Physiology International, Volume 106 (1), pp. 29–38 (2019)
DOI: 10.1556/2060.106.2019.05

Homocysteine-induced inverse expression of tissue factor and DPP4 in endothelial cells is related to NADPH oxidase activity

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Received: November 9, 2018
Accepted: February 2, 2019

Purpose: We previously found that homocysteine (Hcy)-induced apoptosis in endothelial cells coincided with increased NADPH oxidase (NOX) activity. In addition, in ischemic endothelial cells present in the heart, we showed that loss of serine protease dipeptidyl peptidase IV (DPP4) expression was correlated with induction of tissue factor (TF) expression. Since Hcy can initiate thrombosis through the induction of TF expression, in this study, we evaluated whether the inverse relation of TF and DPP4 is also Hcy-dependent and whether NOX-mediated reactive oxygen species (ROS) is playing a role herein. *Methods:* Human umbilical vein endothelial cells (HUVECs) were incubated with 2.5 mM Hcy for 3 and 6 h. The effects of Hcy on DPP4 and TF expression and NOX2/p47^{phox}-mediated nitrotyrosine (ROS) production were studied using digital-imaging microscopy. *Results:* In HUVECs, high levels of Hcy showed a significant increase of TF expression and a concomitant loss of DPP4 expression after 6 h. In addition, NOX subunits NOX2 and p47^{phox} were also significantly increased after 6 h of Hcy incubation and coincided with nitrotyrosine (ROS) expression. Interestingly, inhibition of NOX-mediated nitrotyrosine (ROS) with the use of apocynin not only reduced these effects, but also counteracted the effects of Hcy on TF and DPP4 expression. *Conclusion:* These results indicate that the inverse relation of TF and DPP4 in endothelial cells is also Hcy-dependent and related to NOX activity.

Keywords: homocysteine, tissue factor, DPP4, endothelial cells, NADPH oxidase

Introduction

Elevated homocysteine (Hcy) blood levels constitute a metabolic risk factor for vascular disease (2, 5, 31). Namely, high levels of Hcy promote oxidative stress in the vasculature,

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which has been strongly implicated in the development of atherosclerosis (15, 28, 33, 40, 42, 44) and thrombosis (7, 12, 45). Hcy causes endothelial injury, dysfunction, and activation (17, 24). Moreover, Hcy can initiate a procoagulant state through the induction of tissue factor (TF) expression, inducing thrombosis (14, 25).

Reactive oxygen species (ROS) are known contributors in Hcy-induced injury in endothelial cells (34, 43). Several studies have suggested a role for NADPH oxidase (NOX) enzymes, which are important sources of ROS, in Hcy-induced endothelial injury and/or dysfunction (3, 8, 11, 13). In previous studies, we have shown that Hcy-induced apoptosis in human endothelial cells coincided with NOX activity (38, 39). This coincided with (peri) nuclear NOX2 expression that colocalized with the activating subunit $p47^{phox}$ and local ROS production. In addition, inhibition of NOX-mediated ROS was shown to reduce Hcy-induced apoptosis, suggesting involvement of NOX activity (38, 39).

Previous studies have reported involvement of NOX-mediated ROS in endothelial TF expression. In Human umbilical vein endothelial cells (HUVECs), xanthine (19) and lipopolysaccharide (LPS)-induced ROS (30) were found to increase intracellular TF expression and NOX activity. We previously showed that ischemia induces endothelial TF expression in intramyocardial blood vessels of patients who died of acute myocardial infarction and *in vitro* studies of human endothelial cells (23). This increased TF expression coincided with the loss of serine protease dipeptidyl peptidase IV (DPP4) expression, suggesting an inverse relation that can affect the antithrombotic nature of the endothelium. Furthermore, the ischemia-induced endothelial TF upregulation and DPP4 downregulation could be inhibited by apocynin (apo), an inhibitor of NOX-derived ROS (9, 22), implicating NOX-derived ROS in both events (23).

Since Hcy has been shown to increase TF activity in endothelial cells, in this study, we evaluated TF expression in relation to DPP4 expression and NOX-mediated ROS in HUVECs.

Materials and Methods

Cell culture

HUVECs were isolated from umbilical cords and cultured in Medium 199 (BioWhittaker, Verviers, Belgium) supplemented with 10% heat-inactivated fetal calf serum (BioWhittaker, Verviers, Belgium), 10% heat-inactivated human serum (Sanquin, Amsterdam, The Netherlands), 5 μg/ml heparin (Leo Pharma BV, Breda, The Netherlands), 50 μg/ml endothelial cell growth factor (Sigma, St. Louis, MO, USA), 100 IU/ml penicillin (Yamanouchi Europe BV, Leiderdorp, The Netherlands), and 100 μg/ml streptomycin (Radiopharma-Eisiopharma, Palomonte, Italy) at 37 °C in a humidified 5% CO_2/95% air atmosphere. Experiments were performed after two passages and at 100% confluence of the cells. The cells were incubated for 3 or 6 h with 2.5 mM D,L-Hcy (Sigma). Apo (100 μM; Sigma) was used to inhibit NOX-derived ROS. The duration and/or concentration of Hcy and apo was based on our previous study (38).

Detection of DPP4, TF, NOX2, $p47^{phox}$, and nitrotyrosine expression

HUVECs were grown in four-well chamber slides (Nalge Nunc International, Naperville, IL, USA). After treatment, the cells were fixed with 4% formaldehyde for 10 min at 37 °C and subsequently permeabilized with acetone–methanol (70%–30%) for 10 min at −20 °C. The cells were then incubated with the primary antibodies monoclonal mouse-anti-CD26 (DPP4) (1:50; Serotec, Oxford, UK), monoclonal mouse-anti-CD142 (TF) (1:50; Acris, Herford,

Germany), polyclonal rabbit-anti-gp91^{phox} (NOX2) (1:50; Upstate, North Billerica, MA, USA), polyclonal goat-anti-p47^{phox} (1:50; Santa Cruz Biotechnology Inc., Heidelberg, Germany), and polyclonal rabbit-anti-nitrotyrosine (ROS) (1:50; Invitrogen, Carlsbad, California, USA), as an indirect marker of ROS production (10), for 60 min at room temperature followed by incubation overnight at 4 °C. The next day, the cells were washed and incubated with the secondary antibodies Cy3-labeled goat-anti-mouse (1:40; Invitrogen), Cy5-labeled goat-anti-rabbit (1:40; Jackson Immuno Research, West Grove, PA, USA), and Cy3-labeled donkey-anti-goat (1:40; Invitrogen) for 30 min at room temperature. Negative controls with only secondary antibody were included to assess non-specific binding. All negative controls showed no staining (data not shown).

Before visualization, mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; H-1500, Vector Laboratories Inc., Burlingame, CA, USA) was added to visualize nuclei and the cells were covered.

Digital-imaging fluorescence microscopy

Digital images were acquired and analyzed with a 3I Marianas^{TM} digital-imaging microscopy workstation (Zeiss Axiovert 200 M inverted microscope; Cark Zeiss, Sliedrecht, The Netherlands) equipped with a nanostepper motor (Z-axis increaments: 10 nm) and a thermoelectrically cooled EM-CCD camera (QuantEM: 512C, 512 × 512 pixels; Photometrics, Tucson, AZ, USA). Exposures, objectives, and pixel binning were automatically recorded with each image and stored in memory (Dell Dimension workstation: 3.0 GHz Xeon dual processor, 4 GB RAM). The microscope, camera, and all other aspects of data acquisition as well as data processing were controlled by Slidebook^{TM} software (version 4.2; Intelligent Imaging Innovations, Denver, CO, USA).

Quantification of mean intensity of fluorescence was performed based on software-generated, threshold-derived cell masks (regions of interests). For the analyses of nuclei, the masks were set on DAPI and for the cytoplasm the cell edges were drawn manually where after the nuclei were deducted from this. Ten images per condition were used on average and only cells that were fully visualized were included in the analyses.

Statistical analyses

The GraphPad Prism program (GraphPad Prism 6, GraphPad Software Inc., San Diego, CA, USA) was used for statistical analysis. To evaluate whether observed differences were significant, one-way analysis of variance with *post hoc* Tukey's test was used. All values are expressed as mean ± standard deviation. The p values (two-sided) ≤ 0.05 were considered significant.

Results

TF and DPP4 expression in Hcy-treated HUVECs

In order to investigate the effect of Hcy on TF and DPP4 expression in endothelial cells, we assessed the presence and (sub)cellular localization of TF and DPP4 in HUVECs that were incubated with 2.5 mM Hcy for 3 and 6 h using digital-imaging microscopy.

We found in control cells a low basal presence of TF (Fig. 1A–I). After 3 and 6 h of Hcy incubation, an increased presence of TF in the cytosol and plasma membrane was detected (Fig. 1A–II and III). Quantification showed a significantly increased expression of TF after 6 h of Hcy stimulation compared to both control cells and 3 h of Hcy stimulation: 125.6% ± 19.1% ($p < 0.05$; Fig. 1B).

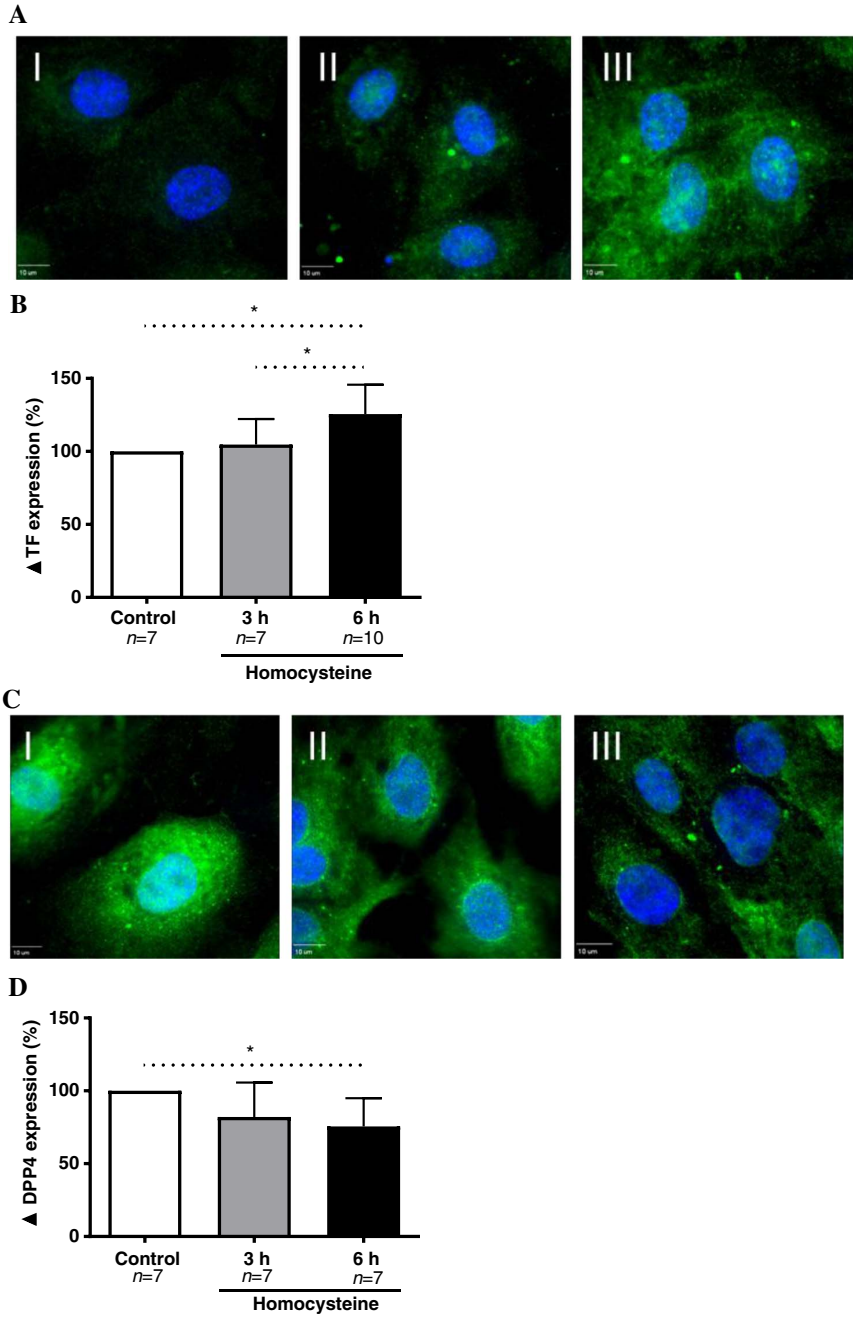

Fig. 1. TF and DPP4 expression in Hcy-treated HUVECs. Digital-imaging microscopy analysis of TF (A and B) and DPP4 (C and D) in fixed and permeabilized HUVECs, subjected to control conditions or subjected to 3 or 6 h of Hcy incubation. Presence of TF and DPP4 (light signal) (A and C) in control (I), 3 h (II), and 6 h (III) of Hcy incubation. Nuclei were stained with DAPI (light signal). Original magnification: 40×. Quantification of TF (B) and DPP4 (D) expression. The results are depicted as the difference in mean intensity using Slidebook^{TM} software analysis. The changes are shown as the difference (Δ) in the percentage compared to control cells set to 100%. *Corrected $p \leq 0.05$

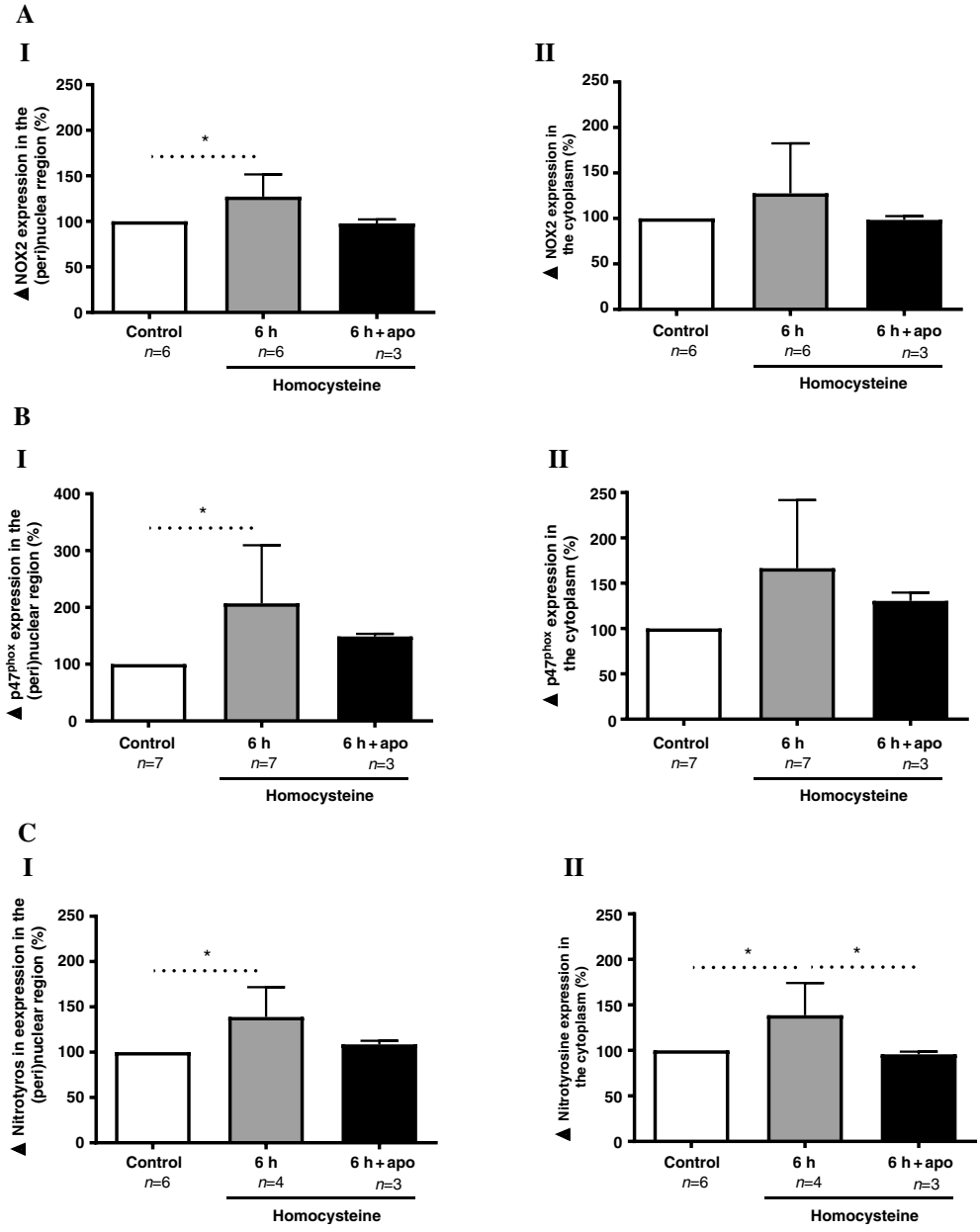

Fig. 2. NOX2, p47^{phox}, and nitrotyrosine expression in Hcy-treated HUVECs in the presence or absence of NOX inhibitor apocynin. Digital-imaging microscopy analysis of NOX2 (A), p47^{phox} (B), and nitrotyrosine (C) in the (peri) nuclear region (I) as well as in the cytoplasm (II) in fixed and permeabilized HUVECs, subjected to control conditions or subjected to 6 h of Hcy incubation with or without the presence of NOX inhibitor apocynin. The results are depicted as the difference in mean intensity using Slidebook^{TM} software analysis. The changes are shown as the difference (Δ) in the percentage compared to control cells set to 100%. *Corrected $p \leq 0.05$

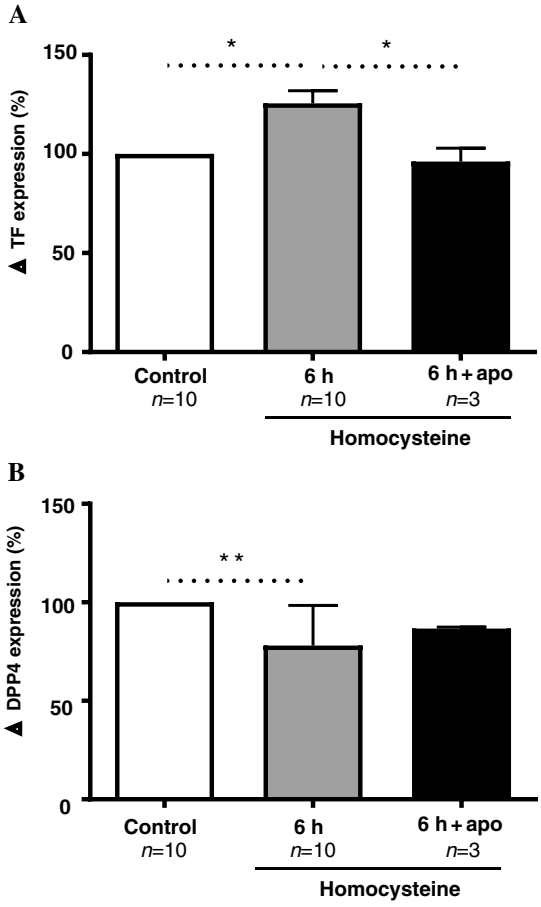

Fig. 3. TF and DPP4 expression in Hcy-treated HUVECs in the presence or absence of NOX inhibitor apocynin. Digital-imaging microscopy analysis of TF (A) and DPP4 (B) expression in fixed and permeabilized HUVECs, subjected to control conditions or subjected to 6 h of Hcy incubation with or without the presence of the NOX inhibitor apocynin. The results are depicted as the difference in mean intensity using Slidebook™ software analysis. The changes are shown as the difference (Δ) in the percentage compared to control cells set to 100%. *Corrected $p \leq 0.05$. **Corrected $p \leq 0.01$

In contrast with TF, in control cells, the presence of DPP4 in the cytosol and plasma membrane was found to be abundant (Fig. 1C–I), which was reduced after 3 and 6 h of Hcy stimulation (Fig. 1C–II and III). Quantification of DPP4 expression showed a significant decrease after 6 h of Hcy incubation compared to control cells with 75.6% ± 17.9% ($p < 0.05$; Fig. 1D).

NOX activity in relation to TF and DPP4 expression

It is known that ROS, which are produced by NOX2/p47^{phox} under stimulation of Hcy (38, 39), can be involved in TF expression in endothelial cells (19). Therefore, we analyzed whether NOX2-mediated ROS are involved in the Hcy-induced TF upregulation and DPP4 downregulation in HUVECs. For this purpose, we analyzed the effect of Hcy after 6 h in the absence or presence of the NOX inhibitor apo on NOX2, p47^{phox}, the indirect ROS marker nitrotyrosine (10), TF, and DPP4 expression using digital-imaging microscopy.

In line with our previous findings (38), the results of the present study revealed that Hcy incubation after 6 h induced a significant increase in NOX2, p47^{phox}, and nitrotyrosine expression in the (peri)nuclear region with 127.3% ± 22.2%, 207.2% ± 94.8%, and 138.9% ± 28.5% ($p < 0.05$), respectively (Fig. 2A and C–I), and also significant increase in nitrotyrosine expression in the cytoplasm with 138.6% ± 30.6% ($p < 0.05$; Fig. 2C–II),

compared to control. NOX2 and $p47^{phox}$ were also slightly increased in the cytoplasm after 6 h of Hcy incubation; however, this elevation of NOX2 and $p47^{phox}$ was statistically not significantly different compared to control.

In addition, the Hcy-induced NOX2, $p47^{phox}$, and nitrotyrosine induction could be counteracted by the presence of apo (Fig. 2). This effect was only significant for the nitrotyrosine expression in the cytoplasm with $95.8\% \pm 38.0\%$ ($p < 0.05$; Fig. 2C–II), compared to 6 h of Hcy stimulation.

Furthermore, apo also counteracted the effects of Hcy on TF and DPP4 expressions (Fig. 3). Again, Hcy stimulation induced a significant increase in TF expression to $125.6\% \pm 19.1\%$ of controls ($p < 0.01$) and a significant decrease in DPP4 expression to $78.1\% \pm 19.3\%$ of controls ($p < 0.01$) after 6 h (Fig. 3A and B). This Hcy-induced increase in TF expression was significantly and completely reduced by apo to $96.2\% \pm 9.5\%$ of controls ($p < 0.05$), whereas the DPP4 expression was completely restored, although this was not statistically different.

Discussion

Elevated blood levels of Hcy form a vascular risk factor due to adverse effects on platelets, clotting factors, and the endothelium (7). ROS are important mediators in the progression of Hcy-induced vascular disease, in which NOX, as a major source of ROS, has been suggested to play an important role (34, 43).

We observed high levels of Hcy-induced TF expression in HUVECs and a concomitant loss of DPP4 expression. Both these effects appeared to depend on NOX-derived nitrotyrosine [indicative for ROS (10)], as apo counteracted both events. The effect of Hcy in increasing TF expression has previously been demonstrated in endothelial cells. Hcy has been shown to increase TF mRNA levels *in vitro* in bovine aortic endothelial cells at 4 h and to increase functional activity at 6 h (37). In HUVECs, Hcy treatment has been demonstrated to increase TF activity at 8 h (6, 14), suggesting altered endothelial cell function.

We previously showed, in the hearts of patients with myocardial infarction and in ischemic endothelial cells *in vitro*, that loss of DPP4 expression was correlated with induction of TF expression (23). The inverse relation of TF and DPP4 was suggested to affect the antithrombotic nature of the endothelium, as TF may behave as a procoagulant protein (26, 35) and DPP4 as an anticoagulant protein (18, 29, 32).

We previously implied a direct relation between TF expression and DPP4 activity in ischemic endothelial cells, because the inhibition of DPP4 activity by diprotin A induced TF expression and induced the adherence of platelets under flow conditions (23). However, prior to our present results, the relation of Hcy-induced TF expression to DPP4 expression was largely unknown.

DPP4 is expressed by endothelial cells and is involved in a wide range of biological functions (27, 32). DPP4 has been demonstrated to be involved in inflammatory and immune responses (1, 41), angiogenesis (4,21), and extracellular matrix degradation (36). It has already been suggested that DPP4 also can behave as an anticoagulant protein, which degrades fibrin (18, 29). Elevated levels of Hcy in patients with established atherosclerotic disease are related to markers of coagulation activation and endothelial cell activation (e.g., fibrinogen, fragment F1+2, factor VII, factor VIIa, factor XIIa, and von Willebrand factor) (15, 16, 20, 28, 42, 45), reflecting a hypercoagulable state. Our present demonstration of Hcy-induced TF repression and Hcy-reduced DPP4 expression is related to a

prothrombotic state of the endothelium. Both TF and DPP4 expressions appear to depend on NOX-derived ROS, because apo counteracted both events.

Involvement of NOX-mediated ROS in TF expression has been reported previously, and our present results show the effects of Hcy on TF expression. In HUVECs, xanthine-induced ROS was previously found to increase intracellular TF expression that coincided with increased NOX activity (19). A role for NOX-mediated ROS in the induction of TF expression in LPS-stimulated HUVECs was also suggested (30). In a study by Miyoshi et al. (30), increased NOX1 mRNA levels were found, whereas no differences were found in NOX2 or NOX4 mRNA levels. In the present study, we found that Hcy increased NOX2, $p47^{phox}$, and ROS using the indirect ROS marker nitrotyrosine (10), which was also reduced using apo. Apo acts by inhibiting the association of $p47^{phox}$ with the membrane-bound components of NOX (9, 22). On the whole, a role for NOX2 in the effect of Hcy on TF and DPP4 may therefore be suggested. However, we cannot exclude that other NOX isoforms or ROS sources may also play a role.

In conclusion, our results indicate that the inverse relation of TF to DPP4 in endothelial cells is Hcy-dependent and is related to NOX activity.

Acknowledgements

This study was financed by the Dutch Burns Foundation (DBF), Beverwijk, Project 13.104.

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