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Characterisation and comparison of temporal release profiles of nitric oxide generating donors

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HIGHLIGHTS

- Nitric oxide release profiles were characterised for commonly used donors.
- Released NO differs greatly between donors and depends on storage conditions.
- High release donors (NOC-5, PAPA NONOate) decay quickly.
- SNP and GSNO show greater stability releasing consistent lower NO levels.
- This comprehensive characterisation provides knowledge to define NO concentrations released *in vitro*.

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ABSTRACT

Background: Nitric oxide (NO) is a vital signalling molecule in a variety of tissues including the neuronal, vascular and reproductive system. However, its high diffusibility and inactivation make characterisation of nitrergic signalling difficult. The use of NO donors is essential to characterise downstream signalling pathways but knowledge of donor release capacities is lacking, thus making comparisons of donor responses difficult.

New method: This study characterises NO profiles of commonly used NO donors. Donors were stored under defined conditions and temporal release profiles detected to allow determination of released NO concentrations.

Results: Using NO-sensitive microsensors we assessed release profiles of NO donors following different storage times and conditions. We found that donors such as NOC-5 and PAPA-NONOate decayed substantially within days, whereas SNP and GSNO showed greater stability releasing consistent levels of NO over days. In all donors tested, the amount of released NO differs between frozen and unfrozen stocks.

Comparison with existing method(s): Fluorescent and amperometric approaches to measure NO concentrations yield a wide range of levels. However, due to a lack of characterisation of the release profiles, inconsistent effects on NO signalling have been widely documented. Our systematic assessment of release profiles of a range of NO donors therefore provides new essential data allowing for improved and defined investigations of nitrergic signalling.

Conclusions: This is the first systematic comparison of temporal release profiles of different NO donors allowing researchers to compare conditions across different studies and the use of defined NO levels by choosing specific donors and concentrations.

1. Introduction

Nitric oxide (NO) is a fundamental and conserved signalling molecule across different species and plays important roles in a myriad of physiological processes within the cardiovascular, nervous, reproductive and other systems (Ignarro et al., 1987; Knowles and Moncada, 1992; Bogdan, 2001; Garthwaite, 2008). Physiologically, NO signals are generated by neuronal NO synthase (nNOS) or endothelial NO synthase (eNOS) and additionally by the immune system-relevant inducible NO synthase (iNOS). In

Abbreviations: GSNO, S-nitrosoglutathione; MNTB, medial nucleus of the trapezoid body; NOC-5, 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene; PAPA NONOate, 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine; PKG, protein kinase G; SNAP, S-nitroso-N-acetylpenicillamine; SNP, sodium nitroprusside; WPI, World Precision Instruments Ltd.

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target cells, through specialised receptors possessing an intrinsic soluble guanylyl cyclase (sGC), NO accumulation results in cyclic guanosine monophosphate (cGMP) production which leads to activation of downstream signalling molecules such as protein kinase G (PKG). Furthermore, higher levels of NO production results in post-translational modifications of proteins through either S-nitrosylation of thiol groups or *via* generation of peroxynitrite leading to tyrosine nitration of proteins (Knott and Bossy-Wetzel, 2009). Unlike conventional neurotransmitters, NO is not constrained by cellular membranes and diffuses in three dimensions from its source of production (Garthwaite and Boulton, 1995). Particularly in the nervous system, the concentration gradient associated with this diffusion is important in signalling mechanisms such as regulation of plasticity (Hardingham and Fox, 2006; Hardingham et al., 2013) and development (Bradley et al., 2010; Jay et al., 2014). Among the many unknowns are the exact levels of NO generated by different sources (*i.e.* neuronal or endothelial), how far it diffuses in active concentrations, the molecular targets other than sGC-coupled receptors and how it is inactivated, particularly in many neurodegenerative and cardiovascular diseases where enhanced NO levels are reported (Naseem, 2005; Nakamura and Lipton, 2008, 2009; Steinert et al., 2010a; Wolin et al., 2010).

Various attempts have been made to determine levels of produced NO. Generally, fluorescent probes such as diamino-fluorescein (DAF)- or dichloro-fluorescin (DCF)-related compounds (fluorescein framework) (Gunasekar et al., 1995; Kasim et al., 2001) or recently developed diamino-rhodamine probes (DAR, rhodamine based chromophore) have been used to characterise NO production and concentration profiles (Takata et al., 2005; Ye et al., 2008; Steinert et al., 2010b). Although these fluorescent probes allow some spatial and temporal characterisation, they do not directly react with NO. Instead a change in fluorescence occurs when non-specific oxidation of the fluorophore leads to the transformation of an amino group to an NH radical which then binds NO. Therefore a simple change in the redox state could lead to changes in oxidation of the fluorophore and thus availability of radical species available for NO binding resuls in problematic quantification of fluorescent signals (Wardman, 2007; Hall and Garthwaite, 2009). However, recent studies using a fluorescent cGMP biosensor, δ-FlincG (Nausch et al., 2008), reported an improved and physiologically relevant way of measuring NO profiles in neurons (Wood et al., 2011).

The use of electrodes provides additional tools to detect direct temporal changes in NO profiles (Finnerty et al., 2012; Jensen et al., 2013). Nevertheless, detection of NO levels yielded values that span over 4 orders of magnitude, from the low picomolar to the micromolar range, with a similar variability observed in different tissues (Hall and Garthwaite, 2009). This is largely a consequence of the selectivity and sensitivity of the recording sensor surface or inaccurate calibration (Bedioui and Villeneuve, 2003).

Investigations of nitrergic signalling also require the use of NO-releasing compounds. In countless publications various NO donors, at various concentrations, have been applied leading to not always reproducible and even controversial findings. The main drawback of using NO donors is the unknown release capacity of each donor which depends on the concentration applied and the environment. In order to identify profiles of NO release, we measured NO concentrations of various donors at different concentration in standard phosphate buffered saline (PBS, pH 7.4) over time using NO sensing electrodes. The NO microsensor chosen for this study (NOPF100 NO microsensor; WPI) possess a multi-layered selective coating that eradicates non-specific detection of other species related to NO research such as arginine, ascorbic acid, cysteine, dopamine, nitrate, nitrite, N_2, O_2 among others and shows reliable NO measurements (Hurst and Clark, 2003). Therefore our data provide a systematic and comprehensive comparison of NO release by different donors in standard *in vitro* conditions, which provides important insight to study nitrergic signalling and allows a better evaluation of reported nitrergic signalling outcomes.

2. Materials and methods

2.1. Nitric oxide donors

During this study the following NO donors were used: 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5, 5–20 μM, Santa Cruz Biotechnology, Inc. and Enzo Life Sciences), S-nitrosoglutathione (GSNO, 100–300 μM, Santa Cruz Biotechnology, Inc.), propylamine propylamine NONOate (PAPA NONOate, 5–20 μM, Santa Cruz Biotechnology, Inc. and Enzo Life Sciences), S-nitroso-N-acetylpenicillamine (SNAP, 5–20 μM, Life Technologies) and sodium nitroprusside dehydrate (SNP, 100–300 μM, Sigma). NO concentrations are expressed as mean ± SEM and displayed in figures as box and whisker plots to indicate median value and interquartile range.

2.2. Apollo 1000 Free Radical Analyser

NO levels were captured using the Apollo 1000 Free Radical Analyser and the NOPF100 NO microsensor (WPI). Data were recorded using Labscribe v3 (WPI) and analysed in Prism v6 (GraphPad) software. Before use, microsensors were polarised by immersing in copper (II) sulphate solution (0.1 M $CuSO_4$, Merck Millipore) under continuous stirring. This provides a potential difference between the recording electrode relative to the reference electrode, which is amplified and recorded when NO is oxidised on the probe membrane. Polarising also provides a reduction in background current. The poise voltage on the Apollo 1000 was set to 865 mV, the current range was set at 10 nA and data were sampled at 10 Hz. The microsensor was left undisturbed for 2 h until a stable baseline was reached.

2.3. Calibration

As the microsensors measure very small voltage changes following oxidation of NO on the sensor, they are very sensitive to external noise, temperature fluctuation and drift and probe sensitivity can change significantly over time (Simonsen et al., 1999). Calibration following polarisation was conducted on a daily basis before and after measurements. The method chosen for calibration involves using the NO donor SNAP and $CuSO_4$, as recommended in the user manual provided by WPI. The probe was placed in a beaker containing 20 ml $CuSO_4$ (0.1 M) with a stirring bar to ensure constant mixing of solution. Once a stable baseline voltage was reached, 2, 4, 8, 16 and 32 μl of 100 μM SNAP solution containing 44 μM ethylenediaminetetraacetic acid (EDTA, EMD Millipore Chemicals) was subsequently added. Upon addition of each volume of SNAP the voltage increased rapidly before reaching a plateau, before plateau decay the next volume of SNAP was added. Each addition of SNAP resulted in released NO as calculated: the conversion efficiency of SNAP to NO in $CuSO_4$ is 0.6 (60%), therefore for every mole of SNAP, 0.6 mole of NO is liberated. From the known amount of NO released from SNAP a calibration curve of voltage response *vs.* NO concentration was constructed.

2.4. NO donor release profiles

The microsensor probe was inserted into PBS solution (pH 7.4), under constant stirring, and the voltage response was allowed to settle over a period of 5–10 min. 10 mM GSNO stock was made in PBS (pH 7.4) with limited exposure to light and oxygen. Once the baseline voltage was stable, 100 μM