FEBS Letters

FEBS PRESS science publishing by scientists

REVIEW ARTICLE

Nitrite-dependent nitric oxide synthesis by molybdenum enzymes

Daniel Bender[1,2] and Guenter Schwarz[1,2,3]

1 Department of Chemistry, Institute for Biochemistry, University of Cologne, Germany
2 Center for Molecular Medicine Cologne (CMMC), University of Cologne, Germany
3 Cologne Cluster of Excellence in Cellular Stress Responses in Aging-associated Diseases (CECAD), University of Cologne, Germany

Correspondence
G. Schwarz, Department of Chemistry, Institute for Biochemistry, University of Cologne, Zuelpicher Straße 47, 50674 Cologne, Germany
Fax: +49 221 470 5092
Tel: +49 221 470 6440
E-mail: gschwarz@uni-koeln.de

(Received 27 April 2018, accepted 2 May 2018, available online 27 May 2018)

doi:10.1002/1873-3468.13089

Edited by Wilhelm Just

Nitric oxide (NO) is an important gasotransmitter involved in numerous intra- and intercellular signaling events. In addition to the oxidative pathway of NO generation, which includes three NO synthase (NOS) isoforms in mammals, a reductive pathway contributes to NO generation. In this pathway, nitrite is reduced to NO by various metal-containing proteins. Among these, all members of the eukaryotic molybdenum (Mo)-dependent enzyme family were found to be able to reduce nitrite to NO. This Review focuses on the current state of research in the field of Mo-dependent nitrite reduction in eukaryotes. An overview on the five eukaryotic Mo-enzymes is given, and similarities as well as differences in their nitrite reduction mechanisms are presented and discussed in the context of physiological relevance.

Keywords: aldehyde oxidase; mARC; molybdenum cofactor; nitrate reductase; nitric oxide; nitrite; sulfite oxidase; xanthine oxidoreductase

Nitric oxide (NO) was the first gasotransmitter to be identified in mammals in the context of blood vessel relaxation [1] and macrophage activation [2,3]. Although carbon monoxide and hydrogen sulfide (H_2S) were later found to extend the physiological repertoire of gaseous signaling molecules in the human body, NO-related biological processes still dominate the field of gasotransmitter research. From the early discoveries in the 80's until today, NO has become the most versatile and complex second messenger involved in numerous cellular processes. NO-dependent nitrosylation of proteins has been recognized as an apparent post-translational modification (PTM) of mainly cysteine thiols with tremendous physiological importance [4]. Moreover, setting NO apart from other PTMs, NO's unique feature is its ability to bind to metal cofactors, which either leads to enzyme activation or inactivation [5,6]. Furthermore, NO not only modulates protein function but it is also able to modify a variety of different products of enzymatic reactions, which increases the spectrum of available metabolites [7]. In this respect, its potential to cross biological membranes, its high diffusion coefficient [8] and its indolent reaction with molecules [7] other than radicals and metals allows NO to function as fundamental cell-to-cell messenger within different tissues.

In pioneering efforts to identify the cellular source of NO in animals, neuronal NO synthase (nNOS) was first discovered [9] followed by the identification of endothelial NOS (eNOS) [10] and inducible NOS (iNOS) enzymes [11]. All three NOS isoforms use molecular oxygen and a guanidine nitrogen of L-arginine to produce L-citrulline and NO [12]. This is enabled by shuttling

Abbreviations
AO, aldehyde oxidase; cGMP, cyclic guanosine monophosphate; cyt *c*, cytochrome *c*; eNOS, endothelial NOS; EPR, electron paramagnetic resonance; FeS, iron sulfur cluster; H_2S, hydrogen sulfide; iNOS, inducible NOS; mARC, mitochondrial amidoxime reducing component; Mo, molybdenum; Moco, molybdenum cofactor; NADH, nicotinamide adenine dinucleotide; nNOS, neuronal NO synthase; NO, nitric oxide; NOS, nitric oxide synthase; NR, nitrate reductase; PTM, post-translational modification; SO, sulfite oxidase; XDH, xanthine dehydrogenase; XO, xanthine oxidase; XOR, xanthine oxidoreductase.

electrons from NAD(P)H at the C-terminal reductase domain through FAD and FMN to the oxygenase domain. Here, a tetrahydrobiopterin-dependent reduction of heme-bound molecular oxygen allows the oxygenation of L-arginine leading to NO release and citrulline formation. Electron flux from the reductase to oxygenase site is regulated *via* Ca^{2+}/calmodulin binding to a motif located between both domains. The iNOS enzyme is different from the other two isoforms as it is neither constitutively expressed nor calcium dependent [11,13]. Bacterial lipopolysaccharides as well as endogenous cytokines induce iNOS transcription [3,13,14]. Cytotoxic NO production from iNOS in macrophages is one major principle of the innate immune response in the clearance of pathogens and tumors [15]. nNOS is the predominant isoform in the brain and peripheral nervous system, however, its transcription was also recognized in skeletal muscle [16], kidney macula densa cells [17] and in the vascular smooth muscle [18]. In the brain, nNOS fundamentally regulates synaptic transmission and modulates learning, memory and neurogenesis [19–21]. Here, one mode of action is based on the transient S-nitrosylation of synaptic proteins [22,23]. In endothelial cells, eNOS acts as the master regulator of the cardiovascular system. A key function of eNOS-derived NO is arterial vasodilation *via* NO-dependent activation of heme-containing soluble guanylate cyclase in smooth muscle cells. In addition, eNOS-derived NO is important in inhibiting leukocyte adhesion [24], platelet aggregation [25] and vascular inflammation [26]. Furthermore, endothelial NO controls smooth muscle proliferation and stimulates angiogenesis [27].

Interestingly, although a plant NOS enzyme was proposed early on [28] up to now no plant homologue to mammalian NOS was identified in any plant genome [29]. The best described enzymatic source of NO in plants is the molybdenum (Mo)-dependent nitrate reductase (NR), a cytosolic enzyme catalyzing the rate limiting step in plant nitrogen assimilation by reducing nitrate to nitrite [30]. The enzyme is restricted to autotrophs and cannot be found in animals. Hence, for a long time NOS enzymes have been recognized as the only source of *de novo* synthesized NO in humans. In contrast, the oxidation products of NO, nitrate and nitrite, were considered as inert and no physiologic function was assigned, although both molecules were detected in serum and tissue [31–34].

Nitrite reduction complements NOS-dependent NO release in mammals

In 1994, nitrite was first demonstrated to function as physiological source of NO in the gastric lumen of humans. Here, the constant supply of nitrite was shown to derive from commensal facultative anaerobic bacteria in the oral cavity of the host that reduce highly abundant nitrate to nitrite in saliva by NR-dependent reduction of nitrate [35]. The swallowed nitrite-containing saliva increased the NO concentration of expelled stomach air 100-fold in comparison to expelled air from the oral cavity [36]. The pretreatment with a proton-pump inhibitor reduced the amount of NO in gastric air significantly, which suggested a low pH in the stomach as indispensable for efficient nitrite reduction. This high NO concentration in the gastric lumen has been associated with a first organismic defense mechanism against swallowed pathogens [37,38].

In mammals, nitrate and nitrite circulate in the blood and are abundant within different tissues, although humans lack an NR enzyme. While serum nitrite concentrations are in average around 0.3 μM in humans [31], the concentration of nitrate can reach up to 40 μM in serum [32,33]. In contrast, nitrite concentration in tissue was found to be increased in comparison to its serum concentration by approximately one order of magnitude [34,39]. Responsible for that is the high resorption capacity toward nitrite as shown by intraperitoneal injection of nitrite into rats and the organismic distribution of nitrite within 5 min postinjection into all rat organs [40]. The intrinsic nitrate–nitrite pools are fueled by two distinct mechanisms: (a) dietary intake by the small intestine and the saliva glands and (b) oxidation of endogenously produced NO (Fig. 1) [41]. The latter involves the oxidation of NO in the blood due to its reaction with oxyhemoglobin, which leads to the formation of nitrate and methemoglobin [42]. A second enzyme which reacts with NO is the multicopper enzyme ceruloplasmin, which oxidizes NO to nitrite (Fig. 1) [43]. Circulating nitrite derived from NO oxidation accounts for up to 70 % of total nitrite in the human vasculature [41].

First experimental evidence that nitrite may function as endogenous source of NO was provided by the application of isotopically labeled ^{15}N-nitrite in ischemic rat hearts [44]. The observed reduction of nitrite to ^{15}NO was proposed to derive from tissue acidosis, which occurs during ischemia that leads to nonenzymatic disproportionation of nitrite and release of NO [45] similar to the effect of low pH on nitrite in stomach. Underlining this, nitrite-dependent NO release was simulated in an *in vitro* approach where the nitrite-dependent dilatation of rat aortic sections was strictly dependent on low buffer pH [46]. The hypothesis of hypoxia-induced NO release from nitrite as backup mechanism for NOS-derived NO is

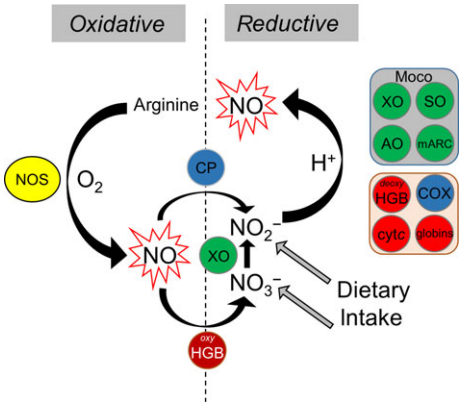

Fig. 1. Enzymes of eukaryotic NO homeostasis. Cellular NO generation *via* the arginine-dependent oxidative route or *via* the nitrate-nitrite-dependent reductive route. O_2-dependent NOS enzymes produce NO from arginine. The produced NO can be oxidized enzymatically *via* oxyhemoglobin (*oxy*HGB) to nitrate or *via* ceruloplasmin to nitrite. Dietary intake supports the systemic nitrate-nitrite pools. XO can reduce nitrate to nitrite. The latter can be reduced to NO in the presence of protons *via* two groups of metal-dependent enzymes/proteins. Mo-dependent XO, SO, AO and mARC constitute one group, while deoxyhemoglobin (*deoxy*HGB), cytochrome *c* oxidase (COX), cyt *c* and other globins represent the other group.

conclusive, as NOS enzymes are strictly dependent on molecular oxygen as cosubstrate. Nonetheless, the question arose whether nitrite reduction predominantly occurs nonenzymatically or if dedicated enzymes reduce nitrite to NO under conditions of compromised NOS activity. Indeed, evidence against a solely nonenzymatic nitrite reduction is the low pKa = 3.4 of nitrite, which does not allow efficient disproportionation to occur even at an extended hypoxia-derived acidosis [47–49].

Within the field of NO research, a variety of enzymes have been reported to reduce nitrite to NO, such as deoxyhemoglobin, cytoglobin, myoglobin [50,51], as well as cytochrome *c* (cyt *c*) [52] and neuroglobin in their penta-coordinate state [53,54]. The physiologic potential of deoxyhemoglobin to reduce nitrite to NO has been studied in detail and is generally widely accepted and the interested reader is referred to another review [50,51]. In this review, we will emphasize on the contribution of Mo-enzymes on the reduction of nitrite to NO.

Eukaryotic Mo-enzymes

Reduction of inorganic nitrogen to ammonia for the synthesis of organic matter is an evolutionary ancient concept, which is linked to the enzyme-bound trace element Mo. Both routes of nitrogen assimilation, the bacterial nitrogen fixation as well as assimilatory and dissimilatory nitrate reduction require Mo-dependent enzymes [30]. From this evolutionary perspective, it is conclusive to assume that also Mo-enzymes might participate in eukaryotic nitrite reduction and NO homeostasis. While bacteria host a large variety of Mo-dependent enzymes, eukaryotic life is restricted to five members of this enzyme family, which are the aforementioned NR, mitochondrial amidoxime reducing component (mARC), sulfite oxidase (SO), aldehyde oxidase (AO) and xanthine oxidoreductase (XOR). In all these enzymes Mo is coordinated *via* an organic tetrahydropyranopterin-based backbone, which presents a unique cofactor utilized by all eukaryotic Mo-enzymes, the so-called Mo-cofactor (Moco) [55]. In eukaryotes, two distinct variants of Moco were found solely differing in their Mo coordination sphere. The square pyramidal coordination of Mo and its substituents is composed of the enedithiolate of the pterin and one oxo-ligand in the equatorial plane. One oxo-ligand is axially coordinated and the fifth sulfur substituent differentiates both Moco variants in the equatorial plane. In case of the SO-family of Moco enzymes the sulfur coordinates *via* a thioester derived from a highly conserved protein-derived cysteine residue (Fig. 2D), while in case of xanthine oxidase (XO)-family members the sulfur is a terminal sulfido ligand originated from an additional modification introduced into Moco before it is inserted into enzymes (Fig. 4B) [56].

NR reduces nitrate to nitrite and further to NO

Nitrate reductase is exclusively found in autotrophs and fungi catalyzing the reduction of nitrate to nitrite [57,58]. NR belongs to the SO-family of Moco enzymes coordinating Mo with a highly conserved cysteine-derived thiol (Fig. 2D) [57]. Besides Moco, NR harbors two additional cofactors: a b_5-type heme and a FAD where NAD(P)H oxidation supplies electrons for intramolecular tunneling toward Moco, the catalytic site of nitrate reduction (Fig. 2E) [57]. A dedicated C-terminal dimerization domain promotes the assembly of NR as homodimer in solution. Together with the catalytic Moco domain, both domains form a structural core unit within one subunit while the b_5 and FAD domains are connected by surface exposed and flexible linker regions (Fig. 2A).

In plants, the catalytic reaction of NR ensures inorganic nitrogen supply for plastidic nitrite reductase and glutamine synthetases, which incorporate nitrate-

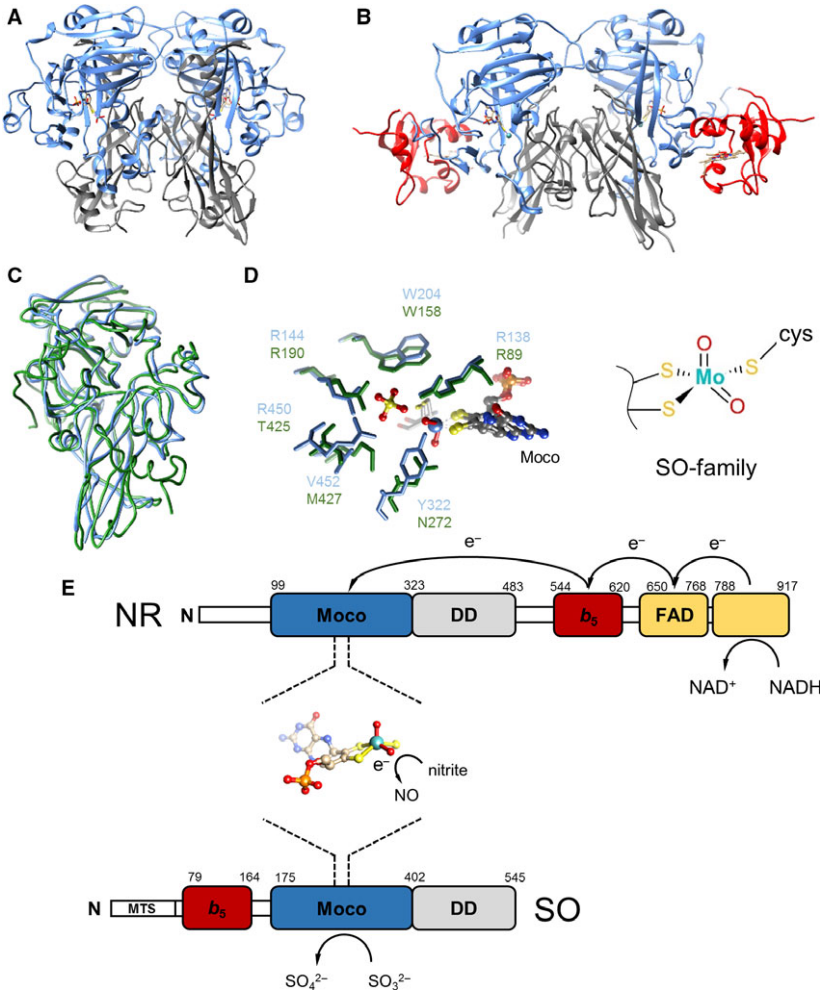

Fig. 2. Eukaryotic members of the SO-family of Moco enzymes. (A) Ribbon representation of homodimeric *Pichia angusta* NR Moco- (blue) and dimerization domain (grey). (B) Ribbon representation of homodimeric chicken SO. The N-terminal heme domain (red) is linked *via* a partially disordered linker to the central Moco domain (blue) followed by the dimerization domain (grey). (C) Structural overlay of Moco- and dimerization domain of *P. angusta* NR (green) and chicken SO (blue) in trace representation of the Cα atoms. (D) Left: Ball and stick representation of the Moco highlighting the Mo atom (teal), the three Mo-coordinated sulfur atoms (yellow) and two Mo-coordinated oxygen atoms (red). The amino acids shaping the active site in chicken SO (blue) and the corresponding *P. angusta* residues (green) are highlighted. Right: The cysteine-derived thioester coordination of Mo defines the SO family of Mo-enzymes. (E) Primary sequence structure of plant NR (upper) and vertebrate SO domain arrangement. Numbers indicate first/last residues of a domain and correspond to *Arabidopsis thaliana* NR and human SO enzyme. Electron flux from NADH *via* FAD (yellow), the b_5-type cytochrome (red) to the Moco domain (blue) is indicated for NR. In SO the N-terminal mitochondrial targeting sequence (MTS) is followed by the b_5-type heme domain (red) which is linked to the Moco domain (blue) followed by the dimerization domain (grey). Nitrite reduction occurs with one electron at the fully reduced Moco active site of both enzymes.

derived nitrogen as ammonium into glutamate [30]. Together with prokaryotic Mo-dependent nitrogenase, both proteins provide the majority of biologically available nitrogen, thus underlining the importance of Mo in the global nitrogen cycle.

As NR catalyzes the reduction of nitrate to nitrite, the reaction product nitrite fits the Moco active site. In 1988, Dean and Harper were the first to report that in crude protein extracts, soybean NR reduces nitrite to NO with a K_M for nitrite one order of magnitude below its K_M for nitrate [59]. The use of inhibitors and different electron donors allowed the conclusion that nitrite reduction takes place at the Moco active site of NR. *In vivo*, several plant species were shown to produce NO. Interestingly, NO emission was dependent on nitrate in the soil and was completely abolished in all species, when plants were grown on ammonium-containing soil, which strongly indicated a role for NR

[60]. Conclusively, isotopically labeled ^{15}N-nitrate was shown to be a major source of emitted ^{15}NO [61]. Using different techniques for direct measurement of NO, corn NR was shown to produce significant amounts of NO not only in the presence of nitrite but also by use of nitrate as an electron donor. This finding suggested that NO can be formed from *de novo* synthesized nitrite despite the presence of high concentrations of nitrate [62]. Finally, a double knockout of both *Arabidopsis thaliana* NR loci (*NIA*1 and *NIA*2) proved a significant contribution of the NR to nitrite-dependent NO release *in vivo* [63].

Since plant NR is known to undergo post-translational regulation, different studies also addressed the NR-dependent NO release in respect to the enzymes' known post-translationally regulated states. NR activity is reduced in the dark *via* its phosphorylation and subsequent 14-3-3 protein binding, which causes a decay in intramolecular electron transfer rate [64]. In line, NR-dependent NO release of leave extracts was abolished under conditions of 14-3-3-dependent inhibition of phospho-NR *in vitro* [65]. Furthermore, the degree of reduction in NO release in plants grown in the dark mirrored the reduction in NR activity [65]. Besides these indirect evidences determined in crude cell extracts, until today a detailed mechanistic and biochemical understanding of NR-catalyzed nitrite reduction is still missing. Nonetheless, the substantial NR-dependent NO production in plants underlines the role of Mo-dependent enzymes in nitrite reduction and thus suggests that mammalian Mo-enzymes may play an important role in NO homeostasis too. Therefore, the closest relative to NR in mammals is SO and has received attention for NO-based research in recent years.

SO exhibits sulfite-dependent nitrite reduction

Structural data of the Moco- and dimerization domain of *Pichia angusta* NR (Fig. 2A) [57], full-length chicken SO (Fig. 2B) [66] and *A. thaliana* SO [67] provide strong evidence for a high degree of structural conservation between NR and SO (Fig. 2C) [68,69]. The chicken SO enzyme serves as a structural blueprint for all vertebrate SOs due to high degree of sequence identity as well as spectroscopic and catalytic similarities [66,70]. Similar to human and murine SO [71], the chicken SO enzyme assembles as homodimer with each subunit consisting of three distinct domains. The N-terminal heme domain harboring a b_5-type cytochrome is connected *via* a flexible tether to the catalytic Moco domain. The C-terminal domain shares homology to a C2-type immunoglobulin fold and confers dimerization of two SO subunits [66].

As evidenced by the X-ray crystal structures, the catalytic Mo centers of NR and SO closely resemble each other (Fig. 2D) which is underlined by electron paramagnetic resonance (EPR) and X-ray absorption spectroscopy studies [72–74]. In both enzymes, solely the catalytically labile equatorial oxo-ligand of Mo faces the active site channel entrance and is proposed to embrace a major role in oxotransfer reactions catalyzed by NR and SO enzymes [68]. In this respect it is important to mention that three residues within the active sites of NRs and SOs are completely conserved, while three additional residues provide specificity in the active site of both enzymes (Fig. 2D) [57,66]. Interestingly, site-directed mutagenesis of two of these residues within SO enabled the enzyme to use nitrate as a substrate with K_M values comparable to those of NR [75] highlighting the evolutionary link between both enzymes. Together with the established role of NR in plant NO homeostasis, a proposal that SO represents a potential vertebrate nitrite-dependent NO synthase is highly conclusive.

In 2015, we reported that SO-catalyzed nitrite reduction contributes to vertebrate NO homeostasis *in vitro* and *in vivo* [76]. By using purified mouse and human SO, nitrite-dependent NO release was demonstrated for sulfite- and dithionite-reduced enzymes. Similar to NR, nitrite reduction occurred at the Moco domain of SO. In case of the strict two-electron donor sulfite, the Mo atom within SO is reduced from the resting state Mo^{VI} to Mo^{IV} upon oxidation of sulfite to sulfate. In this study we showed that solely the sulfite-reduced Mo^{IV} enzyme intermediate is capable to reduce nitrite to NO with a single electron. The resulting Mo^{V} species was inert towards nitrite and could not react any further as revealed by a stable Mo^{V} EPR signal in a nitrite-containing solution [76]. Thus, the SO-dependent NO release appeared stoichiometric rather than catalytic with a low k_{et} of 0.0044 s^{-1} and an apparent K_M of 1.7 mM nitrite.

Interestingly, SO-mediated nitrite reduction was found to be sensitive to pH changes. A decrease in pH to 6.5 increased SO-dependent NO synthesis. Noteworthy, vertebrate SO localizes to the mitochondrial intermembrane space [71,77] where it is known to shuttle sulfite-derived electrons toward cyt *c* thus fueling the respiratory chain [78]. The obligate proximity to cyt *c* ensures a proton-rich environment for SO *in vivo*, which would favor a SO-dependent nitrite reduction.

Further support for SO-dependent release of NO was also provided *in cellulo*. In fibroblasts, which either lacked functional SO or Moco, nitrite-dependent

NO-induced cyclic guanosine monophosphate (cGMP) formation was significantly compromised. The cGMP content of both mutant cell lines leveled to a similar concentration, which indicated that at least in fibroblasts, SO functions as major Mo-dependent nitrite reductase. Future studies are required to develop a better understanding on the underlying active site chemistry that discloses the inert nature of the SO Mo^V species toward nitrite. Furthermore, it remains to be elucidated to which extend SO is able to close the catalytic cycle in the simultaneous presence of sulfite, nitrite and cyt *c*. Interestingly, models for the active site chemistry of nitrite reduction and subsequent catalytic cycles have been proposed for other Mo-enzymes [79,80].

mARC enzymes have a broad substrate specificity

The most recently identified Mo-enzyme is mARC, which was discovered based on its ability to reduce a wide range of hydroxylated amines [81]. The name-giving localization was later refined to the outer mitochondrial membrane, with the active site facing the cytosol [82]. The mARC enzyme is found in all forms of eukaryotes encoded in many species by two different genetic loci translating into two mARC isoforms, termed mARC1 and mARC2 in humans. Interestingly, mARC proteins share no sequence similarity to any of the other four eukaryotic Moco-dependent enzymes but rather resemble the C-terminal domain of Moco-sulfurase, which functions in the transfer of the terminal sulfido ligand of Moco in Mo-enzymes of the XO-family (see below).

Both isoforms of mARC are 35 kDa in size and thus comprise the smallest members of the Moco enzyme family in eukaryotes carrying Moco as only redox active center. While all other eukaryotic Moco enzymes act as homodimers, mARC proteins catalyze their reaction as monomers [83]. They act in concert with FAD-binding NADH:cytochrome b_5 reductase and cytochrome-b_5 forming an intermolecular electron transport chain at the mitochondrial outer membrane to reduce substrates at the mARC active site [83] (Fig. 3A). This arrangement of prosthetic groups resembles the electron transport *via* intramolecular cofactor arrangement of NR. As yet, no physiological substrate of mARC1 or mARC2 was identified, however, a broad substrate specificity toward different *N*-hydroxylated compounds has been reported. Likely candidates for physiological substrates are *N*-hydroxycytosine [84] and *N*-ω-hydroxy-L-arginine [85], the latter being a well-known reaction intermediate of NOS enzymes, which places mARC into the focus of NO research as potential negative regulator of NOS.

Recently, both mARC isoforms were investigated with respect to their nitrite-reducing capacity. In combination with NADH:cytochrome b_5 reductase and cytochrome-b_5 and NADH as electron donor, mARC1 was able to reduce nitrite to NO in a steady-state reaction with a k_{cat} of 0.1 s^{-1} and a K_M for nitrite of 9.5 mM [79]. The reaction was shown to require the Moco active site of mARC and, similarly to SO, was revealed to gain higher efficacy at lower pH. However, at mitochondria, the active site of mARC faces toward the cytosol and thus, the subcellular localization of the enzyme is outside of pH conditions that would favor nitrite reduction *in vivo* at normal physiological conditions.

In contrast to other eukaryotic Mo-enzymes, a structure of mARC is currently lacking. Conflicting findings about the active site architecture point to a cysteine residue important for enzymatic activity and a proposed coordination of Mo [68,86]. Underlining this, the Mo^V EPR signal of mARC isoforms exhibits

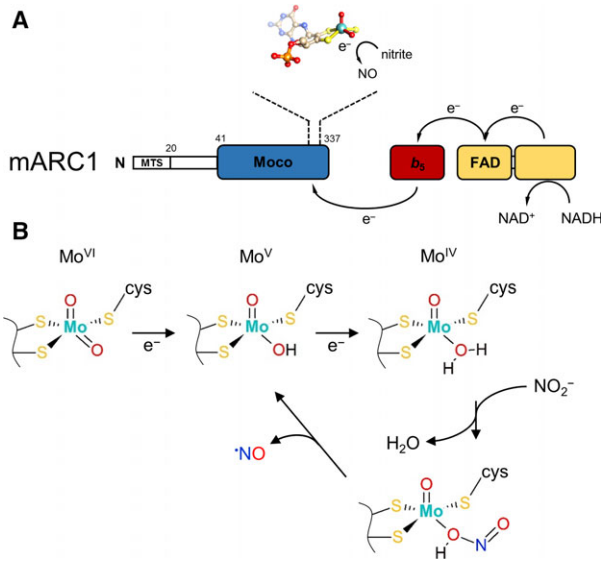

Fig. 3. Domain structure of the eukaryotic mARC complex and the proposed reaction mechanism of nitrite reduction by an SO-type Moco active site. (A) Domain structure of human mARC1. The mitochondrial targeting sequence (MTS) is followed by the Moco domain. Electron flux from NADH to FAD-binding NADH:cytochrome b_5 reductase (yellow) and cytochrome-b_5 (red) to mARC is indicated. Reduction in nitrite to NO requires the Mo^{IV} state of fully reduced mARC. (B) Proposed reaction mechanism of nitrite reduction by an SO-type Mo center according to Yang *et al.* [80]. Upon two-electron reduction of Mo^{VI} to Mo^{IV} nitrite replaced H_2O in the active site giving rise to a Mo-nitrito complex, with a release of NO upon enzyme-assisted protonation of the equatorial oxo ligand of Mo (modified from [80]).

characteristics comparable to those observed for NR and SO proteins, which require a cysteine coordination of Mo [83]. As observed for fully reduced Mo^{IV} SO, the presence of nitrite evoked a stable Mo^{V} EPR signal in mARC indicating that only Mo^{IV} but not Mo^{V} can reduce nitrite to NO [80]. The proposed similarities of the Mo center in mARC to NR and SO were used for molecular dynamics simulations predicting a reaction mechanism of nitrite reduction, which involves a cysteine-coordinated Mo. By using mARC as a model, an inner sphere electron transfer was suggested, which depends on a nitrite coordination to Mo and protonation of the proximal Mo-coordinated oxygen *in silico* (Fig. 3B). In contrast, when this nitrite-derived oxygen atom is deprotonated, a modest reaction barrier was calculated. As a result, the protonation weakens the O-N bond proximal to Mo and thus enables bond fission and NO release. These findings suggest a pH dependence of Mo-catalyzed nitrite reduction, which is in line with empirical observations of increased nitrite reduction efficacy by various Mo-enzymes with decreasing pH [76,79,87,88].

Xanthine oxidoreductase

The mammalian genome encodes for two different types of Moco enzymes, which belong to the XO-family (Fig. 4). The XOR, a key enzyme in purine catabolism, catalyzes the oxidation of hypoxanthine to xanthine and xanthine to uric acid [89]. XOR is a homodimer with a molecular mass of around 300 kDa. Each subunit hosts four different redox active centers (Fig. 4C,D). Substrate oxidation takes place at the Moco active site while two [2Fe-2S] clusters mediate the electron transfer to FAD where NAD^+ serves as electron acceptor (Fig. 4D) [89]. The protein is expressed as xanthine dehydrogenase (XDH), however, reversible or irreversible PTMs may convert XDH into XO [90]. Both isomers catalyze the same oxidation reaction, however, upon conversion to XO, the enzyme becomes highly susceptible to molecular oxygen as terminal electron acceptor thus producing superoxide instead of reducing NAD^+ to NADH [91]. The underlying molecular switch mechanism involves reorientation of a loop region within the NAD^+-binding domain thus blocking the access of NAD+ to FAD [92]. The resulting reactivity of the FAD site toward molecular oxygen renders XO as one major source of reactive oxygen species (ROS) production thus contributing to ischemia/reperfusion injury. Therefore, XO is one of the most pharmacologically targeted enzymes to limit enzymatic ROS formation [93,94]. Interestingly, along with endogenous cytokines,

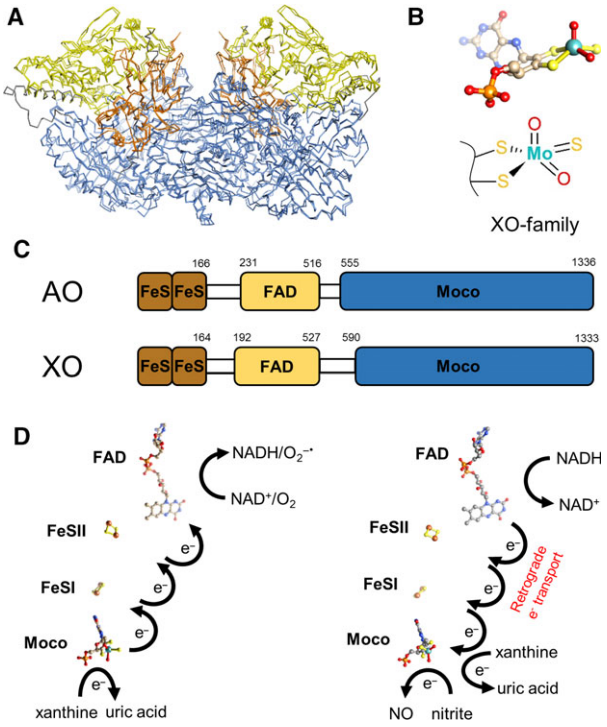

Fig. 4. The XO-type family members of Moco enzymes. (A) Structural overlay of the Cα atoms of XO and AO in ribbon representation. N-terminal FeS cluster domains are colored in orange, FAD-binding domain in yellow and the Moco domain in blue. (B) Ball and stick representation of the Moco highlighting the Mo atom (teal), the three Mo-coordinated sulfur atoms (yellow) and two Mo-coordinated oxygen atoms (red). The terminal sulfido-ligand of Mo defines the XO-family of Mo-enzymes. (C) Primary sequence structure depicts domain arrangement within AO and XO. Numbers indicate first/last residues of a domain and correspond to human AO and bovine XO enzymes. (D) Cofactor arrangement within AO and XO enzymes exemplified for XO. Anterograde electron transport (left) from xanthine oxidation at Moco *via* FeSI, FeSII and FAD to NAD^+ or O_2, serving as electron acceptors in XDH and XO, thereby producing NADH or superoxide, respectively. Retrograde electron transport (right) from NADH *via* the FAD site and the FeS clusters to Moco leads to nitrite reduction at the Moco active site. Alternatively, xanthine can serve as electron donor for nitrite reduction at the Moco active site.

also hypoxia is known to upregulate XDH transcription and activity *in vivo* [95,96]. The vicious circle, which follows XO upregulation and equates XO-derived ROS formation, was considered as molecular hallmark underlying ischemia/reperfusion injury. However, this concept needs to be re-evaluated as XO is likely to capture in addition a beneficial role as physiologic nitrite reductase.

As XO is one of the longest studied enzymes ever [97], it was also the first Moco-dependent enzyme analyzed in respect to nitrite-dependent NO formation

in vitro [98]. Here, bovine XO was incubated with NADH and nitrite under hypoxic conditions while released NO was determined in the gas phase. The use of inhibitors supported the hypothesis of nitrite reduction at the Moco active site rather than at the FAD or FeS cluster sites [98]. In addition to NADH as electron donor (Fig. 4D), xanthine was also revealed to serve as reliable electron source for steady-state nitrite-dependent NO release by XO under hypoxia. However, by use of xanthine, XO-produced NO lead to inactivation of the enzyme by replacement of the sulfido-moiety of Mo with a third-terminal Mo-oxo ligand [88]. Moreover, a xanthine concentration-dependent inhibition of NO production was detected showing that nitrite and xanthine compete for the same binding site at the Mo-active site. In a defined *in vitro* set-up it was possible to reliably determine enzymatic parameters for nitrite-dependent NO release of XO with a high K_M for nitrite of 35.7 mM [88] or 22.8 mM [99] when using NADH as electron source. By measuring urate formation from xanthine, an apparent K_M for nitrite of 149 mM was determined being in line with the competition of xanthine and nitrite for the Moco active site [100]. In contrast, Li *et al.* [101] determined different kinetic parameters by either using xanthine or NADH as electron donors with K_M values of 2.5 and 2.6 mM, respectively. Surprisingly, no competitive effect of xanthine was reported in this study. Noteworthy, similar as seen for mARC and SO, the preferential XO-dependent nitrite reduction was optimal in a pH range between 6 and 7 with k_{cat} values of 0.13 s^{-1} with NADH as electron donor and 2.9 s^{-1} with xanthine [88]. Contrary, Millar *et al.* [99] determined with NADH as electron donor a much higher k_{cat} of 9.14 s^{-1}. In conclusion, although differences in the kinetic behavior of XO toward nitrite reduction were reported by different studies, the conclusive outcome of those investigation showed a XO-dependent reduction of nitrite to NO at the Mo site of the enzyme in the absence of molecular oxygen.

Although similar to SO and mARC, the *in vitro* determined K_M values of XO seem to exclude efficient nitrite reduction within a physiological environment. Nonetheless, *in vivo* evidence supports a function of XO as NO synthase. Alef *et al.* [102] demonstrated a significant role of XO-derived NO in the protection from intima hyperplesia within rats. Moreover, intravenous nitrite injection showed a strikingly protective effect in a rat model for lung injury upon high-pressure ventilation during anesthesia. The mortality of rats subjected to high-pressure ventilation decreased from 48% to 0% with prior injection of 2.5 μmol nitrite/kg body weight. Application of either an NO scavenger or the potent XO inhibitor allopurinol strongly compromised the pharmacological benefit of nitrite injection [103]. In hypoxia-induced rat models of pulmonary arterial hypertension, nitrite-derived NO was revealed to be strongly counteracting vascular smooth muscle proliferation, which was dependent – at least in part – on the nitrite-reducing activity of XO [104]. Similarly, a nitrite-induced protection from ischemia/reperfusion injury was proposed for kidney [105], heart [106] and liver [107,108]: all studies proposing a critical role for XO as endogenous source for protective NO formation.

In addition to nitrite, XO was shown to encompass a function as mammalian NR. *In vitro*, Li *et al.* provided evidence of XO-dependent nitrate reduction to nitrite in the presence of either NADH or xanthine as reductants. Furthermore, by use of the XO inhibitor oxypurinol it was shown that ^{15}N-labled nitrate reduction in different tissues was dependent on XO activity [101]. *In vivo*, this finding was evidenced by use of germ-free rats, which lack symbiont-derived NR. In these rats, administration of nitrate led to significantly increased tissue and serum nitrite levels which could be reversed by the XO inhibitor allopurinol [109]. Evidently, the germ-free living condition alone was sufficient to significantly increase XO protein abundance. Taken together, until today XO has become the most accepted Mo-dependent enzyme involved in mammalian nitrate-nitrite-NO homeostasis. However, it has to be noted that the strict dependence on low pH, high nitrite and hypoxia for efficient NO release collectively provides an ongoing challenge for the assessment of XO in the overall nitrate-to-nitrite-to-NO pathway. Moreover, lack of $XO^{-/-}$ mice and the dependence on XO inhibition might lead to off-target effects on purine metabolism [110]. Along this line, for allopurinol/oxypurinol, effective radical scavenging activities were reported which in turn challenges the differentiation between its intrinsic antioxidant nature and its function as inhibitor of XO [110,111].

Aldehyde oxidase

Aldehyde oxidase represents the second mammalian XO-type Moco enzyme (Fig. 4). Although the X-ray crystal structure of human AO became recently available [112], little is known about the function of this enzyme in mammalian metabolism. Similar to XDH, AO harbors two N-terminal [2Fe-2S] clusters, a central FAD domain and a C-terminal Moco domain and acts as a homodimer of approximately 300 kDa size (Fig. 4). Until today, no primary physiological substrate could be assigned to AO. Instead, the broad

substrate specificity challenges the assessment of such a substrate as AO is capable of oxidizing not only aldehydes but also aromatic N-heterocycles. Moreover, AO-mediated reduction in different functional moieties like S-oxides, N-oxides and nitro groups was also reported [113,114]. Nonetheless, its hepatic localization [115] and the capacity to metabolize exogenous compounds including the activation of prodrugs places AO into the focus of numerous pharmacological investigations [116]. While mice express four AO isoforms, in humans one AO enzyme seems to fulfill all AO-specific functions.

Purified AO was investigated in an NO analyzer in the presence and absence of specific inhibitors. Similarly to SO, mARC and XO, a pH dependence was reported and revealed highest nitrite reduction at low pH [87]. Interestingly, although an artificial electron donor as well as the physiologic electron donor NADH were used to analyze nitrite-reductive capacity of AO, no steady-state reaction of NO release was observed. Instead, the NO release appeared stoichiometric with a maximum release velocity of 0.15 s^{-1} and a K_M for nitrite of approximately 3 mM [87]. Despite the striking sequence identity of 86% between AO and XO in humans, the difference in their nitrite reduction behavior remains enigmatic.

Concluding remarks

Tissue nitrite reduction was recognized in various organs including aorta, heart and liver, the latter having by far the highest capacity to reduce nitrite to NO [117,118]. Interestingly, in liver, Mo-dependent enzymes such as XO, AO, SO and mARC2 are highly abundant. Latest findings concerning the mARC1 expression profile indicate that the enzyme is rather localized in adipose tissue, mammary tissue and thyroid while liver expression was found to be low [119]. However, the propensity of these organs to reduce nitrite was not investigated yet. Most NO release of liver tissue homogenate was strictly dependent on the absence of allopurinol and molecular oxygen [117]. Although considered as specific XO inhibitor, allopurinol is metabolized to oxypurinol by AO *via* the same tautomerization reaction, which inhibits AO in the same way as XO [120]. In this respect, it is interesting to note that within human liver, AO transcripts are one order of magnitude higher than XO transcripts [119]. In liver this might indicate a much higher contribution of AO in nitrite-dependent NO release as initially expected. As cytosolic proteins, AO and XO not only depend on pathologically low pH for effective nitrite reduction, they also require hypoxia. In this respect, the finding that following nitrite infusion or dietary nitrite supplementation a decrease in systemic blood pressure in normoxic humans can be detected, suggests that AO and XO are not the sole Moco-dependent NO synthases within humans [121]. Here, SO needs to be considered as a significant contributor among the Moco-dependent enzymes in the reduction of nitrite to NO.

Sulfite oxidase was shown to reduce nitrite to NO and previous studies revealed human SO as unable to use molecular oxygen as electron acceptor [122] suggesting that SO-dependent nitrite reduction is independent of oxygen tension. SO is compartmentally confined in the mitochondrial intermembrane space and this physiological proton-rich environment makes SO the only Mo-enzyme for which nitrite reduction is conceivable also under less hypoxic conditions. However, whether nitrite transporters exist in higher eukaryotes which distribute nitrite among cellular compartments such as the mitochondrion is not clear yet. Nonetheless, two striking arguments hint toward the existence of highly effective transporters. First, the rapid (5 min) redistribution of nitrite among various rat organs upon intraperitoneal nitrite injection and second, the fact that anionic nitrite is the predominant endogenous form which needs active transport rather than passive diffusion along the lipid bilayers. Interestingly, mitochondria were already revealed to be an intracellular target for nitrite flux *in vivo* [123–125]. In this respect, a linear correlation (R^2 = 0.99) of nitrite-reducing capacity within different organs and the mitochondrial surface area in cells of these organs reveals mitochondria as crucial and indispensable determinants for efficient nitrite reduction [118]. It is already well accepted that mitochondria are physiological targets of NO, which fundamentally regulates respiration, ROS production and cellular signaling [93,126–129]. In this concept, a nitrite reductase activity of SO in close proximity to the respiratory chain complexes is expected to vastly impact a whole variety of mitochondria-induced cellular signaling events and might serve as one missing regulatory link between the tightly connected NO and H_2S signaling pathways.

Acknowledgement

We acknowledge the Center for Molecular Medicine Cologne (CMMC) for funding and support.

References

1 Ignarro LJ, Byrns RE, Buga GM and Wood KS (1987) Endothelium-derived relaxing factor from

pulmonary artery and vein possesses pharmacological and chemical properties that are identical to those for nitric oxide radical. *Circ Res* **61**, 866–879.
2 Hibbs JB, Taintor RR, Vavrin Z and Rachlin EM (1988) Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem Biophys Res Commun* **157**, 87–94.
3 Marietta MA, Yoon PS, Iyengar R, Leaf CD and Wishnok JS (1988) Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry* **27**, 8706–8711.
4 Hess DT, Matsumoto A, Kim S, Marshall HE and Stamler JS (2005) Protein S-nitrosylation: purview and parameters. *Nat Rev* **6**, 150–166.
5 Arnold WP, Mittal CK, Katsuki S and Murad F (1977) Nitric oxide activates guanylate cyclase and increases guanosine 3':5'-cyclic monophosphate levels in various tissue preparations. *Proc Natl Acad Sci USA* **74**, 3203–3207.
6 Stamler JS (1994) Redox signaling: nitrosylation and related target interactions of nitric oxide. *Cell* **78**, 931–936.
7 Beckman JS and Koppenol WH (1996) Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly. *Am J Physiol* **271**, 1424–1437.
8 Vanderkooi JM, Wright WW and Erecinska M (1994) Nitric oxide diffusion coefficients in solution, proteins and membranes determined by phosphorescence. *Biochim Biophys Acta* **1207**, 249–254.
9 Bredt DS, Hwang PM, Glatt CE, Lowenstein C, Reed RR and Snyder SH (1991) Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature* **351**, 714–718.
10 Lamas S, Marsden PA, Li GK, Tempst P and Michel T (1992) Endothelial nitric oxide synthase: molecular cloning and characterization of a distinct constitutive enzyme isoform. *Proc Natl Acad Sci USA* **89**, 6348–6352.
11 Xie Q, Cho H, Calaycay J, Mumford R, Swiderek K, Lee T, Ding A, Troso T and Nathan C (1992) Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science* **256**, 225–228.
12 Marletta MA (1993) Nitric oxide synthase structure and mechanism. *J Biol Chem* **268**, 12231–12234.
13 Xie Q-W, Whisnant R and Nathan C (1993) Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon gamma and bacterial lipopolysaccharide. *J Exp Med* **177**, 1779–1784.
14 Knowles RG (1996) Nitric oxide synthases. *Biochem Soc Trans* **24**, 875–878.
15 Kroencke K, Fehsel K and Kolb-Bachofen V (1998) Inducible nitric oxide synthase in human diseases. *Clin Exp Immunol* **113**, 147–156.
16 Nakane M, Schmidt HHHW, Pollock JS, Förstermann U and Murad F (1993) Cloned human brain nitric oxide synthase is highly expressed in skeletal muscle. *FEBS Lett* **316**, 175–180.
17 Mundel P, Bachmann S, Bader M, Fischer A, Kummer W, Mayer B and Kriz W (1992) Expression of nitric oxide synthase in kidney macula densa cells. *Kidney Int* **42**, 1017–1019.
18 Boulanger CM, Heymes C, Benessiano J, Geske RS, Levy BI and Vanhoutte PM (1998) Neuronal nitric oxide synthase is expressed in rat vascular smooth muscle cells. *Circ Res* **83**, 1271–1278.
19 Hosseini M, Dastghaib SS, Rafatpanah H, Hadjzadeh MA-R, Nahrevanian H and Farrokhi I (2010) Nitric oxide contributes to learning and memory deficits observed in hypothyroid rats during neonatal and juvenile growth. *Clinics* **65**, 1175–1181.
20 Palumbo ML, Fosser NS, Rios H, Zubilete MAZ, Guelman LR, Cremaschi GA and Genaro AM (2007) Loss of hippocampal neuronal nitric oxide synthase contributes to the stress-related deficit in learning and memory. *J Neurochem* **102**, 261–274.
21 Chong C-M, Ai N, Ke M, Tan Y, Huang Z, Li Y, Lu J-H, Ge W and Su H (2018) Roles of nitric oxide synthase isoforms in neurogenesis. *Mol Neurobiol* **55**, 2645–2652.
22 Dejanovic B and Schwarz G (2014) Neuronal nitric oxide synthase-dependent S-nitrosylation of gephyrin regulates gephyrin clustering at GABAergic synapses. *J Neurosci* **34**, 7763–7768.
23 Ho GPH, Selvakumar B, Mukai J, Hester LD, Wang Y, Gogos JA and Snyder SH (2011) S-nitrosylation and S-palmitoylation reciprocally regulate synaptic targeting of PSD-95. *Neuron* **71**, 131–141.
24 Kubes P, Suzuki M and Granger DN (1991) Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci USA* **88**, 4651–4655.
25 Radomski MW, Palmer RM and Moncada S (1990) An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. *Proc Natl Acad Sci USA* **87**, 5193–5197.
26 Rizzo ON, Maloney E, Pham M, Luttrell I, Wessell H, Tateya S, Daum G, Handa P, Schwartz MW and Kim F (2010) Reduced nitric oxide/cGMP signaling contributes to vascular inflammation and insulin resistance induced by high fat feeding. *Arterioscler Thromb Vasc Biol* **30**, 758–765.
27 Jeremy JY, Rowe D, Emsley AM and Newby AC (1999) Nitric oxide and the proliferation of vascular smooth muscle cells. *Cardiovasc Res* **43**, 580–594.
28 Guo F-Q, Okamoto M and Crawford NM (2003) Identification of a plant nitric oxide synthase gene involved in hormonal signaling. *Science* **302**, 100–103.
29 Zemojtel T, Fröhlich A, Palmieri MC, Kolanczyk M, Mikula I, Wyrwicz LS, Wanker EE, Mundlos S, Vingron M, Martasek P *et al*. (2006) Plant nitric oxide

synthase: a never-ending story? *Trends Plant Sci* **11**, 522–524.
30 Schwarz G, Mendel RR and Ribbe MW (2009) Molybdenum cofactors, enzymes and pathways. *Nature* **460**, 839–847.
31 Kleinbongard P, Dejam A, Lauer T, Rassaf T, Schindler A, Picker O, Scheeren T, Gödecke A, Schrader J, Schulz R *et al*. (2003) Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals. *Free Radic Biol Med* **35**, 790–796.
32 Bories PN and Bories C (1995) Nitrate determination in biological fluids by an enzymatic one-step assay with nitrate reductase. *Clin Chem* **41**, 904–907.
33 Cortas NK and Wakid NW (1990) Determination of inorganic nitrate in serum and urine by a kinetic cadmium reduction method. *Clin Chem* **36**, 1440–1443.
34 Rodriguez J, Maloney RE, Rassaf T, Bryan NS and Feelisch M (2003) Chemical nature of nitric oxide storage forms in rat vascular tissue. *Proc Natl Acad Sci USA* **100**, 336–341.
35 Duncan C, Dougall H, Johnston P, Green S, Brogan R, Leifert C, Smith L, Golden M and Benjamin N (1995) Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. *Nat Med* **1**, 546–551.
36 Lundberg JO, Weitzberg E, Lundberg JM and Alving K (1994) Intragastric nitric oxide production in humans: measurements in expelled air. *Gut* **35**, 1543–1546.
37 Dykhuizen RS, Frazer R, Duncan C, Smith CC, Golden M, Benjamin N and Leifert C (1996) Antimicrobial effect of acidified nitrite on gut pathogens: importance of dietary nitrate in host defense. *Antimicrob Agents Chemother* **40**, 1422–1425.
38 Benjamin N, O'Driscoll F, Dougall H, Duncan C, Smith L, Golden M and McKenzie H (1994) Stomach NO synthesis. *Nature* **368**, 502.
39 Bryan NS, Rassaf T, Maloney RE, Rodriguez CM, Saijo F, Rodriguez JR and Feelisch M (2004) Cellular targets and mechanisms of nitros(yl)ation: an insight into their nature and kinetics in vivo. *Proc Natl Acad Sci USA* **101**, 4308–4313.
40 Bryan NS, Fernandez BO, Bauer SM, Garcia-Saura MF, Milsom AB, Rassaf T, Maloney RE, Bharti A, Rodriguez J and Feelisch M (2005) Nitrite is a signaling molecule and regulator of gene expression in mammalian tissues. *Nat Chem Biol* **1**, 290–297.
41 Lundberg JO, Weitzberg E and Gladwin MT (2008) The nitrate–nitrite–nitric oxide pathway in physiology and therapeutics. *Nat Rev Drug Discov* **7**, 156–167.
42 Gow AJ, Luchsinger BP, Pawloski JR, Singel DJ and Stamler JS (1999) The oxyhemoglobin reaction of nitric oxide. *Proc Natl Acad Sci USA* **96**, 9027–9032.
43 Shiva S, Wang X, Ringwood LA, Xu X, Yuditskaya S, Annavajjhala V, Miyajima H, Hogg N, Harris ZL and Gladwin MT (2006) Ceruloplasmin is a NO oxidase and nitrite synthase that determines endocrine NO homeostasis. *Nat Chem Biol* **2**, 486–493.
44 Zweier JL, Wang P, Samouilov A and Kuppusamy P (1995) Enzyme-independent formation of nitric oxide in biological tissues. *Nat Med* **1**, 804–809.
45 Zweier JL, Li H, Samouilov A and Liu X (2010) Mechanisms of nitrite reduction to nitric oxide in the heart and vessel wall. *Nitric Oxide* **22**, 83–90.
46 Modin A, Björne H, Herulf M, Alving K, Weitzberg E and Lundberg JON (2001) Nitrite-derived nitric oxide: a possible mediator of 'acidic-metabolic' vasodilation. *Acta Physiol Scand* **171**, 9–16.
47 Zweier JL, Samouilov A and Kuppusamy P (1999) Non-enzymatic nitric oxide synthesis in biological systems. *Biochim Biophys Acta* **1411**, 250–262.
48 Siesjö BK (1988) Acidosis and ischemic brain damage. *Neurochem Pathol* **9**, 31–88.
49 Rehncrona S (1985) Brain acidosis. *Ann Emerg Med* **14**, 770–776.
50 Cosby K, Partovi KS, Crawford JH, Patel RP, Reiter CD, Martyr S, Yang BK, Waclawiw MA, Zalos G, Xu X *et al*. (2003) Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat Med* **9**, 1498–1505.
51 Tejero J and Gladwin MT (2014) The globin superfamily: functions in nitric oxide formation and decay. *Biol Chem* **395**, 631–639.
52 Basu S, Azarova NA, Font MD, King SB, Hogg N, Gladwin MT, Shiva S and Kim-Shapiro DB (2008) Nitrite reductase activity of cytochrome c. *J Biol Chem* **283**, 32590–32597.
53 Jayaraman T, Tejero J, Chen BB, Blood AB, Frizzell S, Shapiro C, Tiso M, Hood BL, Wang X, Zhao X *et al*. (2011) 14-3-3 Binding and phosphorylation of neuroglobin during hypoxia modulate six-to-five heme pocket coordination and rate of nitrite reduction to nitric oxide. *J Biol Chem* **286**, 42679–42689.
54 Tiso M, Tejero J, Basu S, Azarov I, Wang X, Simplaceanu V, Frizzell S, Jayaraman T, Geary L, Shapiro C *et al*. (2011) Human neuroglobin functions as a redox-regulated nitrite reductase. *J Biol Chem* **286**, 18277–18289.
55 Mendel RR and Schwarz G (2011) Molybdenum cofactor biosynthesis in plants and humans. *Coord Chem Rev* **255**, 1145–1158.
56 Hille R and Bittner F (2011) Molybdenum enzymes in higher organisms. *Coord Chem Rev* **255**, 1179–1205.
57 Fischer K, Barbier GG, Hecht H-J, Mendel RR, Campbell WH and Schwarz G (2005) Structural basis of eukaryotic nitrate reduction: crystal structures of the nitrate reductase active site. *Plant Cell* **17**, 1167–1179.
58 Nicholas DJD, Nason A and McElroy W (1954) Molybdenum and nitrate reductase. *J Biol Chem* **207**, 341–352.

59 Dean JV and Harper JE (1988) The conversion of nitrite to nitrogen oxide(s) by the constitutive NAD(P) H-nitrate reductase enzyme from soybean. *Plant Physiol* **88**, 389–395.
60 Wildt J, Kley D, Rockel A, Rockel P and Segschneider HJ (1997) Emission of NO from several higher plant species. *J Geophys Res* **102**, 5919–5927.
61 Dean JV and Harper JE (1986) Nitric oxide and nitrous oxide production by soybean and winged bean during the in vivo nitrate reductase assay. *Plant Physiol* **82**, 718–723.
62 Yamasaki H and Sakihama Y (2000) Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: in vitro evidence for the NR-dependent formation of active nitrogen species. *FEBS Lett* **468**, 89–92.
63 Desikan R, Griffiths R, Hancock J and Neill S (2002) A new role for an old enzyme: nitrate reductase-mediated nitric oxide generation is required for abscisic acid-induced stomatal closure in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* **99**, 16314–16318.
64 Lambeck I, Chi JC, Krizowski S, Mueller S, Mehlmer N, Teige M, Fischer K and Schwarz G (2010) Kinetic analysis of 14-3-3-inhibited *Arabidopsis thaliana* nitrate reductase. *Biochemistry* **49**, 8177–8186.
65 Rockel P, Strube F, Rockel A, Wildt J and Kaiser WM (2002) Regulation of nitric oxide (NO) production by plant nitrate reductase in vivo and in vitro. *J Exp Bot* **53**, 103–110.
66 Kisker C, Schindelin H, Pacheco A, Wehbi WA, Garrett RM, Rajagopalan KV, Enemark JH and Rees DC (1997) Molecular basis of sulfite oxidase deficiency from the structure of sulfite oxidase. *Cell* **91**, 973–983.
67 Schrader N, Fischer K, Theis K, Mendel RR, Kisker C, Brook S and York N (2003) The crystal structure of plant sulfite oxidase provides insights into sulfite oxidation in plants and animals. *Structure* **11**, 1251–1263.
68 Hille R, Hall J and Basu P (2014) The mononuclear molybdenum enzymes. *Chem Rev* **114**, 3963–4038.
69 Schwarz G and Mendel RR (2006) Molybdenum cofactor biosynthesis and molybdenum enzymes. *Annu Rev Plant Biol* **57**, 623–647.
70 Johnson JL and Rajagopalan KV (1972) Purification and properties of sulfite oxidase from human liver. *J Biol Chem*, **58**, 543–550.
71 Klein J and Schwarz G (2012) Cofactor-dependent maturation of mammalian sulfite oxidase links two mitochondrial import pathways. *J Cell Sci* **125**, 4876–4885.
72 George GN, Kipke CA, Prince RC, Sunde RA, Enemark JH and Cramer SP (1989) Structure of the active site of sulfite oxidase. X-ray absorption spectroscopy of the Mo(IV), Mo(V), and Mo(VI) oxidation states. *Biochemistry* **28**, 5075–5080.
73 Doonan CJ, Wilson HL, Bennett B, Prince RC and George GN (2008) MoV electron paramagnetic resonance of sulfite oxidase revisited: the low-pH chloride signal. *Inorg Chem* **47**, 2033–2038.
74 George GN, Garrett RM, Prince RC and Rajagopalan KV (2004) Coordination chemistry at the molybdenum site of sulfite oxidase: redox-induced structural changes in the cysteine 207 to serine mutant. *Inorg Chem* **43**, 2625–2626.
75 Qiu JA, Wilson HL and Rajagopalan KV (2012) Structure-based alteration of substrate specificity and catalytic activity of sulfite oxidase from sulfite oxidation to nitrate reduction. *Biochemistry* **51**, 1134–1147.
76 Wang J, Krizowski S, Fischer-Schrader K, Niks D, Tejero J, Sparacino-Watkins C, Wang L, Ragireddy V, Frizzell S, Kelley EE *et al*. (2015) Sulfite oxidase catalyzes single-electron transfer at molybdenum domain to reduce nitrite to nitric oxide. *Antioxid Redox Signal* **23**, 283–294.
77 Ito A (1971) Hepatic sulfite oxidase identified as cytochrome b5-like pigment extractable from mitochondria by hypotonic treatment. *J Biochem* **70**, 1061–1064.
78 Schwarz G (2016) Molybdenum cofactor and human disease. *Curr Opin Chem Biol* **31**, 179–187.
79 Sparacino-Watkins CE, Tejero J, Sun B, Gauthier MC, Ragireddy V, Merchant BA, Wang J, Azarov I, Basu P and Gladwin MT (2014) Nitrite reductase and NO synthase activity of the mitochondrial molybdopterin enzymes mARC1 and mARC2. *J Biol Chem* **289**, 10345–10358.
80 Yang J, Giles LJ, Ruppelt C, Mendel RR, Bittner F and Kirk ML (2015) Oxyl and hydroxyl radical transfer in mitochondrial amidoxime reducing component-catalyzed nitrite reduction. *J Am Chem Soc* **137**, 5276–5279.
81 Havemeyer A, Bittner F, Wollers S, Mendel R, Kunze T and Clement B (2006) Identification of the missing component in the mitochondrial benzamidoxime prodrug-converting system as a novel molybdenum enzyme. *J Biol Chem* **281**, 34796–34802.
82 Klein JM, Busch JD, Potting C, Baker MJ, Langer T and Schwarz G (2012) The mitochondrial amidoxime-reducing component (mARC1) is a novel signal-anchored protein of the outer mitochondrial membrane. *J Biol Chem* **287**, 42795–42803.
83 Wahl B, Reichmann D, Niks D, Krompholz N, Havemeyer A, Clement B, Messerschmidt T, Rothkegel M, Biester H, Hille R *et al*. (2010) Biochemical and spectroscopic characterization of the human mitochondrial amidoxime reducing components hmARC-1 and hmARC-2 suggests the existence of a new molybdenum enzyme family in eukaryotes. *J Biol Chem* **285**, 37847–37859.

84 Krompholz N, Krischkowski C, Reichmann D, Garbe-Schönberg D, Mendel RR, Bittner F, Clement B and Havemeyer A (2012) The mitochondrial amidoxime reducing component (mARC) is involved in detoxification of N-hydroxylated base analogues. *Chem Res Toxicol* **25**, 2443–2450.
85 Kotthaus J, Wahl B, Havemeyer A, Kotthaus J, Schade D, Garbe-Schönberg D, Mendel R, Bittner F and Clement B (2011) Reduction of N ω -hydroxy-L-arginine by the mitochondrial amidoxime reducing component (mARC). *Biochem J* **433**, 383–391.
86 Ott G, Havemeyer A and Clement B (2015) The mammalian molybdenum enzymes of mARC. *J Biol Inorg Chem* **20**, 265–275.
87 Li H, Kundu TK and Zweier JL (2009) Characterization of the magnitude and mechanism of aldehyde oxidase-mediated nitric oxide production from nitrite. *J Biol Chem* **284**, 33850–33858.
88 Godber LJB, Doel JJ, Sapkota PG, Blake RD, Stevens RC, Eisenthal R and Harrison R (2000) Reduction of nitrite to nitric oxide catalized by xanthine oxidoreductase. *J Biol Chem* **275**, 7757–7763.
89 Enroth C, Eger BT, Okamoto K, Nishino T, Nishino T and Pai EF (2000) Crystal structures of bovine milk xanthine dehydrogenase and xanthine oxidase: structure-based mechanism of conversion. *Proc Natl Acad Sci USA* **97**, 10723–10728.
90 Kuwabara Y, Nishino T, Okamoto K, Matsumura T, Eger BT, Pai EF and Nishino T (2003) Unique amino acids cluster for switching from the dehydrogenase to oxidase form of xanthine oxidoreductase. *Proc Natl Acad Sci USA* **100**, 8170–8175.
91 Harris CM and Massey V (1997) The oxidative half-reaction of xanthine dehydrogenase with NAD; reaction kinetics and steady-state mechanism. *J Biol Chem* **272**, 28335–28341.
92 Nishino T, Okamoto K, Eger BT, Pai EF and Nishino T (2008) Mammalian xanthine oxidoreductase – mechanism of transition from xanthine dehydrogenase to xanthine oxidase. *FEBS J* **275**, 3278–3289.
93 Granger DN and Kvietys PR (2015) Reperfusion injury and reactive oxygen species: the evolution of a concept. *Redox Biol* **6**, 524–551.
94 Cantu-Medellin N and Kelley EE (2013) Xanthine oxidoreductase-catalyzed reduction of nitrite to nitric oxide: insights regarding where, when and how. *Nitric Oxide* **1**, 19–26.
95 Hassoun PM, Yu FS, Shedd AL, Zulueta JJ, Thannickal VJ, Lanzillo JJ, Fanburg BL, Division CC and England N (1994) Regulation of endothelial cell xanthine dehydrogenase/xanthine oxidase gene expression by oxygen tension. *Am J Physiol* **266**, 163–171.
96 Wang G, Qian P, Jackson FR, Qian G and Wu G (2008) Sequential activation of JAKs, STATs and xanthine dehydrogenase/oxidase by hypoxia in lung microvascular endothelial cells. *Int J Biochem Cell Biol* **40**, 461–470.
97 Dixon M and Thurlow S (1924) Studies on xanthine oxidase. Preparation and properties of the active material. *Biochem J* **18**, 971–975.
98 Zhang Z, Naughton D, Winyard PG, Benjamin N, Blake DR and Symons MCR (1998) Generation of nitric oxide by a nitrite reductase activity of xanthine oxidase: a potential pathway for nitric oxide formation in the absence of nitric oxide synthase activity. *Biochem Biophys Res Commun* **249**, 767–772.
99 Millar TM, Stevens CR, Benjamin N, Eisenthal R, Harrison R and Blake DR (1998) Xanthine oxidoreductase catalyses the reduction of nitrates and nitrite to nitric oxide under hypoxic conditions. *FEBS Lett* **427**, 225–228.
100 Doel JJ, Godber BLJ, Goult TA, Eisenthal R and Harrison R (2000) Reduction of organic nitrites to nitric oxide catalyzed by xanthine oxidase: possible role in metabolism of nitrovasodilators. *Biochem Biophys Res Commun* **270**, 880–885.
101 Li H, Samouilov A, Liu X and Zweier JL (2001) Characterization of the magnitude and kinetics of xanthine oxidase-catalyzed nitrite reduction. *Biochemistry* **276**, 24482–24489.
102 Alef MJ, Vallabhaneni R, Carchman E, Morris SM Jr, Shiva S, Wang Y, Kelley EE, Tarpey MM, Gladwin MT, Tzeng E *et al*. (2011) Nitrite-generated NO circumvents dysregulated arginine/NOS signaling to protect against intimal hyperplasia in Sprague-Dawley rats. *J Clin Invest* **121**, 1–11.
103 Pickerodt PA, Emery MJ, Ph D, Zarndt R, Martin W, Francis RCE, Boemke W and Swenson ER (2012) Sodium nitrite mitigates ventilator-induced lung injury in rats. *Anesthesiology* **117**, 592–601.
104 Zuckerbraun BS, Shiva S, Ifedigbo E, Mathier MA, Mollen KP, Rao J, Bauer PM, Choi JJW, Curtis E, Choi AMK *et al*. (2010) Nitrite potently inhibits hypoxic and inflammatory pulmonary arterial hypertension and smooth muscle proliferation via xanthine oxidoreductase- dependent nitric oxide generation. *Circulation* **121**, 98–109.
105 Tripatara P, Patel NSA, Webb A, Rathod K, Lecomte FMJ, Mazzon E, Cuzzocrea S, Yaqoob MM, Ahluwalia A and Thiemermann C (2007) Nitrite-derived nitric oxide protects the rat kidney against ischemia/reperfusion injury in vivo: role for xanthine oxidoreductase. *J Am Soc Nephrol* **18**, 570–580.
106 Ingram TE, Fraser AG, Bleasdale RA, Ellins EA, Margulescu AD, Halcox JP and James PE (2013) Low-dose sodium nitrite attenuates myocardial ischemia and vascular ischemia-reperfusion injury in human models. *J Am Coll Cardiol* **61**, 2534–2541.
107 Abe Y, Hines I, Zibari G and Grisham MB (2009) Hepatocellular protection by nitric oxide or nitrite in

ischemia and reperfusion injury. *Arch Biochem Biophys* **484**, 232–237.
108 Bjoernsson B, Bojmar L, Olsson H, Sundqvist T and Sandstroem P (2015) Nitrite, a novel method to decrease ischemia/reperfusion injury in the rat liver. *World J Gastroenterol* **21**, 1775–1783.
109 Huang L, Borniquel S and Lundberg JO (2010) Enhanced xanthine oxidoreductase expression and tissue nitrate reduction in germ free mice. *Nitric Oxide* **22**, 191–195.
110 Dao VT, Casas AI, Maghzal GJ, Seredenina T, Kaludercic N, Robledinos-Anton N, Di Lisa F, Stocker R, Ghezzi P, Jaquet V *et al*. (2015) Pharmacology and clinical drug candidates in redox medicine. *Antioxid Redox Signal* **23**, 1113–1129.
111 Moorhouse PC, Grootveld M, Halliwell B, Quinlan JG and Gutteridge MCJ (1987) Allopurinol and oxypurinol are hidroxyl radical scavengers. *FEBS Lett* **213**, 23–28.
112 Coelho C, Foti A, Hartmann T, Santos-silva T, Leimkühler S and Romão MJ (2015) Structural insights into xenobiotic and inhibitor binding to human aldehyde oxidase. *Nat Chem Biol* **11**, 779–786.
113 Obach RS, Huynh P and Allen MC (2004) Human liver aldehyde oxidase: inhibition by 239 drugs. *J Clin Pharmacol* **44**, 7–19.
114 Pryde DC, Dalvie D, Hu Q, Jones P, Obach RS and Tran T (2010) Aldehyde oxidase: an enzyme of emerging importance in drug discovery. *J Med Chem* **53**, 8441–8460.
115 Calzi ML, Raviolo C, Ghibaudi E, de Gioia L, Salmona M, Cazzaniga G, Kurosaki M, Terao M, Garattini E, Natl EP *et al*. (1995) Purification, cDNA cloning, and tissue distribution of bovine liver aldehyde oxidase. *J Biol Chem* **270**, 31037–31045.
116 Garattini E, Terao M, Garattini E and Terao M (2012) The role of aldehyde oxidase in drug metabolism. *Expert Opin Drug Metab Toxicol* **8**, 487–503.
117 Li H, Cui H, Kundu TK, Alzawahra W and Zweier JL (2008) Nitric oxide production from nitrite occurs primarily in tissues not in the blood: critical role of xanthine oxidase and aldehyde oxidase. *J Biol Chem* **283**, 17855–17863.
118 Feelisch M, Fernandez BO, Bryan NS, Garcia-Saura MF, Bauer S, Whitlock DR, Ford PC, Janero DR, Rodriguez J and Ashrafian H (2008) Tissue processing of nitrite in hypoxia: an intricate interplay of nitric oxide generating and scavenging systems. *J Biol Chem* **283**, 33927–33934.
119 GTEx Consortium (2017) Genetic effects on gene expression across human tissues. *Nature* **550**, 204–213.
120 Mirzaei S, Taherpour AA and Mohamadi S (2016) Mechanistic study of allopurinol oxidation using aldehyde oxidase, xanthine oxidase and cytochrome P450 enzymes. *RSC Adv* **6**, 109672–109680.
121 Vitturi DA and Patel RP (2011) Current perspectives and challenges in understanding the role of nitrite as an integral player in nitric oxide biology and therapy. *Free Radic Biol Med* **51**, 805–812.
122 Belaidi AA, Roeper J, Arjune S, Krizowski S, Trifunovic A and Schwarz G (2015) Oxygen reactivity of mammalian sulfite oxidase provides a concept for the treatment of sulfite oxidase deficiency. *Biochem J* **469**, 211–221.
123 Shiva S (2010) Mitochondria as metabolizers and targets of nitrite. *Nitric Oxide* **22**, 64–74.
124 Nohl H, Staniek K, Sobhian B, Bahrami S, Redl H and Kozlov AV (2000) Mitochondria recycle nitrite back to the bioregulator nitric monoxide. *Acta Biochim Pol* **47**, 913–921.
125 Nohl H, Staniek K and Kozlov AV (2005) The existence and significance of a mitochondrial nitrite reductase. *Redox Rep* **10**, 281–286.
126 Iglesias DE, Bombicino SS, Valdez LB and Boveris A (2015) Nitric oxide interacts with mitochondrial complex III producing antimycin-like effects. *Free Radic Biol Med* **89**, 602–613.
127 Clementi E, Brown GC, Feelisch M and Moncada S (1998) Persistent inhibition of cell respiration by nitric oxide: crucial role of S-nitrosylation of mitochondrial complex I and protective action of glutathione. *Proc Natl Acad Sci USA* **95**, 7631–7636.
128 Sarti P, Arese M, Bacchi A, Barone MC, Forte E, Mastronicola D, Brunori M and Giuffrè A (2003) Nitric oxide and mitochondrial complex IV. *IUBMB Life* **55**, 605–611.
129 Burwell LS and Brookes PS (2008) Mitochondria as a target for the cardioprotective effects of nitric oxide in ischemia-reperfusion injury. *Antioxid Redox Signal* **10**, 579–600.