Peroxynitrite-mediated oxidation of dihydrorhodamine 123 occurs in early stages of endotoxic and hemorrhagic shock and ischemia-reperfusion injury

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Abstract To quantify peroxynitrite production during shock, we measured oxidation of dihydrorhodamine 123 in rats. In endotoxic and hemorrhagic shock and splanchic ischemia-reperfusion, dihydrorhodamine oxidation rapidly increased, which was prevented by inhibition of endothelial nitric oxide (•NO) synthase (ecNOS). Thus, peroxynitrite is already formed at early stages of shock from ecNOS-derived •NO. Overproduction of •NO by the inducible NOS at late shock was not associated with additional increases in dihydrorhodamine oxidation. ecNOS inhibition *enhanced* dihydrorhodamine oxidation in control rats. These latter findings may be explained by •NO-mediated inhibition of peroxynitrite-induced dihydrorhodamine oxidation, a phenomenon also observed in vitro.

Key words: Peroxynitrite; Nitric oxide; Endotoxin; Shock; Inflammation; Nitric oxide synthase; Constitutive; Rhodamine; Superoxide

1. Introduction

Peroxynitrite, a potent, reactive oxidant, is formed by the reaction of nitric oxide (•NO) with superoxide anion (O_2^-) [1,2]. The production of peroxynitrite has been demonstrated or proposed in a variety of pathophysiological conditions, including septic shock [3,4], arthritis [5], acute respiratory distress syndrome [6,7], atherosclerosis [8] ischemia-reperfusion [9,10], inflammatory bowel disease [9,11] and MTPT-mediated neurotoxicity [12].

There are limited possibilities for the measurement of peroxynitrite in vivo. Most investigators use semi-quantitative immunohistochemical methods, based on the measurement of nitrotyrosine immunoreactivity, which is based on the specific reaction of peroxynitrite with tyrosine residues of proteins [1,2,6,7,13]. The oxidation of dihydrorhodamine 123 to rhodamine has been used in in vitro experiments to measure the production of peroxynitrite [14]. In the present study, we have made an attempt to utilize the oxidation of dihydrorhodamine 123 to rhodamine to estimate the formation of biologically active peroxynitrite in vivo. This method, coupled with the use of pharmacological inhibitors of •NO synthase (NOS), allowed us to investigate the time course of peroxynitrite formation in rat models of endotoxic and hemorrhagic shock and splanchnic ischemia-reperfusion injury. Our data demonstrate that the formation of peroxynitrite already occurs at early stages of endotoxic and hemorrhagic shock and reperfusion after splanchnic ischemia.

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2. Materials and methods

2.1. Materials

Bacterial lipopolysaccharide (*E. coli*, serotype No. 0127:B8), diethylamine and N^G-nitro-L-arginine methyl ester (L-NAME) were obtained from Sigma (St. Louis, MO). *S*-Methyl-isothiourea sulfate (SMT) was obtained from Aldrich (St. Louis, MO). Diethylamine:NO was obtained from RBI (Natick, MA). Dihydrorhodamine 123 (DHR 123) and rhodamine were obtained from Molecular Probes (Eugene, OR).

2.2. Animals and experimental protocols

Male Wistar rats (Charles River Laboratories, Wilmington, MA) were anesthetized with sodium thiopentol (120 mg/kg, i.p.) and instrumented as described [15]. The trachea was cannulated to facilitate respiration and rectal temperature was maintained at 37°C using a homeothermic blanket. The right carotid artery was cannulated and connected to a pressure transducer for the measurement of phasic and mean arterial blood pressure (MAP) and heart rate which were digitalized using a Maclab A/D converter (AD Instruments, Milford, MA) and stored and displayed on a Macintosh personal computer. The left femoral vein was cannulated for the administration of drugs. Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilize for 10 min.

To induce endotoxin shock, separate groups of animals were injected with *E. coli* endotoxin (15 mg/kg i.v.) for 1, 2 or 3 h. At these time points, separate groups of rats received an i.v. bolus injection of dihydrorhodamine 123 (DHR 123; 2 μmol/kg in 0.3 ml saline). At 20 min after the injection of DHR, animals were sacrificed, blood was collected into heparinized tubes and rhodamine fluorescence was measured as described below. To inhibit ecNOS, the NOS isoform present at the early phase of shock (at 1 h after endotoxin) [15], separate groups of animals were treated with L-NAME (20 mg/kg i.v.) 10 min prior to the injection of DHR 123. At 2 h and 3 h after endotoxin, to inhibit both ecNOS and iNOS, animals received a high dose (10 mg/kg i.v.) of *S*-methyl-isothiourea sulfate (SMT), a potent inhibitor of NOS [16] 10 min prior to the injection of DHR 123. In each group, animals were sacrificed 20 min after the injection of DHR 123.

To induce hemorrhagic shock, blood was withdrawn to reduce MAP to 50 mmHg [17]. MAP was maintained at this level by further withdrawal or re-injection of blood. DHR 123 was injected, in separate groups of rats, at 1 h and at 3 h after the initiation of shock and animals were sacrificed after an additional 20 min. To inhibit ecNOS, the NOS isoform present at 1 h of hemorrhagic shock [17] a separate group of rats were treated with L-NAME (20 mg/kg i.v.), 5 min prior to the injection of DHR 123. At 3 h of hemorrhagic shock, in a separate group of rats, a high dose of SMT (10 mg/kg) was used to inhibit both ecNOS and iNOS. In each group, animals were sacrificed 20 min after the injection of DHR 123.

To induce splanchnic ischemia and reperfusion, the mesenteric artery was clamped for 45 min, followed by release of the clamp (reperfusion) [18]. In this model, DHR 123 was injected at 25 min after the occlusion or at 25 min after the release of the clamp. In separate groups of rats, ecNOS was inhibited with L-NAME (20 mg/kg i.v. as above) 10 min

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prior to the injection of DHR 123. Animals were sacrificed 20 min after the injection of DHR 123.

In separate groups of control rats (without shock), DHR 123 was injected as described and samples were taken 20 min thereafter. This procedure was repeated in control rats pretreated with L-NAME (20 mg/kg i.v.) for 10 min.

In all groups of rats, animals received only one injection of DHR 123 and all animals were sacrificed at 20 min after its injection; $n = 4$–6 rats were used in all groups.

2.3. Measurement of plasma rhodamine levels

After obtaining plasma samples from the various groups of rats, fluorescence of rhodamine (an oxidation product of DHR 123 and a marker of exposure of DHR 123 to peroxynitrite) was measured in a Perkin-Elmer fluorimeter (excitation 500 nm, emission 526 nm, slit widths 2.5 and 3.0 nm, respectively) [14]. In subgroups of the plasma samples (in samples obtained from control rats and in rats after 3 h of endotoxin shock), plasma was exposed to various concentrations of peroxynitrite in vitro to determine maximal fluorescence. We have found that in vitro exposure to peroxynitrite (100 μM, 30 min) was still able to markedly increase fluorescence (to approx. 100 fluorescence units from approx. 4–10 units, $n = 3$), indicating that under the conditions used, the oxidation of DHR 123 was submaximal in vivo. In a separate set of experiments, we have used hydrogen peroxide and peroxynitrite to completely oxidize DHR 123 to rhodamine in vitro in order to determine plasma levels of DHR 123 at 20 min after its injection. Incubation of the plasma samples with 50 μg/ml horseradish peroxidase (HRP) and 250 μM hydrogen peroxide for 2 h at 37°C, followed by incubation with 250 μM peroxynitrite for 1 h at 37°C caused maximal oxidation of DHR 123. Plasma levels of DHR 123 in the rats amounted to 1.07 ± 0.12 μM ($n = 6$), thus providing a concentration comparable for the ones used to measure peroxynitrite in in vitro studies (see also [14]). The amounts of rhodamine formed in vivo were quantified by using a rhodamine standard curve (1–30 nM) prepared in plasma obtained from untreated rats. Background plasma fluorescence was subtracted from all samples.

2.4. Effect of •NO on peroxynitrite-mediated oxidation of DHR 123 in vitro

In a separate set of studies, the effect of •NO on the peroxynitrite-mediated oxidation of DHR 123 was studied in vitro. These studies were performed in phosphate-buffered saline (PBS) containing 100 μM diethylenepentaacetic acid (DTPA), pH 7.4. Nitric oxide was generated by the dissolution of the •NO donor NONO-ate compound diethylamine:NO. The oxidation of DHR 123 by peroxynitrite (1 μM) or hydrogen peroxide (1 μM) plus 25 μg/ml HRP in the presence of various concentrations of the •NO donor to generate •NO at rates of 0.1, 1 μM/min and 10 μM/min, was measured by the change in absorbance at 500 nm ($\varepsilon = 78{,}000\ M^{-1} \cdot cm^{-1}$) after 30 min incubation at 37°C [19].

2.5. Statistical evaluation

Values are expressed as mean±standard error of the mean of n observations. In vivo, $n = 4$–6 animals were used in each time point; in the in vitro studies, $n = 3$–6 samples were used. Student's unpaired t-test was used to compare means between groups. A P-value less than 0.05 was considered to be statistically significant.

3. Results

In normal, control rats, inhibition of ecNOS by L-NAME caused a significant, approximately two-fold increase in the rhodamine fluorescence in the plasma (Fig. 1). Injection of endotoxin caused a significant (approximately six times) increase in rhodamine fluorescence within 1h (Fig. 1a). The increased rhodamine fluorescence persisted at 2 h and 3 h after endotoxic shock (Fig. 1a). The increase in DHR 123 oxidation during 1–3 h following endotoxin treatment was prevented by inhibition of NOS (with L-NAME at 1 h or with SMT at 2 or 3 h) (Fig. 1a).

In hemorrhagic shock, similarly to endotoxic shock, we observed a 9-fold increase in the rhodamine fluorescence in the plasma as early as 1 h after the initiation of bleeding (Fig. 1b), and the increased fluorescence persisted at 3 h after hemorrhagic shock. Inhibition of NOS reduced rhodamine fluorescence both in the early and delayed phase of hemorrhagic shock (Fig. 1b).

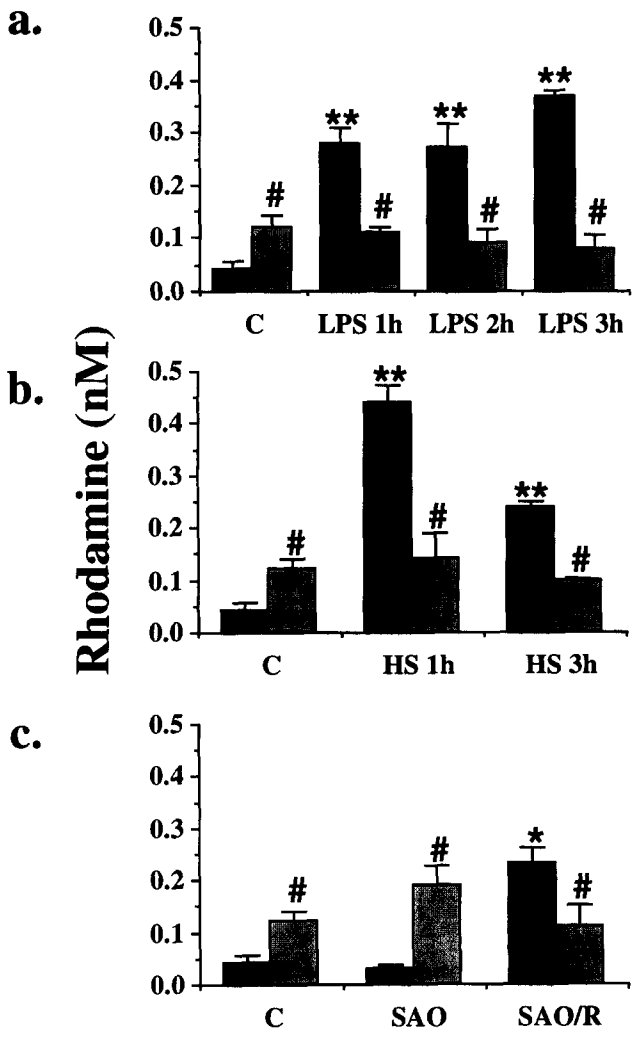

Fig. 1. Plasma levels of rhodamine in control rats (C), in rats at 1, 2 and 3 h after bacterial lipopolysaccharide (LPS) (a), at 1 and 3 h after hemorrhagic shock (HS) (b), and in the ischemic (SAO) and reperfusion (SAO/R) phase of splanchnic artery occlusion/reperfusion shock (c). Closed bars represent the rhodamine levels without NOS inhibitors, open bars depict rhodamine levels in the presence of NOS inhibitors (see Methods). *,**Represent significant increase in rhodamine fluorescence in shock ($P < 0.05$, $P < 0.01$, resp.); #Represents significant effect of the NOS inhibitor at the same time point ($P < 0.05$).

In splanchnic ischemia/reperfusion, no marked increase in the rhodamine fluorescence was observed at the early (ischemic) phase of shock, but there was a four-fold increase in the reperfusion phase, which was inhibited by L-NAME (Fig. 1c).

The finding that L-NAME caused an increase in the fluorescence of rhodamine in the plasma samples of control animals, led us to investigate the effect of •NO on the peroxynitrite-mediated oxidation of DHR 123 in vitro. We observed that a ten-fold excess of •NO (generated by the •NO donor com-

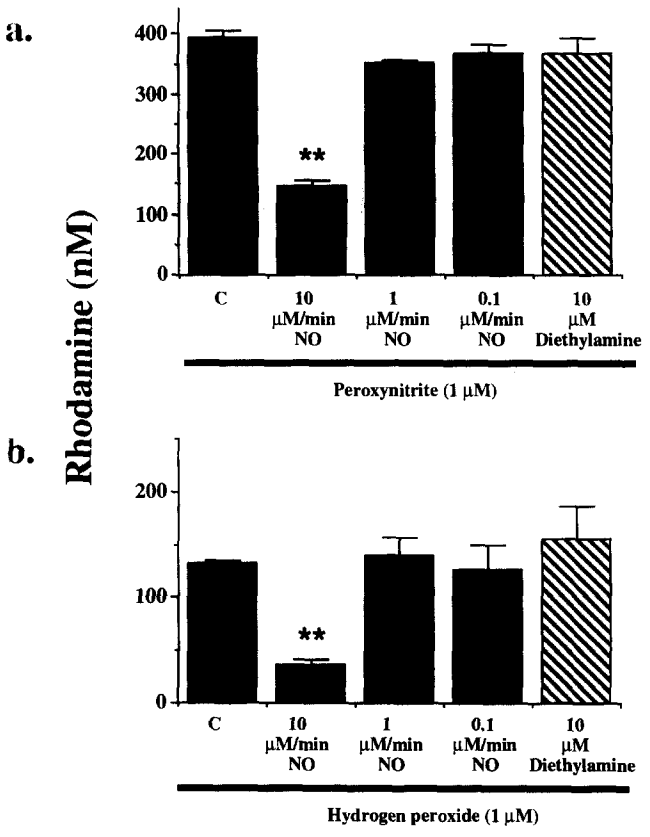

Fig. 2. Effect of •NO in the oxidation of 10 mM DHR 123 by chemically synthesized peroxynitrite (a) or hydrogen peroxide plus HRP (b) in PBS containg 100 μM DTPA, pH 7.4 for 30 min at 37°C. •NO was generated by diethylamine:NO and diethylamine was used as control. The oxidation of DHR 123 to rhodamine was measured by the change in absorbance at 500 nm. **Represents significant inhibition of the oxidation of DHR 123 by •NO ($P < 0.01$).

pound diethylamine:NO) causes a marked suppression of the oxidation of DHR 123 by peroxynitrite (Fig. 2a). Lower concentrations of •NO, however, did not cause such inhibition (Fig. 2a). Similarly, a ten-fold excess of •NO inhibited the hydrogen peroxide/HRP-induced oxidation of DHR 123 (Fig. 2b).

4. Discussion

A substantial increase in nitrotyrosine immunoreactivity, a specific marker of peroxynitrite has been demonstrated in the delayed phase of endotoxic shock [3,4]. The oxidation of DHR 123 by peroxynitrite has been successfully utilized for the measurement of peroxynitrite formation in vitro [4,14]. The method is sensitive and specific, since neither •NO nor superoxide causes DHR 123 oxidation [14]. However, it is noteworthy that, in addition to peroxynitrite, hydrogen peroxide in the presence of horseradish peroxidase, and superoxide plus hydrogen peroxide in the presence of a metal catalyst are known to oxidize DHR 123 to rhodamine [20].

Here we monitored the NOS-inhibitor inhibitable oxidation of DHR 123 in the plasma of rats subjected to shock and we found an increase in the oxidation of DHR 123 to rhodamine already in the early phase. The enhanced formation of peroxynitrite, in these early stages of shock is: (1) due to an •NO-related species, since it is inhibited by NOS inhibitors; (2) but not due to •NO, as •NO alone does not increase oxidation of DHR 123 [14]; and (3) therefore it is presumably due to peroxynitrite. Within 1 h of endotoxic or hemorrhagic shock, peroxynitrite must be formed by a reaction of superoxide (produced by activated neutrophils, macrophages, endothelial cells) with •NO derived from the *constitutive*, endothelial isoform of NOS (ecNOS), since expression of iNOS does not occur until later stages of shock [15–17,21,22]. Thus, peroxynitrite is formed at a very early stage of various forms of shock; its precursor, •NO, is derived, at this stage, from ecNOS rather than iNOS, and thus the rate limiting factor in the production of peroxynitrite in shock may be superoxide, rather than •NO.

At later stages of endotoxic and hemorrhagic shock, such as at 3 h, we have previously confirmed the induction of iNOS in the shock models used in the present study [15–17,21,22]. Increased oxidation of DHR 123 to rhodamine persisted at these later stages of shock and could be inhibited by inhibition of NOS. Unexpectedly, however, we did not observe a massive increase in rhodamine at these later stages.

This latter finding, and the observation that the oxidation of DHR 123 is *enhanced* by L-NAME in control animals, may be explained, in part, by our in vitro results, demonstrating that the peroxynitrite-mediated and the hydrogen peroxide/HRP-mediated oxidation of DHR 123 is inhibited by •NO. A likely explanation is the following: oxidation of DHR 123 by either peroxynitrite or H_2O_2 in the presence of HRP proceeds via a one-electron oxidation to form a radical-DHR 123 intermediate. A second one-electron oxidation will form the final oxidation product rhodamine. The presence of •NO in excess over either peroxynitrite or H_2O_2 would reduce the oxidation of DHR 123 because •NO would react with the one electron intermediate to give back the original compound plus nitrite. Similar, •NO-mediated inhibition of peroxynitrite-mediated lipid peroxidation has been recently described [23]. It is noteworthy that the peroxynitrite-mediated development of endothelial dysfunction can be inhibited by •NO donor compounds [24], and peroxynitrite-mediated cytotoxicity in macrophages is inhibited by •NO donors in vitro (authors' unpublished).

Although NO-mediated inhibition of DHR 123 oxidation is an attractive hypothesis to explain the increase in DHR 123 oxidation by L-NAME in control rats and the lack of massive increase in rhodamine oxidation in later stages of shock, other possibilities also have to be considered. In perfused mesentery, similar to our observation, oxidation of DHR 123 increases after inhibition of ecNOS by L-NAME [25], an effect, which has been attributed to increased leukocyte activation and hydrogen peroxide production [25] In addition, in the delayed state of shock, peroxynitrite and other oxidants may increase cell permeability which may result in an increase of rhodamine trapped in the tissue, thereby reducing the levels of rhodamine detected in the plasma.

Our data suggest that peroxynitrite is present and may be injurious already at earlier stages of shock, when •NO is not yet produced in excessive amounts. Peroxynitrite-mediated injury may explain the protective effect of L-NAME and ecNOS inhibition in conditions associated with superoxide overproduction, such as paraqat mediated lung injury [26], or myocardial ischemia-reoxygenation injury [27], and cerebral ischemia-reperfusion [28] where inhibition of ecNOS has beneficial effects. At later stages of shock, excess •NO may reduce the

peroxynitrite-mediated injury, but, large amounts of •NO may directly cause injury. The complex interactions between •NO and peroxynitrite in vitro and in vivo, require further investigations.

The early formation of peroxynitrite necessitates the introduction of different therapeutic strategies, especially in the early stage of shock. Peroxynitrite has cytotoxic actions that may be, in part, different from that of •NO — involving effects leading to endothelial dysfunction [24], inhibiting key enzymes of the mitochondrial respiratory chain [29], causing DNA strand breaks [30] and depleting intracellular high energy phosphate levels due to activation of energy-consuming DNA repair processes [30].

Thus, the oxidation of dihydrorhodamine is increased in shock and ischemia/reperfusion. Measurement of NOS-inhibitor-inhibitable oxidation of dihydrorhodamine may be useful in investigating peroxynitrite formation in pathophysiological conditions associated with •NO and superoxide production. The method, however, results in under-estimation of the absolute amounts of peroxynitrite because of inhibition of the oxidation of DHR 123 by excess •NO; enhancement of hydrogen peroxide production and oxidation of DHR 123 by NOS inhibitors due to white cell activation, and scavenging of a portion of peroxynitrite prior to its reaction with DHR 123 by plasma components such as uric acid and proteins [14, 31], which are present at concentrations higher than DHR 123.

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