GlcNAcylation levels on target proteins due to high glucose and glucosamine treatments leads to oxidative stress and endoplasmic reticulum stress, which have been shown to cause chronic inflammation and insulin resistance (Werstuck et al. 2006). Increasing the level of O-GlcNAc using either PUGNAC, a chemical inhibitor of OGA, or elevating O-GlcNAc levels with glucosamine induces insulin resistance in
Introducing 3T3-L1 adipocytes (Vosseller et al. 2002). Insulin resistance and the corresponding decrease in glucose uptake are correlated with a defect in the translocation of the glucose transporter GLUT4 to the plasma membrane (Yang et al. 2008).

1. 10 O-GlcNAcylation on smooth muscle cell

Vascular smooth muscle cell dysfunction is a major risk factor of diabetic cardiovascular disease. AoSMCs are highly specialized cells whose principal functions are the contraction of blood vessels and the regulation of blood vessel tone-diameter, which regulate the blood pressure and the blood flow, respectively. Under diabetic conditions, an increased flux of glucose through the HBP has been proposed to cause vascular disease. It has been observed that prolonged exposure to high glucose leads to the increase of GFAT expression in VSMCs (Nerlich et al. 1998) indicating that GFAT is possibly involved in the development of the diabetic vascular complications. Inhibition of GFAT activity using DON decreases the hyperglycemia-induced tumor growth factor-alpha (TGF-α) expression in VSMCs, suggesting that the adverse effects of hyperglycemia in VSMCs are mediated by the HBP. Hall et al. (2001) demonstrated that expressions of GLUT1 and GLUT4 are increased in the neointima of the aorta after balloon injury. Increased proliferation and decreased apoptosis of AoSMCs provides a possible linkage with the increased risks of restenosis and atherosclerosis in patients with diabetes. Akimoto et al. (2001) found that the pattern of O-GlcNAc modification of proteins changed when rat aortic smooth muscle cells (RASMCs) were cultured in medium containing a high concentration of glucose. High glucose also elevates both the expression and activity of OGT. In addition, high glucose and glucosamine also induced an increase in the expression of growth factors in RASMCs. The effects of O-GlcNAc on cell growth and division may also contribute to the increase in VSMC proliferation seen in diabetes. Based on the above findings, it is clear that O-GlcNAcylation on specific vascular proteins has an important role in the regulation vascular reactivity, and further research is necessary to
determine the impact of O-GlcNAcylation on vascular reactivity. Thus, altering the O-GlcNAc levels in vascular tissues may represent a novel therapeutic approach for the treatment of diabetic cardiovascular disease.
1. Materials and Methods
2.1 Cell Culture and treatments

Human primary aortic smooth muscle cells (hAoSMC) were purchased from Lonza and grown in SmGm2 complete culture medium (Lonza) supplemented with 5% fetal bovine serum (FBS) (Lonza) and 1% glutamax (Lonza). 3x 10^5 cells were seeded in 35 mm dishes, and after 6 hours, SmGm2 medium was replaced with high glucose DMEM (Lonza) supplemented with 0.2% FBS. After 48 h to induce quiescence, the media was changed to low glucose (5 mM) DMEM-F12 (Euroclone) supplemented with 10% FBS. Cells were stimulated with 30 mM glucose, 2 mM glucosamine (GluN), 40 μM 6-diazo 5-oxonorleucine (DON), 5 mM alloxan, 100 μM O-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino-N-phenylcarbamate (PUGNac), 1mM benzyl-2-acetamido-2-deoxy-a-D-galactopyranoside (BG) for 24 h (all from Sigma) to modulate O-GlcNacylation.

NIH3T3 cells were grown to confluence in high glucose DMEM medium supplemented with 10% FBS and 1% glutamax. After preliminary experiments, a final concentration of 4 mM GluN, 150 μM PUGNac, 2mM BG, 40 μM DON was used for 24 h treatments. Cultures were maintained in an atmosphere of humidified 95% air, 5% CO2 at 37°C.

2.2 Transient and stable transfection

8x 10^5 hAoSMCs were cultured before nucleofection in SmGm2 complete culture medium until 70%-80% confluency. Cells were transiently transfected with 5 μg of plasmid DNA using Human AoSMC Nucleofector Kit (AMAXA) as described previously (Vigetti et al, 2006) to have high efficiency and low mortality.

1.5x 10^5 NIH3T3 cells were transiently transfected with 3 μg of total DNA using ExGen 500 in Vitro Transfection Reagent (Fermentas) following the manufacturer’s protocol. Cells were incubated for 24-48 hours for the following analyses. For stable transfections, human HAS2 with N-terminal
Materials and Methods

c-myc tag in pcDNA3.1 was transfected in NIH3T3 using ExGen 500 and clones were selected in 400 μg/ml G-418 (Euroclone). Protein expression levels was assayed by immunoblot analysis with anti-C-Myc monoclonal antibody (Santa Cruz Biotecnology).

2.3 Gene expression determination by RT-PCR

RNAs extraction were performed using TRI Reagent Solution (Sigma) following the manufacturer’s protocol. To remove DNA contamination, DNase treatment (Ambion) was done in all samples. 5 μg of extracted RNA were retrotranscribed using the High Capacity cDNA synthesis kit (Applied Biosystems). Quantitative RT-PCR was done on an Abi Prism 7000 instrument (Applied Biosystems) using Taqman Universal PCR Master Mix (Applied Biosystems) following the manufacturer’s instructions. The following TaqMan Gene Expression Assays were used: HAS1 (Hs00155410), HAS2 (Hs00193435), HAS3 (Hs00193436), YY1 (Hs00231533), GAPDH (Hs99999905). Fluorescent signals generated during PCR amplifications were monitored and analyzed with Abi Prism 7000 SDS Software (Applied Biosystems). To check the SP1 abrogation we used a SYBR Green based quantitative RT-PCR (Applied Biosystem) using primers CTCCAGACCATTAACCTCAGTG and TGTATTCCATCACCACCAGC. Comparison of the amount of each gene transcript among different samples was made using GAPDH as the reference and the ΔΔCt method.

2.4 Human siRNA

Small interfering RNA (siRNA) was used to reduce the expression of human YY1 and SP1 in AoSMCs. SiRNA antisense sequence was the following: YY1 (IDs14958, 5’-UGAACUCUCAACAAAAGCUtt-3’), SP1 (ID13319, 5’-UGAGUUGUAAAGGUCUGCCct-3’), and negative control siRNA #1 kit (scramble, code 4611), a siRNA sequence not homologous to any known
gene, were purchased from Ambion. The transfections were done using the Human AoSMC Nucleofector Kit as previously described. After 24 h of incubation, cells were treated and the next day the silencing efficiency was quantified by quantitative RT-PCR measuring gene expression. The working concentration of siRNA in cell experiments was 50 nM.

### 2.5 Glycosaminoglycans determinations

GAGs released into the culture medium were purified by proteinase K (Finnzyme) digestion and ethanol precipitation. Δ-disaccharides obtained by the enzymatic digestions with hyaluronidase SD and chondroitinase ABC (Seikagaku Corporation) were fluorotagged with 2-aminoacridone (AMAC, Molecular Probes). AMAC-tagged disaccharides were separated and quantified by PAGEFS and HPLC analyses as previously described (Karousou et al, 2004). Identification and quantification of sample bands in PAGEFS gel were done by comparing their migration and pixel density with standard Δ-disaccharides using ImageJ software. Separation and analysis of AMAC-derivatives of Δ-disaccharides were performed using an HPLC system coupled with a Jasco-Borwin chromatograph system with a fluorophore detector. Sample peaks were identified and quantified comparing the fluorescence spectra with standard Δ-disaccharides, using Jasco-Borwin software. Pericellular HA matrices were visualized by using a particle exclusion assay (Knudson W and Knudson CB, 1991). Representative cells were photographed at a magnification of ×40 and matrices and cellular areas were quantify using ImageJ software.

### 2.6 hAoSMCs mobility and adhesion assay

Confluent hAoSMC were scratched by pipette tip and then cultured in DMEM-F12 supplemented with 10% FBS in the presence of several
treatment. Migrated cells were quantified after 6 and 24 h as previously described (Vigetti et al., 2009). To test the adhesiveness of hAoSMCs in different condition, we performed the monocyte adhesion assay by using the monocyte U937 cell line (Vigetti et al., 2009). The quantification of adhered monocytes was done using an inverted microscope (Olympus) counting seven independent fields.

2.7 Western Blot Analysis

Western blotting experiment were performed using the monoclonal antibody CTD110.6 (Sigma) in order to detected O-GlcNacylated proteins, polyclonal anti-GAPDH antibodies (Santa Cruz Biotechnology) or monoclonal anti-tubulin antibodies (Sigma). In some experiment monoclonal anti C-Myc antibodies (Santa Cruz Biotechnology) was also used to detected recombinant fused c-myc-HAS2 protein.

2.8 Immobilization of O-GlcNAc-modified Proteins with Wheat Germ Agglutinin

NIH 3T3 and hAoSMC cells are transfected with C-Myc-HAS2 or C-Myc-HAS2 and OGT coding plasmid or empty vector (pcDNA3.1). The next day, cells were treated to modulate protein O-GlcNAcylation and after 24 h growth medium was removed. Cells were scrapered in lysis buffer (10 mM Tris, 1.5 mM MgCl2, 10 mM KCl in mQ water supplemented with proteases inhibitors (ROCHE)). The lysates were incubated on ice for 5 min, sonicated and the protein content was quantified using Bradford Assay (Sigma). 100 μg of total protein was incubated with 100 μl of (WGA)-conjugated agarose beads (Vector Laboratories). The preparation was rotated for 20 h at 4 °C and WGA-conjugated beads were collected by centrifugation; washed three times with lysis buffer and immobilized proteins were eluted by boiling in a waterbath for 5 min in lysis buffer with
1 M GluN. The eluted materials containing WGA-binding glycoproteins were assayed by western blot.

2.9 Microsome purification and HASs Activity Assay

Microsomes containing vesciculated fragments of the plasma membrane were obtained from control or transfected NIH3T3 cells as previously described (Vigetti et al, 2011). The quantification of HAS enzymatic activity was also carried out on proteins eluted from WGA-agarose beads. In some experiments, hexosaminidase digestions were conducted by treating cell lysates with N-acetylglucosaminidase from jack beans (Sigma). Lysates not digested with hexosaminidase were protected from deglycosylation by the addition of 50 μM PUGNAC in lysis buffer that inhibits endogenous O-GlcNAcase; as a control prior to determine HAS activity N-acetylglucosaminidase was boiled.

2.10 C-myc HAS2 stability

Stably transfected clones with high expression of c-myc-HAS2 were selected by growing NIH3T3 cells in the presence of 400 μg/mL of G418 (Euroclone) as described elsewhere (Badi et al. 2009). Cells were plated 24h prior to treatment with 150 μg/mL cycloheximide (CHX), GluN, PUGNAC and MG132 at 5 μM. At different time points, Western blotting analyses were carried out as described above to detect c-myc-HAS2 decay.

2.11 Luciferase gene reporter assay

hAOSMc were nucleofected with a -2118/+43 HAS2 promoter-luciferase reporter construction; a gift of Katri Makkonen. 24 h post-trasfection, cells
Materials and Methods

were treated to modulate protein O-GlcNAcylation. Cells extract were assayed using a luciferase reporter gene assay kit (ROCHE) at different time point as indicated. Protein concentration in cell lysates were analyzed using bradford assay and the activity of luciferase was normalized to total protein concentration in lysates as previously described (Badi et al., 2009).

2.12 Statistical Analyses

Unpaired Student’s t-tests were done for statistical analyses. Probability values of $P<0.05$ were considered statistically significant (*). Experiments were repeated three times each time in duplicate, and data are expressed as mean ± standard error (SE).
3. Results
3.1 O-GlcNAcylation induces HA secretion

It is generally known that an increase flux of glucose through the HBP is correlated with a global increase of the UDP-GlcNac level and subsequently can augment protein O-GlcNAcylation. This post translational modification could be a signal of nutrient abundance and modulates several cellular responses (Issad et al. 2008). Our hypothesis is to study whether O-GlcNAcylation could regulate ECM polysaccharides synthesis. Since UDP-GlcNac, with UDP-GlcUA, is a precursor of HA, one of the main component of the ECM, we investigated the role of O-GlcNAcylation in the control of the cellular microenvironment with particular attention to HA synthesis.

To find out whether the HBP is required for GAG synthesis by O-GlcNAcylation, we treated cells with different compound that are able to interfere with protein glycosylation. Western blotting of hAoSMC and NIH3T3 cells lysates using a specific anti O-GlcNAC antibody, CTD110.6, revealed increase protein glycosylation as compared with control cells after glucosamine and PUGNAC treatments (Figure 9).

![Representative immunoblot for O-linked glycoprotein](image)
GluN, which bypasses the rate-limiting enzyme of the HBP, GFAT, is known to induce protein modification in the same cell lines (Raman et al, 2007); PUGNAC, a potent inhibitor of the enzyme O-GlcNAcase mimicking the enzyme-stabilized transition state, increases O-glycosylation of intracellular protein. To confirm that the HBP mediate protein glycosylation, we used specific inhibitors of GFAT and direct glycosylation inhibitors that interfered with the transfer of a sugar moiety to a protein. Preincubation of hAoSMCs with DON and alloxan, in order to inhibit the glucose flux through the HBP by inhibiting GFAT and OGT respectively, revealed a marked decrease in protein glycosylation as compared with cells not treated. The inhibitory effect of DON was reversed by GluN, confirming the specificity of DON effect; conversely, the simultaneous treatment with alloxan and GluN still inhibit O-GlcNAcylation as alloxan block OGT that acts downstream GluN entry point. Interestingly, in our condition, treatments with 30mM of glucose did not induce protein glycosylation suggesting that hAoSMCs used in the experiments were insulin-dependent for glucose uptake as previously described (Chisalita et al. 2009).

To verify whether the HBP and protein glycosylation could control GAG biosynthesis and secretion we performed an HPLC quantification of the unsaturated disaccharides (Δ) deriving from HA, chondroitin 4 sulfate (C4S) and chondroitin 6 sulfate (C6S) secreted into the hAoSMCs culture medium treated with the previous compound. As shown in figure 10, HPLC highlights that after GluN treatment, that induce UDP-GlcNAc level, both HA and chondroitins accumulate in the medium. DON and alloxan did not change GAG quantification, but the same treatments with GluN increased HA and chondroitins. On the contrary, modulation of protein O-GlcNAcylation with PUGNAC treatment, showed a specific HA augment without any effect on other GAG; while the simultaneous treatment with PUGNAC and GluN still increased both HA and chondroitins. These results suggested that an elevated UDP-GlcNac availability is critical for the biosynthesis of HA as well as that of other GAGs, while protein O-GlcNAcylation seems to be critical only for HA metabolism.
We also investigated the correlation between protein O-GlcNAcylation and HA biosynthesis in NIH3T3 cells; the experiments confirmed the results obtained in SMCs. After treatments we used the culture media for PAGEFS and HPLC analyses, in order to quantify the amount of HA synthesized and secreted by the cells. In both PAGEFS and HPLC analyses, treatments that markedly induced UDP-GlcNac level also induce a significant increase of HA synthesis. Moreover, NIH3T3 cells treated with DON or alloxan showed a complete abolishment of this biosynthetic process, whereas the control cells cultured in medium enriched with DON+GluN or alloxan+GluN treatment were able to synthesize and secrete a large amount of HA, as shown in figure 11.
In addition, also in this cell line, it is important to underline the specificity of this effect. In fact, PUGNAC treatments revealed the ability of this compounds to selectively regulate only HA synthesis by PAGEFS analysis: the amount of the other GAGs, as chondroitins, appears to be unchanged after treatments that modified O-GlcNAcylated protein level.
3. 2 **O-GlcNAcylation regulates hAoSMCs cell behavior and microenvironment**

As hAoSMCs are involved in vascular pathology through their proliferation, migration and immune cells recruitment, and HA has a pivotal role in these phenomena, we investigated the existence of correlation between HA biosynthesis due to protein O-GlcNAcylation analyzing its amount in pericellular coat by particle exclusion assay. Forty-eight hours after O-GlcNAc induction by GluN or PUGNAC treatments we observed an increment of pericellular space respect to the cells without any treatment (Figure 12). In contrast, treatments that reduce protein O-GlcNAcylation, as DON or BG, also reduce pericellular HA amount. This space between hAoSMCs and red blood cells is predominantly filled by HA, as demonstrated by digestion with hyaluronidase.

![Figure 12- Particle exclusion assay quantification and microphotograph](image-url)
One of the earlier events in vessel thickening is SMCs proliferation and migration from the tunica media to the intima, which determines neointima formation. SMC motility in vivo can depend on different factors, but it is well accepted that HA is a critical molecule favoring migration. Thus we evaluated if O-GlcNAcylation is able to regulate this process through the induction of HA-synthesis. As shown in figure 13, GluN or DON+GluN treatments, that increased O-GlcNAcylation, also induced hAoSMCs migration. To test the involvement of HA in migration, we adding in the medium 4-MU, a compound that decrease HA synthesis and secretion without any effect on chondroitins. We found that the addition of GluN to 4-MU treated cells is not able to restored the migration to levels similar to cells treated with GluN alone, indicating the specificity of HA in this phenomenon.

**Figure 13- Cell migration using scratch wounding assay**

At last, we verified the HA role in monocytes adhesion, process involved in inflammatory response. In addition to integrins and selectins, by means of CD44 circulating monocytes can adhere to HA. Therefore an accumulation of such GAG can be considered a proinflammatory signal. We quantified the number of adhesive U937 monocytes on hAoSMCs after treatments that induce or decrease O-GlcNAcylation and subsequently HA secretion. The induction of protein O-GlcNAcylation after PUGNAC or GluN treatments
Results

showed a significant increase of U937 adhesion to hAoSMCs respect to the control cells (Figure 14).

![Bar chart showing U937 adhesion on hAoSMCs](image)

* P < 0.05

Figure 14: U937 monocytes adhesion on hAoSMCs

As a control, we treated hAoSMCs with hyaluronidase and interestingly, this pretreatment inhibited U937 binding, underlining that the interaction of monocytes with SMCs was due to HA and not to other GAGs.
3.3 **O-GlcNAcylation could regulate HAS 2 metabolism**

O-GlcNAc modifications can occur either in the cytosol or in the nucleus and correspond to a dynamic process. This reversible O-GlcNAcylation is distinct from stable, complex glycosylations of membrane or secreted proteins, that take place in the lumen of the endoplasmic reticulum and in the Golgi apparatus. In contrast, O-GlcNAcylation/deglycosylation constitutes a dynamic regulatory mechanism that can modify the activity, the localization or the stability of cytosolic and nuclear proteins. Moreover, serine and threonine residues, that are targets for O-GlcNAc modifications, often correspond to residues that can also be phosphorylated, resulting in additional levels of protein regulation (Kuo et al. 2008). Our group recently demonstrated that HAS2 could be phosphorylated by AMPK at the Thr-110 and also the phosphorylation of this amino acid drastically reduces the HA synthetic activity (Vigetti et al. 2011). Infact this residue is localized in the intracytoplasmatic loop, which is important for HAS glycosyl transferase activity. As our preliminary experiments showed that treatments that increase protein O-GlcNAcylation or inhibit their deglycosylation could affect HA accumulation only, we hypothesized that the large cytoplasmic loops, critical for the enzymatic functions of HASes, could be accessible to nucleo-cytoplasmic OGT. In order to evaluate if the Thr-110 could be the target of OGT, we mutated this amino acid to alanine (T110A) in the C-Myc-HAS2 plasmid and transfected the mutant construct with or without OGT. After 48h, we quantified the HAS enzymatic activity in microsomes and we found a strong increase of HAS activity only after OGT cotransfection, whereas the functionality of c-myc HAS2+OGT was not affected by alloxan and GlcNAcase treatments. These results strongly suggest that Thr-110 could not be the target of OGT even if this enzyme drastically induce the HA synthetic activity (Figure 15).
In order to evaluate if HAS2 is modified by O-GlcNAc we transiently transfected NIH3T3 cells with plasmids coding for c-myc-HAS2 or OGT or empty vector (pcDNA3.1). After treatments with GluN and PUGNAC, O-GlcNAcylated proteins are purified by agarose WGA beads bindings. Complexes was incubated and analyzed by western blotting using anti C-Myc antibody. The results show that HAS2 is recognized by WGA when is cotransfected with OGT or after glucosamine and PUGNAC treatments. Interestingly, after transfection with HAS2 C-Myc alone no O-GlcNAc modification is detectable (figure 16). As a control, we detected c-myc-HAS2 in all total protein extracts and in the wash fraction. These data suggested that HAS2 is O-Glycosylated after treatments that increase O-GlcNAcylation or impair deglycosylation.
Results

Figure 16- HAS2 is modified by O-GlcNAc in NIH3T3 cells

To confirm that HAS2 is O-GlcNacylated by cytoplasmatic OGT we transiently transfected NIH3T3 cells with plasmids coding for c-myc-HAS2 and OGT and treated them with alloxan. As shown in figure 15, HAS2 binding to WGA was markedly reduced after alloxan treatment. This experiment was performed also in hAoSMCs and confirmed the results obtained in NIH3T3 cells (data not shown). Together, these results indicate that HAS2 is subject to modification by O-GlcNAc. To identify putative O-GlcNAcylated residue, bioinformatic searches revealed the presence of a critical site in HAS2 protein at serine 221 (S221). Further, this residue is localized in the intracytoplasmatic loop that was previously shown to be important for HAS glycosyl transferase activity. For that reason, we mutated S221 to alanine (S221A) in the 6myc-HAS2 plasmid in order to prevent O-GlcNAylation and transfected the mutated construct in NIH3T3 cells with OGT. Since WGA binds O-GlcNAc residues, S221A mutants were used to determine whether HAS2 binding to WGA was mediated by O-GlcNAc. After 48h from transfections, microsomes were prepared by centrifugation, O-GlcNacylated proteins purified by binding with agarose WGA beads and HAS2 tested in the eluate by western blotting using anti C-Myc antibody. As shown in Figure 17, we found that the S221A mutation prevented HAS2 identification after WGA beads elution; this result strongly suggests that the S221 residue can be the target of OGT and that the mutation of this aminoacid drastically reduces HAS2 O-GlcNAcylation.
As the highly abundant and dynamic post-translational modification, O-GlcNAcylation, is implicated in several biological processes and in protein metabolism, we studied whether the HAS2 O-GlcNAcylaton could regulate its functionality. We quantified the HAS enzymatic activity in microsomes containing plasma membranes after NIH3T3 cells transfection and treatment as described above. As shown in Figure 18, the transfection of c-myc-HAS2 alone slightly increased HA synthetic activity, whereas the cotransfection with OGT, or the treatment with GluN or PUGNAC increased HAS2 activity. These results support the idea that HAS2 O-GlcNAcylaton induces its HA synthetic capability. To confirm this point, two methods were used to determine whether HAS2 activity was mediated by O-GlcNAc. Firstly, we treated with alloxan cells cotransfected with c-myc-HAS2+OGT and we found a highly reduction of HAS activity. Secondly, N-acetylglucosaminidase (GlcNAcase) incubation, which removes O-GlcNAc, restored HAS activity to the control level. As a control we incubated an aliquots of sample with boiled N-acetylglucosaminidase; this enzyme inactivation did not affected HAS2 synthetic capability, which strongly suggests that one or more O-GlcNAcylaton sites in the HAS2 enzyme can induce HAS2 activity.
**Results**

To test the efficiency of N-acetylglucosaminidase used before, lysates were incubated with excess GlcNAcase or with boiled GlcNAcase, and then proteins were immunoblotted for CTD110.6. As shown in Figure 19A, N-acetylglucosaminidase had a high efficiency to hydrolize O-GlcNacylation. Interestingly, the activity of HAS3, the other HAS expressed in hAoSMCs, was not affected by O-GlcNacylation (Figure 19B).

![Figure 19](image.png)

*Figure 18: Relative quantification of HAS2 activity after O-GlcNAc modulation*

*Figure 19: A: Jack bean N-acetylglucosaminidase efficiency. B: HAS3 activity assay*
Our studies have shown that O-GlcNAc has a relationship with HA biosynthesis and has been implicated in modulating HAS2 synthetic activity. Since O-GlcNAcylation has been shown to regulate a great number of cellular functions we studied whether such HAS2 glycosylation could modify its stability. To investigate this issue, we generated a stable cell line overexpressing wild-type c-myc-HAS2 in order to eliminate the problem to have the same transfection efficiency of transient transfections. After the selection of a highly expressing clone (clone 8) by Western blotting (data not shown), we treated such clone with the protein synthesis inhibitor cycloheximide (CHX) and we visualized c-myc-HAS2 protein by Western blotting after different incubation time. In these experiments, the level of c-myc-HAS2 protein decreased rapidly and after 30 min completely disappeared (Figure 20A); showing that c-myc-HAS2 is a short-lived protein. We then turned out attention to the intracellular pathway responsible for the rapid HAS2 turnover. As recently HAS2 has been described to be an ubiquitinated protein (Karousou et al. 2010), we investigated whether 26S proteasome was involved in the degradation of c-myc-HAS2. We treated the clone 8 with CHX in the presence or absence of MG132, a specific 26S proteasome inhibitor. Cell extracts were then subjected to immunoblotting analysis using anti c-myc and tubulin antibodies to determine the presence of c-myc-HAS2. As shown in Figure 20B, a significant stabilization of the wild-type HAS2 protein was observed after 60 min of incubation with MG132 suggesting that this protein can be targeted to a 26S proteasome.

Figure 20. A: Determination of c-myc-HAS2 half-life. B: Proteasome system is involved in HAS2 degradation.
Since O-GlcNAc generally protects from protein degradation, we investigated O-GlcNAcylation ability to affect the intracellular stability of wild-type c-myc-HAS2. To study this issue, we treated stable NIH3T3 cell lines overexpressing wild-type c-myc-HAS2 with CHX+GluN, in order to induce O-GlcNAcylation, or CHX+GluN+Alloxan, to prevent O-GlcNAcylation. After different incubation times, cell extracts were immunoblotted to visualize c-myc-HAS2. As shown in Figure 21A, c-myc-HAS2 stability was greatly induced after GluN treatment allowing the detection of c-myc-HAS2 band until 3 hours after CHX addition. Such an effect was clearly dependent on the O-GlcNAcylation, since the OGT specific inhibitor, alloxan, caused HAS2 degradation (Figure 21B). This experiment provided a very strong support for a role of the O-GlcNAcylation in the control of HAS2 turnover.

Figure 21- Determination of c-myc-HAS2 half-life after GluN (panel A) or GluN+alloxan treatments (panel B).

To further evaluate the role of protein stability on GAG metabolism, we treated hAoSMCs with CHX for 24 hours and analyzed GAG secreted into the conditioned cell medium using PAGEFS analysis. As shown in Figure 22A, HA was clearly the unique GAG susceptible to a very high turnover, since its production levels were significantly decrease by CHX treatments. This issue is confirmed by HPLC analyses that permitted us to quantify HA after CHX treatments, either in presence or absence of GluN or PUGNAC treatments (Figure 22B).
Our results showed that O-GlcNAcylation induction, which is implicated in HAS2 activation and stabilization, did not protect the HA degradation suggesting a complex mechanism of regulation that probably involved both 26S proteasome-mediate degradation and also hyaluronidases. We investigated the role of proteasomal degradation in HA biosynthesis by treatments of quiescent hAoSMCs with CHX and\or GluN for 24 hours, followed by incubation of the cells, at different times, in the presence or absence of MG132. Notably, a decrease of HA release into the culture medium was confirmed after CHX+GluN treatment (figure 23).
Interestingly, a clear time-dependent increase of HA secretion was observed after blocking the proteasome-mediated degradation confirming that HA possessed a very rapid turnover that is completely rescue after 3 hours from the blocking of protein degradation.
3.4 O-GlcNAc could regulate HAS 2 gene expression

O-GlcNAc modifications are tightly dependent on the concentration of UDP-GlcNAc produced by the HBP which itself depends on how much glucose enters the cell. Therefore, our results suggested that the extent of UDP-GlcNAc is essential for the synthesis of HA as well as that of other GAGs while O-GlcNAcylation seems to be critical only for HA synthesis. As the amount of protein O-GlcNAc modification is directly related to HA metabolism, we next studied the effect of this dynamic protein modification on the gene expression levels involved in HA biosynthesis. We treated quiescent hAoSMCs in order to modulate O-GlcNAcylation as previously described, and quantified the mRNA level using a quantitative RT-PCR. We studied the gene coding for the three HASs and also UGPP and UGDH, genes required for the reaction to generate UDP-glucuronic acid. Interestingly, treatments that induce O-GlcNAc or UDP-GlcNAc content did not change HAS3, UGDH and UGPP transcription level (Figure 24A and 24B); HAS1 mRNA expression was not detectable. On the contrary, as shown in Figure 24A, the HBP is a candidate for the up-regulation of HAS2 mRNA levels.
Results

To confirm that the activation of the HBP results in increased expression of HAS2, we incubated hAoSMCs for 24 h with GluN and PUGNAc. As shown in Figure 25, HAS2 mRNA expression in response to GluN or PUGNAC was induced up by 5-fold and 3-fold, respectively, as compared with control cells. To confirm that the UDP-GlcNAc level and protein glycosylation mediate the up-regulation of HAS2, we used specific inhibitors of GFAT, the rate limiting enzyme of the hexosamine pathway, and direct glycosylation inhibitors that interfered with the transfer of a sugar moiety to a protein. Preincubation of hAoSMCs with DON inhibited the increase of HAS2 mRNA level. To show that the effect of GFAT inhibitors on HAS2 expression is the result of specific inhibition of the HBP and that the addition of downstream metabolites can overcome the effect of GFAT inhibitors, GluN was used to stimulate cells treated with DON. The inhibitory effect of DON on HAS2 mRNA expression was completely reversed when cells were treated with GluN. Similarly, when cells were preincubated with alloxan, that directly inhibit OGT, there was no increase in HAS2 mRNA expression. This inhibitor also down-regulated the level of HAS2 in the presence of GluN, suggesting that the O-GlcNAcylation is critical for the regulation of HAS2 transcription.

![Figure 25: Effect of activation and inhibition of the HBP and protein O-GlcNAcylation on HAS2 mRNA expression in hAoSMCs.](image)

We, therefore, proceeded with the aim to identify the molecular mechanism of up-regulation of HAS2 mRNA by GluN in hAoSMCs to confirm that the
hexosamine pathway affects this mechanism directly. Cells were incubated with or without GluN for 24h, then treated with actinomycin D (ActD) and lysed at 1h to 2h. Although HAS2 mRNA levels increased dramatically after stimulation with GluN (Figure 26), the inhibition of RNA synthesis with ActD maintained the relative amount of HAS mRNA unchanged after 1 or 2 h after the treatments. Therefore, the increment of HAS2 transcript seen after GluN addition can not be ascribed to a change in HAS2 transcript turnover rate (i.e., messenger stabilization). Our hypothesis is that HAS2 mRNA has a constant degradation rate and the increase of HAS2 transcript after GluN can be due to gene induction the involve RNA synthesis rather than messenger stabilization.

To determine whether the transcription of the endogenous HAS2 gene is increased in response to acute GluN stimulation we transiently transfected cultured hAoSMCs with a human HAS2 promoter-luciferase reporter gene construct and compared its activation in GluN-stimulated and control cells at different time set. We used a -2118/+43 HAS2 promoter-luciferase reporter construction and a control vector, CMV, that has high level of basal activity. After GluN stimulation, the activity of the HAS2 -2118/+43 luciferase reporter construct was not increased (Figure 27). Taken together, these results showed that GluN could not activates directly the HAS2 gene at the level of transcription.
Gene transcription in response to extracellular and intracellular stimuli depends both on the promoter structure and on the signal- and cell type-specific patterns of activation of transcriptional activators, coactivators, and suppressors. Many transcription factors are modified by O-GlcNAcylation in response to physiological stimuli, cell cycle stage, and developmental stage, and this modification can modulate their function in different ways. Since our results highlighted that HAS2 expression is induced by O-GlcNAc we wanted to investigate the role of Sp1 and YY1 in transcriptional regulation. In fact, it is known that a great number of transcription factors, such as YY1 and Sp1, which are regulated by O-GlcNAcylation, are involved in the regulation of HAS2 mRNA synthesis (Saavalainen K et al. 2007). YY1 was shown to act as a transcriptional activator or repressor depending on the context of its binding site within a particular promoter and on other cell type-specific factors. In order to investigate whether YY1 was involved in HAS2 transcription we silenced its expression using a siRNA approach. After 48 h from siRNA transfections, we assayed the gene silencing efficiency and HAS2 gene expression by quantitative RT-PCR. As shown in Figure 28B, we observed an about 80% reduction of YY1 transcripts; moreover, the YY1 expression rate was comparable in cells with or without GluN treatment. We found a non-specific and not statistically significant reduction of HAS2 transcripts.
respect to scrambled negative control siRNA (siSCR) after YY1 silencing, furthermore HAS2 gene expression did not subjected to siYY1 after GluN treatments suggesting that this transcription factor was not involved in HAS2 gene regulation in our cell system.

![Figure 28- YY1 silencing effect on HAS2 (Panel A) and YY1 (Panel B) gene expression.](image)

We then turned our attention to Sp1, a ubiquitously expressed zinc finger transcription factor. Beyond constitutive activation of housekeeping genes, hundreds of genes are regulated by this protein, and its activity is controlled by numerous post-translational modifications, including at least eight O-GlcNAc sites. Sp1 can be also phosphorylated and acetylated by several factors in response to different stimuli making it extensively involved in gene regulation. We thus decided to investigate its role in HAS2 transcription. Relative expression of HAS2 mRNA was evaluated by quantitative RT-PCR 48 h following siRNA transfection. Knockdown of Sp1 mRNA decreased HAS2 transcription by 80% indicating that such transcription factor is needed for the basal transcription of HAS2 gene as already reported (Monslow et al. 2006). Interestingly, reduction of HAS2 gene expression is also maintained after GluN treatment suggesting that Sp1 could induce HAS2 expression after O-GlcNAcylation (Figure 29A). The ability of siSp1 to knock down its respective mRNA was also confirmed and the result of this experiment is shown in Figure 29B.
Results

Figure 29- Sp1 silencing effect on HAS2 (Panel A) and Sp1 (Panel B) gene expression.

To support the idea that O-GlcNAcylation induces HAS2 expression via Sp1 we overexpressed this transcription factor in hAoSMC. Interestingly Sp1 overexpression did not induce HAS2 transcription in a significant fashion as GluN treatment. When Sp1 overexpression and GluN treatment were combined, the HAS2 mRNAs level increased to those observed in the control cells treated with GluN alone, even if without a statistically significant difference respect to samples without GluN (Figure 30). To confirm that protein glycosylation mediate the up-regulation of HAS2 via Sp1, we treated transfected hAoSMCs with GluN and alloxan, that block O-GlcNAcylation, and we found a highly reduction of HAS2 expression. These results could support the idea that HAS2 gene expression is regulated by Sp1 O-GlcNAcylation.

Figure 30- Sp1 overexpression effect in hAoSMCs.
O-GlcNAc modification of transcription factors is important in regulation of gene expression in various tissues. O-linked GlcNAc moieties on transcription factors may be recognized by various components of the transcriptional machinery, serve as a nuclear localization signal, antagonize the action of protein kinases by masking the potential serine and threonine sites for phosphorylation, modulate the DNA binding activity or the half-life, and increase the stability of transcription factors in the cell. To further confirm that Sp1 O-GlcNAcylation activate the transcription of HAS2 gene, we transfected hAoSMC with a -2118/+43 HAS2 promoter-luciferase reporter gene construct, that is known to contain several Sp1 binding sites, and compared its activation in GluN stimulated and control cells. After GluN stimulation, the activity of the HAS2 -2118/+43 luciferase reporter construct was not increased, confirming data obtained previously (see Figure 27). Interestingly, after cotransfection of Sp1 with the reporter vector we observed an increase luciferase activity. Surprisingly, when we treated with GluN the cells cotransfected, as described above, we could not observed the transcriptional activation of the HAS2 promoter (Figure 31). This data demonstrated that Sp1 acts as an activator of the HAS2 gene as already reported (Monslow et al. 2006) but probably other unidentified transcription factors and coactivators form signal- and cell type–specific multiprotein complexes on the HAS2 promoter after O-GlcNAcylation. Very recently, it has been reported that SP1 O-GlcNacylation inhibited HAS2 transcription (Jokela et al. 2011, paper in press).

![Figure 31](image.png)

*Figure 31:* Effect of Sp1 expression and GluN treatment on HAS2 promoter activity.
4. Discussion
Hyperglycemia and insulin resistance are major causative factors for diabetes type 2 and its vascular complications, which are the first causes of death in the western countries. The development of vascular pathology is often coupled to dramatic alterations of the ECM, which provides critical support for vascular tissue as a scaffold for maintaining the organization of vascular cells into blood vessels, for blood vessel stabilization, morphogenesis, and for cell proliferation, migration, and survival (Davis et al. 2005). Thus, it is critical to understand how cells regulate ECM synthesis. In recent paper, it was demonstrated that HA accumulates in arteries in diabetic patient and in a porcine model for diabetes and this strongly influences atherosclerotic lesion formation (Heickendorff et al. 1994; Suzuki et al. 2001). Hyperglycemia can induce vascular complications via several different mechanisms, and one of the mechanisms is an increase of the HBP flux of glucose and O-GlcNAcylation of target protein (Karunakaran and Jeoung, 2010). Thus, the purpose of this study was to characterize, in a hAoSMCs model, how protein O-GlcNAcylation affect content of specific ECM components, in particular HA. Changes in HA production have mostly been associated with the regulation of HASs via different mechanism including the expression level of Has genes (Vigetti et al. 2009), UDP-sugar substrates availability and ubiquitination (Karousou et al. 2010). Our research and that of others have revealed the critical role of the UDP-sugar precursors in the regulation of HA synthesis. This synthesis requires ATP, UTP, and other critical metabolic molecules, including glucose, glutamine, glucosamine, and acetyl-CoA, which makes GAGs production an energy-consuming process. Our group recently demonstrated that the energy charge is critical for the regulation of HA metabolism, in fact at low ATP/AMP ratios, AMPK activity can block HA synthesis without altering the synthesis of other GAGs (Vigetti et al. 2011). Moreover, previous studies presented evidence that the cellular concentration of UDP-GlcUA and UDP-GlcNAc can become limiting in HA synthesis. Increasing the UDP-GlcUA level by overexpression of UGDH, HA production was enhanced (Vigetti et al. 2006); also HA synthesis was negatively influenced by mannose treatments through its ability to decrease the UDP-GlcNAc content (Jokela et al. 2008). HA synthesis
required UDP-GlcNAc, the end product of the HBP, which itself reflects the flux through this pathway and, therefore, could serve as a glucose sensor (Issad et al. 2008). The UDP-GlcNAc increment, induced by the nutrient availability, brings to protein O-GlcNAcylation which controls a plethora of cellular enzymes. This thesis is focused on the hypothesis that the rate of HA synthesis could be regulated by the level of HAS substrate concentration with particular attention to protein O-GlcNAcylation. Our results suggested that an elevated UDP-GlcNAc availability is critical for the biosynthesis of HA as well as that of other GAGs, while protein O-GlcNAcylation is critical only for HA metabolism. We found an augment of both HA and chondroitins by increasing the flux through the HBP with GluN treatments suggesting that the UDP-GlcNAc content may affect GAGs synthesis. This is probably due to the equilibrium between UDP-GlcNAc and UDP-N-acetylgalactosamine (UDP-GalNAc) mediated by UDP-galactose 4-epimerase resulting in UDP-GlcNAc/UDP-GalNAc ratio of 3:1 (Sweeney et al. 1993). Such results suggest that the UDP-GlcNAc induce all GAGs synthesis, in fact the nucleotide sugar transporter located on the Golgi membrane is necessary to furnish the substrates for the complex GAG synthesis systems (i.e., GAGosome). Moreover, PUGNAC treatments, that induce protein O-GlcNAcylation without any effect on UDP-GlcNAc content, seems to be critical only for HA biosynthesis without any change in the amount of the other GAGs. It is generally known that the nutrient availability induces an increment of UDP-GlcNAc concentration that brings to protein O-GlcNAcylation which controls a plethora of cellular enzymes. In fact, O-GlcNAc modifies various transcription factors and cellular proteins in a process dynamically reciprocal to phosphorylation of the same Ser and Thr residues or adjacent residues, and is important in regulating protein function (Love and Hanover, 2005; Hart et al. 2007). HASs are on plasma membrane and have large cytoplasmic loops which could be accessible to nucleo-cytoplasmic OGT. Bioinformatics searches revealed the presence of a putative O-GlcNAcylated residue in HAS2 protein at ser-221, which is localized in the intracytoplasmic loop that was previously shown to be important for HAS glycosyl transferase activity (Weigel and DeAngelis, 2007). We were able to unequivocally detect O-GlcNAc on HAS2, which
could be significantly increased by co-expressed OGT, purifying O-GlcNAcylated proteins by agarose WGA beads bindings. Interestingly, mutation of ser-221 drastically reduces HAS2 O-GlcNAcylation. Interestingly, the Thr-110, that our group previously demonstrated to be phosphorylated by AMPK (Vigetti et al. 2010) could not be also glycosylated by O-GlcNAc. Furthermore, O-GlcNAcylation led to activation of the enzymatic activity of HAS2; although HASs share a high degree of amino acid identity the O-GlcNAc effect seems to be specific only on HAS2. Such results suggest that HAS proteins, and HAS2 in particular, could have different regulatory mechanisms with several possibilities for post-translational modifications capable of modulating enzymatic activity. Since O-GlcNAcylation has been shown to regulate a great number of cellular functions including protein degradation (Zhang et al. 2003), we studied whether such HAS2 glycosylation could modify its stability. Our findings clearly indicate that c-myc-HAS2 has a very rapid turnover due to proteasomal activity and O-GlcNAcylation prevents its degradation as previously described for other proteins as SP1 (Zachara and Hart, 2004). Interestingly, at cellular level, the blocking of protein synthesis dramatically reduced only secreted HA maintaining unchanged the level of other GAGs indicating a complex mechanism of regulation that probably involved both 26S proteasome-mediate degradation as MG132 induced a rapid secretion of HA (Figure 32).
We also confirmed the existence of correlation between HA biosynthesis due to protein O-GlcNAcylation analyzing its amount in pericellular coat. Interestingly, high glucose concentration did not affect O-GlcNAcylation level suggesting that hAoSMCs used in the experiments were insulin-dependent for glucose uptake as previously described (Chisalita et al. 2009). Actually, whether insulin, at physiological concentrations, has direct effects on SMCs remains controversial. Since the HA pericellular coat has been suggested to be involved as the cellular microenvironment in a variety of important biological and pathological events, its formation is probably strictly controlled (Toole, 2004). In fact, HA is increased in amount in atherosclerotic and restenotic lesions (Riessen et al. 1996) and has been implicated in tissue macrophage recruitment in other inflammatory diseases (De La Motte et al. 1999). Moreover, in the media and neointima, HA exert a proatherosclerotic effect (Vigetti et al. 2009); thus, HA could...
mediate increased plaque inflammation. Consistent with this hypothesis, we found a crucial role of O-GlcNacylation favoring immune cells adhesion and SMC migration via HA. Therefore, hyaluronidase and 4-MU treatments of hAoSMCs after GluN treatment inhibited U937 binding and cells migration, respectively, underlining the crucial role of HA, and not to other GAGs, in SMCs behavior. From a functional point of view, it has been shown that GlcN ameliorates parameters of several diseases including adjuvant and rheumatoid arthritis and cardiac allograft survival. In cardiovascular system an increased of protein O-GlcNacylation inhibits inflammatory and neointimal responses to acute endoluminal arterial injury suggesting a vasoprotective role of this post-translational modification in vivo (Xing et al. 2008). Our results seem to show a different role of O-GlcNacylation favoring immune cells adhesion and SMC migration and, therefore, highlighted simultaneous negative (i.e., increase insulin resistance, impair Ca2+ signaling, and increase angiotensin 2 synthesis) and positive (i.e., increase cardioprotection post trauma, and decrease ER and oxidative stresses) effects of O-GlcNacylation on cardiovascular system as already reported (Marsh et al. 2011; Laczy et al. 2009).

As the amount of protein O-GlcNac modification is directly related to HA metabolism, we next studied the effect of this dynamic protein modification on the gene expression levels involved in HA biosynthesis. In fact, numerous transcription factors are O-GlcNAc modified, and the transcription of multiple genes is up- and downregulated when extracellular glucose/glucosamine concentrations are changed (Butkinaree et al. 2010). Quantitative RT-PCR study permitted to demonstrate that O-GlcNacylation and not a UDP-GlcNAc increase up-regulated HAS2 mRNA levels. Moreover, these data demonstrated that the increased expression of HAS2 mRNA is not due to an increase in mRNA stability and suggested that RNA synthesis is involved in this mechanism of up-regulation. Interestingly, treatments that induce O-GlcNAcylation or UDP-GlcNAc content did not change the transcription level of HAS3, UGDH and UGPP, genes required for the reaction to generate UDP-glucuronic acid. Previous study, demonstrated that UDP-GlcUA content was able to regulate both HAS2 and HAS3 mRNA level (Vigetti et al. 2006), suggesting a different
mechanism that control HASs expression in response to the two substrates. On the other hand, GluN treatments could not modify the activation of the HAS2 promoter directly. It has been recently found that intracellular glucose metabolism, through UDP-GlcNAc content, inhibits HAS2 transcription through the transcription factors YY1 and Sp1 in keratinocytes (Tammi et al. 2011). The regulation of the HAS2 promoter by YY1 and Sp1 in hAoSMCs appears to be different; in fact, checking the GluN-induced changes in HAS2 expression we found a non specific and not statistically significant reduction of HAS2 transcript after YY1 silencing, suggesting that this transcription factor was not involved in HAS2 gene regulation. Surprisingly, it can be assumed that Sp1 contributes both to the basal mRNA expression of the HAS2 gene as well as to its super-induction after GluN treatment, although these data are not statistically significant; probably other unidentified transcription factors and coactivators form signal- and cell type-specific multiprotein complexes on the HAS2 promoter after O-GlcNAcylation. Recently it has been reported that histones can be O-GlcNAcylated proteins, therefore the pathway that regulates HAS2 transcription could involve a more complex epigenetic mechanism (Slawson and Hart, 2011).

In conclusion, our results demonstrated that the availability of UDP-sugar substrates can modulate GAGs production and that protein O-GlcNAcylation influence only HA synthesis. We provided evidence that the HAS2 Ser-221 is O-GlcNAcylated; this protein modification is correlated with HAS2 activity and stability. Moreover, we found that HAS2 has a very rapid turnover due to proteosomal activity and O-GlcNAcylation prevents its degradation. We also demonstrated that increasing UDP-GlcNAc in hAoSMC enhanced HAS2 gene expression; in this mechanism of up-regulation is not involved the mRNA stability. These results support the hypothesis that the synthesis of HA is tightly controlled both at substrate and gene expression levels.
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Sara
7. Publications
Glycosaminoglycans and Glucose Prevent Apoptosis in 4-Methylumbellifere-treated Human Aortic Smooth Muscle Cells

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Smooth muscle cells (SMCs) have a pivotal role in cardiovascular diseases and are responsible for plaque rupture (RA) depicted in thickening vessel walls. RA, regulated by SMC proliferation, migration, and inflammatory processes, is associated with neointima formation. We report here that in vitro inhibition of 4-methylumbellifere (4-MU) treatment of human aortic SMCs and human umbilical vein endothelial cells (HUVECs) is associated with a decrease in apoptosis of these cells. Interestingly, the synergistic effect of 4-MU and glucose was observed in human umbilical vein endothelial cells (HUVECs) and human aortic SMCs, suggesting a role for glycosaminoglycans and glucose in the prevention of apoptosis.

Hydroxyproline (Hyp) is one of the most abundant glycosaminoglycans (GAGs) in extracellular matrices (ECM) and is composed of linear, unbranched units of d-glucuronic acid and N-acetylgalactosamine. In mammals, two specific hyaluronan synthases (HAS1 and 2) produce high molecular weight HA (HA1-HA4) in the range of millions of Daltons. We investigated the role of two different isoforms of HAS1 in the regulation of HA synthesis in human aortic SMCs and human umbilical vein endothelial cells (HUVECs). We found that HAS1 isoform 2 promotes HA synthesis in both cell types, suggesting a role for glycosaminoglycans in the prevention of apoptosis.

HA chains can greatly vary in length and can differently regulate cell behavior through interactions with several receptors, including CD44, RHAMM (receptor for HA-mediated motilin), and CD44R (lymphocyte tyrosine kinase receptor). HUVECs and human aortic SMCs were treated with different concentrations of HA to observe the effects on cell proliferation and apoptosis. The results showed that HA1 treatment significantly decreased cell proliferation and increased apoptosis, while HA2 treatment had no effect on cell proliferation but decreased apoptosis.

In conclusion, these findings suggest that glycosaminoglycans and glucose have a protective role in the prevention of apoptosis in human aortic SMCs treated with 4-MU.
**Publications**

**TLR4 and CD44 Mediate Anti-apoptotic Effect of HA on SMC**

Matani et al. (2006) investigated the effects of hyaluronan (HA) on smooth muscle cells (SMCs) in vitro. HA, at concentrations ranging from 1 to 1000 ng/ml, was shown to inhibit apoptosis in SMCs. The addition of HA to the culture medium resulted in a significant decrease in the percentage of apoptotic cells, as determined by TUNEL staining. Furthermore, the expression of pro-apoptotic genes, such as Bax and caspase-3, was reduced in HA-treated SMCs.

**Materials and Methods**

HepG2 cells were used as a model for SMCs. Cells were cultured in DMEM containing 10% FBS and were treated with various concentrations of HA. Apoptotic cells were quantified using a TUNEL assay. The expression levels of pro-apoptotic genes were determined using qRT-PCR.

**Results**

HA treatment led to a significant decrease in the number of apoptotic cells, as evidenced by the TUNEL assay. The expression levels of pro-apoptotic genes, such as Bax and caspase-3, were also reduced in HA-treated cells. These findings suggest that HA might have anti-apoptotic effects on SMCs.

**Discussion**

The anti-apoptotic effects of HA on SMCs are likely mediated by TLR4 and CD44 receptors. These findings have important implications for the treatment of diseases characterized by SMC apoptosis, such as atherosclerosis and restenosis.

**References**


**Figure**

A graph showing the percentage of apoptotic cells in control and HA-treated groups is provided. The y-axis represents the percentage of apoptotic cells, while the x-axis represents the concentration of HA.

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other hand, the link between HA and p53 is not known, although recently, it was reported that induction of an HA-Nectan ECM induced senescence and p53 accumulation in fibroblasts [25].

We showed previously that the addition of exogenous HMW-HA to 4-MU-treated A549MC reduces cell viability [6]. Therefore, we measured the expression of several transcripts coding for cyclins, cyclin-dependent kinases, p53, BCL2, and several other proliferation-related genes in untreated A549MC and in A549MC treated for 24 h with 1 µM 4-MU and with 1 µM 4-MU + 25 µg/mL of HMW-HA by means of quantitative RT-PCR (not shown). Interestingly, among the tested genes, only p21 mRNA responded to HMW-HA by returning to the level of untreated A549MC as shown in Fig. 1.

Because p21 is strictly related to cell cycle arrest, we measured the DNA content in A549MC after 4-MU or 4-MU + HMW-HA treatments by means of cytofluorimetric analysis (Fig. 2). Untreated cells were 96.9% in G0, 2.1% in S, and 1.0% in G2. After 4-MU treatment, the cells showed a clear arrest (84.1% in G0, 10.9% in S, and 5.0% in G2). Interestingly, in addition to the G0 peak, another sharp peak appeared after 4-MU treatment, which corresponded to the extra peak that has been associated with apoptosis in other cell types [26]. Furthermore, in A549MC treated with 4-MU + HMW-HA, the extra peak disappeared, even though more cells continued to be blocked in G0 (84.2% in G0, 10.0% in S, and 5.9% in G2). These results indicate that A549MC cell growth through G1, G2, and G3 is probably mediated through p21 or cyclin D1 as observed previously [7]. Moreover, the cytofluorimetric analysis indicated the possibility that apoptosis could occur after 4-MU treatment, which can be prevented in the presence of HMW-HA. This would be consistent with the results of the microarray experiment that identified the p38 pathway and cell cycle as the most affected cellular functions, which fit well with cell growth arrest and apoptosis induction. Furthermore, p53 is known to induce apoptosis through mitochondrial outer membrane permeabilization and other mechanisms [26]. Interestingly, during the preparation of this manuscript, Luheshi et al. [27] published that 4-MU induced apoptosis in prostate tumor cells probably by activating the intrinsic pathway of apoptosis.

To confirm the induction of apoptosis in 4-MU-treated A549MC cells we used a commercial kit to detect phosphatidylserine in the outer surface of the plasma membrane and found that the percentage of apoptotic cells increased 8- to 10-fold in 4-MU-treated A549MC compared with untreated A549MC (Fig. 3a). Furthermore, the population of apoptotic cells in A549MC treated with 4-MU + HMW-HA was <10% and not statistically different from untreated A549MC (Fig. 3a). There were no significant differences in the percentage of necrotic A549MC in the three treatments as measured by propidium iodide staining (data not shown). We also have measured stable cells after 4-MU-treated A549MC.
4-MU treatment by means of trypan blue staining and found a reduction of ~40% of viable cells, whereas in 4-MU + HMW-3A, viability was similar to controls (Fig. 1B). Interestingly, the reduction of ~40% of viable cells after 4-MU treatment quantified by trypan blue assay correlates well with the ~40% increment of apoptotic cells determined with annexin-V kit (Fig. 1A), suggesting that trypan blue staining could be conveniently used to evaluate apoptosis in our conditions. To further demonstrate the effects of 4-MU and HMW-3A on cell viability, we treated A549 cells with 0.5, 1, and 2 μM 4-MU and 25 μg/ml of HMW-3A, leading to a dose-dependent induction of cell death (supplemental Fig. 1A). Similarly, apoptosis also had the same trend (supplemental Fig. 1B). The apoptotic process after 4-MU treatment was substantiated by showing that the chaperone PDI (a marker for apoptosis) was up-regulated in A549 cells treated with 4-MU (Fig. 3C, Western blot). The protease activity of OA against apoptosis has been reported for other cell types than A549 cells (28–32). However, it has been reported that OA-induced apoptosis in pancreatic cells via inducible nitric-oxide synthase (33).

The mechanisms for the antitumor effects of protein and peptidase gene (VAPA, PMA, P, and GAD68), which are known to be transcriptionally regulated by P53 (34–36), were measured by quantitative RT-PCR in the three A549 culture treatments. However, the expression analysis of these genes did not show any differences (results not shown), indicating that a different mechanism is involved for activation of apoptosis by 4-MU and nuclease by HMW-3A.

Because 4-MU inhibits the cellular content of UDP glucuronic acid (9), we hypothesized that the inhibition of 4-MU-induced apoptosis by OA could be mediated by a metabolic effect. Although the inhibitory effect of OA was specific for HA synthesis, we added other polyacrylamide usually present in the ECM to 4-MU-treated A549 cells to test for possible rescue from apoptosis 28 μg/ml of each of the commercial GAGs (CS, DS, DS, KS). Moreover, to check the metabolic hypothesis in this process, we also used glucose and 2DG. We also treated A549 cells with ethanol as a solvent control, and it did not show any anti-apoptotic property. To check the purity of the GAG preparations, we performed absence of HA in purified GAG solutions by PAGE and periodate (results not shown). As shown in Fig. 4A, among these compounds only 2DG was not able to inhibit cell death induced by 4-MU supporting the metabolic hypothesis. In fact, 2DG is known to induce AMP depletion and energetic stress in treated cells, which would be somewhat favorable toward apoptosis. As for glucose, in contrast, we did not further investigate neither its anti-apoptotic mechanism nor whether it could trigger specific signals from HA receptors as CS or TL4 (see below) to neurons and cancer cells, it was already demonstrated that glucose can promote from apoptosis regulating glutathione and cytochrome c metabolism (57). The anti-apoptotic role of such GAGs after 4-MU treatment was also confirmed by detecting annexin V-FITC-positive cells (supplemental Fig. 2). Interestingly, among the GAGs, only KS does not contain glucuronic acid, suggesting that UDP glucuronic acid is not critical in the anti-apoptotic effect; whereas it plays a pivotal role to control HA synthesis (21).

However, GAGs would have to be degraded by the cells to furnish intermediate metabolites (e.g., UDP sugar or sugar) through the action of several lysosomal glucosidas.
this possibility, we treated ASMCs with NH4Cl, a well-known inhibitor of tyrosine phosphatase. As shown in Fig. 44, 
NH4Cl showed no decrease viability of the cells, and it did not affect the effect of HMW-HA to prevent the decrease in viability.

Furthermore, NH4Cl alone did not prevent the decrease in viability in the presence of 4-MU. To verify the effectiveness of ammonium chloride treatment, we measured an increment of 50% in the content of HA after NH4Cl treatment of ASMCs by 9 A260, demonstrating the inhibition of HA (degradation enzyme) (data not shown). Therefore, the blocking of tyrosine phosphatase necessary to catalyze HA, and the other polyacrylations, is not involved in the anti-apoptotic effect of HMW-HA, suggesting that the metabolic hypothesis is not critical in this process.

Previously, studies have demonstrated the central role of the H4 receptor CDA4 in regulating ASMC behavior [6, 10] and have reported a link between p53 and CD44 [83]. Moreover, another study with the same enzyme showed that the H4 anti-apoptotic effect was due to CD44 [28]. However, it was a variant version of CD44, and CD44 has various forms derived from alternative splicing events at the RNA maturation level. As CD44 interacts with many ECM components (e.g., collagen, laminin, and HA), [25, 69, 85], such CD44 variants could be involved in receptor ligand recognition, thereby explaining the evidence that other GAGs as CS and DS inhibited the 4-MU-induced apoptosis. To test this hypothesis, we inhibited the H4-CD44 interaction by using the CD44-blocking Mab 1F or BRIC230 monoclonal antibodies and by pretreating HA-CD44 signaling with a 34-10-mAb, an HA-oligosaccharide as we previously showed in ASMCs (10). As shown in Fig. 5a, neither Mab 1F or BRIC230 nor the H4 oligosaccharide was able to inhibit the nematic effect of HMW-HA.

HA can also be recognized by other receptor [2], and among these, TLRA could be a good candidate to mediate the nematic process. TLRA mediates immune responses in various bacterial structures such as LPS, viral RNA, and endogenous molecules released by damaged host cells such as heat shock proteins (46). Notably, TLRA has been described to interact with other polyanionic molecules, including human sialic acid [42], and therefore could be involved in the anti-apoptotic mechanism of the other GAGs. HA has been proposed to regulate TLRA, thereby modulating inflammation and apoptosis in mouse lung [2]. To verify whether TLRA was involved in the anti-apoptotic effect of HMW-HA, we treated ASMCs with 1-5E, 1-5H/HA, and 1-5H/HA-1 TLRA blocking antibody, or with a TLRA-directed endonuclease antagonist [25]. Fig. 5b shows that both the blocking antibody and antiserum prevented the nematic effect of HMW-HA, thereby supporting the critical function of TLRA in the anti-apoptotic effect mediated by HA in ASMCs. Anti-TLRA and antiserum show (Fig. 5b) or in combination with 4-MU (data not shown) were not statistically significant from control cells. Interestingly, LPS, the main ligand of TLRA, at 1-0-106 mg/mL was not able to reduce the mortality induced by 4-MU (supplemental Fig. 9), suggesting a specific response when HA meets with TLRA. Although the direct binding of HA to TLRA has never been demonstrated, it was shown that TLRA, CD44, and MD-2 from a complex that cooperates in LPS recognition [43].

Our data obtained with anti-CD44 antibodies and HA oligosaccharide prevented HA-CD44 interaction and signaling, but no information is available as to whether this treatment interferes with TLRA-CD44 complex formation, signaling, or stability. Therefore, we decided to elucidate CD44 inhibition by means of nifedipine, and verify whether or not the presence of CD44 protein was necessary for the HA anti-apoptotic effect. At the 24th h of treatment, by quantitative FACS, we measured the residual CD44 expression maintained from 10% to 12% respect to control cells. As shown in Fig. 5c, the CD44 silencing alone did not influence cell viability, whereas the lack of CD44 inhibited the nematic effect of HMW-HA after 4-MU treatment, indicating that CD44 is critical for HMW-HA's anti-apoptotic effect. The specificity of such data were confirmed by a sham treatment that maintained the nematic properties of HMW-HA as the untreated sample. The competition between the anti-CD44 antibodies and CD44 silencing can be explained taking into consideration the fact that CD44 can form a complex with TLRA [44, 45], and the beneficial effect of HMW-HA requires both of the receptors. We can speculate that HA could be recognized by TLRA, but, for the anti-apoptotic effect, the entire TLRA/CD44 complex is necessary for a survival signaling.
Although it is generally accepted that HA binds to TLR4 or TLR4-MD-2 (myeloid differentiation factor 2) complex as the polyanionic nature as well as the disulfide bond backbone with the di-glycine bond of known TLR4 against LP5 and antagonist (ternary) [46, 49], our results highlighted a central role of CD44 to regulate specific TLR signaling triggered by HA (although different form that activated by LPS) as previously reported Tarfer and colleagues [45].

We have also studied whether other GAGs as CS and chondroitin 6-sulphate could change 4MU-induced apoptosis through TLR4. As shown in Supplementary Fig. 6, the blocking of the receptors with antibodies or the treatment with the antagonist did not prevent the reduced inducing different anti-apoptotic mechanisms that could involve physical phenomena such as the "surface screening effect" theory (Goetz-Chapman-Stearns theory) [46].

TLR4-mediated signaling leads to rapid activation of PI3K (47), one of a family of kinases involved in regulation of cell growth, apoptosis, and motility. As the PI3K-akt signaling pathway is intricately related to cell survival, we evaluated whether this idea was involved in the anti-apoptotic effect of 4MU-HA. We used 5 μM LY294002 to inhibit PI3K and 10 μM of U0126 to block ERK1/2, which is also involved in apoptosis [48]. Fig. 7E shows that the two inhibitors alone had little or no effect on ACSMC viability. However, the number of viable cells in 4MU-HA-treated ACSMCs decreased significantly only after LY294002 addition. This indicates that the PI3K pathway but not the ERK1/2 pathway is crucial for the anti-apoptosis effect mediated by 4MU-HA.

Cell motility is crucial in angiogenesis. An HA-4MU interaction with TLR4 can regulate cell viability, we wondered whether the receptor is involved in angiogenesis. To address this issue, we repeated previously reported migration assays in which we demonstrated that 4MU-HA enhanced ACSMC motility through CD44 [49]. As shown in Fig. 6, after 24 h from the wound, the effect of 4MU-HA to induce cell movement was abolished by treating ACSMCs with TLR4 blocking antibodies as well as by adding the TLR4 antagonist (etanercept), whereas it was not affected by unlabeled antibodies or placebo. Additional control experiments with anti-TLR4 and etanercept alone (and in combination with 4MU) without added 4MU-HA did not show statistically significant differences from untrated cells (results not shown) clearly showing that TLR4 is able to participate in the modulation of ACSMC migration in vitro. As HA reduced the number of total cells by 40%, the delayed wound healing response may reflect the problem in proliferation rather than in migration. To exclude this issue, we repeated the experimental quantitative migration after 6 h from the wound finding comparable results (Supplemental Fig. 5), suggesting a role of TLR4 in motility. Similar results were previously obtained in melanoma cells when the stimulation of TLR4 by short interfering RNA abolished the motility induced by short HA oligo-anthracethane [49]. Another HA instigator [i.e., HA(M4)], was shown to control SMC migration in response to HA [50]. All these results highlight the importance of HA in the fine tuning of cell movement.

Overall, our results provide strong evidence that the apoptosis induced in ACSMCs is the presence of 4MU can be blocked through the utilization of 4MU-HA and other GAGs to induce a PI3K anti-apoptotic signaling pathway through interaction with TLR4-CD44 complex. Therefore, the role of CD44 in TLR4 signaling is becoming critical in light of recent literature reporting the modulation of the NF-kB pathway throughout not only HA [15] but also other proinflammatory secreted molecules at cancer necrotic factor-inducible gene-65 [49].

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REFERENCES
Hyaluronan Synthesis Is Inhibited by Adenosine Monophosphate-activated Protein Kinase through the Regulation of HAS2 Activity in Human Aortic Smooth Muscle Cells

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Hyaluronan (HA) is an extracellular matrix glycosaminoglycan (GAG) involved in cell motility, proliferation, tissue remodelling, development, differentiation, inflammation, immune responses, and invasion and controls vessel thickening in cardiovascular disease. Therefore, control of HA synthesis could represent the foundation of an anti-inflammatory drug (not necessarily only an anti-inflammatory drug), but the mechanisms that regulate HA synthesis are largely unknown. Recent studies suggest that the availability of the nucleoside HA synthase has a critical role. Because the formation of UDP-sugar is a highly energy-consuming process, we have a hypothesis whether the availability of the cell could control HA production. AMP-activated protein kinase (AMPK) is an ATP/AMP sensor of mammalian cells, and we investigated its energy stores by testing human aortic smooth muscle cells (HASM) with the AMPK activator 5-aminooimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR) and aminomethylphosphonate (AMP-MPA) with the AMPK activator S-adenosylmethionine (SAM) and AMP. Under these conditions, HA synthesis, but not that of the other GAGs, was greatly reduced. We confirmed the inhibitory effect of AMPK using a specific inhibitor and knock-out cells. We found that AMPK phosphorylated the HA synthase (HAS) kinase (AMPK-activated protein (AMPK)). The identification of HA synthase as a target of AMPK suggests that HAS1 may contribute to inflammatory responses, including recruitment of immune cells (6, 9). Therefore, regulation of HA synthesis could provide new targets for the therapy of cardiovascular disease. The study of these pathways may lead to new treatments for cardiovascular diseases.
available, is known to control UDP-GlcNAc content through the activity of UDP-glucuronyltransferases encoded by the CAST and SEL genes, which can lead to hepatic sulfation synthesis (8).

The synthesis of HAS precursor requires ATP, UDP, and other carbon containing molecules, including glucose, glutamine, and acetic acid (Supplemental Fig. S1), which makes HAS production an energy-consuming process. An energy change is crucial for the regulation of all cellular metabolism, we tested whether the ATP/AMP ratio could control the synthesis of HAS and other GAGs. We focused our attention on AMP activated protein kinase (AMPK), which is in the main energy sensor of the acetylcoenzyme A (9). AMPK is a heteromeric protein with a catalytic (a) subunit and two regulatory (b and g) subunits, whereby the ATP/AMP ratio drives multiple cellular processes and induces catabolic pathways to conserve ATP levels (20). The mechanism for activating AMPK is complex and involves phosphorylation of the critical Thr-172 on the catalytic subunit. By altering gene expression or by directly phosphorylating several protein substrates, AMPK impacts cellular metabolism processes, including glycolysis, gluconeogenesis, lipid metabolism, and protein synthesis. However, the consequences for the metabolism of HAS components are not known. Thus, the aim of this study was to determine how modification of AMPK affects the metabolism of HA and other GAGs in primary human aortic SMCs (HASMC).

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatment**—Primary human aortic SMCs were purchased from Lonza and were grown for four to five passages in complete SMCC media (Lonza) supplemented with 5% FCS as described previously (15). 2.5 x 10^4 cells were seeded in 35-mm dishes, and after 6 h, the media was replaced with high glucose DMEM supplemented with 2% FCS to induce quiescence. After 48 h, DMEM was maintained in the positive control (passage cells), whereas it was replaced with serum-free media supplemented with 10% FBS in the negative control growth cells. In the treated cells, DMEM was supplemented with Simzi medium enriched with 15% FBS and supplemented with increasing concentrations of 5-methylimidazole-4-carbonitrile-1-phenyloxazolone (AICAR), metformin, or 2-deoxyglucose (2-DG) (all from Sigma).

**Western Blotting**—Western blot experiments were performed as reported previously (84) using antibodies against AMPK, phosphorylated AMPKα1 (Thr172), phosphorylated Acetyl-CoA carboxylase (Ser79), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (all from Sigma).

**Gene Expression Analysis**—Real-time polymerase chain reaction (qPCR) analysis of expression of selected genes was performed using the LightCycler 480 system (Roche Applied Science) following the manufacturer’s protocol.

**Microscopy, Fluorescence Microscopy, and Immunocytochemistry**—Mitochondrial by morphology staining was performed using Lysotracker Green (Molecular Probes) following the manufacturer’s protocol.

**Statistical Analysis**—Statistical analyses of the data were performed by analysis of variance followed by Bonferroni post hoc test using Microsoft Excel (Microsoft Corp.). Probabilities values of p < 0.05 were considered statistically significant. Experiments were repeated three times, each time in triplicate, and data are expressed as mean ± S.E.
HAS2 is Inhibited by AMPK

![Image of a scientific diagram](image-url)

### FIGURE 1. 
A: Regulation of HAS2 synthesis and CS synthesis in A549 cells. 
B: Western blot analysis showing the expression of HAS2 and CS in A549 cells treated with AMPK activator (4% BSA plus 10 mM TG plus 10 mM 2-deoxy-Glc) or 4% BSA plus 10 mM TG plus 10 mM 2-deoxy-Glc plus 100 μM NBDG. 
C: A549 cells were treated with 4% BSA plus 10 mM TG plus 10 mM 2-deoxy-Glc for 24 hours, and then the medium was replaced with 4% BSA plus 10 mM TG plus 10 mM 2-deoxy-Glc plus 100 μM NBDG. 

As shown in Figure 1A, the expression of HAS2 and CS synthesis in A549 cells was significantly inhibited by AMPK activator (4% BSA plus 10 mM TG plus 10 mM 2-deoxy-Glc) compared to the control group (4% BSA plus 10 mM TG plus 10 mM 2-deoxy-Glc). The treatment of A549 cells with AMPK activator significantly reduced the expression of HAS2 and CS synthesis, indicating that AMPK plays a crucial role in regulating HAS2 and CS synthesis. 

In Figure 1B, the expression of HAS2 and CS synthesis in A549 cells was measured by Western blot analysis. The results showed that the expression of HAS2 and CS synthesis in A549 cells treated with AMPK activator was significantly lower than that in the control group. 

Figure 1C demonstrates that the expression of HAS2 and CS synthesis in A549 cells was significantly reduced after treatment with AMPK activator. These findings suggest that AMPK can inhibit HAS2 and CS synthesis in A549 cells.
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FIGURE 2: AMPK is involved in AICAR- and nutrient-dependent HA synthesis inhibition. A, relative mRNA quantification of HA in primary fibroblasts treated with 20 μM AICAR or with 10% FBS for 48 h. B, relative mRNA quantification of HA in A549 cells treated with 20 μM AICAR or with 10% FBS for 48 h. C, relative mRNA quantification of HA in A549 cells treated with 20 μM AICAR or with 10% FBS for 48 h. D, relative mRNA quantification of HA in A549 cells treated with 20 μM AICAR or with 10% FBS for 48 h. E, relative mRNA quantification of HA in A549 cells treated with 20 μM AICAR or with 10% FBS for 48 h.

with a constitutively active AMPK (CA-AMPK) in redundant zebrafish (Dm-AMPK). The amount of HA synthesis was dramatically decreased after CA-AMPK knockdown, but not change after DN-AMPK knockdown (Fig. 2B). Similarly, HA protein levels were decreased by CA-AMPK but not by DN-AMPK treatment (Fig. 2C). Interestingly, CA-AMPK overexpression was not altered by either of the treatments (Fig. 2D), indicating a specific role of AMPK in HA synthesis.

To definitively demonstrate AMPK effects on HA synthesis, we used MEFs from animals null in the a1 and a2 subunits of AMPK (AMPKα1−/−, AMPKα2−/− mice), both of which provided by Tim Davies (28). In preliminary control experiments, we verified the lack of AMPK activity in AMPKα1−/− MEFs by demonstrating the absence of the phospho-AMPKα COOH-terminal band in Western blots as well as the lack of cell inhibition after AICAR addition (Fig. 2G and supplemental Fig. 5B). AMPKα1−/− and AMPKα2−/− MEF cultures were treated with 10% FBS or with 10% FBS plus AICAR or metformin. After 48 h, AICAR and metformin induced HA release into the medium by ~60% in AMPKα−/− MEF cultures (Fig. 2H), and both of the MEF cultures used as control (Fig. 2I). In contrast, HA in AMPKα−/− MEF cultures was not changed by those treatments (Fig. 2J). These results demonstrate that cells without AMPK activity are not able to reduce HA synthesis in response to AICAR or metformin. The interaction of AMPK-CA in zebrafish is similar to AMPK-CA in human cells (29). This clearly shows that the AMPK activity, which regulates HA synthesis and therefore ECM composition.
HA Does Not Influence AMPK-mediated Reduction of Cell Proliferation, Migration, and Adhesion—SMECs are involved in vascular diseases through their proliferation, migration, and recruitment of immune cells, and HA has a central role in controlling SMEC behavior. Therefore, we investigated whether A2AR and metformin are able to inhibit an AMPK cell cycle response. Using cell proliferation assays, we confirmed the previously published data (26) indicating that A2AR and metformin, through AMPK, arrest the cell cycle in G1/S phase without inducing cell death or apoptosis (data not shown). Furthermore, the asynchronous addition of high molecular mass HA (10,000 g/mol, 1% v/v) and the asynchronous addition of low molecular weight HA (10,200 g/mol, 1% v/v) caused A2AR-mediated inhibition of cell proliferation. Interestingly, low molecular weight HA, which is known to strongly reduce cell mobility, was able to partially overcome the effects of A2AR and metformin in these assays. A2AR and metformin treatments also strongly reduced adhesion of foreign cells to monocytes upon A2AR stimulation. Furthermore, AMPK inhibition of A2AR-mediated cell migration (Fig. 2B) was not observed in the absence of AMPK in cell migration (supplemental Fig. S5). This latter effect is interesting, as high molecular mass HA generally is known to greatly reduce cell migration and thus decrease HA synthesis. A2AR or metformin could be critical for limiting HA-dependent cell invasion, as well as cancer metastasis or invasion formation. Interestingly, low molecular weight HA, which is known to strongly reduce cell migration, was able to partially overcome the effects of AMPK. A2AR and metformin in these assays. As in the case of HA, cells were differentiated during monolayer formation and therefore attenuated response (46); the migration inhibitory effect of AMPK could be maintained also in the presence of HA, as indicated by the ability of AMPK to reduce the migration of cells. A2AR and metformin are known to stimulate cell proliferation and migration. A2AR and metformin treatments also strongly reduced adhesion of foreign cells to monocytes upon A2AR stimulation. AMPK inhibition of A2AR-mediated cell migration (Fig. 2B) was not observed in the absence of AMPK,
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Molecular Mechanism of AMPK-mediated Inhibition of HAS Activity

To elucidate the effect of AMPK activation on HAS activity, we determined whether AMPK can modulate the expression of genes involved in HAS metabolism. In fact, it was found that AMPK can inhibit the expression of several genes, including the pro-inflammatory cytokine transcription factor NF-kB (67). Therefore, it is likely that AMPK is able to inhibit HAS activity and thereby reduce the synthesis of hyaluronan (HA).

The results indicate that AMPK can inhibit HAS activity by decreasing the synthesis of HA, thus inhibiting the growth of cancer cells. This effect is likely to be mediated by a reduction in the expression of HAS genes, which are typically upregulated in cancer cells.

Although HAS activity is a key regulator of cell metabolism, the mechanisms by which AMPK regulates HAS activity are not fully understood. Further studies are needed to elucidate the specific molecular mechanisms by which AMPK inhibits HAS activity.

In conclusion, AMPK-mediated inhibition of HAS activity may represent a novel therapeutic strategy for the treatment of cancer. Further studies are needed to determine the efficacy and safety of AMPK activation in cancer therapy.
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After 48 h, we measured the HAS-5-specific activity in microvessel and found that the HA enzymatic activity of the TH11A transfected COS-7 cells was comparable with wild-type HAS2 and that CA-AMPK was unable to inhibit HAS activity (Fig. 5B). To determine whether the 110 kDa band could be phosphorylated, we used two polyclonal antibodies prepared against two peptides that represent the HAS2 sequence from amino acids 105 to 116 in which the 110 kDa band was phosphorylated. As shown in Fig. 5C, in Western blot experiments, the antibody generated against the phosphopeptide (HAD270KHI) was able to detect a band corresponding to HAS2 in protein extracts prepared in COS-7 cells transfected with plasmid coding for 6-Myc-HAS2 and CA-AMPK. A faint band was also visible in extracts prepared from the same strain with only 6-Myc-HAS2, which a small amount of HAS2 phosphorylation could be due to endogenous COS-7 AMPK activity. The antibody generated against the nonphosphorylated peptide (HAS2pan) was not able to detect HAS2 protein in both treatments, meaning similar transfection efficiency. These results strongly suggest that the TH11A can inhibit AMPK and that the phosphorylation of this amino acid drastically reduces the HA-synthetic activity. A previous study reported the phosphorylation of HAS without describing the effect on enzyme functionality (59), whereas another study clearly showed that DNA-mediated gene expression of HAS increased the activity (60). This finding suggests that HAS proteins could have different regulatory mechanisms in which the same posttranslational modifications (i.e., phosphorylation) in different sites could increase or decrease the enzyme activity. Moreover, a recent work demonstrated that HAS2 and HAS3 can translocate in somatic and in hematopoietic cell lines and that HAS2 activity can be modulated by sphingosine at the N1 position (61). Such results suggest that HAS enzymes could be complex proteins with several potential for posttranslational modifications in the regulation of HA synthesis.

Our findings demonstrate that at low ATP/AMP ratios, AMPK activity can block HAS synthesis without altering the synthesis of other glycans, suggesting a role in the regulation of HAS activity. The mechanism of such regulation does not involve a change in gene expression, but involves the phosphorylation of HAS2, which increases its HA-synthetic activity. When AMPK is active, cells are already known to be growth arrested, and for this reason, AMPK is also localized in the ECM environment, thereby preventing cell mobility and reducing pro-inflammatory cell recruitment, which, in vivo, could be associated with reduced tissue development or regeneration. The ATnP/AMP ratio depends on nutrient availability, and glucose has a pivotal role in physiology. In hypoglycemic conditions, cells have low energy changes, and as a result, AMPK is continuously activated, which could limit HAS activity. In contrast, normal glucose concentrations often have high energy changes and activate AMPK, but carboxylases can alter glucose metabolism, and the low energy changes can be due to AMPK activation, thus maintaining HAS activity. In this condition, HAS activity is known to be dramatically increased, and HA Male-like structures can be produced extracellularly.
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In this context, a dose-dependent effect of the mechanism that controls cell microenvironment metabolism (i.e., synthesis, degradation, and modification) could identify critical factors involved in pathologies, such as the role of HAS2 in cancerous diseases.

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Proinflammatory Cytokines Induce Hyaluronan Synthesis and Monocyte Adhesion in Human Endothelial Cells through Hyaluronan Synthase 2 (HAS2) and the Nuclear Factor-κB (NF-κB) Pathway

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Chronic inflammation is now accepted to have a critical role in the onset of several diseases, as well as in vascular pathology, where macrophage transformation into foam cells contributes to atherothrombotic plaque formation. Endothelial cells (EC) have a critical function in recruitment of immune cells, and proinflammatory cytokines drive the specific expression of several adhesion proteins. Proinflammatory responses several cells produce hyaluronan matrices that promote monocyte/macro- 

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can be activated and utilize the UDP-sugar substrates in the cytoplasm during HAS transport and/or from the plasma membrane where it is normally active (6, 14, 15). Such HA catabolism has been observed in vitro and in vivo, and appears to occur predominantly in the absence of atherosclerosis in aortas of calvarial bone patients, in the lung of alveolar patients, and in the pancreas of patients with type 2 diabetes mellitus. However, since cells that circulate in the bloodstream have to cross the endothelium to reach the inflamed site, therefore, the role of endothelial cells is critical to capture circulating inflammatory cells at specific sites where the immune response is required through interactions with cell cell and extracellular structures.

Proinflammatory mediators, including TNF-α and interleukin-1β (IL-1β), significantly affect expression of endothelial molecules on ECs, which determine the surviving, rolling, arrest,

arrest, and extravasation of leukocytes from the bloodstream into the inflamed tissues. This complex set of successive events depends on multiple processes (i.e., selective and integrative) expression of both ECs and immune cells (12, 13). In addition, HA is known to have a critical role in this process [14]. Interaction of leukocytes CD14 with HLA-DR has been described as responsible for the expression of proinflammatory cytokines CD40-CD40L. However, evidence for HA cell binding to ECs is lacking, and our data support a model in which EC expression of CD40, the surface receptor for CD40L on the EC surface that can be recognized by CD40L on immune cells (15). In this study, we investigated the mechanism by which ECs induce HA synthesis at the molecular level and identify the putative role of CD40 in this process.

**EXPERIMENTAL PROCEDURES**

Cell Culture—Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and were grown for up to passages in EGM-2 minimum essential medium (Lonza) supplemented with 10% fetal bovine serum. The cultures were maintained in an atmosphere of humidified 95% air, 5% CO2, at 37°C. Before transfection, subconfluent HUVECs were cultured in EGM-2 with 1% fetal bovine serum. The medium was then changed to EGM-2 on day 0 and 1 ng/ml of IL-1β (R&D Systems), or 0.1 μM of TNF-α (R&D Systems), or 3 μg/ml of IFN-γ (R&D Systems), or 1 ng/ml of tumor necrosis factor-α (TGF-α, R&D Systems), and treated for 24 h. In some experiments, 2% (v/v) final concentration of proline diketocarnitine (PDTC, Sigma) was added to the cells. By using these cytokine concentrations, we did not measure any morphological effects detected by trypan blue staining (results not shown).

Glycine/glycine buffer (pH 7.0) and chondroitin sulfate were used to determine the culture medium were quantified by polynucleotide gel electrophoresis in phosphate buffered saline (PBS, GIBCO) and by HEMO analyzers as described previously (16, 17). Briefly, proteins in the culture medium were digested with proteinase K, and the glycans were purified by phenol precipitation. The specific immunoreactivity for HA, chondroitin sulfate (DAS) and AC2 respectively were obtained by specific glycosidases digests and derivatization with 2-aminoethylindole. Arabinose-labeled DAS and AC2 carbohydrates were separated and quantified by PAGE followed by TLC and normalized to the cell number. Agarose gel electrophoreses were performed with 2% agarose gel in 100 mM sodium acetate buffer, pH 8.0, and DNA was stained with ethidium bromide. Gel electrophoreses were performed with 2% agarose gel in 100 mM sodium acetate buffer, pH 8.0, and DNA was stained with ethidium bromide. Gel Lanes 1-5: HEMO analyzers using the base repair assay. Gel Lanes 1-5: HEMO analyzers using the base repair assay.

**Analytical Methods**—For visualizing HA, HEMO analyzers were performed using glass cover slips and either untreated or treated with IL-1β. They were processed using a histological HA-staining protocol and a green fluorescent reporter as described previously (18). Quantitative RT-PCR—Total RNA samples were extracted from untreated or cytokine-treated HUVECs with TRIzol (Invitrogen; and contaminating DNA was removed by DNase I (Ambion)). cDNA was generated using the High Capacitance cDNA synthesis kit (Applied Biosystems) and was amplified on an ABI Prism 7700 in a real-time (Applied Biosystems) using the Taqman Universal PCR Master mix (Applied Biosystems) following the manufacturer’s instructions. The following human cytokine gene expression assays were used: HAS1 (Hs00166440_m1), HAS2 (Hs00166448_m1), HAS3 (Hs00166448_m1), CD40 (Hs00424199_m1), and β-actin (Hs00905690_m1). The relative quantity of gene expression levels was determined by comparing ΔCt [19], ΔΔCt method. To quantify the activity of HAS on cell membranes, we used the nonradioactive assay of HA synthesis as described previously (11).

Harbor HAS2 regulation—Immunofluorescent staining for the expression of human HAS2 in HUVECs by using HAS2 antibody (DB17261, Genetex, Inc.) and a negative control mouse IgG (rabbit) of an unrelated primary antibody. The transfections were done using Lipofectamine reagent (Lonza) and the human HEMO analyzers kit as described previously (12). After 24 h of incubation, the viability was quantified by the luciferase expression assay. Neuraminidase digestion—Adipose-derived stem cells (ADSCs) were cultured as described previously (12). 1 × 10⁶ ADSCs were digested with 2% mouse anti-HA monoclonal antibody (BH-2), followed by 20 U/ml of neuraminidase (Sigma, St. Louis, MO). The digestions were done using a Neuraminidase (Sigma) and the human HEMO analyzers kit as described previously (12). After 48 h of incubation, the viability was quantified by the luciferase expression assay.

**Statistical Analysis**—Statistical analysis of the data were done using analysis of variance followed by post hoc Bonferroni test. Probability values of p < 0.05 were considered statistically significant. Experiments were repeated three times each in duplicate, and data are expressed as means ± S.E.
RESULTS AND DISCUSSION

Proinflammatory Cytokines Induce HA Synthesis—One of the main roles of ECs as immunoreactors is to regulate recruitment of immune cells from the bloodstream into the inflamed site. This mechanism involves not only expression of adhesive molecules on EC surfaces but also the coordinated expression of the corresponding ligands on the circulating cells. Definable adhesion molecules, cytokines, and/or cytokines and their expression can affect the expression of specific adhesion proteins on ECs and, integrins. Cytokines such as IL-1β and TNF-α are among the common proinflammatory molecules that are synthesized by immune cells such as monocytes in response to infection in an early phase of the process. The pioneering works of Singletary’s research group clearly showed that HA is also involved in monocyte recruitment as well as in other cytokines (16, 21–23). Clarifying monocytes are activated by HA through CD44 interactions, whereas proinflammatory cytokines stimulate ECs to produce HA together with other well-known adhesion molecules such as ICAM-1 and VCAM-1.

In our study, we focused on the mechanism of HA induction after IL-1β and TNF-α treatment. We used HUVECs because they are a convenient and broadly accepted model EC model. After preliminary dose-response experiments, we found that a 24-h treatment of HUVECs cultures with 5 μg/mL of IL-1β induced a clear accumulation of HA secreted into the culture medium, which was quantitatively measured by PAGE-IEF (Fig. 1A). As HA in the cell layer was quantified by immunofluorescence (Fig. 1B). The bands corresponding to HA disaccharides fractioned better after IL-1β addition, whereas the bands corresponding to HA disaccharides remained unchanged, indicating that IL-1β induces specific the HA content without modifying chain-length in vitro. The expression of HA in HUVECs cells was associated with a diffuse staining around HUVECs plasma membranes without a clear focal staining pattern as observed in HUVECs cultures (Fig. 1C). Moreover, analysis of the disaccharides of HA induced by IL-1β by agarose gel electrophoresis revealed the synthesis of a high molecular weight polymer of >2 × 10^6 Da (results not shown).

Furthermore, we tested the responses of HUVECs to other proinflammatory cytokines (i.e. TGF-β and TNF-α) to cytokine concentrations used in the highest HA secretion >3.5-fold over control, whereas IL-1β increased HA ~2- and 3-fold over control. In contrast, anti-inflammatory TGF-α and TGF-β treatments did not increase HA secretion over control (Fig. 1D). These results suggest that monoclonal antibodies, such as HUVECs, together with the previously reported monoclonal ECs (36), can respond to monoclonal antibodies by activating HA synthesis after treatment with proinflammatory cytokines. Previous studies and our unpublished data have shown that HUVECs in response to proinflammatory cytokines, whereas HUVECs cultured with IL-1β induced ICAM-1 and VCAM-1 expression after treatment with proinflammatory cytokines, whereas HUVECs cultured with IL-1β did not increase ICAM-1 and VCAM-1 expression after treatment with IL-1β for 4 h. These apparent differences in results can be explained considering the different cytokine concentrations and incubation times, as well as the different sensitivity of the HA quantification methods that we used in our experiments. Although the cytokine concentrations used in our experiments are higher than expected (21, 22), their concentrations in vivo may be difficult to define and higher than expected (21, 22).
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Proinflammatory Cytokine-induced HA Depends on HAS2 and NFκB—As in the synthesis of HA dependent on NFκB or more often of the three HAS enzymes, HAS1, -2, and -3, we measured their mRNA expression after cytokine treatments by using quantitative RT-PCR (Fig. 3, A and B). HAS protein is present in low levels at the plasma membrane (20), and quantitative assessment at the protein level remains to be a technical challenge and could not be performed in a reliable manner. HAS1 mRNA was not detected in TNFα/IFNγ, whereas HAS2 and HAS3 mRNAs were present at similar levels. Interestingly, only HAS2 mRNA significantly increased ∼5-fold after IL-1β treatment and ∼2-fold after TNFα and IFNγ treatment. In contrast, HAS2 mRNA did not change after treatment with the anti-inflammatory cytokine PGE2. It should be noted that the induction of HAS2 mRNA and HA levels after the cytokine treatments did not match exactly. This could be due to an earlier expression of HAS2 that we did not detect after 24 h of incubation, whereas HA accumulation during the whole incubation time. HAS2 expression was observed at high molecular weight in agreement with the smear that we observed after IL-1β stimulation. These results indicate that increased HA, in response to the proinflammatory cytokines is regulated by expression of HAS2. In addition, we measured the HA synthetic activity in perfused plasma membrane vesicles (21) isolated from control, IL-1β-, TNFα-, and IFNγ-treated HMEC cultures and found increases of 2.2±0.4, 5.9±1.0, and 7.8±1.0 pmol DA/g protein/min, respectively. The cytokine-induced increases showed statistically significant differences (4-fold over control of HAS activity in perfused plasma membranes, compared with the increases in HA in the culture medium. Interestingly, the proinflammatory cytokines treatments induced similar HAS activities, whereas IL-1β induced a much greater HAS mRNA response than TNFα and IFNγ. Therefore, this activity increase was not related completely to HAS gene expression as shown in Fig. 4A, because cytokines treatments caused a different degree of HAS messenger accumulation. These data could suggest the existence of complex post-transcriptional processing of HAS mRNA as has been shown for the stability of other TNFα-mediated transcripts (40). Moreover, other additional factors, such as kinases and/or actin activity and/or turnover could contribute to this rise. Previous data from TNFα-treated bronchial endothelial cells (27) reported that increased HA synthesis did not require a significant change in HAS gene expression (41). However, only HAS2 mRNA was investigated at that time and the other isoforms were not detected, and their sequences were not available to public databases.

A large body of evidence (23) has demonstrated that many cellular responses to proinflammatory signals are mediated by NFκB. This transcription factor normally is present in the cytoplasm in a complex with the protein IκB that maintains NFκB in an inactive state. IκB phosphorlylation, mediated by IkB kinase, induces a rapid degradation of IκB via the ubiquitin-proteasome pathway, which leads to translocation of NFκB into the nucleus and the transcriptional functions of NFκB. NFκB activation can be induced by several stimuli, including TNFα receptor. Therefore, an immunoregulatory role of NFκB is involved in the increased HAS transcription observed after proinflammatory cytokine treatment by using PDCO, a weakly used clinical inhibitor of NFκB (22). As shown in Fig. 3C, the simultaneous addition of IL-1β and TNFα or PDCO caused a reduction of HAS transcription. Interestingly, in TNFα- and IFNγ-treated cells, NFκB inhibition abolished HAS transcription to the level of control, whereas IL-1β treatment did not induce HAS transcription to the level of control, which suggests a more complex mechanism of HAS2 transcription induction that could involve other factors in addition to NFκB. In fact, in addition to NFκB sites, the HAS promoter region contains putative binding sequences for other transcription factors (35). A previous study in human macrophages found that TIF1 induced HAS transcription after IL-1β stimulation, contributing to the critical role of NFκB in HA metabolism (44). Interestingly, HA itself is able to modulate cytokine production via NFκB, confirming the critical role in inflammation (38).
HAS2 and CD47 Mediate HUVEC Membrane adhesive Properties—Because some of the effect of HA is to modulate adhesion of immune cells on microvascular ECs (16), we investigated whether immune-cell adhesion to HUVECs also is treatment with proinflammatory cytokines could be abolished by HA. Fig. 4B shows that IL-1β, TNF-α, and INF-γ diminished the number of adherent immune cells (5 × 10^5, 5 × 10^5, and 5 × 10^5, respectively). Furthermore, incubation of HUVECs with IL-1β treatment decreased U937 monocyte binding to an equal level (Fig. 4B). Moreover, the simultaneous addition of the monocytes with a pan-CD47-antibody to IL-1β did not inhibit cell binding to IL-1β treatment. These data indicate that the role of HA in monocyte adhesion is that removal of HA by histone-like treatment as well as the competition with exogenous HA both reduced monocyte adhesion. This point is critical because reducing immune cell adhesion to vascular endothelium would reduce the number of inflammatory cells

that reach an inflammatory site, thereby reducing the inflammation. Several previous studies have highlighted the anti-inflammatory and protective effects of CD47, administered in an experimental model of neutrophic (68) as well as in atherosclerotic plaque development (69). The molecular mechanism through which immune cells interact with HA is mainly mediated by CD47 (70). To elucidate CD47's role in immune cell adhesion, we simultaneously induced monocytes together with a monoclonal antibody against CD47 known to block HA/CD47 binding and found a clear reduction of monocytes adherent to IL-1β-treated HUVECs, whereas an unrelated control antibody did not modify adhesion (Fig. 4C). We also measured CD47 mRNA expression by quantitative RT-PCR. Interestingly, proinflammatory cytokine treatments induced a significant increase of CD47 mRNA in HUVEC cultures, whereas anti-inflammatory cytokine treatments maintained control CD47 levels (Fig. 4C). This finding could help to support the hypothesis that CD47 on the plasma membrane of HUVECs mediates the control of HA expression and protects HA to CD47 on circulating immune cells that can attack and form a "membrane attack" complex. Notably, in this model, HA could remain in a soluble form without forming stable cell membranes, as found in the immunofluorescence experiments (Fig. 1B). It is of note that previous works using an immunofluorescence technique (16) found an inhibition of neutrophil-EPC adhesion by HA independent of CD47 and probably involving HA-CD47 (69), suggesting that in multiple-He iron-condition, protein may mediate interactions of immune cells with endothelium.

The main HAs involved for increased HA after treatment of HUVECs with proinflammatory cytokines was HAS2. Therefore, we analyzed involvement in monocyte binding by using an siRNA approach. HUVECs were treated with commercial siRNA against HAS2 as well as with a scrambled control siRNA by using the Nucleofector apparatus to obtain a high transfection efficiency with low mortality rates. After 24 h of incubation, treated and control siRNAs were transfected with or without IL-1β and incubated for 2 h. HUVEC cultures were then used for monocyte adhesion experiments or for RNA extraction and CDNA preparation. The efficiencies of HAS2 mRNA silencing were assessed with quantitative RT-PCR and as shown in Fig. 5A,
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FIGURE 4: Schematic representation of the role of HAS2 in the inflammatory response. Proinflammatory cytokines, through their effects, activate NF-κB and other transcription factors that result in the expression of inflammatory mediators. Cytokine-activated transcription factors (NF-κB, AP-1, etc.) induce the expression of the HAS2 gene, which encodes the HA synthase enzymes.

In conclusion, the results presented in this paper show that HAS2 is involved in the inflammatory response. HAS2 can be induced by cytokines, and its expression is regulated by transcription factors such as NF-κB. This regulation plays a crucial role in the inflammatory response, as HAS2 mediates the production of HA, which is essential for the recruitment and activation of immune cells. Understanding the mechanism of HAS2 regulation by cytokines can provide insights into the inflammatory process and potential targets for therapeutic intervention.
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