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Chapter 5

Early NOX2 activation is crucial in phenylephrine-induced hypertrophy of H9c2 cells

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Abstract

Increased production of reactive oxygen species (ROS) is implicated in the process of cardiomyocyte hypertrophy, known to be induced by different stimuli, like angiotensin II, pressure overload and phenylephrine (PE). Although targeting adrenoreceptors and therefore sharing common signaling processes, recent studies point to different mechanisms activated by these stimuli, in which different NOX isoforms, as important sources of ROS, might play a role. As such, NOX2 was shown to be the main isoform involved in angiotensin II-induced hypertrophic responses, whereas NOX4 was shown to be essential in pressure overload. The role of NOX isoforms in PE-induced cardiomyocyte hypertrophy, however, is unknown.

Rat neonatal cardiomyoblasts (H9C2 cells) were incubated with 100 μM PE to induce hypertrophy. The effects of PE-induced hypertrophy on the expression of different NOX proteins and NOX-mediated ROS production were studied via electron microscopy and digital-imaging microscopy.

Incubation of H9C2 cells with PE significantly induced hypertrophy after 24 and 48 hours. However, at these time points no significant differences in NOX/ROS expression levels were found. Interestingly, in contrast to NOX1 and NOX4, NOX2 expression increased significantly up to 4 hours after PE stimulation, coinciding with ROS production in the cytoplasm as well as the nucleus. Furthermore, inhibition of NOX-mediated ROS production with apocynin, diphenylene iodonium (DPI) or gp91 docking sequence (gp91ds)-tat peptide during these first 4 hours of PE stimulation significantly inhibited hypertrophy of H9C2 cells, both after 24 and 48 hours of PE stimulation. Early NOX2-mediated ROS production is crucial in PE-induced hypertrophy of H9C2 cells.

Introduction

Cardiac hypertrophy is a (patho)physiological adaptive response of the heart to pressure overload.[1] However, after prolonged periods, this initial adaptive response may become maladaptive, leading to increased mortality and morbidity from heart failure.[2]

Left ventricular hypertrophy can be the result of many pathogenetic factors, including hypertension.[3] However, in contrast to pressure overload, angiotensin II was shown to cause cardiac hypertrophy *in-vivo,* independent of hypertension,[4] suggesting that different stimuli can independently induce cardiac hypertrophy. The same is true for phenylephrine (PE). It namely was shown that, although angiotensin II and PE are coupled to similar downstream signaling pathways and hypertrophic

responses in cardiomyocytes, PE was a more effective inducer of hypertrophy.[5/6] As such, incubation of PE during 48 hours was more effective than angiotensin II in increasing cell size in isolated neonatal rat cardiomyocytes.[5/6] In addition, an angiotensin II receptor antagonist blocked the angiotensin II effect but did not suppress the PE effect,[6] underlining different stimulus-dependent pathways.

Redox-sensitive mechanisms play an essential role in mediating the development of cardiac hypertrophy, in which NADPH oxidase (NOX) is particularly important.[7] Different NOX isoforms, NOX1, NOX2, NOX4, NOX5 and DUOX1/DUOX2 meanwhile have been identified either in cardiomyocytes and/or vascular cells.[8-12] In particular NOX2 and NOX4 were shown to be involved in pro-apoptotic[9/10/13] but remarkably also pro-hypertrophic[14/15] signaling in cardiomyocytes. As such, angiotensin II-induced hypertrophy coincided with an increased left ventricular NOX activity that was reduced in NOX2 knock-out mice compared to wild-type controls.[16] In contrast, in a pressure overload model, induced by aortic constriction, identical cardiac hypertrophy was found both in wild-type mice as well as NOX2 knock-out mice.[16] In these NOX2 knock-out mice, however, increased NOX4 expression was detected in the left ventricle, whereas NOX1 expression was not found.[16] In cardiac specific NOX4 knock-out mice indeed a decrease in ROS and cardiac hypertrophy was found after aortic constriction. In these NOX4 knock-out mice expression levels of NOX2 were not affected compared to wild-type controls and NOX1 was not found.[10]

Tanaka *et al.*[17] have shown in isolated rat cardiomyocytes that PE induced phosphorylation of $p47^{phox}$, increased NOX activity and ROS in heart homogenates of rats that was inhibited by the flavoenzyme inhibitor diphenylene iodonium (DPI). Although they did not analyze hypertrophy, neither did they differentiate between the different NOX isoforms.[18] Therefore, in the present study we have analyzed the role of the different NOX isoforms in PE-induced cardiomyocyte hypertrophy.

Materials and Methods

Cell culture

Rat cardiomyoblasts cells (H9c2 cells) were obtained from the American Type Culture Collection ((ATCC), Manassas, VA, USA) and cultured in culture medium: Dulbecco's modified eagles medium (DMEM, Cambrex Corporation, East Rutherford, NJ, USA) with addition of 10% (v/v) heat inactivated fetal calf serum (FCS, Bio-Whittaker, Walkersville, MD, ASU), 100 IU/ml penicillin (Yamanouchi Europe BV, Meppel, The Netherlands), 100 µg/ml streptomycin (Radiopharma Fisiopharme, Palomonte, Italy) and 2 mM L-glutamine (Invitrogen Corporation, Carlsbad, CA, USA). H9c2 cells were cultured at a 5% CO_2 atmosphere at 37°C.

To induce hypertrophy, cells were starved for 18 hours in DMEM containing 1% FCS and subsequently incubated with 100 µM PE (Sigma, Milwaukee, USA) up to 48 hours. Different inhibitors of NOX were studied herein, namely apocynin (100 µM, Sigma;[19] an inhibitor of the association of cytosolic and membrane-bound components of NADPH oxidase), diphenylene iodonium (DPI: 10 µM, Sigma;[19] a flavoenzyme inhibitor) and NOX2 docking sequence tat peptide (NOX2ds-tat: 50 µM;[20] a specific inhibitor of the NADPH oxidase 2).

Determination of cell size

Digital-imaging microscopy

The area of the cell was determined via quantification of the total surface. To specify between stretching of cells and hypertrophy, the size of the nucleus was determined via both quantification of the square area and its volume via 3D stack images.

Electron microscopy

Cells were fixed in 2% glutaraldehyde for 30 minutes and 1.5% osmium tetraoxide for 10 minutes. Cells were then dehydrated with acetone and embedded in Epon 812. Ultra thin sections were collected on 300-mesh Formavar-coated Nickel grids. The sections were contracted with uranyl acetate and lead citrate and were examined in a Jeol-1200 EX electron microscope. Five electron microscopy pictures of control cells or PE-stimulated cells were analyzed to determine the area of the nucleus (magnification 9000×, 5 cells per picture) and the area of the whole cell (magnification 4500×, 10 cells per picture).

Automatic Cell Counter

The diameter of cells in suspension was quantified with a Scepter 2.0 Handheld Automated Cell Counter (Millipore, Billerica, MA, USA) according to standard operating procedure.

Digital-imaging microscopy

After treatment, H9c2 cells were fixed with 4% paraformaldehyde for 10 minutes at 37°C and permeabilized with acetone-methanol (30%-70% (v/v)) for 10 minutes at RT. The cells were subsequently incubated with the primary antibodies for 1 hour at RT followed by incubation overnight at 4°C. Primary antibodies used were goat-anti NOX1 (1:50, Santa Cruz, CA, USA), rabbit-anti $p91^{phox}$ (NOX2: 1:50, Upstate, North Billerica, MA, USA) and goat-anti NOX4 (1:50, Santa Cruz). The cells were then

incubated with the secondary antibodies Alexa Fluor 568-labeled donkey-anti goat (1:40, Invitrogen) and Alexa Fluor 647-labeled donkey-anti rabbit (1:40, Invitrogen) for 30 min at RT in the dark. Negative controls with only secondary antibody were included to assess nonspecific binding. These controls were all negative (data not shown). Before visualization, mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; H-1500, Vector Laboratories Inc, Burlingame, CA, USA) was added and the cells were covered.

2D/3D optical sections were acquired and analyzed with a 3I Marianas™ digital-imaging microscopy workstation (Zeiss Axiovert 200M inverted microscope; Cark Zeiss, Sliedrecht, The Netherlands). Exposures, objectives and pixel binning were automatically recorded with each image. Data processing was controlled by Slidebook™ software (version 4.2; Intelligent Imaging Innovations, Denver, CO, USA). Expression levels of NOX1, NOX2 and NOX4 were determined via quantification of the mean intensity.

Imaging of ROS production

H9C2 cells were cultured in chamber slides and after treatment either fixed and stained with rabbit-anti nitrotyrosine (1:50, Invitrogen), as an indirect marker of ROS generation[21] (1:50, Invitrogen) or loaded with 10M 5-(6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA), as a marker of H_2O_2 generation (Molecular Probes, Leiden, The Netherlands). As described above, cells were analyzed by the use of 3I Marianas™ digital-imaging microscopy workstation. Cells were scored by determining the number of nitrotyrosine/H_2O_2-positive H9C2 cells.

Detection of caspase 3 activity

Caspase 3 activity in H9C2 cells was measured using a caspase 3 assay kit (Homogenous caspase 3/7 assay kit, Roche, Mannheim, Germany). Equal cell numbers were lysed and incubated with DEVD-rhodamine 110 substrate (Roche, Mannheim, Germany) for one hour at 37°C. The amount of free rhodamine was determined using a microplate fluorescence reader (TECAN spectra Fluor, Switzerland) with an absorption filter of 492 λ, an emission filter of 535 λ and a gain of 93.

Statistics

The SPSS statistics program (Windows version 9.0) was used for statistical analysis. To evaluate whether observed differences were significant, the paired T-tests and One-way ANOVA with post hoc Bonferroni test was used. In the relevant figures and in the text values are given as mean ± SE. A two-sided p value of 0.05 or less was considered to be significant.

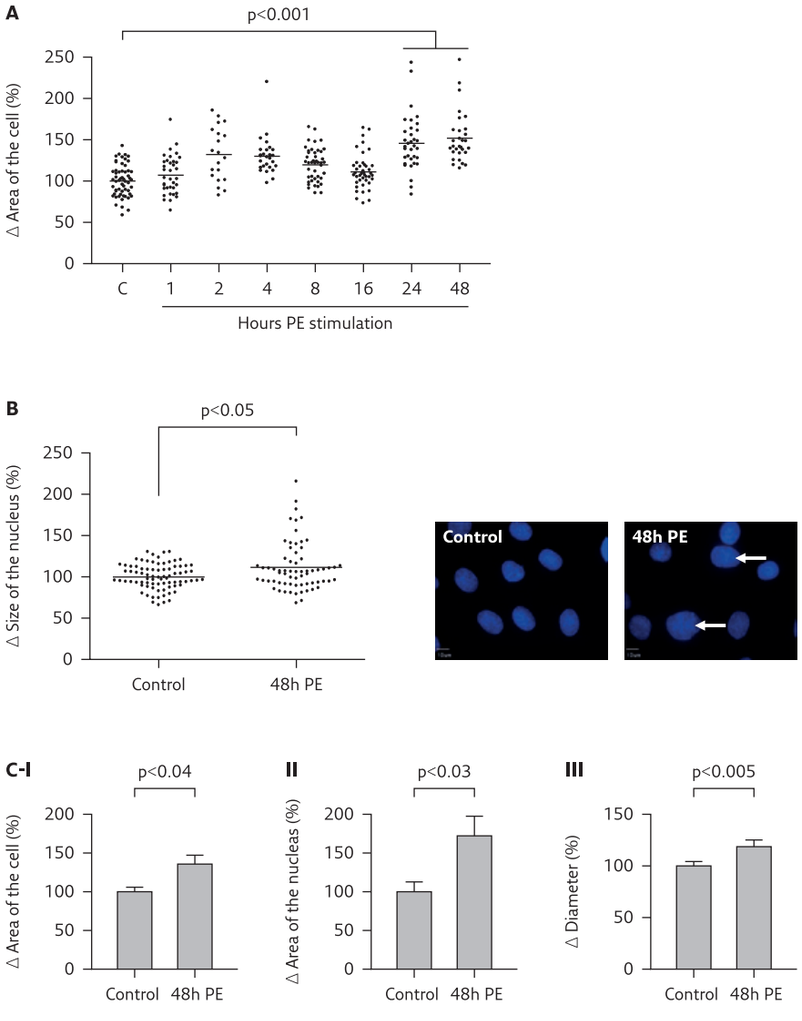
Results

Phenylephrine induced cardiomyocyte hypertrophy

PE-induced hypertrophy in attached H9C2 cells was analyzed using digital-imaging microscopy. Stimulation with PE after 1, 2, 4, 8 and 16 hours induced a non-significant increase in cell area, respectively with 7±4%, 30±7%, 27±3%, 20±3% and 11±3%, compared to control cells (figure 1A). Only after 24 and 48 hours of PE stimulation a significant increase in cell area was induced, respectively with 52±8% ($p<0.001$) and 56±5% ($p<0.001$), compared to control cells. In line with this, stimulation with PE after 48 hours also significantly increased the size of the nucleus (figure 1B, arrows) with 12±4% ($p<0.05$), compared to control cells (figure 1B, graph).

This PE-induced hypertrophy effect was also verified in H9C2 cells in suspension, since attached cells as analyzed above, can stretch, as such mimicking hypertrophy. 48 hours of PE stimulation then induced a significant increase in total cell area (figure 1C-I, with 36±11% $p<0.04$) and nuclear area (figure 1C-II, with 72±25% $p<0.03$) as measured via electron microscopy. Also a significant increase in cell diameter was found (figure 1C-III, with 19±6% $p<0.005$) as measured via the Automated Cell Counter, underlining induction of cell hypertrophy.

These results thus show that PE induced hypertrophy of H9C2 cells in time.



1. Phenylephrine induced hypertrophy of H9c2 cells | Digital-imaging microscopy analysis of phenylephrine (PE)-stimulated H9c2 cells after different time intervals. Analysis of (A) area of the cell (n=6) and (B) size of the nucleus (n=4) of attached H9c2 cells. Images of nuclei are stained with DAPI (blue signal), representative for n=4. Arrows indicate increased area of the nucleus. (C) Analysis of phenylephrine (PE)-stimulated H9c2 cells after 48 hours. Electron microscopy analysis of (I) area of the cell (n=5), (II) area of the nucleus (n=5) and Automated Cell Counter analysis of (III) diameter (n=3) of H9c2 cells in suspension. The changes are shown as the difference (△) in the percentage compared to control cells set to 100%.

Inhibition of NOX-mediated ROS production reduced cardiomyocyte hypertrophy

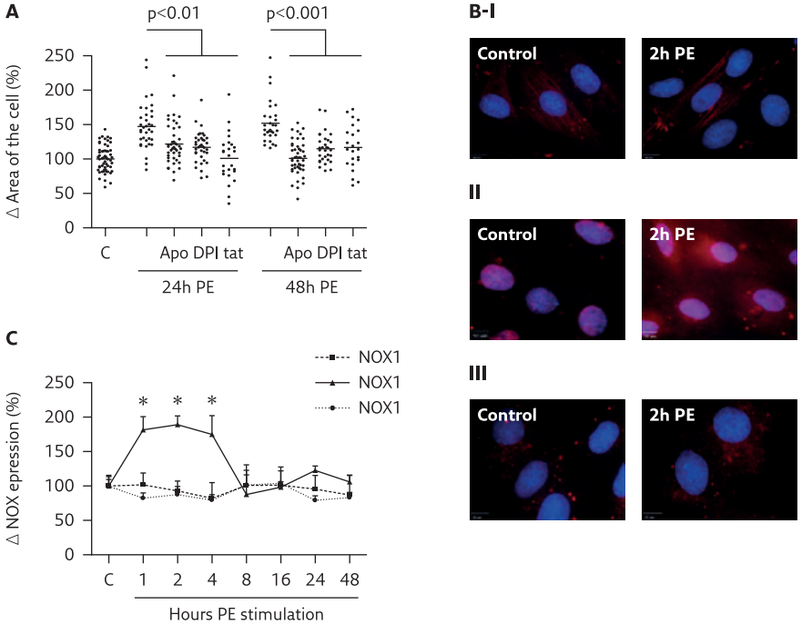
To verify whether NOX-mediated ROS production indeed plays a role in PE-induced cardiomyocyte hypertrophy,[15/22/23] the effect of the different NOX inhibitors apocynin, DPI[19] and NOX2ds-tat[20] on the induction of hypertrophy was analyzed in attached H9c2 cells using digital-imaging microscopy.

Stimulation of H9c2 cells with PE during 24 hours in the presence of apocynin, DPI or NOX2ds-tat resulted in a significant decreased cell area, respectively of 25±5% ($p<0.01$), 30±4% ($p<0.01$) and 46±7% ($p<0.01$), compared to 24 hours PE stimulation only (figure 2A). The same was true for 48 hours of PE stimulation, namely a significant reduction in total cell area of 51±4% (apocynin, $p<0.001$), 37±4% (DPI, $p<0.001$) and 35±7% (NOX2ds-tat, $p<0.001$), compared to 48 hours PE stimulation only. Although the mechanisms of inhibition of apocynin, DPI and NOX2ds-tat differ,[24] no significant differences in inhibitory effect between these inhibitors at 24 and 48 hours of PE stimulation was found. This thus proves a role for NOX-mediated ROS production in PE-induced cardiomyocyte hypertrophy.

NOX2 expression coinciding with ROS is increased early in phenylephrine stimulation

To verify which NOX isoforms could be related to PE-induced cardiomyocyte hypertrophy, we analyzed at different time intervals of PE stimulation the expression levels and subcellular localization of NOX isoforms that have been shown in the heart, e.g. NOX1, NOX2 and NOX4,[25] that theoretically could be involved in PE-induced hypertrophy, using digital-imaging microscopy. Since NOX5 is not expressed in rodents, and DUOX1/DUOX2 play an important role in the generation of thyroglobulin,[11] we excluded these NOX isoforms in this analysis.

We found that PE stimulation had no significant effect on the expression levels, nor the subcellular localization of NOX1 (figure 2B-I) and NOX4 (figure 2B-III) up to 48 hours (figure 2C). In contrast, NOX2 expression levels were significantly increased after 1, 2 and 4 hours of PE stimulation, respectively with 50±6% (*p<0.001), 69±6% (*p<0.001) and 40±5% (*p<0.001), compared to control cells (figure 2C). However, after 8, 16, 24 and 48 hours of PE stimulation, NOX2 expression levels reduced significantly back to control levels, respectively with 50±4% (p<0.001), 55±2% (p<0.001), 40±3% (p<0.001) and 40±2% (p<0.001). It has to be noticed that in control cells NOX2 expression was found in the cytoplasm and (peri)nuclear regions of H9c2 cells (figure 2B-II). PE however did not induce a difference in the subcellular localization of NOX2.

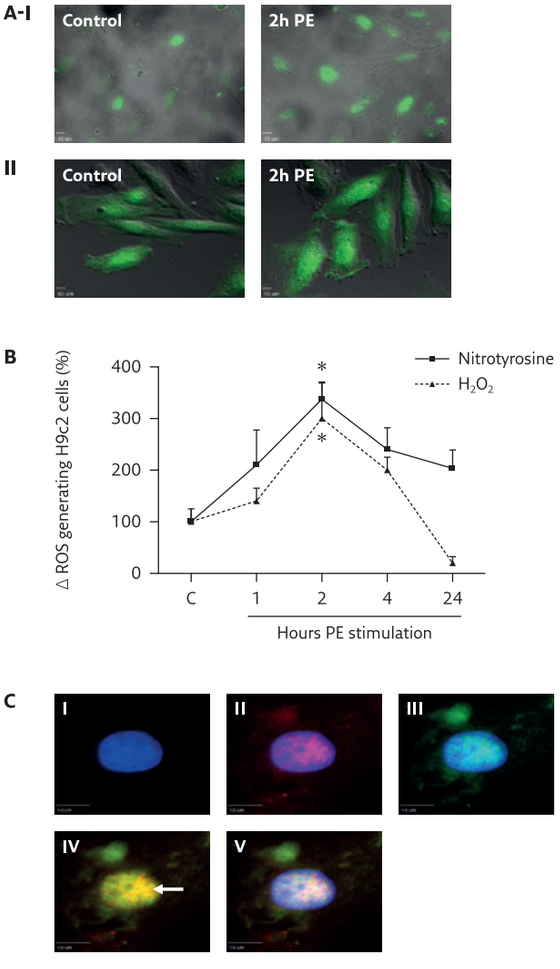


2. Time-dependent role for NOX2 in phenylephrine-induced hypertrophy of H9c2 cells
Digital-imaging microscopy analysis of phenylephrine (PE)-stimulated H9c2 cells after different time intervals. (A) Analysis of the effect of apocynine (Apo), diphenylene iodonium (DPI) and gp91 docking sequence tat peptide (tat) after 24 and 48 hours (n=3). (B) Subcellular analysis of NOX1 (I, red signal), NOX2 (II, red signal) and NOX4 (III, red signal). Images of nuclei are stained with DAPI (blue signal), representative for n=4. (C) NOX1, NOX2 and NOX4 expression levels after slide book analysis (n=4). The changes are shown as the difference (△) in the percentage compared to control cells set to 100%. *p<0.001.

We subsequently analyzed whether this NOX2 expression did coincide with the generation of ROS (as measured via nitrotyrosine expression) or H_2O_2 (as measured via CM-H_2DCFDA fluorescence). In line with NOX2, generation of ROS was found in the cytoplasm and (peri)nuclear regions of control H9c2 cells (figure 3A-I/II). Also here PE did not induce any differences in the subcellular localization of both nitrotyrosine and H_2O_2. On the other hand, PE increased the expression levels of nitrotyrosine after 1, 2, 4 and 24 hours, respectively with 110±67%, 237±31%, 140±42% and 84±44%, compared to control cells (figure 3B). However, this was only significant after 2 hours of PE stimulation (*p<0.01). In line herewith, PE increased H_2O_2 levels after 1, 2 and 4 hours, respectively with 40±25%, 200±71% and 100±25%, compared to control cells (figure 3A), which again was only significant after 2 hours PE stimulation (*p<0.01). However, after 24 hours of PE stimulation, H_2O_2 expression levels were reduced significantly, even below control levels with 180±13% (p<0.001). In addition, (peri)nuclear NOX2 was found to colocalize with (per)nuclear nitrotyrosine (figure 3C-IV, yellow signal), suggesting PE-induced (peri)nuclear NOX2 activity.

It has to be noticed that nuclear NOX2-mediated ROS has been shown to play an important role in the induction of apoptosis in H9c2 cells, in response to ischemia[19/26] and homocysteine.[13/27] To analyze whether the PE-induced NOX2 expression and ROS generation in (peri)nuclear regions was also related to apoptosis, we measured caspase 3 activity. We then found no significant differences in active caspase 3 concentration after 1, 2, 4, 8, 16, 24 or 48 hours of PE stimulation compared to control cells (data not shown).

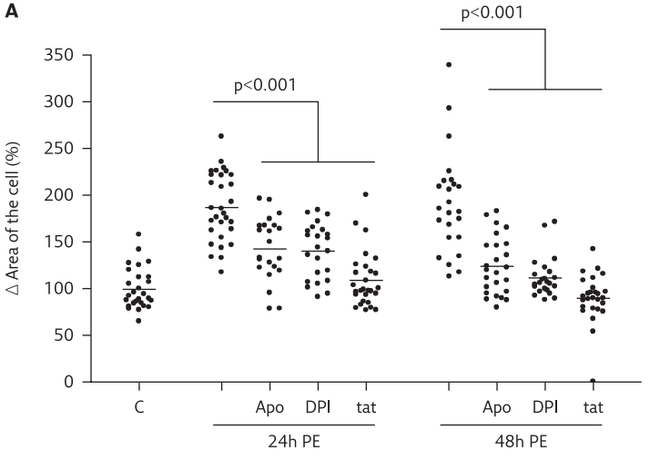
These data thus indicate that NOX2 coinciding with ROS plays a role in PE-induced hypertrophy of H9c2 cells.



3. Time-dependent role for ROS in phenylephrine-induced hypertrophy of H9c2 cells
Digital-imaging microscopy analysis of phenylephrine (PE)-stimulated H9c2 cells after different time intervals. (A) Subcellular analysis of nitrotyrosine (I, green signal) and H_2O_2 (II, green signal), representative for n=3. (B) Nitrotyrosine and H_2O_2 expression levels after slide book analysis (n=3). Changes are shown as the difference (△) in the percentage compared to control cells set to 100%. *p<0.01. (C) Subcellular localization of NOX2 and nitrotyrosine in phenylephrine-stimulated H9c2 cells. Cells were stained for NOX2 (II, red signal) or nitrotyrosine (III, green signal). Nucleus was stained with DAPI (blue signal). Pictures IV and V demonstrate that under 2 hours phenylephrine (peri)nuclear NOX2 focally coincides with nitrotyrosine (yellow signal, arrow) and DAPI (white signal).

Early inhibition of NOX-mediated ROS production decreased phenylephrine-induced hypertrophy

To prove whether this time-dependent NOX2 activation indeed is crucial in PE-induced hypertrophy, we studied the effect of early inhibition of NOX/ROS hereon. For this, apocynin, DPI or NOX2ds-tat were added during the first 4 hours of 24 hours of PE stimulation only. Then a significant decrease in total cell area was found with respectively 44±7% (p<0.001), 47±6% (p<0.001) and 79±6% (p<0.001), compared to 24 hours of PE stimulation alone (figure 4). Also when cells were stimulated with PE during 48 hours, addition of apocynin, DPI or NOX2ds-tat during the first 4 hours only, induced a significant decreased cell area, respectively with 67±6% (p<0.001), 80±5% (p<0.001) and 101±5% (p<0.001), compared to 48 hours PE stimulation alone. There was no significant difference between the different NOX/ROS inhibitors at both 24 and 48 hours of PE stimulation. However, it has to be noticed that only NOX2ds-tat inhibited the cell area to control levels after 24 and 48 hours of PE stimulation.
Thus, early activation (during the first 4 hours) of NOX-mediated ROS plays an important role in PE-induced hypertrophy of cardiomyocytes at the longer term (at 24 and 48 hours).



4. Early inhibition of NOX-mediated ROS production | Digital-imaging microscopy analysis of phenylephrine (PE)-stimulated H9c2 cells in which apocynine (Apo), diphenylene iodonium (DPI) and gp91 docking sequence tat peptide (tat) were added during the first 4 hours only, when stimulated with PE during 24 hours and 48 hours (n=3). The changes in cell area are shown as the difference (△) in the percentage compared to control cells set to 100%.

Discussion

ROS are important mediators in the induction of cardiac hypertrophy. Interestingly, different hypertrophic stimuli (e.g. angiotensin II, pressure overload and PE) were shown to exert their hypertrophic effects via different intracellular signal transduction cascades,[4/6/28] suggesting stimuli-specific responses. Moreover, a NOX isoform-specific role herein was suggested, at least in angiotensin II and pressure overload induced hypertrophy.[10/15/16/29] Knowledge of the role of the different NOX isoforms in PE-induced cardiac hypertrophy, however, still is lacking.

We now found that the isoform NOX2 plays a role in PE-induced hypertrophy of H9c2 cells. Indeed, induction of hypertrophy in H9c2 cells via PE stimulation during 24 and 48 hours coincided with a significant ROS production after 2 hours, and a significant increase in expression of NOX2, but not NOX1 and NOX4, from 1 to 4 hours. In line with this, inhibition of NOX-mediated ROS using different NOX inhibitors, namely apocynin, DPI and NOX2ds-tat, added during the first 4 hours of PE stimulation, significantly reduced PE-induced hypertrophy after 24 and 48 hours. Increased NOX activity has been described in total rat heart homogenates in PE-induced hypertrophy, however, without specifying the NOX isoform and/or whether this was related to NOX activity in cardiomyocytes in particular.[18] We now found that the NOX inhibitors apocynin and DPI did inhibit PE-induced hypertrophy of H9c2 cells, underlining the role of NOX herein. This of course still does not specify which particular NOX isoform is involved, as these inhibitors do not differentiate between the different NOX isoforms.[24] On the other hand, the inhibitory effect of NOX2ds-tat on PE-induced hypertrophy does point to a role of NOX2 herein. This because NOX2ds-tat was shown to specifically inhibit NOX2-mediated ROS production, by mimicking a sequence in the cytosolic B-loop of NOX2, but not the NOX1 and NOX4 oxidase systems.[20] In line with this, Tanaka *et al.*[17] did find increased phosphorylation of $p47^{phox}$, an important cytosolic subunit/activating protein of NOX2,[19] in total heart homogenates in PE-induced hypertrophy. As we did find increased expression of NOX2 colocalizing local ROS production after 2 hours of PE stimulation, while NOX1 and NOX4 then remained at their basal expression levels, this strongly points to an important role for NOX2 activity in PE-induced cardiomyocyte hypertrophy. It has to be noticed that NOX5 does not play a role in PE-induced hypertrophy in these H9c2 cells, as NOX5 is lacking in rats. However, as we recently did find NOX5 to be present in human cardiomyocytes,[30] we can not exclude a role of NOX5 in hypertrophy induction in the human heart.

Interestingly, the early induction of NOX2-mediated ROS, after 2 hours PE stimulation, resulted in long-term hypertrophy. This corresponds to data found in isolated adult rat cardiomyocytes where it was shown that PE induced elevated levels of intracellular ROS after 5 minutes of stimulation, based on oxidation of dichlorofluroescein (DCF), that declined to basal levels after 60 minutes.[17] However, although this ROS induction was inhibited by DPI, a direct effect on hypertrophy was not studied, neither did they study the different NOX isoforms herein.[17]

It was shown *in-vitro* that angiotensin II induced expression of immediate early genes (e.g. *c-Fos, c-Jun,* and activator protein 1 (AP1)) within 30 minutes, whereas late markers of cardiac hypertrophy (e.g. skeletal alpha-actin and atrial natriuretic peptide (ANP) expression) were induced after 6 hours.[31/32] Since the ROS scavenger N-acetyl-l-cysteine (NAC) was shown to almost completely abolish the angiotensin II-induced increase of AP1 binding, a role for ROS in the intracellular transduction of angiotensin II for immediate early gene induction was suggested.[33] However, this was analyzed in mouse myogenic C2C12 cells, not in cardiomyocytes. In isolated rat cardiomyocytes PE also did induce early changes in gene expression (e.g. *c-Jun* and connective tissue growth factor (CTGF)) within 1 hour and subsequently changes in expression of structural genes associated with hypertrophy (increased over 4 to 24 hours).[34/35] Although redox regulation of *c-Jun* activity and expression has been described,[36/37] a role for NOX/ROS-mediated early changes has not yet been studied in relation to PE.

In conclusion, our results indicate a time-dependent role of NOX2-mediated ROS production in PE-induced hypertrophy of H9c2 cells.

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