Can Summary Nitrite+Nitrate Content Serve as an Indicator of NO Synthesis Intensity in Body Tissues?

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Studies with the use of a highly specific enzymatic sensor demonstrated that, contrary to the common opinion, normally nitrate is in fact not present in the most important physiological fluids. NO metabolites in the amniotic fluid and semen are mainly presented by NO donor compounds. Therefore, the intensity of NO synthesis can be evaluated by the total content of all its metabolites, but not by the widely used summary nitrite+nitrate content.

Key Words: *nitric oxide (NO); nitrite; nitrate; NO donors*

Summary nitrite+nitrate (NOx) content in living tissues is regarded as an indicator of NO synthesis intensity, because these compounds are assumed to be the final and most stable products of its metabolism, quantitatively predominating over the rest metabolites [4,7]. However, nitrate is consumed in large amounts with food and water. If nitrite is a normal product of NO oxidation in tissues, the next logical question is how can NO generation be paralleled by the formation of nitrite, a toxic product? In addition, it is not clear, how the specificity of NO activity is attained. It seems that there are some mechanisms preventing or minimizing NO reactions with oxygen and other physiologically negligible targets (Fig. 1).

All methods for NO metabolite measurements used up to the present time were either not sufficiently selective or required preliminary purification of the sample, which could add significant artifacts distorting the qualitative and quantitative composition of the measured substances [9,11]. The enzymatic sensor we have created is devoid of these drawbacks and opens new vistas in the studies of NO metabolism [1,2].

We analyzed the composition of nitro and nitroso compounds of the most important physiological fluids in health and inflammatory processes characterized, according to published data, by intense NO synthesis [3,8].

MATERIALS AND METHODS

Experiments were carried out with monopotassium phosphate, sodium chloride, sodium nitrate (Laverna), catalase, potassium nitrite, glutathione, N-methyl-D-glutamine dithiocarbamate (MGD; Sigma), sulfanilic acid, N-(1-naphthyl)-ethylenediamine dihydrochloride (NED), and mercury dichloride (Merck). Nitro and nitroso compounds were measured using an enzymatic sensor, described in detail in our previous reports [1,2]. The sensor was based on the unique capacity of all nitroso compounds to inhibit catalase in the presence of halogen ions with about the same efficiency. Other known catalase inhibitors could not react this way and were normally not present in biological objects in concentrations which can add artifacts [1,2]. Nitrosyl iron complexes, containing thiol ligands (INC/SH), lost the inhibitory activity in medium containing iron chelator (o-phenanthroline, EDTA) and NO trap (oxyhemoglobin, sulfanilic acid), capturing free NO. S-Nitrosothiols (RSNO) were evaluated as compounds transforming into ICN/SH under the effect of Fe^{2+} and thiols [6,12] and acquiring their characteristics. Nitrite (NO_2^-) and N-nitroso compounds (RNNO) did

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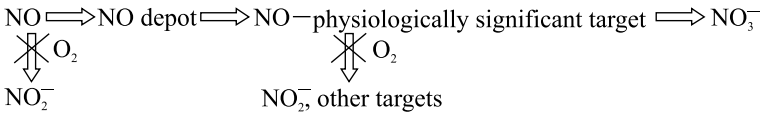

Fig. 1. Scheme of physiological effects of NO [12,14]. NO depots: S-nitrosothiols, nitrosyl iron complexes [12,14], some organic nitrites [1].

not produce ICN/SH in neutral medium and retained the inhibitory activity in the presence of Fe^{2+}, glutathione, NO trap, and iron chelator added in succession [1,2]. Nitrosyl iron complexes, containing no thiols (Fe-(NO)n), were evaluated as compounds acquiring the (ICN/SH) characteristics after addition of glutathione into reaction medium [1,2]. The total pool of nitrocompounds was evaluated after reduction with vanadium trichloride to nitrosostatus and inhibition of catalase. High-molecular nitrates (RNO_2) producing nitrosyl iron complexes were defined as compounds acquiring the capacity to inhibit catalase under the effects of Fe^{2+} and thiols [2]. The method required no pretreatment of the sample; the measurements were carried out in neutral medium. The sensitivity of the method was 40 nM [1,2].

In parallel with this, classical Griess method was used to measure nitrite and its modified form was used for evaluation of RSNO [1].

The total pool of NO-producing compounds was evaluated by Fe-MGD spin trap forming an MINC-MGD mononitrosyl complex with the released NO and characterized by specific EPR signal [14]. Radiopan EPR spectrometer was used.

Blood specimens were collected in volunteers and patients with inflammatory diseases (Department of Surgery, Biomedical Faculty; Department of Neurology, N. I. Pirogov Medical University). Blood clotting was prevented by heparin (0.1 ml 1% solution/10 ml blood). Cow milk specimens were collected at farms of (Sergiev Posad District, Moscow Region). Specimens of the amniotic fluid and seminal fluid of birds were a gift from Gene Fund Firm.

The results were statistically processed by BIOSTAT software. The data were presented as the means± standard deviations. The differences were considered significant at $p<0.05$.

RESULTS

According to the data obtained by the Fe-MGD spin trap, NO-producing compounds were present in the milk, but not plasma (Table 1). However, it was impossible to identify precisely the nature of these compounds by means of the spin trap. According to Griess method, nitroso compounds in normal cow milk were presented by NO_2^- and RSNO, according to enzymatic sensor by RSNO alone (Table 1). Normally NO_2^- in concentrations of up to 1.8 μM was detected in human plasma by Griess method (sensitivity of 100 nM), which was in line with published data [9,11,13], but not by the enzymatic sensor (sensitivity up to 40 nM) (Table 1). Commercial NO_2^- added to the plasma and milk specimens was detected by the enzymatic sensor as NO_2^- in estimated concentration (Table 1). This result confirmed that the studied objects contained no NO_2^- in concentrations detectable by the sensor.

TABLE 1. Levels of RSNO, ICN/SH, and NO_2^- in Specimens of Milk from Clinically Healthy Cows (n=4) and Normal Human Plasma (n=4)

Method	Object	Concentrations of measured compounds, μM			
		RSNO	NO_2^-	ICN/SH	total content of NO and NO_2^--donors
Griess	Plasma	0	1.20±0.07	-	1.20±0.07
	Milk	0.15±0.08	0.30±0.15	-	0.45±0.23
Enzymatic sensor	Plasma	0	0	6.9±1.8	6.9±1.8
	Plasma+0.15 μM NO_2^-	0.16±0.02	6.9±1.8	7.06±2.00	
	Milk	7.50±0.08	0	0	7.50±0.08
	Milk+0.2 μM NO_2^-	7.50±0.08	0.19±0.02	0	7.69±1.00
Spin trap	Plasma	-	-	-	0
	Milk	-	-	-	0.90±0.15

TABLE 2. Content of Nitro and Nitroso Compounds in Physiological Fluids in Health, Measured by the Enzymatic Sensor (*n*=50)

Object	RSNO, μM	ICN/SH, μM	Fe-(NO)n, μM	RNO_2, μM	NO_2^-+RNNO, μM	NO_3^-, μM
Normal human plasma	<0.05	12.3±8.1	<0.05	<0.05	<0.05	52.0±30.0
Milk (cow)	8.5±4.2	<0.1	<0.1	<0.1	<0.05	84.0±51.0
Colostrum (cow)	17±3	<0.1	153±48	1600±300	<0.05	300±100
Semen (cock)	<0.1	<0.1	9.2±3.5	145±62	<0.05	<0.1
Amnion (cow)	21.2±0.8	<0.1	167±42	2400±500	<0.05	<0.1
Chicken embryo amnion	<0.1	<0.1	21.5±4.8	210±30	<0.05	<0.1

Note. Exogenous nitrate added to chicken embryo amnion in a concentration of 0.3 μM was detected as nitrate in a concentration of 0.30±0.03 μM.

According to the enzymatic sensor measurements, normal plasma contained ICN/SH (NO donors) [12,14] (Tables 1, 2). Disagreement between the data obtained by EPR and enzymatic sensor (Table 1) was presumably explained by the fact that an appreciable portion of MICN–MGD in the plasma was oxidized (diamagnetic), this leading to the absence of EPR signal [14]. Presumably, the presence of ICN/SH was responsible for detection of NO_2^- in normal human plasma in concentrations from hundreds of nanomoles to several micromoles by methods involving the sample purification and modification [9,11,13].

Embryogenesis and spermatogenesis are accompanied by intensive production of NO [5,10]. However, according to the enzymatic sensor measurements, NOx were present in the seminal and amniotic fluid in just trace amounts, while the nitro and nitrosocompound pool was presented by RSNO, Fe-(NO)n, and RNO_2 (Table 2).

Hence, evaluation of NO production intensity by the summary content of nitrite and nitrate is not correct. This is particularly demonstrative for the seminal and amniotic fluids. Presumably, there are mechanisms preventing oxidation of synthesized NO to NO_2^- (Fig. 1). Compounds with inhibitory activity characteristic of NO_2^- and RNNO appear in the plasma during inflammatory diseases and disappear after recovery. A similar phenomenon has been observed with the milk of cows with mastitis [2]. No significant and specific changes in nitrate content have been detected over the course of recovery (Table 3). It seems that NO_2^- and RNNO appear in the plasma as a result of production of active oxygen and nitrogen species by stimulated leukocytes, which results in the formation of peroxynitrite, N_2O_3, NO_2^-, and RNNO [2]. It remains unclear whether free NO is oxidized or NO donor compounds are subjected to oxidative destruction. Hence, emergence of NO_2^- is a result of failure of the mechanism preventing oxidation of synthesized NO to NO_2^- (Fig. 2), but not the indicator of NO synthesis intensity.

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TABLE 3. Changes in Plasma Concentrations of Nitro and Nitroso Compounds in Patients at Admission and Discharge from the Hospital

Patient No.	Diagnosis	Concentration, μM					
		ICN/SH		NO_2^-+RNNO		NO_3^-	
		admission	discharge	admission	discharge	admission	discharge
1	Acute maxillary sinusitis	15.0±0.9	7.5±0.2	1.5±0.04	<0.05	80.0±5.0	75.0±4.0
2	Acute appendicitis	2.50±0.15	8.1±0.3	4.3±0.2	<0.05	64.0±2.8	57.0±2.7
3	Acute cholecystitis	5.5±0.2	4.0±0.2	2.20±0.07	<0.05	38.0±2.8	55.0±2.5
4	ARD	10.00±0.35	7.5±0.3	1.20±0.08	<0.05	32.5±1.6	54.0±3.0

Note. Mean values of 4 independent measurements are presented.

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