Canadian Journal of Physiology and Pharmacology

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Journal:	<i>Canadian Journal of Physiology and Pharmacology</i>
Manuscript ID	cjpp-2019-0055.R1
Manuscript Type:	Brief Report
Date Submitted by the Author:	14-Feb-2019
Complete List of Authors:	Bkaily, Ghassan; University of Sherbrooke, Anatomy and Cell Biology, Faculty of Medicine Najibeddine, Wassim; University of Sherbrooke, Anatomy and Cell Biology, Faculty of Medicine Jacques, Danielle; University of Sherbrooke, Anatomy and Cell Biology, Faculty of Medicine
Is the invited manuscript for consideration in a Special Issue:	2018 IACS Slovakia
Keyword:	NOX, free radicals, hereditary cardiomyopathy, heart failure, ROS

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Increase of NOX3 in heart failure of hereditary cardiomyopathy

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Short title: NADPH oxidase in heart failure

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Abstract

During the development of heart failure in humans and animal models, an increase in reactive oxygen species (ROS) levels were observed. However, there is no information whether this increase of ROS is associated with an increase in the density of a specific isoform of NADPH oxidases (NOX1-5). The objective of this study was to verify whether the densities of NOXs 1-5 change during the development of heart failure. Using the well-known model of cardiomyopathic hamsters, the UM-X 7.1 line, a model that strongly resembles the pathology observed in humans from a morphological and functional point of view, our studies showed that as in humans, the different NOXs, 1-5, are present in both normal and UM-X7.1 hamsters' hearts. Even though the densities of NOXs 2 and 5 were unchanged, the levels of both NOXs 1 and 4 significantly decreased in UM-X7.1 hamster hearts during heart failure. These changes were accompanied with a significant increase in NOX3 level. These results suggest that, during heart failure, NOX3 plays a vital role in compensating the decrease of NOXs 1 and 4. This increase in NOX3 may also be responsible, at least in part, for the reported increase in ROS levels in heart failure.

Keywords: NOX, free radicals, hereditary cardiomyopathy, heart failure, ROS

Introduction

In the heart, reactive oxygen species (ROS) can be generated endogenously by numerous mechanisms including oxidative phosphorylation in the mitochondria, xanthine oxidase (XO), cytochrome P450, catecholamines autooxidation, NO synthase (NOS) decoupling as well as by NADPH oxidases (NOXs) (Giordano, 2005; Steinhorn et al., 2018). Their functions in the cell are diverse. They are involved in signaling pathways leading to cardiomyopathy and heart failure (Kwon et al., 2003; Giordano, 2005; Dhalla et al., 1996; Kinugawa et al., 2000; Sabri et al., 2003; Suematsu et al., 2003; Takimoto et al., 2007; Ray et al., 2012; Ide et al., 1999; Ide et al., 2000; Kuroda et al., 2010a; Deo et al., 2014) as was shown in the hereditary cardiomyopathy of the hamster (Wu et al., 2012; Mollnau et al., 2005; Escobales and Crespo, 2006).

NOXs are the primary generators of ROS in cells, and they appear to be expressed in all cells of the cardiovascular system (Ahmarani et al., 2013), suggesting that they play a role at this level (Lassègue et al., 2012). Mounting evidence points to the involvement of NOXs in heart failure. Several studies showed increases in the expression and/or activity of different NOXs in human heart failure (Borchi et al., 2010; Heymes et al., 2003; Maack et al., 2003; Zhang 2013).

In vascular pathologies, ROS production, as well as mRNA and protein levels of NOXs 1,2 and 4, were increased in a mouse model developing atherosclerosis induced by a high-fat diet (Fenyo et al., 2011). However, in hypertensive rat aortas, an increase in ROS production was accompanied by an increase in protein density of NOXs 1 and 2 but not NOX4 (Wind et al., 2010).

In cardiac pathologies, an increase in NOX2 density was observed in humans (Krijnen et al., 2003) and animal models (Fukui et al., 2001; Sirker et al., 2016) with heart infarction. Besides, Byrne et al. showed that NOX4 mRNA levels were increased when hypertrophy was induced by aortic constriction, suggesting a role of NOX4 in cardiac pathophysiology (Byrne et al., 2003). Like NOX2, NOX4 appears to be also involved in heart failure (Kuroda et al., 2010b; Ago et al., 2010). However, this is not shared by other studies in the field (Ago et al., 2010). An increase in mRNA and protein expression of NOX5 was observed in the human myocardium following acute myocardial infarction (Hahn et al., 2012). Nevertheless, there is no information whether this type of NOX is implicated in hypertrophy and heart failure.

For NOXs 1 and 3, very few cardiac studies are available and more particularly in heart failure. The reason for the lack of data for these two types of NOXs is their low density reported in cardiac cells of rats and mice (Matsuno et al., 2012). It should be noted that the absence of NOX1 in transgenic mice suggests a beneficial effect on the performance and survival of cardiomyocytes (Matsuno et al., 2012).

The hereditary cardiomyopathic hamster is one of the best models for hypertrophy and heart failure associated with increased ROS levels (Dhalla et al., 1996; Wu et al., 2012; Mollnau et al., 2005; Escobales and Crespo, 2006). Hence, in this study, using the well-known animal model of hypertrophy and heart failure, the UM-X7.1 hereditary cardiomyopathic hamster (Jacques and Bkaily, 2018; Bkaily and Jacques, 2017; Bkaily et al., 2015), we verified whether the density of certain types of NOXs particularly NOXs 1 and 3 undergo changes during the development of heart failure.

Our results showed that, in heart failure, there is an increase in the density of NOX3 that was accompanied by a decrease of NOXs 1 and 4.

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Materials and methods

Protein extraction

The method of protein extraction was described previously (Chahine et al., 2005; Bkaily et al., 2015). Briefly, we used normal hamsters (NH) aged 11 to 14 months (Charles River, Montreal, Qc, Canada) and age-matched UM-X7.1 cardiomyopathic hamsters (HCMH), from our colony at the Faculty of Medicine of the University of Sherbrooke. The animals were sacrificed as per the standards of the animal protection committees of our institution and following the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care). The beating hearts were rapidly dissected, the atria were removed, and the ventricles were washed from blood. The ventricles were then homogenized on ice using a Polytron (Kinematica GmbH, Switzerland) in a homogenization buffer (Trizma base 125 mM pH 6.8, phenylmethylsulfonyl fluoride (PMSF) 0.1 M, leupeptin 0.5 mg/ml, aprotinin 0.5 mg/ml and pepstatin 0.7 mg/ml) (10 ml of buffer for 1g of tissue). An equal volume of Laemli 2X buffer (2.3% sodium dodecyl sulfate (SDS), 5% beta-mercaptoethanol, 10% glycerol and 0.001% bromophenol blue and 62.5 mM Trizma base pH 6.8) was then added and the whole was centrifuged at 10,000 RPM for 10 min at room temperature. The supernatant containing the proteins was recovered and stored at -20 ° C. The protein dosage was performed using the BCA ("Bicinchoninic acid protein assay reagent") method (Pierce Chemical Co., Rockford, IL). A standard curve was established with bovine serum albumin (BSA) performed in parallel for each assay. All samples were assayed in triplicate.

Western blot

The method used was similar to that previously reported (Bkaily et al., 2003, 2015; Chahine et al., 2005). Briefly, equivalent amounts of proteins (70 µg) were separated by polyacrylamide gel electrophoresis (7.5%) and transferred onto a nitrocellulose membrane (Amersham Life Science, Piscataway, NJ, USA). The proteins were then stained with a Ponceau red solution to ensure transfer efficiency and the membranes were incubated for 2 hours at room temperature in a blocking solution composed of PBS 1X (NaCl 137 mM, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH = 7.4), 5% non-fat dry milk (NFD, Carnation, Smucker Foods Canada Inc., Ontario, Canada) and 0.05% Tween-20 (Bio-Rad Laboratories, Ontario, Canada). The membranes were then incubated overnight at 4 °C on a shaker with one of the following primary goat-derived antibodies: polyclonal MOX1 (N-15) antibody, polyclonal gp91-phox (E-19) antibody, polyclonal NOX3 (F-12) antibody, polyclonal NOX5 (W-15) antibody (Santa Cruz Biotechnology, California, USA) or with a rabbit monoclonal NOX4 antibody (ab109225, Abcam, Ontario, Canada). In order to confirm equal loading of proteins, the blots were also probed with a mouse monoclonal antibody against GAPDH (6C5) (Abcam, Ontario, Canada). The membranes were then washed three times and incubated with one of the following secondary antibodies: peroxidase-conjugated anti-goat IgG monoclonal antibody (Santa Cruz Biotechnology, California, USA), peroxidase-conjugated anti-rabbit IgG monoclonal antibody (GE Healthcare Ltd., Little Chalfont, UK), or peroxidase-conjugated anti-mouse IgG monoclonal antibody (GE Healthcare Ltd., Little Chalfont, UK). Also, negative controls were carried out in the presence of the blocking peptide for each of the NOXs (Santa Cruz Biotechnology, California, USA) and clearly show the absence of the corresponding bands.

After three washes, the immune complexes were detected by chemiluminescence (Western Lightning Plus-ECL, Perkin-Elmer, MA, USA) and visualized by autoradiography on BIOMAX type film (Kodak, Rochester, NY, USA).

Densitometry

The films were digitized using an "Imaging Research Inc." system equipped with an "MTI CCD72 camera". The densities of the bands were obtained using the MCID Basic-M5 software (Imaging Research Inc., Ontario, Canada). The density value obtained for each band represents its density multiplied by its surface area followed by subtracting the background ($[\text{density} \times \text{surface}] - \text{background noise}$). Then, the ratio of the NOX band density to the GAPDH band density was calculated, and this ratio was used to compare the density of the different NOXs between normal and cardiomyopathic hamsters.

Statistics

All values were expressed as mean $\pm$ SEM where n is the number of Western blot wells of at least three different animals. Statistical comparisons were performed using Student's t -test (GraphPad Prism software) and ANOVA followed by Bonferroni post-hoc analysis where applicable. p -values less than 0.05 (95% confidence interval) were considered significant.

Results

Negative and Positive Controls of NOXs 1-5

All the antibodies used in this study were previously used by our laboratory for immunocytochemistry studies and they were found to be specific (Ahmarani et al., 2013). In this series of experiments, we used Western immunoblotting in order to confirm the specificity of the antibodies further. Since most studies on different NOXs were done in the A10 rat smooth muscle cell line and in rat heart tissue extracts (NOX1-3 and 5) (Ahmarani et al., 2013) as well as in the HK cell line (NOX4), we used these three preparations as positive controls. Besides, we performed negative controls in the presence of control peptides provided by the supplier and against which the primary antibodies were raised.

Figure 1A is a representative Western blot showing the specificity of the NOX1 antibody used as well as the positive control. As seen in this figure, using the NOX1 antibody preadsorbed with its control peptide (negative control) demonstrated no labeling of NOX1. However, the positive control of protein extracts from A10 cells showed a small band of NOX1. Furthermore, protein extracts of ventricular heart muscles showed a very dense band of NOX1 in normal hamsters (Fig. 1A).

Figure 1B shows a negative control and a positive control for the NOX2 antibody used. As seen in this figure, the negative control using NH cardiac ventricular muscle showed the presence of a dense band. However, the positive control in A10 cells showed the presence of two bands and the absence of the high-density band seen in the heart muscle in the negative control. Also, as seen in this figure, in NH, three bands of NOX2 were present:

the large band present in the negative control and the other two bands found in A10 cells. Thus, the dense band present in NH cardiac ventricular extracts that is surrounded by the two other bands was considered non-specific and was not taken into account in our measurements.

Figure 1C shows the absence of the NOX3 band in the negative controls using cardiac ventricular extracts of NHs and rats as well as extracts from A10 cells. However, NOX3 is present in NH heart muscle. These results confirm the specificity of the antibody used.

For NOX4, the negative controls in NHs and rats, as expected, showed low labeling which suggests specificity of the antibody. Using the HK cell line as a positive control for this NOX isoform showed the presence of a band whose density depends on the quantity of proteins used. A dense band for NOX4 can be seen in NH cardiac ventricular extracts.

A positive control (figure 6) and a negative control (not shown) for the NOX5 antibody in rat hearts confirmed the specificity of the antibody used (Ahmarani et al., 2013).

Decrease of NOX1 density in HCMH hearts.

Using Western blot, figure 2A shows examples of the level of NOX1 in the protein extracts of normal and hereditary cardiomyopathic (HCMHs) hamsters. As can be seen in this figure, the NOX1 bands were denser in NH ventricular extracts compared to those obtained from HCMHs. This example of Western blot also showed an apparent lower density of NOX1 in rat ventricular extracts compared to those from NHs. Densitometric measurements confirm that there is a significant decrease ($p < 0.0001$) in the density of NOX1 in ventricular extracts from HCMHs compared to NHs (figure 2B). This figure also

demonstrates a significantly higher level ($p < 0.0001$) of NOX1 in NH hearts compared to those from rats.

Absence of change in NOX2 density in HCMH hearts

Figure 3 shows examples of the absence of changes of NOX2 density in HCMH hearts. As seen in the Western blot examples, there are no apparent changes in the density of the two bands corresponding to NOX2 (figure 3A). Figure 3B confirms that the relative density of the band at 63 and 72 KD did not undergo changes in HCMH hearts compared to those from NHs.

Increase of NOX3 density in HCMH hearts.

As was shown in figure 1C, the density of NOX3 is very low in NHs heart extracts (figure 4A), and this was confirmed by densitometric measurements (figure 3B). As seen in figure 3A-B, the density of this NOX isoform in NH hearts is significantly lower ($p < 0.01$) than that measured in rat heart extracts. This figure also showed a highly significant increase ($p < 0.001$) in the relative density of NOX3 in HCMH hearts compared to NH hearts. The density level of NOX3 was also significantly higher ($p < 0.05$) in HCMHs when compared to rats.

Decrease of NOX4 density in HCMH hearts.

In this series of experiments, we verified whether NOX4 density changes in hereditary cardiomyopathy. As seen in figure 5A, there was a decrease in the density of NOX4 in HCMH hearts compared to those of NHs. Figure 5A also shows that the apparent density of the NOX4 band in rat hearts was similar to that of NHs. Densitometric measurements confirmed that there is a decrease in the density of NOX4 in HCMH hearts compared to

NHs (figure 5B). In addition, our measurements showed that the level of NOX4 in NH hearts is similar to that in rats.

Absence of change in NOX5 density in HCMH hearts

Using an antibody directed against NOX5 showed that the level of NOX5 density was similar in both NH and HCMH ventricular heart tissue (figure 6A). The level of this NOX isoform was also similar in NH and rat ventricular muscles.

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Discussion

NOXs 1-5 are involved in several cardiovascular pathologies, but they do not all play the same role. Our group has shown the presence of different NOXs in human cardiovascular cells (Ahmarani et al., 2013). The density of these different types of NOXs is cell type dependent (Ahmarani et al., 2013).

Our results showed that NOXs 1-5 are present in ventricular cardiomyocytes of normal and cardiomyopathic hamsters. These results are in agreement with those previously reported by our group in human cardiovascular cells (Ahmarani et al., 2013) and confirm that NOX3 presence is not limited to the inner ear (Lambeth et al. 2000; Paffenholz et al. 2004; Petry et al. 2010), macrophages (Sasaki et al. 2009) and lung endothelium of mice (Zhang et al. 2006). Our results showed that NOXs 2 and 5 densities did not change in heart failure of cardiomyopathic hamsters. Thus, these two NOX isoforms do not contribute to the increase in ROS levels reported in hypertrophy and heart failure of hereditary cardiomyopathic hamsters (Wu et al., 2012; Dhalla et al., 1996; Wu et al., 2012; Mollnau et al., 2005; Escobales and Crespo, 2006). However, NOX2 was reported to be implicated in induced cardiac hypertrophy in mice (Kuroda et al., 2010; Ago et al., 2010; Sirker et al., 2016; Byrne et al., 2003). The differences between our results and those reported in mice could be due, at least in part, to the animal model used and induced versus naturally occurring hypertrophy (Byrne et al., 2003). However, our results are dealing with late stages of heart failure. This may imply that the contribution of a specific NOX isoform may depend on the stage of the heart pathology as well as the age of the animal (Fukuchi et al., 1991).

Our results also showed that in heart failure of HCMHs there was a decrease in NOXs 1 and 4. These results showed for the first time the involvement of NOX1 in cardiac pathologies, particularly in heart failure.

In vitro overexpression of NOX4 had no effect on hypertrophy but induced apoptosis of cardiomyocytes (Ago et al., 2010). Thus, the decrease in NOX4 density reported in this study supports the concept that NOX4 contributes little to hypertrophy and heart failure.

Our results also showed that the density of NOX3 in the hearts of NHs was very low. This very low density of NOX3 in the heart could be one of the reasons why it was never detected before in the cardiovascular system using Western blot. Our results clearly showed that there was a highly significant increase in NOX3 in heart failure of HCMHs. The increase in the density of NOX3 in heart failure of HCMHs makes it easier to be detected. This increase in the density of NOX3 could be responsible, at least in part, for the reported increase in ROS generation in cardiac hypertrophy (Kwon et al., 2003; Giordano, 2005; Dhalla et al., 1996; Kinugawa et al., 2000; Sabri et al., 2003; Suematsu et al., 2003; Takimoto et al., 2007; Ray et al., 2012; Ide et al., 1999; Ide et al., 2000; Kuroda et al., 2010; Deo et al., 2014) including that in the cardiomyopathic hamster (Wu et al., 2012; Dhalla et al., 1996; Wu et al., 2012; Mollnau et al., 2005; Escobales and Crespo, 2006). This NOX isoform was also reported to play a key role in mediating superoxide production in cytokine promoted lipolysis in adipocytes (Issa et al., 2018).

The decrease in NOX1 and NOX4 densities could be a compensation mechanism for the increase in NOX3 density or vice versa. This should be verified in the future. Such a compensation mechanism between NOXs 2 and 4 was reported to take place in a NOX2

knock-out mouse model (Byrne et al., 2003). This mechanism may help limit heart damage by decreasing programmed cardiomyocyte death (Matsuno et al., 2012).

Finally, there is a lack of agreement in the literature concerning the presence of NOX1-5 and their implication in intracellular ROS level. One reason could be that their presence and densities are cell type- (Ahmarani et al., 2013) and species-dependent. This aspect is clearly shown by our results that the level of cardiac NOX1 in NHs is higher than that in the rat. However, the level of cardiac NOX3 is lower in NHs compared to that found in rat hearts. In addition, the levels of NOXs 4 and 5 were similar in both NH and rat hearts. It is worth mentioning that the contribution of a single NOX isoform to hypertrophy and more particularly to heart failure may also depend on the method by which these cardiac pathologies were induced (Byrne et al., 2003). Since the hypertrophy and heart failure in the UM-X7.1 cardiomyopathic hamster, as in humans, is naturally occurring, then some of the discrepancies in the literature concerning the role of the different types of NOXs in hypertrophy and particularly in heart failure are in part due to the animal model used and to the method of induction of these pathologies. It is also noteworthy that it is difficult to relate the level of ROS to the activity and density of a single NOX isoform because ROS level is also determined by the activities of antioxidants such as glutathione peroxidase.

In conclusion, our results demonstrated, for the first time, an important role of NOX3 in the increase in ROS generation during the development of hypertrophy and heart failure in hereditary cardiomyopathy. This increase in NOX3 density could be due, at least in part, to a compensatory mechanism for the decrease in NOXs 1 and 4. More studies should be done in order to further elucidate this mechanism.

Acknowledgements

This study was supported by a grant from the Canadian Institutes of Health Research (CIHR) and the Natural Sciences and Engineering Research Council (NSERC).

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Figure legends

Figure 1. Western blots showing the positive and negative controls of NOXs 1-4 antibodies. Autoradiographs showing total NOX1 (A), NOX3 (C) and NOX4 (D) densities in cardiac ventricles from rats and normal hamsters (NH) as well as in A10 cells. In panel B, the autoradiograph shows the total NOX2 density at the cardiac ventricle level in NHs as well as in A10 cells. In panel D, HK cell extracts were used as positive controls for the NOX4 antibody. Negative controls were performed by incubating the membranes in the presence of the respective antibody blocked with the corresponding NOX peptide from the supplier. The band corresponding to the reference protein GAPDH shows the presence of proteins in the negative control wells.

Figure 2. Decrease in NOX1 density during the development of heart failure in the hereditary cardiomyopathic hamster. (A) Autoradiographs showing NOX1 density in NH, HCMH and rat hearts. (B) Bar graph showing the decrease in NOX1/GAPDH ratio density in HCMH hearts compared to age-matched NH hearts as well as a lower NOX1/GAPDH ratio in rat hearts compared to NH hearts. The values are expressed as mean $\pm$ SEM where n is the number of Western blot wells of at least three different animals. *** P < 0.001.

Figure 3. Absence of change in NOX2 density during the development of heart failure in the hereditary cardiomyopathic hamster. (A) Autoradiographs showing NOX2 density in NH and HCMH hearts. (B) Bar graph showing that the NOX2/GAPDH ratio does not change in HCMH hearts compared to those from age-matched NHs. The values are expressed as mean $\pm$ SEM where n is the number of Western blot wells of at least three different animals.

Figure 4. Increase in NOX3 density during the development of heart failure in the hereditary cardiomyopathic hamster. (A) Autoradiographs showing NOX3 density in NH, HCMH and rat hearts. (B) Bar graph showing the increase in NOX3/GAPDH ratio in HCMH hearts compared to those from age-matched NHs as well as a higher NOX3/GAPDH ratio in rat hearts compared to NH hearts. The values are expressed as mean $\pm$ SEM where n is the number of Western blot wells of at least three different animals.

Figure 5. Decrease in NOX4 density during the development of heart failure in the hereditary cardiomyopathic hamster. (A) Autoradiographs showing NOX4 density in NH, HCMH and rat hearts. (B) Bar graph showing the decrease in NOX4/GAPDH ratio in HCMH hearts compared to age-matched NH hearts. However, there is no difference in NOX4/GAPDH ratio between rat and NH hearts. The values are expressed as mean $\pm$ SEM where n is the number of Western blot wells of at least three different animals. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 6. Absence of change in NOX5 density during the development of heart failure in the hereditary cardiomyopathic hamster. (A) Autoradiographs showing the density of NOX5 in NH, HCMH and rat hearts. (B) Bar graph showing that the NOX5/GAPDH ratio does not change in HCMH hearts compared to those from age-matched NHs. This ratio in rat hearts is not different from that in NH hearts. The values are expressed as mean $\pm$ SEM where n is the number of Western blot wells of at least three different animals.

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113x234mm (150 x 150 DPI)

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178x149mm (150 x 150 DPI)

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174x143mm (150 x 150 DPI)

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