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Broad regulation of matrix and adhesion molecules in THP-1 human macrophages by nitroglycerin

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ABSTRACT

Although nitroglycerin (NTG) is effective for the acute relief in coronary ischemic diseases, its long-term benefits in mortality and morbidity have been questioned. The possibility has been raised that NTG may increase the activity of matrix metalloproteinases (MMP), which could lead to disruption and dislodging of atherosclerotic plaques. This study examined the broad effects of acute NTG exposure on the expression and activity of genes encoding MMP-9, as well as an array of ECM and adhesion molecules in THP-1 human macrophages. Gene array studies identified that while NTG exposure (100 μM, 48 h) did not significantly increase MMP-9 gene expression, genes encoding testican-1, integrin α-1, thrombospondin-3, fibronectin-1 and MMP-26 were significantly down-regulated. On the other hand, genes encoding catenin β-1 and vascular cell-adhesion molecule-1 were up-regulated. Real-time PCR studies confirmed significant down-regulation of testican-1 gene expression, but its protein expression was not significantly altered. NTG exposure, caused a significant increase in total MMP-9 protein expression (1.96-fold) and active MMP-9 (3.7-fold) concentrations. Recombinant MMP-9 was significantly activated by NTG and its dinitrate metabolites, indicating post-translation modification of this protein by organic nitrates. These results indicate that NTG exposure could broadly affect the gene expression and activity of proteases that govern the ECM cascade, thereby potentially altering atherosclerotic plaque stability.

Introduction

Nitroglycerin (NTG), a prototypical organic nitrate, has been widely used for over 130 years to treat patients with stable and unstable coronary syndromes [1]. Bioactivation of NTG is believed to release nitric oxide (NO) and/or related species, activating soluble guanylyl cyclase to produce cyclic guanosine monophosphate *in vivo* and resulting in vasodilation. The clinical benefits of short-term use of nitrates is undisputable [2]. However, continuous prolonged use of NTG produces pharmacological tolerance (see reviews [2,3]), as well neutral [4–7] or even negative clinical effects in patients with stable coronary artery disease [8–11]. A suggested mechanism for these negative outcomes was attributed to plaque destabilization through the activation of matrix metalloproteinases (MMPs) by macrophages, thus counterbalancing the beneficial effects of vasodilatation by these drugs [9,12].

MMPs are zinc-endopeptidases that regulate cell–matrix interactions and are critical for the breakdown and remodeling of the extracellular matrix, and are involved in many physiological processes such as development, wound healing, embryogenesis, repair of blood vessels, as well as pathological processes such as atherosclerotic plaque destabilization, tumor metastasis, infections, inflammation, arthritis, angiogenesis [13,14]. The activity of these tissue destructive enzymes is tightly regulated by gene expression, producing the latent forms of these enzymes and co-secretion of tissue inhibitors [13,15,16]. A balance between synthesis and degradation of the matrix components is critical for the stability of the atherosclerotic plaque. An increase in macrophage density increases the concentrations of MMPs, which weakens the fibrous cap of the plaque and may lead to plaque rupture [17].

MMP-9, the major MMP secreted by the macrophages in the vulnerable plaque region [14,18], has been linked to plaque rupture [19,20]. Structurally, MMP-9 consists of a prodomain, catalytic domain, hinge region and hemopexin domain. The conserved sequence, PRCGVPD, containing the so-called "cysteine switch" in the prodomain and the zinc binding motif in the catalytic domain, is the signature of MMP family [21]. This cysteine residue, at position-99, binds to a zinc atom to maintain the enzyme in its latent state [22]. Activation of proMMPs can be effected by several enzymatic/non-enzymatic mechanisms that reacts with this cysteine residue, leading to dissociation of the cysteine switch from the active zinc atom [21].

We have shown recently that NTG can cause significant cellular protein thiol oxidation, including *S*-glutathionylation [23]. Because of this pro-oxidant property, we hypothesize that NTG may alter the expression of MMPs and other ECM proteases and adhesion molecules. Here, we investigated the broad *in vitro* transcriptional

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effects of NTG on MMP-9 and other ECM proteases and adhesion molecules. Follow up studies to verify the mRNA changes of selected genes with real-time quantitative reverse transcriptase real-time PCR reactions (RTQ RT-PCR) were carried out. Further, the post-transcriptional effects of NTG on protein expression and activity of MMP-9 were examined.

Materials and methods

Materials

THP-1 cells were obtained from the American Type Culture Collection (Manassas, VA). RPMI medium with supplemented glutamine, fetal bovine serum (FBS), 0.05 mM 2-mercaptoethanol, nitrocellulose membranes, sterile phosphate buffered saline (PBS), 100 U/ml penicillin, 100 μg/ml streptomycin and TOPO cloning products were purchased from Invitrogen Corporation (Carlsbad, CA). NTG solution was obtained from American Reagent Laboratories Inc. (Shirley, NY). The sources of other reagents were: RNA extraction kit and SV Total RNA® isolation system from Promega (Madison, WI); Oligo GEArray® (OHS-013) from SABiosciences (Frederick, MD); PCR primers of β-actin, GAPDH, cyclophilin, MMP-9, MMP-2 and testican-1 from Operon (Huntsville, AL); DNA purification system from Promega (Madison, WI); recombinant human MMP-9 and testican-1, anti-human testican-1 and anti-mouse IgG-HRP antibody and quantikine human MMP-9 immunoassay kit from R&D system (Minneapolis, MN); Biotrak MMP-9 activity assay system from GE Healthcare (Piscataway, NJ); glyceryl 1,2- or 1,3-dinitrate (GDN) from Cerilliant (Round Rock, TX); ECL-enhanced chemiluminescence system from Pierce (Rockford, IL). All other chemicals were obtained from Sigma (St. Louis, MA).

THP-1 cell culture and differentiation into macrophages

THP-1 (human monocytic leukemia) cells were cultured in RPMI-1640 cell medium supplemented with glutamine 10% FBS, 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in an incubator with 5% CO_2. The cells were counted using trypan blue staining after centrifugation (2000*g* for 10 min at 4 °C) and aliquots of 1 ml (1×10^6 cells) cell suspension were placed into six well plates. Cell viability was ⩾95%, as determined by trypan blue staining. Cells were differentiated using phorbol 12-myristate 13-acetate (PMA) at a final concentration of 100 ng/ml over 24 h [24].

Nitroglycerin incubation

The medium was removed and each well was washed with sterile PBS. Fresh cell medium (1 ml) containing RPMI-1640 cell medium supplemented with glutamine, 0.2% FBS, 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin was added. NTG (final concentration 100 μM) or vehicle (30% propylene glycol v/v and 30% ethanol v/v) was added in respective wells (10 μl) and the plates were incubated at 37 °C for 48 h. The medium then was collected, centrifuged (2000*g* for 5 min at 4 °C) and stored at −80 °C for MMP-9 activity and protein determinations. The cells were then washed twice with 1 ml of cold and sterile PBS and lysed using a lysis buffer for total RNA extraction.

Total RNA isolation and gene array analysis

Total RNA isolation was carried out using SV Total RNA® isolation system, according to the recommended protocol. The integrity of the extracted RNA was determined by formaldehyde-gel electrophoresis and quantified spectrophotometrically at 260 nm. The samples were stored at −80 °C until the microarray assay by Oligo GEArray® (OHS-013) which contained 118 genes encoding proteins that play key roles in mediating cell–cell, cell–tissue and cell–extracellular matrix interactions, housekeeping genes including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-2-microglobulin, β-heat shock 90 kDa protein-1 and β-actin and biotinylated artificial sequence-2 control for chemiluminescent detection. Hybridizations of labeled cRNAs to arrays were conducted according to the manufacturer's protocols. Briefly, the total RNA from each treatment was first converted to cDNA, followed by cRNA synthesis, labeling and amplification (True Labeling-AMP™ 2.0). The arrays were hybridized overnight and a chemiluminescent detection kit was used to detect the gene signal. Image acquisition was performed using a Kodak Imager 2000MM (Carestream Health Molecular Imaging, New Haven, CT) and analyzed with software Kodak ID (ver. 3.6.3).

Microarray data analysis

A gene-stability index [25] was used to determine the expression stability of the housekeeping genes. The principle behind this index is that ideally the expression levels of control genes should remain unchanged irrespective of the exposure or type of cell. The stability of total array intensity and the geometric mean of the four housekeeping genes, as a normalizing factor, was also tested using this index. The internal control gene-stability index is defined as the "average pairwise variation of a particular gene with all other control genes" [25]. Thus, a lower gene-stability index indicates more stable expression. Unpaired *t*-test was not used because of its low statistical power and assumption of normality and equal variance. In addition, to assess the false discovery rate, a non-parametric approach based on resampling techniques, the permutation adjusted *t*-test (PATS), was applied to the gene expression data [26]. The resampling method is a statistical approach that identifies all possible outcomes within the same empirical data set via repeated sampling. The observed test statistics are then compared against the test statistics from all possible data sets. This approach has been used by several groups [26–29]. In this study, there were 252 possible permutations of the expression data using five replicates for each treatment group (10!/5!∗5! = 252). However, only 126 unique permutations of the data sets were considered since the other 126 permutations are mirror permutations with equal magnitude but with a negative sign. The changes in gene expression were considered significant if the given *t*-statistic from the data set was the highest value amongst the *t*-statistic distribution from all possible permutated data sets.

Real-time PCR

RTQ RT-PCR was performed on the house keeping genes; β-actin, GAPDH, cyclophilin and the genes of interest; MMP-9, MMP-2 and testican-1, to verify the results of the gene array study. The PCR primers and reaction conditions are listed in Table 1. Total RNA was reverse-transcribed at 65 °C for 5 min, at room temperature for 10 min, 42 °C for 60 min and 90 °C for 5 min. RTQ RT-PCR was performed using Stratagene's MX4000 quantitative PCR system. Each PCR reaction contained 5 μl of cDNA and 45 μl of master mix containing 0.5 μl each of SYBR green (1/500 dilution) and rhodamine X (1/750 dilution). Amplification was carried out at 95 °C for 4 min, 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min by repeating the complete cycle 40 times. PCR products of all genes were cloned using pCR®2.1-TOPO® vector, into one shot® *E. coli* cells. Plasmids with PCR inserts were extracted using Wizard plus SV minipreps DNA purification system, quantified spectrometrical-