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Doxorubicin triggers endothelial dysfunction by downregulating KLF2 expression: Strategies for pharmacologic intervention.

Master thesis of F.J. Voskuil, s1862294, in partial fulfillment of the requirements for the degree of Master of Science

Department of Pathology, Center for Excellence in Vascular Biology, Brigham and Women's Hospital, Harvard Medical School, Boston, USA

Supervisor Boston, USA
S.G.Tullius, MD PhD
Chief Division of Transplant Surgery
Associate Professor Harvard Medical School
Harvard Medical School, USA
stullius@partners.org

Supervisor Groningen, the Netherlands
Prof. Dr. W.J. van Son
Member of Gips-M
Interne Geneeskunde
University of Groningen,
The Netherlands
w.j.van.son@umcg.nl

Supervisor Boston, USA
G. Garcia-Cardena, PhD
Associate Professor of Pathology
Harvard Medical School, USA
Director, Laboratory for Systems Biology
Brigham and Women's Hospital
ggarcia-cardena@rics.bwh.harvard.edu

Supervisor Groningen, the Netherlands
Dr. H.G.D. Leuvenink
Principal Investigator
Surgical Research Laboratory
University of Groningen,
The Netherlands
h.g.d.leuvenink@umcg.nl

Abstract

Introduction

Doxorubicin (DOX) is a widely used chemotherapeutic agent. Despite its high anticancer capacities, DOX is notorious for its strong side effects. Common DOX associated side effects are cardiotoxicity and palmar-plantar erythrodysesthesia (PPE). Recently, it's determined that DOX causes endothelial injury, however, the exact mechanisms remain unknown. Since it's known that Kruppel-like factor 2 (KLF2) plays a crucial role in maintaining endothelial function, there might be a potential role for KLF2 in the DOX-induced side effects.
In this study, we examined the role of KLF2 in the DOX-induced endothelial damage, and the possibility for pharmacological intervention by statins.

Materials and Methods

Primary human umbilical vein endothelial cells (HUVECs) were used to determine the expression of KLF2 and its target genes after DOX treatment. To investigate if DOX treatment leads to apoptosis in ECs, we used both a MTT and flow cytometry assay to quantify apoptosis in DOX-treated cells. In addition, we evaluated the expression of vasoprotective genes under vasoprotective shear stress conditions, using a previously developed Dynamic Flow System (DFS). Moreover, we evaluated the effect of Simvastatin treatment prior and simultaneously on DOX treatment.

Results

The expression of KLF2 and its vasoprotective transcriptional target genes, as well as other endothelial vasoprotective genes were significantly altered after DOX treatment at different concentrations under static culture conditions. The MTT assay showed that there was no significant difference between the used concentrations and the vehicle in regard to cell death. In addition, flow cytometry analyses documented that there was no difference in apoptotic cells between DOX treated and untreated cells. The expression of KLF2 in the vasoprotective shear stress environment was curtailed after DOX treatment, and angiopoietin-2 (Ang-2) was strongly upregulated. Importantly, we also showed that the effect of DOX on vasoprotective genes can be rescued after statin treatment.

Conclusions

DOX treatment in HUVECs leads to an important change in the expression of vasoprotective genes. Moreover, we show that statins could rescue the gene alterations when administrated simultaneously, which might be an easy implementable addition to DOX treatment in the clinical setting. However, *in vivo* experiments, and human trials need to be done to draw final conclusions.

Samenvatting

Introductie

Doxorubicine (DOX) is een vaak gebruikt chemotherapeuticum. Ondanks de goede anti-kanker eigenschap is DOX berucht om de endothiale toxiciteit dat het veroorzaakt. Cardiotoxiciteit en de palmar-plantar-erythrodysesthesia (PPE) zijn vaak geobserveerde bijwerkingen na behandeling met DOX. Recentelijk is het ontdekt dat DOX endotheleel schade veroorzaakt, echter, het exacte mechanisme blijft onduidelijk. Sinds het bekend is dat KLF2 een belangrijke rol speelt in het behoud van endotheel functie, is er een mogelijke rol voor KLF2 in de DOX veroorzaakte bijwerkingen. In deze studie hebben we de rol van KLF2 onderzocht in de DOX geïnduceerde endotheel schade. Daarnaast hebben we gekeken naar de mogelijkheid om dit effect te kunnen blokkeren doormiddel van statines toe te dienen.

Materiaal en Methoden

Primaire human umbilical vein endothelial cells (HUVECs) zijn gebruikt om de expressie van KLF2 en de door KLF2 gereguleerde genen te bepalen. Om te onderzoeken of DOX behandeling leidt tot apoptose, hebben we zowel een MTT test als een flow cytometry assay uitgevoerd om het aantal apoptotische cellen te bepalen. Daarnaast hebben we de expressie van vasoprotectieve genen in endotheel cellen bepaald na behandeldeling met DOX tijdens blootstelling aan een vasoprotectieve, shear stress omgeving. Hierbij werd gebruikt gemaakt van een eerder ontwikkelend Dynamic Flow System (DFS). Tevens hebben we het effect van Simvastatine behandeling voor en tijdens DOX behandeling bepaald.

Resultaten

De expressie van KLF2 en de door KLF2 gereguleerde vasoprotectieve genen, en tevens andere vasoprotectieve genen bleek significant veranderd na behandeling met DOX in verschillende concentraties onder statische groei condities. De MTT test liet zien dat er geen sprake is van apoptotische cellen wanneer de verschillende concentraties vergeleken worden met de controle groep. Daarnaast liet ook de flow cytometry test zien dat er geen sprake was van apoptose in de DOX behandelde groep. De expressie van KLF2 na DOX behandeling in de vasoprotectieve shear stress omgeving was zelfs meer verlaagd vergeleken met de statische condities. Ditzelfde gold voor de toename in Angiopoietin 2 (Ang-2) expressie. Daarnaast hebben we aangetoond dat Simvastatine het effect van DOX op vasoprotectieve genen kan beperken.

Conclusies

DOX behandeling leidt tot belangrijke verandering in de expressie van vasoprotectieve genen. Daarnaast tonen we aan dat statines deze verandering kunnen beperken wanneer ze tegelijkertijd worden toegediend. Dit zou een makkelijk uitvoerbare toevoeging kunnen zijn aan DOX behandeling in de kliniek. Desalniettemin moeten er *in vivo* experimenten worden uitgevoerd om definitieve conclusies te kunnen trekken.

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List of abbreviations

DOX: Doxorubicin
EC: Endothelial cell
PPE: Palmar-plantar erythrodysesthesia
CHF: Congestive heart failure
ROS: Reactive oxygen species
CVD: Cardiovascular disease
KLF2: Kruppel-like factors 2
Ang-2: Angiopoietin-2
ET-1: Endothelin 1
VCAM-1: Vascular cell adhesion molecule
SMC: Smooth muscle cell
HUVEC: Human umbilical vein endothelial cell
MTT: Thiazolyl Blue Tetrazolium Bromide
DFS: Dynamic Flow System
PBS: Phosphate buffered saline
PCR: Polymerase chain reaction
Ang-1: Angiopoietin-1

List of tables and figures

Preface and acknowledgements

This paper is part of my research internship for my study Medicine in Groningen, as well as the GIPS-M P2 program, performed in Boston, USA, at the Brigham and Women's Hospital, Harvard Medical School. Dr. Tullius and Dr. Garcia-Cardena were so kind to give me the great opportunity to perform my research internship at there laboratories. As a result of my past months, I wrote this research report of my experiments.

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Introduction

Doxorubicin (DOX), also known as Adriamycin, is one of the most common used chemotherapeutic drugs for the treatment of leukemia's, sarcomas, and breast cancers (1-3). Besides, although less prescribed, DOX is also used in cancers of the stomach, lungs, ovaries, thyroid as in soft tissue sarcoma's and multiple myeloma's. Commonly used DOX-containing regimens are CA (cyclophosphamide, Adriamycin), TAC (Taxotere, CA), ABVD (Adriamycin, Bleomycin, Vinblastine, Dacarbabzine), CHOP (Cyclophosmphamide, Adriamycin, Vincristine, Prednisone) and FAC (5-Fluorouracil, Adriamycin, Cyclophosphamide) (4).

Historical perspective

The history of DOX goes back to the early 1950's, when both an Italian and French research group, independently began to look into soil-based microbes to find new anticancer compounds at the same time. A soil sample was isolated which contained a new strain of Streptomyces Peucetius. The antibiotic produced from this strain seemed to have a good activity against mouse tumours. Together, both groups named the compound Daunirubicin, where *Dauni* referred to a pre-Roman tribe that occupied the area where the compound was isolated, and ruby, *rubis,* referred to the color (5). From the 1960's, clinical trails began and showed hopeful results in the treatment of acute leukemia and lymphoma. However, while undetected in animal studies (6), by 1967, clinical trails showed that Daunirubicin could lead to fatal cardiac toxicity. After these results, modifications were made to the compound, what resulted in a different, red-colored antibiotic. This compound was named Adriamycin, after the Adriatic Sea, and the name was later changed to Doxorubicin, to establish naming convention. Daunirubicin was known to be highly effective against acute lymphoblastic and myeloblastic leukemia's, while DOX became interesting since it had a much broader anticancer spectrum, which includes several solid tumours in addition to hematological malignancies. DOX showed a better activity to tumours and a higher therapeutic index compared to Daunirubicin. However, the cardiotoxicity remained. From then on, studies established the cumulative dose of anthracyclins, also known as anthracycline antibiotics, the class of drugs where DOX belongs to, as the main risk factor for cardiotoxicity (7,8). Dauni- and Doxorubicin can be seen as the first anthracycline anitibiotics, and today, it is estimated that there are more than 2,000 known analogs of DOX. However, only a few of them are approved for clinical use (9).

Side effects, clinical relevance

Nowadays, DOX is widely used in the treatment of several cancers, both for adults and children. The American Cancer Society estimated that in 2010, 1,150,000 people received DOX as a first choice treatment of breast cancer and soft tissue carcinoma in the US (4).
Often seen side effects of DOX are cardiotoxicity and palmar-plantar erythrodysesthesia (PPE), which could limit the administration, which in turn, might lead to undesirable delays in the treatment. Recently, it's shown that DOX may have serious injurious effects on the vascular endothelium, including apoptosis of endothelial cells (ECs), which leads to endothelial injury(10-12).

Endothelial dysfunction

The last years, there has been a growing interest in the role of DOX on the vascular endothelium. In a recent study of Jenei et al. it's found that anthrycyclines lead to an impaired endothelial and vascular function (13). Besides, it's known that DOX leads to both

cardiomyocyte and endothelial cell apoptosis, which may play an important role in DOX-induced cardiomyopathy (12). This impaired endothelial function is an important and recently recognized toxicity since endothelial dysfunction is an early event in atherogenesis (14). These unfavorable changes can be considered not only as risk factors but as earliest subclinical markers of development of clinically relevant CVD also (13).

Cardiotoxicicty

Cardiotoxicity is a life threatening side effect and depends on the cumulative dosage of DOX, and is divided into four forms. The *acute form* is the most common, with typical complications as vasodilation, hypotension and transient cardiac rhythm disturbances (15), however these symptoms resolve usually shortly after the end of administration. *Subchronic cardiotoxicity* is extremely rare and happens within 1-3 days after administration and occurs as pericarditis-myocarditis. *Early chronic cardiotoxicity* develops weeks to months after the completion of the chemotherapy and is characterized by dilated cardiomyopathy, which can lead to left ventricular contractile dysfunction and congestive heart failure (CHF) (9).
The *delayed cardiotoxicy* due to anthracycline treatment, is known for the fact that it can cause cardiotoxicity even decades after the anthracycline treatment (16-19).
In a recent study from Eckman et al., it's found that DOX treatment leads to increased wall thickness of the medial and adventitial layers in the microcirculatory coronary wall, which, in turn, possibly could lead to myocardial ischemia. In addition to this, it's already shown that anthracycline exposure increases the incidence of vascular events, including myocardial ischemia and stroke (20).
Moreover, although in a small trail, it's determined that even low to moderate dose of DOX treatment can cause left ventricular dysfunction and aortic stiffness early after exposure, and this remained at least 6 months after the treatment was stopped (21).
Despite the fact that authors can not agree on the incidence of DOX-induced cardiotoxicity, it is clearly a major problem in the treated population since numbers vary between 7-26% of patients which develop CHF after treatment of a cumulative dosage of 550 mg/m^2 DOX (8,22). At this moment, there is no curative treatment of this DOX-induced cardiotoxicity besides the aim to prevent existing cardiomyopathy from worsening (23).

Palmar-plantar erythrodysesthesia

PPE is a cutaneous drug reaction that is mostly induced by chemotherapeutic agents (24), and DOX is one of the most common causers. Despite the fact that the actual incidence is very difficult to determine, estimations vary between 22-89% of patients that develop PPE after DOX administration, dependent on different treatment regimens (25-27). Despite the chemotherapeutic agent, the symptoms are very similar, and seem to be dose-dependent. The majority of the patients develop a prodrome of dysesthesia of the palms and soles. Within a few days, this progresses to burning pain with swelling (edema) and erythema. The pain may be so severe that daily activities are limited (24). PPE can occur within days (24h to 2-3 weeks) or after months (2-10 months), dependent on the treatment regimen. The cause of PPE remains unknown, and unfortunately, there is at the time of writing not a proven therapy for PPE other than dose reduction, lengthening of the drug administration interval and ultimately drug withdrawal (24).

Mechanism of action

In today's cancer research, it is an important challenge to understand how DOX works, and what the mechanistic pathways are associated with its therapeutic effects and toxicity. Despite their extensive use, the exact therapeutic mechanism of DOX remains unclear and apparently it is a combination of several different mechanisms, which in turn, account for the high

efficacy of this class of drugs (9). However, recent studies have determined that DOX acts on tumor cells in two independent manners; by the inhibition of topoisomerase I and II, and by the intercalation into the DNA double helix to interfere with its uncoiling, ultimately inducing cell death (28,29).

Two independent groups have shown that these two potential mechanisms might be able to explain the anti-tumour effects of DOX. Zhang et al. showed that Top2b gene lacking mice have a diminished cardiotoxicity after DOX administration (28). They state, in accordance to previous studies, that DOX helps to stabilize the complexes of double-stranded DNA and topoisomerase II enzyme and that topoisomerase seems to be responsible for both the toxicity to normal cells as the tumour cells (Figure 1 right).

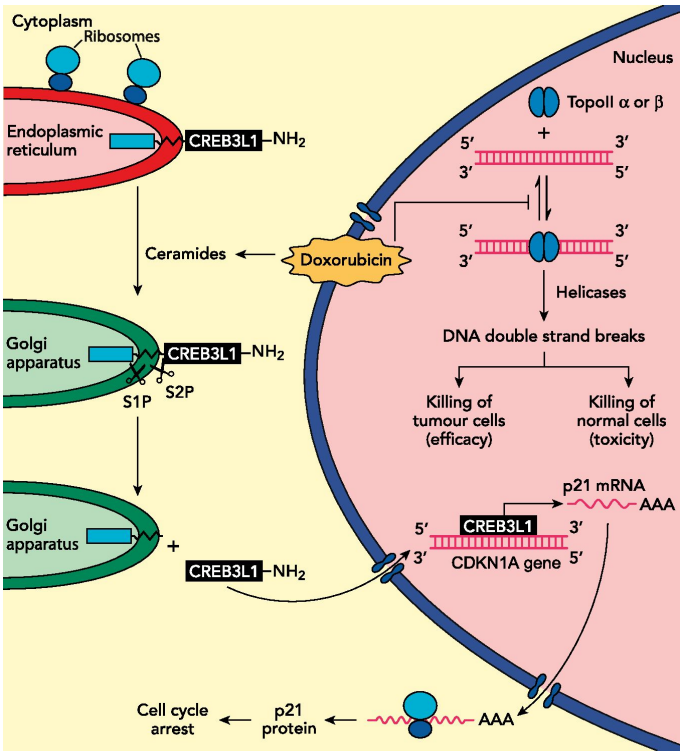


Figure 1:

Right: Topoisomerase II cuts both of the DNA strands, which results in death of both normal cells, mostly through topoisomerase II-*beta*, and tumour cells that are susceptible to the drug, mostly through topoisomerase II-*alfa*.

Left: DOX increases the production of ceramides in the cells, which leads to a translocation of the CREB3BL1 transcription factor. The S1P and S2P proteases then cut the CREB3L1 protein, which leads to the migration of the amino-terminal to the nucleus. Here, it acts as a transcription factor to activate the CDKN1A locus and some additional genes. Ultimately, this leads to an increased p21 protein expression, as well as some other related proteins, that inhibit the proliferation of tumour cells.

Figure adapted from Patel et al. (30).

The second mechanism, determined by Denard et al., involves the fact that DOX treatment leads to an increased production of ceramides inside the cells, which in turn, leads to the translocation of the latent transcription factor CREB3BL1, and ultimately to cell cycle arrest (29) (Figure 1 left).

As both authors conclude, these mechanisms are only the first steps in the discovery of the DOX pathway and the DOX-induced toxicity, which remains not fully understood (30). Furthermore, DNA strand breaks may trigger apoptosis of cancer cells, apparently via the p53-dependent pathway (31).

Another theory is that increased oxidative stress due to reactive oxygen species production (ROS) may explain the undesirable side effects of DOX. Many studies attribute ROS to be a major factor in DOX-induced cardiotoxicity. The type of cell death due to DOX administration is dependent on the dose and cell line, however, previous studies have shown that cardiomyocytes are susceptible for all four types of cell death (apoptosis, autopsy, necrosis and senescence) (32).

However, it remains unclear if oxidative stress acts as the main trigger or the key executor of anthracycline induced cardiotoxicity, and ROS formation might be a secondary consequence of previous cellular and mitochondrial damage (9).

As written, many studies have determined that DOX leads to apoptosis, and therefore when acting in vascular cells may cause vascular leakage (33,34). These studies found that this

endothelial dysfunction may be the result from independent effects on ATP and/or GSH metabolism (33). It's also thought that DOX-induced, metal-catalyzed production of H_2O_2 plays a role both in the toxicity to ECs and cardiomyocytes (35).

Despite the fact that there is a broad consensus about DOX and its cardiovascular side effects, it remains unclear by what mechanism DOX treatment causes endothelial dysfunction. Concluding all the previous work, the shared idea is that DOX induces vascular injury due to cell death.

DOX induces vascular damage, the role of KLF2

It is determined that anthracyclines damage the vascular endothelium, which leads to endothelial dysfunction (10,11,36). This results in a dysfunctional vascular homeostasis, which will lead to edema and compromising organ dysfunction. In regard to this, two recent articles were published which found that DOX causes impaired vascular function in long-term survivors of cancer, which is an early marker of cardiovascular diseases (CVD) (13,37).

Kruppel-like factors (KLFs), are a subclass of the zinc finger transcription family of transcription factors that can regulate multiple cellular functions and tissue development (38). KLF2 is expressed in the endothelium of the vasculature and is required for normal vessel development (39,40). Two of the most powerful known inducers of KLF2 expression in the endothelium are blood flow-derived shear stress and statins (41-43). It's known that KLF2 expression involves activation of a MEK5/ERK5/MEF2 pathway, and that MEK5 activation is necessary for up-regulation of KLF2 in the vascular endothelium (44). Moreover, KLF2 attenuates the cytokine-mediated induction of proinflammatory molecules (e.g. E-selectin and VCAM-1), which induces gene expression of antithrombotic molecules (e.g. Thrombomodulin) (42,45,46).
In contrast, decay in KLF2 expression plays a key role in the loss of endothelial barrier function, which results in increased vascular permeability, tissue edema and inflammation (47). Because the majority of these events are also seen as serious side effects after DOX administration, we suggest that there might be an important role for KLF2 in the vascular changes after DOX treatment.

Statins, also known as HMG-Coa reductase inhibitors, have beneficial effects on atherogenesis in addition to their lipid-lowering action. Examples of these effects include the up-regulation of the production of NO in ECs, decreased proliferation of vascular smooth muscle cells (SMCs), inhibition of platelet activation, and increased fibrinolytic activity (48,49). In the recent past, investigators gained an increased interest for statins in the field of (renal) transplantation, and it's shown that statins can rescue the KLF2 expression after flow cessation (50). Furthermore, it's found that Simvastatin-treated kidneys showed a diminished EC-EC gap formation after transplantation (47).

As written earlier, it's thought that the DOX-induced vascular injury is induced by apoptosis of endothelial cells. In this work we focused on the expression of the endothelial vasoprotective KLF2 gene, and used clinical, non-apoptotic DOX concentrations, to assess endothelial dysfunction instead of endothelial injury. Moreover, we looked for the possibility of pharmacologic intervention using statins. Therefore, our research question was: Does Doxorubicin lead to a downregulated KLF2 expression, and could this alteration be rescued by statins? ***We hypothesize that the DOX-induced vascular dysfunction occurs through a KLF2 dependent mechanism, which could be ameliorated by statins***.

Material and Methods

DOX treatment on HUVECs (analysis of different concentrations)

Primary human umbilical vein endothelial cells (HUVECs) were isolated and cultured as previously described (42,51). Briefly, HUVECs cultured in Medium-199 (BioWhittaker, Lonza Inc, Basel, Switserland) supplemented with 50mg/ml EC growth supplement (BT-203, Biomedical Technologies Inc., Stoughton, MA), 100mg/ml heparin (Sigma, Saint Louis, MO, USA), 100units/ml penicillin plus 100ug/ml streptomycin (BioWhittaker), 2mM L-glutamine (GIBCO, Carlsbad, CA), and 20% fetal bovine serum (BioWhittaker) were plated on 0.1% gelatin (Difco, BD, Sparks, MD)-coated plastic C6 wells and maintained at 37°C 5% CO_2 in the incubator for 24hr of static culture. After 24h, the medium was changed and Doxorubicin Hydrochloride (Sigma-Aldrich, Saint Louis, MO, USA) in DMSO (Sigma-Aldrich, Saint Louis, MO, USA) was added in different concentrations and cultured for 24h.

Analysis of apoptosis of HUVECs after DOX treatment using the MTT assay

To determine if the used concentrations do not lead to apoptosis of the endothelial cells, we performed a MTT cell viability assay. After 24h of static culture in a 96-well plate as described above, we changed the medium and added DOX in different concentrations, including a positive control (0ng/ml, 10 ng/ml, 100ng/ml, 250ng/ml and 2500ng/ml) and cultured ECs again for 24h. After 24h, the medium was aspirated and Thiazolyl Blue Tetrazolium Bromide (MTT) (Sigma-Aldrich, Saint Louis, MO, USA) (5mg/ml in medium) was added. After 3h, the MTT was aspirated and the Formazan (MTT metabolic product) was resuspended in isopropanol for 5min. The absorption was read in a plate reader at 560nm with a 670nm background. Blank wells served as negative control.

Quantification of apoptosis using Annexin V and propidium iodide

To validate and further characterize our apoptosis data, we performed FACS analyses to quantify the apoptosis in ECs after DOX treatment. After 24h of static culture, we changed the medium and added DOX (250ng/ml) and cultured the ECs again for 24h. After 24h, floating cells in the treatment media and in the wash-effluent of DPBS (- Ca^{++}/Mg^{++}) were saved and pooled with the adherent cells that had been removed with a brief treatment of trypsin/EDTA (BioWhittaker) + DPBS. Cells were spun down, resuspended, and double stained with propidium iodide / Annexin V-FITC and counted by flow cytometry according to the manufacturer's instructions (BD Pharmingen 556547).

DOX treatment on HUVECs using a Dynamic Flow System

To mimic physiological conditions, we exposed cells to shear stress using a Dynamic Flow System (DFS). This DFS is a multicomponent cone and plate device that mimics the physiological shear stress in the human vessels. The design consists a microscope stage, with a transparent cone and plate surface, which gives the ability that the cells can be easily visualized under dynamic flow via microscopy. This system is previously developed in our laboratory (42,51).

The machine is able to mimic different waveforms that are generated by the drive system and controlled with microstepper motor technology (Parker Hannifin, Rohnert Park, CA). With software (Compumotor 6000, Rohnert Park, CA), the different waveforms can be simulated, ranging from laminar flow to more complex arterial waveforms.

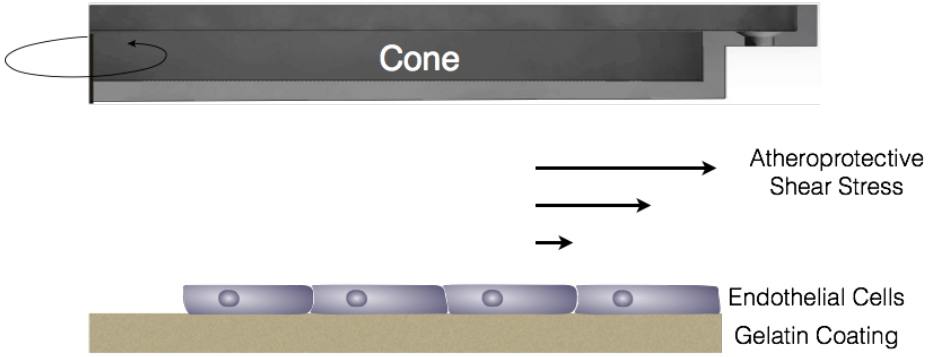


Figure 2: Schematic view of DFS. Endothelial cells, plated on 0.1% Gelatin coating are exposed to atheroprotective flow.

To mimic a physiological environment, the device includes two access ports in the well that permit continues exchange of fresh media. Another benefit of these ports is that there is the ability to introduce pharmacological agents without disturbing the experiment or flow conditions. The wells are heated by an external heater (Atlanti Thermal Co., Hopedale, MA), which controls the temperature at 37°C. The device is covered with a transparent litter which maintains the environment at 5% CO2 in humidified air.

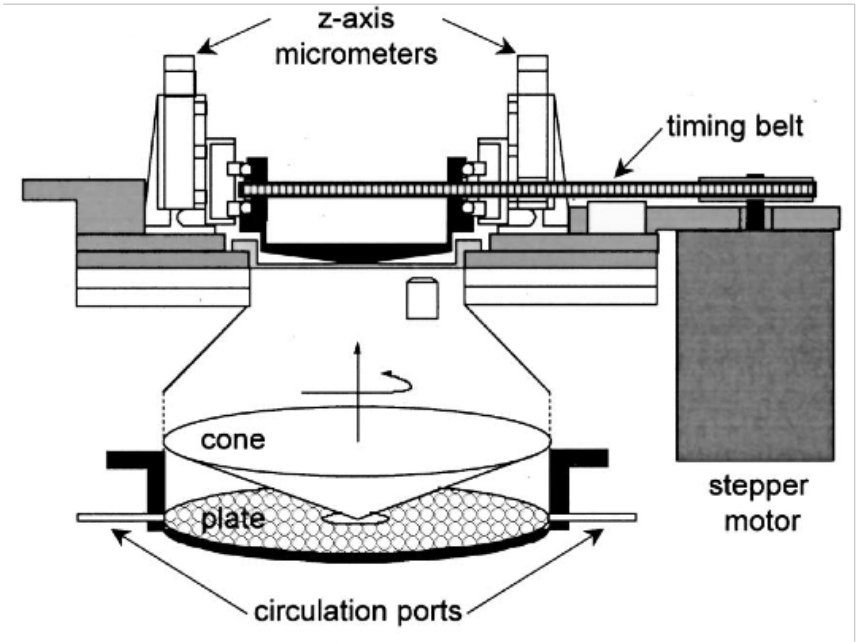


Figure 3: Schematic of the DFS. The DFS consists a transparent cone and plate attached on a microscope stage. The rotation of the cone is controlled by an programmed stepper motor using a timing belt connection.

Figure adapted from Blackman et al. (70).

Primary HUVECs were isolated and cultured as described above. After 24h, the static EC growth medium was replaced for shear medium (*Medium- 199, 20% fetal bovine serum, 2 mM L-glutamine, 100units/mL penicillin plus 100ug/mL streptomycin, and 2% dextran (Sigma)).* To achieve physiologic levels of KLF2 expression in cultured ECs, which is previously shown by our group (42), we exposed ECs to vasoprotective flow (atheroprotective shear stress waveform (42)) using a DFS. After 24h, the shear stress medium was changed for fresh shear stress medium with 250ng/ml DOX and we exposed the ECs to shear stress for another 24h. For each set of experiments, two identical flow devices were used at the same time. One of them was used for the DOX addition, the other one was used a control. At the same time, to plates were cultured under static (no flow) conditions

with the same experimental set up and the same isolate of cells, to compare static vs. shear stress.

Simvastatin treatments

Primary HUVECs were isolated and cultured as described above. After 24h of static culture, culture medium was changed for fresh culture medium with Simvastatin (10uM in DMSO) (Merck KGaA, Darmstadt, Germany). After 24h, medium was exchanged for fresh medium with Simvastatin (10uM) and DOX (250ng/ml).

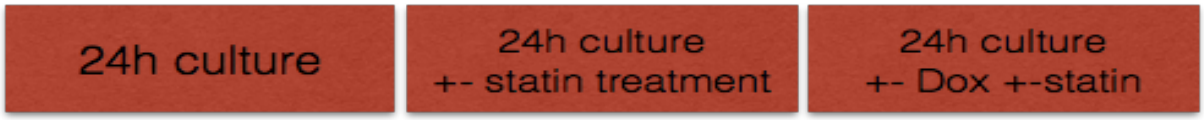


Figure 4: Timeline of static Simvastatin experiment.

RNA isolation and RT-TaqMan PCR Analysis

ECs were rinsed twice with cold phosphate buffered saline (PBS) and the total RNA from ECs was isolated and purified using the Prism Nucleic Acid Prep-Station (Applied Biosystems, Foster City, CA) according to manufacturer's protocol. RNA quality was verified using Agilent's 2100 Bioanalyzer. RNA was reverse transcribed to cDNA using a MultiScribe-based reaction (Applied Biosystems). cDNA templates were amplified by real-time TaqMan polymerase chain reaction (PCR) on an ABI Prism 7900HT Fast Detection System (Applied Biosystems). Expression of KLF2, eNOS, TM, CNP, VEGF, Ang-2, TIE2, PTGDS, ASS, VEGFR2 was analyzed using predesigned gene expression assays obtained from Applied Biosystems according to the manufacturer's protocol and reported relative to endogenous control GAPDH. All PCR reactions were performed in duplicate and using nuclease-free water as no template control.

Statistical analysis

Statistical analysis was performed using Microsoft Excel Software. Student's *t*-test was used for comparing differences between two groups. Differences were considered significant at $P < 0.05$.

Results

DOX treatment affects the expression of KLF2, eNOS and ANGPT2

To determine the effects of DOX treatment on KLF2 expression, we first generated a dose response curve. In patients, the plasma concentration of DOX after administration shows a peak concentration of approximately 500-1500ng/ml, with a rapid decrease in the first hour. During the first hour, the concentration decreases to 150-500ng/ml (52,53). To look into clinical relevant concentrations, without leading apoptosis of the cells, we used DOX concentrations in the range between 10-250ng/ml.
Although it's difficult to choose a single clinical relevant concentration, we assume that this range will cover the plasma concentrations after the first hours of administration. We cultured ECs, and after 24h we added DOX in different concentrations and cultured ECs again for 24h. As shown in Figure 5A, ECs treated with DOX react in a dose dependent manner on KLF2 expression compared to the vehicle. Simultaneously, we looked for several other genes that play a role in the vasoprotective phenotype of the vessels. eNOS mRNA levels, a downstream-regulated target gene of KLF2, are also significantly decreased by DOX (Figure 5B). Remarkably, DOX treatment increases Ang-2 mRNA levels, a vessel-destabilizing gene (Figure 5C). VEGF, CNP and Thrombomodulin did not show any significance difference (data not shown).

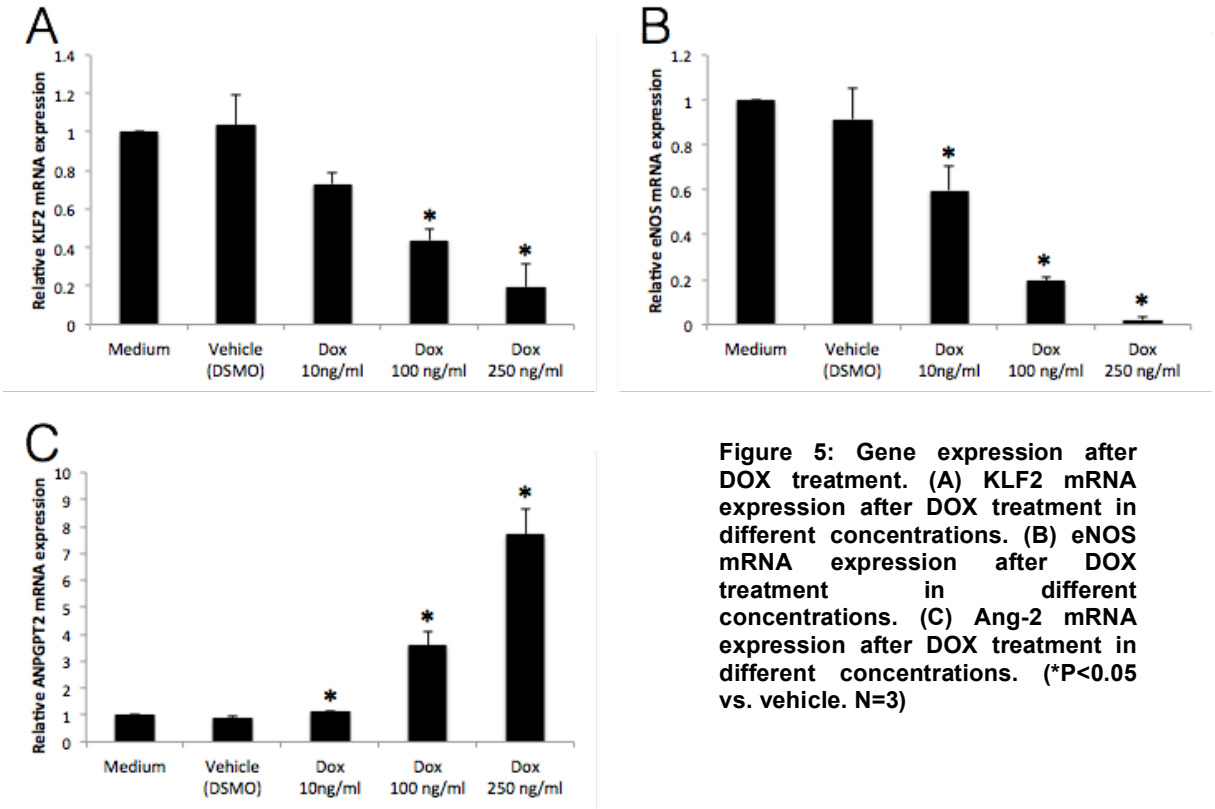


Figure 5: Gene expression after DOX treatment. (A) KLF2 mRNA expression after DOX treatment in different concentrations. (B) eNOS mRNA expression after DOX treatment in different concentrations. (C) Ang-2 mRNA expression after DOX treatment in different concentrations. (*P<0.05 vs. vehicle. N=3)

DOX treatment does not decrease cell viability

Previously, authors demonstrated that high doses of DOX (>500ng/ml) lead to apoptosis in endothelial cells (34,54). To validate our data, we want to determine if the used concentrations in our experiments did not lead to cell death. To begin documenting this, we performed a cell viability MTT assay. As shown in Figure 6, there is no significant difference between the vehicle and the different used concentrations, except for the positive control.

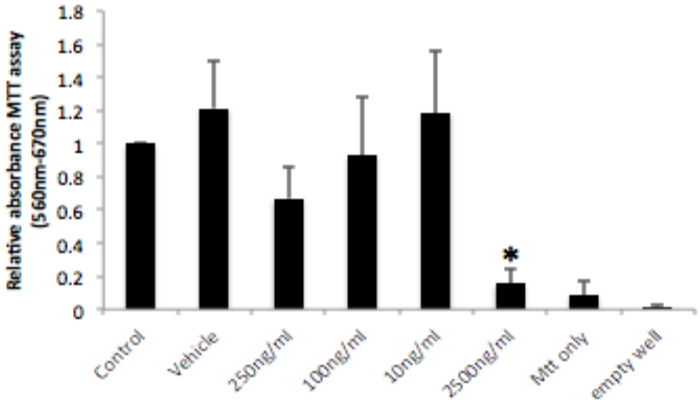
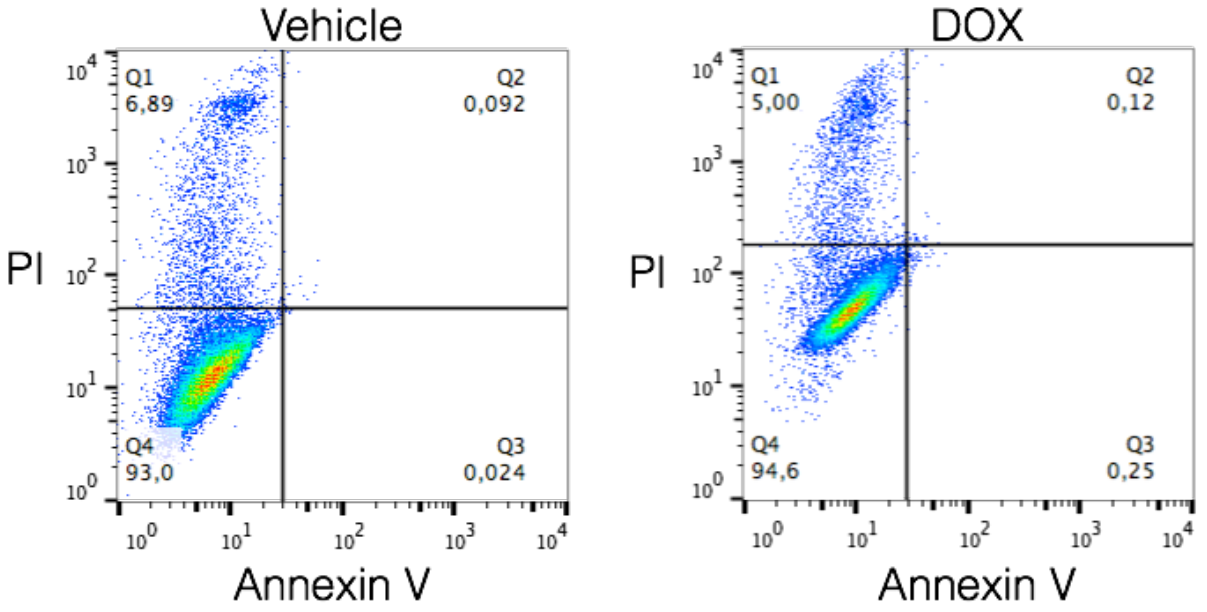


Figure 6: Cell viability assay. Relative absorbance of Formazan (product of MTT). (*P<0.05 vs. vehicle. N=3)

DOX treatment does not lead to apoptosis using flow cytometry

To validate and further characterize our earlier obtained data where we have shown that the used concentrations did not lead to apoptosis, we performed a specific apoptosis assay using flow cytometry. After 24h culture, we treated cells with DOX or its vehicle (DMSO). After 24h, we collected both floating cells in the treatment media and in the wash-effluent, together with the adherent cells. We double stained the cells with propidium iodide / Annexin V-FITC and counted the cells by flow cytometry. As shown in Figure 7, there was no significant difference in the expression of PI and Annexin V between DOX treated cells or its vehicle.



	Control PI	DOX PI	Control Annexin V	DOX Annexin V
Average % of cells	8.833	3.98	0.054	0.163
Standard Deviation	1.688	0.891	0.027	0.076

Figure 7: TOP, representative PI and Annexin V expression after DOX treatment. BOTTOM, flow cytometry quantitvative analysis of three independent experiments.

DOX treatment leads to an altered gene expression under shear stress

Our lab and others have previously shown that flow-mediated shear stress induces the vasoprotective transcription factor KLF2, that plays a role in the maintenance of the vasoprotective phenotype of the endothelium (41,42), besides, it is required for normal vessel development (39,40). After the KLF2, eNOS and Ang-2 alterations in our static experiment, we asked the question if these changes, or maybe even bigger changes, could be found under shear stress as well, since we know from earlier studies that the total KLF2 expression is at least 10x higher during flow-mediated shear stress (55). After we plated the cells for 24h on the DFS plates under static conditions, we exposed the cells for 24h to shear stress, to achieve physiological levels of vasoprotective genes. After 24h, shear medium was changed and DOX was added. As shown in Figure 8, ECs treated with DOX show a more than 7x decrease in KLF2 expression when exposed to vasoprotective laminar shear stress. Furthermore, although not significant, Ang-2 mRNA shows the same trend as seen in static cultures after DOX treatment after shear stress exposure (p=0.06). eNOS, TM, CNP and VEGF did also not show any significance difference.

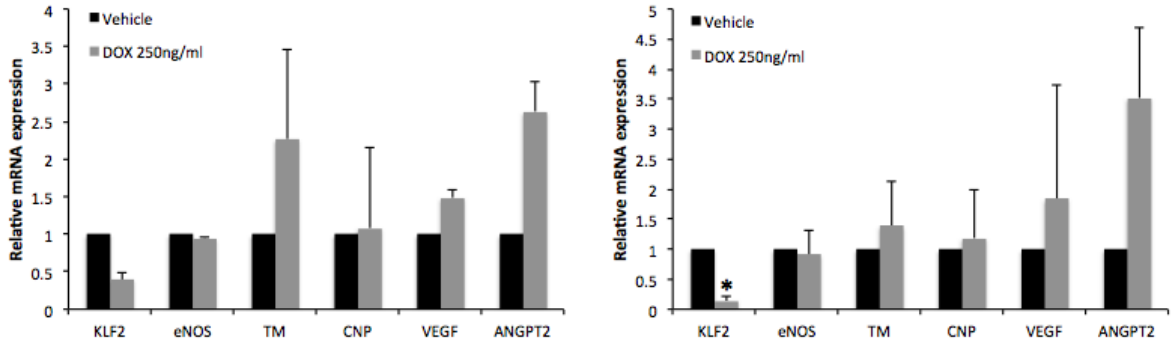


Figure 8: Gene expression after DOX treatment under shear stress. LEFT: Gene expression after DOX treatment under static conditions (N=2). RIGHT: Gene expression after DOX treatment under laminar shear stress conditions (*P<0.05 vs. vehicle. N=3)

Statins can rescue the KLF2 drop after DOX treatment

It is previously demonstrated that KLF2 expression is induced by shear stress, and that KLF2 expression an important factor is for the maintenance of vasoprotective phenotype in the vessels (41,42). Gracia-Sancho et al. have recently demonstrated that the KLF2 decay due to flow cessation could be rescued with Simvastatin treatment, as well as its downstream target genes. Besides, they concluded that KLF2 mediates the Simvastatin induced maintenance of the vasoprotective phenotype (55). Because we showed that DOX significantly decreases KLF2 expression in ECs, as well as some of its downstream target genes, we aimed to test the effect of Simvastatin on the expression of KLF2 and the KLF2-dependent target genes. We cultured ECs for 24h to achieve a confluent monolayer. After 24h, we added Simvastatin, to augment the KLF2 expression, which leads to more physiological levels of KLF2 expression. Finally, we added DOX for another 24h. As shown in Figure 9A, KLF2 mRNA expression is significant reduced compared to its control. Moreover, this reduce is significantly rescued with Simvastatin treatment. We next asked the question if Simvastatin also could rescue the vasoprotective target genes. As shown in Figure 9B, both the eNOS drop, and the Ang-2 increase could be rescued with Simvastatin treatment (Figure 9C). To show that the alterations in gene expression are specific for some genes, we here show that PTGDS mRNA does not change after DOX treatment (Figure 9D).

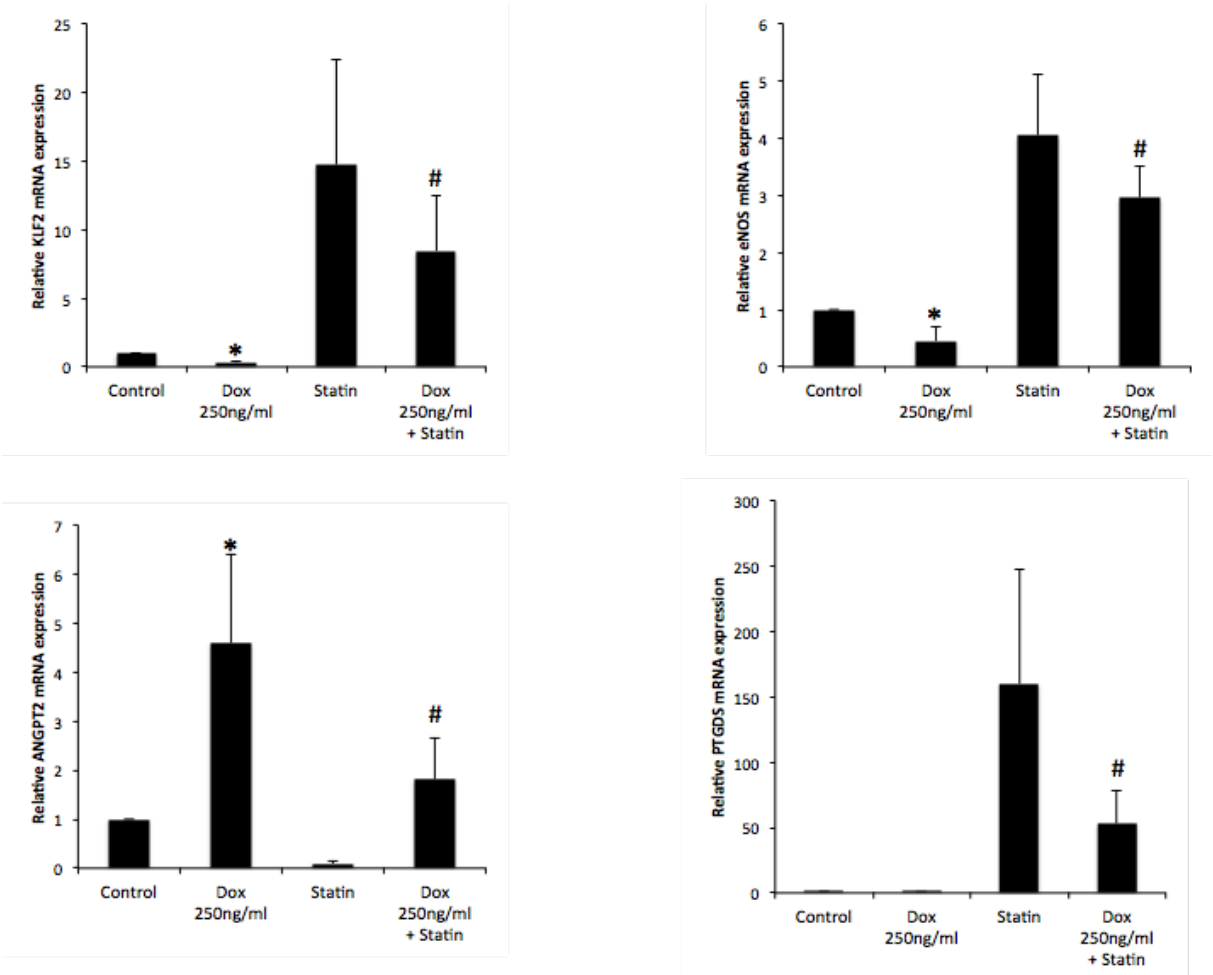


Figure 9: Statins can rescue the KLF2 drop and the KLF2-mediated vasoprotective genes after DOX treatment. (A) KLF2 mRNA expression in endothelial cells (ECs) in media supplemented with Simvastatin (10uM) after DOX treatment. (B) eNOS mRNA expression in endothelial cells (ECs) in media supplemented with Simvastatin (10uM) after DOX treatment. (C) Ang-2 mRNA expression in endothelial cells (ECs) in media supplemented with Simvastatin (10uM) after DOX treatment. (D) PTGDS mRNA expression in endothelial cells (ECs) in media supplemented with Simvastatin (10uM) after DOX treatment. (*P<0.05 vs. vehicle. N=4)

Discussion

Over the last 50 years, the treatment of cancer patients has significantly improved, and more patients survive this disease. However, the improvements in treatment regimens are accompanied by short and long term effects of the treatment, impacting quality of life (37). In regard to this, there is paid a lot attention to the side effects of cancer treatment with anthracyclines. Anthracyclines are notorious for their cardiotoxicity, whether causing acute (characterized by tachyarrhythmia and it occurs during the course of high dose administration) or chronic heart failure (seems to be dose-dependent). Eventually, both have the ability to induce or contribute to cardiomyopathy, dysfunction and ultimately heart failure (56). In the present study, we focused on DOX, an anthracycline that is known for over 60 years for its anticancer treatment.
Several studies have attempted to find the mechanism of DOX-induced cardiotoxicity, and many studies attribute ROS to be a major contributor in this cardiotoxicity, however, there is no consensus reached about the role of ROS in the DOX-induced cardiotoxicity (9). It is also thought that many other mechanisms such as the p53 pathway with a possible role for ERK2, the Bcl-2/Bax pathway (9) and the caspase-3 family are involved (12,57,58). Besides the cardiovascular side effects, DOX is also a well known inducer of PPE, a frequent toxic reaction of the skin related to chemotherapeutic agents. PPE presents with variable severity and can necessitate modification of the chemotherapy treatment schedule(24). One of the shared features of these side effects is the vessel damage, which is induced by DOX.
Many articles have been published about the DOX-induced endothelial damage, and the shared idea of these studies is that endothelial dysfunction occurs through apoptosis by several mechanisms (59-64).

In the present study, we show that DOX treatment of HUVECs leads to endothelial dysfunction, but not to apoptosis when using clinical relevant concentrations, which could contribute to new insides of the development of the DOX related side effects.

In our first experiment, we show that KLF2 mRNA and its downstream target gene eNOS mRNA are both significantly decreased, in a dose-dependent manner. Moreover, Ang-2 mRNA expression is, again in a dose-dependent manner, upregulated after Ang-2 expression.
KLF2, eNOS and Ang-2 show a respectively 5x, 10x and 8x altered gene expression after DOX treatment.
Our laboratory and others have demonstrated that KLF2 acts as a critical mediator for the establishment of vasoprotective phenothype (41,42,50).
Ang-2 is a member of the angiopoietin family. Angiopoietins play an essential role in angiogenesis. Angiopoietin-1 (Ang-1) stabilizes blood vessels and acts as an anti-permeability factor to prevent vascular leakage (65). In contrast, Ang-2 serves as an inhibitor of Ang-1 activity, and thus leads to disruption of vessel integrity as well a disassembly of cellular compounds (66). Zan et al. found that Ang-2 increases in parallel with a vascular permeability increase (67). When taken together, these data could potentially explain the vessel damage after DOX treatment since two *'endothelial protection'* genes (KLF2 and eNOS) are downregulated, and a vessel-destabilizing gene (Ang-2) is upregulated after DOX treatment.

Many studies have attributed apoptosis as a key factor in the vascular damage that DOX treatment entails. In a broad range of used concentrations, DOX causes apoptosis both in vitro and in vivo (34,36,54). To determine if our findings in regard to the earlier shown gene alterations can be contributed to endothelial dysfunction, we performed an endothelial cell

apoptosis assay. Our experiments demonstrated that DOX treatment in cultured ECs did not lead to apoptosis, which proves that DOX attenuates the EC function but not kill the cells.

To verify our earlier obtained data in regard to the apoptosis, we performed a more precise flow cytometry assay, to quantify the amount of necrotic and apoptotic cells.
Our results show that there is no difference in the amount of necrotic and apoptotic cells between untreated and DOX treated ECs. With these data, we show again that DOX treatment in ECs did not lead to apoptosis, and again, from which we can conclude that there is a possible role for endothelial dysfunction after DOX treatment, instead of endothelial cell apoptosis.

It's previously shown that KLF2 is selectively induced in ECs that are exposed to a biomechanical stimulus. KLF2 is identified for the first time as a gene regulated by steady laminar shear stress in ECs by Dekker et al. 2005 (41).
Parmar et al. showed that in the atheroprotected regions of the human carotid, but not in atheroprone regions, KLF2 is upregulated via a MEK5/ERK5/MEF2 signaling pathway. Moreover, MEK5 seems to be both necessary and sufficient for the upregulation of KLF2 (42). In a recent study from our lab, it's shown that KLF2 mRNA in ECs under shear stress is more than 10x higher compared to static conditions (50). In the present study, we show that KLF2 is more than 7x downregulated after DOX treatment in a vasoprotective shear stress environment. Besides, we show that Ang-2 is, again, although not significant, upregulated.
As shown, the vasoprotective KLF2 gene is almost completely knocked down after DOX treatment, even when the cells are exposed to the vasoprotective shear stress environment, which could explain the undesirable side effects of DOX. It can be speculated that this KLF2 drop plays an important role in the vessel damage, since we know that KLF2 decay can lead to EC-EC gap formation, which participates in the formation of edema (47). Moreover, as discussed earlier, an Ang-2 upregulation, might explain the vascular leakage what is observed after DOX treatment.

It's previously shown by our lab and others that statins have beneficial effects on atherogenesis in addition to their lipid-lowering action. Statins induce the expression of KLF2 in ECs, which leads to the establishment of a vasoprotective phenotype (43). It is found that KLF2 acts as a key regulator of several endothelial functions, including but not limited to blood vessel formation, control of vascular tone, inflammation, and coagulation (43). However, the exact mechanism how KLF2 is induced by statins remains not fully understood. It's thought that signaling molecules such as MEF2 and Akt are necessary for statin-mediated KLF2 expressin (68). Nevertheless, three often used statins, namely Simvastatin, Cerivastatin and Lovastatin, showed an increased KLF2 mRNA expression (43). Recently, Gracia-Sancho et al. founded that statins, when used as a supplement in an organ preservation solution, are capable of maintaining the expression of important vasoprotective genes in HUVECs through a KLF2-dependent mechanism (55). To see if we could rescue the KLF2 drop, and its downstream regulated target genes, after DOX administration, we performed an experiment where we treated HUVECs before and during DOX administration with Simvastatin. Here, we report that Simvastatin leads to a significant rescue of KLF2 mRNA and eNOS mRNA, as well as a significant Ang-2 mRNA decrease. These findings are in line with a recent published clinical study, where it was shown that co-administration of statins during DOX treatment could alleviate the delayed cardiotoxicity after DOX administration (69). Our findings, together with the previous work what is done, may have important implications for the implementation for statins during, or before, the treatment of cancers with anthracyclines.

A limiting factor of this study is that all observations are done at mRNA levels. There might be a serious difference in expression at protein levels. However, the mRNA levels show an excessive change after DOX treatment compared to the control, thus it's assumable that there is also a different expression at protein level. To make sure that these alterations are also expressed at protein level, future experiments will quantity proteins encoded by the studied genes after DOX treatment.

Furthermore, in this study we have used exclusively HUVECs, thus we only have looked at the venous endothelial cells. People could argue that there might be a different expression in the arterial side, since there is a different environment in the oxygenized blood system. To look if our observed effects are independent of the type of cells, it's necessary to use different endothelial cells types in the future.

Besides, although obvious, another limiting factor of this study if the fact that all experiments are done *in vitro*, to see if these effects are also clearly seen *in vivo,* it's necessary to validate our findings in a mouse model. The mouse model allows us to determine if we could prevent the DOX related side effects after statin treatment at the same time.

Another crucial experiment what needs to be done to complete our data is to determine by which pathway DOX regulates KLF2, in our case downregulates KLF2. From previous studies we know that KLF2 is regulated via the MEK5/ERK5/MEF2 pathway (44), and statins act via depletion of GGPP-dependent signaling pathway to upregulate KLF2 (43). If we are able to show that DOX acts through one of these mechanisms, we could possibly prevent this downregulation in the near future. Otherwise, there might be a new mechanism by which DOX acts on the KLF2 expression.

To verify if the KLF2 decay after DOX treatment actually leads to the earlier mentioned side effects, such as an increased permeability, we need to investigate if the used concentrations lead to an increased EC-EC gap formation. Wolf et al. already showed that DOX causes an increased permeability in the vessels and an increased LDH leakage (54). However, they used significant higher concentrations.

As we showed that statins could save the altered KLF2 expression after DOX treatment, it would be of great value to repeat this experiment under shear stress conditions. If we are able to show that this rescue also exists under shear stress, we can conclude that KLF2 upregulation via either shear stress or statins acts via a different mechanism.

Conclusion

Our study documents that there is an important role for dysregulation of vascular genes in the development of DOX-induced side effects. This is, to the best of our knowledge, the first study that identified key molecular determinants of endothelial dysfunction, after DOX treatment. In particular, this study demonstrated that DOX downregulates the endothelial protective KLF2 gene, and several of its downstream target genes. Moreover, we prove that statins could rescue the gene alterations significantly when administrated simultaneously, which represents a potential implementable option in the clinical setting in the near future.

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