Short-time non-enzymatic nitric oxide synthesis from L-arginine and hydrogen peroxide induced by shock waves treatment

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Abstract The evidence that nitric oxide (NO) production is possible by a non-enzymatic pathway has already been shown under restrictive experimental conditions. Here we show that NO can non-enzymatically be formed with short-time kinetics (min), by 'bombing' with shock waves a solution containing 1 mM hydrogen peroxide and 10 mM L-arginine. This procedure is widening its medical application with surprisingly positive effects in tissue regeneration and our finding could be one of the first steps for the understanding of the biochemical responsible for these therapeutical effects. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Shock waves; Non-enzymatic nitric oxide

1. Introduction

A shock wave (SW) [1] consists of an acoustic wave that generates a pressure impulse during 0.1–0.2 ms and is capable of determining a pressure gradient between +100 and −10 Mpa. Conveyed by an appropriate generator to a specific target area (focal area), the power created can be modulated in the range of 0.003–0.890 mJ/mm^2. In the last 15 years the clinic use of this technique has been significantly enlarged, especially for kidney and urinary calculus lithotripsy. Technological evolution in energy level control and in target area focusing has improved the clinical results, reducing at the same time undesired side-effects. Besides this 'primary' use, a few years ago a secondary anti-inflammatory effect in tendon and muscle tissues [2] and a complete recovery even in pseudoarthrosis pathologies were detected [2–5]. In particular, treatment of the tendon and muscle tissues was found to induce a long-time (1–4 months) tissue regeneration effect [5,6], besides a more immediate anthalgic and anti-inflammatory effect. This regenerative pathway seems to involve free radical production and revascularization events taking place in the SW-treated area [6].

Our idea is that one of the most important molecules involved in these therapeutic effects might be nitric oxide (NO). NO, normally produced in eukaryotes from L-arginine by different isoforms of NO synthase (NOS), exerts a potent and immediate vasodilatory action and modulates the subsequent angiogenesis [7,8]. Based on the evidence that the vasodilatory effect detectable in the area treated with SW is almost immediate, we decided to start our studies in an in vitro system, to verify first of all whether SW application could induce rapid and non-enzymatic formation of NO.

Information describing that NO can be produced without the catalytic activity of NOS has been already reported [9–12]. It includes both the non-enzymatic in vivo formation of NO due to the reaction of dietary/salivary nitrites with gastric acid [9], and the in vitro synthesis of nitrites in the solution containing L-arginine (10–20 mM) and hydrogen peroxide (10–50 mM) [12].

The present study was aimed at verifying if SW treatment elicits the non-enzymatic production of NO even under milder conditions than those previously described. Indeed, we performed the experiments at physiological pH in the presence of the amounts of H_2O_2 lower than those used by Nagase et al. [12] and in a shorter time (8 min) than that used in their system (up to 5 days) [12].

2. Materials and methods

2.1. Materials

L-Arginine, L-citrulline, glycine, urea were from Fluka; H_2O_2 was from Carlo Erba; nitrate reductase was from Roche; glutamate dehydrogenase from Boehringer, HCl from Merck; NaOH, sodium phosphate (NaP), ammonium acetate, α-ketoglutarate, naphtylethylenediamine dihydrochloride, sulfanilamide, carbon disulfide, *N*-methyl-D-glucamine and ferrous sulfate were from Sigma. *N*-methyl-D-glucamine dithiocarbamate (MGD) sodium salt was synthesized by the method of Shinobu et al. [13]. Stock solutions of Fe^{2+}–$(MGD)_2$ were prepared by dissolving MGD sodium salt and ferrous sulfate in nitrogen-purged double distilled water, molar ratio 5:1.

2.2. SW treatment

An electromagnetic MODULITH SLK SW device (provided by Storz Medical AG) was used throughout. The instrument was equipped with a support for Greiner 2-ml test-tubes, to allow for the correct alignment of the focal point with the test-tube. All experiments were performed in solutions buffered to pH 7 with NaP.

Samples were thermally regulated at 37°C before and during the SW treatments (500–2000 SW shots; two shots/s, so the time course ranged between 4 min 10 s and 32 min), and put in ice after the end of the treatment, to stop the course of the reaction, and kept at −20°C

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Abbreviations: SW, shock wave; NOS, nitric oxide synthase; MGD, *N*-methyl-D-glucamine dithiocarbamate; DAN, 2,3-diaminonaphthalene

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Table 1
SW-elicited production of nitrites

Parameters	Samples series				
	1	2	3	4	5
[L-Arg], mM	1	10	1	10	10
[H_2O_2], mM	0.1	0.1	1	1	10
[NO_2^-], nM ± S.D.	(traces) < 5 (n = 10)	11 ± 5 (n = 10)	0 (n = 10)	86 ± 21 (n = 15)	0 (n = 10)

The amounts of nitrites produced in the solution containing L-arginine and H_2O_2 were measured by fluorometric method after 1000 shots of SW at the energy level of 0.89 mJ/mm^2. n = number of experiments.

or in liquid nitrogen until use. The blank samples (without SW treatment) were thermally treated in the same way.

2.3. Spectroscopic measurement of NO_2^- and NO_3^-

Nitrites, NO_2^-, were determined either spectrophotometrically with the Griess reactant [14], forming a colored product that can be optically detected at 541 nm, or fluorometrically, following the formation of a fluorescent product, due to the reaction of NO_2^- with 2,3-diaminonaphthalene (DAN) [15,16]. Nitrates, NO_3^-, were determined with the Griess reagent [14] after their enzymatic reduction to nitrites.

2.4. Measurement of NO

NO was measured by the spin trapping technique, using the Fe^{2+}–$(MGD)_2$ complex [17]. Briefly, 2 mM Fe^{2+}–$(MGD)_2$ (final concentration expressed in terms of iron content) was added to the incubation mixtures just before performing the SW treatment. The mixtures were immediately frozen at the end of the experiment and lyophilized. They were then redissolved in a smaller volume to increase sensitivity, and EPR spectra were recorded at 100 K to observe the typical nitrosyl–iron triplet signal arising from trapped ^{14}NO. X-band EPR spectra were measured on a Bruker EMX spectrometer at the Nicox Research Institute, Bresso, Milan, Italy.

3. Results and discussion

To evaluate the non-enzymatic synthesis of NO elicited by SW treatment, solutions containing 1–10 mM L-arginine and 0.1–10 mM H_2O_2 were treated with 1000 shots of SW, and nitrite levels in the solution were estimated by a fluorometric method [16]. The rationale for choosing millimolar concentrations of H_2O_2 is based on the assumption that hydrogen peroxide transiently rises to such levels during inflammatory events, as it can be deduced by several data existing in the literature [18], although H_2O_2 is rapidly dismutated by catalase under physiological conditions. As shown in Table 1, SW elicited a rapid (8 min of SW treatment) and significant production of nitrites (86 ± 21 nM) only in the solution containing 10 mM L-arginine and 1 mM H_2O_2 (sample 4), suggesting that SW treatment could lead to the non-catalytic synthesis of physiopathologically relevant amounts of NO under appropriate conditions. It should be noted that these conditions are far milder than those tested by Nagase et al. [12] and may more reliably mimic the early phase of an acute inflammatory process.

In order to determine the entire production of NO, nitrates were estimated after their enzymatic reduction to nitrites. Since the fluorometric standard measurements gave no reproducible results, probably because of the presence of a 'quencher' in the system used, the estimation of nitrites plus nitrates was made with the Griess method [14]. Unfortunately, the

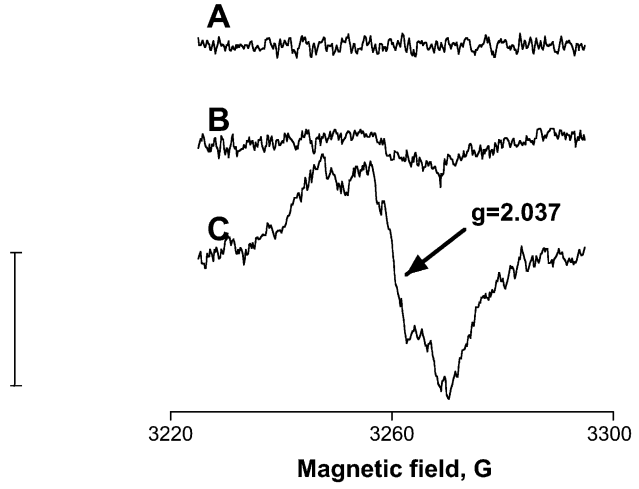

Fig. 1. EPR spectra of a solution of (A) 1 mM H_2O_2, 2 mM Fe^{2+}–$(MGD)_2$; (B) 10 mM L-arginine, 1 mM H_2O_2, 2 mM Fe^{2+}–$(MGD)_2$; (C) 10 mM L-arginine, 1 mM H_2O_2, 2 mM Fe^{2+}–$(MGD)_2$ after treatment with SW (1000 shots, 0.89 mJ/mm^2). EPR settings were: frequency, 9.31 GHz; microwave power, 1 mW; modulation amplitude, 2 G; temperature, 100 K.

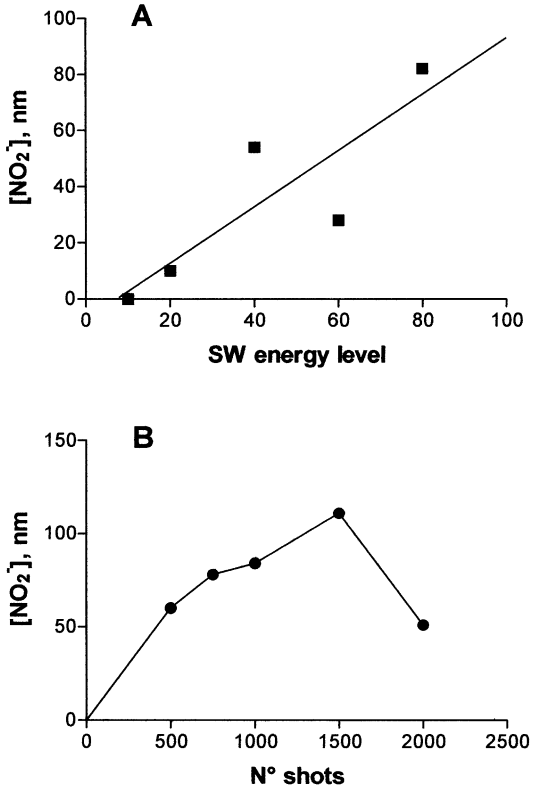

Fig. 2. Production of nitrites in the the presence of 10 mM L-arginine and 1 mM H_2O_2 at constant number (1000) of shots of increasing energy levels of SW (A), and at constant energy (0.89 mJ/mm^2) of increasing number of shots (B). The experiments were performed using three identical samples for each condition tested (triplicate measurements/sample). Standard deviations were comprised in the range of 8.5–19.4%, for all values.

spectrophotometric assay turned out to be not enough sensitive to detect the production of nitrites.

A direct production of nitrites from arginine without the intermediate formation of NO could not be ruled out a priori under our experimental conditions. Therefore, the experiment was also performed in the presence of Fe^{2+}–$(MGD)_2$, an established water-soluble NO spin trap. As shown in Fig. 1, a signal centered at $g = 2.037$ and with a definite triplet lineshape, typical of the nitrosyl adduct between ^{14}NO and Fe^{2+}–$(MGD)_2$ [19] was observed in the sample containing 10 mM L-arginine and 1 mM H_2O_2 and subjected to SW treatment (Fig. 1C). On the other hand, only a very weak resonance was measured in the absence of SW treatment (Fig. 1B), and no signal at all was present when L-arginine was omitted from the mixture (Fig. 1A).

To further investigate if NO could be formed not only from L-arginine but also from other potential nitrogen donors, solutions containing 10 mM L-citrulline, L-valine, glycine or urea were treated, in the presence of 1 mM H_2O_2, with 1000 shots of SW at the highest energy level (0.89 mJ/mm^2). The results obtained indicated that SW treatment was unable to produce detectable amounts of nitrites in any of these solutions (data not shown). Given that the presence of nitrites reveals the formation of NO, we can conclude that SW-elicited production of NO appears to require, as does NOS, L-arginine as a specific nitrogen donor. The understanding of the chemical pathways leading to the non-enzymatic synthesis of NO from L-arginine in the presence of H_2O_2 after SW treatment awaits further elucidation.

Finally, the estimation of non-enzymatic formation of NO was performed in the solution containing 10 mM L-arginine and 1 mM H_2O_2 to study the effect caused by applying (i) different SW energy levels, keeping constant the number of shots (1000), and (ii) different number of shots, at constant SW energy level (0.89 mJ/mm^2). As shown in Fig. 2, SW treatment elicited energy-dependent production of nitrites (Fig. 2A), and SW-elicited production of nitrites increased in dependence on the number of shots up to 1500 (Fig. 2B).

The present study demonstrates that the fluorometric DAN method is sensible enough to estimate the concentrations of nitrites up to 10 nM, and that SW treatment elicits a rapid (8 min of treatment) production of NO (as unequivocally shown by EPR) in the solution containing 10 mM L-arginine and 1 mM H_2O_2. The estimated amounts of nitrites (86–108 nM) are compatible with those of NO under physiopathological conditions, although this value should probably be underestimated, because of the possible transformation of nitrites to nitrates during the experimental process. These data are, on the one hand, in line with those reported by Nagase et al. [11], indicating the non-catalytic synthesis of NO in the solution containing L-arginine and H_2O_2. On the other hand, they have been obtained under far milder experimental conditions (higher concentration in both L-arginine and H_2O_2) than the results of Nagase et al. [12].

In conclusion, SW treatment, under conditions mimicking physiopathological situations (i.e. 10 mM L-arginine and 1 mM H_2O_2), induced non-enzymatic production of physiologically relevant amounts of NO (108 ± 16 nM, 1500 shots at the highest energy applied in 12.5 min, see Fig. 2B), as a function of both the number of shots and the applied energy levels. Therefore, the results presented here, even if obtained by using an extremely simplified in vitro system, may represent, mutatis mutandis, a first step to understand the biochemical events that underlie the clinically observed beneficial effects of SW treatment in some inflammatory syndromes.

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