Green Fluorescent Protein Selectively Induces HSP70-Mediated Upregulation of COX-2 Expression in Endothelial Cells

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ABSTRACT

Reporter genes, including green fluorescent protein (GFP), have been used to monitor the expression of transgenes introduced into vascular cells by gene transfer vectors. Here, we demonstrate that GFP by itself can selectively induce expression of certain genes in endothelial cells. Elevation of the cytoplasmic concentration of GFP in endothelial cells, specifically, resulted in a robust upregulation of heat shock protein 70 (HSP70). GFP induced both messenger RNA and protein expression of HSP70 in a dose-dependent manner. GFP-mediated upregulation of HSP70 resulted in induction of cyclooxygenase-2 (COX-2) followed by prostaglandin E$_2$ (PGE$_2$) production. GFP-mediated upregulation of HSP70 is independent of MAP-kinase and PI3-kinase signaling cascades as inhibition of these pathways had no effect upon HSP70 increases. Adenoviral delivery of GFP into murine vasculature significantly enhanced blood flow, suggesting that sufficient PGE$_2$ is produced to induce vasodilation. Identification of the molecular partners that interact with GFP will increase our understanding of the vascular-specific factors that regulate stress angiogenesis and hemostasis.
INTRODUCTION

Endothelial cells (ECs) are ideal targets for gene therapy since they are readily accessible to gene transfer vectors via the circulation and are involved in a number of normal and pathophysiologic conditions, including angiogenesis, atherosclerosis, tumor growth, myocardial infarction and limb and cardiac ischemia\(^1\)\(^-\)\(^4\). However, little is known about the direct biological effects of gene delivery vectors on the function of endothelial cells. Moreover, the global transcriptional perturbations that may result from overexpression of reporter genes in endothelial cells have not been well studied.

Green fluorescent protein (GFP), is an \textit{in vivo} real-time reporter molecule cloned from the jellyfish Aequorea victoria\(^5\). It has attracted widespread interest due to its potential in the studies of gene expression and regulation\(^6\). GFP has been used to track gene expression in a wide variety of mammalian cells\(^7\), including endothelial cells. However, whether GFP as a foreign molecule, could potentially alter the biology of endothelial cells is not known.

We have shown that E1\(^{-}\)E4\(^+\) adenoviral vectors can directly induce genes that alter the inflammatory response of the endothelial cells\(^8\)\(^-\)\(^10\). Introduction of E1\(^-\)E4\(^+\), but not E1\(^-\)E4\(^-\) adenoviral vectors, resulted in induction of factors that promote survival and prevent apoptosis of endothelial cells\(^9\). In addition, E1\(^-\)E4\(^+\) vectors induce expression of pro-inflammatory adhesion molecules including ICAM1, VCAM1 and alter the chemokine expression profile\(^8\). The effect of E4\(^+\) adenoviral vectors is specific to vascular endothelial cells, as E4\(^+\) vectors fail to induce similar types of changes on other cell types including monocytes, epithelial cells or cancer cells. These data have set forth the intriguing possibility that vascular endothelial cells may be specifically sensitive to the introduction of gene transfer vectors or, potentially the reporter genes such as GFP, that are expressed by these genes.
Our data indicate that while the GFP-expressing adenoviral E1⁻E4⁻ vector does not induce any overt phenotypic changes on endothelial cells, it can specifically upregulate gene of HSP70, which may alter the physiology of endothelial cells. The heat shock proteins (HSPs) are encoded by genes whose expression is substantially increased during stress conditions\textsuperscript{11-13}, such as heat shock, viral infections, alcohol, ischemia, oxidative stress, fever or inflammation. In humans, there are at least 11 distinct genes that code for HSP70 isoforms, which are located on several different chromosomes\textsuperscript{14}. The 73-kDa protein (HSP73 or HSC70) is present at constitutive levels, whereas the 72-kDa protein (HSP72 or HSP70) is highly inducible and its synthesis is increased in response to multiple stressors. The transcription of inducible forms of HSP70 and HSP72 are under the control of the heat shock factor (HSF)\textsuperscript{15}, as well as a variety of physiological processes, such as cell cycle control, proliferation and differentiation\textsuperscript{16}. The major HSPs are molecular chaperones, which play an essential role in directing the folding and assembly of polypeptides in addition to coordinating regulation of protein translocation. HSPs also limit cellular damage following stress by their ability to prevent protein aggregation and to restore the function of denatured proteins. Moreover, HSP70 can function as a chaperone and cytokine\textsuperscript{17} by upregulating the expression of tumor necrosis factor-α, interleukin-1β and IL-6 on human monocytes, and stimulate cells of the innate immune system\textsuperscript{18}.

In the present study, we demonstrate that introduction of GFP by various gene transfer vectors into the endothelial cells selectively induces HSP70 mRNA and protein resulting in the upregulation of COX-2 expression, leading to enhanced production of the vasoactive prostaglandin E\textsubscript{2}. These studies suggest that GFP used as a reporter gene should be used with caution, since it may alter the physiology and stress response of endothelial cells during gene transfer experiments. Identification of the endothelial specific factors that GFP interacts with will also increase of our understanding of the mechanism endothelial cells respond to physiological stressors.
MATERIALS AND METHODS

Cell culture and treatment conditions

Human umbilical-vein endothelial cells (HUVEC) and human umbilical smooth muscle cells (HuSMC) were isolated by treating HUVECs with collagenase and were cultured in EC medium [M199 medium containing 10% (v/v) fetal bovine serum, 20 \( \mu \text{g/ml} \) EC growth factor, 90 \( \mu \text{g/ml} \) pig intestinal heparin, 100 \( \mu \text{g/ml} \) penicillin and 100 \( \mu \text{g/ml} \) streptomycin] in a humidified incubator at 37 °C with air/5% CO\(_2\) \(^{19}\). HUVEC monolayers from passages 2–4 were used in these studies. A549 (human lung carcinoma cells), HL60 (leukemic cell lines) and HeLa (human cervix carcinoma cells) were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Human pulmonary microvascular endothelial cells (HMVEC) and human dermal microvascular endothelial cells (HDMEC) were obtained from Clonetic (Clonetics, NJ). Bone marrow endothelial cells (BMEC) were obtained from an immortalized human bone marrow endothelial cell line. Cell viability was assayed by the trypan blue exclusion method, indicating that fewer than 5% of the cells took up the dye both before and after the infection of Advectors.

Construction of Advectors

The Ad vectors used in this study included: E1\(^{-}\)E4\(^{+}\) AdNull (E1, E3, E4\(^{+}\), cytomegalovirus early-immediate promoter/enhancer [CMV], no transgene in the expression cassette)\(^{20}\); E1\(^{-}\)E4\(^{+}\) Ad\(\beta\)gal (E1, E3, E4\(^{+}\); CMV promoter driving the Escherichia coli \(\beta\)-galactosidase [\(\beta\)-gal] gene)\(^{21}\); E1\(^{-}\)E4\(^{+}\) AdGFP (identical to E1\(^{-}\)E4\(^{+}\) Ad\(\beta\)gal, but with a modified form of the Aeguorea victoria green fluorescent protein cDNA [GFP] in place of \(\beta\)-gal)\(^{22}\); E1\(^{-}\)E4\(^{+}\) AdYFP(E1, E3, E4\(^{+}\); CMV promoter driving the
*Escherichia coli* yellow fluorescent protein cDNA [YFP] gene); E1E4– AdGFP (same as AdGFP, but with a complete deletion of the E4 gene, using the green fluorescent protein cDNA gene as a spacer in the E4 region); and AdLuciferase (E1, E3, E4⁺; CMV promoter driving the luciferase gene)²³. Ad vector stocks were purified by cesium chloride centrifugation and dialysis and quantified by plaque forming units (pfu) in 293 cells, as previously described²⁴. All Ad vectors had a particle/pfu ratio of approximately 100.

**Microarray Analysis**

Cells were harvested and total RNA was extracted by RNeasy Mini Kit (Qiagen). cDNA was synthesized by using Superscript choice kit (Invitrogen) with a T7-(dT)₂₄ primer incorporating a T7 RNA polymerase promoter. The cRNA was prepared and biotin-labeled by in vitro transcription (Enzo Biochemical). Labeled cRNA was fragmented by incubation at 94 °C for 35 min in the presence of 40 mM Tris acetate, pH 8.1, 100 mM potassium acetate, and 30 mM magnesium acetate. Fifteen µg of fragmented cRNA was hybridized 16 h at 45 °C to an U95a array (Affymetrix, Santa Clara, CA). After hybridization, the gene chips were automatically washed and stained with streptavidin-phycoerythrin by using a fluidics station. Finally, probe arrays were scanned at 3-µm resolution using the Genechip System confocal scanner (Aligent). Affymetrix Microarray Suite 4.1 was used to scan and analyze the relative abundance of each gene from the average difference of intensities.

**Western Blot Analysis.**

Cells were lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate, and 10 g/ml aprotinin). Insoluble debris was pelleted, and the protein concentration of the supernatant was determined with a DC protein assay kit (Bio-Rad). Fifty micrograms of each protein
sample was separated on an SDS-PAGE gel. The protein samples were transferred to nitrocellulose membrane. Protein expression was confirmed by immunoblotting with the following antibodies: HSP70, HSC70, Hemo Oxgenase-1 (HO-1) (StressGen, Victoria, BC, Canada), HSP90, COX-2 (Santa Cruz, CA), and GFP (Invitrogen). After incubation with the appropriate primary and horseradish peroxidase-conjugated secondary antibodies, the membranes were developed with enhanced chemiluminescence reagent (Amersham Pharmacia Biotechnology, Piscataway, NJ).

**Northern blot analysis.**

Total RNA (10 µg) was extracted from HUVECs with TRIzol reagent (Invitrogen) and was separated on 1.0% agarose gels containing 2.2 M formaldehyde, transferred to nylon membranes and UV-cross-linked. Probes for cDNA of HSP70b (StressGen, Victoria, BC, Ca) or GAPDH were radiolabeled with \( ^{32}P \)dCTP using a random primers DNA labeling kit. Northern hybridization was done using QuickHyb (Stratagene, Carlsbad, CA) as per the manufacturer’s protocol. After hybridization, the membranes were washed twice in 0.2% SSC containing 0.1%(w/v) SDS at room temperature for twice 20 min and finally in 0.1% SSC/0.1% SDS at 42°C for 45 min. The membranes were exposed to a Kodak film. To verify equivalency of RNA loading in the different lanes, the blot was stripped of radioactivity and rehybridized to determine the levels of GAPDH.

**Transfection and Assays of Reporter Gene Activity**

HUVEC monolayers were plated in six-well plates 24 h before transfection. Cells were transfected with the reporter plasmid HSP70b promoter β-Galactosidase using Polyfect transfection reagent (Qiagen Inc.) according to the manufacturer's directions. After overnight incubation with the transfection mixture, cells were infected with control, Adnull or AdGFP vectors. Cells were harvested
48 h after infection with Advectors. Galactosidase activity was assayed with a luminescent substrate (Galacton Plus) according to the manufacturer's instructions (TROPIX, Inc.). Results represent means of 3 experiments ± S.D.

**PGE$_2$ Release**

HUVEC monolayers were cultured in X-vivo medium 48 hrs after infection with and without AdGFP or AdNull. The supernatants were harvested and stored at –70 °C until they were assayed. The amount of PGE$_2$ in the supernatant was measured by ELISA$^{25}$.

**Laser-Doppler Measurements Mice Ear Blood Flow**

The pinna of the ear of the mice were injected with AdGFP (1x10$^8$MOI), AdNull (1x10$^8$MOI) or PBS (control). Three days after injection, blood flow of the ear was assessed by laser-Doppler with a laser flowmeter (model ALF21/21D, Advance, Tokyo, Japan).
RESULTS

To identify genes that were induced by GFP expression, we compared the gene expression profiles of control uninfected endothelial cells with AdNull- or AdGFP-infected HUVEC by using Affymetrix U95 high-density oligonucleotide genechips. AdGFP infection of endothelial cells for 48 h resulted in an 80 fold increase in HSP70 mRNA. AdGFP also induced, to a lesser degree, other stress-related genes including heme oxygenase-1 (HO-1)(table 1).

AdGFP-induced HSP70 expression is endothelial cell specific

The expression of HSP70 by the endothelial cells after AdGFP infection was assessed by immunoblot analysis. As shown in Fig.1 a, AdGFP effectively induced HSP70 protein expression in HUVEC. However, AdNull, Adβ-galactosidase and AdLuciferase failed to induce HSP70 expression in HUVECs (Fig.1a). AdGFP infection of confluent monolayers of HUVEC induced a dose-dependent expression of HSP70 protein with a peak effect at 400 MOI (Fig.1b). Although AdGFP also induced the expression of HO-1, it had no effect on the expression of HSP90 and HSC70 (Fig.1b). The time course of HSP70 induction by AdGFP was assessed by Western blot analysis. Maximal induction of HSP70 protein occurred after 2-3 days of infection with AdGFP (Fig.1c) and consistently high expression of HSP70 was detected after 6 days in parallel with GFP expression (Fig.1c). Of note, there was a slight upregulation of HSC70 expression 2-3 days after AdGFP infection. These data suggest introduction of GFP in endothelial cells results in a robust upregulation of HSP70 whereas enhanced synthesis of HSC70 is less prominent and transient.

To assess whether the induction of HSP70 by AdGFP is only restricted to endothelial cells, HSP70 protein expression was evaluated in HuSMC, A549, HL60 and Hela cell lines following infection with AdGFP. Although AdGFP was efficient in transducing more than 90% of the cells, there
was no induction of HSP70 in HuSMC, A549 (Fig.2A and 2B), HL60 or HeLa cell line (data not shown). Moreover, GFP not only induced HSP70 expression on the HUVECs, but also other microvascular endothelial cells, including human dermal microvascular endothelial cells (HDMEC) (Fig. 2C), bone marrow endothelial cells (BMEC) (Fig. 2D) and human pulmonary microvascular endothelial cells (HMVEC) (Fig. 2E). These data suggest that AdGFP induction of HSP70 is endothelial cell specific, and is not dependent upon the origin of endothelial cells.

**GFP directly induces HSP70 transcription.**

The expression of HSPs is regulated at both transcriptional and post-transcriptional levels. To further elucidate the mechanism responsible for the changes in amounts of HSP70 protein, we measured the mRNA levels of HSP70 by Northern blotting after endothelial cell infection with AdNull or AdGFP. As shown in Fig. 3a, HSP70b mRNA levels were upregulated in response to AdGFP, but not by AdNull. This result was consistent with the GFP-mediated induction as detected by microarray analysis (Table 1) and Western blotting (Fig. 1). We next investigated the effect of GFP overexpression on HSP70b promoter activity in endothelial cells. Endothelial cells were transfected with reporter vector expressing β-Galactosidase (β-gal) from HSP70 promoter and then infected with AdGFP for 48 hours. Fig. 3b demonstrates that in comparison to control vectors, there was an 8 fold increase in the β-gal activity in the AdGFP treated endothelial cells.

**GFP induction of HSP70 is independent on gene transfer vectors.**

We have previously shown that the E4 gene of adenoviral gene transfer vectors can alter the survival and apoptotic potential of endothelial cells. To rule out the possibility that the E4 gene or any other adenoviral gene(s) can directly induce HSP70 expression, we examined HSP70 expression in
HUVEC cells infected with lentivirus expressing GFP. Exposure of HUVEC to lentivirus-GFP induced the synthesis of protein of HSP70 (Fig. 4a) in endothelial monolayers. Moreover, we found that yellow fluorescent protein (YFP)\textsuperscript{26}, a structurally similar molecule to GFP, at 200 MOI also strongly induced HSP70 protein expression in HUVEC monolayers (Fig. 4b). These data suggest that GFP and its homologues can induce expression of HSP70 independent of the gene transfer vectors.

Since E4\textsuperscript{+} Ad vectors are known to modulate endothelial cells survival and activation status\textsuperscript{9}, we compared the effect of E1′E4\textsuperscript{−} AdGFP with that of E1′E4\textsuperscript{+} AdGFP on HSP70 expression in HUVEC. Both forms of AdGFP induced HSP70 protein expression and this induction correlated with the levels of GFP expression (Fig 5). These data suggests that GFP induction is not dependent on the co-expression of the E4 gene products.

**GFP induces HSP70 independent of MAPK and PI3K signaling cascades.**

To dissect the mechanism by which GFP induces HSP70 expression, we explored whether GFP activates signaling cascades which lead to enhanced HSP70 transcription. MAPK (mitogen-activated protein kinase) a Ser/Thr protein kinase has been shown to participate in heat stress response\textsuperscript{27,28}. The c-Jun NH\textsubscript{2}-terminal kinase (JNK) represents one subgroup of MAP kinases that is activated primarily by cytokines and exposure to environmental stress in mammalian cells\textsuperscript{13}. However, our evaluation of treatment with MEK inhibitor PD98059, p38 inhibitor SB203580, JNK inhibitor SP600125 or phosphatidylinositol 3-kinase (PI-3-K) inhibitor LY 294002 showed no significant disruption of AdGFP induced HSP70 protein expression, indicating that MAPK and PI3K pathway are not affected by GFP in HUVECs (Fig. 6).

**GFP induces COX-2 expression and PGE\textsubscript{2} production**
Among the known factors that regulate endothelial cell physiology, COX-2 induction has been shown to modulate endothelial cell function, including angiogenesis and inflammation. To identify the molecular pathways activated by overexpression of HSPs that alters endothelial cell function, we examined the effect of GFP on the expression of COX-2 levels. Fig. 7A shows that AdGFP, but not AdNull, induced COX-2 protein expression in a dose-dependent manner. Moreover, HUVEC treatment with AdGFP significantly increased the synthesis of PGE2, which is catalyzed by COX-2 (Fig. 7B). To determine whether HSP70 causes induction of COX-2 expression in HUVEC, we evaluated the effect of HSP70 protein on COX-2 expression. As shown in Fig. 7C, HSP70 protein induced COX-2 protein expression in HUVEC. These data suggest that GFP induced-HSP70 augments COX-2 expression and PGE2 production.

To examine the physiological significance of GFP-mediated PGE2 upregulation, the pinna of the ear of the mice were injected with AdGFP and the blood flow was measured by doppler after 24 hours. As compared to AdNull treated mice, AdGFP treated mice showed significant increase in blood flow most likely mediated by an increase in GFP upregulation of PGE2 expression (Fig. 8). Collectively, these data suggest that elevation of GFP induce production of sufficient PGE2 to alter the regional blood flow.

Discussion

Successful gene therapy strategies targeted to the blood vessels, depend on delivering the transgene of interest without inflicting unnecessary vascular toxicity. In this regard, identifying the factors or reporter genes that may alter the biology of the endothelial cells, promises to improve the reliability of the gene therapy strategies. In the present study, we demonstrate that introduction of the GFP reporter gene by adenoviral or lentiviral vectors selectively induces expression of HSP70 in human endothelial cells. Upregulation of HSP70 is associated with induction of COX-2 and synthesis of PGE2.
in vitro and regional increase in blood flow in vivo. The GFP-induced expression of HSP70 is only observed on the endothelial cells and not on other cell types such as smooth muscle cells or cancer cell lines. These data suggest that expression of GFP, specifically results in induction of genes in the vascular endothelial cells which alters the biological response of endothelial cells.

GFP-mediated induction of HSP70 in endothelial cells was not due to the introduction of the E4 adenoviral gene as the E4 vectors (AdE4-GFP) also induced HSP70 expression. Moreover, lentiviral vectors expressing GFP induced the expression of inducible HSP70. The induction of HSP70 was dose-dependent with a higher level of HSP70 being induced as escalating doses of GFP were used. It is intriguing that the constitutive isoform of HSP70 was also slightly induced when high doses of GFP were introduced.

HSP70 has been shown to protect against TNF-induced lethal inflammatory shock and to have a protective effect on the myocardium against more severe physiological stressors\(^{12,29,30}\). Substantial evidence indicates that heat shock proteins are capable of protecting cells, tissues, organs, and animals from a subsequent, normally lethal heating, as well as from other types of noxious conditions, including hypoxia and ischemia/reperfusion\(^{31,32}\). Here, we demonstrate that HSP70 can also induce the expression of COX-2 in the endothelial cells. Recombinant HSP70 induced the expression of COX-2 when the endothelial cells where incubated with this protein. Upregulation of COX-2 was associated with increased expression of PGE\(_2\).

Exogenous HSP70 can bind with high affinity to the cell surface\(^{17}\), and activate nuclear factor (NF)-κB and upregulate the expression of pro-inflammatory cytokines TNF-α, interleukin(IL)-1β and IL-6. COX-2 can be regulated in response to pro-inflammatory cytokines. This could be a possible mechanism for the exogenous HSP70-mediated effects on COX-2 expression in endothelial cells.
The mechanism by which GFP promotes the expression of HSP70 may be mediated through induction of HSP70 transcription. This hypothesis is supported by the data demonstrating that GFP can turn on the HSP70 promoter. HSP70 is regulated at the transcriptional level by heat shock transcription factor 1 (HSF1). Activated HSF1 binds to a specific DNA recognition sequence (heat shock element) in the HSP70 promoter\(^{13}\). HSP70 may interact with important kinases of the mitogen-activated signal cascades, such as Src kinases, tyrosine receptor kinases, Raf and MAP-kinases\(^{33}\). To rule out the possibility that GFP may alter the HSP70 expression through activation of signaling cascades, we challenged the cells expressing GFP with several inhibitors that selectively block Erk1, p38 MAPK, PI3 kinase and cJun N-terminal kinase (Jnk). None of these inhibitors blocked the GFP-mediated upregulation of HSP70 suggesting that GFP may not interact with those signaling pathways. It is possible that HSF1 is activated by mechanisms that are independent of phosphorylation, or that dephosphorylation of inhibitory phosphorylation sites stimulates the transcriptional activity of HSF1\(^{13}\).

We have shown that the effect of E4\(^+\) adenoviral vectors in mediating survival and proliferation is restricted to endothelial cells\(^9\). Presently, the exact mechanism whereby E4\(^+\) genes selectively alter the biology of vascular cells is not known. Similarly, GFP failed to induce HSP70 in smooth muscle cells and carcinoma as well as the leukemia cells suggesting that expression of certain permissive factors may be unique to the endothelial cells. It is conceivable that GFP, through interaction with endothelial specific proteins, induces the expression of HSP70.

GFP has been used in many \textit{in vivo} gene transfer experiments without any apparent toxicity. Based on our data, it is possible that the dose of GFP may be the major determinant in induction of HSP70. Indeed, we show that only at relative high doses of GFP is there an induction of HSP70. This high dose can only be achieved when high-copy adenoviral vectors or lentiviral vectors with strong
promoter drive GFP expression\textsuperscript{34}. In this regard, the toxicity of GFP can be diminished by adjusting the dose of the adenoviral or lentiviral constructs used in gene therapy studies.

Nonetheless, as COX-2 and PGE\textsubscript{2} can modulate vasodilator properties of vascular endothelial cells\textsuperscript{35} \textsuperscript{36} \textsuperscript{37}, the use of GFP as a reporter gene in adenoviral vectors that carry angiogenic factors should be used with extreme caution. As GFP-mediated upregulation of COX-2 and PGE\textsubscript{2} may act synergistically with the angiogenic factor expressed as a transgene, such as Adenoviral vectors expressing VEGF, then the outcome of these studies may not represent the true potential of the transgene of interest being tested.

Our data also sets the stage for studies to understand the mechanism by which GFP alters the physiological repertoire of endothelial cells. If GFP, by promoting expression of HSP70 and COX-2, conveys signals that may increase the survival of endothelial cells, GFP may be used to identify molecular factors that support survival, and angiogenic potential of endothelial cells.

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REFERENCES


Fig. 1. GFP Ad vector induced HSP70 protein expression in HUVEC. (A) Cells were treated with culture medium (control), AdNull 200 MOI, AdLuciferasre 200 MOI, AdGFP 200 MOI and Adβ-galactosidase (Adβ-gal) 200 MOI, and cultured in growth factor-free medium for 48 hours. Cellular extracts were prepared from HUVECs, and HSPs were detected by Western blot analysis using antibodies specific for HSP70 and β-actin. (B) HUVEC were infected for 48 hours with control or AdGFP at the indicated concentrations ranging from 50 to 400 MOI. Immunoblots were probed with antibodies specific to HSP70, HSC70, HSP90, HO-1 and β-actin. (C) HUVEC were infected with control, AdNull (200 MOI) or AdGFP (200 MOI) for the indicated time periods. Immunoblots were probed with antibodies specific for HSP70, HSC70, GFP and β-actin. The results shown represent one sample experiment from a total of three independent experiments.
**Fig. 2. GFP induced HSP70 protein expression specific in endothelial cells.** Non-endothelial cells: HuSMC (A) or A549 cell line (B) were infected with AdGFP (50-500 MOI) for 48 hours. Endothelial cells: HDMEC (C), BMEC (D) or HMVEC (E) were infected with AdGFP or AdNull at the indicated concentrations ranging from 50 to 200 MOI for 48 hours. Cell lysates were then assayed for HSP70, GFP and β-Actin by Western blotting.
Fig. 3. AdGFP-induced transcriptional activation of HSP70 promoter and RNA expression.

(A) Northern analysis of HSP70 gene expression in endothelial cells. Total RNA (10 µg/lane) was isolated from uninfected control cells and cells infected with AdNull (200 MOI) or AdGFP (50-200 MOI) for 48 hours. The blot was hybridized to a $^{32}$P-labeled human HSP70b cDNA probe and control $^{32}$P-labeled GAPDH probes to normalize RNA loading. (B) AdGFP activates HSP70 promoter activity. HUVEC were transfected with 2 µg HSP70 reporter plasmid containing the β-galactosidase for 12 hours, and then treated for 48 hours under the following conditions: control, AdNull 200 MOI or AdGFP 200 MOI. β-galactosidase reporter activities were measured in cellular extract. The columns are the means, and the bars are the S.D. *, $p < 0.001$ compared with control. The results are representative of three separate experiments.
Fig. 4. Increased HSP70 expression in HUVEC after engraftment with Lentivirus transduced GFP or AdYFP. (A) HUVEC infected with lentivirus GFP (MOI 50) for 48 hours. (B) HUVEC infected with AdNull (200 MOI), AdYFP (200 MOI), AdYFP (400 MOI) and AdGFP (200 MOI) for 48 hours. Immunoblots were probed with antibody specific for HSP70, GFP and β-actin.
Fig. 5. Effect of E4\(^+\) GFP Ad vector or E4\(^-\) GFP Ad vector on HSP70 expression in HUVEC.

Confluent endothelial cells were infected with the E1E4\(^+\) AdGFP, E1E4\(^+\) AdGFP vector or uninfected as controls, cultured in growth factor-free medium for 48 hours. Immunoblots were probed with antibody specific for HSP70, GFP and \(\beta\)-actin.
Fig. 6. Effects of kinase inhibitors on the HSP70 protein expression in HUVEC.

Cell were infected with AdGFP (200 MOI) or uninfected control and cotreated with either PD98059 (PD), SB203580 (SB), SP600125 (SP) or LY294002 (LY) for 48 hours. Cells were harvested and lysed for Western blot analysis of HSP70.
Fig. 7

A

Control  AdGFP 50 MOI  AdGFP 100 MOI  AdGFP 200 MOI  AdNull 200 MOI

COX-2

β-Actin

B

PGE2 (pg/ml)

Control  AdNull 200MOI  AdGFP 100MOI  AdGFP 200MOI

C

Control  inactivated HSP70  5 µg

COX-2

β-Actin
Fig. 7. Effects of GFP on COX-2 expression and PGE2 production in endothelial cells

(A) Control uninfected cells and cells infected with AdGFP (50-200 MOI) or AdNull (200 MOI) for 48 hours. Cell lysates were probed for COX-2 expression by Western blotting and (B) culture supernatants were analyzed for PGE2. Production of PGE2 was determined by enzyme immunoassay as described in MATERIALS AND METHODS. The columns are the means, and the bars are the S.D. (n = 4), *, p<0.05 compared with Control; **, p<0.01 compared with Control. (C) Cells were treated without or with recombinant human HSP70 (5 µg/ml) or heat-inactivated HSP70 (5 µg/ml) for 24 hours. Immunoblots were probed with antibody specific for COX-2.
Fig. 8

A

Flow (ml/min/100 g)

Control  AdNull  AdGFP

B

AdNull

C

AdGFP
Fig. 8. Effects of AdGFP on ear blood flow and vasodilation.

(A) AdGFP (1x10^8 MOI/ear), AdNull (1x10^8 MOI/ear) or control (PBS) were injected in the pinna of the mice’s ear. After 3 days following injection, blood flow was measured using a laser-Doppler. The columns are the means, and the bars are the S.D. (n = 3), *, p<0.05 compared with Control or AdNull. As compared to the AdNull injected mice (B), three days after AdGFP injection (C) there was an increase in the number of grossly vasodilated vessels.
Table: Stress-related genes altered after infection AdGFP

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Control uninfected endothelial cells and cells infected with AdGFP (200 MOI) for 48 hours. Microarray analysis was determined by Affymetrix genechip as described in the Material and Methods. * AdGFP v.s Control.
Green fluorescent protein selectively induces HSP70-mediated upregulation of COX-2 expression in endothelial cells

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