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Temporal patterns of tyrosine nitration in embryo heart development

Liliana Viera¹, Milka Radmilovich², Marcelo R. Vargas³, Cassandra N. Dennys⁶, Landon Wilson⁴, Stephen Barnes⁴, Maria Clara Franco⁶, Joseph S. Beckman⁵, and Alvaro G. Estévez⁶

¹Laboratory of Motor Neuron Biology, Burke Medical Research Institute, White Plains, NY 10605

²Departamento de Histología y Embriología, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay

³School of Pharmacy, University of Wisconsin, Madison, WI 53705

⁴Department of Pharmacology and Toxicology, University of Alabama at Birmingham, Birmingham, AL 35294

⁵Linus Pauling Institute, Environmental Health Sciences Center, Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97330

⁶Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida

Abstract

Tyrosine nitration is a biomarker for the production of peroxynitrite and other reactive nitrogen species. Nitrotyrosine immunoreactivity is present in many pathological conditions including several cardiac diseases. Because the events observed during heart failure may recapitulate some aspects of development, we tested whether nitrotyrosine is present during normal development of the rat embryo heart and its potential relationship in cardiac remodeling through apoptosis. Nitric oxide (NO) production is highly dynamic during development, but whether peroxynitrite and nitrotyrosine are formed during normal embryonic development has received little attention. Rat embryo hearts exhibited strong nitrotyrosine immunoreactivity in endocardial and myocardial cells of the atria and ventricles from E12 to E18. After E18, nitrotyrosine staining faded and disappeared entirely by birth. Tyrosine nitration in the myocardial tissue coincided with elevated protein expression of nitric oxide synthases (eNOS and iNOS). The immunoreactivity for these NOS isoforms remained elevated even after nitrotyrosine had disappeared. Tyrosine nitration did not correlate with cell death or proliferation of cardiac cells. Analysis of tryptic peptides by MALDI-TOF shows that nitration occurs in actin, myosin, and the mitochondrial ATP synthase alpha chain. These results suggest that reactive nitrogen species are not restricted to pathological conditions but may play a role during normal embryonic development.

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Corresponding Author: Alvaro G. Estévez, Ph.D., Burnett School of Biomedical Sciences, College of Medicine, University of central Florida, 6900 Lake Nona Blvd, Orlando, FL 32827, 407 266 7097, alvaro.estevez@ucf.edu.

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INTRODUCTION

Nitric oxide was first described as the endothelial-derived relaxing factor, which modulates blood vessel relaxation by activating soluble guanylate cyclase [1]. Nitric oxide also modulates many other physiological processes, including neurotransmission, immune response, bone remodeling and muscle regeneration. Nitric oxide is produced through the oxidation of L-arginine to L-citrulline by three isoforms of the enzyme nitric oxide synthase (NOS), frequently identified as neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) isoforms [2]. In addition to activating the cGMP pathway, nitric oxide also reacts at diffusion-limited rates with superoxide to form peroxynitrite that thereby initiates radical-mediated oxidation of lipids, DNA and proteins [2,3].

Nitration of tyrosine is a major footprint left by peroxynitrite and by myeloperoxidase-mediated oxidation of nitrite. It has emerged as a hallmark of oxidative tissue injury during acute and chronic inflammatory diseases in adult animals as well as in neurodegeneration. Studies of cardiac ischemia/reperfusion have found nitrotyrosine staining in the ischemic tissue and in myocarditis [4–6], whereas tissue from control animals is generally devoid of nitrotyrosine immunoreactivity. In addition, elevated serum levels of protein-bound nitrotyrosine can be predictive of cardiovascular disease in humans [7]. An increase in cardiac protein nitration occurs during both experimental and clinical settings of decompensated cardiac failure and may correlate with disease severity [8]. Production of reactive oxygen and nitrogen species has also been linked to apoptotic cell death [9,10]. For example, tyrosine nitration by peroxynitrite can kill motor neurons and PC12 by activating apoptotic pathways [11–13], but does not necessarily induce cell death under all conditions [14,15]. Peroxynitrite has also been shown to mediate protective responses to endothelial shear stress by activating JNK and other signal transduction pathways [16,17].

In the course of examining whether tyrosine nitration would be found during the normal pruning of motor neurons in the spinal cord during embryogenesis, we were surprised to find tyrosine nitration present in the developing heart and other organs. It was well established that all three NOS isoforms are differentially expressed during normal heart embryonic development [18], consistent with nitric oxide having multifaceted roles in heart development. However, a role for nitric oxide producing reactive nitrogen intermediates is unexpected during early development. Tyrosine nitration is known to occur during the regenerative processes induced by heart injury, which have been suggested to recapitulate embryogenesis [19,20]. Because nitrotyrosine is present during heart disease, we investigated whether tyrosine nitration was associated with the regions of remodeling involving cell death occurs during heart development [21] or was associated with other developmental events.

MATERIALS AND METHODS

Pregnant female Sprague-Dawley rats of different gestational ages (6 rats per group, gestational ages from E10 to E21) were sacrificed with 100 mg/kg i.p. sodium pentobarbital (Abbott Laboratories, Chicago, IL) and the embryos obtained. We also used six newborn animals in each group from post-natal day 1 to 5. The embryos and newborn pups were then prepared for use in each of the techniques detailed below. The investigation conforms to the policies set forth in the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

Tissue preparation

For light microscopy studies, whole embryos harvested from day 10 to the end of the gestational period, were rapidly transferred to freshly prepared 4% paraformaldehyde, fixed

overnight at 4°C, dehydrated and embedded in paraffin. Serial sections of 5 µm in thickness were cut parallel to the longitudinal axis of the heart including the trabeculated and compact parts of the wall of both ventricles and ventricular cavities. The sections were mounted on glass slides and stored at 4°C until further use.

Immunohistochemistry

Nitrotyrosine, iNOS and eNOS—Tissue sections from the hearts of embryos from different ages (E12-E19) and of newborns were immunostained as previously described [22]. Briefly, paraffin sections of the specimens were immunostained with a polyclonal anti-3-nitrotyrosine antibody [22,23], eNOS and iNOS antibodies (BD Biosciences, Franklin Lakes, NJ). Tissue sections were preincubated with 0.3% hydrogen peroxide in absolute methanol, washed in PBS, blocked with 10% goat serum, and incubated with the primary antibody. Immunoreactivity was visualized with DAKO Envision Kit (DAKO Corporation, Carpinteria, CA) according to the manufacturer's instructions and then developed with diaminobenzidine (DAB). The slides were counterstained with hematoxylin. The specificity of the antibodies was examined by pre-absorbing the antibody with excess target protein or reducing agent dithionite for nitrotyrosine and by omitting the primary antibody for iNOS and eNOS.

Bromodeoxyuridine (BrdU)—For each pregnant rat of different gestational ages, 100 mg of BrdU (Sigma-Aldrich Corporation, St. Louis, MO) was dissolved in 8ml of saline and a total of four doses of 2 ml each were injected in 3-h intervals over a 12-hour period. The animals were sacrificed twenty-four hours after the last injection, the embryos were obtained, fixed by immersion in 4% paraformaldehyde (the duration of the fixation process depended on the size of the embryos), embedded in paraffin and sectioned. Slides were incubated overnight at 4°C with an antibody anti-BrdU (Dako Corporation, Carpinteria, CA), and the immunohistochemistry performed using a Dako Envision Doublestain System according to the manufacturer's instructions. The colorimetric reaction was done using DAB/Fast Red.

Immunoprecipitation and Western Blotting

Nitrotyrosine—Ten to twelve hearts of the same age embryos (E10 to E21) as well as from postnatal (PN1-5) and adult rats were homogenized generating separated samples from each age group. They were sonicated in lysis buffer pH 7.4 (20mM Tris Base, 150mM NaCl, 10% glycerol, 1% Triton X-100, 4mM EDTA) containing protease inhibitors (Sigma Protease Inhibitor Cocktail, Sigma-Aldrich Corporation, St. Louis, MO) plus 1mM PMSF and 1mM orthovanadate. Lysates were microcentrifuged and supernatants were precleared using Gamma-Bind Plus Sepharose beads (Transduction-GE Healthcare Bio-Sciences Corp. Piscataway, NJ) for 1 hour at 4°C. Samples were incubated overnight with AminoLink Coupling Gel (Amersham-GE Healthcare Bio-Sciences Corp. Piscataway, NJ) and then labeled with anti-nitrotyrosine monoclonal antibody (UBI-Millipore, Billerica, MA). Proteins were electrophoresed, blotted and probed overnight with anti-nitrotyrosine polyclonal antibody and then for one additional hour with goat anti-rabbit HRP-conjugated secondary antibody (BioRad Laboratories, Hercules, CA). Blotted proteins were visualized using enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL). The samples did not were in contact with hydrogen peroxide until the development using enhanced chemiluminescence, long after incubation with the antibodies.

Nitrated HSP90—Ten E15 heart embryos, ten two day-old post-natal hearts (PN-2) and three adult hearts were homogenized generating separated samples from each age group. They were sonicated in lysis buffer pH 7.4 (20mM Tris Base, 150mM NaCl, 10% glycerol, 1% Triton X-100, 4mM EDTA) containing protease inhibitors (Sigma Protease Inhibitor

Cocktail, Sigma-Aldrich Corporation, St. Louis, MO) plus 1mM PMSF and 1mM orthovanadate. Lysates were centrifuged and supernatants were precleared using Gamma-Bind Plus Sepharose beads (Transduction-GE Healthcare Bio-Sciences Corp. Piscataway, NJ) for 1 hour at 4°C. Samples were incubated overnight with AminoLink Coupling Gel (Amersham-GE Healthcare Bio-Sciences Corp. Piscataway, NJ) and then labeled with anti-HSP90 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were electrophoresed, blotted and probed overnight with anti-nitrotyrosine monoclonal antibody and then for one additional hour with goat anti-rabbit HRP-conjugated secondary antibody (BioRad Laboratories, Hercules, CA). Blotted proteins were visualized using enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL).

Myeloperoxidase, Xanthine-Oxidase and NADPH-oxidase—Samples were prepared as described above for western blotting. Proteins were separated into three different SDS-polyacrylamide gels: of 8% for XO, 10% for NADPH-oxidase, and 15% for MPO. Then they were electrophoresed, blotted and probed overnight with the corresponding primary antibody: anti-XO polyclonal antibody (Rockland Immunochemicals, Gilbertsville, PA), anti-NADPH-oxidase gp91 phox subunit polyclonal antibody (gift from Dr. M. Quinn, Montana State University), anti-MPO polyclonal antibody (Biomedica, Foster City, CA) and then incubated for one additional hour with goat anti-rabbit HRP-conjugated secondary antibody (BioRad). Blotted proteins were visualized using enhanced chemiluminescence (Pierce).

iNOS in situ Hybridization

A 405bp of rat iNOS cDNA was amplified with the following primers 5'-CTGGAATTCCCAGCTCATCC-3' y 5'-TCCTCCAGGATGTTGTAGCG-3' and cloned into a pGEM-T vector (Promega, Madison, WI). Following sequencing and linearization, sense and anti-sense biotin labeled-riboprobes were synthesized with SP6/T7 RNA polymerases, using a DIG RNA labeling kit (Roche, Indianapolis, IN) according to manufacturer instructions.

Paraffin was removed from tissue sections from E15 rat embryos before they were pretreated with 20µg proteinase K (Sigma-Aldrich) for 30 minutes at 37°C. In order to inhibit endogenous peroxidase activity specimens were treated with 3% hydrogen peroxide in absolute methanol for 10 minutes at -20°C. The slides were incubated in a hybridization buffer for 30 minutes at 55°C and hybridized overnight with 20ng/ml iNOS biotinylated probe at the same temperature. The probe was detected using the Tyramide Signal Amplification-Cy3 Fluorescence System (NEN-Perkin Elmer, Boston, MA), counterstained with DAPI and mounted with Slow-Fade (Molecular Probes-Invitrogen, Eugene, OR).

Apoptosis

Serial sections of rat embryos of varying ages were labeled with DeadEnd Colorimetric Apoptosis Detection System (Promega, Madison, WI), a modified TUNEL assay, according to the manufacturer's instructions. Briefly, sections were fixed with 4% paraformaldehyde in 0.1M PBS, permeabilized with 20µg proteinase K for 25 minutes, incubated with a mixture of biotinylated nucleotides and TdT enzyme for one hour at 37°C. HRP-labeled streptavidin were then bound to these nucleotides that had been detected using hydrogen peroxide and DAB. Apoptotic nuclei stained dark brown.

Mass Spectrometry

Tissue homogenates were immunoprecipitated with antibody anti-nitrotyrosine as described above and separated by SDS-page. The protein was identified by analysis of tryptic fragments and comparing them with those of the NCBI database. The procedure used was

adapted from that developed at UCSF (<http://donatello.ucsf.edu/ingel.html>). Briefly, the bands were excised from a polyacrylamide gel, digested overnight with trypsin, and the peptides were extracted with 50% acetonitrile/5% formic acid. The extracts were concentrated and re-dissolved in 10 μ l 50% acetonitrile/5% formic acid. The extracted peptides were mixed with the matrix α -cyano-4-hydroxycinnamic acid (CHCA) (Sigma-Aldrich, St Louis, MI), spotted onto a MALDI plate and analyzed with a Voyager DePro mass spectrometer (Applied Biosystems, Foster City, CA). The peptide masses were searched via Mascot (<http://www.matrixscience.com/>) and the NCBI database to identify immunoprecipitated proteins.

Actin peptide sequencing and detection of nitrotyrosine was performed using a mass spectrometer Waters-Micromass Qtof II. Doubly- or triply-charged parent ions were detected in the first quadrupole and if above a predefined threshold, the ion is selected, carried into a collision cell where fragmentation occurs, and the ions are detected by an orthogonal TOF detector to produce the tandem mass spectrum. Manual de novo sequencing was the principal method used to interpret the spectra. The MSMS spectra were processed to remove noise and then processed via MaxEnt3 software. This processing allows for rapid identification of ions in y-ion and b-ion series. The peak lists from each MS-MS spectrum were analyzed by PROWL (Rockefeller University) to determine their peptide sequence.

RESULTS

Nitrotyrosine in Normal Embryonic Heart Development

Rat embryonic hearts showed a striking and specific anatomical and temporal distribution of tyrosine nitration. Immunoreactivity for nitrotyrosine was localized in the cytoplasm of endocardial and myocardial cells mainly in the ventricle wall from E11 to E18 (Fig. 1). At E11, the compact wall of the ventricles is thin and the first short *trabeculae carneae* forms in the spongy zone. At this stage, nitrotyrosine immunoreactivity was found almost exclusively in endocardial cells lining the *trabeculae* of the ventricular wall. The ventricular *trabeculae* were the primary sites of nitration during the mid gestation period. As the myocardium develops during E12-14, the thickness of the compact wall and the number of *trabeculae* increases. During this time, nitrotyrosine staining declined in the endothelial cells and became more predominant in the myocytes of the ventricular *trabeculae* (Fig. 2). Around E15 the compact myocardium also showed nitrotyrosine immunoreactivity, which remained evident until E18. The immunoreactivity for nitrotyrosine decreased markedly at the end of the gestational period. Starting at E18, only weak staining in the myocardium was observed and no nitrotyrosine immunoreactivity could be found in the heart in the last few days of gestation or after birth. Nitration was also observed in other organ systems, such as the developing vertebra.

Mass Spectroscopy Analysis of Nitrated Cardiac Muscle Proteins

To identify what proteins were nitrated, E15 heart homogenates were immunoprecipitated with nitrotyrosine antibodies and resolved by SDS-PAGE. The MALDI-TOF analysis of tryptic peptides revealed that the cytoskeletal proteins α -actin and β -myosin heavy chain were the main targets for protein nitration as well as the α -chain of Ca^{2+} ATP synthase (Table I). These three protein targets have been previously reported as nitrated in heart disease and aging [2,24]. Further analysis of α -actin trypsin fragments by MALDI-TOF-TOF showed that the tyrosine residues 55 and 200 were nitrated (Fig. 3). The nitration of these proteins was also confirmed by immunoprecipitation of the protein followed by Western blot using nitrotyrosine antibody. Western blot analysis of nitration of β -myosin heavy chain showed a temporal pattern identical to that revealed by immunohistochemistry (Fig. 4).

Not all proteins were nitrated in the embryonic heart. Previously, we have shown that Hsp90 was nitrated in several pathological conditions including amyotrophic lateral sclerosis [12,25]. Nitrated Hsp90 was not detected in heart from E15 embryos, in two-day old post-natal pups or in control adults, but was nitrated in hypertrophic adult hearts. Thus, certain proteins such as Hsp90 may be preferentially nitrated in pathological conditions, but not necessarily during normal heart development (Fig. 5).

Unexpectedly, an opposing pattern of localization was observed between apoptotic nuclei and nitrotyrosine immunoreactivity. Apoptotic nuclei were found mainly in the compact myocardium of the left and right ventricles and in the infundibular section of the right ventricle where cells proliferate. Nitrotyrosine was confined mainly to the *trabeculae* of both atria and ventricles, where most of the cells had fully differentiated (Fig. 6). Proliferating cells identified with bromouridine did not show nitrotyrosine immunoreactivity. In summary, nitrotyrosine immunoreactivity was principally located in terminally differentiated cells and was not associated with either proliferation or apoptosis.

iNOS and eNOS are Present During Cardiac Development

The developing heart was immunoreactive for the endothelial and inducible isoforms of NOS. Immunoreactivity for endothelial NOS was localized in endocardial and myocardial cells in the compact myocardium as well as the *trabeculae* (Fig. 2 and 7). Immunoreactivity for the inducible NOS was localized in the atrial and ventricular myocardium of the embryonic heart (Fig. 2 and 8). Both NOS isoforms were present throughout development, including the latest stages of heart formation and even after birth. The protein expression and distribution of iNOS was confirmed by *in situ* hybridization in E15 hearts. The iNOS mRNA and protein immunoreactivity were distributed identically (Fig. 9).

Myeloperoxidase, Xanthine-Oxidase And NADPH Oxidase Protein Expression

To test whether the decrease in nitrotyrosine is due to changing levels of superoxide or hydrogen peroxide formation, the protein expression of XO, MPO and NADPH-oxidase was investigated during heart development (Fig. 10). Western blot analysis of protein extracts from embryo hearts showed that MPO become detectable starting at E16 up until birth and post-natal ages, whereas XO started protein expression at E19 and continued to increase its levels after birth. In contrast, the gp91 phox subunit of the NADPH-oxidase was not expressed until adulthood (Fig. 10).

DISCUSSION

The temporal and anatomical distribution patterns of nitrotyrosine during heart development reveal that the generation of reactive nitrogen species responsible for nitration is spatially restricted and highly dynamic. At certain stages of development, apoptosis occurs in well-defined segments of the heart to help shape its final structure. Surprisingly, nitrotyrosine levels did not correlate with apoptotic heart remodeling but was rather associated with myocyte differentiation. Supporting this conclusion, two proteins of the contractile cardiac sarcomere, β -myosin heavy chain and α -actin were nitrated in the embryonic heart. Nitration of these proteins may potentially modify contractile force during the embryonic period. A growing body of evidence indicates that tyrosine nitration of contractile proteins can reduce myocardial contractility. In isolated human myocardial cells, nitration of proteins such as α -actinin alters their contractile properties [26]. Exposure of rat ventricular *trabeculae* to physiologically relevant concentrations of peroxynitrite impairs the maximal trabecular force generation and leads to the nitration of several myofibrillar proteins including myosin heavy chain [8].

Myosin heavy chain (MHC), a major contractile protein that converts chemical energy from ATP into mechanical force, has two isoforms. The beta isoform is predominant during embryonic development and is replaced at birth by the alpha isoform [27]. The alpha isoform increases the maximum sarcomere shortening velocity in the adult heart. The embryonic beta isoform is characterized by lower ATPase activity and slower filament sliding velocity, but it can generate a cross-bridge force with a higher economy of energy consumption than the alpha isoform. In failing mouse and human adult hearts, severe cardiovascular stress triggers a shift from α - to β -MHC, essentially causing expression to revert to an embryonic pattern [28]. Our results show that the nitration occurred in the β -MHC but disappeared at birth as the expression of α -MHC increased, suggesting that β -MHC could be a selective target for nitration. The adaptive significance of β -MHC nitration could be the reduction in cardiac pump function, because the amount of nitration corresponds to the decrease in cardiac pump function in isolated hearts [29].

Interestingly, the pattern of embryonic gene expression shares features with cardiac hypertrophy [21]. The embryonic proteins, β -MHC and skeletal actin, are induced following aortic stenosis [30] and in cardiac hypertrophy [19]. The “reactivation” of the fetal gene expression is also supported by the similarities in myofibrillar formation in re-differentiating adult cardiomyocytes with their embryonic counterparts during development [31–33].

The expression of two nitric oxide synthases isoforms (eNOS and iNOS) during development is consistent with previous reports [18,34,35]. NO production by eNOS alone has been implicated in promoting angiogenesis and vasculogenesis. It also mediates the activities of many angiogenic factors such as VEGF [36,37]. Inhibition of NO production significantly reduced the number of mature myocytes, directly implicating NO in cardiomyocyte differentiation. While nitrotyrosine and NOS isoforms colocalize at early embryonic ages, NOS isoforms remain detectable in post-natal stages long after nitrotyrosine immunoreactivity disappeared [34]. These results are consistent with nitric oxide itself not being responsible for nitration, but rather requiring the formation of other reactive nitrogen species to nitrate tyrosine in proteins [2].

The gene deletion of each of the NOS isoforms leads to specific sets of functional and morphological abnormalities. Particularly relevant to the development of the embryonic cardiovascular system, eNOS knock-out mice exhibit hypertension, atherosclerosis, abnormal aortic valve development and heart failure and congenital septal defects [38–41]. While the deletion of the eNOS gene does not block heart development, it causes developmental abnormalities that are still compatible with embryonic survival. This may be due to compensatory mechanisms utilizing the other NOS isoforms [42].

Tyrosine can be nitrated by a number of different mechanisms that involve reactions with superoxide and hydrogen peroxide [2]. The reaction of peroxynitrite with carbon dioxide to form carbonate radical and nitrogen dioxide is a major contributor to nitration in vivo [43,44]. Tyrosine nitration can also be catalyzed from hydrogen peroxide and nitrite by peroxidases such as MPO [45,46]. In the developing heart, nitrotyrosine immunoreactivity was present long before myeloperoxidase expression was detectable. Because NOS is expressed both during embryonic development and after birth, nitrotyrosine might be controlled by increased superoxide production. Xanthine oxidase and NADPH oxidase are major sources of superoxide in events associated with inflammation and ischemia/reperfusion. However, their expression increased as nitrotyrosine decreased, suggesting that alternative sources of superoxide or hydrogen peroxide exist in the embryonic heart.

Taken together, our results lead to the surprising conclusion that tyrosine nitration, most likely mediated by peroxynitrite, is a physiological phenomenon occurring during

embryogenesis. The nitration of proteins observed in adult diseased hearts could be the recapitulation of events found normally in embryogenesis to modulate tissue repair processes. However, physiological nitration is not restricted to the embryo heart and was previously described in aging heart mitochondria and in the placenta. In both conditions, the levels of nitration were greatly increased in disease conditions [15,47]. Nitrotyrosine in the normal chorioallantoic membrane chick embryo suggest that tyrosine nitration may be a conserved evolutive trait [48]. Tyrosine nitration also was found in the normal development of the tunicate *Ciona intestinalis* supporting role for tyrosine nitration early in the evolution [49]. The long-term consequences of modifying tissue proteins by such a highly reactive oxidant are likely minimized by restriction of nitration to terminally differentiated tissues, which may serve to reinforce terminal differentiation.

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Highlights

- Nitrotyrosine is formed during normal heart embryo development
- Nitrotyrosine is present in differentiated cells
- Nitrotyrosine is not detected after day E18 and remains absent in adult heart
- Tyrosine nitration disappears even though eNOS and iNOS are expressed
- Only nitrated Hsp90 was identified in heart pathology but not in development

Fig 1. Nitrotyrosine Immunoreactivity in Rat Embryonic Cardiac Development

Immunohistochemistry for nitrotyrosine was performed on sections of hearts at different embryonic ages. From E12 to E17 a strong immunoreactivity is observed as a brown precipitate located mostly in the ventricular trabeculae and atria. In later stages of development from E18 to newborn, the immunoreactivity decreased markedly and disappeared at birth. The specificity of binding was verified by pre-absorbing the antibody with 10 μ M of the synthetic nitrated tri-peptide glycine-3-nitrotyrosine-alanine (not shown). Scale bar: 2 mm.

Fig. 2. Nitrotyrosine, eNOS and iNOS immunoreactivity in E12 Rat Embryo Heart Development
 The panels show serial images of sagittal sections of the wall of the left ventricle in the E12 rat embryo. The nitrotyrosine immunoreactivity is mainly found in the trabecular area of the ventricles, while the compact myocardium shows a lower intensity. The next panel shows the antibody blocking control to demonstrate the specificity of the staining. Endothelial NOS is found in both compact and trabecular myocardium and endocardium while iNOS is mostly present in the compact myocardium and trabeculae. Scale bar: 300 μ m.

Fig. 3. Identification of Nitrated Tyrosine Residues in cardiac muscle α -actin.

Homogenates of E15 embryo hearts were immunoprecipitated as described in Table I. A. Sequence corresponding to the amino acids 53–63 showing nitrotyrosine in position 55. The top right panel shows the expected b and y ion for the different fragments. B. Same than A but for amino acids 199–208 showing nitrotyrosine in position 200.

Fig. 4. Western Blot analysis of nitrated proteins in the embryo heart

Western Blot analysis of nitrotyrosine in different age embryonic rat hearts homogenates. The tissue was homogenized, prepared for nitrotyrosine immunoprecipitation with an anti-nitrotyrosine monoclonal antibody and subjected to SDS-PAGE (100 μ g/lane) as detailed in Material and Methods. The blot was probed with a polyclonal anti-nitrotyrosine antibody. The molecular weight shown in the figure (in Kd) corresponds to the myosin heavy chain molecular weight. A positive control was performed by using a heart homogenate of tissue treated with 0.5 mM peroxynitrite for 10 seconds.

Fig. 5. Comparison between nitrotyrosine immunostaining versus apoptosis and DNA synthesis in rat embryonic cardiac development

The upper three panels show high magnification images from the ventricular wall at the compact myocardium level of an E15 embryo. Nitrotyrosine immunostaining is negative in this area of the heart while apoptotic nuclei and BrdU positive cells are observed. In the lower three panels, the images were from the trabecular myocardium of the same heart sections. Nitrotyrosine immunoreactivity was positive in the myocardial cells while there is a conspicuous absence of apoptotic nuclei and BrdU positive cells. Scale bar: 50 μ m.

Fig. 6. Hsp90 nitration in heart

Heart homogenates from normal adult (Ad), new born (NB) and E15 rat embryo were immunoprecipitated using an antibody against Hsp90. The immunoprecipitates were processed by electrophoresis and blotted in PVDF membranes. The membranes were stained using monoclonal antibodies to nitrotyrosine. Samples from fistula aorto-cava heart were processed following the same procedure.

Fig. 7. Endothelial NOS immunohistochemistry in the rat cardiac development

Transverse sections of the cardiac area of rat embryos at different ages (E12-E18) and a newborn, showing positive immunoreactivity for eNOS. There is a strong positive staining in the atria and ventricles of both compact and trabecular zones that includes both myocytes and endothelial cells. Scale bar: 2 mm.

Fig. 8. Inducible NOS immunohistochemistry in the rat cardiac development

Adjacent sections to those shown in figure 5 were stained for iNOS demonstration. The immunoreactivity is localized in atria and ventricles, predominantly in the myocytes. Scale bar: 2 mm.

Fig. 9. In situ hybridization for NOS II in rat embryonic hearts

Serial sections of the free left ventricular wall of an E15 rat embryo heart. In panel A, endocardial and myocardial cells showing a red precipitate indicating a positive hybridization signal (anti-sense iNOS probe). In panel B, the negative control using a sense iNOS probe. Hybridization was performed at 55°C overnight and developed with the TSA-CY3 System. DAPI counterstaining. Scale bar: 40 μ m

Fig. 10. Western blots analysis for MPO, XO and NADPH-oxidase in rat embryonic hearts
The tissue was homogenized and prepared for SDS-PAGE analysis (50 μ l/lane) as detailed in Materials and Methods. The blots were probed with anti-myeloperoxidase, anti-xanthine oxidase and anti-NADPH-oxidase gp91 phox subunit polyclonal antibodies respectively. Blotted proteins were visualized by enhanced chemiluminescence. NB: newborn; PN-4: post-natal 4 days old

Table I
Identification of nitrated proteins by MALDI-TOF analysis of tryptic peptides

Proteins from E15 rat embryo hearts cells immunoprecipitated with an antibody anti- nitrotyrosine and separated by SDS-PAGE. The gels were stained with GelCode Blue. The protein bands were excised and subjected to in-gel trypsin digestion followed by MALDI-TOF mass spectrometry for peptide mass fingerprint analysis. The peptide masses were matched using the Mascot database search engine (<http://www.matrixscience.com>).

Protein	Score	Peptides matched
ATPsynthase chain	97	10
Myosin heavy chain β , cardiac	86	7
Actin , cardiac	63	10