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Stable isotope-assisted LC–MS/MS monitoring of glyceryl trinitrate bioactivation in a cell culture model of nitrate tolerance

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ABSTRACT

The nitric oxide (NO) metabolites nitrite (NO_2^-) and nitrate (NO_3^-) can be quantified as an endpoint of endothelial function. We developed a LC–MS/MS method of measuring nitrite and nitrate isotopologues, which has a lower limit of quantification (LLOQ) of 1 nM. This method allows for isotopic labeling to differentiate newly formed nitrite and nitrate from nanomolar to micromolar background levels of nitrite and nitrate in biological matrices. This method utilizes 2,3-diaminonaphthalene (DAN) derivatization, which reacts with nitrite under acidic conditions to produce 2,3-naphthotriazole (NAT). NAT was chromatographically separated on a Shimadzu LC System with an Agilent Extend-C_{18} 5 μm 2.1 × 150 mm column and detected using a multiple reaction monitoring (MRM) method on an ABSciex 3200 QTRAP mass spectrometer operated in positive mode. Mass spectrometry allows for the quantification of ^{14}N-NAT (m/z 170.1) and ^{15}N-NAT (m/z 171.1). Both nitrite and nitrate demonstrated a linear detector response (1 nM – 10 μM, 1 nM – 100 nM, respectively), and were unaffected by common interferences (Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), phenol red, and NADPH). This method requires minimal sample preparation, making it ideal for most biological applications. We applied this method to develop a cell culture model to study the development of nitrate tolerance in human endothelial cells (EA.hy926).

1. Introduction

Nitric oxide (NO) is a signaling molecule and free radical that plays a diverse role in cardiovascular, immune, and nervous systems [1,2]. NO is produced from L-arginine by NO synthase (NOS) in mammals [1], yet it can also be produced via the reduction of nitrite (NO_2^-) and nitrate (NO_3^-) by xanthine oxidase (XO) [3–7]. Nitrite and nitrate are present in the environment and diet. NO is released by cells in the picomolar to nanomolar range [8], and has an estimated in vivo half-life of 3–4 s in human blood [9]. It is therefore very difficult to accurately measure, so its nitrite and nitrate metabolites are often measured as surrogates [10]. Measurement of nitrite and nitrate is the most suitable and practical method to assess NO synthesis in vivo [11]. Given that nitrite and nitrate can be reduced to NO by XO, nitrite and nitrate can be viewed as bioavailable storage pools for NO [12].

1.1. Nitrate tolerance

Glyceryl trinitrate (GTN), more commonly known as nitroglycerin, is used for the treatment of angina pectoris, coronary artery disease, acute myocardial infarction, hypertension, and congestive heart failure. GTN is a prodrug that must be bioactivated in endothelial cells to produce NO (Fig. 1), which activates soluble guanylyl cyclase (sGC) to release the second messenger cyclic guanosine monophosphate (cGMP), stimulating the relaxation of smooth muscle cells [13,14]. Continuous treatment with GTN results in nitrate tolerance after only a few weeks, which limits the long-term use and benefits of treatment [15]. Currently, nitrate tolerance is managed by implementing nitrate-free intervals, but this strategy could become obsolete when co-therapies that effectively prevent nitrate tolerance become available. The cause of nitrate tolerance and the mechanism of GTN bioactivation remain relatively unknown. In the present study we measure nitrite and nitrate as

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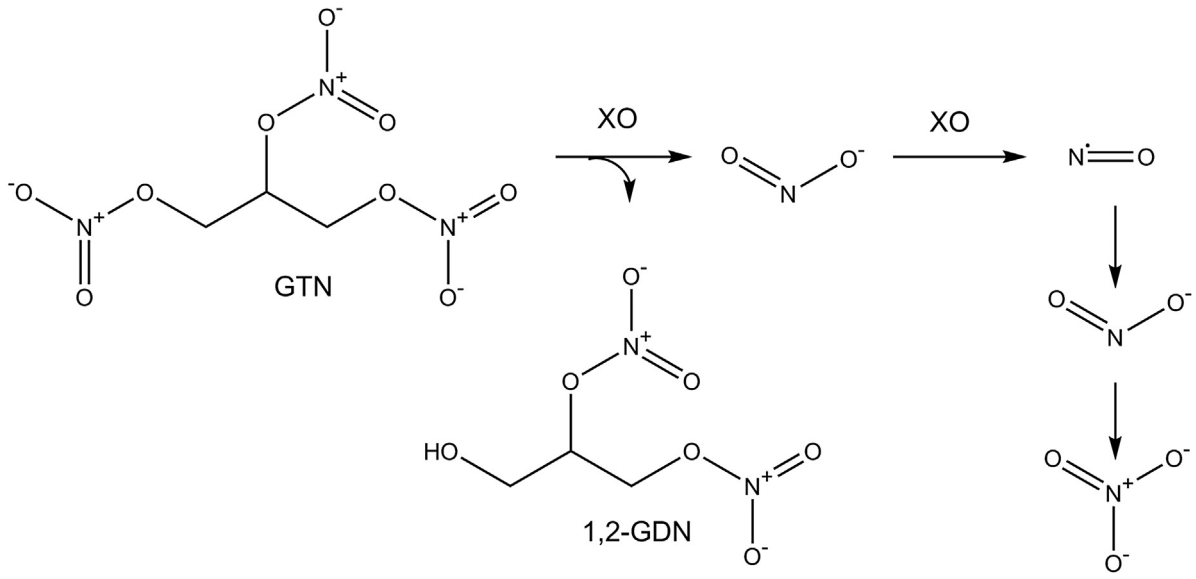

Fig. 1. Glyceryl trinitrate (GTN) is denitrified by xanthine oxidase (XO) in endothelial cells to produce 1,2-glyceryl dinitrate (1,2-GDN) and NO_2^-, which is reduced into NO and subsequently non-enzymatically oxidized to NO_2^- and NO_3^-.

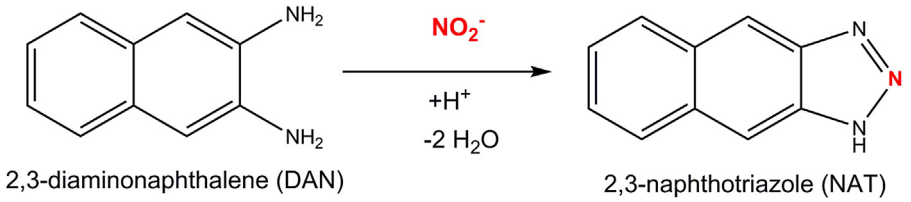

Fig. 2. NO_2^- reacts with 2,3-diaminonaphthalene (DAN) under acidic conditions to produce 2,3- naphthotriazole (NAT).

endpoints of GTN bioactivation in endothelial cells. Our intent is to determine whether cell culture and the measurement of nitrite and nitrate isotopologues can be used as an appropriate model to determine the mechanism of GTN bioactivation and the cause of nitrate tolerance.

1.2. Quantification of nitrite and nitrate

There are over 200 analytical methods for the measurement of nitrite and nitrate, yet a small portion of these can be applied to human biological fluids [11]. Current methods that are used for nitrite and nitrate analysis include fluorescence, chemiluminescence, high performance liquid chromatography (HPLC), capillary electrophoresis, colorimetric and ultraviolet (UV) spectrophotometric methods, and gas chromatography-mass spectrometry (GC–MS). The current methods have been extensively reviewed [8,11,16–18]. Mass spectrometry is the only analytical technique that is able to measure isotopologues of nitrite and nitrate, and is an indispensable analytical tool for reliable quantitative analysis of nitrite and nitrate [8]. In the present study, we modify a method of measuring nitrite with LC–MS/MS [19,20], and expand upon it to include quantification of nitrate. We utilized the well-established 2,3-diaminonaphthalene (DAN) derivatization, which reacts with nitrite under acidic conditions to produce the highly fluorescent 2,3-naphthotriazole (NAT)[21](Fig. 2). Measuring NAT with LC–MS/MS, instead of by fluorescence, allows for the use of isotope labeling which reduces the impact of high background levels of nitrite and nitrate in biological matrices.

This LC–MS/MS method is a significant contribution to the field because it is a sensitive and reproducible method for quantifying nitrite and nitrate isotopologues as metabolic products of GTN. This method is used to investigate the mechanism of nitrate tolerance in human endothelial cells, which is made possible with the use of $^{15}N_3$-GTN.

2. Materials and methods

2.1. Chemicals

2,3-diaminonaphthalene (DAN) was procured from TCI America (Portland, OR). $^{15}N_3$-glyceryl trinitate (GTN) in acetonitrile (^{15}N, 98%+), sodium ^{15}N-nitrite (^{15}N, 98%+), and sodium ^{15}N-nitrate (^{15}N, 98%+) were from Cambridge Isotope Laboratories (Tewksbery, MA). GTN, NADPH, sodium hydroxide, and nitrate reductase were from Sigma–Aldrich (St. Louis, MO). Dulbecco's Modified Eagle Medium (DMEM), Hank's Buffered Sodium Salt (HBSS), trypsin-EDTA, fetal bovine serum (FBS), penicillin, and streptomycin were procured from Invitrogen (Carlsbad, CA). Ammonium bicarbonate, LCMS-grade water and methanol were purchased from J.T Baker (Center Valley, PA).

2.2. Endothelial cell culture and treatments with glyceryl trinitrate (GTN)

EA.hy926 human hybrid endothelial cells (American Type Culture Collection; Manassas, VA) were maintained in culture in T75 flasks with DMEM medium containing 4.5 g/L glucose and 84 mg/L L-arginine, and supplemented with 10% FBS and 1% penicillin-streptomycin (PS) in a humidified incubator at 37 °C with 5% CO_2. Endothelial cells were chosen because they have been previously shown to produce NO from nitrite, which caused changes in markers of intracellular nitrosation reactions [22]. Endothelial cells