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Effects of pO_2 on the activation of skeletal muscle ryanodine receptors by NO: A cautionary note

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Abstract

Eu et al., reported that O_2 dynamically controls the redox state of 6–8 out of 50 thiols per skeletal ryanodine receptor (RyR1) subunit and thereby tunes the response of Ca^{2+}-release channels to authentic nitric oxide (NO) [J.P. Eu, J. Sun, L. Xu, J.S. Stamler, G. Meissner, The skeletal muscle calcium release channel: coupled O_2 sensor and NO signaling functions, Cell 102 (2000) 499–509]. A role for O_2 was based on the observation that RyR1 can be activated by submicromolar NO at physiological (∼10 mmHg) but not ambient (∼150 mmHg) pO_2. At ambient pO_2, these critical thiols were oxidized but incubation at low pO_2 reset the redox state of these thiols, closed RyR1 channels and made these thiols available for nitrosation by low NO concentrations. Eu et al., postulated the existence of a redox/O_2sensor that couples channel activity to NO and pO_2 and explained that "the nature of the 'redox/O_2 sensor' that couples channel activity to intracellular redox chemistry is a mystery".

Here, we re-examined the effect of pO_2 on RyR1 and find that incubation of RyR1 at low pO_2 did not alter channel activity and NO (0.5–50 μM) failed to activate RyR1 despite a wide range of pO_2 pre-incubation conditions. We show that low levels of NO do not activate RyR1, do not reverse the inhibition of RyR1 by calmodulin (CaM) even at physiological pO_2. Similarly, the pre-incubation of SR vesicles in low pO_2 (for 10–80 min) did not inhibit channel activity or sensitization of RyR1 to NO. We discuss the significance of these findings and propose that caution should be taken when considering a role for pO_2 and nitrosation by NO as mechanisms that tune RyRs in striated muscles.

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Keywords: Nitric oxide; Nitrosothiol; Oxygen tension; Ryanodine receptor; Transnitrosation; Calmodulin

1. Introduction

Eu et al., reported that O_2 dynamically controls the redox state of 6–8 out of 50 thiols per skeletal ryanodine receptor (RyR1) subunit and thereby tunes the response of Ca^{2+}release channels to nitric oxide (NO) [1]. The main finding was that submicromolar concentrations of NO activate the channel by *S*-nitrosation of a single cysteine residue but only at low physiological oxygen tension ($pO_2 \sim 10$ mmHg). At ambient pO_2, Eu et al. observed neither activation nor *S*-nitrosation of RyR1 by NO [1]. The importance of these findings was underscored by stating: "We uncovered a striking plasticity of RyR1's redox state that was dictated by O_2 tension", and further that "Exposure of native sarcoplasmic reticulum (SR) vesicles to ambient air resulted in oxidation of 6–8 free thiols per RyR1 subunit (or 24–32 per channel) that reverted to the reduced state upon lowering of the pO_2 into the physiological range (∼10 mmHg). Thus, pO_2 appears to be the main determinant of the redox status of 6–8 RyR1 thiols, which are thereby identified with an O_2 sensing function. Evidently, this thiol-based 'O_2 sensor' is coupled to a redox system within the SR that provides the reducing equivalents for switching between

Abbreviations: RyR1, skeletal muscle ryanodine receptor; NO, nitric oxide gas; Cys-SNO, *S*-nitrosocysteine; GSNO, *S*-nitrosoglutathione; SR, sarcoplasmic reticulum; CK, creatine kinase; CP, creatine phosphate; CaM, calmodulin

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'oxidized' and 'reduced' states of the channel. It is also intimately linked to NO function; that is, only at physiological pO_2 is the channel responsive to physiological concentrations (submicromolar) of NO. Channel activation is the result of *S*-nitrosylation of one cysteine. Thus, the redox state of the channel (i.e., its responsiveness) is set by O_2 tension, whereas redox regulation of the channel is mediated by NO" [1].

Eu et al. recognized the complexity of the redox biochemistry of RyR1 by stating that "The nature of the 'redox/O_2 sensor' that couples membrane excitability to intracellular redox chemistry is a mystery." [1]. The enigma was caused by several observations and assumptions:

(1) In rabbit skeletal SR isolated at ambient pO_2, approximately 30% of the thiols on the native RyR1 were nitrosated while the thiols in the 'redox/O_2'-sensing site of the channel were oxidized (but not nitrosated). Upon shifting to low pO_2, the 'redox/O_2 sensor' was automatically reduced by an undefined factor that, acted ex vivo exclusively at low pO_2. Furthermore, this factor acted without consumption of reducing equivalents from cofactors.
(2) Once reduced by the unknown factor, the NO sensor became highly sensitive to *S*-nitrosation by low exogenous NO (~0.75 μM). Unexplained is why the 'redox/O_2 sensor' failed to be nitrosated in vivo whereas noncritical (i.e. less reactive) thiols were readily nitrosated. Since in microsomal preparations exogenous NO selectively nitrosated the 'redox/O_2 sensor' at low pO_2, one is compelled to conclude that the unknown factor cannot reduce the 'redox/O_2 sensor' once it is in its SNO state. Mechanistically, these assumptions do not appear to be compatible with the proposed redox regulation of the channel by NO under physiological conditions.
(3) A novel derivative chemistry of NO was postulated where NO exhibits a remarkable regional selectivity in nitrosating RyR1 at low pO_2, whereas at ambient oxygen tension, NO efficiently de-nitrosates RyR1-SNO (see Table 2 in reference [1]).
(4) Irrespective of pO_2, in vitro nitrosation of the critical thiol on RyR1 by NO did not occur in the presence of glutathione (GSH; see Table 2 in ref. [1]). These findings are incompatible with the endogenous *S*-nitrosation of RyR1 unless (a) GSH cannot protect/de-nitrosate RyR1 thiols in intact cells, (b) nitrosation of RyR1 occurred during the preparation of RyR1, and/or (c) measurements of the number of SNO and SH functions on RyR1 were unreliable.

The interpretation of these redox paradigms is further complicated by the likely oxidation of RyR1 by transition metal ions. The latter oxidation reactions are often responsible for the ex-vivo oxidation of biological thiols, and perhaps for the decomposition of *S*-nitrosothiols [2–4]. Mechanistic explanation(s) of these issues were not proposed in the first or in following reports by this group on the same topic [5–8].

Given that these findings represented an interesting chemical as well as physiological paradigm, we re-examined the influence of pO_2 on the activation of RyR1 by NO. In particular, the effects of pO_2 on RyR1 were studied under conditions that were recommended to activate the 'unknown factor' and to reset the redox state of critical thiols on RyR1. Skeletal muscle SR vesicles were incubated in reaction media at physiological or ambient pO_2 at different temperatures and for different periods of time then the rates and amounts of SR Ca^{2+} uptake were measured in the presence or absence of submicromolar concentrations of NO. Single channel activity and changes in the open probability of RyR1 incorporated in planar bilayers were also examined for RyR1 incubated at low and ambient pO_2 followed by additions of NO. *S*-Nitrosocysteine was used as a control reagent that is known to activate RyR1 [9].

2. Materials and methods

2.1. Materials

Phospholipids, 1,2-diphytanoyl-sn-glycero-3-phophoehtanolamine (PE), 1,2-diphytanoyl-sn-glycero-3-phosphoserine (PS) and 1,2-diphytanoyl-sn-glycero-3-phosphocholine (PC) were purchased from Avanti Polar Lipids (Alabaster, AL); Cys-SNO was synthesized as previously described [10]. Antipyrylazo III (AP III) was purchased from ICN Biochemicals (Cleveland, OH). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Preparation of heavy SR vesicles (HSR)

Skeletal SR vesicles were isolated from rabbit white muscle taken from the hind legs, as previously described [11]. The vesicle preparations were suspended in 0.29 M sucrose plus 20 mM histidine buffer at pH 7.0 and kept in liquid nitrogen until use. To obtain heavy SR vesicles, SR vesicles were centrifuged on a discontinuous sucrose gradient and HSR vesicles were collected from the 35 to 40% interface [12]. SR proteins were protected from proteolytic degradation by adding the protease inhibitors leupeptin (1 μg/ml) and phenylmethylsulfonyl fluoride (PMSF = 0.2 mM) as previously described [13]. Protein concentrations were determined with the Bio-Rad Protein Assay kit with bovine serum albumin as a standard.

2.3. Preparation and measurement of NO saturated solution

Solution of 0.1 M phosphate buffer (pH 7.20) saturated with NO gas was prepared by first deoxygenating the medium with N_2 gas for 20 min and then gassing with authentic NO gas that was passed through a gas trap containing KOH (4N). NO was produced by dropping 6N sulfuric acid to sodium nitrite, as described in [14]. NO was quantified with

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