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Putting xanthine oxidoreductase and aldehyde oxidase on the NO metabolism map: Nitrite reduction by molybdoenzymes☆

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ABSTRACT

Nitric oxide radical (NO) is a signaling molecule involved in several physiological and pathological processes and a *new* nitrate-nitrite-NO pathway has emerged as a physiological alternative to the "classic" pathway of NO formation from L-arginine. Since the late 1990s, it has become clear that nitrite can be reduced back to NO under hypoxic/anoxic conditions and exert a significant cytoprotective action *in vivo* under challenging conditions. To reduce nitrite to NO, mammalian cells can use different metalloproteins that are present in cells to perform other functions, including several heme proteins and molybdoenzymes, comprising what we denominated as the "non-dedicated nitrite reductases". Herein, we will review the current knowledge on two of those "non-dedicated nitrite reductases", the molybdoenzymes xanthine oxidoreductase and aldehyde oxidase, discussing the *in vitro* and *in vivo* studies to provide the current picture of the role of these enzymes on the NO metabolism in humans.

1. Introduction

Nitrite is presently recognized as a relevant source of nitric oxide radical (˙NO, herein abbreviated as NO) for human cell signaling and survival under challenging conditions [1–7]. In plants and bacteria, nitrite is also gaining grounds as a source of signaling NO (reviewed in [8–10]), suggesting that nitrite-derived NO would be relevant in all forms of life! This (*at first sight surprising*) ubiquity of the nitrite-derived NO is not unexpected at all: the so-called "human" nitrate-nitrite-NO pathway is, in fact, part of ancient "respiratory" prokaryotic pathways of the biogeochemical cycle of the nitrogen (Fig. 1) [8,11–13]. Hence, the nitrite reduction to NO can be thought as a heritage from a distant pre-aerobic past, that has been reused every since (evolutionary convergence).

2. "Classic" pathways of NO formation

In mammals, NO controls a plethora of functions, including vasodilation (through the well-known activation of guanylate cyclase), neurotransmission, platelet aggregation, apoptosis, gene expression, immune response, and mediates a wide range of both anti-tumor and anti-microbial activities [14]. In humans (Fig. 2), three tissue-specific isoforms of NO synthase (NOS; neuronal, endothelial and inducible NOS) catalyze the formation of NO from L-arginine and dioxygen [15–17]. Because of this dioxygen dependency, the onset of hypoxia/anoxia hampers the NOS catalytic activity and the NO formation can become compromised. The specificity of the NO signaling is guaranteed by the NOS tight regulation and by the limited NO life time, which is achieved through its rapid oxidation to nitrate (by the well known reaction with oxy-hemoglobin and oxy-myoglobin [18–29]) and to nitrite (by ceruloplasmin [30], cytochrome *c* oxidase [31] or dioxygen [32–34]).

3. Nitrite-derived NO

At the same time as our knowledge about the physiological roles of NO in humans was growing exponentially, nitrate and nitrite were ignored and considered "useless" end-products of NO metabolism. This

Abbreviations: AO, aldehyde oxidase; AFR, activity-to-flavin ratio; Cb, cytoglobin; Cc, cytochrome *c*; CcO, cytochrome *c* oxidase; DAF-FM, 4-amino-5-methylamino-2′,7′-difluorofluorescein; DAF-FM DA, DAF-FM diacetate; DPI, diphenyleneiodonium chloride; EPR, electron paramagnetic resonance; Hb, hemoglobin; HepG2, human epithelial cells from liver carcinoma; HL, human liver; HMEC, human microvascular endothelial cells; L-NAME, N^{ω}-nitro-L-arginine methyl ester hydrochloride; mARC, mitochondrial amidoxime reducing component; Mb, myoglobin; MGD_2-Fe, iron-*N*-methyl-*D*-glucamine dithiocarbamate; Nb, neuroglobin; NO, nitric oxide radical (˙NO); NOS, NO synthase; RL, rat liver; SO, sulfite oxidase; SOD, superoxide dismutase; XD, xanthine dehydrogenase; XO, xanthine oxidase; XOR, xanthine oxidoreductase

☆ EC according to International Union of Biochemistry and Molecular Biology, Enzyme Nomenclature Committee (www.chem.qmul.ac.uk/iubmb).

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dogma changed in the early XXI century, when it became clear that nitrite can be reduced back to NO under hypoxic conditions (Eq. (1)) and it was re-discovered that nitrite administration can be cytoprotective during *in vivo* ischemia and other pathological conditions (Fig. 2) [35–69] (*interestingly, the physiological action of nitrite had already been described in 1880* [70]). Since then, a novel concept emerged and nitrite began to be thought as a "storage form" of NO, that can be used to maintain the NO formation and ensure cell functioning under conditions of hypoxia/anoxia, precisely when the dioxygen-dependent NOS activity is impaired and a "rescue" pathway would be needed to form NO. Through the nitrite/NO "recycling" pathway, an organ under ischemia can maintain (or even increase) the blood flow, modulate the dioxygen distribution and the reactive oxygen species formation and, at the same time, maintain an anti-inflammatory and anti-apoptotic environment.

$$NO_2^- + 1e^- + 2H^+ \longrightarrow {}^{\bullet}NO + H_2O \quad (1)$$

4. "Who" is reducing nitrite to NO?

Simultaneously to the emergence of the new paradigm of nitrite-derived NO, the search for the human (mammalian) protein(s) responsible for the nitrite reduction to NO began. (*Following the studies in mammals, more recently, a similar quest began in plants* [8–10]).

The reduction of nitrite to NO is known for a long time in prokaryotic organisms, where it is catalyzed by copper-containing or heme-containing nitrite reductases, in "respiratory" pathways, as part of the biogeochemical cycle of the nitrogen (Fig. 1) [8,11,12]. However, to date, no "dedicated" (true) mammalian nitrite reductase was ever identified (*what definitively contributed to consider nitrite as a "useless" molecule in the earlier years*). Hence, the scientific community searched for the nitrite reductase activity in proteins that perform other (already well known) functions in the cell.

In the recent years, several mammalian metalloproteins, with different molecular features, subcellular localization and cellular roles (enzymes, metabolite transporters and electron transfers), were shown to be able to reduce nitrite to NO -comprising what we denominated as the "**non-dedicated nitrite reductases**" [8] (Fig. 2). The long list of "non-dedicated nitrite reductases" includes all the known mammalian molybdenum-containing enzymes (xanthine oxidoreductase (XOR), aldehyde oxidase (AO), sulfite oxidase (SO) [71] and mitochondrial amidoxime reducing component (mARC) [72]), and a growing number of heme-containing proteins, where hemoglobin (Hb) and myoglobin (Mb) stands out in number of publications, but including also neuroglobin (Nb) [73], cytoglobin (Cb) [74], cytochrome *c* (Cc) [75], cytochrome P_{450} [76], cytochrome *c* oxidase (CcO) [77–79], and several other proteins [80–83]. *At this pace, other mammalian nitrite reductases will probably be identified in the next years*. In addition, some protein-independent pathways were also proposed for the nitrite reduction in ischemic tissues, stomach and brain [84–93]. Noteworthy, the protein-independent nitrite reduction to NO in the stomach (the pioneering work of Lundberg's and Benjamin's groups [84,85,87]) and in ischemic tissues (Zweier's group work [86,88]) were the first pathways through which nitrite was suggested to be a relevant source of bioactive NO, in the 1990s.

In all those pathways, the nitrite reduction to NO was described to occur under hypoxic or anoxic and acidic conditions, but the level of characterization of each pathway is very dissimilar. So far, only Mb and XOR have been demonstrated to be directly involved in the cytoprotective action of nitrite *in vivo* or *ex vivo* [38,45,94–96], but only the XOR nitrite reductase activity was thoroughly characterized. The nitrite reductase activity of Hb, on it is turn, has been extensively characterized *in vitro*, with several mechanisms being proposed to explain how it would be possible for NO to escape being trapped by the heme (reviewed, *e.g.*, in [8]). The characterization of the other mammalian nitrite reductases is more limited. Yet, regardless of the knowledge so far accumulated, the nitrite "recycling" to NO is still a complex subject, overshadowed by several (bio)chemical constrains, of which we highlight: (i) In the case of enzymes, how can nitrite compete with the "classic" oxidizing substrates? (ii) In the case of the heme proteins, how can the formed NO avoid being rapidly trapped by the heme itself? (iii) How can we reconcile the *in vivo* observed nitrite effects with the *in vitro* knowledge of nitrite reduction through those diverse pathways? (iv) How are all those pathways orchestrated *in vivo*? Are all equally relevant? Are tissue-specific? Have different triggering levels/conditions?

Herein, we will review our current knowledge about the nitrite reductase activity of the mammalian molybdoenzymes XOR and AO, discussing the *in vitro* and *in vivo* studies to provide the best possible current picture of the role of these enzymes on the NO metabolism.

5. Human XOR and AO

XOR is a key enzyme in purine catabolism, where it catalyzes the oxidation of both hypoxanthine and xanthine to the terminal metabolite, urate [97–102]. AO catalyzes the oxidation of aldehydes into the respective carboxylates and, although its physiological function remains a matter of debate, it seems to be a probable partner in the metabolism of some neurotransmitters and retinoic acid [103–108]. Both enzymes contribute also to the xenobiotic metabolism (due to their low substrate specificity) and are allegedly involved in signaling (physiological conditions) and oxidative stress-mediated pathological conditions (due to their ability to form reactive oxygen species, superoxide anion radical and hydrogen peroxide) [109–144]. *In vivo*, AO exists exclusively as an oxidase (reduces dioxygen; EC 1.2.3.1; Eq. (2)), whereas XOR exists predominantly as a NAD^+-dependent dehydrogenase, named xanthine dehydrogenase (XD; EC 1.17.1.4; Eq. (3)) [97–102,104,106,108,145,146]. Yet, XD can be rapidly converted into a "strict" oxidase form that reduces dioxygen instead of NAD^+ – the very well documented xanthine oxidase (XO; EC 1.17.3.2; Eq. (4)). (*Note*[1] *gives more details for readers interested in the nature of the two XOR forms.*) Overall, XO, XD and AO catalyze the transfer of one oxygen atom from a water molecule to a carbon center of the substrate (as indicated by the red oxygen atoms in Eqs. (2)–(4); Fig. 3(D)), through a reaction mechanism that is identical in all enzymes, with dioxygen or NAD^+ acting as electron acceptors [97–102,152,153].

Structurally, XOR (Fig. 3(A), (B)) and AO are also very similar. Both are complex homodimeric molybdoenzymes that harbor (per monomer) one identical molybdenum center, where the hydroxylation reactions occur, two [2Fe-2S] centers and one FAD, responsible for the reduction of dioxygen (XO, XD, AO) and NAD^+ (XD) (Fig. 3(C))

[1] Mammalian XO and XD are two forms of the same protein (same gene product). Mammalian XOR enzymes are synthesized as a NAD+-dependent dehydrogenase form, the XD, and are believed to exist mostly as XD under normal physiological conditions [97–102]. However, the XD form can be readily converted into a "strict" oxidase form, the XO. This conversion can be either reversible, through oxidation of Cys535 and Cys992, or irreversible, by proteolysis after Lys551 or Lys569 (bovine XOR numbering) [146–151]. The only "functional" distinction between XD and XO lies in the electron acceptor used by each form: while XD transfers the electrons preferentially to NAD+, XO fails to react with NAD+ and uses exclusively dioxygen. During the XD into XO conversion process, the protein conformation at the FAD center is modified and this conformational alteration is responsible for the differentiated oxidizing substrate specificity displayed by XO and XD [146–153] (note that both dioxygen and NAD+ react at the FAD center). On the other hand, the protein structure at the iron/sulfur and molybdenum centers is not changed during the conversion and, in accordance, the two enzyme forms are virtually identical in respect to the binding and catalysis of substrates at the molybdenum center, as is the case of oxidation of xanthine and other heterocyclic compounds and aldehydes [97–102]. For these reasons, XO and XD can be considered as one unique enzyme in what concerns the overall structural organization of the molybdenum domain and the molybdenum center reactivity.