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Magnetic Resonance Study of the Transmembrane Nitrite Diffusion

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Abstract

Nitrite (NO_2^-), being a product of metabolism of both nitric oxide (NO^*) and nitrate (NO_3^-), can accumulate in tissues and regenerate NO^* by several mechanisms. The effect of NO_2^- on ischemia/reperfusion injury was also reported. Nevertheless, the mechanisms of intracellular NO_2^- accumulation are poorly understood. We suggested significant role of nitrite penetration through biological membranes in the form of undissociated nitrous acid (HNO_2). This hypothesis has been tested using large unilamellar phosphatidylcholine liposomes and several spectroscopic techniques. HNO_2 transport across the phospholipid bilayer of liposomes facilitates proton transfer resulting in intraliposomal acidification, which was measured using pH-sensitive probes. NO_2^- -mediated intraliposomal acidification was confirmed by EPR spectroscopy using membrane-impermeable pH-sensitive nitroxide, 2,2,5,5-tetramethyl-1-oxyl-2,5-dihydro-1H-imidazol-3-ium-4-yl)-aminomethanesulfonic acid (pK 5.25), and by ³¹P-NMR spectroscopy using inorganic phosphate (pK 6.9). Nitrite accumulates inside liposomes in concentration exceeding its concentration in the bulk solution, when initial transmembrane pH gradient (alkaline inside) is applied. Intraliposomal accumulation of NO_2^- was observed by direct measurement using chemiluminescence technique. Perfusion of isolated rat hearts with buffer containing 4 μM NO_2^- was performed. The nitrite concentrations in the effluent and in the tissue, measured after 1 minute perfusion were close, supporting fast penetration of the nitrite through the tissue. Measurements of the nitrate/nitrite showed that total concentration of NO_x in myocardium increased from initial 7.8 μM to 24.7 μM after nitrite perfusion. Physiological significance of passive transmembrane transport of NO_2^- and its coupling with intraliposomal acidification are discussed.

Keywords

nitrite; phospholipids' membranes; diffusion; pH

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Introduction

As a product of metabolism of both nitric oxide (NO) and nitrate (NO_3^-), nitrite (NO_2^-) has been observed in all biological tissues that are able to generate nitric oxide, the universal biological messenger. Nitrite has been shown to exhibit vasodilating activity *in vitro* at high concentrations [1-7].

Several chemical and enzymatic mechanisms of nitrite conversion to NO have been reported [8-11], supporting an important role of nitrite as a source of NO production and thus, it is expected that NO_2^- may exert cardioprotection in a manner similar to one observed with NO donors [12-14]. NO can protect the heart [15], and knockout of endothelial NO synthase (NOS) has been reported to render the hearts of mice more sensitive to ischemic insults [16,17]. Indeed, in some *in vitro* and *in vivo* models of infarction, nitrite has been shown to protect against ischemia–reperfusion (I/R) damage [18-20]. However, contradictory findings have been reported in this regard. Other studies suggest that NO_2^- -derived NO may contribute to the damage [11,21] and thus the effects of NO derived from NO_2^- in myocardial injury are uncertain [13,14] and may depend on the levels of NO_2^- and the amount of NO formed. Plasma and tissue nitrite levels vary widely [22-25]. Nitrite concentrations reported in plasma are from 0.15 to 1 μM [25,26], the concentration in aortic tissue is above 10 μM [25,27,28] and in heart tissue 1-50 μM [21].

Nitrite levels are controlled by a number of factors including diet and concentration of ambient NO in the inhaled air as well as by production from NOS or other enzymes [29-32]. The nitrite level can be raised by high dietary ingestion, pharmacological administration of organic nitrates, or other NO-donating drugs. In addition, pathological conditions associated with high levels of stress and inflammation such as sepsis that are accompanied by high levels of NOS induction are also markedly increased nitrite levels [33-35].

In spite of growing interest in the role of NO_2^- as a precursor of NO, the mechanisms of the nitrite transport through the cell membranes and its intracellular accumulation are poorly understood and poorly described in the biomedical literature. The data obtained from the studies with the pig red blood cells [36] and horse erythrocyte ghosts [37] support the nitrite transmembrane transport in the form of the anion, NO_2^- , via heteroexchange with other monovalent anions [37] or by conductive mechanism [36]. Interestingly, nitrite diffusion through the bulk non viable muscle tissue (meat) was quantitatively characterized and diffusion coefficient was estimated to be $3.8 \times 10^{-9} \text{ m}^2/\text{s}$ [38]. These results cannot be directly applied to living biological tissues, though they strongly suggest that nitrite may penetrate through cell membranes also by passive diffusion mechanism. However, it should be noted, that passive diffusion of NO_2^- in the anion form across the hydrophobic lipid bilayer, main structural component of the biomembranes, must be extremely slow. For comparison, the permeability coefficient across phospholipid membrane for Cl^- anion is $\sim 10^{-10} \text{ cm/s}$ which is ten orders of magnitude less than corresponding value, 2 cm/s, for the neutral molecule, undissociated HCl [39]. Membrane permeability coefficient for the nitrate anion was found to be in the same low range ($10^{-10} \text{ cm sec}^{-1}$ to $10^{-9} \text{ cm sec}^{-1}$) [40]. Therefore, transmembrane nitrite transfer by passive diffusion seems to be more plausible to proceed in the form of undissociated nitrous acid (HNO_2), rather than in the form of NO_2^- anion. A low fraction of the undissociated nitrous acid ($\text{pK}_a = 3.14$) at physiological pH, being equal to about 0.01%, can be well compensated by its significantly higher membrane permeability compared with nitrite anion. In particular, this mechanism will be facilitated in acidic medium where the fraction of the undissociated acid is increased. Indeed, pH-dependent nitrite transfer has been demonstrated in chloroplast inner envelope vesicles [41,42]. Nitrite-induced acidification of both chloroplast inner envelope vesicles and vesicles prepared from asolectin, a protein-free lipid mixture, was observed [42], but only in the presence of initial proton gradient. We also reported [39]

NO_3^- -induced proton gradient dissipation followed by intraliposomal acidification at pH 3.0, for egg phosphatidylcholine vesicles. Estimated nitric acid membrane permeability coefficient was about 10^{-4} cm/s. Taking into account significantly higher $\text{pK}_a(\text{HNO}_2)=3.14$ compared with $\text{pK}_a(\text{HNO}_3)=-1.62$, it can be assumed that transmembrane HNO_2 transfer might be important even at physiological pH. Moreover, the experimental observation of the NO_2^- and NO_3^- -dependent intravesicular acidification only in the presence of transmembrane proton gradient [39,42], might be the result of the experimental conditions aimed to the higher sensitivity of the approach. Therefore, in the current paper we tested the role of NO_2^- penetration through biological membranes in the form of undissociated nitrous acid at physiological pH independent on the existence of a transmembrane pH gradient. The importance of this mechanism has been confirmed using large unilamellar phosphatidylcholine liposomes by several spectroscopic techniques, including direct measurement of the nitrite accumulation in the inner aqueous volume of the liposomes using chemiluminescence technique. Nitrite-induced intraliposomal acidification was measured using a pH-sensitive paramagnetic EPR probe [43] and by ^{31}P -NMR spectroscopy using inorganic phosphate (pK 6.9) [21].

Materials and methods

Chemicals

pH sensitive probe for EPR spectroscopy (2,2,5,5-tetramethyl-1-oxyl-2,5-dihydro-1H-imidazol-3-ium-4-yl)aminomethanesulfonic acid (AMS) was synthesized according to procedure described in [43]. Structure of AMS is presented in fig.1.

Egg phosphatidylcholine and other chemicals were purchased from Sigma-Aldrich.

Isolated Heart Perfusion and sample preparation

Female Sprague-Dawley rats (250–300 g) were heparinized and anesthetized with intraperitoneal pentobarbital. The hearts were excised rapidly and immediately were placed in an ice-cold Krebs–Henseleit solution saturated with oxygen. The heart then was mounted rapidly on a perfusion system and was perfused retrogradely via the aorta at 37°C and at a pressure of 80 mm Hg. The Krebs–Henseleit perfusion solution (17 mM glucose, 120 mM NaCl, 25 mM NaHCO_3 , 2.5 mM CaCl_2 , 0.5 mM EDTA, 5.9 mM KCl, 1.2 mM MgCl_2) had a pH of 7.35–7.4 when gassed with 95% O_2 and 5% CO_2 . The base of the pulmonary artery was incised to allow efficient drainage of the right ventricle. Coronary flow (average over 1 min) was measured by collections of the effluent in polystyrene tubes. Average flow during the perfusion was 16 ± 0.6 ml/min. We did not observe any significant effect of nitrite on the coronary flow. Afterwards hearts were perfused with 2 ml of nitrite free buffer to remove nitrite from intravascular volume. Note that this brief period of perfusion with nitrite-free buffer did not result in significant washout of nitrite/nitrate species from the heart (see Table 1). Then, hearts were freeze-clamped, ground in liquid nitrogen, combined with two volumes of distilled water, homogenized on ice, and centrifuged at 3000 g for 10 min. Supernatant was separated and analyzed for nitrite and nitrate. Obtained concentrations were recalculated to initial myocardium volume.

Chemiluminescence measurements of nitrite and nitrate were performed by quantitative conversion of nitrite to NO by acidified potassium iodide in a purging vessel by method of Garside [44] with detection of released NO gas using a NOA 270 Sievers nitric oxide analyzer. Quantification was performed by comparison of the amount of NO released with the amount released from NO_2^- standard. Nitrate/nitrite ($\text{NO}_3^- + \text{NO}_2^-$) measurements were performed similarly by using vanadium chloride as reducing agent.

Liposome preparation

Large (200 nm diameter) unilamellar liposomes from egg phosphatidylcholine (PC) were prepared by extrusion using small-volume extrusion apparatus by LiposoFast (Avestine, Inc., Ottawa, Canada) with method similar to that described previously [45]. Lipid (50 mg/mL) was dissolved in chloroform and dried on the walls of rotating cylinder under the nitrogen flow and then kept under the vacuum for 0.5 h. For EPR experiments, the lipid film was hydrated for 4 hours in 10 mM phosphate buffer containing 10 μ M AMS. After that, pH was adjusted to the required value. In order to remove spin label from outer liposomal volume, the liposome suspension was diluted to twice the initial volume by the buffer and centrifuged at $10,000 \times g$ for 0.5 h; the supernatant was removed and sedimented liposomes were resuspended in the buffer. Procedure was repeated three times before liposome suspension was brought back to original volume of 1 ml. The concentration of AMS in last supernatant was less than 0.1 % of concentration inside liposomes estimated by EPR signal intensity.

Liposomes for NMR experiments were prepared in a similar way with the exception that AMS was omitted, concentration of phosphate buffer was 70 mM, and outside buffer was replaced by pyrophosphate buffer, 31 mM, with the same pH and osmolarity.

EPR measurements were performed in 50 μ L capillary tubes with the Bruker ESP300 X-band spectrometer equipped with HS resonator. Parameters of the acquisition were: modulation amplitude 0.5 G, modulation frequency 100 kHz, scans time 43 sec. In order to reduce the time interval between sample preparation and the acquisition of the high field peak of the AMS (the most pH sensitive peak of the AMS EPR spectrum) magnetic field scans were started from high field, allowing to register spectral changes in less than 30 sec after sample preparation.

NMR measurements

^{31}P NMR spectra were acquired at 202 MHz using DRX-500 spectrometer (Bruker) with 5 mm broadband probe. Typical acquisition and processing parameters include 0.8 s acquisition time, 40 kHz spectral width, 256 scans, 0.4 s delay, 300 K temperature, 10 μ s (60°) pulse, 4 Hz exponential line broadening prior to Fourier transform. All samples contained 10 % D_2O for shimming and lock stabilization.

Results and discussion

Nitrite consumption by the perfused isolated hearts

With perfusion of isolated rat hearts with buffer containing 4 μM NO_2^- for one minute, the NO_2^- effluent concentration was observed to be lower than 4 μM . Also increase of the NO_2^- and NO_3^- in the myocardium was observed (see Table 1). Interestingly, the nitrite concentrations in the effluent and in the tissue, measured after this short-time perfusion, were close, supporting comparatively fast penetration of the nitrite through the tissue. Measurements of the nitrite/nitrate showed that total concentration of NO_x in myocardium increased from initial 7.8 μM to 24.7 μM after nitrite perfusion. These experiments suggest that NO_2^- efficiently penetrates through the cell membranes. The initial step of nitrite/nitrate accumulation in the myocardium is nitrite penetration through the vessel walls from the intravascular compartment into the tissue. We made the rough estimate of the capillary permeability coefficient for nitrite according to the following equation which is based on the increase of the NO_x concentrations over the time of perfusion:

$$P = \frac{\Delta[\text{NO}_x]^h \cdot m^h}{([\text{NO}_2^-]^p - [\text{NO}_2^-]^h) \cdot S_v \cdot V^h \cdot \Delta t}$$

where $\Delta[\text{NO}_x]^h$ (h denotes to the heart) is difference between concentrations of NO_x in the hearts before and after nitrite perfusion; m^h is weight of the heart; Δt is time of perfusion; $([\text{NO}_2^-]^P - [\text{NO}_2^-]^h)$ is averaged difference between nitrite concentrations in the perfusate, $[\text{NO}_2^-]^P$ and in the heart, $[\text{NO}_2^-]^h$; S_V is estimate for the capillary surface area (vasculature area) per unit of volume; and V^h is volume of the heart. Note that NO_x refers to total concentration of the nitrite and nitrate, the latter formed upon nitrite oxidation and accumulated in the heart during nitrite perfusion. Substituting the numerical values to the above equation ($\Delta[\text{NO}_x]^h = 16.3 \mu\text{mol/kg}$, see table 1; $m^h \sim 1.1 \times 10^{-3} \text{ kg}$; $\Delta t = 60 \text{ sec}$; $([\text{NO}_2^-]^P - [\text{NO}_2^-]^h) \approx 2 \mu\text{M}$; $S_V = 272 \text{ cm}^{-1}$ being average of values of $308 \pm 13 \text{ cm}^{-1}$ and $235 \pm 32 \text{ cm}^{-1}$ for subepicardium and subendocardium of rat hearts, correspondingly [49]); $V^h = 1.1 \text{ cm}^3$ as estimated from m^h assuming the density being equal to 1 g/cm^3) we obtained the value of $p = 6 \times 10^{-4} \text{ cm} \cdot \text{sec}^{-1}$. This estimate seems to be too high for the passive diffusion of the nitrite anion and may be attributed to nitrite transfer through the specific protein channels or/and to the passive diffusion of nitrous acid. Myocardium accumulation of nitrate, formed upon nitrite oxidation, suggests that permeability of cellular membranes is significantly higher for nitrite, than for nitrate. This is in agreement with the passive diffusion mechanism since fraction of undissociated HNO_2 ($\text{pK}_a = 3.14$) is almost five orders of magnitude higher compared with that for HNO_3 ($\text{pK}_a = -1.62$). Our data demonstrate that about 85 % of the myocardium-consumed NO_2^- was converted to NO_3^- . The most probable mechanism for this conversion is via participation of oxymyoglobin which presents in the heart tissue in significant amount and is able to convert nitrite to nitrate [46-48]. The amount of nitrite/nitrate accumulated in the heart was in a good agreement with the amount of nitrite consumed from perfusate. Namely, typical nitrite uptake by the heart (as calculated from NO_x increase in the heart) was $16.3 \mu\text{M} \times 1.1 \times 10^{-3} \text{ l} = (17.9 \pm 4) \text{ nmol}$. Nitrite uptake calculated from nitrite concentration difference in perfusate and effluent was found to be equal to $1.1 \mu\text{M} \times 16 \times 10^{-3} \text{ l} = (17.6 \pm 2) \text{ nmol}$. The balance between accumulated nitrite/nitrate in the heart and the amount of nitrite absorbed from perfusate demonstrates that $17.9 \pm 4 \text{ nmol}$ of nitrite was absorbed by the heart for 1 min of perfusion. The activity of NOS in the rat heart produces about 1 pmol/min/mg of protein of NO [50] or about 0.1 nmol by total heart for the same time. This amount is two orders of magnitude lower than observed nitrite absorption. Therefore change in nitrite/nitrate concentrations may be solely attributed to nitrite transport into myocardium.

Nitrite transport through the phosphatidylcholine liposomal bilayer

The phospholipid bilayer, the main structural component of the biomembrane, provides a hydrophobic barrier for the transport of hydrophilic and ionic species. The ability of the NO_2^- to penetrate through the phospholipid bilayer in the form of nitrous acid was tested using large unilamellar phosphatidylcholine liposomes. Nitrite transfer across the biomembrane in the form of nitrous acid followed by acid dissociation has to be accompanied by intraliposomal acidification. Nitrite-induced acidification of inner aqueous medium of large unilamellar vesicles can be measured by several spectroscopic approaches utilizing pH sensitive molecular probes. Figure 1 shows the structures of two hydrophilic membrane-impermeable pH probes which were previously utilized for intraliposomal pH measurement by EPR and NMR spectroscopies.

EPR studies of the nitrite-induced intraliposomal acidification

pH Sensitive nitroxide, AMS ($\text{pK}_a = 5.25$ [43]), does not penetrate the phospholipid bilayer and, therefore, was encapsulated in the inner aqueous space of phospholipid vesicles as described in Materials and Methods. Figures 2a and 2b demonstrate EPR spectra of the liposome-encapsulated AMS probe before and 30 min after addition of sulfuric acid in amount corresponding to decrease of extraliposomal pH from $\text{pH} = 6.2$ to $\text{pH} = 5.2$. Absence of appreciable EPR spectral changes of AMS after more than half an hour confirm (i) negligible leakage of the probe from the inner liposomal space, and (ii) an ability of the liposomes to hold

the pH gradient. The latter is in agreement with our previous data and is explained by blocking the proton transport due to the establishment of transmembrane electric potential of about 60 mV upon initial electrogenic proton transfer [43]. However, subsequent addition of 2 mM nitrite to the liposomes caused immediate increase of the EPR spectral component of the protonated form of the radical, RH^+ , (Fig. 2c), indicating acidification of the intraliposomal compartment apparently due to the transport of nitrous acid.

These results are in agreement with the data of Shihngles et al. [42] who demonstrated nitrite-induced acidification of the inner space of the asolectin vesicles but only in the presence of initial transmembrane proton gradient. Transmembrane proton gradient, pH being more acidic outside, obviously promotes inward NO_2^- transport through the lipid bilayer by increasing the fraction of the undissociated nitrous acid in the outer space. This might be of physiological importance for the mitochondria where significant transmembrane gradient, with pH being higher inside, is known. Nevertheless, the absence of transmembrane proton gradients for many organelles or slightly lower values of intracellular pH compared with extracellular space are very common. Normally perfused hearts have pH 7.1 [21] which reflects mainly intracellular pH. Measured pH of perfusate was 7.35-7.4. To demonstrate that transmembrane nitrite transfer via HNO_2 diffusion mechanism does not require pH gradient, we measured nitrite-induced intraliposomal acidification in the absence of initial ΔpH (see Figure 3). Dose-dependent increase of the fraction of protonated form of AMS was observed, indicating subsequent decrease in intraliposomal pH (fig. 3). Therefore, the establishment of the transmembrane pH gradient, being more acidic inside, was observed, supporting the mechanism of the transmembrane nitrite transfer in the form of nitrous acid followed by its dissociation in the inner aqueous space. The results obtained support the hypothesis that nitrite may penetrate cellular membranes in the form of nitrous acid.

^{31}P NMR studies of the nitrite-induced intraliposomal acidification

EPR spectroscopy using the AMS probe clearly demonstrates rapid nitrous acid diffusion across the phosphatidylcholine membrane of the liposomes at pH in the range 5.2-6.2. However, this probe does not allow acidity measurements at higher pH due to insignificant contribution of its protonated form at $pH \gg pK_a(AMS)$. Meanwhile, the measurement of the transmembrane nitrite transport at pH around 7.0 may be important because of its physiological relevance. It is also worth noting that the rate of the nitrite transfer in the form of conjugated acid is proportional to the concentration of nitrous acid, and, as a consequence, decreases with pH as the term 10^{pK_a-pH} .

^{31}P NMR spectroscopy of inorganic phosphate is a commonly used approach for pH measurements in biological fluids and tissues in the physiological pH range. Fig.4 shows the pH titration curve of the chemical shift of inorganic phosphate centered on $pK_{a2} = 6.9$ of phosphoric acid. Phosphate anion does not penetrate through lipid bilayer and is an ideal NMR probe for the study of nitrite-induced transmembrane proton transfer in the physiological pH range. In the liposomal preparations in phosphate buffer, an accurate measurement of the small phosphate peak from the intraliposomally located phosphate was strongly affected by overwhelmingly larger phosphate signal from extraliposomal volume (data not shown). To enhance spectral and functional (pH) resolution, phosphate buffer in outer volume was replaced with mixture of pyrophosphate and phosphate of the same pH and osmolarity. Figure 5A shows the ^{31}P NMR spectrum of typical liposome preparation with external buffer replaced with pyrophosphate. Two small peaks around chemical shift of 2 ppm were assigned to the inner (I) and outer (O) phosphates while much larger signal around -7 ppm (signal P) belongs to pyrophosphate. The minor splitting between inner and outer phosphate signals was due to imperfection of buffer preparation and corresponded to a small difference in pH of about 0.1 pH unit between inner and outer liposome phases. Addition of sulfuric acid, which does not

diffuse through the lipid bilayer, to the liposome suspension shifted strongly pyrophosphate signal (P) together with only one small signal (O) of phosphate to the right. At the same time another phosphate signal (I) did not change the position (fig.5B). This allows us to assign leftmost peak on panel B (I), to the phosphate encapsulated in the liposomes.

Addition of 4 mM NaNO_2 solution did not alter the position of the (O) signal of the extraliposomal phosphate but caused the shift in the position of signal (I) corresponding to acidification of the intraliposomal compartment (fig. 5, E and F). The effect was larger for the samples prepared with initial transmembrane pH gradient (Fig. 5C and D, changes in intraliposomal acidity, $\Delta\text{pH}_i = 0.40$) compared with that in the absence of initial ΔpH (Fig. 5E and F, $\Delta\text{pH}_i = 0.12$), in agreement with the EPR data.

Chemiluminescence detection of the intraliposomal nitrite accumulation

The above results support the ability of the nitrite to diffuse through the lipid bilayer in the form of nitrous acid. Chemiluminescence technique was used for the direct measurements of nitrite concentration in inner and outer liposome volume after nitrite addition. The results in Table 2 show significant accumulation of the nitrite in the inner aqueous space of the liposomes supporting its ability to diffuse through the lipid bilayer.

Moreover, higher accumulation of nitrite in the alkaline media supports its diffusion in the protonated form. Indeed, in this case, partitioning of the nitrite between inner and outer liposomal volume at the equilibrium is determined by the equality of the concentrations of nitrous acid across the lipid bilayer, namely $[\text{NO}_2^-]_o/[\text{NO}_2^-]_{in} = [\text{H}^+]_{in}/[\text{H}^+]_{out} = 10^{\text{pH}_{out}-\text{pH}_{in}}$ (See figure 6). This is in qualitative agreement with the observed nitrite accumulation in the areas with higher pH (Table 2). Quantitative agreement with calculated nitrite partitioning is hardly expected due to substantial amount of interliposomal media (supernatant) in liposomal pellet. This apparently causes underestimation (or overestimation, in case of lower intraliposomal pH) of the nitrite content in the pellet, but still correctly reflects the tendency of higher nitrite concentration in compartments with higher pH.

Thus, the nitrite penetration through biological membranes in the form of undissociated nitrous acid was experimentally verified for large unilamellar phosphatidylcholine liposome by several spectroscopic techniques. Nitrite-mediated intraliposomal acidification was confirmed by EPR spectroscopy using membrane-impermeable pH-sensitive nitroxide and by ^{31}P -NMR spectroscopy using inorganic phosphate. Direct measurement of the nitrite accumulation in the inner aqueous volume of the liposomes was performed using chemiluminescence technique. Note that nitrite-induced acidification for the chloroplast inner envelope vesicles and vesicles prepared from asolectin was reported previously [42] only in the presence of initial proton gradient. Our data demonstrate that pH gradient is not required for the transmembrane nitrite diffusion. However, transmembrane nitrous acid diffusion and equilibration promote nitrite accumulation in the areas with higher pH. The latter effect might have physiological relevance for the nitrite distribution in intracellular compartments, e.g. its preferable accumulation in mitochondria. Intracellular nitrite accumulation may be also affected by ischemia induced acidosis [21] contributing to the enhanced nitrite toxicity in ischemia reperfusion [11]. The diffusion of small nonelectrolytes across a lipid membrane varies with the membrane fluidity [39,51,52]. Liposomal membrane composed of egg phosphatidylcholine has comparably high fluidity and thus is easily permeable for the HNO_2 . On the other hand, the membrane permeability to HNO_2 could be significantly decreased in many cell membranes with high level of cholesterol[39,53], like the plasma membrane of erythrocytes. In this case other mechanisms of the nitrite transport may play a role. Many cell membranes have a protein (AE1) which typically exchanges bicarbonate for chloride [54], and supports the transmembrane exchange of other mono anions like NO_3^- and NO_2^- [37]. Proton-facilitated nitrite diffusion is proportional to the gradient of HNO_2 , and will transport HNO_2 into more alkaline

compartment, while AE1-assisted transmembrane exchange proportional to the gradient of NO_2^- and will shuttle NO_2^- back. Thus, the combination of the anion exchange mechanism with proton-facilitated HNO_2 diffusion could shuttle proton from more acidic compartments. In abnormal situations, e.g. high nitrite concentrations, ischemia-induced acidosis, etc., the diffusion/AE1 assisted transport couple may overcome the capacity of cellular regulatory mechanisms and result in loss of transmembrane pH gradient. This might be of physiological importance, e.g. resulting in dissipation of proton gradient in mitochondria contributing to the toxicity of the NO_2^- during ischemia-reperfusion [11,21].

The rate of the intraliposomal acidification is proportional to the inward flux of nitrous acid and is inversely proportionally to buffer capacity of intraliposomal compartment. In turn, the flux of nitrous acid is controlled by nitrous acid gradient, total surface area of the liposomes and membrane permeability coefficient, (P_{HNO_2}). Our data on heart perfusion with nitrite and nitrite-induced intraliposomal acidification provide low estimate of P_{HNO_2} being about 10^{-3} cm/s or larger. The published data [42] for the nitrite-induced dissipation of proton gradient in asolectin vesicles provides rough estimate of the permeability coefficient of the HNO_2 being about 10^{-2} cm/s. Therefore, not surprisingly, in our experiments we did not observe the kinetics of nitrite-induced intraliposomal acidification which proceeds within the time resolution of the applied analytical approaches.

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Figure 1.
pH sensitive membrane-impermeable probes for EPR (AMS) and ³¹P NMR (phosphate) spectroscopies.

Figure 2.

EPR spectra of 10 μ M AMS encapsulated into liposomes. **A.** Initial liposome suspension prepared in 10 mM phosphate buffer, pH, 6.2; **B.** Liposome suspension (**A**) 30 min after addition of 2.5 mM sulfuric acid; **C.** Liposome suspension (**B**) 0.5 min after addition of 2 mM sodium nitrite. Symbols RH^+ and R indicate high-field spectral components of the protonated and unprotonated radical forms, respectively. Dotted line shows calculated spectra as superposition of pure RH^+ and R forms with the fraction of RH^+ form equal to 0.6 (**C**) corresponding to pH value 5.2;

Figure 3.

EPR spectra of 10 μ M AMS encapsulated into liposomes. **A.** Initial liposome suspension prepared in 10 mM phosphate buffer, pH, 6.2; **B.** 30 sec after addition of 2 mM nitrite solution in 10 mM phosphate, with pH adjusted to 6.2; estimated from the EPR spectrum intraliposomal pH is equal to 6.0; **C.** 30 sec after addition of 10 mM nitrite solution in 10 mM phosphate, pH 6.2, to the sample (**A**), estimated intraliposomal pH is equal to 5.9; **D.** 30 sec after addition of 50 mM nitrite solution in 10 mM phosphate, pH 6.2, to the sample (**A**), estimated intraliposomal pH is equal to 5.8.

Figure 4.
Calibration curve for the ^{31}P NMR chemical shift of inorganic phosphate measured in 70 mM phosphate buffer.

Figure 5.

^{31}P NMR spectra of the liposome suspension prepared as described in Materials and Methods: (A) liposomes alone; (B) liposomes after addition of 9 mM sulfuric acid; (C) the same as (B); (D) 4 mM of sodium nitrite added to (C); (E), initial liposome suspension, the same as (A); (F) 4 mM of sodium nitrite added to (E). Large signal, P, corresponds to extraliposomal pyrophosphate; two small signals corresponding to outside and inside phosphate are shown in expanded view in the insert. The values of pH_i and pH_o calculated from the shifts of the corresponding NMR signals are shown on the panels.

Figure 6.

Schematic diagram of the nitrite partitioning across the phospholipid membrane assuming its diffusion in the form of undissociated acid. Added nitrite concentration (calculated over the total volume of the sample) is 4 mM, total liposome volume is 3% and initial pH equals 7.72. Inner and outer concentrations calculated based on pK_a of nitrous acid.

Table 1
Concentrations of the nitrite/nitrate in the myocardium perfused with 4.0 μM of NO_2^- in the perfusion buffer and effluent

	$[\text{NO}_2^-]^b$ in buffer, μM	$[\text{NO}_2^-]^c$ in effluent μM	$[\text{NO}_2^-]^b$ in heart, μM	$[\text{NO}_3^-]^b$ in buffer μM	$[\text{NO}_3^-]^c$ in effluent μM	$[\text{NO}_3^-]^b$ in heart, μM
Control heart	0.31 ± 0.05	0.21 ± 0.05	0.6 ± 0.1	0.63 ± 0.05	0.70 ± 0.1	7.8 ± 0.9
Nitrite-perfused heart	4.0 ± 0.05	2.9 ± 0.3	2.7 ± 0.3	0.65 ± 0.1	0.9 ± 0.4	22.0 ± 4

Table 2

pH values in inner and outer space of the liposome suspensions and corresponding nitrite concentration determined in the supernatant and in liposome pellet after centrifugation.

	$[\text{NO}_2^-]_{\text{in}}$, mM (pellet)	$[\text{NO}_2^-]_{\text{out}}$, mM (supernatant)	pH _{in}	pH _{out}
Liposomes + 4 mM NO_2^-	3.00±0.02	4.12±0.03	7.49	7.72
Liposomes + 4 mM NO_2^- + 9 mM H_2SO_4	5.29±0.03	3.86±0.01	7.17	6.72