Detail Materials and Methods

Cell cultures

HCAECs and endothelial growth medium-2 (EGM-2) were purchased from Cambrex BioWhittaker Inc. (Walkersville, MD). Cells were used at passage 4 to 6. When HCAECs grew to 80%-90% confluence in 6-well plates, they were divided into four groups. Group I, cells were treated with different concentrations (0.2, 2 or 10 μg/ml) of recombinant cHSP60 or heat-inactivated cHSP60 (HI-cHSP60, 10 μg/ml) for different times (6, 24 or 48 hours). Heat inactivation of cHSP60 was performed by boiling the protein at 100^{o}C for 30 minutes. Recombinant cHSP60 was obtained as a gift from Dr. Grant N. Pierce at the Division of Stroke and Vascular Disease, St Boniface General Hospital Research Centre, Manitoba, Canada.

Some of the cells were then treated with actinomycin D (2.5 μg/ml, Sigma, St. Louis, MO) for additional 0.5, 1, 3, 6 and 12 hours to test the eNOS mRNA stability. Group II, cells were pre-treated with antibodies against human HSP 60 (Abcam, Cambridge, MA), cHSP60 (Affinity BioReagents, Golden, CO), isotype control antibody (rabbit IgG, 4 μg/ml), toll-like receptor 2 (Abcam, Cambridge, MA) or toll-like receptor 4 (Abcam, Cambridge, MA) for 1 hour before adding cHSP60 (2 μg/ml) for 24 hours. Group III, cells were transfected with various plasmids containing basic vector (pGL2) without eNOS promoter, vector with optimal length of enhancer and eNOS promoter (-1193/+109) or vector with shortened, nonfunctional enhancer and eNOS promoter (-49/+109) for 48 hours, and then treated with cHSP60 (2 μg/ml) for another 24 hours. Group IV, cells were cultured with or without antioxidants seleno-L-methionine (SeMet, 20 μM) or SOD mimetic MnTBAP (AG Scientific, San Diego, CA, 2.5 μM) and cHSP60 (2 μg/ml) for 24 hours. Group V, cells were treated with cHSP60 (2 μg/ml) for 5, 10, 20, 30, 45, 60 or 90 minutes. Group

VI, cells were pre-treated with 1 μM p38 MAPK inhibitor (SB239063), 40 μM JNK inhibitor (SP600125) or 40 μM ERK1/2 inhibitor (PD98059) for 1 hour and then co-cultured with cHSP60 (2 μg/ml) for another 24 hours. In the separate group, adenovirus vectors (AD) containing dominant-negative forms (DN) of p38, JNK1 or ERK2 were used to infect HCAECs for 24 hours, and then treated with cHSP60 (2 μg/ml) for another 24 hours. In all groups, cells cultured in the plain medium were used as negative controls. The concentration of cHSP60 was selected based on the previous publications.[1] All other chemicals were reagents with molecular biology grade and were obtained from Sigma Chemical Co. (St. Louis, MO).

Myograph tension system. Fresh porcine hearts were harvested from young adult farm pigs (6-8 months old) at a local slaughterhouse. The right coronary artery was carefully dissected and cut into multiple 5-mm rings, which were then incubated at 37°C in DMEM as controls or in DMEM containing 0.2, 2, or 10 μg/ml of cHSP60 for 24 hours. On the next day, the vasomotor function of these rings was tested using the myograph tension device as previously described in our publications.[17,18] Briefly, rings were suspended between the wires of the organ bath myograph chamber (Danish Myo Technology Organ Bath 700 MO, Aarhus, Denmark) in 6 ml of Kreb's solution, maintained at 37ºC and oxygenated with pure oxygen gas. Rings were slowly subjected stepwise to a predetermined optimal tension of 30 mN and allowed to equilibrate for at least 30 minutes. Following equilibration, each ring was precontracted with 20 μl of thromboxane A2 analogue U46619 ($3X10^{-8}$ M). After 60-90 minutes of contraction and stabilization, the relaxation curve was generated by adding 60 μl of five cumulative concentrations of the endothelium-dependent vasodilator bradykinin (10^{-9} to 10^{-5} M) every 3 minutes. In addition, 60 μl of SNP (10^{-6} M) was added and endothelium-independent vasorelaxation was recorded. Contractility and

percentage of relaxation were calculated based on the tension change. The data of porcine coronary artery rings from each heart were averaged and represented as one data point for statistical analysis.

Real-time PCR. The endothelial cells of the porcine artery ring were collected by scraping the luminal surface of the ring. Total RNA from porcine endothelial cells and HCAECs was isolated using tri-reagent kit (Sigma). cDNA was generated by reverse transcription (RT) from mRNA using the iScript cDNA Synthesis Kit (Bio-Rad). iQ SYBR Green SueperMix Kit (Bio-Rad) was used for real-time PCR reaction. Master mixture was used to reduce variability in primer and reagent concentrations. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to account for variations in mRNA loading. The same total RNA (1 μg) was loaded for all samples. Human eNOS, GAPDH, NOX1, NOX4, p22, CAT, SOD1, GPX1, GPX7, TLR2 , and TLR 4 and porcine eNOS and GAPDH primers (Table I) were designed by Beacon Designer (Bio-Rad), and synthesized by Sigma Genosys (Woodlands, TX). Real-time PCR was performed in the iCycler iQ real-time PCR detection system (Bio-Rad). Thermal cycle condition used for RT was as follows: 5 minutes at 25ºC, 30 minutes at 42ºC, and 5 minutes at 85ºC. The condition used for real-time PCR was as follows: 3 minutes at 95ºC, 40 repeats of 20 seconds at 95ºC, and 1 minute at 60ºC. Controls were performed with no RT (mRNA sample only) or no mRNA (water only) to demonstrate the specificity of the primers and the lack of DNA contamination in samples. Sample cycle threshold (Ct) values were determined from plots of relative fluorescence units (RFU) versus PCR cycle number during exponential amplification so that sample measurement comparisons were possible. Each gene expression in each sample was calculated as $2^{(40 - Ct)}$ and further normalized to GAPDH expression as $[2^{(Ct[GAPDH] - Ct[gene\ of\ interest])}]$.

Western blot. Total proteins were isolated from HCAECs using tri-reagent kit. Protein concentration was determined using the Bradford protein assay with bovine serum albumin as standard. The same amount of endothelial proteins (6 μg) was resolved electrophoretically by one-dimensional SDS-PAGE (10% polyacrylamide) for approximately 1 hour at 150 V. Subsequently, the gel was equilibrated in transfer buffer (48 mM Tris, 39 mM glycine, 0.03% SDS, and 20% methanol) and the proteins were electrophoretically transferred to the nitrocellulose filter for overnight at 30 V. Filter was blocked using 5% nonfat dried milk in Tris buffer saline (TBS) with 0.05% Tween 20 (TBS-T) for 1 hour at room temperature. eNOS was detected using a mouse anti-human eNOS monoclonal antibody (BD Biosciences, San Jose, CA) diluted 1:1000, and β-actin was detected using a mouse anti-human β-actin monoclonal antibody (Sigma) diluted 1:10000. The eNOS and β-actin primary antibodies were detected with a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody diluted 1:2000. Blots were developed using ECL-plus kit (Amersham Biosciences, Piscataway, NJ) and analyzed with gel documentation system and analysis software (Alpha Innotech Co., San Leandro, CA).

eNOS enzyme activity assay. An eNOS fluorimetric assay kit (Sigma) was used to determine eNOS activity. A cell-permeable diacetate derivative of 4,5-diaminofluorescein (DAF-2 DA) penetrates cells rapidly where it is hydrolyzed by intracellular esterase to DAF-2 that, in turn, reacts with NO produced by eNOS to form a fluorescent triazolo-fluorescein. The fluorescent product can be quantitated using an excitation filter at 492 nm and an emission filter at 515 nm. eNOS activities in cHSP60-treated cells were normalized with untreated cells, and data are shown as mean ± SEM (% of controls).

eNOS promoter activity assay. eNOS promoter plasmid constructs were kindly provided by Dr. Philip A. Marsden (University of Toronto, Toronto, Ontario, Canada).[2] The promoter fragment from -1193 to +109 bp (optimal length of enhancer and eNOS promoter) or from -49/+109 bp (non-functional short enhancer and eNOS promoter) was subcloned into the pGL2-basic vector carrying firefly luciferase (Promega, San Luis Obispo, CA.). HCAECs were co-transfected with pGL2 construct and pRL-SV40 vector carrying Renilla luciferase (as an internal control reporter, Promega, Madison, WI) by using Lipofectin reagent (Invitrogen). HCAEC cultures were plated at 3.3×10^4 cells/ml (3.5 ml) and grown on 60-mm dishes for 48 hours. DNA-Lipofectin complexes (2:1 mass/mass ratio) were incubated for 60 minutes at 22°C and then added to the cells at 37°C in serum-free Opti-MEM I. The transfection mix was replaced at 6 hours with EMG-2 medium. The pGL2-basic vector, lacking both a eukaryotic promoter and enhancer sequences, was used as a negative control. After transfection for 48 hours, cells were treated with cHSP60 (2 μg/ml) for additional 24 hours. After brief wash with PBS, luciferase activities were measured using Dual Luciferase Reporter assay system (Promega, Madison, WI) and a luminometer. Luciferase activities were normalized by the ratio of firefly and Renilla luciferase activities. Data are shown as mean ± SEM. All experiments were carried out in triplicate.

Flow cytometry. Cells were harvested with 0.025% Trypsin/EDTA and adjusted to $1X10^6$ cells per FACS tube. For eNOS staining, cells were pre-treated with 500 μl of cytofix/cytoperm buffer (BD Biosciences) for 20 minutes at 4°C, followed by primary antibody (1 μg/10^6 cells, BD Biosciences) and secondary antibody (1 μg/10^6 cells, Molecular Probes, Eugene, OR) for 30 minutes each at 4°C. For O_2^- and NO staining, dihydroethidium (DHE, 3 μM, Molecular Probes) and 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF–FM DA, 10 μM, Molecular Probes) were, respectively, added to the cells and incubated in 37°C for 30 minutes. Finally, the cells were

collected in 500 µl staining buffer (BD Biosciences) and stored in 4°C. The cells were analyzed using FACScan and Cell Quest software (Becton Dickinson, Franklin Lakes, NJ) within 24 hours of preparation. In each experiment, at least 10,000 events were analyzed.

Lucigenin-enhanced chemiluminescence. Levels of the superoxide anion produced by porcine endothelial cells were detected by using the lucigenin-enhanced chemiluminescence method.[3,4] Porcine coronary artery rings were cut open longitudinally and trimmed into approximately 5 x 5 mm^2 pieces. They were then rinsed briefly in a modified Krebs HEPES buffer solution (KHBS, 120 mM NaCl, 4.7 mM KCl, 1.18 mM K_2HPO_4, 20 mM HEPES, 2.5 mM $CaCl_2$, 1.17 mM $MgSO_4$, and 25 mM $NaHCO_3$). Assay tube (12 X 75 mm) was filled with 500 µl of Krebs HEPES buffer solution and 25 µl of lucigenin for a final concentration of 50 µM. After gently vortexing, the vessel segments were placed endothelium-side-down in the tubes. Time-based reading of the luminometer was recorded by FB12 software (Berthold Detection Systems GmbH, Bleichstr, Germany). The data in relative light units per second (RLU/second) for each sample were averaged between 5 and 10 minutes. Values of blank tubes containing the same reagents as the vessel ring samples were subtracted from their corresponding vessel samples. The area of each vessel segment was measured using a caliper and used to normalize the data for each sample. Final data are represented as RLU/second/mm^2.

Assessment of mitochondrial membrane potential (Δψm). The $\Delta\psi m$ was assessed using flow cytometry analysis of cells stained with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazole-carbocyanide iodine (JC-1, MitoScreen kit, BD Biosciences). Mitochondria with a normal $\Delta\psi m$ concentrate JC-1 into aggregates (red fluorescence), but with a de-energized or depolarized $\Delta\psi m$, JC-1 forms monomers (green fluorescence). Cells ($5x10^5$) were incubated with 10 µg/ml JC-1 for

12 minutes at 37°C and analyzed by flow cytometry using a FACScan and Cell Quest software (Becton Dickinson). The analyzer threshold will be adjusted on the FSC channel to exclude most of the subcellular debris. Photomultiplier settings will be adjusted to detect JC-1 monomer fluorescence signals on the FL1 detector (green fluorescence, centered at 390 nm) and JC-1 aggregate fluorescence signals on the FL2 detector (red fluorescence, centered at 340 nm). Data analyses were performed with Paint-a-Gate Pro Software (Becton Dickinson). In each experiment, at least 20,000 events were analyzed.

Measurement of ATP content. ATP levels were measured with an ATPLite kit (PerkinElmer, Wellesley, MA). The ATPLite assay system is based on the production of light caused by the reaction of ATP with added luciferase and D-luciferin. The emitted light is proportional to the ATP concentration within certain ranges. Cells were seeded on 96-well black plates (5,000 cells/well) and cultured with or without cHSP60 (2 μg/ml) for 24 hours. Lysis solution and substrate solution (PerkinElmer) were added to each well of the plate, respectively. The luminescence was measured by TopCount Microplate Scintillation and Luminescence Counter (PerkinElmer).

Assessment of NADPH oxidase activity. The activity of NADPH oxidase (NOX) was measured using the methods previously described.[5,6] HCAECs were seeded in the 96-well plate (CulturPlate, PerkinElmer) at the density of 10,000/well. When cells grew to 80%-90% confluence, they were treated with EMG-2 containing cHSP60 or LY83583 (10 μM) in the presence or absence of MnTBP (2.5 μM) for 24 hours. After washed in ice-cold PBS, cells were incubated in Krebs-HEPES buffer (KHB, pH 7.4) for 10 minutes. Dark-adapted lucigenin (5 μM) was added and the plate was sealed with an adhesive sealing film and loaded into a TopCount NXT microplate scintillation and luminescence counter (PerkinElmer). Chemiluminescence was recorded for 10 seconds with the

counter running in Single-Photon-Count mode. Responses of the cells to the β-NADPH (100 μM) with or without the presence of Tiron (cell-permeable O_2^- scavenger, 20 mM), DPI (flavoprotein inhibitor, 5 μM) were also tested. A buffer blank background was subtracted from each respective reading. Final data were presented as RLU/second.

Measurement of CAT and SOD activity. Treated HCAECs were homogenized and centrifuged in HEPES buffer (pH 7.4) containing 1 mM EDTA. CAT and SOD enzyme activities were measured with commercial enzyme assay kits (Cayman Chemical, Ann Arbor, MI) following manufacturer's protocols. CAT and SOD enzyme activities were calculated from the average absorbance of each sample using the equations provided in the kit manuals. Final data was represented as mean ± SEM (nmol/minute/ml) for CAT activity and U/ml for SOD activity.

Bio-Plex immunoassay. BioPlex 2200 luminex system (Bio-Rad) combines the Luminex multianalyte profiling technology with unique antigen-coated fluoromagnetic bead chemistry and versatile software. HCAECs were cultured with cHSP60 (2 μg/ml) for 0, 5, 10, 20, 30, 45, 60 or 90 minutes. Cell lysate was prepared using the kit obtained from Bio-Rad. Detection of phospho- and total ERK2, JNK and p38 was performed using A manufacturer's protocol. Each test included three positive controls obtained from Bio-Rad to monitor detector stability, specimen and sample integrity. Final data were analyzed and presented as a ratio of phosphoprotein/total protein for each MAPK (average of triplicates).

Statistical analysis. Maximal contraction and endothelial-dependent and independent vasorelaxation of the porcine coronary artery rings were compared between the control and treatment groups using one-way ANOVA. Data from other experiments were compared using the

paired Student's t test (two-tails). Significance was considered if $P<0.05$. Data are reported as mean ± SEM.

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Table I. Sequence of real-time PCR primers

Gene	Genbank No.	Forward primer	Reverse primer
Human eNOS	NM_000603	5'-AGGAACCTGTGTGACCCTCA-3'	5'-CGAGGTGGTCCGGGTATCC-3'
Human GAPDH	NM_002046	5'-TGCACCACCAACTGCTTAGC-3'	5'-GGCATGGACTGTGGTCATGAG-3'
Human NOX-1	NM_007052	5'-GCCTCCATTCTCTCCAGCCTATC-3'	5'-CACATACTCCACTGTCGTGTTTCG-3
Human NOX-4	NM_016931	5'-TGGCTTTGGATTTCTGGACCTTTG-3'	5'-ATGATGGTGACTGGCTTATTGCTC-3'
Human p22	BT006861	5'-CAGATCGAGTGGGCCATGTG-3'	5'-AAAGTACCACTGGGTGAAGCG-3'
Human CAT	NM_001752	5'-GCGGAGATTCAACACTGCCAATG-3'	5'-CTGTTCCTCATTCAGCACGTTCAC-3'
Human SOD1	NM_000454	5'-ATGACTTGGGCAAAGGTGGAAATG-3'	5'-GTTAAGGGGCCTCAGACTACATCC-3'
Human GPX1	NM_000581	5'-CGAGGGAGGAACACCTGATCTTAC-3'	5'-GGGAAACTCGCCTTGGTCTGG-3'
Human GPX7	NM_015696	5'-GACAGCAACAAGGAGATTGAGAGC-3'	5'-ACATGGGGAATGAGACACTGTAGG-3'
Human TLR2	NM_003264	5'-GCCTCTCCAAGGAAGAATCC-3'	5'- TCCTGTTGTTGGACAGGTCA-3'
Human TLR4	NM_138554	5'- TACAAAATCCCCGACAACCTCC-3'	5'- GCTGCCTAAATGCCTCAGGG-3'
Porcine eNOS	AY266137	5'-CCCTACAACGGCTCCCCTC-3'	5'-GCTGTCTGTGTTACTGGATTCCTT-3'
Porcine GAPDH	AF017079	5'-TGTACCACCAACTGCTTGGC-3'	5'-GGCATGGACTGTGGTCATGAG-3'

Additional Discussion

Since we demonstrated that cHSP60 induces oxidative stress, we further tested the blocking effects of antioxidants SeMet and SOD mimetic MnTBAP on cHSP60-treated cells. SeMet was chosen because it is a dietary antioxidant with unique mechanisms different with other types of antioxidants and it could be ready for clinical use. Indeed, SeMet effectively blocked cHSP60-induced eNOS downregulation and decrease of CAT and SOD activities. SeMet was chosen in the current study because it is a dietary antioxidant with unique mechanisms different with other types of antioxidants and it could be ready for clinical use. Selenium is an essential nutritional element for human health. Dietary selenium includes both inorganic (selenite and selenate) and organic (SeMet, selenocysteine and selenomethylselenocysteine) compounds.[1] SeMet is the most prevalent form of dietary selenium. The Recommended Dietary Allowance (RDA) for selenium for both men and woman is 55 μg/day and the Tolerable Upper Intake Level (UL) for adult is set at 400 μg/day (Food and Nutrition Board—USA Institute of Medicine, 2000). Normal serum concentrations in healthy

individuals range from 70 to 125 µg/ml. Absorption rate of all forms of dietary selenium is relatively high (70–95%) although it may vary according to the source and the selenium status of the subject.[2] SeMet is absorbed by the same active transport mechanism used by methionine because selenium can substitute for sulphide atoms due to its similar ionic radius. Although Selenium metabolism in the body is not completely understood, selenocystein is the major selenocompound present in selenoproteins of body tissues.[3] SeMet can be converted to hydrogen selenide and selenocystein, which are ready for incorporation into proteins specific UGA codons. Currently, 25 selenoproteins have been found including glutathione (GSH) peroxidases (GPX) 1–5, iodothyronine deiodinases 1–3, thioredoxin reductases, selenoprotein P, selenoprotein H, and other proteins with mostly unknown functions.[4,5] GPX recycle GSH and reduce lipid peroxidation by catalyzing the reduction of peroxides including hydrogen peroxide. Iodothyronine selenodeiodinases catalyse the extrathyroidal production of tri-iodothyronine. In addition, SeMet is able to directly interact with some oxidant molecules or oxidant generating ions.[6-8] It is regenerated from Se-oxide (Se(O)Met) nonenzymatically by GSH. This mechanism also occurs for SeMet in proteins.[9] SeMet competes with Met for metabolism including protein synthesis. SeMet can charge tRNAMet, resulting in substitution for Met in peptides.[10] SeMet is more effective than Met as a substrate for Met-adenosyl transferase,[11] forming selenium-adenosyl SeMet (Se-SAM), which can serve as methyltransferase substrate in the methylation of RNA, phosphotidyl lipids, creatine, histamine and thiols. Physiological significance of these SeMet properties is largely unknown. Selenium deficiency has been linked to human diseases. For examples, two endemics diseases have been reported from the low-selenium areas in China and Easther Siberia originating:[12] Keshan disease, an endemic cardiomyopathy, and Kashin-Beck disease, a deforming arthritis. A form of cretinism associated with hypothyroidism appears also be related to selenium deficiency. Furthermore, epidemiologic studies have indicated that selenium levels are negatively associated

with several cardiovascular diseases, cancers, information and immune disorders.[13-17] Thus, selenium has been tested as a pharmacological agent or supra-nutritional supplement for several disease conditions. It is hypothesized that selenium could prevent and treat several types of cancers, cardiovascular disease and other disorders.[13-17] Further investigations in both basic science research and clinical trials are warranted to confirm this hypothesis.

In the current study, we have used 20 μM SeMet in cell cultures. It is not clear how this concentration can be translated into the clinical use. In a clinical trial of use of oral SeMet (200 μg/day), serum levels of SeMet could reach to 147±17 ng/ml (0.75 μM) after 2 hours.[17] It is hard to achieve 20 μM in humans by oral administration because of consideration of potential cytoxicity of SeMet. Since development of endothelial dysfunction and vascular disease in humans is a long term process, long term oral supplement of the relative small amount of SeMet could have a protective effect against vascular pathogenesis due to its antioxidant activity. Thus, enhanced serum levels of SeMet (0.75 μM) may still be effective even though it is much lower than 20 μM used in current study, in which SeMet treatment was 24 hours.

It is well known that the low level of GSH is a critical factor contributing oxidative stress, while antioxidant activity of SeMet can act at GSH levels. However, this mechanism is not investigated in the current study. It is not clear whether cHSP60 could reduce GSH levels or SeMet could increase GSH levels in endothelial cells. A recent study demonstrated that decreased endothelial GSH was partly responsible for the age-related loss of eNOS activity and endothelium-dependent vasorelaxation function in elderly rats, and (R)-α-lipoic acid can effectively increase cellular GSH levels and improve age-related endothelial dysfunction.[18] (R)-α-lipoic acid (LA) is able to induce GSH synthesis.[18]

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