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REVIEW ARTICLE

A review and discussion of platelet nitric oxide and nitric oxide synthase: do blood platelets produce nitric oxide from L-arginine or nitrite?

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Abstract The NO/sGC/cGMP/PKG system is one of the most powerful mechanisms responsible for platelet inhibition. In numerous publications, expression of functional NO synthase (NOS) in human and mouse platelets has been reported. Constitutive and inducible NOS isoforms convert L-arginine to NO and L-citrulline. The importance of this pathway in platelets and in endothelial cells for the regulation of platelet function is discussed since decades. However, there are serious doubts in the literature concerning both expression and functionality of NOS in platelets. In this review, we aim to present and critically evaluate recent data concerning NOS expression and function in platelets, and to especially emphasise potential pitfalls of detection of NOS proteins and measurement of NOS activity. Prevailing analytical problems are probably the main sources of contradictory data on occurrence, activity and function of NOS in platelets. In this review we also address issues of how these problems can be resolved. NO donors including organic nitrites (RONO) and organic nitrate ($RONO_2$) are inhibitors of platelet activation. Endogenous inorganic nitrite (NO_2^-), the product of NO autoxidation, and exogenous inorganic nitrite are increasingly investigated as NO donors in the circulation. The role of platelets in the generation of NO from nitrite is also discussed.

Keywords sGC · cGMP · Nitric oxide · Mass spectrometry · Nitric oxide synthase · Pitfalls · Platelets

Abbreviations

CA	Carbonic anhydrase
DAF	Diaminofluorescein
DAN	Diaminonaphthalene
ECNICI	Electron-capture negative-ion chemical ionization
EI	Electron ionization
GC	Gas chromatography
sGC	Soluble guanylyl cyclase
cGMP	Cyclic guanosine monophosphate
IP	Immunoprecipitation
LOD	(Lower) limit of detection
LOQ	(Lower) limit of quantitation
MS	Mass spectrometry
NAT	Naphthotriazol
NO	Nitric oxide
NOS	Nitric oxide synthase
eNOS	Endothelial NOS
iNOS	Inducible NOS
nNOS	Neuronal NOS
PDE	Phosphodiesterase
PFB	Pentafluorobenzyl
PFB-Br	Pentafluorobenzyl bromide
PKG	cGMP-dependent protein kinase
RSD	Relative standard deviation
SIM	Selected-ion monitoring
VASP	Vasodilator stimulated phosphoprotein
WF/R	von Wildebrandt factor/ristocetin

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Platelets and NOS

Platelets are small anucleate cells derived from the cytoplasm of megakaryocytes in the bone marrow by the extension of cytoplasmic processes into microvessels, which are sheared from their trans-endothelial stems by flowing blood (Italiano et al. 2007; Machlus and Italiano 2013). Platelets play a key role in hemostasis through their ability to rapidly adhere to activated or injured endothelium and to sub-endothelial matrix proteins (platelet adhesion), and to other activated platelets (platelet aggregation) (Jurk 2015; Ruggeri 2007). The NO/sGC/cGMP/PKG system is one of the most powerful mechanisms responsible for platelet inhibition. NO donors are well known inhibitors of platelet activation (Lohmann and Walter 2005; Smolenski 2012; Walter and Gambaryan 2009).

NO is synthesized from L-arginine by the family of NO synthases (NOS), which includes endothelial (eNOS), neuronal (nNOS), and inducible (iNOS) isozymes (Davis et al. 2001; Daff 2010; Förstermann and Sessa 2012). By diffusion across the plasma membrane NO can exert its biological effects by several different mechanisms. Soluble guanylyl cyclase (sGC) which regulates platelet cGMP synthesis is one of the main receptors of NO (Friebe and Koesling 2003). cGMP effects in platelets are mediated predominantly by the cGMP-dependent protein kinase (PKG). Activation of PKG inhibits almost all agonist-induced events, including the increase of intracellular calcium levels (Geiger et al. 1994), integrin activation (Subramanian et al. 2013), cytoskeletal reorganization, and platelet granule secretion (reviewed in Smolenski 2012; Walter and Gambaryan 2009). In addition to the activation of sGC, NO and other NO metabolites such as *S*-nitrosothiols (Tsikas et al. 1999) exerts cGMP-independent functions, albeit at much higher concentrations than needed for sGC activation.

Numerous data from the literature reported on expression and function of two NOS isoforms, i.e., eNOS and iNOS, in platelets. However, the expression, regulation, and function of eNOS and iNOS in platelets are highly controversial. There are three main problems that underlie these controversies. The first problem relates to the detection of NOS protein or mRNA expression in platelets and regulation of NOS activity. The second problem is cGMP which is often used as a surrogate of NOS activity. Yet, measurement of cGMP is also associated with unusual analytical problems of commercially available cGMP assays (Fig. 1). Third, there are serious pitfalls in the measurement of NOS activity. These issues are outlined in detail as follows in the sections that follow.

NOS expression in platelets

In most of the reported studies, platelet NOS expression has not been reliably validated, for instance by including a positive control (e.g., endothelial cells) and negative control

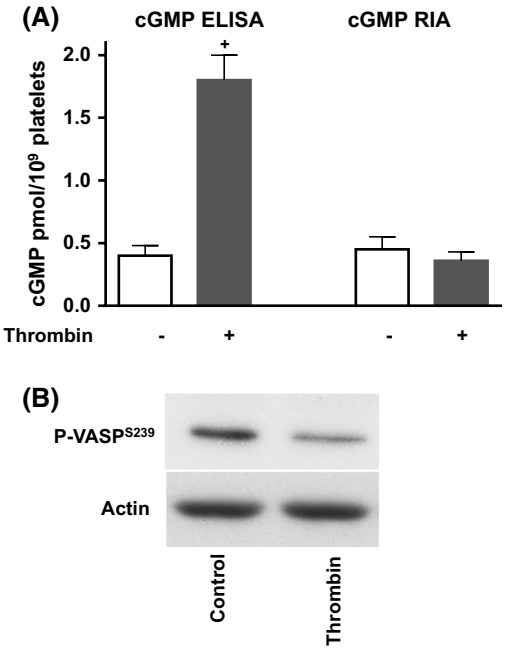

Fig. 1 Measurement of cGMP in platelets reveals diametrically opposite results and conclusions. Human platelets (1×10^9/mL) were stimulated with thrombin (0.01 U/mL, 1 min). Samples were collected for cGMP analysis by ELISA and RIA (**a**) and for VASP phosphorylation by Western blot analysis of (**b**). ELISA yielded unexpected more than a threefold increase of cGMP content, which was not reproduced by RIA. RIA data clearly correlate with VASP phosphorylation which decreases upon thrombin stimulation. Actin blot served as loading control. Data are mean ± SEM from three independent measurements; the symbol + means $P < 0.01$ compared to the control. Washed platelets were prepared from blood donated by three healthy untreated subjects after informed consent

(e.g., cells from NOS knock-out (KO) mice). The main problem with NOS protein expression detected by Western blot technique is connected with the specificity of the antibodies used. In 2008, we (Gambaryan et al. 2008) described this problem in detail and showed that many commercially available eNOS antibodies can recognise some proteins with similar molecular weight even in eNOS-KO mouse tissues and platelets. Additionally and more importantly, use of a phospho-eNOS^{S1177} antibody, a widely accepted indicator of activated eNOS, has detected increase of eNOS^{S1177} phosphorylation in thrombin-stimulated platelets isolated from eNOS-KO mouse (Gambaryan et al. 2008). This finding clearly indicates that the phospho-eNOS^{S1177} antibody recognizes at least one additional phosphoprotein which has a molecular weight similar to eNOS and is phosphorylated upon platelet activation. Therefore, before this selectivity-related problem is not resolved, this phospho-eNOS^{S1177} antibody should not be used for identification of eNOS activity in platelets and, probably, in other tissues as well. Misleadingly, phosphorylation of eNOS at Ser1177 is generally regarded as the main criterion of platelet NOS activation.

eNOS expression and activity in human platelets has also been reported by using two sequential affinity chromatography procedures, i.e., adenosine 2′,5′-bisphosphate (2′,5′-ADP)-Sepharose and calmodulin Sepharose 4B, and by the oxyhaemoglobin assay (Muruganandam and Mutus 1994). The K_M and V_{max} values for L-arginine and eNOS, respectively, obtained by this assay were determined to be 0.2 μM and 3.5 μmol NO per mg eNOS and per minute, respectively. The K_M value of 0.2 μM is one of the lowest reported, whereas the V_{max} value of 3.5 μmol NO per mg eNOS and per minute is about 18 times higher than that of an isolated recombinant nNOS (Tsikas et al. 2000b), about 1000 times higher than that of a recombinant iNOS and almost 1,000,000 times higher than the V_{max} value of a recombinant human eNOS (Böhmer et al. 2014b). In contrast, another group reported on the presence of eNOS in blood platelets of healthy humans with a V_{max} value of 0.122 pmol per mg eNOS per minute in homogenate of washed human platelets by using the L-citrulline assay (Kawamato et al. 2002). This inconsistency and the effects seen upon variation of the concentration of Ca^{2+}, for instance, inhibition of eNOS activity upon addition of 1.25 mM Ca^{2+} (Kawamato et al. 2002), argue against the presence of an active eNOS in the platelets used in those studies (see "Pitfalls with the measurement of NOS activity").

eNOS expression in human platelets was demonstrated by immunoprecipitation (IP), which is more sensitive than the Western blot technique, and by mass spectrometry (MS) analysis of precipitated proteins (Ji et al. 2007). IP in combination with the MS method is highly sensitive and, in this case, contamination with other blood cells in platelet preparation is likely to have been the source of the detected eNOS. Recently, in a carefully and thoroughly performed MS-based proteomics study on highly purified human platelets, we described more than 4000 proteins, yet not a single peptide was detected, which would correspond to an NOS protein (Burkhart et al. 2012, 2014).

Contamination with other blood cells in platelet preparations is also a main reason for inconsistencies in the literature regarding eNOS/iNOS mRNA expression. mRNA of eNOS and iNOS was reported to be expressed in washed platelet preparations (Mehta et al. 1995; Chen and Mehta 1996). Interestingly, in pulmonary hypertension patients in comparison to normal controls eNOS protein expression was down regulated, whereas eNOS mRNA was not detectable in platelets from both groups (Aytekin et al. 2012). We compared conventionally prepared washed platelets with our original method which yields highly purified platelets and which do not contain any other blood cells (Birschmann et al. 2008). The comparison clearly demonstrated that neither eNOS mRNA nor iNOS mRNA are detectable by RT-PCR in highly purified platelets (Gambaryan et al. 2008). It is worth mentioning, that in genome-wide RNA-seq analysis of human and mouse platelet transcriptomes no NOS mRNA transcripts were found (Rowley et al. 2001, http://www.plateletomics.com).

Regulation of NOS activity

Shortly after the discovery of the L-arginine/NOS/NO pathway, Moncada's group reported in 1990 (Radomski et al. 1990a, b) that NOS activity is present in agonists-stimulated platelets. Yet, Vane´s group reported in 1989 that other contaminating blood cells may falsely contribute to NOS activity in humans (Salvemini et al. 1989). Subsequently, numerous groups reported on platelet NOS activation in various conditions. In 2005, however, Özüyaman's group (Özuyaman et al. 2005) questioned NOS expression in mouse platelets. Later, we (Gambaryan et al. 2008; Böhmer et al. 2013, 2014a, b; Tsikas and Gambaryan 2014; Gambaryan and Tsikas 2015) and the group of Emerson (Tymvios et al. 2009) presented experimental data supporting the lack of functional NOS proteins in human and mouse platelets. Unfortunately, previous and recent reports on the lack of NOS expression and the potential interference by contaminating NOS-expressing blood cells are rarely considered in the current research on platelet NOS.

NOS activity is regulated by elevation of intracellular calcium, phosphorylation at Ser^{633}, Ser^{1177}, and Thr^{495}, as well as by the interaction with other proteins like caveolin, Hsp70, and Hsp90 (Butt et al. 2000; Boo et al. 2002). All these and other mechanisms of NOS activation have been described for platelets as well. Activation of platelet eNOS by the β_2-adrenoceptor (Queen et al. 2000), insulin (Fleming et al. 2003), and acetylsalicylic acid (O'Kane et al. 2009) was reported to be calcium-independent and phosphorylation-dependent. β_2-Adrenoceptors- and adenosine-dependent (Anfossi et al. 2002a, b, c; Russo et al. 2004) platelet NOS activation is mediated by the cAMP/protein kinase A (PKA) pathway. Insulin acts through AMP kinase and the PKB pathway (Fleming et al. 2003; Trovati et al. 1996), whereas acetylsalicylic acid acts via NOS protein acetylation (O'Kane et al. 2009). Other investigators demonstrated calcium-dependent platelet eNOS activation (Radomski et al. 1990a, b), as well as eNOS stimulation by tyrosine de-phosphorylation (Patel et al. 2006). Many other substances such as estradiol (Jana et al. 2014; Wu et al. 2010), alpha-tocopherol (Freedman et al. 2000), dehydroepiandrosterone (Munoz et al. 2012), pyridoxine (Wu et al. 2010), the endocannabinoids 2-arachidonoylglycerol and anandamide (Signorello et al. 2011a, b), homocysteine (Signorello et al. 2009), glucose (Massucco et al. 2005), lipopolysaccharide (Zhang et al. 2009), and andrographolide, an NF-kappa B inhibitor (Lu et al. 2011) have also been described to enhance platelet NOS activity.

Interestingly, the same authors (Massucco et al. 2005) reported that high glucose rapidly activated platelet NOS, but that the same high glucose treatment inhibited aspirin-induced NOS activation (Russo et al. 2012). Regulation of NOS activity by globular β-actin has been described (Ji et al. 2007), but this study is compromised by many inaccurate results especially related to demonstration of NOS expression by IP. Similarly, activation of platelet NOS by integrin αIIb/β3 inhibition has been reported (Chakrabarti et al. 2004); however the authors showed in their study that expression of eNOS in platelets after 15 min of αIIb/β3 inhibition increased at least five times.

Alterations of eNOS activity have also been described in several clinical studies. In most of these papers platelet NOS expression and activity was shown to be down-regulated in arterial hypertension (Gkaliagkousi et al. 2009; Moss et al. 2010), in hypertension associated with hypercholesterolemia (Alexandru et al. 2011), in pulmonary arterial hypertension (Aytekin et al. 2012), in advanced Huntington disease (Carrizzo et al. 2014), and in heart failure (Shah et al. 2011). However, in coronary artery disease (Garelnabi et al. 2014) and acute coronary syndromes (Bergandi et al. 2010), which are also associated with endothelial dysfunction, platelet NOS activity was shown to be up-regulated. In the above mentioned papers, NOS activity was assessed by Western blot detection of eNOS, $eNOS^{S1177}$ phosphorylation and/or cGMP determination. The discrepancies among these reports are therefore likely to be due to the issues discussed above.

cGMP assays

Measurement of cGMP is one of the common methods to detect NOS activity. Here, we present data that may explain false NOS activation in stimulated platelets previously described in many publications. An increase of cGMP was detected in thrombin- and fatty acid-stimulated platelets already in 1977 (Glass et al. 1977a, b). More recently, it was shown that almost all platelet agonists, including thrombin, collagen, thromboxane, ADP (Gkaliagkousi et al. 2007; Naseem 2008; Naseem and Riba 2008), and von Wildebrandt Factor/ristocetin (VWF/R) can also increase platelet cGMP concentration (Gambaryan et al. 2004; Marshall et al. 2004; Li et al. 2003). Increase of intracellular cGMP may be mediated by the activity both of sGC and particulate guanylyl cyclase (pGC). However, platelets do not contain any pGC (Gambaryan et al. 2013). Eventually, the cGMP content in platelets is regulated by its synthesis rate by sGC and its degradation rate by phosphodiesterases (PDE).

We tested several commercially available ELISA kits for cGMP from different suppliers and found that in some cases stimulation of platelets with thrombin gave a false increase of cGMP. This was especially the case, when platelets were suspended in HEPES buffer which is the most commonly used buffer for platelet preparation, and when the reaction was stopped by ethanol. We compared the data obtained by an ELISA kit with those obtained by using a commercially available cGMP RIA assay, where the reaction is terminated by trichloroacetic acid. In contrast to the cGMP ELISA approach, we detected only small decreases of cGMP by means of the RIA method, which correlated with a decrease of the phosphorylation on the PKG preferential site (Ser^{239}) of the vasodilator stimulated phosphoprotein (VASP) (Fig. 1). The reasons for such differences between cGMP ELISA and RIA data are not yet known. In thrombin-stimulated platelets, cGMP was not increased in contrat to VASP phosphorylation which seems to be more sensitive than cGMP as indicated by the decrease of phospho-VASP in Western blot (Fig. 1b).

Some other possible mechanisms might be the reason of NO/NOS-independent increase of platelet cGMP induced by various substances. One explanation may be interference by contaminating substances which may release NO and/or activate sGC. For example, azide (N_3^-), which is a common preservative of various samples, stock solutions, and buffers of commercially available assays for cGMP and other biomarkers, is a well-known strong activator of sGC (Russo et al. 2008). Further, NO-independent regulation of sGC activity may be mediated by protein–protein interaction of sGC with Hsp70, Hsp90, and PSD95, as well as by Ser/Thr and/or Tyr phosphorylation of sGC (Balashova et al. 2005; Meurer et al. 2005). Also, several compounds including thrombin inhibitors (Kobsar et al. 2012), gemfibrosil (Sharina et al. 2014), VWF/R (Naseem and Riba 2008; Gambaryan et al. 2004; Marshall et al. 2004; Li et al. 2003) can moderately and NO-independently increase sGC activation and thus cGMP concentration. Also, apparent increase of platelet cGMP might be the reason of phosphodiesterase (PDE) inhibition, without any sGC activation. In platelets, PDE5 is the major PDE isoform responsible for cGMP hydrolysis (Haslam et al. 1999; Schwarz et al. 2001), and inhibition of PDE5 activity results in increase of platelet cGMP (Apostoli et al. 2014). Therefore, increase of platelet cGMP per se, even if cGMP measurement is specific, without detailed analysis of the underlying mechanisms, is not an unequivocal evidence for NO formation by intra-platelet NOS.

Pitfalls with the measurement of NOS activity

NOS (EC 1.14.13.39) catalyses the oxidation of one of the terminal nitrogen atoms of the guanidine group of L-arginine (1) to produce L-citrulline (2) and NO (3). Each one

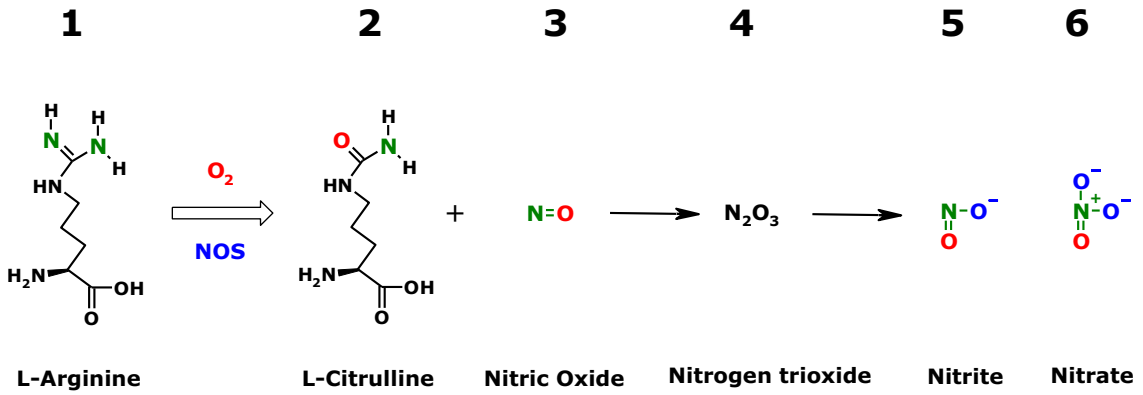

Fig. 2 Schematic and simplified presentation of the L-arginine/NOS/NO/L-citrulline pathway and of the NO reaction products N_2O_3, nitrite and nitrate

O atom from the co-substrate molecular oxygen (O_2) is incorporated into NO and the ureide group of L-citrulline (Tsikas 2005a, b) (Fig. 2). NO is further oxidized to nitrite (NO_2^-) (5) and nitrate (NO_3^-) (6), presumably via intermediate formation of nitrogen trioxide (N_2O_3) (4). Formation of nitrite from NO is likely to occur by autoxidation of NO and hydrolysis of intermediate(s) to nitrite (see reactions R1a, R1b). Being a heme protein, NOS (NOS–FeO_2) is able to oxidize its own NO to NO_3^- (see reaction R2) (Böhmer et al. 2014a), presumably by a reaction resembling the oxidation of NO to NO_3^- by oxyhaemoglobin. Thus, in incubation mixtures of recombinant NOS isoforms, nitrite and nitrate are always detected, albeit in different molar ratios, commonly 1:1 or higher.

$$4\ NO + O_2 \rightarrow 2\ ON{-}O{-}NO \qquad \text{(R1a)}$$

$$2\ ON{-}O{-}NO + 2\ H_2O \rightarrow 2\ ONO^- + 2\ ONO^- + 4\ H^+ \qquad \text{(R1b)}$$

$$NOS{-}Fe^{(2+)}O_2 + NO \rightarrow NOS{-}Fe^{(3+)} + NO_3^- \qquad \text{(R2)}$$

In theory, NOS activity can be determined by measuring the decrease of the concentration of its substrate L-arginine, the increase of its primary reaction products L-citrulline and NO, and/or the increase of the reaction products of NO, N_2O_3, NO_2^- and NO_3^- (Fig. 2). In practice, however, the number of suitable assays to accurately measure NOS activity is strongly limited. As platelets and erythrocytes have been reported to express endothelial-like NOS, we focus our review and discussion in this article on the measurement of eNOS activity.

Assessment of eNOS activity by measuring L-arginine or L-citrulline

Compared to nNOS and iNOS, the activity of eNOS is very low. Even for purified recombinant eNOS and in the presence of optimal concentrations of its substrate L-arginine (e.g., 20 μM), its cofactors and prosthetic groups, eNOS activity is of the order of 150 pmol NO or L-citrulline per mg eNOS and min (Böhmer et al. 2014b; Tsikas 2015a). In incubation mixtures of recombinant eNOS the concentration of L-citrulline, nitrate and nitrite are below 1000 nM (Böhmer et al. 2014a, b; Tsikas 2015a). Assessment of eNOS activity by measuring the concentration of L-arginine is therefore practically impossible. One of the most commonly used NOS activity assays is based on the measurement of L-citrulline, the so called citrulline assay (Marletta 1993). However, the citrulline assay is associated with severe shortcomings when used for eNOS in vivo and in vitro in cell systems (Fig. 3). This is because much less than 0.1 % of L-arginine is converted by eNOS to L-citrulline and NO (Hibbs et al. 1992), while a major fraction of L-arginine is converted to L-citrulline via the urea cycle. Furthermore, L-citrulline is produced from asymmetric dimethylarginine (ADMA) by the catalytic action of dimethylarginine dimethylaminohydrolase (DDAH) isoforms 1 and 2 in liver, kidney and other organs at rates by far higher than those of the eNOS activity (Leiper and Vallance 2006). DDAH2 has been identified in platelets of healthy humans by mass spectrometry-based proteomics (Burkhart et al. 2012). It is worth mentioning that enzymes of the urea cycle such as arginase are physiologically present in blood platelets (Villanueva and Giret 1980). Thus, even if platelets are poor in pathways that contribute to L-citrulline from L-arginine, ADMA and possibly from other sources when compared with other types of cells, the likelihood for measuring L-citrulline from pathways others than the L-arginine/NOS pathway is very high and is likely to have occurred in the past.

The difficulties associated with the measurement of L-citrulline formation as a surrogate of eNOS activity in biological samples such as intact or lysed platelets and even in immuno-precipitates can only partly be overcome

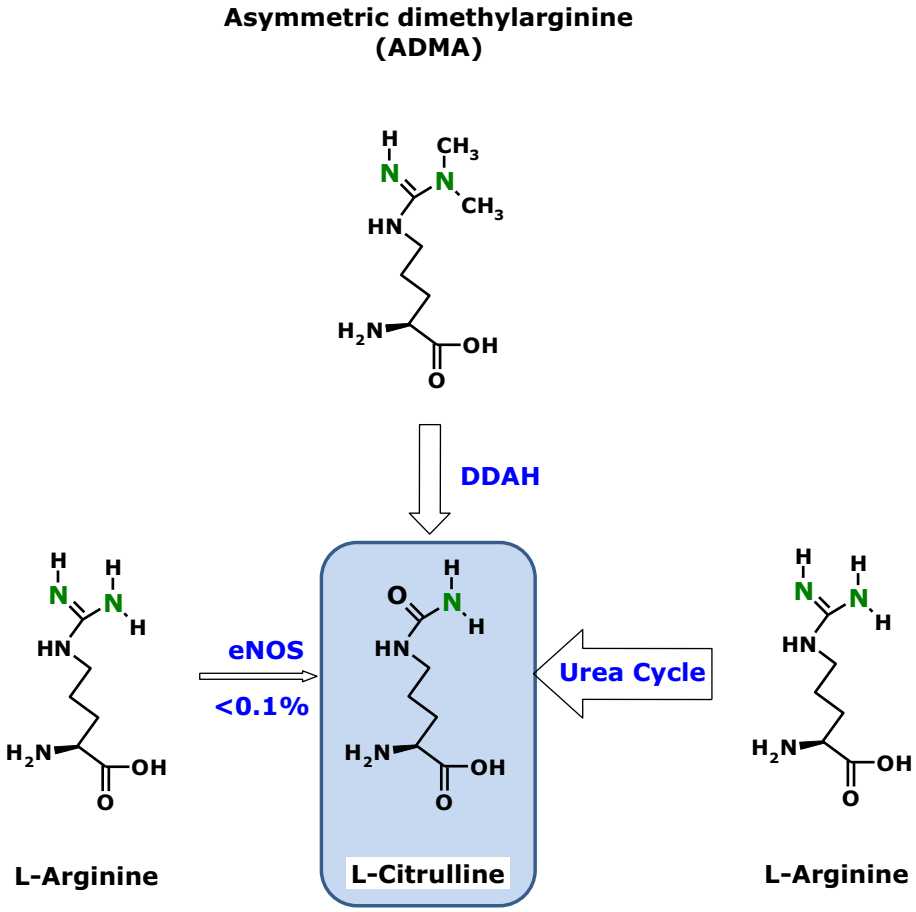

Fig. 3 Contribution of three possible pathways to L-citrulline in platelets. The thickness of the arrows is a rough measure of the extent of contribution of the individual pathways

by using radio-labelled or stable-isotope labelled L-arginine as the substrate, e.g., L-arginine labelled with ^{3}H, ^{14}C, or ^{15}N. Usually, radio-labelled L-arginine is contaminated with radio-labelled L-citrulline and many other metabolites including polyamines and unknown contaminants that may include tritiated water (Barilli et al. 2012). Complete chromatographic separation of labelled L-citrulline from the very high excess of labelled L-arginine seems to be almost impossible (Böhmer et al. 2014a). In case of simply counting radioactivity, which is the most widely used method to measure NOS activity, without preceding adequate chromatographic separation of L-citrulline from L-arginine, for instance by high-performance liquid chromatography or thin-layer chromatography, eNOS activity will be highly overestimated. Moreover, counting unspecific radioactivity will even simulate eNOS activity. Measurement of ^{15}N-labelled L-citrulline requires use of mass spectrometry-based methods such as LC–MS/MS, which nevertheless is problematic due to incomplete separation of ^{15}N-labelled L-citrulline from ^{15}N-labelled L-arginine (Böhmer et al. 2014a).

In this context, of particular interest is the finding that the L-citrulline NOS assay indicated NOS activity in the supernatant but not in the pellet of red blood cells of healthy subjects (Kang et al. 2000). Interestingly, radioactivity was not suppressible by NOS inhibitors, but was suppressed by norvaline, an inhibitor of arginase activity with no known effect on NOS. Arginase activity was not inhibited by NOS inhibitors, but was inhibited by norvaline. Eventually, thin-layer chromatography revealed that the isolated radioactivity was tritiated L-ornithine, the product of arginase activity. It is also of interest, that this group detected eNOS and iNOS proteins by immunoblot (Kang et al. 2000). Our interpretation of these findings is that the monoclonal antibodies to eNOS and iNOS used in that study were not specific and falsely indicated presence of eNOS/iNOS in red blood cells, rather than that these enzymes were inactive.

Assessment of eNOS activity by measuring NO or higher oxides of nitrogen such as N_2O_3

Given the high reactivity of NO and of other higher oxides of nitrogen including N_2O_3, assessment of eNOS activity by measuring NO and for example N_2O_3 is challenging.

NO-specific electrodes have been used by many groups to measure NO in biological samples including stimulated platelets (Freedman et al. 1997). From own experience

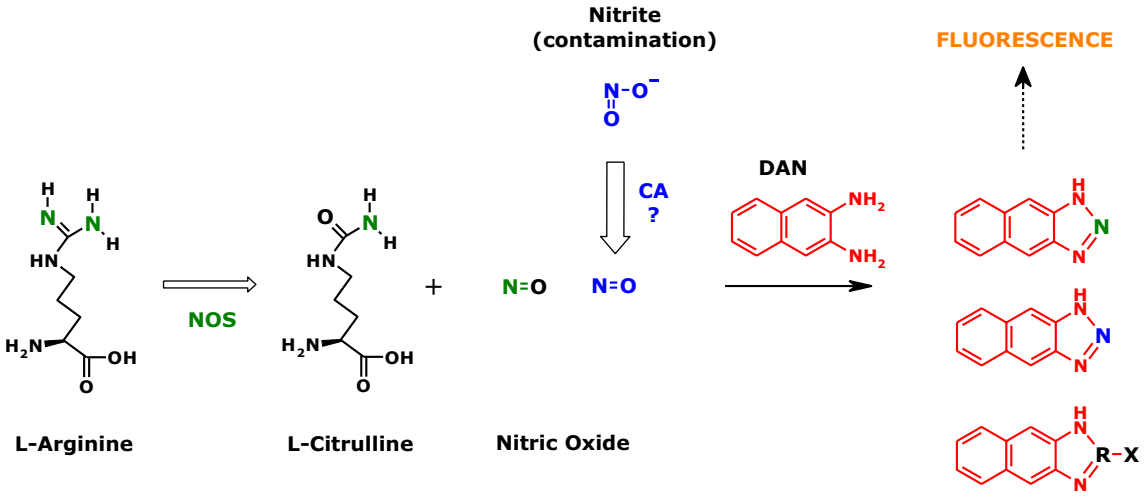

Fig. 4 Contribution of the L-arginine/NOS pathway to NO, of the CA-catalyzed conversion of nitrite to NO, and of other unknown proteins/enzymes (indicated by the symbol *question mark*) able to reduce/convert inorganic nitrite to NO. NO from these pathways and other substances (RX; e.g., Se, MDA, ascorbic acid) may react with DAN to form fluorescent derivatives. Other reagents such as DAF and its derivatives behave analogous to DAN towards NO ad RX

(Tsikas et al. 1999, 2011; Tsikas 2005b) measurement of NO by means of commercially available NO-specific electrodes is very challenging for several reasons. They include poor sensitivity, accuracy and precision, as well as multiple interferences such as proteins (Tsikas et al. 2011). Furthermore, detection of NO by NO-specific electrodes does not provide information about the origin of NO, which may include *S*-nitrosothiols on the outer surface of the platelets. In addition, it is worth mentioning that even in aqueous buffered solutions of purified nNOS (e.g., at 5 µg) and all of its cofactors at optimal concentrations, NO cannot be detected by Clark-type NO-specific electrodes in the absence of externally added superoxide dismutase (Mayer et al. 1995).

NO itself can also be measured by using highly purified oxyhaemoglobin ($HbFe^{2+}O_2$). By this assay, eNOS activity is assessed by measuring spectrophotometrically methemoglobin ($HbFe^{3+}$) formation (see reaction R3). Thus, oxyhaemoglobin has to be used in great excess over eNOS in order to oxidise all NO molecules generated by eNOS. Potential shortcomings of this assay are multiple endogenous and exogenous redox-active substances which are able to oxidise oxyhaemoglobin to methemoglobin in biological samples such as lysed platelets.

$$HbFe^{(2+)}O_2 + NO \rightarrow HbFe^{(3+)} + NO_3^- \quad \text{(R3)}$$

eNOS activity is increasingly measured by using diamino-containing fluorogenic substances which may react with N_2O_3 and other higher oxides of nitrogen to form cyclic fluorescent derivatives (Fig. 4). Examples for this type of substances are 2,3-diaminonaphthalene (DAN) and diamino-fluorescein (DAF) and its derivatives. Such reagents provide high analytical sensitivity which enables DAN- and DAF-based assays for measuring eNOS activity. Indeed, such assays are very popular and widely used, yet mostly to "visualize" rather than to measure eNOS activity (Cortese-Krott et al. 2012; Cortese-Krott and Kelm 2014; Momi et al. 2014; Cozzi et al. 2014). However, the use of DAN, DAF and DAF-like substances to assess eNOS activity is also associated with major analytical difficulties, notably lack of selectivity in biological samples (Planchet and Kaiser 2006; Rümer et al. 2012; Uhlenhut and Högger 2012). It is well-known that many species different from NO and N_2O_3, such as malondialdehyde (MDA) and even selenium (Cukor and Lott 1965), can react with these reagents to produce fluorescent species (Tsikas et al. 2011). It is worth mentioning that MDA is an endogenous compound that is present in nM-to-µM concentrations in biological samples. Ascorbic acid and dehydroascorbic acid have been also shown to interfere with the analysis of NO by 4,5-diaminofluorescein (DAF-2) (Zhang et al. 2002). In summary, use of DAN- and DAF-based assays in a batch-fashion, i.e., without any chromatographic separation, will overestimate and even simulate eNOS activity.

Another potential interference in DAN- and DAF-based assays is that NO/N_2O_3 may arise from nitrite which is not formed from L-arginine/NOS during the eNOS assays. Even if DAN- and DAF-based assays involve chromatographic separation of derivatives of DAN or DAF-reagents with NO/N_2O_3 from other derivatives not arising from NO such as with MDA, the following interference will be not eliminated: Nitrite is a ubiquitous contaminating species. In the presence of isoforms of carbonic anhydrase (CA) which are also ubiquitous and abundant in biological samples,

nitrite can be converted to NO-species (Aamand et al. 2009), which in turn generate fluorescence thus simulating eNOS activity (Fig. 4). It is worth mentioning that blood platelets of healthy humans were found to physiologically express active CA isoforms (Belloni and Turpini 1957; Siffert and Gros 1984; Siffert et al. 1984a, b; Ritter et al. 1990; Gende 2005). Whether other proteins and enzymes present in red blood cells and being able to reduce nitrite to NO are also present in human platelets is unknown (Fig. 4).

Assessment of eNOS activity by mass spectrometry-based assays

Lack of specificity of eNOS assays due to the problems and pitfalls that were discussed above can be overcome under certain conditions by mass spectrometry (MS) coupled with gas chromatography (GC) or liquid chromatography (LC), such as GC–MS and LC–MS/MS, respectively. As NO, N_2O_3 and other possible oxides of NO (N_xO_y) are short-lived and poor analytes for GC–MS and LC–MS/MS, their stable oxidation products nitrite and nitrate are by far easier accessible for quantitative analysis. Thus far, native nitrite and nitrate cannot be sensitively analyzed by LC–MS/MS. Therefore, sensitive quantitative determination of nitrite and nitrate requires derivatization both in GC–MS and in LC–MS/MS.

We have developed a highly sensitive and specific GC–MS method for the simultaneous measurement of nitrite and nitrate in many different biological samples (Tsikas et al. 2000b) and for the measurement of the activity of all NOS isoforms in vitro and in vivo (Tsikas et al. 2000b; Tsikas 2004). The concentration of contaminating nitrite and nitrate may exceed by far the concentration of nitrite and nitrate which are formed from NO by the catalytic action of eNOS on L-arginine. This problem can be overcome by measuring stable-isotope labelled nitrite and nitrate, i.e., nitrite and nitrate labelled with ^{15}N, ^{18}O or with both. As ^{18}O is exchangeable in nitrite and nitrate to a considerable degree, measuring of ^{15}N-labelled nitrite and nitrate is the best solution. The nitrogen (N) atom occurs in nature in the form of two stable isotopes, i.e., ^{14}N with an abundance of 99.63 %, and ^{15}N with an abundance of 0.37 %. These numbers mean that, in the presence of example of nitrite and nitrate at a concentration of 1000 nM each, for instance in incubation mixtures in which eNOS activity has to be measured, each about 996 nM will be ^{14}N-nitrite and ^{14}N-nitrate, whereas the remaining 4 nM will be ^{15}N-nitrite and ^{15}N-nitrate. Thus, the molar ratio $^{15}N/^{14}N$ will be theoretically each about 0.004 for nitrite and nitrate. This ratio will not change, if eNOS activity will produce unlabelled NO from unlabelled L-arginine. Because measurement of the $^{15}N/^{14}N$ ratio for nitrite and nitrate is indispensable for quantitative analysis, ^{15}N-labelled L-arginine must be added to samples to be tested for eNOS activity (Fig. 5).

Commercially available are since several decades differently ^{15}N-labelled L-arginine analogs. The specificity of the eNOS activity assay is greatly enhanced by using as substrate L-arginine labelled with ^{15}N in the guanidine (N^G) group, because it cannot be excluded that the other amino groups including the α-amino group of L-arginine could also be oxidized to finally generate nitrite and nitrate, even if such an oxidation is expected to be of low extent. Although nitrite and nitrate can be sensitively detected by methods based on other principles such as chemiluminescence, it must be emphasized that ^{14}N and ^{15}N can only be discriminated by mass spectrometry. eNOS-catalysed formation of ^{15}NO from ^{15}N-labelled L-arginine, for instance from L-[guanidine-$^{15}N_2$]-arginine, will be oxidized to ^{15}N-nitrite ($^{15}NO_2^-$) and ^{15}N-nitrate ($^{15}NO_3^-$) (Fig. 5a). Because the concentration of contaminating ^{14}N-nitrite ($^{14}NO_2^-$) and ^{14}N-nitrate ($^{14}NO_3^-$) will not change during the eNOS assay, $^{15}NO_2^-$ and $^{15}NO_3^-$ derived from ^{15}NO formed L-[guanidine-$^{15}N_2$]-arginine will add to the contaminating $^{15}NO_2^-$ and $^{15}NO_3^-$ and will increase the total concentration of $^{15}NO_2^-$ and $^{15}NO_3^-$, for example from about 4 to 400 nM, unequivocally indicating eNOS activity (Fig. 6). Provided the concentration of contaminating nitrite and nitrate is constant during the eNOS assay, the concentration of $^{15}NO_2^-$ and $^{15}NO_3^-$ derived from ^{15}NO formed L-[guanidine-$^{15}N_2$]-arginine can be calculated and used for the calculation of the eNOS activity (Tsikas et al. 2000b). As L-arginine is ubiquitous in biological samples as well, endogenous (unlabelled) L-arginine will also be converted by eNOS to (unlabelled) nitrite and nitrate. This potential shortcoming can be overcome by using externally added L-[guanidine-$^{15}N_2$]-arginine at high molar excess over endogenous (unlabelled) L-arginine (Böhmer et al. 2012, 2014a, b).

At present, there is only one derivatization reaction that can be utilized for the simultaneous derivatization of nitrite and nitrate and their simultaneous analysis by mass spectrometry. In this reaction, pentafluorobenzyl bromide (PFB-Br) is used as the derivatization reagent, and the reaction is performed preferably in acetone as the organic solvent which is miscible with water. The major advantage of using acetone as the solvent in this method is its low boiling point which enables its rapid removal after the derivatization. The reaction of nitrite and nitrate with PFB-Br is a nucleophilic substitution of bromide by nitrite and nitrate (see reactions R4 and R5; Fig. 5b). In case of GC–MS analysis of the pentafluorobenzyl (PFB) derivatives PFB-NO_2 (from nitrite) and PFB-ONO_2 (from nitrate), acetone is quickly removed under a stream of nitrogen and the PFB derivatives are rapidly and quantitatively extracted with toluene of which 1-μL aliquots are injected into the apparatus. GC–MS analysis in the electron-capture negative-ion chemical ionization (ECNICI) mode yields the anions nitrite ($^{14}NO_2^-$, *m/z* 46;

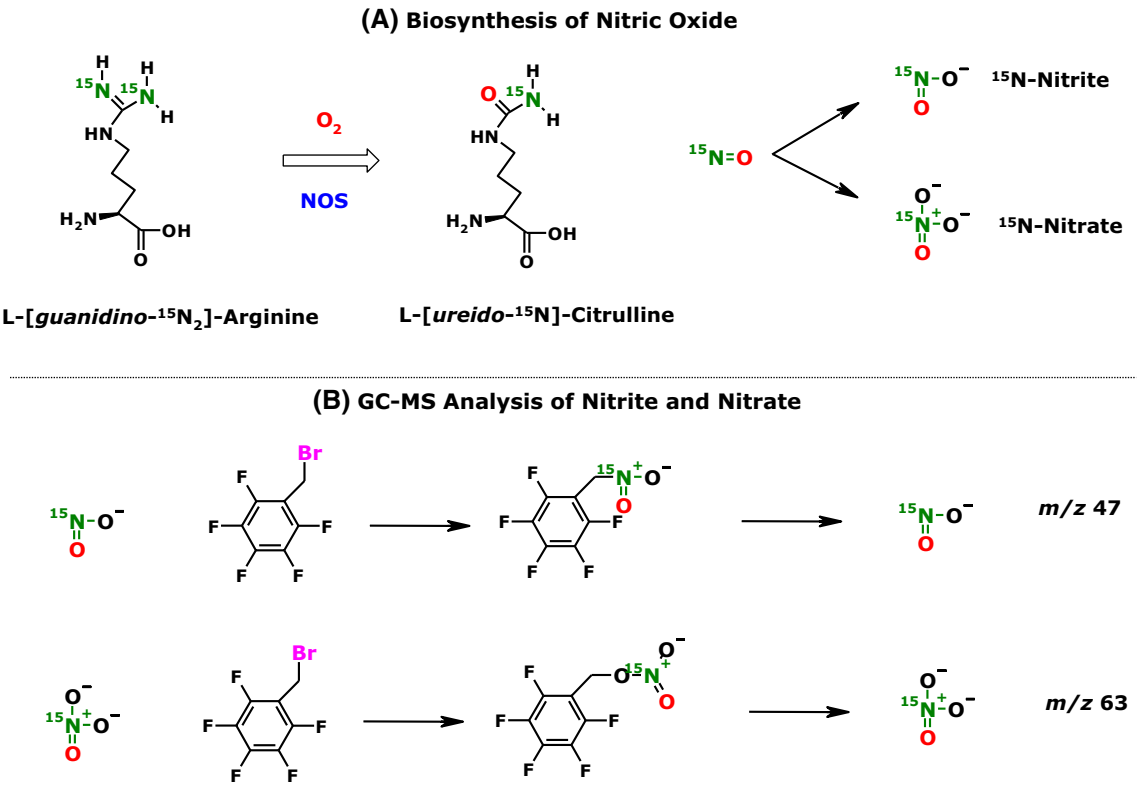

Fig. 5 Schematic of a GC–MS assay based **a** on the use of L-[guanidine-$^{15}N_2$]-arginine as the substrate for NOS, and **b** on the derivatization of [^{15}N]nitrite and [^{15}N]nitrate with pentafluorobenzyl bromide

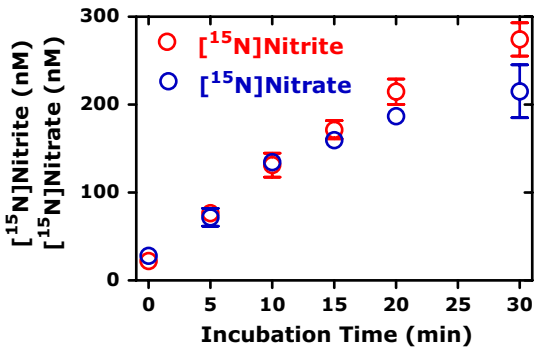

Fig. 6 Formation of [^{15}N]nitrite and [^{15}N]nitrate upon incubation of L-[guanidine-$^{15}N_2$]-arginine (20 μM) with a recombinant eNOS in potassium phosphate buffer of pH 7.4 at 37 °C together with all cofactors and prosthetic groups. Data are shown as mean ± standard deviation of four independent experiments. This figure was re-constructed using previously reported data (Tsikas 2015a)

$^{15}NO_2^-$, *m/z* 47) and nitrate ($^{14}NO_3^-$, *m/z* 62; $^{15}NO_3^-$, *m/z* 63). These anions are then separated mass spectrometrically. Quantification of nitrite and nitrate is performed by selected-ion monitoring (SIM) of *m/z* 46, *m/z* 47, *m/z* 62, and *m/z* 63 (Tsikas et al. 2000a, b; Tsikas 2004).

$$PFB{-}Br + NO_2^- \rightarrow PFB{-}NO_2 + Br^- \quad (R4)$$

$$PFB{-}Br + ONO_2^- \rightarrow PFB{-}ONO_2 + Br^- \quad (R5)$$

Alternatively, nitrite, but not nitrate, can be analyzed by GC–MS, LC–MS/MS or HPLC (Jobgen et al. 2007) after derivatization with DAN under acidic conditions. The reaction of nitrite with DAN is a cyclic (intra-molecular) diazotization reaction which produces the fluorescent 2,3-naphthotriazol (NAT). In the ECNICI mode, the NAT derivative of nitrite ionizes to form the most intense ions at *m/z* 169 [$(C_{10}H_7^{14}N_3)^-$] for $^{14}NO_2^-$ and *m/z* 170 ([$C_{10}H_7^{14}N_2^{15}N]^-$) for $^{15}NO_2^-$. In the electron ionization (EI) mode, the NAT derivative of nitrite ionizes to form intense ions at *m/z* 169 ([$C_{10}H_7^{14}N_3]^-$), *m/z* 141 ([$C_{10}H_7^{14}N]^-$) and *m/z* 114 ([$C_9H_6]^-$) for $^{14}NO_2^-$, and *m/z* 170 ([$C_{10}H_7^{14}N_2^{15}N]^-$), *m/z* 141 ([$C_{10}H_7^{14}N]^-$) and *m/z* 114 ([$C_9H_6]^-$) for $^{15}NO_2^-$ (Tsikas et al. 2011). Thus, quantification of nitrite is performed by SIM of *m/z* 169 and *m/z* 170 in both ionization modes. Nitrate cannot be analyzed by this method because under moderately acidic conditions nitrate does not react with DAN to form NAT. Furthermore, the GC–MS analysis of nitrite as NAT derivative is compromised both by poor chromatography (i.e., broad tailing peaks) and disadvantageous mass spectrometry behaviour for the following reason. Unlike nitrite (NO_2^-) and nitrate (NO_3^-) which are detected in our GC–MS assay, the NAT

derivative of nitrite comprises 10 carbon atoms ions (i.e., $C_{10}H_7N_3$) which consist of the stable isotopes ^{12}C (natural abundance, 98.89 %) and ^{13}C (natural abundance, 1.11 %). Therefore, when L-[guanidine-$^{15}N_2$]-arginine-derived ^{15}NO would be analyzed as NAT derivative, i.e., as the ion *m/z* 169 [$(C_{10}H_7^{14}N_3)^-$], the ^{13}C isotope of the NAT derivative of unlabelled nitrite would contribute by about 11 % to the ion at *m/z* 170 [$(C_{10}H_7^{14}N_2^{15}N)^-$] of the ^{15}N-labelled nitrite. For comparison, the contribution of the ^{15}N (0.37 %) and ^{17}O (0.04 %) isotopes of unlabelled nitrite and nitrate to ^{15}N-labelled nitrite and nitrate is not higher than about 0.5 %. Therefore, analysis of nitrite and nitrate as PFB derivatives has a much lower limit of quantitation (LOQ) and a larger linear dynamic range in addition to the much lower limit of detection (LOD), the latter due to the high ionization yield of PFB-NO_2 and PFB-ONO_2 in the ECN-ICI mode. The "sensitivity" of the analysis of $^{15}NO_2^-$ as PFB derivative is approximately 22 times higher than its analysis as NAT derivative. As a numerical explanation for the high "sensitivity" or "discrimination power" of the GC–MS analysis of nitrite and nitrate as PFB derivatives, we mention the following example. A urine sample with a nitrate concentration of 400 μM contains about 398 μM $^{14}NO_3^-$ and 2.24 μM $^{15}NO_3^-$. Addition of 1 μM $^{15}NO_3^-$ to this urine sample, which is about 0.25 % of the basal nitrate concentration and about 50 % of the basal $^{15}NO_3^-$ concentration of 2.24 μM, can be measured with high precision (relative standard deviation (RSD), 1.5 %) and high accuracy (recovery, 103 %) (Tsikas 2009). The LOD of this method is 20 amol of $^{15}NO_2^-$ and was determined with RSD of 4.8 % and a signal-to-noise ratio of 20:1 (Tsikas et al. 2000b). The LOQ of the method in phosphate buffer with an average nitrite concentration of 280 nM is 4 nM $^{15}NO_2^-$ and was determined with a mean recovery of 93 % and a mean precision (RSD) of 5.4 % (Tsikas et al. 2000b).

By means of the GC–MS method that measures simultaneously $^{14}NO_2^-$, $^{15}NO_2^-$, $^{14}NO_3^-$, and $^{15}NO_3^-$ we measured specifically and sensitively the activity of eNOS (e.g., Thum et al. 2003), nNOS and iNOS in vitro and in vivo, in animals and in humans (Tsikas 2005a, 2008). In platelets isolated from blood of healthy humans we did not find any NOS activity (Böhmer et al. 2014a, b). In red blood cells isolated from blood of healthy humans we also did not find any NOS activity (Böhmer et al. 2012). However, addition of recombinant NOS isoforms to intact and lysed blood platelets and red blood cells and measurement of $^{15}NO_2^-$ in platelets and $^{15}NO_3^-$ in red blood cells indicated NOS activity. This observation strongly suggests that presence of physiological and active NOS in these preparations would have yielded formation of ^{15}NO from the externally added L-[guanidine-$^{15}N_2$]-arginine which can penetrate both the platelet membrane (Böger et al. 2004) and the erythrocyte membrane (Böhmer et al. 2012). In contrast to us, other groups have detected NOS proteins and NOS activity in red blood cells of healthy and ill subjects (Kleinbongard et al. 2006; Cortese-Krott et al. 2012) and of mice (Wood et al. 2013).

Inorganic nitrite and platelets

We have previously shown that incubation of platelet-rich human plasma with *S*-[^{15}N]nitroso-L-cysteine (L-S^{15}NC) or *S*-[^{15}N]nitroso-D-cysteine (D-S^{15}NC) but not with [^{15}N]nitrite or *S*-[^{15}N]nitrosoglutathione (GS^{15}NO) resulted in accumulation of [^{15}N]nitrite in the platelets (Tsikas et al. 1999). As L-S^{15}NC and D-S^{15}NC are potent ^{15}NO donors, while GS^{15}NO and [^{15}N]nitrite are not and they cannot penetrate the platelet membrane (Tsikas et al. 1999), this observation suggests that accumulation of [^{15}N]nitrite in the platelets is due to ^{15}NO released by L-S^{15}NC and D-S^{15}NC which are potent ^{15}NO donors. The concentration of inorganic nitrite in plasma/serum of healthy, fasted, non-medicated humans is in the range 200–2000 nM (Tsikas 2008). Oral intake of therapeutically relevant doses of organic nitrates such as ISDN or PETN increases several fold the basal nitrite concentration (Keimer et al. 2003). There is evidence that inhibition of platelet aggregation by inorganic nitrite requires the presence of red blood cells and deoxygenation (Srihirun et al. 2012; Akrawinthawong et al. 2014; Park et al. 2014), a situation that facilitates reduction and/or decomposition of nitrite to freely diffusible NO. However, the concentration of extra-platelet NO that can reach the cytosol of platelets is considered very low and of the order of 1 nM (Hall and Garthwaite 2009). Thus, intra-platelet nitrite derived from extra-platelet NO is expected to be of the same order of magnitude. Enzymes and proteins in the platelets being able to reduce or convert presumably a minor portion of nitrite to NO, are likely not to play a major role in the regulation of platelet function via nitrite/NO. There are findings which suggest that CA is present on the outer surface of human platelets (Ritter et al. 1990). On the assumption that the concentration of nitrite in plasma is much higher than in the platelets, extra-platelet nitrite may serve as a substrate for CA isoforms on the outer surface of blood platelets, which may convert nitrite to NO (Aamand et al. 2009) that in turn activates intra-platelet sGC and inhibits platelet aggregation, thus simulating NOS activity in platelets (Fig. 4).

In the absence of nitrite or the potent NO donor L-*S*-nitrosocysteine (SNC) (Tsikas et al. 1999) washed human platelets incubated for 30 min at 37 °C produced 0.08 ± 0.05 nM cGMP (Fig. 7). Addition of nitrite at the suprapharmacological concentration of 100 μM yielded 0.39 ± 0.12 nM cGMP. In the presence of 100 μM nitrite and the very high concentration of 5 mg/mL erythrocytic bovine CAII, 5.62 ± 2.66 nM cGMP were formed.

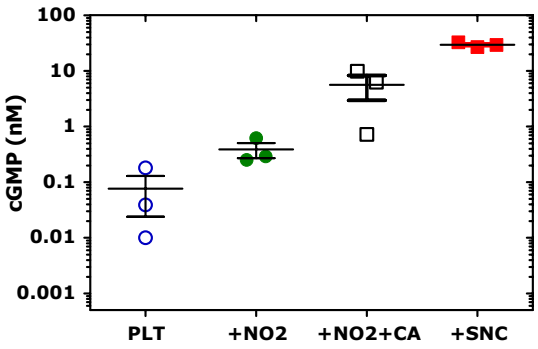

Fig. 7 Effect of nitrite, carbonic anhydrase (CA) and the NO donor L-*S*-nitrosocysteine on the formation of cGMP in washed human platelets. Washed human platelets (PLT, 8×10^8/mL) were incubated alone (PLT), with 100 μM nitrite ($+NO_2$), 100 μM nitrite +CA II (5 mg/mL) ($+NO_2$ +CA) or with 1 μM L-*S*-nitrosocysteine (+SNC) for 30 min at 37 °C. Data are shown as mean ± SD from 3 independent measurements. The release rate of NO from SNC is about 50 % (Tsikas 2005b). Note the logarithmic scale on the *y* axis

However, addition of 1 μM SNC increased the cGMP concentration to 29.5 ± 1.68 nM (Fig. 7). These results indicate that at on a molar basis and on average, extra-platelet nitrite is about 7500 times less active than extra-platelet SNC in activating sGC in human platelets. Extra-platelet CAII in the presence of the extraordinarily high concentration of 5 g/L leads to only a weak stimulation of platelet sGC.

Conclusions and perspectives

The literature is rich in contradictory reports on the expression and functionality of NOS in blood platelets and erythrocytes. That human blood platelets express functional eNOS and an intra-platelet L-arginine/eNOS/NO/sGC/cGMP pathway exists and regulates platelet function has been reported immediately after the discovery of this pathway in other blood cells early in the 1990s. Despite early indications that other blood cells expressing iNOS which produces NO at millionfold higher rates than eNOS and can be present as contamination in platelet preparations used to measure NOS activity and platelet aggregation, the existence and the role of the L-arginine/NOS/NO/sGC/cGMP pathways in humans platelets is generally accepted. However, there is recent convincing evidence by mass spectrometry proteomics that platelets lack NOS proteins. This observation is in line with findings by other approaches which indicate that detection of eNOS proteins in platelets is artefactual most likely due to the use of commercially available unspecific eNOS antibodies. This observation is also in line with the lack of [^{15}N]nitrite and [^{15}N]nitrate formation upon incubation of intact or lysed platelets with L-[guanidine-$^{15}N_2$]-arginine, the substrate of all NOS isoforms. The most likely explanation for this discrepancy is the use of unspecific assays to measure NO (and/or N_2O_3), its metabolites nitrite and nitrate, and L-citrulline, the second reaction product.

Increases in L-citrulline concentration may be due to other pathways such as the urea cycle which have not been considered and entirely neglected because of their low activity when compared to other cells. However, renunciation of such pathways does not consider that eNOS also possesses low activity and much less than 0.1 % of L-arginine is converted to L-citrulline and NO by the healthy human organism. More recently, inorganic nitrite turned out to be an abundant reservoir of NO. Several proteins and enzymes were found to reduce or convert nitrite to NO. Reported proteins and enzymes include hemoglobin and xanthine oxidoreductase of which the activity is increased manifold under certain conditions such as hypoxia. The family of the carbonic anhydrases is also able to produce NO or other NO-species from nitrite, which can inhibit platelet aggregation and simulate NOS activity by not yet uncovered mechanisms. Carbonic anhydrases are also expressed in the platelets, albeit in much lower abundance when compared with red blood cells. There is increasing evidence that other blood cells, notably the erythrocytes, may be of relevance for the nitrite/NO-dependent regulation of platelet function at pathologically and pharmacologically high concentrations of nitrite in the blood as they may occur in diseases such as sepsis and administration of organic nitrates or inorganic nitrate. Yet, these issues remain to be investigated in forthcoming experimental and clinical studies.

Given the plethora of papers published in the topic of platelets related to NO, we were not able to consider all relevant work. Nevertheless, our analysis unequivocally revealed that the research in this topic is highly challenging and heavily encumbered with numerous analytical shortcomings. They concern the detection of NOS proteins and the measurement of their activity with regard to NO and L-citrulline in platelets. These shortcomings are not unique to platelets but they also apply to red blood cells. We hope that our review and discussion of the literature dealing with the NO-related physiology and pathology of the platelets and the pharmacological modulation of their function will make scientists more attentive and critical in their work as investigators, authors and reviewers.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The studies reported in this article were approved by the Ethics Committee of the Hannover Medical School.

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