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Original Contribution

Nox regulation of smooth muscle contraction

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Abstract

The catalytic subunit gp91*phox* (Nox2) of the NADPH oxidase of mammalian phagocytes is activated by microbes and immune mediators to produce large amounts of reactive oxygen species (ROS) which participate in microbial killing. Homologs of gp91*phox*, the Nox and Duox enzymes, were recently described in a range of organisms, including plants, vertebrates, and invertebrates such as *Drosophila melanogaster*. While their enzymology and cell biology are being extensively studied in many laboratories, little is known about *in vivo* functions of Noxes. Here, we establish and use an inducible system for RNAi to discover functions of dNox, an ortholog of human Nox5 in *Drosophila*. We report here that depletion of dNox in musculature causes retention of mature eggs within ovaries, leading to female sterility. In dNox-depleted ovaries and ovaries treated with a Nox inhibitor, muscular contractions induced by the neuropeptide proctolin are markedly inhibited. This functional defect results from a requirement for dNox-for the proctolin-induced calcium flux in *Drosophila* ovaries. Thus, these studies demonstrate a novel biological role for Nox-generated ROS in mediating agonist-induced calcium flux and smooth muscle contraction.

Keywords: Nox; Duox; dNox; dDuox; NADPH Oxidase; Reactive Oxygen; Drosophila; Nox1; Nox2; Nox3; Nox4; Nox5; Duox1; Duox2; gp91; gp91phox

Introduction

Reactive oxygen species (ROS) are generally thought to be broadly reactive, mutagenic, and cytotoxic entities that are produced largely as an "accidental" by-product of aerobic metabolism. While such "unintentional" production of ROS clearly occurs, it is also known that ROS can be produced in a regulated manner and serve useful biological purposes. The classical example of this is the NADPH oxidase of professional phagocytes, of which gp91*phox* is the catalytic subunit. This enzyme produces large amounts of ROS that form an important part of the phagocyte's bactericidal machinery [1,2]. Observations of the regulated production of ROS by NADPH oxidase-like enzymatic sources in many other cell types in addition to phagocytes led to the discovery that gp91*phox* is in fact a member of a family of NADPH oxidases termed the Nox/Duox family, which have now been identified in a wide range of organisms including plants, and vertebrate and invertebrate animals [3].

The human genome encodes seven homologs in the Nox/Duox family termed Nox1-5, Duox1, and Duox2 [3]. Nox1-4 consist of six transmembrane α helices that bind two hemes plus FAD domain containing NADPH binding site [3]. Nox1-3 are activated by regulatory subunits, while Nox4 is constitutively active [1,4,5]. The N terminus of Nox5 contains an additional EF-hand-containing calcium-binding domain that mediates its calcium-dependent activation [6,7]. Duoxes build further upon the Nox5 structure with an additional N-terminal peroxidase domain [8].

While the enzymology and cell biology of Noxes are being extensively studied, little is known about their *in vivo* functions. In recent years, studies using cultured mammalian cells have supported a role for reactive oxygen species as potential signaling molecules. ROS are produced by cells in a regulated manner in response to a wide range of growth factors and hormones. Oxidation by ROS of key amino acid residues such

Abbreviations: ROS, reactive oxygen species; DPI, diphenylene iodinuim; IR, inverted repeat; PTPs, protein tyrosine phosphatases; VSMCs, vascular smooth muscle cells; Ang II, Angiotensin II.

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as low-pK_a cysteines may mediate the signaling by ROS by altering the activity of downstream enzymes and transcription factors. NADPH oxidases are implicated as the source of agonist-induced ROS in a variety of cells, including insulin stimulation of adipocytes [9] and Angiotensin II stimulation of vascular smooth muscle cells [10]. While cell culture experiments support a role for Noxes in cell signaling, so far there are very few *in vivo* data on signaling or other nonimmune functions for these enzymes.

The *Drosophila* genome encodes only two Noxes rather than the seven seen in humans: dNox (an ortholog of h-Nox5) and dDuox. The relative simplicity of the *Drosophila* genome and the availability of genetic tools for these flies provided an opportunity to explore the biological functions of Noxes in an intact animal. We used RNAi coupled to the GAL-UAS binary system to conditionally knock down the expression of dNox in *Drosophila*. RNAi ofdNox resulted in a marked deficit in female egg-laying caused by a defect in ovulation, which was due to the failure of ovarian muscles to contract in an agonist-dependent manner. We also show herein that dNox regulates an agonist-stimulated calcium flux in ovarian tissue. This study provides some of the first definitive *in vivo* evidence of a role for Nox enzymes in cell signaling.

Materials and methods

Fly stocks and crosses

Drosophila were cultured on standard cornmeal food at 25 °C. Transgenic UAS-dNox-IR flies were generated by subcloning a 1000-bp portion of the dNox coding sequence in an inverted repeat orientation, separated by a 330–bp spacer sequence downstream of the UAS element in the pUAST vector [11]. The first 1000 bp of the dNox coding sequence were amplified by polymerase chain reaction (PCR) using primer sets 5′-gcatgaattc-atggactttgccgagcaaatt-3′ 5′-gcataagcttggacaacccacgacccctg-3′ and 5′-gcatggtaccatggactttgccgagcaaatt-3′ 5′gcatctcgaggga-caacccacgacccctg-3′; 330–bp of green fluorescent protein sequence was amplified by PCR for use as spacer sequence using primers 5′-gcataagctttgcttcagccgctaccccga-3′ and 5′-gcatctcgagggcgagctgcacgc-3′. The resulting PCR fragments were digested using appropriate restriction enzymes and cloned sequentially into equivalent sites in Bluescript SK. The completed dNox sense-spacer–dNox antisense fragment was restricted from Bluescript using *Eco*RI and *Kpn*I and inserted into the equivalent sites in pUAST. Transformation of *Drosophila* embryos was carried out in the w^{1118} stock using standard techniques [12]. All insertions in transgenic stocks were mapped to a chromosome and either made homozygous or balanced with appropriate balancer chromosomes. GAL4 drivers TubP, c179, and c855a and 49A UAS-aequorin flies were obtained from the Bloomington Stock Center. The Nrv1-GAL4 driver was a gift from Paul Salvaterra. Adult flies carrying one copy of a UAS-dNox-IR construct and one copy of a GAL4 driver were used for the assays. For aequorin assays, male flies carrying one copy of UAS-dNox-IR and one copy of Nrv1-GAL4 were generated and subsequently crossed with females homozygous for the UAS-aequorin insertion. From this cross, all progeny carried a copy of UAS-aequorin, and flies also carrying one copy of both Nrv1-GAL4 and UAS-dNox-IR were selected based on an easily recognized head morphology defect that occurs when dNox RNAi is driven by Nrv1-GAL4.

Fertility and ovulation assays

To determine fertility, virgin females of the indicated genotype were collected and crossed 5 per vial with 5 w^{1118} males. After 24 h, flies were cleared from vials and the total number of progeny resulting from crosses was determined 14 days later. To determine egg-laying ability, 50 virgin females of the appropriate genotype were crossed to w^{1118} males on grape juice medium with fresh yeast, and the total number of eggs laid was determined 24 h later. For ovulation assays, 4- to 5-day-old virgin females were crossed individually with w^{1118} males for 1 h and then placed on standard medium. At the indicated times, the females were dissected and scored positive for ovulation if an egg was present in the common oviduct or uterus.

Ovary contraction assays

Virgin females of the appropriate genotype were collected and placed on standard medium with freshly added yeast for 3–5 days. Ovaries were dissected in Schneider's *Drosophila* medium (Invitrogen). Where indicated, ovaries were preincubated with 20 μM diphenylene iodinium (DPI) (Sigma) for 15 min prior to the addition of 1 μM proctolin (Phoenix Pharmaceuticals). Movements of ovaries were recorded as QuickTime movies using a Nikon Coolpix 4500 camera. Movies were analyzed for ovarian movement using Videopoint software. The tip of one ovary at t0 was defined as the origin and the location of that same ovarian tip relative to the origin was measured every 0.1 s. Data were recorded as distance from origin in pixels and were graphed as a function of time using GraphPad Prism 4 software.

Superoxide generation assays

Virgin w^{1118} females were collected and placed on standard medium with freshly added yeast for 3–5 days. Ovaries were dissected in Schneider's *Drosophila* medium and placed three to a well in a white 96-well plate and 50 μM L-012 [13] (Wako Chemicals) was added. Where indicated M40403 (gift from Altana Pharma), BAPTA-AM (Calbiochem), and DPI were preincubated with tissue for 15 min prior to the start of the assay. L-012 luminescence was read using a FLUOstar Optima (BMG Labtech) plate-based luminometer. Proctolin and ionomycin (Sigma) were injected automatically at the times indicated.

Aequorin assays

Virgin females were collected and placed on standard medium with freshly added yeast for 3–5 days. Ovaries were dissected in Schneider's *Drosophila* medium and placed three to