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Pivotal participation of nitrogen dioxide in L-arginine induced acute necrotizing pancreatitis: protective role of superoxide scavenger 4-OH-TEMPO

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Abstract

For the first time, a direct sensitive method of $^{\cdot}NO_2$ detection and measurement in biological material has been established. It is based on the interaction of this radical with the coordination compound of Cr(III) with aminodeoxysugar as biosensor. Our new method makes it possible to precisely assess $^{\cdot}NO_2$ level in experimental acute necrotizing pancreatitis induced by L-arginine, where oxidative and nitrosative stresses are supposed to play a key role in the pathomechanism of the disease. As much as 20 nmol of $^{\cdot}NO_2$/mg protein was detected which correlated with severe deterioration of pancreatic acinar cell ultrastructure. Protective effect of superoxide radical scavenger 4-OH-TEMPO expressed as $^{\cdot}NO_2$ level decrease confirmed by preserved acinar cell ultrastructure and decreased pancreatic amylase release to blood serum is demonstrated. This study reveals a possible pathomechanism of L-arginine induced acute pancreatitis.

Keywords: Nitrogen dioxide; NO_2; L-Arginine; Acute pancreatitis; Superoxide; 4-OH-TEMPO; Kinetics; Mechanism; Chromium(III) complexes; Aminodeoxysugars

It has been demonstrated that excessive doses of L-arginine (Arg) induce acute necrotizing pancreatitis (AP) [1]. The mechanism of selective destruction of pancreatic acinar cells by Arg has not been fully elucidated yet. There is accumulating evidence that reactive oxygen species (ROS) [2] and reactive nitrogen species (RNS) [3] trigger the development of the disease.

A particular role played by various oxidants in AP is still poorly understood. It is not clear whether ROS in AP serve principally as upstream or downstream inducers or effectors. It is also not known if ROS act alone or synergistically with RNS. Moreover, reactive nitrogen and oxygen species (e.g., $^{\cdot}NO$, $^{\cdot}NO_2$, and $O_2^{\cdot-}$) may be generated at different times during cellular response or in different compartments within acinar cells. Very little is known of the spatial and temporal regulation of RNS and ROS in AP.

Nitric oxide ($^{\cdot}NO$), synthesized from the guanidine group of Arg, has been identified as a possible mediator

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in inflammation. Oxidation products of ·NO such as NO_2^- and NO_3^- while elevated after pretreatment of rodent pancreas with lipopolysaccharide and Arg have been decreased in the presence of D-arginine or N^G-nitro-L-arginine (L-NNA)—·NO synthase inhibitor, respectively [4].

Nitrogen dioxide ($^{\cdot}NO_2$) belongs to a group of powerful oxidants [5] and possessing an unpaired electron can be classified as the free radical [6]. It is identification as a key biological oxidant provided enormous implications in the understanding of the chemistry of cellular oxidative and nitrosative stresses [7]. $^{\cdot}NO_2$ can stimulate protein tyrosine oxidation primarily generating phenoxyl radical. The phenoxyl radical can react further with $^{\cdot}NO_2$ to form nitrotyrosine which was found to be a predominant final product in neutral solution. $^{\cdot}NO_2$ induced oxidation of cysteine–thiolate involving the transient formation of cysteinyl radical anions even faster than that of tyrosine [8]. $^{\cdot}NO_2$ has been found to inhibit the dimeric cytosolic creatine kinase (CK) by interacting with the crucial SH group of cysteine 282 of CK [9]. It is not yet clear which targets of attack of $^{\cdot}NO_2$ on protein molecule are the most important in vivo. It is supposed to be biologically important for the development of Arg induced AP. $NO_2^{\bullet}$ concentration depends critically on the steady-state concentration of ·NO and $O_2^{\bullet -}$. Reaction between them yields peroxynitrite which as conjugated acid dissociate (pK_a close to 7) at near diffusion controlled rate, to yield in part $^{\cdot}NO_2$. The estimated lifetime of $^{\cdot}NO_2$ is <10 μs, reflecting both the concentration and compartmentalization of reactive targets and the reactivity of $^{\cdot}NO_2$ towards them [10].

Our previous study demonstrated the protective effect of superoxide scavenger 4-OH-TEMPO on free radical toxicity in experimental pancreatitis [11]. It has been found that 4-OH-TEMPO reduces dinitrobenzene sulfonic acid-induced colitis and appearance of nitrotyrosine as a marker of nitrosative stress [12]. In rodent model of carrageenin-induced pleurisy 4-OH-TEMPO scavenges and inactivates superoxide anions thereby preventing the formation of peroxynitrite [13].

The highly protective effect of 4-OH-TEMPO is poorly defined at mechanistic level so in order to substantiate the necessity of superoxide for the formation of precursor of $^{\cdot}NO_2$ namely $ONOO^-$, we decided to detect $^{\cdot}NO_2$ directly in the model of Arg induced AP. Consequently, in the present study the formation of $^{\cdot}NO_2$ during Arg induced AP and implication of superoxide elimination on $^{\cdot}NO_2$ level were investigated.

The primary purpose of these studies was to design a convenient biosensor making possible the direct assessment of $^{\cdot}NO_2$ in the biological sample in the presence of ·NO in order to establish a real impact of $^{\cdot}NO_2$ on the development of acute necrotizing pancreatitis induced by L-arginine. The kinetic measurements of the nitrogen dioxide uptake carried out by using the stopped-flow technique for *cis*-$[Cr(C_2O_4)(AaraNH_2)(OH_2)_2]^+$ ion allowed us to establish the mechanism of substitution reactions of this ionic compound in aqueous solution.

The kinetic model developed in this way was reproduced in successive measurements in which $^{\cdot}NO_2$ was generated from SIN-1 [14–16] as a direct source of nitrogen dioxide. The use of this sole reagent enabled us to approximate our experiment to conditions of $^{\cdot}NO_2$ generation in biological systems.

Materials and methods

Reagents. Methyl 3-amino-2,3-dideoxy-α-D-*arabino*-hexopyranoside ($AaraNH_2$) and *cis*-$[Cr(C_2O_4)(AaraNH_2)(OH_2)_2]^+$ were synthesized according to procedures described in [17,18] and [19], respectively. The final uptake product, *cis*-$[Cr(C_2O_4)(AaraNH_2)(ONO_2)]^0$ was characterized according to procedure described in [20]. Buffer solutions for spectrophotometric measurements were prepared according to procedures described elsewhere [19,21].

Spectroscopic measurements. Electronic absorption spectra were recorded using a Perkin–Elmer Lambda 18 Instrument with the scan accuracy of 1 nm, 1 nm slit width, at a scanning rate of 120.00 nm min^{-1}. Kinetic measurements were carried out using a stopped-flow technique and an Applied Photophysics SX-17 MV spectrophotometer. The observable rate constants were computed using a "Glint" program based on global analysis [22–25].

Determination of nitric dioxide concentration. Solution of the complex was prepared by mixing *cis*-$[Cr(C_2O_4)(AaraNH_2)(OH_2)_2]^+$ (0.5 mL, 1 mmol) with solutions of Mes (2 mL, 0.2 M) and $NaClO_4$ (2 mL, 2 M). The temperature was maintained at 20 °C with an accuracy of ± 0.1 °C. The concentrations of $^{\cdot}NO_2$ uptake in the studied probes containing cytosol rate were defined on the UV–Vis absorption basic, at an ionic strength of $I = 1$ M ($NaClO_4 + HClO_4$ and $[^{\cdot}NO_2] \gg [Cr]_T$). Linear relationship between absorption and concentration of $^{\cdot}NO_2$ in phosphate buffers at pH 7.3 was determined. Samples were prepared by addition of appropriate amounts of the SIN-1 solution and completion of the appropriate buffer to final 3 mL volume. The concentrations of $^{\cdot}NO_2$ were computed by Origin 6.0 program, based on absorbance variations at a selected 541 nm wavelength, using non-linear least squares method [26]. Global analysis of the data acquired was performed for different reaction models.

Animals, experimental groups. The experiments performed were approved by the Animal Care Ethic Committee of Medical University of Gdańsk. Male Wistar rats weighing 250 g were used. The animals were kept at a constant temperature of 25 °C with a 12 h light–dark cycle, and they were allowed free access to water and standard laboratory chow. The rats were fasted for 16 h before injections, while access to water was kept until euthanasia. Four experimental groups were established:

control ($n = 9$) received 0.9% NaCl (*ip*);

injected (*ip*) with solution of Arg/HCl (pH 7.4) at dose 3 g of free base/kg body weight (b.w.) ($n = 8$);

4-OH-TEMPO 20 μg in 0.5 mL of 0.9% NaCl and 30 min after Arg 3 g/kg b.w. as previous ($n = 12$),

4-OH-TEMPO only at dose 20 μg ($n = 5$). After 24 h, under anesthesia, blood samples were collected from the heart and animals were killed by exsanguination.

Samples for microscopic examination. The pancreata were removed and immediately cooled on ice, cleaned from fat and lymphatic nodes, and weighed. Pancreatic tissue fragments measuring about 1 mm in