Dopamine induces lipid accumulation, NADPH oxidase-related oxidative stress and a pro-inflammatory status of the plasma membrane in H9c2 cells

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ABSTRACT

Background: Excess catecholamine levels are suggested to be cardiotoxic and to underlie stress-induced heart failure. The cardiotoxic effects of noradrenaline and adrenaline are well recognized. However, although cardiac and circulating dopamine levels are also increased in patients with stress-cardiomyopathy, knowledge regarding putative toxic effects of excess dopamine levels on cardiomyocytes is very scarce. Here, we have studied the effects of elevated dopamine levels in H9c2 cardiomyoblasts.

Methods: H9c2 cells were cultured and treated with dopamine (200 μM) for 6, 24 and 48 hours. Subsequently, the effects on lipid accumulation, cell viability, flippase activity, reactive oxygen species (ROS) production, subcellular NOX protein expression and ATP/ADP and GTP/GDP levels were analysed.

Results: Dopamine did not result in cytotoxic effects after 6 hours. However after 24 and 48 hours dopamine treatment induced a significant increase in lipid accumulation, nitrotyrosine levels, indicative of ROS production, and cell death. In addition, dopamine significantly reduced flippase activity and ATP/GTP levels coinciding with phosphatidylserine exposure on the outer plasma membrane. Furthermore, dopamine induced a transient increase in NOX1 and NOX4 expression in the cytoplasm and (peri)nucleus after 24 hours which subsided after 48 hours. Moreover, while dopamine induced a similar transient increase in NOX2 and p47phox expression in the cytoplasm, in the (peri)nucleus this increased expression persisted for 48 hours where it co-localized with ROS.

Conclusion(s): Our data show that exposure of H9c2 cells to elevated dopamine levels induced lipid accumulation, oxidative stress, and a pro-inflammatory status of the plasma membrane. This can, in part, explain the elevated inflammatory response in patients with stress-induced heart failure.
INTRODUCTION

Stress-induced heart failure, also known as catecholamine-induced myocarditis, including Takotsubo cardiomyopathy, is related to sustained exposure of the heart to excess levels of catecholamines. Excess cardiac catecholamine levels can result from an increase in catecholamine blood levels. The main catecholamines noradrenalin (norepinephrine), adrenalin (epinephrine) and dopamine are released in the blood at baseline plasma levels of 100-450 pg/ml for noradrenaline, <100 pg/ml for adrenaline and <20 pg/ml for dopamine in humans. However, in response to conditions such as pheochromocytoma, burn wounds, sepsis or emotional stress their plasma levels can rise severely. Such increased plasma catecholamine levels were shown to be associated with stress-cardiomyopathy in patients wherein plasma levels of 1709-2910 pg/ml for noradrenaline, 916-1374 pg/ml for adrenaline and 106-146 pg/ml for dopamine were measured one day after hospitalization and were still elevated after nine days. In addition, evidence for local catecholamine release in the heart was found in patients with Takotsubo-like left ventricular dysfunction, as higher catecholamine levels were measured in blood taken from the coronary sinus (maximum values of 5719 pg/ml for noradrenaline, 356 pg/ml for adrenaline and 158 pg/ml for dopamine) than in blood taken from the aortic root (maximum values of 4238 pg/ml for noradrenaline, 423 pg/ml for adrenaline and 118 pg/ml for dopamine).

Experimental research into the cardiotoxic effects of catecholamines has focused almost exclusively on noradrenaline and adrenaline and multiple adverse cardiac effects have been ascribed to excess levels of these two catecholamines, including microvascular dysfunction, inflammation, fibrosis, and cardiac hypertrophy. Moreover, excess levels of these two catecholamines were shown to directly induce cardiomyocyte damage and death in vitro and in animals in vivo. In contrast, although cardiac and circulating dopamine levels are also increased in patients with stress-cardiomyopathy, knowledge regarding putative adverse effects of excess dopamine levels on the heart and on cardiomyocytes in particular is very scarce. Recently, infusion of dopamine in rats induced Takotsubo-like cardiac dysfunction indicating a role for dopamine also in the induction of stress-induced heart failure.

Important factors in catecholamine-induced cardiomyocyte damage and death appear to be oxidative stress and lipotoxicity, i.e. the intracellular accumulation of lipids. Both adrenaline (500 μM) and noradrenaline (2 and 100 μM) induced reactive oxygen species (ROS) production in H9c2 rat cardiomyoblasts and in isolated adult rat cardiomyocytes in vitro. Moreover, increased ROS levels were found in cardiomyocytes in patients with Takotsubo cardiomyopathy. In addition, high levels of isoprenaline, a synthetic adrenaline-like catecholamine, induced severe lipid accumulation in cardiomyocytes in mice and rats in vivo, that in the rats coincided with necrosis, fibrosis, and inflammation. Indeed, infiltration of inflammatory cells, i.e. macrophages, neutrophils, and lymphocytes, has been observed in catecholamine-induced heart failure both in humans and in rats.
Since the putative adverse effects of excess dopamine levels on cardiomyocytes are unknown, in the present study we have analyzed the effects of elevated levels of dopamine on cell viability, lipid accumulation, and oxidative stress in H9c2 cells.

**MATERIAL AND METHODS**

**Cell culture**

Rat cardiomyoblasts (H9c2 cells, ATCC (LGC standards), Wesel, Germany) were cultured in Dulbecco’s Modified Eagles Medium (DMEM, Lonza, Breda, the Netherlands) supplemented with 10% heat inactivated fetal calf serum (FCS, Hyclone), 2 mM L-glutamine (Invitrogen, Breda, the Netherlands), 100 IU/ml penicillin (Invitrogen) and 100 μg/ml streptomycin (Invitrogen) and grown at 37°C in a humidified 5% CO₂/95% air atmosphere. Experiments were performed with cells grown to a confluency of 60-90%.

In all experiments the cells were incubated with 200 μM (38 μg/ml) dopamine (Sigma, Zwijndrecht, the Netherlands) in growth medium for 6, 24 or 48 hours at 37°C in a humidified 5% CO₂/95% air atmosphere. Control cells were cultured without dopamine. In order to maintain elevated dopamine levels, the culture medium and dopamine (200 μM) were refreshed after 6, 24 and 30 hours (dependent on the final incubation time). Cells that detached during the treatment (mostly dead or dying cells) were collected, resuspended in the fresh culture medium, and added back to the cell culture (unless mentioned otherwise).

**Dopamine concentration measurements in growth medium**

After treatment with dopamine, the culture medium was collected from the H9c2 cells, centrifuged at 280×g for 5 minutes, and acidified with concentrated HCl to pH 2. The dopamine concentration was then measured in the acidified supernatant using high-performance liquid chromatography, as described before. Briefly, dopamine was isolated from the supernatant by cation exchange on Amberlite CG 50 (Serva, Heidelberg, Germany). Analysis was performed by ion-pair reversed phase high-performance liquid chromatography. For detection of the amine, the native fluorescence emitted at 313 nm on excitation at 285 nm was monitored.

**Lipid accumulation analysis**

After treatment only the adherent H9c2 cells were trypsinized, collected by centrifugation, and resuspended in PBS to a final concentration of 500000 cells/ml (detached cells were removed prior to collection). From each sample an equal amount of cells (25000 cells) was transferred to a glass slide using a cytospin centrifuge (Thermo Scientific, Waltham, USA). The cells were then fixed in a 1.76% (w/v) calcium/9.25% (v/v) formalin solution for 15 minutes, rinsed in water, washed in in 60% isopropanol for a few seconds, and incubated in
Dopamine induces cytotoxic effects in H9c2 cells

a 0.5% (w/v) Oil Red O (Klinipath, Duiven, the Netherlands) solution in 60% isopropanol for 15 minutes. Next, the slides were rinsed in 60% isopropanol for a few seconds, washed in water, counterstained with heamatoxylin for 5 minutes, and covered. Finally, the number of lipid-accumulating H9c2 cells was counted in each slide and used as the final score for that particular slide.

Electron microscopy
After treatment, H9c2 cells were trypsinized, collected by centrifugation and the pellet was fixed in 3% (v/v) gluteraldehyde for 1.5 hour and subsequently in 2% (w/v) osmium tetraoxide for 20 minutes. The cell pellet was then dehydrated with alcohol and embedded in Epon 812. Ultra-thin sections were collected on 75-mesh hexagonal formvar-coated Copper grids. The sections were stained with 5% uranyl acetate and lead citrate and were examined in a Jeol-1200 EX electron microscope.

Flow cytometry
Phosphatidylserine (PS) exposure of the plasma membrane was assessed with FITC-labeled human recombinant annexin-V (Bender Med Systems, Vienna, Austria), while propidium iodide (PI)(Bender Med Systems) was used to determine plasma membrane permeability. After treatment, H9c2 cells were washed with phosphate buffered saline (PBS), trypsinized, collected by centrifugation, and resuspended in serum free DMEM containing annexin-V (1:20), and incubated for 30 minutes in the dark at 37°C in a humidified 5% CO₂/95% air atmosphere. PI (1:20) was added 1 minute prior to analysis. The cells were analyzed using a FACS Calibur (Becton Dickinson, Breda, the Netherlands) and the results were analyzed using Cell Quest Pro software (Beckton Dickinson).

To determine flippase activity the H9c2 cells were trypsinized, collected by centrifugation, and resuspended in serum free DMEM. NBD-labeled PS (PS-NBD: (1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl]-sn-glycero-3-phosphoserine)(Avanti Polar Lipids) was added in a final concentration of 1 μM at 37°C for 30 min. Cells were then transferred to ice and 5 ml ice-cold 1% (w/v) BSA in PBS was added to wash away excess PS-NBD. After 15 minutes, the cells were pelleted, resuspended in serum free DMEM, and analyzed by flow cytometry using a FACSCalibur.

Active caspase-3 measurements
H9c2 cells were cultured in a 96-wells plate. After treatment, the concentration of active caspase-3 was determined using a fluorimetric homogeneous caspase assay (Roche, Almere, the Netherlands), according to the manufacturer’s instructions. Briefly, cells were lysed and incubated with DVED-rhodamine 110 substrate for 1h at 37°C. Subsequently, the amount of free rhodamine was determined at a microplate fluorescence reader (TECAN spectrafluor,
TECAN, Männedorf, Switzerland). The developed fluorochrome was proportional to the concentration of activated caspase-3 and could be quantified by a calibration curve of diluted free rhodamine.

**Nucleotide measurements**

After treatment, only the adherent H9c2 cells were used for nucleotide extraction and measurements that were performed as described before\(^1^8\). Briefly, H9c2 cells were extracted with 200 μl of ice-cold 0.4 M perchloric acid and centrifuged at 10,000 g at 4°C for 3 minutes. The supernatant was removed, neutralized with K₂CO₃, and used for HPLC analysis. The pellet containing total protein was dissolved in 500 μl of 0.2 M NaOH. Protein content was determined using bicinchonic acid solution containing 0.1% CuSO₄, as described before\(^1^8\). The final nucleotide concentration was calculated as the amount of nucleotide divided by the total amount of protein.

**Digital imaging fluorescence microscopy**

H9c2 cells were grown in Lab-tek II 4-well chambers slides (Nalge Nunc International, Rochester, USA). After treatment, cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature and permeabilized with 70% (v/v) methanol/30% (v/v) aceton solution for 10 minutes at -20°C. Next, the cells were incubated overnight at 4°C with rabbit-anti-gp91\(^{phox}\) (NOX2, 1:50, Upstate, Lake Placid, USA), goat-anti-p47\(^{phox}\) (1:50, Santa Cruz), rabbit-anti-nitrotyrosine, as an indirect marker of reactive oxygen species (ROS) production\(^1^9\), goat-anti-MOX1 (NOX1, 1:50, Santa Cruz, Heidelberg, Germany) and goat-anti-NOX4 (1:50, Santa Cruz). Antibodies against gp91\(^{phox}\), p47\(^{phox}\) and nitrotyrosine were co-incubated to assess co-localization. The cells were then incubated with the secondary antibodies (all from Life Technologies, Bleiswijk, the Netherlands) donkey-anti-goat cy3 (1:50), donkey-anti-goat cy5 (1:50) and donkey-anti-goat FITC (1:50) for 30 minutes at room temperature in the dark. Negative controls with only the secondary antibody were included to assess non-specific binding. All negative controls showed no staining (data not shown). Before visualization, slides were covered with vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories Inc., Peterborough, England). 2D optical sections were acquired and analysed with a 3i Marianas™ (Intelligent Imaging Innovations, Göttingen, Germany) digital-imaging microscopy workstation (Zeiss Axiocam 200M inverted microscope, Carl Zeiss, Sliedrecht, the Netherlands) equipped with a nanostepper motor (z-axis increments: 10 nm) and a thermo-electrically cooled EMCCD camera (Quantum: 512C, 512x512 pixels, Photometrics, Tucson, USA) and analyzed using Slidebook™ software (version 5.0, Intelligent Imaging Innovations, Göttingen, Germany).
Peroxidase measurements
Culture medium (DMEM + 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin) was supplemented with a single dose of dopamine (200 μM) or without dopamine (control) and placed at 37°C in a humidified 5% CO₂/95% air atmosphere. After 48 hours, the presence of peroxidase was determined using the Oxiselect™ Peroxidase Fluorometric Assay Kit (Cell Biolabs Inc., San Diego, USA), according to the manufacturer’s instructions. Briefly, the samples were incubated with an ADHP(10-Acetyl-3,7-dihydroxyphenoxazine, 100 μM)/horseradish peroxidase(HRP, 0.2 U/ml) assay buffer for 30 minutes at room temperature in the dark. In the presence of HRP, ADHP reacts with peroxidase in a 1:1 stoichiometry producing highly fluorescent Resorufin. The amount of Resorufin was determined in a microplate fluorescence reader (TECAN spectrafluor). The developed fluorochrome was proportional to the concentration of peroxidases within the sample and could be quantified using a calibration curve of diluted Resorufin.

pH measurements
Culture medium (DMEM + 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin) was supplemented with a single dose of dopamine (200 μM) or without dopamine (control) and placed at 37°C in a humidified 5% CO₂/95% air atmosphere. At several time points (after 0, 5 and 30 minutes and 1, 2, 6, 24, 30 and 48 hours), the pH of the treated DMEM was measured using a pH210 microprocessor-based bench pH-meter (HANNA Instruments, Nieuwegein, the Netherlands).

Statistics
Statistics were performed using the SPSS statistics program (Version 20.0, IBM, Amsterdam, the Netherlands). Data were analyzed using the non-parametric Mann-Whitney U test. Two-sided p-values ≤0.05 were considered significant.

RESULTS
Dopamine concentration decreases over time
We stimulated the H9c2 cells with 200 μM dopamine, according to an in vivo mouse model of LPS-induced systemic inflammation in which the same dopamine concentration was used successfully. Catecholamines have a short half-life, therefore, we first analyzed the effect of time-dependent degradation of dopamine up to 48 hours. Already after 6 hours a significant but minor decrease to 80% in dopamine concentration was found in the growth medium of treated H9c2 cells (p=0.050), that decreased to less than 10% after 24 hours (p=0.050), and completely disappeared after 48 hours (figure 1A, p=0.037). Catecholamines can be elevated for several days after the initial stressor in patients.
with stress-cardiomyopathy, therefore, we refreshed the growth medium with dopamine at several time points during the incubation as described in the materials and methods section.

Figure 1: Levels of dopamine in time and its effect on lipid accumulation
A. The level of dopamine in supernatant of cultured H9c2 cells at 0, 6, 24 and 48 hours after addition of dopamine at a starting concentration of 200 μM (mean is set to a 100%; n = 3). Median and interquartile range are shown. * significance compared to 0 hours. B. Number of lipid accumulating cells in control H9c2 cells (C) and H9c2 cells treated with 200 μM dopamine for 6, 24 and 48 hours (hrs)(n = 4). Data is a box-plot with min to max whiskers. * significance compared to controls. C-D. Representative pictures of lipid accumulating cells (arrows) in controls (C) and dopamine-treated cells (200 μM) for 48 hours (D). Magnification x20. E. Electron microscopical picture of a dopamine-treated cell showing lipid droplets (L) within the cytoplasm. Magnification x60000.

**Dopamine induces lipid accumulation**
Recently, in catecholamine-induced heart disease it was shown that the synthetic catecholamine isoprenaline induced lipid accumulation in cardiomyocytes coinciding with
myocardial dysfunction, necrosis, and cardiac stunning\(^\text{13, 14}\). Therefore, we analyzed the effect of dopamine treatment (200 µM) on lipid accumulation in H9c2 cells in time. Remarkably, after 6 hours the number of lipid containing cells decreased significantly compared to controls (figure 1B, C, \(p=0.001\)). However, a marked and significant increase in lipid accumulating cells was found after 24 and 48 hours compared to controls (figure 1B, D, \(p=0.001\)). The accumulation of lipids was ultrastructurally verified as lipid-containing vesicles within the cytoplasm (figure 1E).

**Dopamine reduces cell viability and induces a pro-inflammatory status of the plasma membrane**

Next, we analyzed whether this dopamine (200 µM)-induced lipid accumulation coincided with a decrease in H9c2 cell viability and phosphatidylserine (PS) exposure visualized via annexin V binding and/or PI staining. In line with the lipid accumulation, a small but significant increase in the percentage of viable cells was observed after 6 hours (\(p<0.001\)), whereas a marked significant decrease was found after 24 (\(p<0.001\)) and 48 hours (\(p<0.001\)) dopamine incubation compared to controls (figure 2A). The percentages of single annexin V-positive cells (representing either early apoptosis or reversible PS exposure\(^\text{22}\); figure 2B) and annexin V/PI-positive cells (representing late apoptosis or necrosis; figure 2C) were significantly decreased after 6 hours (\(p<0.001\) and \(p=0.011\)), but were both increased significantly after 24 hours and especially 48 hours (all \(p<0.001\)) compared to controls. Remarkably, this coincided with a minor, but significant decrease in active caspase-3 concentration after 48 hours (figure 2D, \(p<0.001\)). This, therefore, indicates that the single annexin V-positive cells likely are not apoptotic, but reversibly expose PS which is associated with a pro-inflammatory status of the plasma membrane\(^\text{22, 23}\).

An important regulator of keeping PS in the inner plasma membrane leaflet, thereby preventing PS exposure, is flippase\(^\text{24}\). Indeed, both 24 hours and 48 hours of dopamine (200 µM) treatment resulted in a significant increase of cells wherein flippase is inactive (figure 2E, both \(p=0.050\) compared to controls). Since flippase activity is ATP dependent, we analyzed whether dopamine (200 µM) affected cellular nucleotide levels. After 6 hours, a limited but significant decrease in the percentage of cellular ATP (73%, \(p=0.021\)), GTP (72%, \(p=0.021\)), ADP (77%, \(p=0.043\)) and GDP (71%, \(p=0.021\)) levels was observed compared to controls (set to 100%; figure 2F). Also after 24 and 48 hours the ATP (24h: 86%, 48h: 72%), GTP (24h: 90%, 48h: 77%) and ADP (24h: 77%, 48h: 75%) levels were decreased moderately compared to controls, that was only significant for ATP after 24 and 48 hours (both \(p=0.021\)) and for GTP after 48 hours (\(p=0.021\); figure 2F). Although it is unknown below which threshold ATP levels affect flippase activity in cardiomyocytes, it is unlikely that the minor decrease in ATP levels we observed explains the dramatically reduced flippase activity as studies performed in erythrocytes showed that flippase activity was only severely decreased after ATP was almost depleted\(^\text{25, 26}\).
Figure 2: The effect of dopamine on cell viability, flippase activity and nucleotide levels

A-C. Percentages of viable (A), single annexin V-positive (B) and annexin V/PI-positive (C) cells in control (white bars) and dopamine-treated cells (200 μM; grey bars) for 6 (n = 5), 24 (n = 10) and 48 hours (hrs)(n = 11).

D. Caspase activity in cells treated with dopamine (200 μM) for 24 and 48 hours (hrs; grey bars) is given relative to controls (mean set to 100%; n = 3).

E. Percentage of flippase inactive cells (n = 3) in control (C) and dopamine-treated cells (200 μM) for 24 and 48 hours (hrs).

F. Percentage of cellular ATP, GTP, ADP and GDP levels in control (mean is set to 100%) and dopamine-treated cells (200 μM) for 6, 24 and 48 hours (hrs) (n = 4). Data are medians and interquartile ranges. * significance compared to controls.
Dopamine induces cytotoxic effects in H9c2 cells

Dopamine reacts with growth medium
It has been described that dopamine (50-1000 μM) could have a cytotoxic effect on cells in culture via induction of H$_2$O$_2$ in the culture medium. For this, we determined H$_2$O$_2$ concentrations in DMEM either or not supplemented with dopamine (200 μM). Dopamine added to DMEM for 48 hours resulted in a significant increase in H$_2$O$_2$ concentration up to 5.9 μM (range: 0.3-27.5 μM, p<0.001; figure 3A). Therefore, we analyzed whether H$_2$O$_2$ at such concentration had a cytotoxic effect on H9c2 cells. Addition of H$_2$O$_2$ in final concentrations of 6 μM (mean of all samples) or 10 μM (3rd quartile) for 48 hours, however, had no significant effects on the percentages of viable (figure 3B), single annexin V-positive (figure 3C) or annexin V/PI-double positive cells (figure 3D) compared to controls, while dopamine (200 μM) did result in a significant decrease in viability (figure 3B, all p=0.004 vs control and both H$_2$O$_2$ concentrations) coinciding with a significant increase in single annexin V-positive cells (figure 3C, p=0.01 vs control, p=0.025 vs both H$_2$O$_2$ concentrations) and annexin V/PI-positive cells (figure 3D, p=0.004 vs control and both H$_2$O$_2$ concentrations). The dopamine-formed H$_2$O$_2$ theoretically could affect the pH of the growth medium. However, no significant effect of dopamine on the pH of the growth medium was found (figure 3E). These results thus indicate that the effects of dopamine on the viability of H9c2 cells were related to a direct effect of dopamine rather than an effect on the growth medium.

Dopamine increases intracellular reactive oxygen species (ROS)
Elevated catecholamine levels are associated with oxidative stress induction in cardiomyocytes, both in vitro with 500 μM adrenaline and 100 μM noradrenaline, as well as in patients with Takotsubo cardiomyopathy. Therefore, we analyzed the effect of dopamine (200 μM) on intracellular ROS using the indirect ROS marker nitrotyrosine. After 6 hours, no effects on cellular nitrotyrosine levels were found (figure 4A). In contrast, after 24 hours a significant increase in (peri)nuclear (p<0.001) and cytoplasmic (p<0.001) nitrotyrosine was found (figure 4B), which persisted after 48 hours in the (peri)nuclear region (figure 4C, p<0.001), but decreased significantly in the cytoplasm (figure 4C, p<0.001). These results thus indicate that dopamine induces oxidative stress in H9c2 cells.
Figure 3: Dopamine interaction with growth medium

A. Concentration of H$_2$O$_2$ in growth medium without (control (C)) and with dopamine (DA) (200 μM; n = 4). Box-plot with min to max whiskers is shown.

B-D. Percentage of viable (B), single annexin V-positive (C) and annexin V/PI-positive (D) cells in control (C), dopamine (DA) (200 μM), and H$_2$O$_2$ treated cells for 48 hours (n = 3). Data are medians and interquartile ranges. * significance compared to control, + significance compared to peroxide treated cells.

E. pH of growth medium with and without (control) dopamine (200 μM) added measured at 0 min, 5 min and 30 min and 1h, 2h, 6h, 24h, 30h and 48h. Data is the median and error range.
Dopamine induces cytotoxic effects in H9c2 cells

**Figure 4**

A-C. Percentage of (peri)nuclear and cytoplasmic nitrotyrosine (ROS) intensity after 6h (A; n = 3), 24h (B; n = 7) and 48h (C; n = 5) in control (white bars) and dopamine (DA) treated (200 μM; grey bars) H9c2 cells. Data are medians and interquartile ranges. * significance compared to control.

**Dopamine increases intracellular expression of NOX-proteins**

An important source of ROS in H9c2 cells are the NOX proteins, of which NOX1, NOX2, NOX4 and p47phox, the activating subunit for NOX2, have been described in H9c2 cells. Because the increased ROS levels were found after 24 hours and 48 hours, NOX expression levels were analyzed at these time-points after dopamine incubation (200 μM) only. After 24 hours, significant increases in both (peri)nuclear and cytoplasmic NOX1 (figure 5A, both: p<0.001) and NOX4 (figure 5A, both: p<0.001) were observed, which decreased again significantly after 48 hours (figure 5B, all p<0.001). In addition, after 24 hours significant increases in (peri)nuclear and cytoplasmic presence of NOX2 (figure 5C, (peri)nuclear: p<0.001; cytoplasm:
p=0.003) and of p47\textsuperscript{phox} (figure 5C, both: p<0.001) were observed. Interestingly, significantly increased (peri)nuclear presence of NOX2 (p<0.001) and p47\textsuperscript{phox} (p<0.001) persisted after 48 hours (figure 5D), while their presence in the cytoplasm decreased significantly to below control levels (figure 5D, NOX2: p<0.001 and p47\textsuperscript{phox}: p<0.001). Moreover, nitrotyrosine, NOX2 and p47\textsuperscript{phox} were found to co-localize in the (peri)nuclear region after both 24 (figure 6 B-E-H-K) and 48 hours (figure 6 C-F-I-L) of dopamine treatment, but not in controls (figure 6 A-D-G-J).

These results thus suggest a putative role for NOX1, NOX2, and NOX4 in dopamine-induced cardiotoxicity.

Figure 5: Dopamine increases intracellular expression of NOX-proteins and p47\textsuperscript{phox}

A-B. Percentage of (peri)nuclear and cytoplasmic NOX-1 and NOX-4 expression after 24h (A) (both n = 4) and 48h (B) (both n = 4) after dopamine (DA) (200 μM) incubation. C-D. Percentage of (peri)nuclear and cytoplasmic NOX-2 and p47\textsuperscript{phox} expression after 24h (C) (NOX-2: n = 5, p47: n = 4) and 48h (D) (NOX-2: n = 5, p47: n = 4) after dopamine (DA) (200 μM) incubation. White bars represent control cells, grey bars represent dopamine treated cells. Data are medians and interquartile ranges. * significance compared to control.
Figure 6: Dopamine induces (peri)nuclear co-localisation of NOX-2, ROS and p47 A-L. Representative images of (peri)nuclear expression of NOX-2 (A-C), nitrotyrosine (ROS; D-F), p47 (G-I) and merged figures of all three fluorescent signals (J-L) in control (A-D-G-J) and dopamine (200 μM) treated cells for 24h (B-E-H-K) and 48h (C-F-I-L). Magnification x40.

DISCUSSION

Elevated catecholamine levels play an important role in the pathophysiology of stress-induced heart failure. The cardiotoxic effects of noradrenaline and adrenaline are well recognized. However, although dopamine levels are also severely increased in patients with stress-cardiomyopathy\(^1,5\) and were shown to induce Takotsubo-like cardiac dysfunction in rats\(^10\), knowledge regarding the adverse effects of excess dopamine on cardiomyocytes is very
scarce. We now show that elevated levels of dopamine (200 μM) induce lipid accumulation, oxidative stress, and a pro-inflammatory status of the plasma membrane in H9c2 cells. Intracellular accumulation of lipids is associated with cellular dysfunction and cell death, that can eventually contribute to organ failure in a process called lipotoxicity\textsuperscript{29}. Lipid accumulation in cardiomyocytes in response to the synthetic adrenaline-like catecholamine isoprenaline was shown before in the hearts of mice, rats in vivo, and HL-1 cardiomyocytes in vitro, coinciding with necrosis, decreased cardiac function, and electrophysiological disturbances\textsuperscript{13,14}. We now found that dopamine induced lipid accumulation in H9c2 cells, coinciding with a loss of viability and oxidative stress indicative for a lipotoxic effect of this catecholamine also. The isoprenaline-induced lipid accumulation manifested itself already after 2 hours in the mouse heart and HL-1 cardiomyocytes\textsuperscript{15}, while the dopamine-induced lipid accumulation became evident only after 24 hours in the H9c2 cells and was even lower than controls after 6 hours. Whether this difference depends on the research models used or indicates a difference in the mechanisms whereby these catecholamines induce lipid accumulation we do, as yet, not know. Interestingly, in the mouse model no lipid accumulation was found 7 days after isoprenaline treated mouse heart\textsuperscript{13}. Similarly, subcutaneous infusion of noradrenaline (43 μg/ml/day) in rats did not result in lipid accumulation in the heart after 7 days, although earlier time-points were not analyzed\textsuperscript{30}. These data suggest that catecholamine-induced lipid accumulation may be a reversible process, although we did not test this for dopamine yet. Next to lipotoxicity, catecholamine-induced cardiotoxicity has also been attributed to excess ROS formation. Indeed, increased ROS levels were detected in cardiomyocytes in patients with Takotsubo cardiomyopathy\textsuperscript{31}, and both adrenaline (500 μM) and noradrenaline (2 and 100 μM) were found to increase ROS levels in the heart after 7 days, although earlier time-points were not analyzed\textsuperscript{30}. We now observed that also dopamine (200 μM) significantly increased intracellular nitrotyrosine levels indicative of ROS production. Moreover, dopamine induced increased (peri)nuclear and cytoplasmic expression levels of NOX1, NOX2, NOX4 and p47\textsuperscript{phox} within these cells, pointing to the NOX proteins as a source of dopamine-induced ROS production. However, what the involvement is of these three NOX isoforms in the observed dopamine-induced effects remains to be established. Previously, it was found that treatment with noradrenaline (10 μM) for 48 hours led to increased NOX1 expression and ROS production in fetal rat hearts and H9c2 cells\textsuperscript{12}, whereas NOX4 expression was unaltered and NOX2 could not be detected in these cells. This NOX1-dependent oxidative stress led to PKCɛ gene repression which may lead to diminished cardioprotection against cellular stress. In addition, increased lipid accumulation in the rat heart after angiotensin II infusion coincided with ROS production and increased mRNA expression of NOX1 and NOX4\textsuperscript{30} and it was suggested that this NOX1- and NOX4-dependent ROS generation altered gene expression involved in lipid homeostasis resulting in lipid accumulation\textsuperscript{30}. The increases in NOX1 and NOX4 expression and ROS production in response to dopamine we observed here, may have similar effects.
Dopamine induces cytotoxic effects in H9c2 cells

Moreover, lipid accumulation within cardiomyocytes in turn might also induce oxidative stress. In contrast to NOX1 and NOX4, NOX2 as well as p47phox remained significantly increased (peri)nuclear after 48 hours dopamine application, the time point of the most profound cell death, perhaps suggesting a role for NOX2 in dopamine-induced cell death. Indeed, increased (peri)nuclear localized NOX2 activation in response to ischemia was shown by us to induce apoptosis in H9c2 cells. However, the lack of concomitant caspase-3 activation in response to dopamine suggests a different mechanism. Indeed, increased (peri)nuclear localized NOX2 activation in response to ischemia was shown by us to induce apoptosis in H9c2 cells. Whether this mechanism is relevant in the dopamine-induced death in H9c2 cells and whether NOX-induced ROS are involved herein remains to be elucidated.

Alternatively, absence of caspase-3 activation can indicate absence of apoptosis. Previously, we have shown in H9c2 cells and adult ventricular rat cardiomyocytes that exposure of phosphatidylserine (PS) on the outer plasma membrane leaflet, previously considered a hallmark of apoptosis, can occur independent of apoptosis and be reversible. This non-apoptotic PS exposure was related to inactivation of flippase, the plasma membrane transporter responsible for shuttling PS from the outer to the inner membrane leaflet, thereby maintaining plasma membrane phospholipid asymmetry. Importantly, we now found that dopamine induces PS exposure with concomitant flippase inactivation in the absence of caspase-3 activation indicative of non-apoptotic PS exposure. This corresponds to the absence of caspase-3 activity, TUNEL-positive, and complement-9 positivity that were found in cardiomyocytes of patients with stress-induced cardiomyopathy. Exposure of PS in the outer plasma membrane layer allows the binding of inflammatory mediators, such as sPLA₂-IIA and CRP and inflammatory cells and thereby facilitates inflammation. The dopamine-induced pro-inflammatory status of the plasma membrane may explain inflammation that was found in animal models and patients with stress-induced heart disease.

A limitation of our study is the relative high concentration of dopamine, namely 200 μmol/L (38 μg/ml), that we have used in our in vitro model. This because in patients with stress-induced heart failure, 106-146 pg/ml was found in the blood plasma, while in blood taken from the coronary sinus a dopamine concentration of 158 pg/ml was found in patients with Takotsubo-like left ventricular dysfunction. However, it has to be noticed that (200 μM) dopamine was used successfully in an in vivo mouse model of LPS induced systemic inflammation, in which it inhibited NLPR3 inflammasome activation, while infusion of 375 μg/ml dopamine did induce Takotsubo-like cardiac dysfunction in male wistar rats.

In conclusion, our data show that elevated dopamine levels also have cytotoxic effects, as it induces lipid accumulation, oxidative stress, increased cell death, and importantly, a pro-inflammatory status of the plasma membrane. These cytotoxic effects and pro-inflammatory status may explain the inflammation found in patients with stress-induced heart failure.
REFERENCES