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Is *S*-nitrosoglutathione formed in nitric oxide synthase incubates?

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In nitric oxide synthase (NOS) incubates, superoxide ($O_2^{\bullet-}$) may be produced in addition to nitric oxide ($^{\bullet}NO$). $^{\bullet}NO$ and $O_2^{\bullet-}$ react together to form peroxynitrite ($ONOO^-$). The reaction of $^{\bullet}NO$ and $ONOO^-$ with GSH may lead to low-yield formation of *S*-nitrosoglutathione (GSNO) and *S*-nitroglutathione ($GSNO_2$) [1], respectively. Consequently, it can be expected that in the presence of GSH, formation of GSNO and $GSNO_2$ would occur in NOS incubates. Indeed, on the basis of HPLC and electrochemical detection, formation of GSNO in NOS incubates has been reported [2,3]. However, it is not unequivocally evident that GSNO had been formed in these studies, because GSNO and $GSNO_2$ possess almost identical physicochemical properties including co-elution in various HPLC systems [1]. Independent of this, quantitation by HPLC revealed that GSNO formation amounted to 5% at 2 mM GSH and 47% at 7 mM GSH with respect to L-citrulline [2]. GSNO has been reported to being formed enzymatically at 3 μM in NOS incubates in the presence of 1 mM GSH, and quantitation by an $^{\bullet}NO$ electrode yielded a GSNO production rate of 25–45% with respect to that of L-citrulline [3]. Thus, the reported GSNO production is considerably higher than the expected GSNO/$GSNO_2$ formation from GSH and $^{\bullet}NO$ or $ONOO^-$. This discrepancy was explained by the assumption that not $ONOO^-$ but a species like $^{\bullet}NO/O_2^{\bullet-}$ produced by NOS was very efficient in producing GSNO from GSH [3].

Analysis of GSNO and other *S*-nitroso compounds in nitrite- and thiol-containing matrices, such as in NOS incubates, is associated with many problems stemming from their facile artifactual formation under acidic conditions [5], which usually prevail both during sample preparation [4] and HPLC analysis [2,3,5]. The potential artifactual formation of GSNO in GSH- and nitrite-rich matrices prompted us to investigate formation of GSNO in NOS incubates. We used experimental conditions almost identical with those described for NOS incubates [2,3] and quantitated by GC-MS [^{15}N]nitrite and GS^{15}NO formation in incubates of a neuronal NOS using L-[$^{15}N_2$]arginine.

NOS-dependent [^{15}N]nitrite amounted to (mean ± S.D., $n = 2$) 0.59 ± 0.15 μM (at 5 μg/ml NOS) and 1.29 ± 0.10 μM (at 10 μg/ml NOS). Analysis by HPLC system A (pH 7) and GC-MS showed [^{15}N]nitrite as the major and GS^{15}NO as the minor product (29 nM and 42 nM, respectively) resulting in molar ratios of [^{15}N]nitrite to GS^{15}NO of 20:1 and 31:1, respectively (Fig. 1A). This finding disagrees with that previously reported [3]. Analysis of an NOS incubate by HPLC system B (pH 2) and GC-MS revealed significant formation of GS^{15}NO besides [^{15}N]nitrite in dependence on L-[$^{15}N_2$]-arginine concentration (Fig. 1B). At 10 μM of L-[$^{15}N_2$]-arginine, [^{15}N]nitrite and GS^{15}NO were measured at 3.7 μM and 0.8 μM, respectively, resulting in a [^{15}N]nitrite to GS^{15}NO molar ratio of 4.6:1. At 100 μM of L-[$^{15}N_2$]arginine, [^{15}N]nitrite and GS^{15}NO were measured at 34.2 μM and 8.5 μM, respectively, resulting in a [^{15}N]nitrite to GS^{15}NO molar ratio of 4.0:1. Injection of a buffered solution of GSH (3 mM) and [^{15}N]nitrite (34 μM) into HPLC system A (pH 7) did not result in detectable amounts of GS^{15}NO, but analysis by HPLC system B (pH 2) resulted in formation of 7.7 μM of GS^{15}NO, i.e. 23% of [^{15}N]nitrite was converted to GS^{15}NO. These findings strongly suggest that the greatest part of GS^{15}NO measured in the second experiment (i.e. B) was formed artifactually. The possibility that the major part of GS^{15}NO formed in the first experiment (i.e. A) could had been decomposed during incubation is refuted, because GS^{15}NO is relatively stable in NOS incubates (Fig. 1C).

Ammonium sulfamate is frequently used to eliminate nitrite prior to detection of *S*-nitroso compounds under acidic conditions [3,4], under which artifactual formation of GSNO from GSH and unremoved nitrite has been observed [4]. We confirm this finding. Acidification of 50 mM phosphate buffered solutions of GSH (1 mM), nitrite (100 μM) and ammonium sulfamate (0.1, 1.0 or 10 mM final concentration) (pH 7) with *o*-phosphorous acid to pH 3 followed by analysis with HPLC system A revealed time-dependent formation of GSNO (48, 27 and 5 μM, respectively, 20 min after acidification).

Our results demonstrate that GSNO/$GSNO_2$ are formed in NOS incubates in the presence of GSH, however, at concentrations considerably lower than those reported previously by other groups [2,3]. Our results also show potential artifactual formation of GSNO both during sample treatment and HPLC analysis under acidic conditions. GSNO and $GSNO_2$ exhibit $^{\bullet}NO$-like activities, but GSNO and $GSNO_2$ are several orders of magnitude more stable than $^{\bullet}NO$. Thus, the concentration of long-lived GSNO and $GSNO_2$ in NOS incubates, and expectedly in NOS-producing cells, too, could be higher than that of the short-lived $^{\bullet}NO$. Therefore, the GSNO/$GSNO_2$ pathway could be more significant than the $^{\bullet}NO$ pathway. More detailed investigation of the (bio)chemical mechanisms of the GSNO/$GSNO_2$ generation from NOS and its quantitative relationship to $^{\bullet}NO$ using artifactual-free analytical methods and detection techniques such as HPLC at neutral or alkaline pH and mass spectrometry should increase our understanding of the biological significance of the GSNO/$GSNO_2$ pathway. According to the present knowledge, a prerequisite for the formation of $GSNO_2$ in NOS incubates is the simultaneous formation of $^{\bullet}NO$ and $O_2^{\bullet-}$. The NOS-catalyzed formation of $O_2^{\bullet-}$ has been, however, questioned [6]. The significance of $O_2^{\bullet-}$ formation is enormous and its origin requires pressing clarification. Performance of careful experiments including positive and negative control experiments is self-evident. The most crucial point, however, is the use of unobjectionable, highly specific, sensitive and interference-free analytical methods. In our opinion, these are the most challenging tasks in the field of $^{\bullet}NO$ research.

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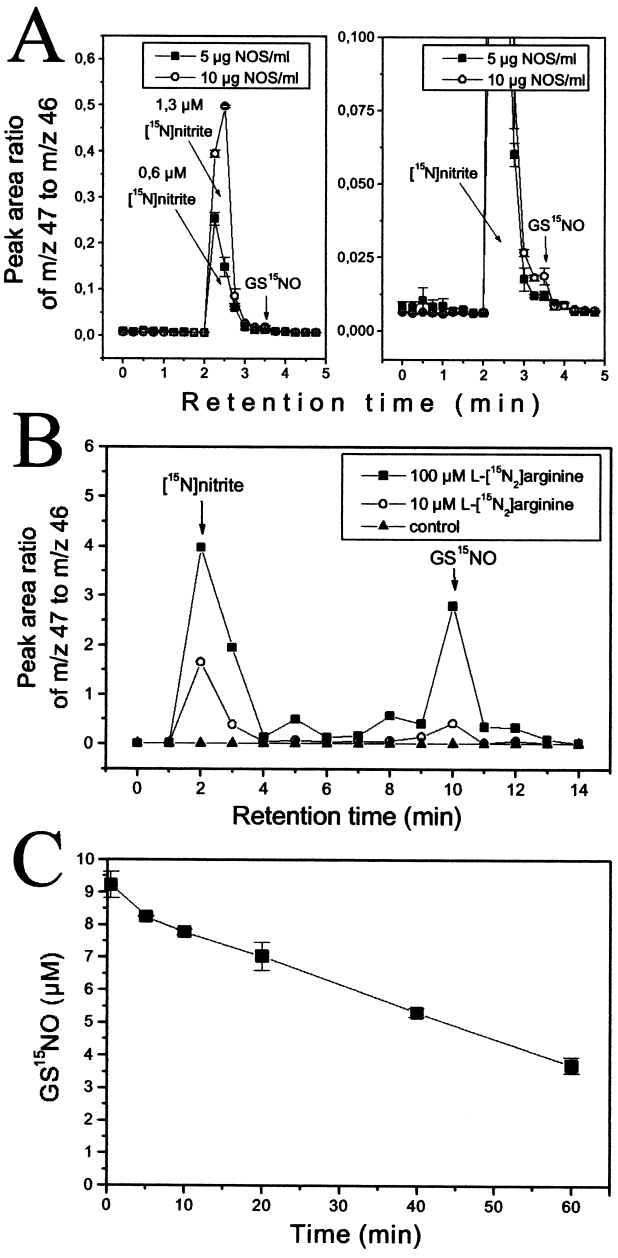

Fig. 1. A: Peak area ratio of *m/z* 47 (for [^{15}N]nitrite and GS^{15}NO) to *m/z* 46 (for nitrite and GSNO) determined in 0.25-min HPLC fractions collected from the analysis of a 200-μl aliquot of an NOS incubate (pH 7; 37°C) containing: NOS, 5 or 10 μg/ml; L-[$^{15}N_2$]arginine, 100 μM; NADPH, 50 μM; calmodulin, 600 nM; $CaCl_2$, 600 μM; and GSH, 1 mM. The incubation time was 10 min. HPLC system A with a mobile phase pH of 7.0 was used. Values are shown as mean ± S.D. from two experiments. In the right panel, the *y*-axis of the chromatogram is amplified by a factor of six as compared with that of the left panel. B: Peak area ratio of *m/z* 47 to *m/z* 46 measured in 1-min HPLC fractions collected from the analysis of a 200-μl aliquot of an NOS incubate (pH 7; 37°C) containing: NOS, 13 μg/ml; L-[$^{15}N_2$]arginine, 10 or 100 μM; NADPH, 800 μM; calmodulin, 500 nM; $CaCl_2$, 500 μM; FAD, 5 μM; FMN, 5 μM; H_4B, 10 μM; and GSH, 1 mM. The incubation time was 30 min. The control sample contained all compounds except for NOS. HPLC system B with a mobile phase pH of 2.0 was used. C: Stability of synthetic GS^{15}NO in an NOS incubate (pH 7; 37°C) that contained all compounds as described in B. The flow rate was 1 ml/min in both HPLC systems.

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Note from the Editorial Office: *FEBS Letters* has recently published several articles of Correspondence regarding the technical difficulties of measuring byproducts formed from NOS incubates. In order to make space for Correspondence on other topics, we are not interested in continuing the NOS discussion further at this point in time.

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Platelet activation: a new vascular activity of anandamide

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Endocannabinoids are amides and esters of long chain polyunsaturated fatty acids, which act as mediators in brain and peripheral tissues through the binding to cannabinoid receptors. Anandamide (*N*-arachidonylethanolamine, AEA) is a major endocannabinoid, showing cardiovascular activity [1] by induction of vasorelaxation [2]. Although AEA has been described as an endothelium-derived hyperpolarizing factor (EDHF), this hypothesis was debated and recent data suggest that EDHF is instead a cytochrome P450-metabolite [3]. Whether or not an EDHF, AEA is likely to play an important role in the control of vascular tone, as supported also by the observation that both rat endothelial cells and macrophages can release it [4].

We found and published in this journal [5] that AEA (≤1.2 mM) activates human platelets by a cannabinoid receptor-independent mechanism, which involves a rise in intracellular calcium and does not depend on the arachidonate cascade. In the same paper we showed that human platelets have the biochemical machinery to degrade AEA, i.e. a high affinity transporter and a fatty acid amide hydrolase (FAAH). Afterwards, these data have been largely confirmed by Braud et al. [6], who found that AEA in rabbit platelets was active at physiological concentrations (≤10 μM) when used in combination with $CaCl_2$ and fibrinogen. These authors attributed the aggregating effect of AEA to its cleavage into arachidonic acid

Table 1
Kinetic parameters of FAAH activity in human peripheral cells

Human cells	K_m (μM)	V_{max} (pmol min^{-1} mg protein^{-1})	Reference
Platelets	10 ± 1.0	270 ± 30	[5]
Lymphoma cells (U937)	6.5 ± 0.6	520 ± 50	[7]
Lymphocytes	8.0 ± 1.0	187 ± 20	[8]
Endothelial cells (HUVEC)	7.0 ± 0.7	25 ± 3	[9]
Mast cells (HMC-1)	5.0 ± 0.5	160 ± 15	[10]

by an AEA-degrading enzyme [6]. At any extent, these data suggest that AEA is an unlikely physiological agonist of platelets, but it can rather act in vivo as a co-agonist in combination with other 'classical' aggregating molecules such as arachidonic acid, fibrinogen or thrombin [5,6]. However, the role of endocannabinoids in the cross-talk between platelets and endothelium, which might be crucial in thrombosis, still awaits clarification.

Platelet activation by AEA appears of interest also because it can be released from endothelial cells and macrophages in rats [4]. This suggests an interplay between different blood cells in regulating the peripheral endocannabinoid system, hence the cardiovascular activities of these newly discovered lipid mediators [1–4]. In keeping with this concept, we showed that peripheral human cells such as lymphoma (U937) cells [7], lymphocytes [8], endothelial (HUVEC) cells [9] and mastocytes (HMC-1) [10] take up AEA and degrade it through FAAH. The apparent kinetic constants of FAAH in human blood cells (Table 1) suggest that a similar enzyme is expressed to different extents, showing similar K_m but different V_{max} values (Table 1). Finally, it should be stressed that platelet activation is paralleled by a decrease in nitric oxide, which stimulates AEA uptake by human cells [7–10]. Therefore, platelets can affect endocannabinoid degradation by the neighboring blood cells, contributing to the control of these compounds.

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