Basic research

Assessment of the correlation between oxidative stress and expression of *MMP-2*, *TIMP-1* and *COX-2* in human aortic smooth muscle cells

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Abstract

Introduction: Smooth muscle cells (SMCs) are considered to be the main producer of matrix metalloproteinase-2 (MMP-2) participating primarily in extracellular matrix (ECM) remodeling. Any disturbances in ECM structure may underlie the pathogenesis of many cardiovascular diseases and contribute to angiogenesis, cancer development, invasion or metastasis. The purpose of the study was to examine the effect of oxidative stress on the expression of MMP-2, its tissue inhibitor type 1 (TIMP-1) and cyclooxygenase-2 (COX-2) in human aortic smooth muscle cells (HASMCs).
Material and methods: HASMCs were treated with exogenously applied H_2O_2 or TNF-α. N-acetylcysteine (NAC) was used as an antioxidant. Gene expression levels were measured by real-time PCR and the protein levels were determined using ELISA assay.
Results: The studies revealed no association between oxidative stress and either mRNA quantity or protein secretion of MMP-2 and TIMP-1. However, we found markedly reduced ($p < 0.001$) MMP-2 secretion in cells incubated with NAC. HASMCs stimulated with TNF-α demonstrated a significantly increased COX-2 mRNA level as well as enzyme activity. H_2O_2-induced cells showed lowered COX-2 activity in comparison to untreated cells. MMP-2 and TIMP-1 expression did not change after COX-2 inhibition with DuP-697.
Conclusions: We did not find any effect of oxidative stress on expression of MMP-2 and TIMP-1 in HASMCs. However, COX-2 mRNA and protein level were elevated in these conditions. There was no correlation between COX-2 activity and MMP-2 and TIMP-1 mRNA expression or protein secretion.

Key words: hydrogen peroxide, oxidative stress, tumor necrosis factor-α.

Introduction

Metalloproteinases (MMPs) belong to a group of zinc-dependent endopeptidases produced and secreted by multiple cell types, including inflammatory cells and vascular smooth muscle cells (SMCs), both in physiological and pathological states. They possess the capacity for proteolytic degradation of extracellular matrix (ECM) proteins such as collagens, proteoglycans, elastin and fibronectin. SMCs constitute a major cellular component of the arterial wall and play a crucial role in regulating vascular tone and producing the ECM framework of the vessel wall. Vascular SMCs are generally surrounded by and embedded in a variety of ECM proteins. Any modifications in ECM structure, in particular resulting from

MMP activity, lead to vascular remodeling. Therefore, MMPs can participate in pathogenesis of many cardiovascular diseases, such as atherosclerosis or aneurysms. They also play a major role in angiogenesis, cancer development, invasion and metastasis. Expression and activity of MMPs are regulated at the transcriptional level by various cytokines, at the protein level by proenzyme activation, and at the active enzyme level by exogenous and endogenous inhibitors [1, 2]. The understanding of the factors influencing the expression and activity of MMPs and their tissue inhibitors seems to be very important to clarify the pathomechanism of many disorders. One such factor could be reactive oxygen species (ROS), involved in numerous processes occurring inside the cells – both physiologic and pathologic.

The goal of the study was to elucidate the correlation between externally applied hydrogen peroxide (H_2O_2), which is a cell-permeable oxidant, or endogenously generated H_2O_2 after tumor necrosis factor α (TNF-α) stimulation, and the expression of MMP-2 and TIMP-1 in human aortic smooth muscle cells. MMP-2 belongs to the gelatinases that degrade type IV collagen in basal membranes, as well as collagen V, VII, X, elastin and gelatin. The changed activity of this proteolytic enzyme or its tissue inhibitor, TIMP-1 under pathological conditions may contribute to the development of many cardiovascular diseases [3–6] or cancers [7–10].

Additionally, in this research we investigated the effect of H_2O_2 and TNF-α on cyclooxygenase-2 (COX-2) expression level and activity, as well as the possible relationship between COX-2 activity and MMP-2 and TIMP-1 expression. COX-2 is an inducible enzyme converting arachidonic acid to prostaglandins (PGs) at the site of inflammation and is linked to several physiological and pathological pathways related to angiogenesis, inflammation or invasiveness [11, 12].

Material and methods

Cell culture and treatment

The cryopreserved human aortic smooth muscle cells (HASMCs) pooled from a single donor were purchased as cryopreserved samples from Gibco (Life Technologies). The cells were grown in Medium 231 (Gibco) supplemented with 5% smooth muscle growth supplement (SMGS, Gibco) and 1% gentamicin/amphotericin B solution (Gibco) at 37°C in a 95% humidified atmosphere of 5% CO_2. The medium was replaced every 2–3 days. At confluence, cells were subcultured by trypsinization (using 0.025% trypsin – 0.01% EDTA solution, Gibco), after which the cells were seeded with a split ratio of 1 : 3. Cell viability was evaluated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Sigma Aldrich) performed in 96-well plates. Measurements were taken by the multiwell plate reader (Multiskan Ascent) with an appropriate filter set (570 nm). Cultures at 4–7 passages were used in the experiments. Before all experiments the cells were synchronized by 24 h starvation in SMGS- and antibiotic-free Medium 231. Then the cells were preincubated for 30 min with or without 1 mol/l N-acetylcysteine (Sigma-Aldrich) and/or 10 nmol/l DuP-697 (Cayman Biochemicals) followed by treatment with hydrogen peroxide (50 and 150 µmol/l) or TNF-α (50 ng/ml) for 24 h. Untreated cells constituted a control.

Real-time PCR

Cell lysis, cDNA synthesis and real-time PCR were performed using the Power SYBR Green Cells-to-Ct Kit (Ambion/Thermo Fisher Scientific) according to the manufacturer's protocol. Briefly, treated and untreated smooth muscle cells were directly lysed for 5 min at room temperature with Lysis Solution (provided in the kit) in 96-well culture plates after washing with phosphate-buffered saline (PBS). Cell lysates were reverse transcribed to synthesize cDNA using the RT Enzyme Mix and SYBR RT Buffer. Then cDNA was amplified by real-time PCR on the Stratagene Mx3005P System (Agilent Technologies) using Power SYBR Green PCR Master Mix and the specific primers as follows: MMP-2 forward primer 5′ ATGAATACTGGATCTACTCAGC 3′ and reverse primer 5′ GTATCTCCAGAATTTGTCTCC 3′, TIMP-1 forward primer 5′ CACCTTATACCAGCGTTATG 3′ and reverse primer 5′ TTTCCAGCAATGAGAAACTC 3′, COX-2 forward primer 5′ TGGAATTACCCAGTTTGTTG 3′ and reverse primer 5′ TGCGGTACTCATTAAAAGAC 3′, GAPDH forward primer 5′ CTTTTGCGTCGCCAG 3′ and reverse primer 5′ TTGATGGCAACAATATCCAC 3′ (Sigma-Aldrich). The amount of target mRNA was normalized to GAPDH, and the relative gene expression was calculated by the formula $2^{-\Delta\Delta Ct}$ using samples from the control group as calibrator samples [13, 14].

Enzyme-linked immunosorbent assay (ELISA)

For the measurement of MMP-2 and TIMP-1 protein levels in cell culture supernatants, Human MMP-2 ELISA and Human TIMP-1 ELISA kits (Sigma-Aldrich) were used. The quantity of prostaglandin E2 (PGE2), the product of COX-2 activity being released into the cell culture medium, was determined by the ELISA Kit from Abnova. Before the experiments, the HASMCs were grown in 6-well plates and were treated with experimental reagents as described above. Additionally, in the case of PGE2 level determination, one hour before the end of the incubation period, arachidonic acid

(substrate for COX-2) was added to the cell culture medium at the final concentration of 15 µmol/l. Cell culture supernatants were collected, centrifuged and frozen at –80°C until analysis. The assays were carried out following the instructions provided by the manufacturers. The absorbances were measured at 450 nm (for MMP-2 and TIMP-1 assays) or at 405 nm (for PGE2) on the Glomax Multi Detection System (Promega). The obtained concentration values were normalized to the total protein content as measured by the Micro BCA Protein Assay Kit (Thermo Fisher Scientific) from each cell lysate.

Statistical analysis

Statistical analysis was performed using Statistica StatSoft Version 12. Data are expressed as the mean ± standard deviation. The results were analyzed by one-way ANOVA. When differences were indicated, Tukey's *post-hoc* test for multiple comparison was used to determine the significance. *P*-value < 0.05 was considered statistically significant.

Results

The effect of H_2O_2 and TNF-α on the mRNA expression level of MMP-2 and TIMP-1 in HASMCs

The oxidative stress originating from exogenously added H_2O_2 or endogenously generated after TNF-α stimulation did not significantly influence the *MMP-2* and *TIMP-1* mRNA amounts in HASMCs in comparison to untreated cells (Figure 1). The preincubation of cells with an antioxidant NAC also did not show any significant differences in mRNA levels of both genes between control and experimental groups of cells. In the case of the TIMP-1 gene the relative mRNA quantity was elevated by more than 30% in cells treated with NAC both alone and treated simultaneously with 50 µmol/l H_2O_2, but the difference did not reach statistical significance. Additionally, the increase of *TIMP-1* mRNA level in cells incubated with NAC and 150 µmol/l H_2O_2 was borderline significant ($p = 0.0499$) compared with control cells.

The effect of H_2O_2 and TNF-α on secretion of MMP-2 and TIMP-1 from HASMCs

The ELISA results showed no significant effect of H_2O_2 and TNF-α on MMP-2 and TIMP-1 release from smooth muscle cells to the culture medium (Figure 2). However, we found markedly ($p < 0.001$) reduced MMP-2 secretion in cells incubated with NAC, both in the absence and the presence of H_2O_2 compared to the control cells. A slight decrease (by about 30%) in MMP-2 release upon NAC administration was also observed in TNF-α-stimulated cells but the difference did not reach the level of statistical significance ($p > 0.05$).

The effect of H_2O_2 and TNF-α on COX-2 mRNA expression and protein activity

HASMCs stimulated with TNF-α expressed about a twofold larger amount of *COX-2* mRNA than unstimulated cells ($p = 0.011$). An increase of mRNA level of this gene was also observed in cells treated with 50 µmol/l H_2O_2 (higher concentration of hydrogen peroxide did not exert any influence), but without statistical significance ($p > 0.05$). Pretreatment of TNF-α-stimulated cells with NAC resulted in lower *COX-2* mRNA expression by about 25%, but the difference did not reach significance. The group of cells incubated with this antioxidant alone did not show any changes in the mRNA level of *COX-2*. The detailed results are presented in Figure 3.

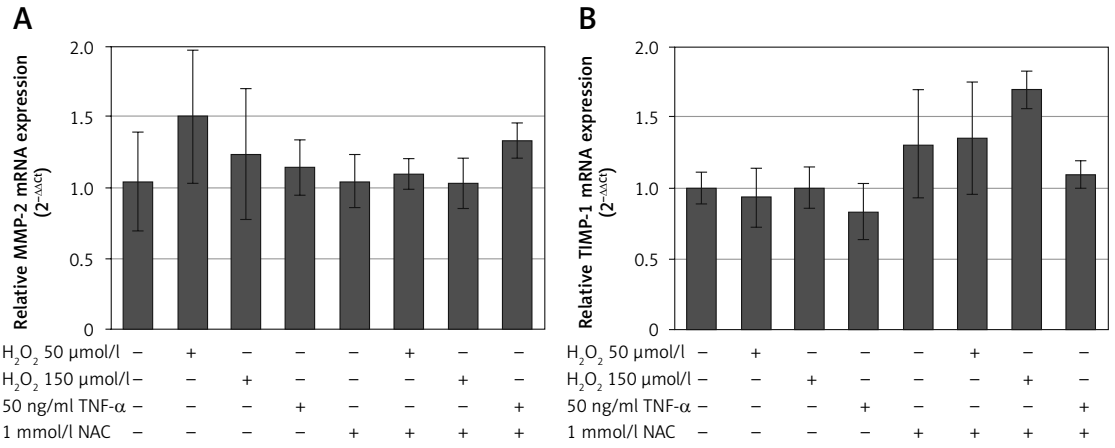

Figure 1. *MMP-2* (**A**) and *TIMP-1* (**B**) mRNA expression levels in HASMCs subjected to oxidative stress. HASMCs were incubated for 24 h with H_2O_2 (50 µmol/l or 150 µmol/l), 50 ng/ml TNF-α or 1 mmol/l NAC, either alone or in combination. Untreated cells constituted the control group. The mRNA levels were measured by real-time PCR. Histograms indicate mean ± SD

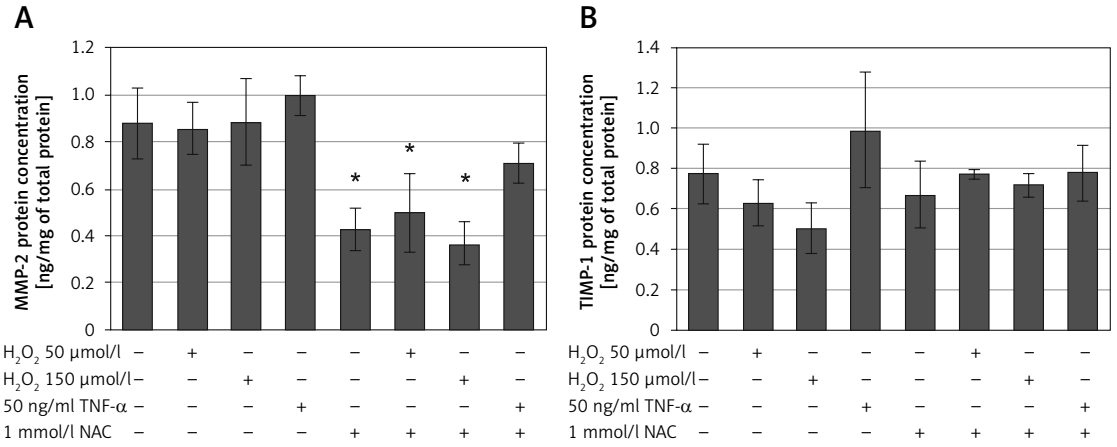

Figure 2. MMP-2 (A) and TIMP-1 (B) protein secretion into the culture supernatants from HASMCs subjected to oxidative stress. HASMCs were incubated for 24 h with H_2O_2 (50 µmol/l or 150 µmol/l), 50 ng/ml TNF-α or 1 mmol/l NAC, either alone or in combination. Untreated cells constituted the control group. The protein levels were measured by ELISA. Histograms indicate mean ± SD. *$p < 0.05$ vs. control (according to Tukey's *post-hoc* test after one-way ANOVA)

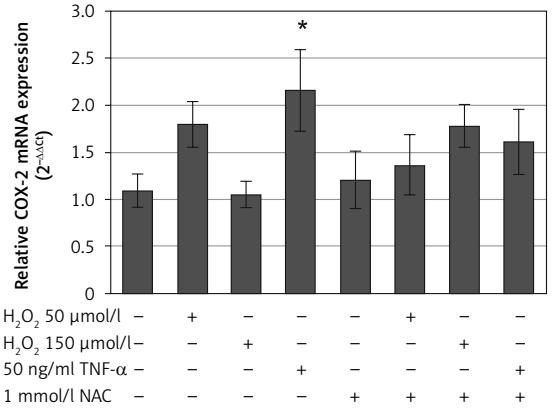

Figure 3. *COX-2* mRNA expression level in HASMCs subjected to oxidative stress. HASMCs were incubated for 24 h with H_2O_2 (50 µmol/l or 150 µmol/l), 50 ng/ml TNF-α or 1 mmol/l NAC, either alone or in combination. Untreated cells constituted the control group. The mRNA levels were measured by real-time PCR. Histograms indicate mean ± SD. *$p < 0.05$ vs. control (according to Tukey's *post-hoc* test after one-way ANOVA)

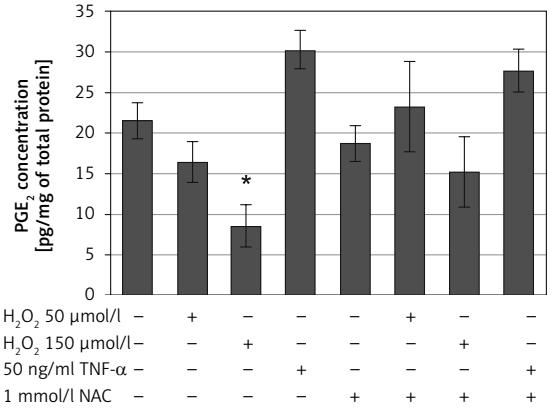

Figure 4. Concentration of prostaglandin PGE_2 in the cell culture supernatants. HASMCs were incubated for 24 h with H_2O_2 (50 µmol/l or 150 µmol/l), 50 ng/ml TNF-α or 1 mmol/l NAC, either alone or in combination. Untreated cells constituted the control group. Prostaglandin levels were measured by ELISA. Histograms indicate mean ± SD. *$p < 0.05$ vs. control (according to Tukey's *post-hoc* test after one-way ANOVA)

The activity of COX-2 enzyme was determined by measuring the level of its reaction product PGE_2, being released outside the cells. Figure 4 shows that treatment of cells with TNF-α increases the activity of COX-2 enzyme by nearly 40%, but the difference is statistically insignificant. In contrast, H_2O_2-induced cells secreted much less PGE2 into cell culture medium than control cells (by about 60% for 150 µmol/l H_2O_2, $p = 0.003$). Preincubation of H_2O_2-treated cells with NAC abolished this effect.

The effect of COX-2 inhibition on expression of MMP-2 and TIMP-1

Inhibition of cyclooxygenase 2 activity using the selective COX-2 inhibitor DuP-697 did not result in changes in *MMP-2* and *TIMP-1* mRNA expression or protein activity measured as the amount of PGE2 secreted outside the cells (Figure 5).

Discussion

Although the influence of oxidative stress on metalloproteinases expression and activity has been studied from many years, the results have not provided conclusive information in this area. Several published articles indicate that ROS contribute to the induction of expression and activity of various metalloproteinases [15]. Here, we investigated the effects of oxidative stress originating from different sources on the expression of MMP-2 and its inhibitor TIMP-1 in human aortic

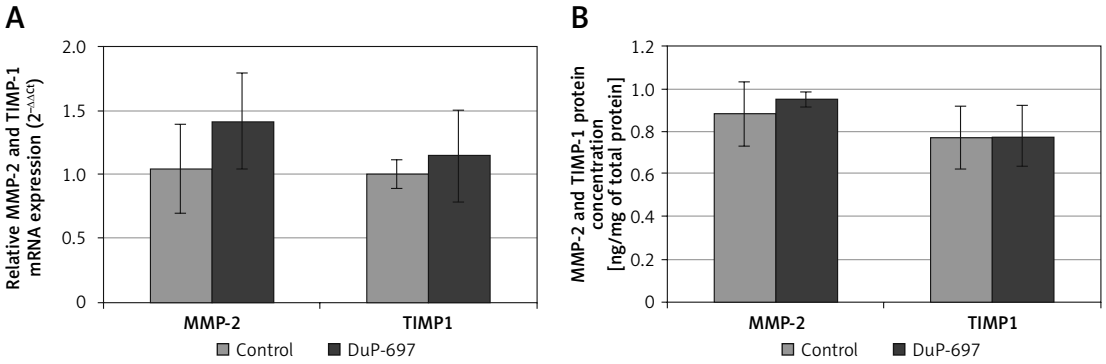

Figure 5. The effect of COX-2 inhibition by DuP-697 on expression of MMP-2 and TIMP-1 at the level of mRNA (**A**) and protein (**B**) release. HASMCs were incubated for 24 h with 10 nmol/l DuP-697. Cells without treatment constituted the control group. The mRNA levels were measured by real-time PCR and the protein secretion into the culture supernatant using ELISA. Histograms indicate mean ± SD

smooth muscle cells. Vascular smooth muscle cells are the cellular components of the normal blood vessel wall that maintain structural integrity and regulate vascular tone. SMCs produce the majority of the extracellular matrix (ECM) structural constituents as well as a large number of proteolytic enzymes (such as matrix metalloproteinases) responsible for the balance between synthesis and degradation of the ECM.

In this research HASMCs were subjected to exogenously supplied hydrogen peroxide or TNF-α in order to assess their influence on the mRNA expression and protein secretion of MMP-2 and TIMP-1. Hydrogen peroxide is thought to be the principal redox signaling molecule in many biological processes such as cell proliferation and differentiation, tissue repair, inflammation, circadian rhythm and aging [16]. This non-radical ROS can modulate gene expression through the regulation of synthesis, stability, subcellular localization and activity of many various transcription factors [17]. H_2O_2 may also influence protein activity via reversible oxidation of its targets including protein tyrosine phosphatases, protein tyrosine kinases, receptor tyrosine kinases and transcription factors [18].

The literature data concerning the regulation of MMP-2 expression level and activity by hydrogen peroxide are ambiguous. It is determined that H_2O_2 increases MMP-2 activity in endothelial cells [19], fibroblasts [20, 21], dental pulp cells [22], cardiomyocytes [23] or vascular smooth muscle cells [24], whereas other results show the inhibitory effect of H_2O_2 on MMP-2 activity [25]. The discrepancies may depend on the hydrogen peroxide concentration used, as was previously observed [26, 27]. Zhang *et al.* [27] examined the effect of 0.2–0.8 mmol/l H_2O_2 on MMP-2 mRNA level in human uterosacral ligament fibroblasts and they found a decrease in MMP-2 mRNA at 0.2 mmol/l and an increase at 0.8 mmol/l. Treatment of the cells with 0.4 mmol/l H_2O_2 did not cause any significant effect in that experiment. In our research we did not find any relevant difference either in *MMP-2* mRNA level or protein release after stimulation of the HASMCs with H_2O_2 at a concentration of 50 and 150 µmol/l. Therefore, extended studies with a wider range of H_2O_2 concentration are still required to obtain more consistent results.

To date, there have been a few studies concerning the involvement of ROS in modulation of TIMP-1 level, but they did not give clear results. In the study conducted by Hemmerlein *et al.* [28] *TIMP-1* mRNA levels remained unaffected in renal cell carcinoma subjected to H_2O_2. Farrokhi *et al.* [29] reported that H_2O_2 diminished the *TIMP-1* mRNA level in vascular smooth muscle cells after 48 h of incubation, but did not cause any changes in *TIMP-1* expression when incubation with H_2O_2 lasted for 24 h. The results are consistent with our observation after 24 h of incubation of HASMCs with H_2O_2.

In this project HASMCs were also stimulated with 50 ng/ml TNF-α, which is known to induce endogenous production of H_2O_2 [30–32]. The results obtained here did not show any significant differences in *MMP-2* and *TIMP-1* mRNA quantity or protein secretion in comparison to untreated control cells. Previous studies conducted by other authors gave divergent results. TNF-α increased *MMP-2* expression and activity in HASMCs in the study by Zhang and Wang [33]. On the other hand, this cytokine had a negligible effect on MMP-2 secretion by myometrium SMCs [34], Fanconi anemia fibroblasts [35] and human melanoma A-2058 cells [36]. Therefore, further studies are needed to better understand these relationships.

Although we did not observe any influence of H_2O_2 or TNF-α on the *MMP-2* and *TIMP-1* expression levels, we noted significantly diminished MMP-2 protein secretion by HASMCs in all groups of cells pretreated with 1 mmol/l N-acetylcysteine. Simultaneously, the *MMP-2* mRNA quantity remained unchanged. There are several published

data proving that various antioxidants and sulfurous compounds may directly inhibit some metalloproteinases [37–40]. NAC contains abundant cysteine residues which can interfere with the process of the "cysteine switch" during MMP activation [41].

Additionally, in this research we examined the influence of oxidative stress on the amount of *COX-2* mRNA and protein activity. We found that H_2O_2 at concentration of 150 µmol/l markedly lowered the PGE_2 secretion from HASMCs, but did not affect the *COX-2* mRNA level. On the other hand, in the cells subjected to TNF-α we observed significantly elevated mRNA and enzyme activity, which may suggest different mechanisms of action of these two compounds in HASMCs. Similarly to our results, the stimulating effect of TNF-α on *COX-2* mRNA and activity has been observed in several other studies conducted on myofibroblasts [42, 43], human FDC-like cell line [44], follicular dendritic cells [45] or human umbilical vein endothelial cells [46]. However, our results demonstrating the influence of hydrogen peroxide on *COX-2* mRNA expression and protein release from the HASMCs are not consistent with previously published data which generally report upregulation of COX-2 upon H_2O_2 treatment in cell lines [47, 48] and animal models [49–51]. This could suggest that the effect may depend on the cell types or H_2O_2 concentration.

Since the current literature demonstrates that COX-2 may be involved in the upregulation of some metalloproteinases [52–54], we attempted to determine a possible relationship between COX-2 activity and MMP-2 and TIMP-1 expression level in HASMCs. For this purpose we measured the concentration of mRNA and protein released from the cells after their pretreatment with the COX-2 selective inhibitor DuP-697. However, the obtained results show no changes in MMP-2 and TIMP-1 expression in these conditions.

Nonetheless, the findings of the research should be interpreted in light of certain limitations. One of them is not taking into account the possibility of coexistence of additional mechanisms stimulating endogenous H_2O_2 production or TNF-α activation. So, in further studies, the levels of H_2O_2 and TNF-α should be measured in cells before and after cells' treatment. The next limitation is the narrow concentration range of tested compounds and lack of analysis showing the effect depending on time of cell incubation. Such experiments could give better insight into the investigated process.

In conclusion, in this study we did not find any effect of oxidative stress, represented by H_2O_2 treatment, or its endogenous generation after exposure of HASMCs to TNF-α on the expression of MMP-2 and TIMP-1. However, we observed markedly reduced MMP-2 secretion in cells incubated with NAC. Moreover, HASMCs stimulated with TNF-α demonstrated significant increases in COX-2 mRNA level and enzyme activity. But there was no correlation between COX-2 activity and MMP-2 and TIMP-1 mRNA expression or protein secretion. We anticipate that the obtained results will provide important insights for understanding the effects of ROS on the ECM remodeling components.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

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