1999

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Marianne Hamel
Lehigh University

Hamel, Marianne

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May 31, 1999

HEPARIN DOWNREGULATES P38 ACTIVATION IN BOVINE ENDOTHELIAL CELLS

By

Marianne Hamel

A Thesis

Presented to the Graduate and Research Committee

of Lehigh University

in Candidacy for the Degree of

Master of Science

in

Biological Sciences

Lehigh University

May 5, 1999

This thesis is accepted and approved in partial fulfillment of the requirements for the Master of Science.

May 10, 1999
Date

Linda J. Lowe-Krentz
Associate Professor of Biological Sciences
Thesis Advisor

Jennifer Swann
Associate Professor of Biological Sciences

Lynne Cassimeris
Associate Professor of Biological Sciences

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ABSTRACT

Heparin has been shown to block proliferation in vascular smooth muscle cells which is an early event in the genesis of atherosclerotic plaques. Heparin's effects on vascular endothelial cells, however, indicate it may downregulate the stress pathways that characterize the initial stages of heart disease. This study tracks the activation of p38 and JNK-1, intermediates in the signal transduction of the stress response in endothelial cells. Results indicate heparin downregulates the phosphorylation of p38, and its effect on JNK-1 is undetermined. These data indicate heparin or its analogs may have uses in the treatment of heart disease.

Introduction

The endothelial basis of atherosclerosis

Atherosclerosis, better known simply as heart disease or "hardening of the arteries", causes over half of all deaths in the United States, Europe, and Japan (1,2,3). Hypercholesteremia, hypertension, nicotine use, diabetes and obesity aggravate the condition, and potentially lead to stroke, myocardial infarction, and gangrene of the extremities (1,2,3). The disease is, however, a cumulative process over the course of a lifetime and may begin as early as childhood (1).

The hallmark of atherosclerosis is the deposition of fatty plaques on the inner surface of the artery wall in response to minor injury (1,2). The forces that injure the endothelium at the cellular level may include fluid shear stress and the tangential component of the hemodynamic force (4). Plaques result from the excessive response of the normal wound repair pathways involving growth factors and cytokines in endothelial cells. These response-to-injury pathways cause cardiac arteries to slowly narrow, resulting in heart disease, myocardial infarction, and eventually, death. Evidence in the literature implicates the signal transduction pathways leading to the activation of stress activated protein kinases (SAPKs) p38 and JNK-1 in this process in both endothelial and smooth muscle cells (SMCs). These pathways affect the stress response of the cell to various extracellular conditions instead of the mitogenic outcome that is the result of activation of the better-studied mitogen-activated protein kinase (MAPK) pathway. Activation of the SAPK pathway can take many forms, with cell

stretching, ceramides, G-protein-coupled receptor agonists, chronic hypoxia, ischemia/reperfusion, integrin clustering, and vasoactive peptides among them (5). While the MAPK pathway may promote varied outcomes, like apoptosis, proliferation, hypertrophy, repair, or growth in different tissues (6,7), we are interested in the response of vascular endothelial cells to stress through activation of the SAPK pathway.

Heparin's role in treatment of heart disease

Heparin, a negatively charged glycosaminoglycan well-known for its anticoagulant properties, has been shown to inhibit the growth and proliferation of SMCs through specific, high-affinity binding sites. Heparin uptake occurs through what appear to be both receptor-mediated and endocytotic pathways (8). Heparin's mechanistic role in blocking or controlling SAPK pathways in endothelial cells is the focus of this research. Other aspects of research currently being conducted in our lab include purification of the heparin receptor in SMCs, upregulation of heparan sulfate proteoglycans, and localization of MAPKs in response to heparin treatment in SMCs.

While heparin itself, due to its side effects in long-term use, may not be used in a clinical protocol to treat atherosclerosis, an understanding of the signal transduction cascade that results from activation of the heparin receptor is beneficial to the study of the excessive wound response that is the hallmark of atherosclerosis. Although heparin's anti-proliferative qualities have proven useful in animal studies of atherosclerosis and restenosis, human trials have been

less successful. This is likely because heparin is sequestered by human blood serum but not by fetal bovine serum, which is commonly used during *in vitro,* cell culture, and animal studies (9). The use of heparin analogs instead during human trials may result in a new clinical protocol for the treatment of heart disease, as the heparin molecule's anticoagulant properties are separate and distinct from its antimitotic qualities (10).

Endothelial injury marks the genesis of fatty arterial plaques

In 1993, Russell Ross put forth the "response-to-injury" hypothesis to explain the development of artery narrowing and blockage, suggesting that the disease begins with an insult to the endothelial cells which line the arteries. This insult causes the endothelium, normally a permeability barrier and an anticoagulant surface, to become dysfunctional. Endothelial changes occur, and can be monitored by polymorphonuclear leukocyte recruitment (PMNs) and expression of adhesion molecules like CAMs on the inner surface of the artery, as well as the accumulation of low-density lipoproteins (LDLs) in the artery. In high blood concentrations, LDLs quickly become oxidized (oxLDLs) and toxic to the endothelial and smooth muscle cells of the affected vessel. oxLDLs cause the secretion of chemoattractants and growth regulatory molecules, which recruit monocytes and T lymphocytes to the site of injury (1,2).

The innermost layer of the artery wall is known as the intima, and it is here that the cells' stress pathway reaches its physiological endpoint. Lesions called "fatty streaks" appear on the intima, and are formed from the aggregation

of lipid-rich macrophages and T-cells (1,2). Macrophages are formed from monocytes which migrate into the subendothelial space of the artery and become transformed (1,2). These white blood cells then ingest the oxLDLs and become foam cells. Foam cells characterize the appearance of the fatty streak, the most obvious precursor of heart disease on the inner surface of the artery (1,2).

Also contributing to the formation of an atherosclerotic plaque are elements of extracellular matrix, including elastic fiber proteins, collagen, proteoglycans, and other necrotic debris (1,3). Proteoglycans contain glycosaminoglycan side chains, and the heparin molecule is structurally related to the heparan sulfate component of proteoglycans (10,11). These components are synthesized in lower concentrations by smooth muscle cells which have migrated from the tunica media to the intima layer. Together, the accumulation of these materials forms a fibrous plaque which may occlude the blood vessel (1,3).

Many molecules modulate the response of endothelial cells to extracellular stimuli. Tumor necrosis factor (TNF-α), interleukin-1 (IL-1β), free oxygen radicals, transforming growth factor β (TGF-β), histamine, thrombin, oxLDL, platelet-derived growth factor (PDGF), and platelet-activating factor (PAF) provoke a procoagulant response (1,3,11). These factors are largely derived from and secreted by smooth muscle cells, platelets, and macrophages (1, 2, 11, 12).

SAPK and MAPK pathways pass extracellular stimuli to the nucleus

The stress message moves from the cell surface inward through a series of kinases, each of which is known by multiple names. The early steps of the cascade are unclear, and may activate GTPases Rac1 and RhoA (13). Other upstream regulators of MAPKs may exist which are directed in a cell-specific manner, and result in different responses to similar extracellular stimuli through differential gene expression (4). Later elements of the cascade, however, are better characterized. One constituent of the MAPK pathway is ras, which helps to activate raf, also known as MAP kinase kinase kinase (MAPKKK). The MAPKKKs activate MAPKKs, including MKK, MEK, SEK, or JNKK in the stress pathways, which then add a phosphate group to the protein JNK-1 or p38, which are MAPK analogs (14, 15) called stress-activated protein kinases (SAPKs). The pathways leading to the activation of p38 and JNK-1 have distinct intermediates. For instance, JNK-1 is phosphorylated by MKK4 and MKK7 (15, 16). p38's pathway may include TAK1 as a MAP kinase kinase kinase candidate, with specificity for MEK homologs MKK3, MKK4 and MKK6 as substrates (reviewed in 17). JNK-1 and p38 translocate from the cytoplasm to the nucleus, where they phosphorylate transcription factors such as jun which then alter transcription (18, 19, 20, 21). Signal transduction through a sequential series of kinases in SAPK pathways allows the cell multiple opportunities for modulation of the stress response, and the presence of different proteins in parallel cascades may alter the kinetics and magnitude of MAPK response to stress (4).

Heparin's effects on signal transduction in MAPK and SAPK pathways

Heparin is well-known for its anti-coagulant properties, and has been shown to curtail the activation of MAPKs in cultured smooth muscle cells, and thereby their growth and migration. Less studied, however, are the molecule's effects on the signal transduction of a stress message in endothelial cells. While the molecule has been shown to consistently return endothelial cells to an anticoagulant state, the specific portion of the pathway it modulates is unclear. Heparin is a glycosaminoglycan consisting of repeating disaccharide sequences of D-glucuronic acid 1,4-linked to N-acetyl-D-glucosamine. The molecule also causes the production of excess tissue factor pathway inhibitor and tissue-type plasminogen activator in endothelial cells. These substances promote anticoagulant processes (22, 23). Heparin-treated endothelial cells express heparan sulfate proteoglycans in a dose-dependent relationship (24,25,26,27). The "wounded" cell layer causes the concentration of the heparan sulfate component to drop, decreasing the concentration of anti-mitotic heparin molecules present in the intimal layer (28). Vascular uptake of LDLs is decreased due to the negative charge these proteoglycans confer on the endothelium (26, 27).

Physiological responses to stress in endothelial cells

One of the physiological endpoints of the endothelial stress response is the expression of adhesion molecules which contribute to the formation of the fibrous plaque. Physiological responses to stress in endothelial cells may include the

formation of complexes to promote clotting and cell adhesion. Recently, it has been suggested that activation of the SAPK pathway with cytokines causes an increase in transcription of genes coding for cell adhesion molecules in conjunction with the clotting response (28, reviewed in 29). Some evidence suggests heparin has the ability to inhibit leukocyte recruitment to the endothelium through the binding of L-selectins (30, 31). Endothelial cells quickly express E-selectin after treatment with IL-1β, TNF-α, and lipopolysaccharide, and these adhesion molecules also recruit leukocytes to the endothelial lesion (28). Heparin has also been found to decrease expression of intercellular adhesion molecules at the transcriptional level in endothelial cells (32).

The role of p38 and JNK-1 in atherosclerosis

The protein kinases p38 and JNK-1 are MAPK analogs in cellular stress pathways, which have been found to be sensitive to environmental stresses like ultraviolet and x-ray radiation, hydrogen peroxide, protein synthesis inhibitors, and osmotic and heat shock (19). p38 is analogous to a protein in the high osmolarity glycerol response (HOG1) pathway in yeast which is responsive to hyperosmolarity and osmotic imbalance. It has been localized to the nucleus of the cell (33, 34). This suggests that p38 plays a role in signal mediation to the nucleus, which is strongly supported by the fact that transcription factors like ATF2, CREB, and CHOP are among the protein's substrates (35, 36, 37, 38). Also, though p38 is weakly activated through the protein kinase C (PKC) pathway, it is strongly activated by LPS and inflammatory cytokines such as

those used in our research like IL-1β and TNF-α (37, 39). Evidence suggests that p38 is linked to heart disease through its role in preconditioning's protection of the artery, and its activation may spur the signal transduction cascade of ischemic preconditioning (40). p38 phosphorylates MAPK-activated protein kinase-2, and plays a role in mediating apoptosis and cytokine production. This intracellular kinase is a well-known mediator of both apoptosis and cell-cycle arrest, and is itself phosphorylated by SAPKK-2.

c-Jun NH_2-terminal kinase (JNK-1) has many similarities to p38, in that it also plays a role in apoptosis, and activates transcription factors like c-Jun, Elk-1, and ATF2 after being translocated into the nucleus, upregulating their activity (41, 42). JNK-1 is activated preferentially by cellular stress, and to a lesser degree, inflammatory cytokines like TNF-α and IL-1β. JNK-1 phosphorylates c-jun at serines 63 and 73, and binds to the adjacent amino acid sequence within the N-terminus of the transcription factor called the "delta region" (4). In addition, the pathway which activates JNK-1 is susceptible to crosstalk from other signaling cascades. For instance, PKC negatively regulates JNK through increased expression of MAP kinase phosphatase-1 (MKP-1). This MAPK is required for cellular processes in the early embryonic development of *Drosophila* and mammalian cells (17) and contributes to apoptotic and immune responses and oncogenic transformation (4). The transformation experienced by endothelial cells in response to altered patterns of blood flow have been linked to the activation of this SAPK (41).

JNK-1 and p38 are both members of a dually-phosphorylated class of kinases generally termed MAPKs. Phosphate groups are added in the motif Thr-Xaa-Tyr, where Xaa is Pro in JNK-1 and Gly in p38 (37, 42, 43). JNK-1 and p38 are activated by mitogen-activated kinase kinases (MKKs); specifically, MKK3 and MKK6 activate p38, while MKK4 activates both proteins. p38 and JNK-1 are part of parallel MAPK cascades activated in response to cellular stress. It has been suggested for JNK-1 in particular that the MAPK's action may promote cell survival and repair (4). Evidence suggests that JNK-1 and p38 play a role in the dismantling of collagen in fibrous plaques through the induction of matrix metalloproteinases like collagenases, stromelysin, and gelatinases (reviewed in 29).

Phosphatases help control the activation state of SAPKs

Our lab has hypothesized that heparin or its analogs may be appropriate for the prevention of heart disease as it blocks the excessive wound response of the MAPK pathway in the endothelium and smooth muscle of artery walls. It is clear that the phosphorylation state of the SAPKs in question is controlled by the competing actions of upstream kinases and expressed MKPs. Two possible mechanisms for heparin's action have been proposed. First, heparin may inactivate p38 and JNK-1 by upregulating a member of the MKP family. MKP-1 is an immediate early gene product whose expression is induced by growth factors (40), and has been shown to inactivate not only JNK-1 and p38, but all members of the MAPK family as well (37, 44) due to its broad substrate

specificity. Secondly, heparin may block the activity of the pathway at an upstream point by inactivating an MKK4 homolog. It is clear that heparin binds and is actively metabolized by endothelial cells and that this binding can prevent the conversion of a nonthrombogenic phenotype to a thrombogenic one. At this time, scientific literature contains no obvious answers to the question of heparin's action in vascular endothelial cells. However, soon after the pathways studied in this work were scrutinized carefully, it was determined that the cell must have a mechanism through which to modify or shut off its response to mitogenic stimulation and stress. Several members of a family of phosphatases were found to have an affinity for SAPK in varying degrees, and their localization in the cell suggests the compartmentalized control of signal transduction. Heparin treatment of endothelial cells upregulates the expression of phosphatases which modify the activation of SAPKs *in vivo*. There are several phosphatases with specificity for constituents of the SAPK pathway, although most have some ability to affect both stress and mitogenic outcomes. Dual specificity phosphatases, like the ones that regulate growth and stress pathways, undergo powerful regulated expression in response to extracellular stimuli (45).

Characterized in 1993, MAP kinase phosphatase-1(MKP-1) is the best known and appears to be the dominant dual specificity phosphatase in vascular smooth muscle cells (VSMCs) (46). Its phosphatase nature was detected in a mitogen-inducible murine gene, 3CH134, which coded for the signature protein-tyrosine phosphatase (PTPase) motif VHCXAGXXR(S/T)G (47) of a novel

family of phosphatases, where X represents any amino acid. This sequence motif proved to be essential for phosphatase activity (44), and an essential cysteine residue is located near the N terminus of the motif (48). A human homolog for the gene has been identified in CL100 (49). It was found that the removal of a phosphate group from either the tyrosine or threonine residue of a MAPK caused its inactivation (50). MKP-1 specificity and phosphatase activity was hypothesized to control unchecked cell growth through the regulation of the mitogenic outcome of the MAPK pathway. Localization experiments proved MKP-1 to be exclusively nuclear, suggesting that the inactivation of its substrates was solely a nuclear event. It was also shown to inhibit cell growth as a G1 cell cycle inhibitor and constitutive expression proved to repress DNA synthesis (51). Constitutive MKP-1 expression was also found to inhibit jun kinase activity (52).

A great deal of controversy exists concerning the specificity of MKP-1 towards MAPKs and SAPKs. The phosphatase has a high degree of substrate specificity for some MAPKs (47), although it has been shown to inactivate the p38 and JNK equally (53), and p38 preferentially over ERK2, a MAPK (49). It has also been shown that the cell needs to produce thirty times more MKP-1 to inactivate ERK2 than either JNK known at the time (44). Inhibition of MKP-1 through the addition of antisense oligonucleotides to the cell system caused prolonged activation of ERK1 and ERK2, despite the evidence that these substrates are not the enzyme's preferred target (47) These data suggest that MKP-1 is not alone in inactivating SAPKs and MAPKs. Furthermore, its broad

substrate specificity suggests crosstalk between the MAPK and SAPK pathways, which is supported by recently observed growth attenuation following SAPK activation. This is most likely because the activation of the SAPK pathway results in MKP-1 expression (54).

Interestingly, MKP-1 has been shown not only to regulate the activation of MAPKs and SAPKs, but also its own production through a negative feedback mechanism (49), demonstrating an additional level of regulation of the SAPK pathway. This may serve to resensitize cells to subsequent extracellular stimuli. MKP-1 may also reduce the expression of not only itself, but other dual-specificity phosphatases as well (49). However, activation of the MAPK pathway through Raf/MEK/ERK has proven insufficient to force the expression of MKP-1. Other factors, such as Ca^{2+}, are needed to mimic the production levels of the phosphatase seen with growth factors. Barring Ca^{2+}-dependent expression of MKP-1 causes the sustained activation of ERK. This suggests MKP-1 is an intersection for crosstalk between several signal transduction schemes, including the ERK pathway (55).

Because certain MAPKs can be inactivated in the cell in the absence of MKP-1 (49), it was logical to search for other candidates with phosphatase activity to explain this phenomenon. MKP-2 was discovered and characterized in 1996. It was isolated by virtue of the structural homology of its catalytic regions to MKP-1 and another phosphatase, PAC-1. The gene coding for the protein, also known as hVH-2, also contains a large 3'-untranslated region of 3 to 6

kilobases which may serve to regulate transcriptional stability (56). The MKP-2 gene was found and isolated from a human mitogen-activated T-cell DNA library. MKP-2 is not expressed following stimulation of growth or stress pathways in human fibroblasts, and has been shown to have little or no affinity for p38, but detectable activity toward JNK-2. Its expression can be forced *in vivo* with the addition of phorbol 12-myrisate 13-acetate (PMA), a phorbol ester tumor promoter (53). This suggests the phosphatases' possible relationship with human immune response and adhesion molecule expression, particularly in the plaque-forming phase of heart disease.

Cloned in 1996, the novel gene product MKP-3 displays a localization, substrate specification, and regulated expression distinct from the other members of its gene family. MKP-3 contains only 36 percent identity to MKP-1. It does, however, display great homology to the cell cycle regulator Cdc25, which is also a phosphatase, in two N-terminal CH2 domains. These domains are shared by all known members of this gene family. Most importantly, MKP-3 is localized in the cytosol of the cell, the first phosphatase to demonstrate extra-nuclear restriction. This suggests that MKP-3 blocks nuclear translocation of activated kinases (57).

MKP-4 exhibits 57 percent identity to MKP-3, and unsurprisingly displays two N-terminal CH2 domains similar to Cdc25. Its cDNA clone is predicted to encode a protein of 41.8 Kdal, and an X-linked human homolog has been identified as Xq28. MKP-4 is present in small quantities in both the nucleus and

the cytoplasm in 10-20 percent of cells. MKP-4 targets equally the SAPKs, and has a slightly higher affinity for ERK1 and ERK2 (46).

Another dual-specificity phosphatase, M3/6, demonstrates a highly specific preference for p38 and JNK, and inactivity versus MAPKs. Like MKP-3, M3/6 is a cytosolic factor. The gene coding for M3/6 contains a complex trinucleotide repeat, which codes for multiple serine and glycine repeats in the C-terminal third of the protein (58).

In this thesis we report the downregulation of activated p38 in endothelial cells through the addition of heparin. Our lab has definitive evidence that the deactivation of MAPKs in a VSMC system is attributed to expression of MKP-1 (Isleib, unpublished results). Also, recent experiments in our lab indicate the increased deactivation of p38 with a concomitant increase in MKP-1 expression in endothelial cells (Moore, unpublished results). We approached the question of heparin's downregulation of the SAPK pathway in two parts. First, the maximal activation of p38 and JNK-1 was studied, and then the change in SAPK phosphorylation upon addition of heparin to activated cells was quantified.

MATERIALS AND METHODS

Materials

Calf pulmonary artery endothelial cells (CPAEs) and capillary bovine vascular endothelial cells (EJGs) were obtained commercially through American Type Culture Collection (Manassas, VA). Cell culture and electrophoresis solutions were made up according to supplier's protocols. Denaturing lysate consisted of 1% SDS, 10 mM Tris Cl (pH = 7.4), and 1 mM $NaVO_4$. 2X sample buffer was made up of 4% SDS, 20% glycerol, 0.125 M Tris (pH = 6.8), and 10% 2-mercaptoethanol. 10X TBS consisted of 60.6 g Tris base and 87.7 g NaCl diluted to 1 L with deionized water (pH = 7.5). Blotto consisted of 50 g Carnation Non-fat dry milk in 1800 mL of Tris Buffered Saline (TBS), and 100 µL of 4% sodium azide, and TBST was composed of 0.1% Tween-20 in 1X TBS. Developing solution for Western blots was made up of 50 µl of 5-bromo-4-chloro-3-indolyl phosphate and 500 µl of nitro blue tetrazolium in 50 ml of 1X alkaline phosphatase buffer. 1X alkaline phosphatase buffer was made up of 50 ml 10X Alkaline phosphatase buffer mixed with 450 ml deionized water. 10X alkaline phosphatase buffer consisted of 121.2 g Tris base, 5804 g NaCl, and 10.1 g $MgCl_2 \cdot 6H_20$. EDTA solution was comprised of 1 g glucose, .025 g Na_2-EDTA, and 100 mL 10X phosphate-buffered saline (PBS) diluted to 1 L with deionized

water. 10X PBS consisted of 2 g KCl, 2 g KH_2PO_4, and 11.5 g Na_2HPO_4 diluted to 1 L with deionized water.

Cell Culture

CPAEs were cultured in minimal essential media (MEM) (Sigma, St. Louis, MO) with a total volume media solution of 450 ml of containing 10 ml of 0.2 M glutamine in deionized water, 5 ml of penicillin-streptomycin (penicillin 1 units/mL, streptomycin 1 μg/mL) (Sigma, St. Louis, MO), and 50 ml of heat-inactivated fetal bovine serum (Sigma, St. Louis, MO, and Atlanta Biologicals, Atlanta, GA). Media was changed every other day in non-confluent plates, and every seven days in confluent plates. Cells were sometimes allowed to become confluent on 100 mm plates, and were sometimes harvested before confluence was achieved. Transfer on to new plates was achieved through the addition of 5 mL of EDTA solution. 5 mL of trypsin was added, and plates were incubated at 37° C for five minutes. Plate contents were centrifuged for five minutes with 5 mL of MEM media, and were resuspended in media. The suspension was equally divided among plates precoated with a 0.5% solution of gelatin.

Experimental Protocols

Plates of cells were treated with TNF-α (Sigma, St. Louis, MO, and R&D Systems, Minneapolis, MN) and incubated for 5 to 45 minutes before harvesting. Addition of TNF-α to 100 mm plates varied from 25-50 μL for a final concentration of 2.5-5 ng/mL of media. Other plates were treated with 100 μl of

a fresh 0.01g heparin/1 ml deionized water solution for ten minutes, followed by treatment with TNF-α before harvesting. Cells were harvested with 1 mL of hot denaturing lysate solution. Harvested cell samples were sometimes stored in the freezer until SDS-PAGE could be performed. This cell solution was mixed with 2X sample buffer in a 1:1 ratio. Samples were then run on a 10% SDS-polyacrylamide gel using Laemmli buffer system (59) and transferred onto nitrocellulose.

Detection of Proteins of Interest

1) *Detection of p38*

Nitrocellulose blots were incubated for two hours at room temperature or overnight in the 37° F cold room with Blotto to block non-specific antibody binding. Blots underwent three 10-minute washes in TBST and were placed into a p38 primary antibody (Santa Cruz Biologicals, Santa Cruz, CA #sc-535) dilution of 1:500 (100 μL antibody in 50 mL of Blotto) for two hours at room temperature or overnight in the cold room. Excess primary antibody was removed with three 10-minute washes in TBST. Incubation with donkey anti-rabbit biotin conjugate secondary antibody (Jackson ImmunoResearch Labs, West Grove, PA) in a 1:5000 dilution (10 μL antibody in 50 mL of TBST) followed a similar incubation to the primary antibody, and then blots were again placed in three 10-minutes washes in TBST. Blots were incubated in a 1:300,000 solution of ExtrAvidin alkaline phosphatase (3 μL ExtrAvidin in 50 mL of

TBST) for two hours at room temperature or overnight in the cold room. ExtrAvidin was obtained from Sigma in St. Louis, MO. Blots were then washed twice for 10 minutes in TBST, and again in an additional 10-minute wash in 1X alkaline phosphatase buffer. Developing solution with NBT and BCIP was then added to visualize the appropriate protein bands, and protein levels were quantified using the densitometer. Some experiments were run with secondary antibody only to identify bands of interest.

2) *Detection of phospho-p38*

Blots were blocked as in p38 protocol. Blots underwent three 10-minute washes in TBST and were placed into phospho-p38 primary antibody (New England Biolabs, Beverly, MA) in a 1:500 dilution (consisting of 40 μL of antibody, 40 μL of 25% Tween 20, 36 mL of deionized water, 2 g of bovine serum albumin (BSA), and 4 mL of 10X TBS) for two hours at room temperature or overnight in the 37° F cold room. Excess primary antibody was washed away with three 10-minute washes in TBST. Incubation with donkey anti-rabbit biotin conjugate secondary antibody (Jackson ImmunoResearch, West Grove, PA) followed a similar incubation to the primary antibody, and then blots were again placed in three 10-minutes washes in TBST. The remaining steps in blot development were identical to those of the above procedure for p38, although phospho-p38 blots were compared to p38 total protein blots for band localization.

3) *Detection of JNK-1*

Blots were blocked as in p38 protocol. Blots underwent three 10-minute washes in TBST and were placed into JNK-1 primary antibody (Santa Cruz Biologicals, Santa Cruz, CA, #sc-571) in a 1:500 dilution (100 μL antibody in 50 mL Blotto) for two hours at room temperature or overnight in the cold room. The remaining steps in blot development were identical to those of the above procedure for p38.

4) *Detection of p-JNK*

Blots were blocked as in p38 protocol. Blots underwent three 10-minute washes in TBST and were placed into p-JNK primary antibody (Santa Cruz Biologicals, Santa Cruz, CA) in a 1:500 dilution (100 μL antibody in 50 mL TBST) for two hours at room temperature or overnight in the 37° F cold room. Excess primary antibody was removed with three 10-minute washes in TBST. Incubation with goat anti-mouse secondary antibody (Jackson ImmunoResearch, West Grove, PA) in a 1:10,000 dilution (5 μL antibody in 50 mL of TBST) followed a similar incubation to the primary antibody, and then blots were again placed in three 10-minutes washes in TBST. The remaining steps in blot development were identical to those of the above procedure for p38, although blots were compared to JNK-1 total protein blots for band localization.

Results

Activation of p38 and JNK-1 with TNF-α in Bovine Endothelial Cells

In order to determine heparin's effect on the SAPK pathway in endothelial cells, the pathway must first be activated. Candidate molecules suspected to set the SAPK pathway in motion include interleukin-1B (IL-1β), lipopolysaccharide (LPS), histamine, and tumor necrosis factor-α (TNF-α). All of these molecules were tested for their ability to induce phosphorylation of p38 and JNK-1 *in vitro* in confluent endothelial cells. LPS (see Figure 1) and IL-1β produced little or no consistent activation of either SAPK. Treatment of confluent cells with histamine resulted in weak activation of both p38 and JNK-1, although the phosphorylation of JNK-1 was much more consistent in both cell types (see Figure 2). TNF-α produced the most consistent and strongest activation over short time courses (see Fig. 3).

A survey of recent literature reveals TNF-α to be a powerful and consistent activator of the stress pathway in bovine cells, a conclusion borne out by this study. Time course experiments with TNF-α were performed on bovine endothelial cells to determine the incubation time which produced maximal activation. Using confluent 100 mm plates of cells we assayed for activation of the stress pathway by immunoblotting. Using standard molecular weight rainbow markers and comparison to p38 total protein blots, we determined that the maximal activation of p38 was achieved through a ten minute incubation of the

stressor with confluent cells (see Figure 3, panel A). Although activation of the enzyme is clearly seen by ten minutes of incubation, no change in total protein was seen over the course of the experiment (see Figure 3, panel B). Quantitative analysis by densitometry of the phospho-38 can be seen in panel C of Figure 3. Activation of p38 was also seen most strongly at ten minute incubations of 50 μL of TNF-α with confluent bovine EJGs (Figure 4, panel A). Again, there was no change in total protein in EJGs (Figure 4, panel B).

Total protein blots in both cell types reveal p38 to be the dominant SAPK enzyme in these cell lines. This may account for the faint banding in many blots stained for p-JNK, as the inactivated protein seems to be expressed in much lower quantities than p38. Although phosphorylation of JNK-1 was achieved in bovine CPAEs, its activation proved less definitive than that of p38. The enzyme appears to be maximally activated also through a ten minute incubation with 50 μL of TNF-α with confluent cells (Figure 5). Total JNK-1 protein remains constant over the time course (data not shown).

Heparin downregulates activated p38

Experiments to demonstrate heparin's ability to decrease the stress response at the SAPK level were performed largely with TNF-α due to its greater capacity for activation and its reliability. These studies revealed a new variable in these experiments which demanded consideration. Despite the demonstrated effect of the addition of heparin ten minutes before incubation with stressors

(Gilotti, unpublished results), blots probing for activated p38 in confluent cells showed little or no downregulation of the stress response in CPAEs (Figure 6). Plates which were incubated for ten minutes with various heparin concentrations and then probed for the presence of the immediate early gene product MKP-1 demonstrated the unexpected constitutive expression of the phosphatase in all lanes, including controls without heparin (Moore, 1999, unpublished results). Figure 7 and the accompanying data analysis of the blot (Graph 2) make this finding evident. Experiments in which cells were only 70 to 85 percent confluent, however, show that heparin induced changes in SAPK activity. Figure 8, panel A, and its accompanying data analysis (panel B) demonstrate heparin's modulation of p38 activation by TNF-α over a short (5 to 15 minute) time course. No evidence of constitutive MKP-1 expression was found in the heparin concentration experiments with non-confluent CPAEs, however (Figure 9, panels A and B). Instead, there is a dose-dependent relationship between heparin concentration and MKP-1 expression. No evidence of downregulation of activated JNK-1, however, was observed. This may be because the total amount of JNK-1 protein is very limited in these cell lines (data not shown). Finally, the addition of only half as much TNF-α in non-confluent cells seemed to produce at least as much activation of p38 as in confluent plates (Figure 10, panels A and B).

Discussion

This study was designed to discern heparin's effects in endothelial cells on the stress pathway which modulates the body's response to the arterial events which lead to atherosclerosis.

The differentiation between heparin's effects in confluent and non-confluent plates of cells makes clear several points. First, the suggestion of redundancy between the MAPK pathway, with its mitogenic outcome, and the signal transduction of an extracellular stress message is valid. Constitutive expression of MKP-1 in confluent cells demonstrates one of the ways in which confluent cells suppress unnecessary growth, and by doing so, dampen the "resensitization" process discussed by Franklin (49). This resensitization may modulate overall levels of SAPKs as well. A consideration of the circumstances under which atherosclerosis begins suggests that a confluent monolayer of cells may not be the best model of the endothelial portion of the human artery with which to study the disease *in vitro*. A saturation of focal contacts causes cells to shut off the MAPK pathway, and also curb signal transduction in the SAPK pathway. Non-confluent cells, however, seem to show both stronger activation of p38 in particular, and may have different levels of SAPK enzymes. Non-confluent cells also reveal heparin's effects on p38 activation. The lack of downregulation at the 15 minute time point in Figure 8 likely depicts a loading inconsistency, as heparin's effects have usually been found to extend beyond ten minutes after addition. Visual inspection suggests that the 15 minute time point

with heparin had decreased despite densitometry data suggesting it had stayed activated. These results should be confirmed. Further experimentation must also assure that proteins of interest compared between non-confluent and confluent cells are considered in proper context and proportion. Also, a study of SAPK pathway activators that proved unsuccessful in this work, like IL-1β and LPS, may prove fruitful in a non-confluent system, as non-confluent cells demonstrated activation at a lower concentration of stressor.

Heparin's failure to clearly block the activation of JNK-1 in endothelial cells may have several root causes. Most obviously, JNK-1 exists at a low level in an inactivated form in confluent endothelial cells, as evidence by the light or absent total protein bands seen in many of our JNK-1 experiments. Non-confluent cells may contain too little of the enzyme to properly assay its decrease in activity upon heparin treatment. This situation may be remedied by different methods of sample harvesting to increase JNK-1 concentration during immunoblotting. Also, SAPKs contain no nuclear localization signal, and the proteins most probably enter the nucleus through the use of chaperone proteins. It is possible that endothelial cells fail to upregulate the chaperone proteins needed to move p-JNK into the nucleus, where the enzyme could be dephosphorylated by MKP-1, a nuclear phosphatase. Although JNK-1 is a known substrate of MKP-1, it may be the preferred substrate of another phosphatase, such as M3/6, which specifically deactivates SAPKs. If this is true, then this

phosphatase is apparently not upregulated in response to heparin treatment, as is MKP-1.

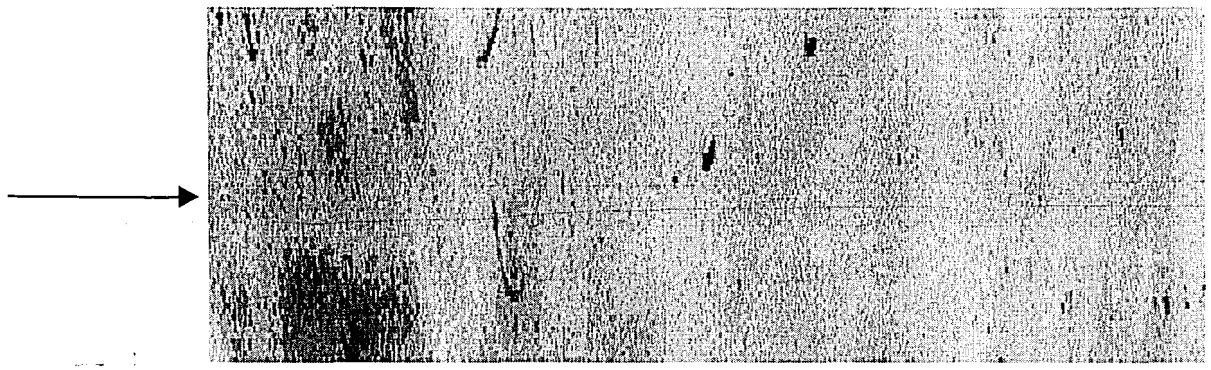
JNK-1 may also be dephosphorylated in the cytosol by a non-nuclear phosphatase, and its possible that sufficient quantities of p-JNK never translocate into the nucleus to become MKP-1's substrate. Another aspect of JNK-1's failure to downregulate upon heparin treatment may concern a downstream constituent of the SAPK pathway which is affected by MKP-1, and not the SAPK itself. In support of this theory, heparin treatment blocks many of the physiological endpoints of atherosclerosis, such as cell adhesion molecule expression, that are known to be associated with JNK-1 activation. Further experimentation may shed light on JNK-1's failure to downregulate in this study, and should include an immunofluorescence study to determine the localization of p38 and JNK-1 upon their activation. Further work will be needed to determine which steps of the stress pathway are altered in confluent cells.

Heart disease remains one the western world's leading killers. Heparin or, more likely, its analogs may prove to be key to developing a new treatment protocol for atherosclerosis. Heparin's ability to downregulate the activation of some SAPKs in response to the minor injury that marks the genesis of the disease is separate from its anti-coagulant properties. With further study, this aspect of heparin's action could be exploited for medical use.

Conclusions

CPAEs and EJGs are stressed by tumor necrosis factor-α, and to a lesser extent, by histamine, as measured by levels of phospho-p38 and p-JNK by immunoblotting. Confluent endothelial cells appear less stressed than sub-confluent cells, most likely due to the constitutive presence of the dominant MAPK phosphatase, MKP-1. Confluent endothelial cells resist heparin's effects on SAPK activity levels. However, our data suggests that sub-confluent cells respond to heparin with lower active phospho-p38 levels in TNF-treated cells, demonstrating a modulation of the pathway that leads to atherosclerosis. While JNK-1 appears not to respond to heparin treatment, levels of JNK in sub-confluent cells may be too low for accurate determination of heparin effects.

CPAES with LPS stained for phospho-p38



Control 15 Minutes 30 Minutes 45 Minutes

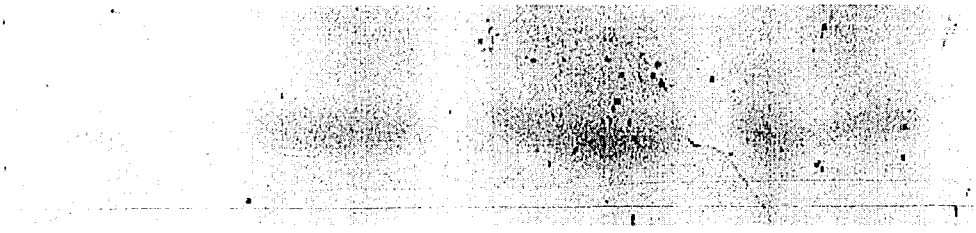
Figure 1.

LPS Fails to Activate p38 in Bovine CPAEs

Bovine CPAEs were incubated with 50 μL lipopolysaccharide (LPS) (final concentration of 5 μg/ mL) for 0 to 45 minutes. The control lane contains no LPS. The blot was stained with phospho-p38 antibody.

No activation was seen at any time point. The arrow indicates the region of the phospho-p38 protein.

CPAEs with histamine stained for p-JNK



Control 15 Minutes 30 Minutes 45 Minutes

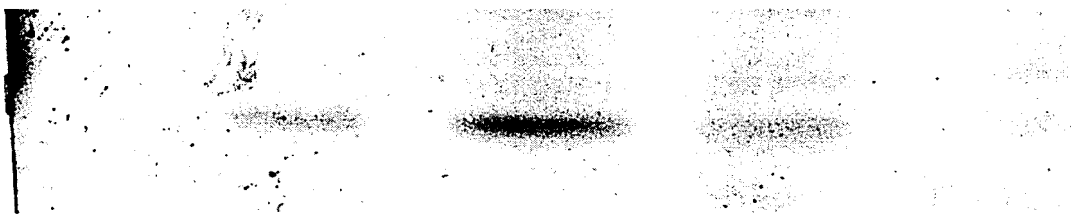
Figure 2.

Histamine activates JNK-1 in CPAEs

Bovine CPAEs were incubated with 50 μL stock histamine (0.001841 mg/mL water) for a final concentration of 9.2 ng/mL for 0 to 45 minutes. Cell samples were harvested and run on 10% SDS-PAGE, and the blot was stained with p-JNK antibody.

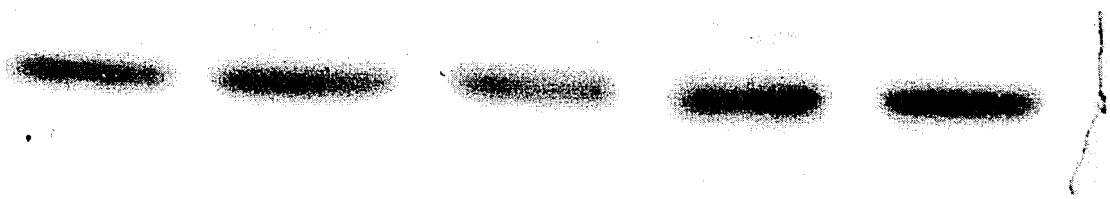
Slight activation can be seen at the 15, 30, and 45 minute time points, with 30 minutes staining most heavily.

A. CPAEs with TNF-α stained for phospho-p38



Control 5 Mins. 10 Mins. 15 Mins. 20 Mins.

B. CPAEs with TNF-α stained for total p38



Control 5 Mins. 10 Mins. 15 Mins. 20 Mins.

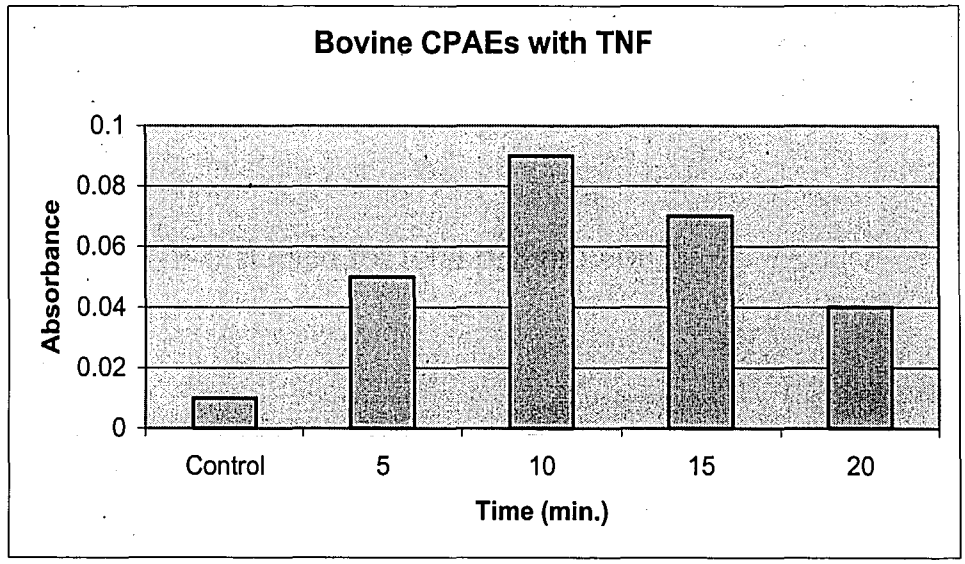
Figure 3.
TNF-α activates p38 in bovine CPAEs

Bovine CPAEs were incubated with 50 μL TNF-α (final concentration of 5 ng/mL) for 0 to 20 minutes. Cell samples were harvested and run on a 10% SDS-PAGE, and the blot was stained with phospho-p38 antibody.

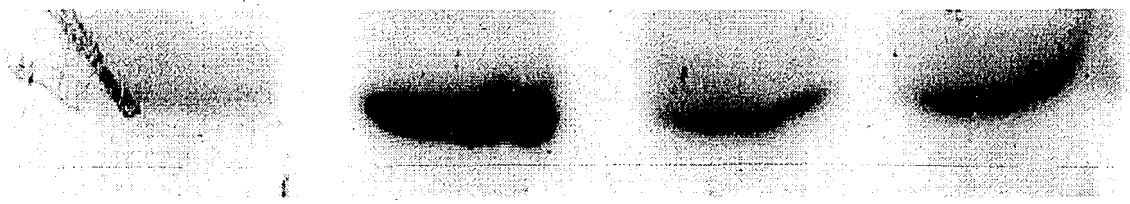
Activation can clearly be seen in the 5, 10, and 15 minute samples, and it peaks at the 10 minute time point.

C. Data Analysis:

Graph 1. Phospho-p38 levels of CPAEs with TNF-α

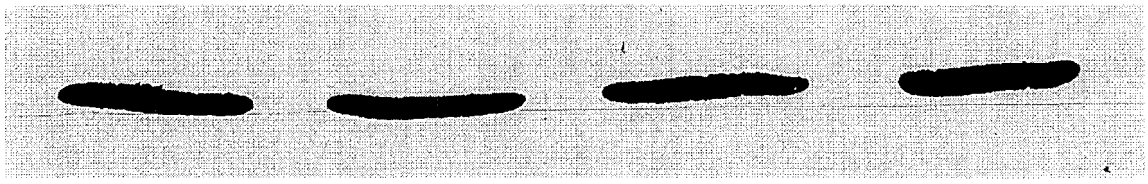


A. EJGs with TNF-α stained for phospho-p38



Control 10 Mins. 20 Mins. 30 Mins.

B. EJGs with TNF-α stained for total p38 protein



Control 10 Mins. 20 Mins. 30 Mins.

Figure 4.

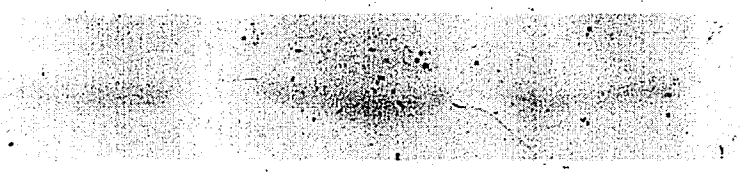
TNF-α activates p38 in bovine EJGs

Bovine EJGs were incubated with 50 μL of TNF-α (final concentration of 5 ng/mL) for 0 to 10 minutes. Cell samples were harvested and run on a 10% SDS-PAGE. The blot was stained with phospho-p38 antibody.

Activation can clearly be seen in the 10, 20, and 30 minute samples, peaking in between the control and 20 minute time points.

Total protein remains constant throughout the time course.

CPAEs with TNF-α stained for p-JNK



Control 15 Minutes 30 Minutes 45 Minutes

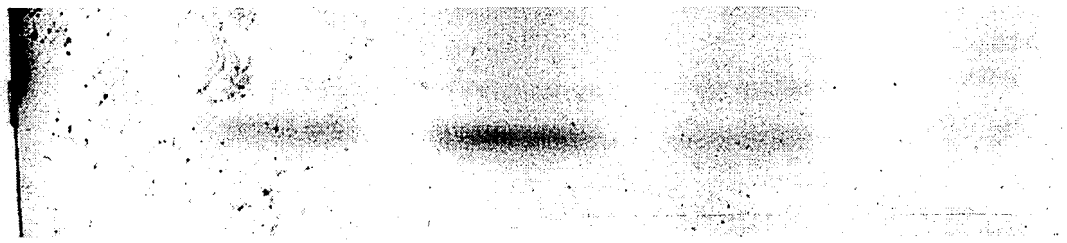
Figure 5.

TNF-α activates JNK-1 in bovine CPAEs

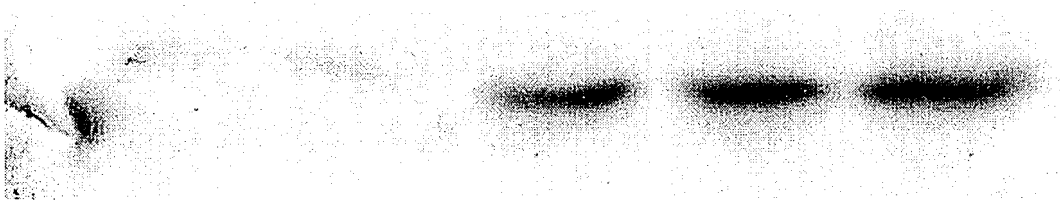
Bovine CPAEs were incubated with 50 μL of TNF-α (final concentration of 5 ng/mL) for 0- to 45 minutes. The control lane contains no TNF-α. The blot was stained with p-JNK antibody.

Activation can be seen in the 15, 30, and 45 minute samples. It peaks in the 30 minute lane.

A. CPAEs with TNF-α only stained for phospho-p38



B. CPAEs with heparin and TNF-α stained for phospho-p38



Control 5 Mins. 10 Mins. 15 Mins. 20 Mins.

Figure 6.

Heparin fails to block p38 activation by TNF-α in confluent bovine CPAEs

Bovine CPAEs were treated with 100 µL of fresh heparin (0.10 mg/mL deionized water) for a final concentration of 100 µg/mL for 10 minutes, and then were incubated with 50 µL of TNF-α (final concentration of 5 ng/mL) for 0 to 20 minutes. Cell samples were harvested and run on a 10% SDS-PAGE. The blot was stained with phospho-p38 antibody.

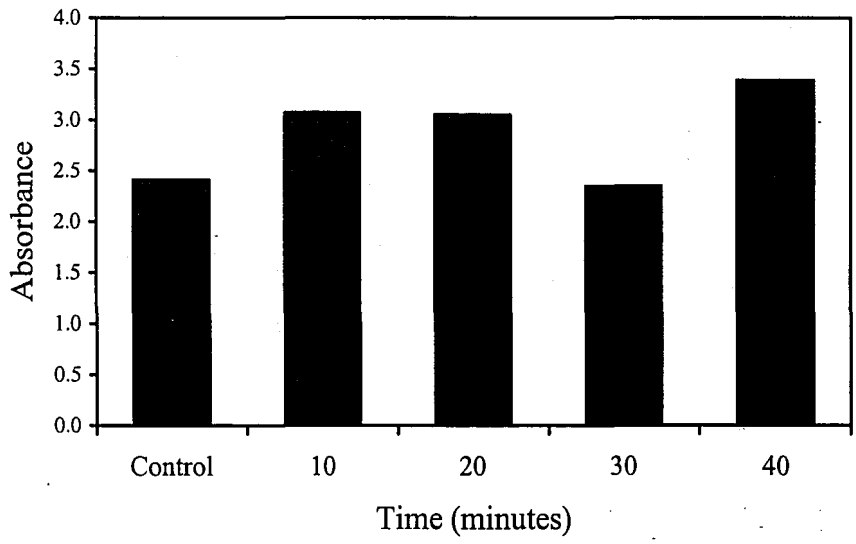
Heparin does not obviously suppress p38 activation at any point.

A. CPAEs with heparin stained for MKP-1

Control 10 min 20 min 30 min 40 min

Figure 7.

MKP-1 levels are constant despite incubation with heparin from 0-40 minutes in confluent bovine CPAEs.



Bovine CPAEs were treated with 100 μL of heparin (0.01 mg heparin/mL of deionized water) for a final concentration of 100 μg/mL. Cells samples were harvested and run on a 10% SDS-PAGE. Blots were stained with MKP-1 antibody. Note MKP-1 appears in the untreated control, and at similar levels throughout each time point.

A. CPAEs with TNF-α and heparin

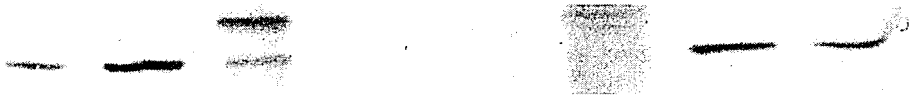


Figure 8.

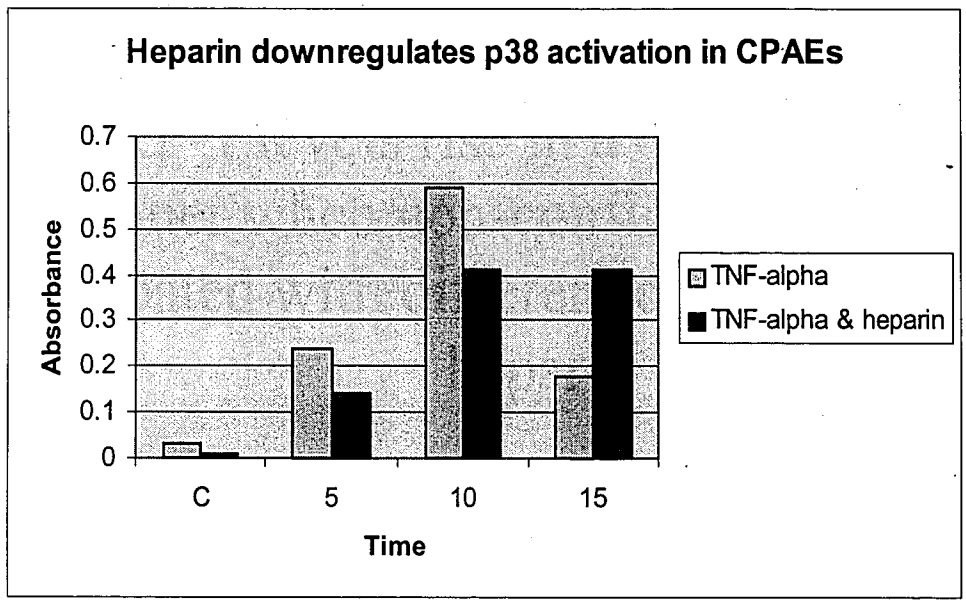
Heparin downregulates activation of p38 in non-confluent bovine CPAEs

Four plates of non-confluent bovine CPAEs were incubated with 25 μL of TNF-α (final concentration of 2.5 ng/mL) for 0 to 15 minutes; four others were treated with 100 μL of fresh heparin (0.01 mg heparin/mL of deionized water) for a final concentration of 100 μg/mL for 10 minutes and were then incubated with 25 μL of TNF-α (final concentration of 2.5 ng/mL) for 0 to 15 minutes. Cell samples were harvested and run on a 10% SDS-PAGE. The blot was stained with phospho-p38 antibody.

Downregulation of p38 activation can be seen at the 5 and 10 minute time points.

Panel B shows the results of densitometry analysis of the above blot.

B. Data analysis by densitometry



A. Bovine CPAEs with heparin stained for MKP-1

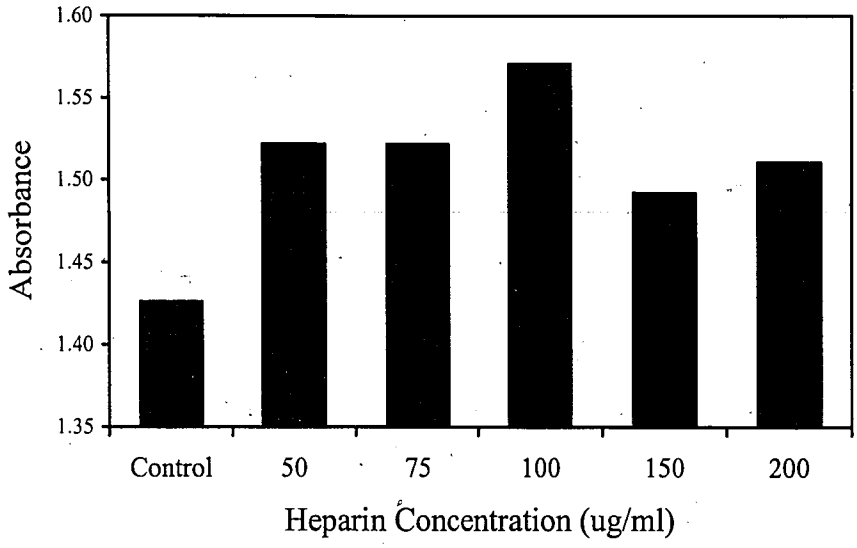
Control 50 μg/ml 75 μg/ml 100 μg/ml 150 μg/ml 200 μg/ml

Figure 9.

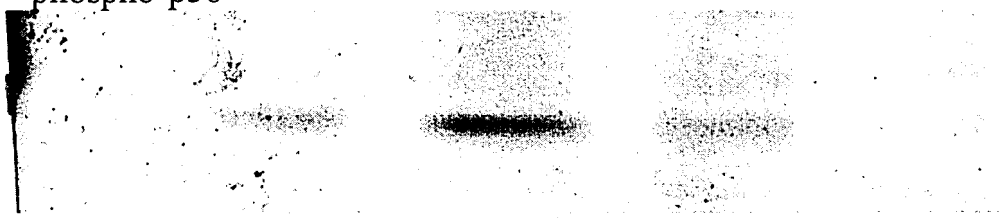
MKP-1 levels rise in a dose-dependent manner when non-confluent CPAEs are incubated with different concentrations of heparin

Non-confluent bovine CPAEs were incubated with varying concentrations of heparin from 0 to 200 μg/mL. Cell samples were harvested and run on a 10% SDS-PAGE, and the blot was treated with MKP-1 antibody. Note the rise in MKP-1 expression which peaks at 100 μg/mL.

B. Data analysis by densitometry

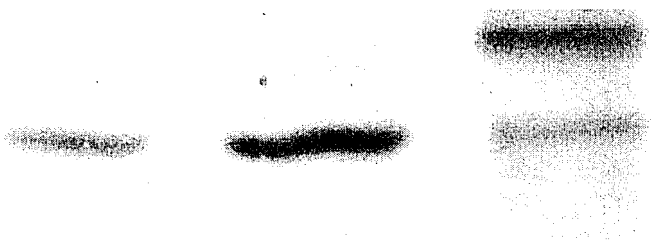


A. Confluent CPAEs treated with 50 μL of TNF-α and stained for phospho-p38



Control 5 Mins. 10 Mins. 15 Mins. 20 Mins.

B. Non-confluent CPAEs treated with 25 μL of TNF-α and stained for phospho-p38



Control 5 Mins. 10 Mins. 15 Mins.

Figure 10.

Non-confluent bovine CPAEs stress more easily than confluent cells

Bovine CPAEs were incubated with either 50 μL for a final concentration of 5 ng/mL (in panel A, confluent cells) or 25 μL for a final concentration 2.5 ng/mL (in panel B, non-confluent cells) of TNF-α over a short time course. Cell samples were harvested and run on a 10% SDS-PAGE. The blots were stained with phospho-p38 antibody.

Panel B demonstrates that non-confluent cells are more highly activated by TNF-α than confluent cells upon the addition of half as much stressor.

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Vita

Marianne Hamel was born to Coleman Rodney Hamel and Elsie Woodruff Hamel on May 21, 1974 in Bethlehem, Pennsylvania. She graduated with highest honors from Liberty high School in Bethlehem in 1992. In 1996, she earned her Bachelor of Science in Biology, Chemistry minor, from the College of William and Mary in Williamsburg, Virginia. Following graduation, she earned certification in phlebotomy from Lehigh County Community College and took a job at Miller Memorial Blood Center. In Fall of 1996, she began classes in Biological Sciences at Lehigh University, and formally entered the graduate school in January 1998. In June of 1998, she began working with Dr. Linda J. Lowe-Krentz on the study of signal transduction and atherosclerosis. She completed her research project for a Master of Science in May1999.

END OF TITLE