Nitroglycerin (NTG) is one of the most frequently used therapeutic agents for the symptomatic relief of stable or unstable coronary artery disease (CAD), having first been used for this purpose in 1879 (1). Nitroglycerin releases nitric oxide (NO) within smooth muscle cells, leading to vasorelaxation. Despite this, there have been no reports that NTG attenuates the progression of atherosclerosis or provides prognostic benefit in subjects with coronary disease; in fact, in acute coronary syndromes, nitrates do not improve outcomes in coronary disease. We now describe evidence that NTG has potentially deleterious effects on plaque stability.

Atherosclerosis is characterized by the formation of arterial plaques consisting of a lipid core with a fibrous cap. The mechanical strength of the plaque cap is a vital component of plaque stability and depends (in part) on the amount of matrix proteins (7). The cap tissue is dynamic, with synthesis of matrix proteins by cells being balanced by degradation of the matrix. A range of proteases, including the metalloproteinases, performs matrix degradation. Metalloproteinases are multidomain, zinc-containing neutral endopeptidases and include the collagenases, stromelysins, gelatinases and membrane-type metalloproteinases (7). The major matrix metalloproteinase (MMP) expressed by macrophage is MMP-9. Nitroglycerin treatment stimulated a dose-dependent increase in MMP-9 mRNA levels (NTG 200 pmol: 193 ± 6% and NTG 2,000 pmol: 372 ± 9% compared to controls, p < 0.005) and MMP-9 activity (NTG 200: 142 ± 5.3% and NTG 2,000: 167 ± 11% compared to controls, p < 0.005). Nitroglycerin 2,000 pmol also increased MMP-2 and MMP-7 mRNA levels to 187 ± 8% and 183 ± 21% of control values, respectively (p < 0.05). Furthermore, tissue inhibitor of metalloproteinase (TIMP)-1 (the major tissue inhibitor of MMPs) mRNA and protein levels were decreased in NTG 2,000 pmol-treated MDMs compared with control cells (mRNA: 67 ± 7%, p < 0.005; protein: 45 ± 5%, p < 0.005).

Nitroglycerin in pharmacologically relevant concentrations activates MMP but represses TIMP expression in human macrophages. The subsequent imbalance in MMP/TIMP expression associated with NTG treatment could promote matrix degradation, with potentially adverse effects on plaque stability.

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Flow centrifugation elutriation at 20°C (Nycomed Pharma, Asker, Norway) followed by counter-gradient separation of the white cells on Lymphoprep in peripheral blood of healthy human volunteers and monocytes.

Isolation of human monocytes and culture of human macrophages. Macrophages are powerful enzymes, and as such their activity is tightly regulated. Regulation occurs at several levels, including gene expression, secretion of pro-enzymes that require activation and inhibition by tissue inhibitors of metalloproteinases (TIMPs), chiefly TIMP-1 (7). Metalloproteinases are not constitutively expressed in most cell types; rather, gene transcription is induced by a variety of factors including growth factors and cytokines. Importantly, MMP expression and activity can be modulated by reactive oxygen species and NO (11). Therefore, we investigated whether NTG affected MMP and TIMP levels in human MDMs.

**METHODS**

Isolation of human monocytes and culture of human MDMs. White cell concentrates were obtained from the peripheral blood of healthy human volunteers and monocytes were removed within 24 h of collection by density gradient separation of the white cells on Lymphoprep (Nycomed Pharma, Asker, Norway) followed by counter-flow centrifugation elutriation at 20°C, as previously described by our group (12), by use of a Beckman J2-21 M/E centrifuge equipped with a JE-6B elutriation rotor and a 4.2 ml elutriation chamber (Beckman Coulter Inc., Fullerton, California). The elutriation buffer was Hanks buffered saline solution without calcium or magnesium (Sigma-Aldrich, Castle Hill, NSW, Australia), supplemented with ethylenediaminetetra-acetic acid (0.1 g/l) and 1% heat-inactivated human serum. The system and tubing were inactivated human serum. The system and tubing were ethylenediaminetetra-acetic acid (0.1 g/l) and 1% heat-inactivated human serum. After priming, the system was allowed to adhere for 1.5 h at 37°C under 5% CO2 in air. The medium was then removed and the adherent monocytes washed twice gently with 1X phosphate-buffered saline before adding RPMI containing 10% human serum, penicillin G (50 U/ml) and streptomycin (50 fg/ml). Media changes occurred every two to three days. On days 8 to 10, cells were treated for 4 or 24 h with NTG (200 and 2,000 pmol; Saphire Bioscience Pty Ltd., Sydney, Australia), 3-morpholinosydnoimine hydrochloride (2,000 pmol; Saphire Bioscience Pty Ltd.), S-nitroso-N-acetylpenicillamine (2,000 pmol; Saphire Bioscience Pty Ltd.) or glycerol (GLY) (200 and 2,000 pmol). Untreated cells acted as controls. The concentrations of NTG were chosen to span the range of concentrations usually seen in vivo in plasma, after NTG administration to humans by the sublingual, transdermal or intravenous routes for the treatment of CAD (13–16). Each experiment was performed at least three times, with triplicate wells for each condition.

**RNA isolation and competitive reverse transcription-polymerase chain reaction (RT-PCR) protocol.** Total ribonucleic acid (RNA) was extracted using TRI reagent (Sigma), normalized to 250 ng/µl by the SYBR Green II nucleic acid stain assay (17) and stored at −70°C. Competitive RT-PCR was performed on the total RNA samples. The protocol is based on multitemplate RNA competitor molecules and has previously been described by our group (18). The competitive RT-PCR protocol involved c-deoxyribonucleic acid synthesis in 10 µl volumes containing 250 ng of total RNA and 1.2 serial dilutions of the RNA competitor molecule in standard Superscript II RNaseH reverse transcriptase reactions (Life Technologies). The reactions were incubated at 25°C for 5 min, 50°C for 30 min and 94°C for 2 min. Polymerase chain reaction amplification was performed in 25 µl reaction volumes containing 10% of the reverse transcription reaction, 1X polymerase chain reaction buffer (PE Biosystems, Foster City, California), 0.2 mM deoxynucleotide triphosphate (Boehringer Mannheim Australia, Castle Hill, Australia), 20 pmol of sense and antisense primers and 2.5 U Taq polymerase (PE Biosystems). The temperature cycling profile was as follows: 94°C for 15 s, 55°C for 20 s, 72°C for 45 s; this profile was cycled 25 times for beta-actin, 30 times for TIMP-1 and MMP-9 and 35 times for MMP-7 and MMP-2 in a Perkin-Elmer 9600 thermal cycler (Wellesley, Massachusetts). Primer sequences for MMP-2 were sense

<table>
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<tr>
<th>Abbreviations and Acronyms</th>
<th>Description</th>
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<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>APMA</td>
<td>p-aminophenyl mercuric acetate</td>
</tr>
<tr>
<td>CAD</td>
<td>coronary artery disease</td>
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<td>GLY</td>
<td>glycerol</td>
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<td>MDM</td>
<td>monocyte-derived macrophage</td>
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<td>MMP</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<td>NF-κB</td>
<td>nuclear factor-kappa B</td>
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<td>NO</td>
<td>nitric oxide</td>
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<td>NTG</td>
<td>nitroglycerin</td>
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<td>RNA</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>RT-PCR</td>
<td>reverse transcription-polymerase chain reaction</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gels</td>
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<td>TIMP</td>
<td>tissue inhibitors of metalloproteinase</td>
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5′ CGC GTG CGC CCA TCA TCA GT and antisense 5′ TGG ATT CGA GAA AAC CGC AGT GG (19). The sequences for MMP-7 were sense 5′ AGA TGT GGA GTG CCA GAT GT and antisense 5′ TAG ACT GCT ACC ATC CGT CC (20). The MMP-9 and TIMP-1 sequences have been described by our group (18).

Polymerase chain reaction products were separated on 2% agarose gels containing 50 ng/ml ethidium bromide and the image was directly digitized (Grab-It; UVPLtd., Cambridge, United Kingdom). The band densities were measured using Phoretix software (Phoretix International, Newcastle Upon Tyne, United Kingdom) and normalized on the basis of the molecular weight of the product (21). The log of the ratio of the band densities within each lane was plotted against the log of the concentration of RNA competitor molecule added per reaction. The concentration of the target messenger ribonucleic acid (mRNA) was determined where the ratio of the competitor and target band densities was equal to 1.

**TIMP-1 protein concentrations.** TIMP-1 protein concentrations in the conditioned media were determined by commercially available enzyme-linked immunosorbent assays (Amersham Pharmacia Biotech, Castle Hill, Sydney, Australia).

**Zymography.** Sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) containing gelatin (1 mg/ml) were used to demonstrate gelatinolytic activity (22) in conditioned media from MDM cell cultures. Conditioned media was normalized to 15 μg or 4.5 μg total protein concentration before electrophoresis. Dilution to 4.5 μg was necessary to obtain optimal band resolution for densitometric analysis. Total protein concentration was determined by a commercially available BCA method (BioRad, Sydney, Australia). For electrophoresis, 20 μl of sample was loaded onto 8 × 10 cm (SDS-PAGE) containing 1 mg/ml of gelatin under nonreducing conditions. The gels were run at 100 V for 45 min. Molecular weight standard proteins (BioRad, Regents Park, NSW, Australia) were run simultaneously. After SDS-PAGE, the gels were washed twice in 2.5% Triton X-100 (2 × 15 min) to remove sodium dodecyl sulfate from the gel and then incubated for 4 h at 37°C in 50 mM Tris-HCl, pH 7.4, containing 10 mM calcium chloride and 0.05% Brij 35 (Sigma). After incubation, the gels were stained in a solution of 0.25% Coomassie blue in methanol:acetic acid:water (30:10:60, v/v/v) and then destained in methanol:acetic acid:water (30:10:60, v/v/v) until clear bands were observed against the blue background of stained gelatin. The gels were digitized directly (Grab-It software, UVPLtd., Cambridge, United Kingdom), and the intensity of the bands was measured using Phoretix software (Phoretix International).

It has been demonstrated that activation of MMPs can be elicited by p-aminophenyl mercuric acetate (APMA) treatment. To see fully active MMP-9, aliquots of some samples were incubated with APMA (1 mM, 37°C, 30 min) to cleave the proenzyme for maximal MMP zymographic activity. After activation, the samples were subjected to zymographic analysis as previously described.

**Data analysis.** Results of the experimental studies are reported as mean ± SEM compared with control conditions. Analysis of variance was used for statistical analysis with Scheffe’s test for post-hoc pairwise comparison. A p value of <0.05 was regarded as significant.

**RESULTS**

**Effect of NTG exposure on MMP-9 protein levels in human MDMs.** Zymographic analysis of conditioned media from cultured human MDMs showed that the major gelatinase secreted by the human MDMs was MMP-9 (Fig. 1A; clear band at ~90 kDa). When 15 μg total protein was analyzed by zymography, both the latent (heavier band) and active (lighter band) forms of MMP-9 were observed. No MMP-2 activity (clear band at ~70 kDa) could be detected. Macrophages exposed to NTG 200 pmol for 24 h (Fig. 1A; Lanes 3 and 4) showed more latent and active MMP-9 activity then cells exposed to equimolar concentrations of GLY (Lanes 1 and 2).

Figure 1B shows recruitable MMP-9 activity present in the MDM samples. These samples were activated in vitro via APMA treatment, and the large clear bands indicate there is abundant MMP-9 activity present in these samples. Incubation of MDMs with NTG 200 pmol for 24 h increased recruitable MMP-9 activity compared to GLY-treated controls (Lanes 3 and 4 vs. Lanes 1 and 2; Fig. 1B).

To quantify differences in MMP-9 levels, samples were diluted to 4.5 μg and cells were exposed to NTG for only 4 h in order to obtain optimal band resolution. Figure 2 shows that incubation of MDMs with NTG (200 and 2,000 pmol) increased the intensity of the MMP-9 band, migrating at ~90 kDa (NTG 200, 142 ± 5.5%; NTG 2,000, 167 ± 11% compared to control, 100 ± 1.34%; p < 0.005 or p < 0.001, respectively). Administration of other NO donors, SIN-1 (200 pmol) and SNAP (200 pmol), also resulted in an increase in MMP-9 activity (134 ± 6% and 123 ± 5.4%, p < 0.05 and NS, respectively). Treatment of cells with GLY (same chemical background as NTG) did not increase MMP-9 activity (gly 200, 79 ± 8.3%; gly 2,000, 102 ± 6.5% compared to control, p = NS).

**NTG increases MMP-9 mRNA levels in human MDMs.** Matrix metalloproteinase-9 mRNA levels were measured by competitive RT-PCR using a MMP-9 specific heterologous RNA template (18). This assay showed that a 4-h exposure of MDMs to 200– or 2,000– pmol NTG increased MMP-9 mRNA levels in a concentration dependent manner. Figure 3 shows NTG 200 pmol increased MMP-9 mRNA levels to 193 ± 6% of control levels (100 ± 7.5%, p < 0.0005). Nitroglycerin 2,000 pmol caused a further induction to 372 ± 9% (p < 0.0001).

**NTG increases MMP-2 and MMP-7 mRNA levels in human MDMs.** To determine whether NTG induced other MMPs at the mRNA level in human MDMs,
levels increased by 187 pmol resulted in a more dramatic increase, with MMP-2 by 148 pmol.

Figure 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) zymography of media conditioned by human monocyte-derived macrophages after 24-h exposure to nitroglycerin (NTG). (a) Cells were exposed for 24 h to glycerol 200 pmol (Lanes 1 and 2) or NTG 200 pmol (Lanes 3 and 4). Aliquots of conditioned media, normalized to 15 µg total protein, were run on SDS-PAGE (10% w/v gel) supplemented with 1 mg/ml gelatin substrate under nonreducing conditions. Zones of clearing at approximately 90 kDa (as measured by simultaneous electrophoresis of protein standards, Lane 5) indicate gelatinolytic activity of both latent (heavier) and active (lighter) matrix metalloproteinase (MMP)-9. Band densities are: Lanes 1 and 2, glycerol (latent 162,160; 167,492: active 176,945; 173,336) and Lanes 3 and 4, NTG 200 pmol (latent 29,515; 22,223: active 39,664; 31,960). (b) Aliquots of conditioned media, normalized to 15 µg total protein, were treated with 1 mM p-aminophenyl mercuric acetate (APMA) for 30 min at 37°C. Treated samples were then run on SDS-PAGE (10% w/v gel) supplemented with 1 mg/ml gelatin substrate under nonreducing conditions. Zones of clearing at approximately 90 kDa (as measured by simultaneous electrophoresis of protein standards, Lanes 3 and 4) indicate gelatinolytic activity of both latent and active MMP-9. Band densities are: Lanes 1 and 2, glycerol (active 269,957; 276,765) and Lanes 3 and 4, NTG 200 pmol (active 321,321; 302,633). MW = molecular weight.

MMP-2 and MMP-7 mRNA levels were also measured by competitive RT-PCR. As seen with MMP-9, NTG treatment led to a dose-dependent increase in MMP-2 and MMP-7 mRNA levels (Fig. 4). Nitroglycerin 200 pmol increased MMP-2 levels by 135 ± 12% and MMP-7 levels by 148 ± 12.5% relative to control. Nitroglycerin 2,000 pmol resulted in a more dramatic increase, with MMP-2 levels increased by 187 ± 8% and MMP-7 levels up by 183 ± 21%, p < 0.005 and p < 0.05, respectively. Importantly, 35 cycles of PCR were required to measure the mRNA levels of both MMP-2 and -7 compared with only 30 cycles for MMP-9 and TIMP-1. This indicated that expression of MMP-2 and MMP-7 in human MDMs is relatively low, as was further demonstrated by an absence of observable MMP-2 activity using gelatin zymography (Fig. 1).

NTG reduces TIMP-1 protein and mRNA levels in human MDMs. To determine whether NTG altered expression of a key TIMP, TIMP-1, TIMP-1 mRNA and protein levels were measured in human MDMs treated with NTG 200 pmol and NTG 2,000 pmol. Nitroglycerin caused a dose-dependent decrease in TIMP-1 mRNA (200 pmol, 85 ± 1%; 2,000 pmol, 67 ± 7%; p < 0.005) and protein expression (200 pmol, 60 ± 5%; 2,000 pmol, 45 ± 5%; p < 0.005), relative to controls (100 ± 11%; Fig. 5).

DISCUSSION

This study was undertaken to investigate the effects of NTG, a commonly used drug in the treatment of obstructive coronary disease, on MMP and TIMP expression in human macrophages. We found that when MDMs were exposed to standard pharmacologic doses of NTG, the agent exerted a striking reciprocal effect on the balance between MMP and TIMP expression, with MMP-9, -2 and -7 expression enhanced and TIMP-1 expression significantly inhibited. These changes are consistent with a pro-inflammatory effect via enhanced matrix degradation.

NTG therapy and mortality in acute coronary syndromes. Overall, nitrates do not appear to alter outcomes in acute coronary syndromes, despite their clear benefits in terms of reducing myocardial preload and dilating coronary arteries (1). Most early studies of intravenous NTG in this setting have been small and uncontrolled (23,24) and initially no randomized controlled trials addressed the efficacy of intravenous NTG for symptom relief or control of cardiac events (25). The recent publications of the large GISSI-3 and ISIS-4 trials, however, involving more than 75,000 randomized patients, clearly show no benefit of oral or transdermal nitrate therapy on mortality in subjects with acute coronary syndromes (2,3). This raises the possibility of potentially deleterious effects of nitrates in this setting, to counterbalance the benefits of coronary dilation. One such possibility is an adverse effect on plaque stability, via NTG-related changes in vascular biology.

NTG enhances MMP expression and activity. In this study, NTG enhanced MMP activity and downregulated TIMP protein expression, via effects at the mRNA levels. These opposing effects of NTG on MMP and TIMP expression effectively amplify the degradation arm of the MMP/TIMP system, which in turn could lead to net breakdown of extracellular matrix in atherosclerotic plaques. Nitroglycerin treatment induced a two- to fourfold increase in MMP-9 expression and activity. Twofold increases in MMP activity have been associated with several cardiovascular disease states, including abdominal aortic aneurysm (26) and dilated cardiomyopathy (27). In a recent study, raloxifene (a selective estrogen receptor-modulating drug) was shown to induce a twofold increase in monocyte...
MMP-1 production, and patients treated with raloxifene showed higher peripheral blood monocyte MMP-1 levels; results interpreted as potentially predisposing to plaque instability (28). Our current results have shown that NTG also induces significant basal and recruitable MMP-9 activity. Although the precise mechanism(s) for the in vivo activation of MMP-9 is not known, MMP-9 is activated by plasmin and trypsin, both of which are found in the atherosclerotic plaque. Thus, increased MMP production by macrophages induced by NTG treatment may have important consequences in the most vulnerable regions of atherosclerotic plaques, where MDMs are known to accumulate preferentially (5).

**NTG regulation of MMP gene expression.** The signaling pathways mediating NTG regulation of MMP gene expression are not completely understood; however, MMP gene expression occurs under tightly regulated mechanisms that include changes in transcription (8). Analysis of the MMP/TIMP promoters has identified essential response elements including activator protein-1 (AP-1) elements, multiple Ets elements and, for MMP-9, a nuclear factor-kappa B (NF-κB) element (29). Regulation of MMP or TIMP gene expression involves interplay between all of the transcription factors that bind these response elements (30). Nitric oxide-generating compounds, including SNAP and SIN-1, have been shown to cause a dose-dependent increase in NF-κB activation in macrophages, which was positively correlated with the level of NO production (31). Similarly, NO has been shown to induce AP-1 deoxyribonucleic acid binding activities in many cell types (32). Thus, the NO

![Figure 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) zymography of media conditioned by human monocyte-derived macrophages exposed to nitroglycerin (NTG) for 4 h. (a) Cells were exposed for 4 h to control media (Lanes 1 and 2), glycerol (GLY) 200 pmol (Lanes 3 and 4), GLY 2,000 pmol (Lanes 5 and 6), NTG 200 pmol (Lanes 7 and 8), NTG 2,000 pmol (Lanes 9 and 10), 3-morpholinosydnoimine hydrochloride 200 pmol (Lanes 11 and 12) or S-nitroso-N-acetylpenicillamine 200 pmol (Lanes 13 and 14). Aliquots of conditioned media, normalized to 4.5 μg total protein, were run on SDS-PAGE (10%[w/v]gel) supplemented with 1 mg/ml gelatin substrate under nonreducing conditions. Zones of clearing indicate gelatinolytic activity of latent matrix metalloproteinase (MMP)-9. (b) Scanning analysis of MMP-9 secreted by human MDMs. Results represent the mean ± SEM for analysis of four independent experiments and do not represent the analysis in (a) alone. *p < 0.05 compared with control cells. **p < 0.005 compared with control cells. ***p < 0.0001 compared to control cells.
donor, NTG, may regulate MMP and TIMP gene expression via activation of AP-1 and/or NF-κB. This study showed that not only MMP-9 expression was upregulated in this study but also two other MMPs were upregulated, namely MMP-2 and MMP-7. Although the low level of expression indicates that these findings may not be of pathophysiologic importance, they demonstrate that NTG upregulates all measurable MMP expression. It is of importance that the promoter region of MMP-2 is completely different to the other MMPs and does not contain AP-1, Ets or NF-κB sites (30). Rather, an AP-2 site predominantly regulates MMP-2 expression (30). It is not known whether NTG increases AP-2 deoxyribonucleic acid bind-

Figure 3. The effect of nitroglycerin (NTG) on matrix metalloproteinase (MMP)-9 messenger ribonucleic acid (mRNA) levels in human monocyte-derived macrophages (MDMs). Cells were exposed to control (CTRL) media (a), NTG 200 pmol (b) or NTG 2,000 pmol (c) for 4 h. Matrix metalloproteinase-9 mRNA levels were measured by competitive reverse transcription-polymerase chain reaction (described in Methods). Competitor mRNA concentrations were 8.56 (Lane 1), 17.13 (Lane 2), 34.25 (Lane 3), 68.5 (Lane 4) and 137 (Lane 5) attomoles. Arrows represent MMP-9 target (lower band) versus competitor (upper band) equivalence points for each condition. (d) Quantification of RT-PCR bands was determined by gel densitometry. Results represent the mean ± SEM for analysis of four independent experiments and do not represent the analysis in (a to c) alone. *p < 0.005 compared with control cells.

Figure 4. The effect of nitroglycerin (NTG) on matrix metalloproteinase (MMP)-2 and MMP-7 messenger ribonucleic acid (mRNA) levels in human monocyte-derived macrophages (MDMs). Cells were exposed to control media, NTG 200 pmol or NTG 2,000 pmol for 4 h. Total ribonucleic acid was extracted and competitive reverse transcription-polymerase chain reaction (RT-PCR) was performed to measure MMP-2 (solid bars) or MMP-7 (open bars) as described in the Methods section, and each PCR reaction mix was subjected to electrophoresis on a 2% (w/v) agarose gel. Quantification of RT-PCR bands was determined by gel densitometry. Results represent the mean ± SEM for analysis of three independent experiments. *p < 0.05 and **p < 0.005 compared with control cells.

Figure 5. The effect of nitroglycerin (NTG) on tissue inhibitor of metalloproteinase (TIMP)-1 expression in human monocyte-derived macrophages. Monocyte-derived macrophages were exposed to control media, NTG 200 pmol or NTG 2,000 pmol for 4 h. Ribonucleic acid was extracted and competitive reverse transcription-polymerase chain reaction performed to measure TIMP-1 messenger ribonucleic acid (mRNA) levels (solid bars). Y-axis represents mRNA levels as % of control. Aliquots of conditioned media were collected and TIMP-1 protein levels measured by enzyme-linked immunosorbent assay (open bars). Y-axis represents protein concentration (ng/ml). Results represent the mean ± SEM for analysis of three independent experiments. **p < 0.005 compared with control cells.
ing activities. Further investigation of the interactions between these regulatory sites in response to NTG is required to provide insights into the mechanisms that control NTG regulation of the MMP/TIMP genes.

**NO per se upregulates MMP gene expression.** As part of our investigation, we used three different NO donors, including two classical direct NO donors, SIN-1 and SNAP, and one indirect NO donor, NTG, for their potencies to generate NO and alter MMP/TIMP expression. All three of the NO donors were effective in altering MMP/TIMP levels, suggesting it was not the bioactivation pathway required for NO formation from NTG that led to the observed effects. Additionally, both NTG and SIN-1, but not SNAP, treatment can lead to reactive oxygen species (ROS) formation (superoxide radicals and peroxynitrite). Nitric oxide then reacts with ROS, and together they are much more active than either ROS or NO alone in modifying and changing the expression and functions of several proteins including MMPs. Indeed, NTG’s effects depend (in part) on the specific balance between the concentrations of NO and ROS formation (33,34). Our finding that all three NO donors in similar concentrations were similarly effective in altering MMP/TIMP levels is consistent with the hypothesis that NO per se appears to be the primary radical inducing the changes in MMP/TIMP expression in macrophages. It is of interest that Rajagopalan et al. (11) could not demonstrate that NO stimulated MMP-2 activity in rabbit lipid-laden macrophages. We could not measure MMP-2 activity in our cell media after 4- or 24-h incubation times. MMP-9 was by far the dominant MMP expressed by human macrophages, and the difference between our results in human cells and the previous animal study may reflect species differences in MMP expression.

**Study limitations.** This investigation suggests that NTG could increase MDM-derived MMP activity in the atherosclerotic plaque. Importantly, the MMP-9 proenzyme is also constitutively expressed at high levels by monocytes and macrophages in fatty streaks (35), long before any question of instability of plaque arises, and thus NTG treatment leading to activation of MMP-9 may also influence plaque development. However, whether we can extend our findings from ex vivo healthy MDMs to those cells harbored in atherosclerotic plaques remains a limitation of this study. Although NO donors may have beneficial effects on some other aspects of vascular biology, such as smooth muscle relaxation or antiadhesive properties for the endothelium (36), the current data suggest potentially deleterious effects, by contrast, as regards MMP production by human macrophages.

**Summary.** In conclusion, NTG in clinically relevant concentrations induces MMP, and reduces TIMP-1, expression by human MDMs. The results reveal a new, heretofore unappreciated action of nitrates that may need to be considered when treating CAD. These results suggest the possibility that MMP inhibition, particularly that targeted towards MMP-9, may be a novel therapeutic target in the management of CAD, which frequently requires long-term administration of NTG. In addition, the vascular biological observations in this study may help explain the lack of a positive association between NTG therapy and beneficial effects on plaque progression or coronary event rates, despite NTG’s powerful vasodilator activity.

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