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ORIGINAL ARTICLE

Sodium nitrite attenuates MMP-9 production by endothelial cells and may explain similar effects of atorvastatin

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Abstract Imbalanced matrix metalloproteinase (MMP) activity promotes cardiovascular alterations that are attenuated by statins. These drugs exert pleiotropic effects independent of cholesterol concentrations, including upregulation of nitric oxide (NO) formation and MMP downregulation. However, statins also increase tissue concentrations of nitrites, which activate new signaling pathways independent of NO. We examined whether atorvastatin attenuates MMP-9 production by human umbilical vein endothelial cells (HUVEC) stimulated with phorbol 12-myristate 13-acetate (PMA) by mechanisms possibly involving increased nitrite, and whether this effect results of NO formation. We also examined whether such an effect is improved by sildenafil, an inhibitor of phosphodiesterase-5 which potentiates NO-induced increases in cyclic GMP. MMP activity and nitrite concentrations were measured by gelatin zymography and ozone-based reductive chemiluminescence, respectively, in the conditioned medium of HUVECs incubated for 24 h with these drugs. Phospho-NFκB p65 concentrations were measured in cell lysate to assess NFκB activation. Atorvastatin attenuated PMA-induced MMP-9 gelatinolytic activity by mechanisms not involving NO, although it increased nitrite concentrations, whereas sildenafil had no effects. Combining both drugs showed no improved responses compared to atorvastatin alone. While sodium nitrite attenuated MMP-9 production by HUVECs, adding hemoglobin (NO scavenger) did not affect the responses to nitrite. Neither atorvastatin nor nitrite inhibited PMA-induced increases in phospho-NFκB p65 concentrations. These findings show that sodium nitrite attenuates MMP-9 production by endothelial cells and may explain similar effects exerted by atorvastatin. With both drugs, the inhibitory effects on MMP-9 production are not dependent on NO formation or on inhibition of NFκB activation. Our findings may help to elucidate important new nitrite-mediated mechanisms by which statins affect imbalanced MMP activity in a variety of cardiovascular disease.

Keywords Endothelial cells · Nitric oxide · Nitrite · Matrix metalloproteinase-9

Introduction

Matrix metalloproteinases (MMPs) are zinc-dependent enzymes critically involved in the pathogenesis of cardiovascular diseases (Yabluchanskiy et al. 2013; Castro et al. 2009a), and MMP inhibition or downregulation has been shown as a relevant strategy to reverse both functional and structural alterations commonly affecting the cardiovascular system (Castro et al. 2009b; Castro and Tanus-Santos 2013). For example, increased MMP-9 activity has been implicated in atherosclerotic plaque instability (Kunte et al. 2008) and an important pharmacological strategy to improve vascular health is by using statins, which are improve vascular function and reverse atherosclerotic lesions by mechanisms that involve downregulation of MMP-9 (Kunte et al. 2008; Souza-Costa et al. 2007a; Souza-Costa et al. 2007b).

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Statins exert a variety of pleiotropic effects independent of lowering cholesterol concentrations, including upregulation of endothelial nitric oxide (NO) expression and antioxidant effects (Davignon 2004), which may result in increased NO activity. This is very important because NO-donor drugs were previously shown to inhibit MMP-9 production by endothelial cells (Meschiari et al. 2013) and therefore may explain how treatment with statins helps to improve vascular health. However, although NO-donor drugs inhibit MMP-9 production by endothelial cells (Meschiari et al. 2013) and statins increase NO activity (Davignon 2004), it is not clear whether statins attenuate MMP-9 release by stimulated endothelial cells by mechanisms directly related to NO. In fact, statins increase tissue concentrations of NO oxidation products including nitrites (Guimaraes et al. 2013) and nitrates (Castro et al. 2004), and although nitrates are more stable and inert products of NO, nitrites may well activate new signaling pathways independent of NO and soluble guanylate cyclase and result in cardiovascular protective effects (Mo et al. 2012). This study showing that nitrite may exert direct effects independent of NO (Mo et al. 2012) supports the idea that this major metabolite of NO may activate a variety of mechanisms unstudied until now, which may add much complexity to the already known mechanisms implicated in the responses to NO including posttranslational protein modifications such as nitrosylation and nitration, or the direct scavenging of other radicals by NO(Toledo and Augusto 2012). Therefore, it is possible that statins exert protective effects as a result of increased nitrite concentrations.

In this study, we examined whether atorvastatin attenuates MMP-9 production by endothelial cells stimulated with phorbol 12-myristate 13-acetate (PMA) by mechanisms involving increased nitrite concentrations and whether this effect results of NO formation. Moreover, we examined whether such an effect is improved by sildenafil, an inhibitor of phosphodiesterase-5 (PDE5) which potentiates NO-induced increases in cyclic GMP (Castro et al. 2004). If nitrite mediates the effects of atorvastatin on MMP-9 production independent of NO formation, treatment with sildenafil would not improve the responses to atorvastatin.

Methods

Drugs and reagents

Atorvastatin calcium salt trihydrate, sildenafil citrate salt, sodium nitrite, PMA, dimethylsulfoxide (DMSO), bovine serum albumin (BSA), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). Modified Dulbecco's modified Eagle medium (DMEM) without phenol red (containing 6 mmol/L L-glutamine and 4.5 g/L D-glucose, and iron (III) sulfate instead of iron (III) nitrate) were purchased from Cultilab (Sao Paulo, Brazil).

Human umbilical vein endothelial cell culture

Human umbilical vein endothelial cell (HUVEC) line was purchased from ATCC (CRL-2873, Manassas, VA, USA) and maintained as previously described (Meschiari et al. 2013). Cells were grown in modified DMEM supplemented with 15 % fetal bovine serum (FBS, Cultilab, Sao Paulo, Brazil), at 37 °C in a 5 % CO_2-humidified atmosphere incubator. The cells were confluent on the day of treatment. All experiments were performed between passages 2 and 5 after unfreezing.

Cell viability determination

Drug treatment toxicity was assessed by using the MTT method (Liu et al. 2009). In 96-well microplates, 8000 cells/well were plated and grown in 100 μL DMEM + FBS for 24 h. Then, the medium was replaced with serum-free DMEM supplemented with 0.1 % BSA (starvation medium), and the cells were incubated for 24 h. A large range of drug concentrations was analyzed for cell viability after incubation in starvation medium for 24 h. Then, the medium was replaced by 200 μL of fresh starvation medium plus 10 μL MTT solution (1 mg/mL). The microplates were incubated at 37 °C in a 5 % CO_2-humidified atmosphere at the incubator for 4 h and centrifuged at 4000*g* for 5 min. The supernatant was discarded, and the precipitated formazan was dissolved in 100 μL DMSO. The optical density of the samples was measured at 492 nm (μQuant, Bio-Tek Instruments Inc., Winooski, VT, USA). HUVEC treated with vehicle (and untreated cells) were used as controls.

Assessment of the effects of atorvastatin and sildenafil on PMA-stimulated MMP-9 production by HUVEC

HUVEC were plated in 24-well plates (48,000 cells/well) with 300 μL DMEM + FBS for 24 h followed by 24 h in starvation medium. The cells were treated with starvation medium with the vehicle (control, untreated cells) or with 10 nmol/L PMA (a well-known MMP-9 inducer) or 10 nmol/L PMA combined with one of the following drugs: atorvastatin (1 or 10 μmol/L) or sildenafil (1 or 10 μmol/L). All treatments were carried out for 24 h (unless otherwise described). Conditioned medium and cell lysates were collected and stored at −80 °C until used in biochemical assays. The concentrations of atorvastatin and sildenafil used were chosen with basis on those reported in previous studies showing significant effects of atorvastatin on MMP production by endothelial cells (Izidoro-Toledo et al. 2011) or on the circulating concentrations of sildenafil achieve when it is used in patients (Bruton et al. 2006).

Measurement of MMP-9 levels in the conditioned medium by SDS-polyacrilamide gel electrophoresis gelatin zymography

MMP-9 gelatinolytic activity was assessed in the conditioned medium by SDS-polyacrylamide gel electrophoresis (PAGE) gelatin zymography as described previously (Souza-Tarla et al. 2005). Briefly, conditioned medium samples were diluted in sample buffer (10 % SDS, 125 mmol/L Tris–HCl; pH 6.8, 20 % glycerol, and 0.01 % bromophenol blue) in a 1:5 ratio (*v*/*v*), and 20 μL of sample were subjected to electrophoresis on a 12 % SDS-PAGE copolymerized with gelatin (0.05 %) as the substrate. Next, the gels were washed twice with 2 % Triton X-100 solution for 30 min and incubated for 18 h on Tris $CaCl_2$ pH 7.4 buffer at 37 °C. Then, the gels were stained with 0.05 % Coomassie Brilliant Blue G-250 solution and destained with 25 % methanol and 7 % acetic acid solution. MMP-9 gelatinolytic activity was detected as unstained band against the background of the Coomassie blue-stained gelatin. Images were captured using the MF-ChemiBIS 1.6 system (DNR Bio-Imaging Systems, Jerusalem, Israel), and the intensity of the bands was measured with the software Image J (NIH, USA). FBS was used in each gel as a molecular weight standard for gelatinases and also as an internal standard to correct for intergel variability.

Measurement of nitrite concentration

Conditioned medium nitrite concentrations were analyzed in duplicate using an ozone-based reductive chemiluminescence assay as previously described (Pinheiro et al. 2014). Briefly, 100 μL of conditioned medium samples were injected into a purge vessel filled with 8 mL of triiodide solution (1.3 g of iodine and 2 g of potassium iodide dissolved in 40 mL of water and 140 mL of acetic acid) in line with a gas-phase chemiluminescence NO analyzer (Sievers Model 280 NO analyzer; Boulder, CO, USA). The data were analyzed using the software Origin Lab 8.5.

Assessment of the effects exerted by atorvastatin or by sodium nitrite on PMA-stimulated MMP-9 production by HUVEC and whether those effects are mediated by NO

Because treatment with atorvastatin may increase NO activity, we examined whether the effects of atorvastatin on MMP-9 production by endothelial cells are mediated by NO. To examine this possibility, we examined the effects of atorvastatin (2 μmol/L) on PMA (10 nmol/L)-stimulated MMP-9 production by endothelial cells in the absence and in the presence of the nitric oxide synthase inhibitor L-NAME (1000 μmol/L), or of the NO scavengers hemoglobin (20 μmol/L) or 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO; 10 μmol/L) for 24 h.

Moreover, because atorvastatin (but not sildenafil) increased nitrite concentrations in the conditioned medium, we wanted to validate the idea that the inhibitory effects of atorvastatin on MMP-9 production could be mediated by nitrite. To test this hypothesis, we used sodium nitrite at 0.1, 0.2, 2, 10, and 20 μmol/L to carry out the same experiments as described above for atorvastatin or sildenafil. The nitrite concentrations used in the present study correspond to physiological nitrite concentrations previously reported in humans (Metzger et al. 2007) or to nitrite concentrations achieved after treatment with atorvastatin (Nagassaki et al. 2006).

In addition, because many of the effects associated with sodium nitrite are attributable to NO generation from nitrite (Pinheiro et al. 2012), we carried out another series of experiments using the same conditions as described above to reassess the effects of sodium nitrite in the presence of the powerful nitric oxide scavenger oxy-hemoglobin (Sertorio et al. 2013). Therefore, the effects of sodium nitrite 2 μmol/L interacting with oxy-hemoglobin 0.2, 2, or 20 μmol/L were examined. To further validate the experiments with oxy-hemoglobin, we carried out similar experiments using the NO scavenger PTIO at 2 and 20 μmol/L.

Measurement of phospho-NFκB p65 concentration by enzyme-linked immunosorbent assays

Because nitric oxide was previously shown to attenuate MMP-9 production by endothelial cells independent of NFkB-mediated mechanisms (Meschiari et al. 2013), we wanted to examine whether the inhibitory effects of increased nitrite concentration (or atorvastatin treatment) on MMP-9 production are not mediated by activation of NFkB, as revealed by the concentrations of phospho-NFκB.

To determine NFκB activation in the cell lysate (Chang et al. 2005), HUVECs were treated with 10 nmol/L PMA combined with vehicle (control) or with 10 μmol/L atorvastatin, or with 2 μmol/L sodium nitrite for 30 min, and the phospho-NFκB p65 concentrations were determined in the cell lysate by using a commercially available enzyme-linked immunosorbent assays (ELISA) kit (eBioscience Inc., San Diego, CA, USA) according to the manufacturer's instructions.

Statistical analysis

The results were expressed as mean ± SEM. The data were normally distributed and were analyzed using one-way analysis of variance (one-way ANOVA) followed by the Tukey test to examine the effects of different drug concentrations. Two-way ANOVA followed by the Bonferroni test were used

to examine interaction between drugs. The probability value <0.05 was considered significant.

Results

Atorvastatin (but not sildenafil) inhibits PMA-induced MMP-9 production by endothelial cells

This study examined how atorvastatin blunts stimulated MMP-9 production by endothelial cells, possibly by mechanisms involving increased nitrite concentrations. Figure 1a–c shows that atorvastatin 3–100 μmol/L, sildenafil 3–100 μmol/L, and sodium nitrite 0.2–200 μmol/L, respectively, have no effects on cell viability as assessed by the MTT method (all $P > 0.05$), and therefore, no toxicity was found with the use of these drugs in the present study.

Figure 2a shows a representative zymography gel of conditioned medium samples, and Fig. 2b shows that 10 nmol/L PMA increases baseline MMP-9 gelatinolytic activity by more than tenfold ($P < 0.05$). Atorvastatin at 10 μmol/L (but not at 1 μmol/L) concentration attenuated PMA-induced increases in MMP-9 gelatinolytic activity by more than 60 % ($P < 0.05$). However, sildenafil (an inhibitor of cGMP specific PDE5) had no effects on PMA-induced increases in MMP-9 gelatinolytic activity when tested at 1 or 10 μmol/L concentrations (Fig. 2; both $P > 0.05$). In addition, no significant improvement in the effects of atorvastatin 10 μmol/L alone was found when sildenafil 10 μmol/L was combined with atorvastatin 10 μmol/L (Fig. 2; $P < 0.05$). These results show that sildenafil alone or combined with atorvastatin exerted no effects on stimulated MMP-9 production by endothelial cells.

Atorvastatin (but not sildenafil) increased nitrite concentrations

We investigated whether atorvastatin or sildenafil treatments increase nitrite concentrations in the conditioned medium. Figure 3 shows that treatment with PMA at 10 nmol/L concentration did not affect nitrite concentration ($P > 0.05$). However, atorvastatin at 10 μmol/L concentration increased nitrite concentration in conditioned medium by approximately 50 % (Fig. 3; $P < 0.05$), whereas treatment with atorvastatin at

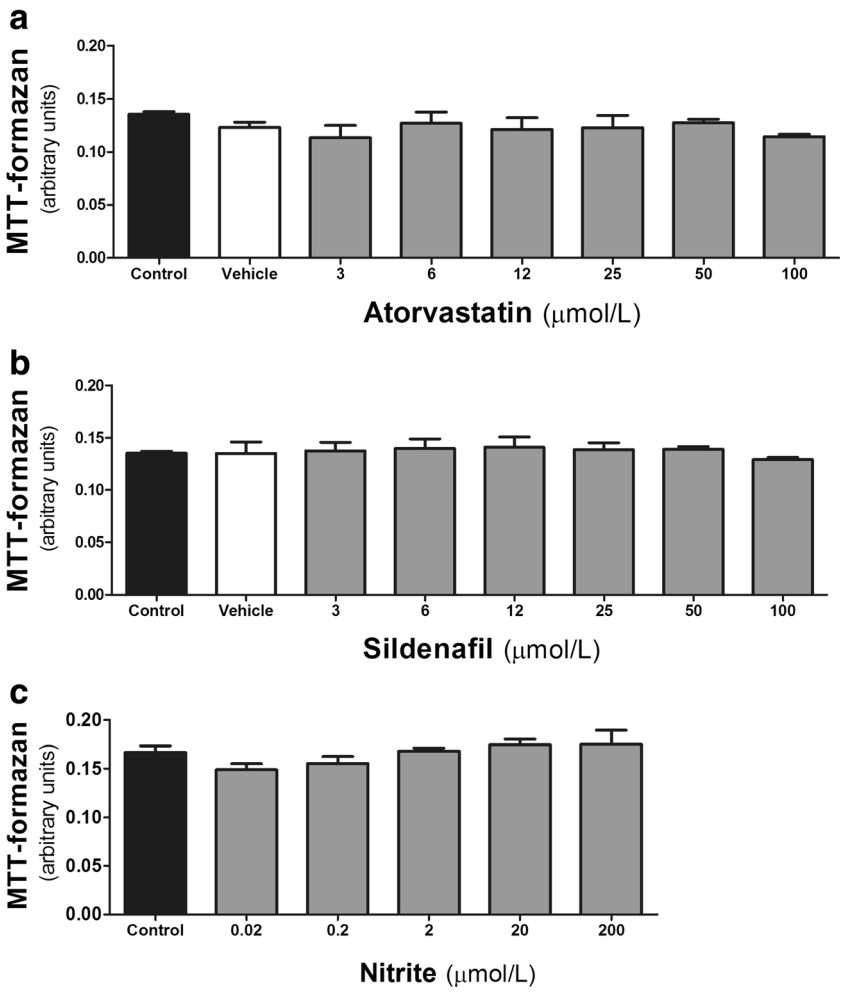

Fig. 1 Atorvastatin, sildenafil, and sodium nitrite showed no significant toxicity at wide concentration range. Results of MTT-formazan cell viability assay to assess cell toxicity of atorvastatin 3–100 μmol/L (**a**), sildenafil 3–100 μmol/L (**b**), and sodium nitrite 0.2–200 μmol/L (**c**). HUVECs were treated with drugs or vehicle (0.1 % DMSO) for 24 h. Control = cells incubated with no drugs. Data are expressed as means ± SEM. (n = 3–5/group)

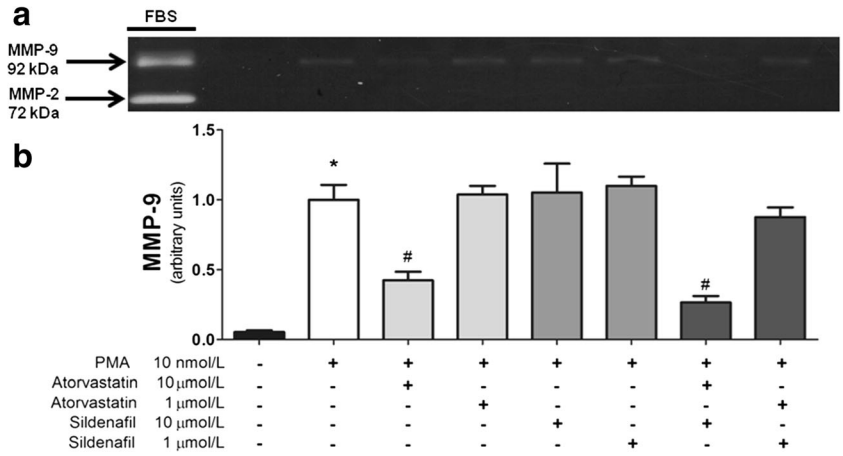

Fig. 2 Atorvastatin (but not sildenafil) attenuates MMP-9 production stimulated by PMA (10 nmol/L). **a** Representative SDS-PAGE zymogram showing bands corresponding to MMP-9 and to MMP-2 secreted into conditioned medium by endothelial cells. Fetal bovine serum (FBS) was used as a standard to normalize the results. **b** MMP-9 gelatinolytic activity in conditioned medium after treatment with different drugs as indicated. MMP-2 was almost undetectable. Data are expressed as mean ± SEM (n = 4–6/group). *$P < 0.05$ versus control (untreated cells). $^{\#}P < 0.05$ versus PMA 10 nmol/L

1 μmol/L or sildenafil at 1 or 10 μmol/L had no effects (Fig. 3; all $P > 0.05$). In line with the MMP-9 results, the combination of atorvastatin and sildenafil at 10 μmol/L exerted similar effects on nitrite concentration as compared to the effects found with atorvastatin 10 μmol/L alone (Fig. 3; $P > 0.05$), thus showing that sildenafil did not improve the effects of atorvastatin.

Atorvastatin inhibits PMA-induced MMP-9 production by endothelial cells by mechanisms independent of nitric oxide

We investigated whether atorvastatin treatment inhibits MMP-9 production by endothelial cells as a result of increased nitric oxide production. Figure 4 shows that treatment with atorvastatin at 10 μmol/L concentration attenuated PMA-induced increases in MMP-9 gelatinolytic activity by more than 65 % ($P < 0.05$). However, treatment with the NO synthase inhibitor L-NAME, or with the NO scavengers oxyhemoglobin or PTIO, did not modify the inhibitory effects of atorvastatin (Fig. 4; all $P < 0.05$ versus PMA only treatment), thus suggesting the effects of atorvastatin are not mediated by increased NO formation.

Nitrite attenuated PMA-stimulated MMP-9 production by HUVEC by mechanisms that are independent of NO

Given the fact that atorvastatin (but not sildenafil) attenuated PMA-stimulated MMP-9 release by endothelial cells and increased nitrite concentrations, we investigated whether nitrite could mediate this inhibitory effect of atorvastatin. Therefore, PMA-stimulated endothelial cells were incubated with sodium nitrite at 0.1 to 20 μmol/L, and we found that sodium nitrite at 0.2 or 2.0 μmol/L exerted maximum inhibitory effect on stimulated MMP-9 production (Fig. 5; both $P < 0.05$). Interestingly, the 0.2-μmol/L nitrite concentration corresponds approximately to the nitrite concentrations found after treatment with atorvastatin, either alone or combined with sildenafil (Fig. 3), which inhibited stimulated MMP-9 production by more than 60 % (Fig. 2b);

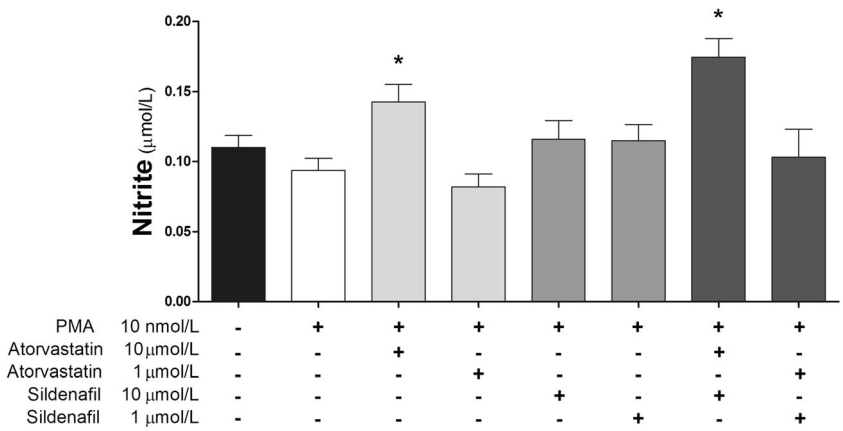

Fig. 3 Atorvastatin (but not sildenafil) increases nitrite concentrations in conditioned medium. Nitrite concentrations measured in conditioned medium after treatment with different drugs as indicated are shown. Data are expressed as mean ± SEM (n = 6–8/group). *$P < 0.05$ versus PMA 10 nmol/L

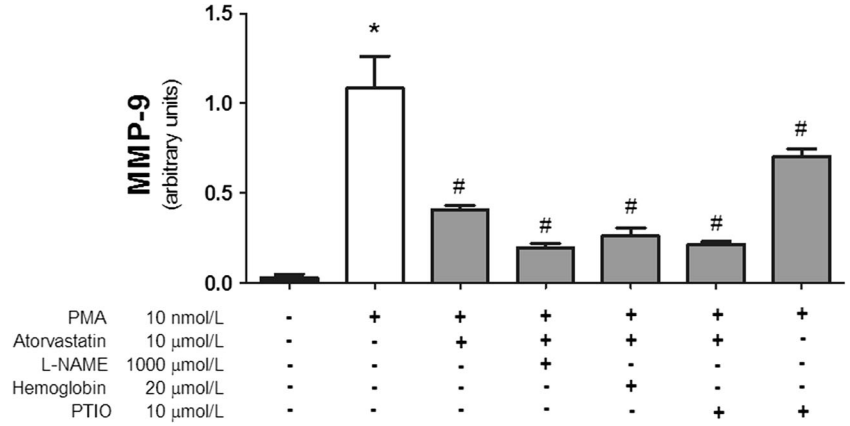

Fig. 4 Atorvastatin attenuates PMA-stimulated MMP-9 production by endothelial cells independent of nitric oxide formation. The effects of atorvastatin (2 μmol/L) on PMA (10 nmol/L)-stimulated MMP-9 production by endothelial cells were examined in the absence and in the presence of the nitric oxide synthase inhibitor L-NAME (1000 μmol/L), or the NO scavengers hemoglobin (20 μmol/L) or 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO; 10 μmol/L) for 24 h. Values shown correspond to MMP-9 gelatinolytic activity in conditioned medium. Data are expressed as mean ± SEM (n = 8/group). *P < 0.05 versus control (untreated cells). $^{\#}P$ < 0.05 versus PMA

In previous studies (Meschiari et al. 2013), we reported that nitric oxide inhibits MMP-9 production by endothelial cells, and therefore, atorvastatin-induced increases in nitrite concentrations could inhibit MMP-9 production as a result of NO generation from nitrite (Pinheiro et al. 2012). To test this possibility, we examined the effects of sodium nitrite 2 μmol/L on PMA-stimulated MMP-9 production during incubation with vehicle (control) or with the NO scavenger hemoglobin (Sertorio et al. 2013) at 0.2, 2, or 20 μmol/L. While nitrite consistently inhibited MMP-9 production by endothelial cells, hemoglobin at 0.2, 2, or 20 μmol/L did not modify this effect (Fig. 6; all P > 0.05), thus ruling out the possibility that nitrite-derived NO would be involved in the effects of nitrite or atorvastatin. These results agree with the lack of effect of sildenafil alone, or lack of improvement of the effects of atorvastatin when sildenafil was combined with this statin (Fig. 2) because sildenafil usually potentiates NO-mediated effects, as both NO and sildenafil increase cGMP concentrations (Dias-Junior et al. 2008).

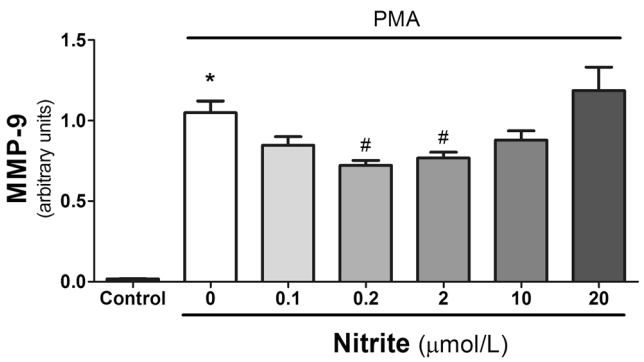

Fig. 5 Nitrite treatment attenuates PMA-stimulated MMP-9 production by endothelial cells. MMP-9 gelatinolytic activity in conditioned medium after endothelials cells were stimulated with PMA (10 nmol/L) and treated with sodium nitrite (from 0.1 to 20 μmol/L) is shown. Data are expressed as mean ± SEM (n = 6/group). *P < 0.05 versus control (untreated cells). $^{\#}P$ < 0.05 versus 0 nitrite concentration

To further confirm that nitrite-derived NO is not involved in nitrite-induced inhibition of MMP-9 production by endothelial cells, we tested whether the NO scavenger C-PTIO affects the responses to nitrite. Supplementary Figure 1 shows that C-PTIO did not affect the responses to sodium nitrite. However, these negative findings may be limited by the fact that control experiments using C-PTIO alone partially inhibited MMP-9 production by endothelial cells to the same extent as sodium nitrite (See Supplementary Figure 1).

Atorvastatin and nitrite attenuated PMA-stimulated MMP-9 production by HUVEC by mechanisms that are independent of NFκB activation

MMPs are transcriptionally regulated NFκB, and inhibition of NFκB markedly inhibits mechanisms upregulating MMPs (Meschiari et al. 2013; Cau et al. 2011). Given that PMA increases MMP-9 expression by activating NFκB (Meschiari et al. 2013), treatment with atorvastatin or nitrite could inhibit NFκB activation and therefore inhibit PMA-induced increases in the concentrations of phospho-NFκB p65. Figure 7 clearly shows that this mechanism is not relevant with both atorvastatin and nitrite because both treatments had no effects on PMA-induced increases in phospho-NFκB p65 concentrations (Fig. 7; both P > 0.05).

Discussion

This is the first study to show that atorvastatin, but not sildenafil, downregulates MMP-9 activity in endothelial cells by mechanisms that involve nitrite signaling and are not dependent on nitrite-derived NO formation and do not involve inhibitory effects on NFκB activation. Our findings may help to explain new mechanisms activated by treatment with

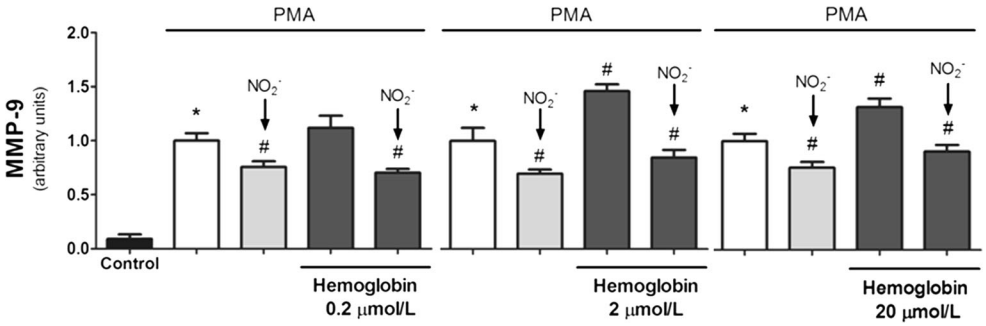

Fig. 6 Nitrite treatment attenuates PMA-stimulated MMP-9 production by endothelial cells independent of nitric oxide formation. The effects of nitrite (2 μmol/L) on PMA (10 nmol/L)-stimulated MMP-9 production by endothelial cells were examined in the absence and in the presence of the nitric oxide scavenger hemoglobin at 0.2, 2, and 20 μmol/L concentrations. Values shown correspond to MMP-9 gelatinolytic activity in conditioned medium. Data are expressed as mean ± SEM (n = 6–8/group). *$P < 0.05$ versus control (untreated cells). #$P < 0.05$ versus PMA in the presence of hemoglobin

atorvastatin and other statins, which are widely used to treat a variety of atherosclerotic and non-atherosclerotic cardiovascular diseases, particularly those involving enhanced MMP-9 expression and activity.

It is widely acknowledged that statins upregulate endothelial NO synthase expression and activity, and this effect has been associated with cardiovascular protection against clinical events (Davignon 2004). However, statins may exert other pleiotropic effects that are equally or more important, such as inhibition of MMP expression (Izidoro-Toledo et al. 2011). Interestingly, a variety of pleiotropic effects of statins probably involve many different mechanisms that remain to be determined and are not dependent on the widely acknowledged inhibition of cholesterol synthesis in the liver that results of blocked conversion of 3-hydroxy-3-methylglutaryl coenzyme A to mevalonate (Liao and Laufs 2005). Our results showing that neither the NO synthase inhibitor L-NAME nor the NO scavengers oxy-hemoglobin or PTIO affected the responses to atorvastatin are consistent with the idea that atorvastatin may inhibit MMP-9 production by endothelial cells by mechanisms not involving increased NO production.

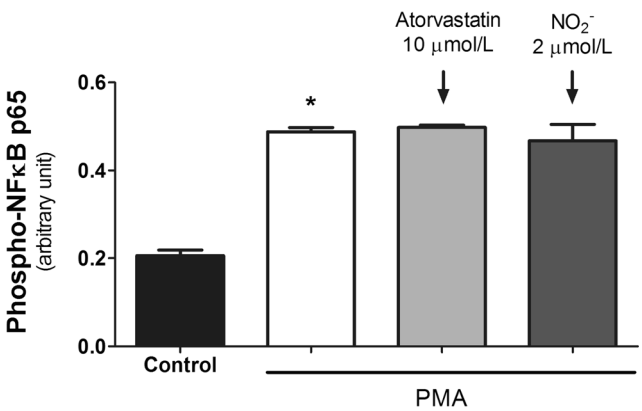

Fig. 7 Neither atorvastatin, nor nitrite treatment attenuates PMA-stimulated NFκB activation. Phospho-NFκB concentrations were measured in cells without stimulation (control) and after stimulation with PMA (10 nmol/L) in the presence of vehicle or atorvastatin (10 μmol/L) or sodium nitrite (2 μmol/L). Data are expressed as mean ± SEM (n = 6–8/group). * $P < 0.05$ versus control (untreated cells)

Atorvastatin increased nitrite concentrations and nitrite inhibited MMP-9 production by endothelial cells in the present study. Because nitrite is reduced to NO under certain conditions (Gladwin et al. 2005; Montenegro et al. 2014), and NO was shown to downregulate MMP-9 production by endothelial cells (Meschiari et al. 2013), we examined whether atorvastatin-induced increases in nitrite concentrations would result in NO-mediated inhibition of MMP-9 production by endothelial cells. Our results support that idea that the inhibitory effects of atorvastatin and nitrite on MMP-9 productions are not mediated by NO generated from nitrite. This is because co-incubation of cells with nitrite and oxy-hemoglobin (a major NO scavenger) at variable concentrations did not modify the inhibitory effects of nitrite on MMP-9 production. Moreover, many effects mediated by NO are potentiated by PDE5 inhibitors such as sildenafil, as NO activates guanylate cyclase enhancing cGMP concentrations and sildenafil inhibits PDE5, which degrades cGMP. Therefore, while sildenafil usually potentiates NO-cGMP-mediated effects, this interaction was not found in the present study (as silfenafil did not enhance the effects of atorvastatin). Together, these findings support the idea that NO does not mediate the effects of atorvastatin and nitrite reported here.

NFκB is a major inducer of MMP expression (He 1996; Deschamps and Spinale 2006; Cau et al. 2011), and therefore, we investigated whether treatment with atorvastatin or nitrite decreases PMA-induced NFκB activation to explain their inhibitory effects on MMP-9 production by endothelial cells. In line with a previous study showing that atorvastatin inhibited MMP-9 production as well as other pro-inflammatory mediators (Han et al. 2012), we found that this statin lowered MMP-9 production by endothelials cells. However, atorvastatin and nitrite exerted no effects on PMA-induced NFκB activation, thus suggesting that other mechanisms than interference with NFκB pathway are involved. Such mechanisms could result from unexplored direct nitrite signaling, as previously discussed (Shiva 2013).

Increasing tissue nitrite concentrations may protect the cardiovascular system, as nitrite may function as a NO reservoir with antioxidant (Montenegro et al. 2011; Montenegro et al. 2012) and antiproliferative properties contributing to maintain functional and morphological integrity of the cardiovascular system (Gladwin 2004; Guimaraes et al. 2013). Examining how statins are useful as a new strategy to increase vascular concentrations of nitrite may help to elucidate their protective effects against cardiovascular diseases (Guimaraes et al. 2013). This is clearly the case of atorvastatin, which increased both plasma and vascular nitrite concentrations and improved endothelial-dependent vascular relaxation in a hypertension model (Guimaraes et al. 2013) associated with increased vascular MMP expression (Guimaraes et al. 2011). While it may be impossible to separate the effects of atorvastatin from those attributable to nitrite, the inhibitory effects of nitrite on MMP-9 production reported here are consistent with this suggestion. Giving further support to this suggestion, the circulating nitrite concentrations were found at inverse relationship with plasma MMP-9 concentrations in humans (Demacq et al. 2008; Metzger et al. 2012). Moreover, treatment with atorvastatin increased plasma nitrite concentrations and decreased plasma MMP-9 levels in humans (Nagassaki et al. 2006; Wu et al. 2012; Souza-Costa et al. 2007b). The results reported in the present study fully support those previous clinical findings in humans and offer a mechanistic perspective directly implicating nitrite as a mediator of the inhibitory effects of statins on MMP-9 production.

This study has some limitations that should be mentioned. We have not clearly defined the details about the mechanism activated by nitrite that may inhibit MMP-9 production by endothelial cells. However, there is very little information regarding molecular mechanisms activated by nitrite (Mo et al. 2012), and this will surely become a rapidly moving field. While we carried out experiments with the NO synthase (N(omega)-nitro-L-arginine methyl ester; L-NAME) to show that inhibiting NO synthesis has no effect on MMP-9 production by endothelial cells (see Supplementary Figure 2) under the conditions detailed here, other mechanisms involving NO such as protein nitrosylation and nitration or other interactions of NO with other reactive oxygen species could have an impact on endothelial cell biology (Toledo and Augusto 2012).

In summary, this study shows that increased nitrite concentrations may mediate the effects of atorvastatin and attenuate MMP-9 production by endothelial cells independent of NO formation or on inhibition of NFκB activation. Our findings may help to elucidate important new nitrite-mediated mechanisms by which atorvastatin and other statins affect imbalanced MMP activity in a variety of cardiovascular disease.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

References

Bruton LB, Lazo JS, Parker KL (2006) Goodman & gilman's the pharmacological basis of therapeutics. 11th ed.

Castro MM, Tanus-Santos JE (2013) Inhibition of matrix metalloproteinases (mmps) as a potential strategy to ameliorate hypertension-induced cardiovascular alterations. Curr Drug Targets 14(3):335–343

Castro MM, Rizzi E, Rascado RR, Nagassaki S, Bendhack LM, Tanus-Santos JE (2004) Atorvastatin enhances sildenafil-induced vasodilation through nitric oxide-mediated mechanisms. Eur J Pharmacol 498(1–3):189–194

Castro MM, Rizzi E, Prado CM, Rossi MA, Tanus-Santos JE, Gerlach RF (2009a) Imbalance between matrix metalloproteinases and tissue inhibitor of metalloproteinases in hypertensive vascular remodeling. Matrix Biol 29(3):194–201

Castro MM, Rizzi E, Rodrigues GJ, Ceron CS, Bendhack LM, Gerlach RF, Tanus-Santos JE (2009b) Antioxidant treatment reduces matrix metalloproteinase-2-induced vascular changes in renovascular hypertension. Free Radic Biol Med 46(9):1298–1307

Cau S, Guimaraes D, Rizzi E, Ceron C, Souza L, Tirapelli C, Gerlach R, Tanus-Santos J (2011) Pyrrolidine dithiocarbamate downregulates vascular matrix metalloproteinases and ameliorates vascular dysfunction and remodeling in renovascular hypertension. Br J Pharmacol 164(2):372–381

Chang MS, Chen BC, Yu MT, Sheu JR, Chen TF, Lin CH (2005) Phorbol 12-myristate 13-acetate upregulates cyclooxygenase-2 expression in human pulmonary epithelial cells via ras, raf-1, erk, and nf-kappab, but not p38 mapk, pathways. Cell Signal 17(3):299–310

Davignon J (2004) Beneficial cardiovascular pleiotropic effects of statins. Circulation 109(23 Suppl 1):III39–III43

Demacq C, Metzger IF, Gerlach RF, Tanus-Santos JE (2008) Inverse relationship between markers of nitric oxide formation and plasma matrix metalloproteinase-9 levels in healthy volunteers. Clin Chim Acta 394(1–2):72–76

Deschamps AM, Spinale FG (2006) Pathways of matrix metalloproteinase induction in heart failure: bioactive molecules and transcriptional regulation. Cardiovasc Res 69(3):666–676

Dias-Junior CA, Montenegro MF, Florencio BC, Tanus-Santos JE (2008) Sildenafil improves the beneficial haemodynamic effects of intravenous nitrite infusion during acute pulmonary embolism. Basic Clin Pharmacol Toxicol 103(4):374–379

Gladwin MT (2004) Haldane, hot dogs, halitosis, and hypoxic vasodilation: the emerging biology of the nitrite anion. J Clin Invest 113(1):19–21

Gladwin MT, Schechter AN, Kim-Shapiro DB, Patel RP, Hogg N, Shiva S, Cannon 3rd RO, Kelm M, Wink DA, Espey MG, Oldfield EH, Pluta RM, Freeman BA, Lancaster Jr JR, Feelisch M, Lundberg JO (2005) The emerging biology of the nitrite anion. Nat Chem Biol 1(6):308–314

Guimaraes DA, Rizzi E, Ceron CS, Oliveira AM, Oliveira DM, Castro MM, Tirapelli CR, Gerlach RF, Tanus-Santos JE (2011) Doxycycline dose-dependently inhibits mmp-2-mediated vascular

changes in 2k1c hypertension. Basic Clin Pharmacol Toxicol 108(5):318–325

Guimaraes DA, Rizzi E, Ceron CS, Pinheiro LC, Gerlach RF, Tanus-Santos JE (2013) Atorvastatin and sildenafil lower blood pressure and improve endothelial dysfunction, but only atorvastatin increases vascular stores of nitric oxide in hypertension. Redox Biol 1(1):578–585

Han L, Li M, Liu Y, Han C, Ye P (2012) Atorvastatin may delay cardiac aging by upregulating peroxisome proliferator-activated receptors in rats. Pharmacology 89(1–2):74–82

He C (1996) Molecular mechanism of transcriptional activation of human gelatinase b by proximal promoter. Cancer Lett 106(2):185–191

Izidoro-Toledo TC, Guimaraes DA, Belo VA, Gerlach RF, Tanus-Santos JE (2011) Effects of statins on matrix metalloproteinases and their endogenous inhibitors in human endothelial cells. Naunyn Schmiedeberg's Arch Pharmacol 383(6):547–554

Kunte H, Amberger N, Busch MA, Ruckert RI, Meiners S, Harms L (2008) Markers of instability in high-risk carotid plaques are reduced by statins. J Vasc Surg 47(3):513–522

Liao JK, Laufs U (2005) Pleiotropic effects of statins. Annu Rev Pharmacol Toxicol 45:89–118

Liu HT, Li WM, Xu G, Li XY, Bai XF, Wei P, Yu C, Du YG (2009) Chitosan oligosaccharides attenuate hydrogen peroxide-induced stress injury in human umbilical vein endothelial cells. Pharmacol Res 59(3):167–175

Meschiari CA, Izidoro-Toledo T, Gerlach RF, Tanus-Santos JE (2013) Nitric oxide attenuates matrix metalloproteinase-9 production by endothelial cells independent of cgmp- or nfkappab-mediated mechanisms. Mol Cell Biochem 378(1–2):127–135

Metzger IF, Sertorio JT, Tanus-Santos JE (2007) Modulation of nitric oxide formation by endothelial nitric oxide synthase gene haplotypes. Free Radic Biol Med 43(6):987–992

Metzger IF, Sandrim VC, Tanus-Santos JE (2012) Endogenous nitric oxide formation correlates negatively with circulating matrix metalloproteinase (mmp)-2 and mmp-9 levels in black subjects. Mol Cell Biochem 360(1–2):393–399

Mo L, Wang Y, Geary L, Corey C, Alef MJ, Beer-Stolz D, Zuckerbraun BS, Shiva S (2012) Nitrite activates amp kinase to stimulate mitochondrial biogenesis independent of soluble guanylate cyclase. Free Radic Biol Med 53(7):1440–1450

Montenegro MF, Amaral JH, Pinheiro LC, Sakamoto EK, Ferreira GC, Reis RI, Marcal DM, Pereira RP, Tanus-Santos JE (2011) Sodium nitrite downregulates vascular nadph oxidase and exerts antihypertensive effects in hypertension. Free Radic Biol Med 51(1):144–152

Montenegro MF, Pinheiro LC, Amaral JH, Marcal DM, Palei AC, Costa-Filho AJ, Tanus-Santos JE (2012) Antihypertensive and antioxidant effects of a single daily dose of sodium nitrite in a model of renovascular hypertension. Naunyn Schmiedeberg's Arch Pharmacol 385(5):509–517

Montenegro MF, Pinheiro LC, Amaral JH, Ferreira GC, Portella RL, Tanus-Santos JE (2014) Vascular xanthine oxidoreductase contributes to the antihypertensive effects of sodium nitrite in l-name hypertension. Naunyn Schmiedeberg's Arch Pharmacol 387(6):591–598

Nagassaki S, Sertorio JT, Metzger IF, Bem AF, Rocha JB, Tanus-Santos JE (2006) Enos gene t-786c polymorphism modulates atorvastatin-induced increase in blood nitrite. Free Radic Biol Med 41(7):1044–1049

Pinheiro LC, Montenegro MF, Amaral JH, Ferreira GC, Oliveira AM, Tanus-Santos JE (2012) Increase in gastric ph reduces hypotensive effect of oral sodium nitrite in rats. Free Radic Biol Med 53(4):701–709

Pinheiro LC, Amaral JH, Ferreira GC, Montenegro MF, Oliveira-Paula GH, Tanus-Santos JE (2014) The antihypertensive effects of sodium nitrite are not associated with circulating angiotensin converting enzyme inhibition. Nitric Oxide 40C:52–59

Sertorio JT, Neto-Neves EM, Dias-Junior CA, Sousa-Santos O, Kiss T, Muhl D, Tanus-Santos JE (2013) Elevated plasma hemoglobin levels increase nitric oxide consumption in experimental and clinical acute pulmonary thromboembolism. Crit Care Med 41(7):e118–e124

Shiva S (2013) Nitrite: a physiological store of nitric oxide and modulator of mitochondrial function. Redox Biol 1(1):40–44

Souza-Costa DC, Figueiredo-Lopes L, Alves-Filho JC, Semprini MC, Gerlach RF, Cunha FQ, Tanus-Santos JE (2007a) Protective effects of atorvastatin in rat models of acute pulmonary embolism: involvement of matrix metalloproteinase-9. Crit Care Med 35(1):239–245

Souza-Costa DC, Sandrim VC, Lopes LF, Gerlach RF, Rego EM, Tanus-Santos JE (2007b) Anti-inflammatory effects of atorvastatin: modulation by the t-786c polymorphism in the endothelial nitric oxide synthase gene. Atherosclerosis 193(2):438–444

Souza-Tarla CD, Uzuelli JA, Machado AA, Gerlach RF, Tanus-Santos JE (2005) Methodological issues affecting the determination of plasma matrix metalloproteinase (mmp)-2 and mmp-9 activities. Clin Biochem 38(5):410–414

Toledo Jr JC, Augusto O (2012) Connecting the chemical and biological properties of nitric oxide. Chem Res Toxicol 25(5):975–989

Wu YW, Kao HL, Huang CL, Chen MF, Lin LY, Wang YC, Lin YH, Lin HJ, Tzen KY, Yen RF, Chi YC, Huang PJ, Yang WS (2012) The effects of 3-month atorvastatin therapy on arterial inflammation, calcification, abdominal adipose tissue and circulating biomarkers. Eur J Nucl Med Mol Imaging 39(3):399–407

Yabluchanskiy A, Ma Y, Iyer RP, Hall ME, Lindsey ML (2013) Matrix metalloproteinase-9: many shades of function in cardiovascular disease. Physiology (Bethesda) 28(6):391–403