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OXIDATIVELY MODIFIED PLASMA PHOSPHOLIPIDS CONTAINING REACTIVE CARBONYL FUNCTIONS MEASURED BY HPLC: EVIDENCE FOR PHOSPHATIDYLCHOLINE-BOUND ALDEHYDES IN PLASMA OF BURN PATIENTS

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A HPLC method has been developed to measure phosphatidylcholine (PC) containing reactive carbonyl functions in the sn-acyl residue in order to study processes in which such reactive carbonyls can be formed due to e.g. oxidative fragmentation. The method has been applied to determine PC-bound carbonyls as 2,4-dinitrophenylhydrazones (DNPH) in plasma of burn patients. Plasma from healthy volunteers served as controls. Additionally, *in vitro* oxidation experiments (A: plasma, buffer diluted; B: plasma + iron-EDTA complex and C: plasma + iron-EDTA complex + H_2O_2) have been performed to obtain and to identify 2,4-dinitrophenylhydrazine derivatizable carbonyl functions in plasma PC. Both, the PC-aldehydes and PC-aldehyde DNPH derivatives were cleavable with phospholipase C. Quantification was based on thin-layer chromatography purified soybean phosphatidylcholine, which was identically oxidized and derivatized as the plasma lipids *in vitro*.

KEY WORDS: Phospholipid-bound aldehydes, phosphatidylcholine, oxidized phosphatidylcholine, lipid peroxidation, cation-exchange HPLC, burn patients.

Abbreviations: PC: phosphatidylcholine; oxPC: oxidatively modified PC; BHT: butylated hydroxytoluene; EDTA: ethylenediaminetetraacetic acid; DNPH: 2,4-dinitrophenylhydrazone; H_2O_2: hydrogen peroxide; TLC: thin-layer chromatography; HPLC: high performance liquid chromatography.

INTRODUCTION

In a variety of pathophysiological conditions such as in ischemia/reperfusion, shock, burn injury or polytrauma in general, the involvement of oxygen-derived free radicals seems evident.[1-7] It has been assumed that one of the possible mechanisms of free radical induced cellular damage occurs via peroxidation of phospholipid fatty acids in the cell membrane bilayer. A consequence of such oxidative events on polyunsaturated fatty acids is the formation of lipid hydroperoxides. These lipid hydroperoxides can decompose to initiate new radical reactions or generate a large number of non-radical degradation products.[8] A main mechanism of this decomposition

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(often catalyzed by traces of transition metals[9]) is the beta-scission via the lipid alkoxyl radical to form a variety of cytotoxic aliphatic aldehydes, derived from the methyl-terminus of the fatty acid chain.[10] Beside such aldehydes as malondialdehyde, n-alkanals, alkenals, hydroxyalkenals (e.g. 4-hydroxynonenal) and other water-soluble carbonyls, it is also of interest to examine reactive aldehydes alternatively formed during the beta-cleavage of lipid hydroperoxides, where the aldehyde function remains attached to the parent lipid molecules e.g. phospholipids[11-14] or cholesteryl esters.[15] For short and medium chain aldehydes the high cytotoxic potential (i.e. reaction with cell membrane proteins and many other cell constituents) has been extensively investigated by Esterbauer *et al.*[8,16] Such biological reactivity may be also relevant for sn-diacyl phosphatidylcholine (PC)-bound aldehydes derived from PC, a major constituent of membranes and in particular of plasma phospholipids. In *in vitro* experiments reactivity has been shown for oxidatively fragmented phosphatidylcholine, in which resulting aldehydes in the sn-2 acyl residue have platelet activating factor (PAF)-like activity.[17]

We have therefore developed a HPLC method to measure oxidatively modified PC containing reactive carbonyl functions in the sn residue which are derivatizable with 2,4-dinitrophenylhydrazine. The method has been applied to measure PC-carbonyls in plasma samples from burn patients. The measurement of PC-carbonyls in plasma samples from healthy volunteers served as controls. In addition, an *in vitro* plasma oxidation system was used to confirm the obtained results.

PATIENTS AND METHODS

A total of ten patients with severe burn injury was studied (ranging in age from 22 to 52 years with a mean age of 32 ± 8 years). Mean (±SD) total burn extension was 54 ± 14% (range: 38–81%) with 27 ± 19% of third-degree of burn (range: 2–60%), and the mean Hospital Trauma Index – Injury Severity Score was 22 ± 6 (range: 16–31).

Materials

Analytical or spectroscopical grade solvents, chemicals and reagents, silica gel 60 for TLC and Adsorbex-NH_2 (aminopropyl) columns were purchased from Merck (Darmstadt, Germany). Soybean phosphatidylcholine was from Sigma Chemie (Deisenhofen, Germany) and phospholipase C (Bacillus cereus) and catalase (beef liver) from Boehringer Mannheim (Mannheim, Germany).

Plasma Sampling

Whole blood from healthy volunteers (controls) as well as from burn patients was collected by venipuncture with vacutainers containing 2 mg EDTA/ml, centrifuged (2200 × g, 4°C, 10 min.) and the plasma was immediately extracted or stored at −28°C until further processing.

In Vitro Oxidation Systems

a) Control plasma was diluted (1:4) with oxygen saturated phosphate buffer (20 mM, pH 7.4). After addition of a freshly prepared iron-EDTA complex solution[18] and

hydrogen peroxide to initiate oxidation, the dilution of plasma was 1:4.2. The final concentrations in the solution were 2 mM Fe^{2+}; 2.4 mM EDTA and 25 mM H_2O_2. As measured in previously performed oxidation experiments the concentration of H_2O_2 (measured according to Takayama *et al.*[19]) decreased to zero within 20 minutes. Then a second portion of H_2O_2 was added to yield a final concentration of 25 mM. After an incubation period of 2 hrs at 37°C the reaction was terminated by addition of catalase (40 U) and through addition of the extraction solvents. Three incubation experiments were performed : (A) plasma, diluted with buffer only, (B) plasma + iron-EDTA and (C) plasma + iron-EDTA + H_2O_2.

b) TLC purified soybean PC (2.5 mg) was suspended in phosphate buffer and treated under identical conditions as in the plasma incubation experiment (C). Using the same derivatisation and purification steps (TLC or solid-phase extraction) as described below, the concentration of oxPC-DNPH derivatives was estimated at 370 nm with an average extinction coefficient of 25500 $M^{-1} cm^{-1}$ [12,23] prior to HPLC analysis.

Isolation of PC-bound Reactive Carbonyls

Lipids from plasma or incubated plasma samples where extracted with a 4-fold volume of $CHCl_3$:MeOH (2:1, v/v, containing 0.05% BHT) in principal according to the method of Folch.[20] The upper phase was then re-extracted with 2.5 volumes $CHCl_3$ (containing 0.005% BHT). The pooled $CHCl_3$ extract was washed with 150 mM saline containing 1% EDTA: $CHCl_3$:MeOH (47:3:48, v/v/v), concentrated to 0.3 ml under a stream of N_2 and treated with 0.2 ml MeOH and 0.1 ml 2,4-dinitrophenylhydrazine solution (freshly prepared, 0.35 mg/ml in 1 N HCl;[21]). After vigorously shaking (10 min.) the reaction was allowed to proceed for 3 hrs at 25°C in the dark. For phase separation 0.2 ml bidistilled H_2O was added and the $CHCl_3$ layer was applied onto an Adsorbex-NH_2 column (aminopropyl, 1 ml bed volume) preconditioned with n-hexane. Lipids were subsequently eluted using solvents as described by Kaluzny.[22] Neutral lipids and excess of 2,4-dinitrophenylhydrazine reagent were eluted with $CHCl_3$/2-propanol (2:1), phospholipids and oxidatively modified phospholipid-DNPH derivatives with methanol (5 × 100 μl; containing 0.001% BHT). Aliquots (50μl) were analyzed by cation-exchange HPLC.

For recovery, concentrations of soybean oxPC-DNPH derivatives were determined by HPLC-measurements before and after Adsorbex-NH_2 solid phase extraction. The yield was about 95%.

Cation-Exchange HPLC

PC and oxPC-DNPH derivatives were separated from the other neutral phospholipids by cation-exchange HPLC on a Partisil 10 SCX column (250 × 4 mm, Whatman, Maidstone, U.K.) using a linear gradient elution from 12.5% to 22.5% water in acetonitrile within 16 min. at a constant flow rate of 1.5 ml/min. A Beckman System Gold HPLC apparature (Beckman Instruments, Fullerton, CA, U.S.A.) equipped with a Model 168 diode array detector was used. The intact phospholipids were monitored at 205 nm and the DNPH derivatives at 370 nm. Each HPLC fraction of PC was collected and the content measured by phosphorus analysis.[24]

Quantification of plasma oxPC-DNPH derivatives was based on comparison of

the peak heights with a calibration curve of standard soybean oxPC-DNPH derivatives.

Thin Layer Chromatography

Lipid extracts were applied onto a silica gel 60 plate (precleaned with $CHCl_3$: MeOH = 1:1) and developed with $CHCl_3$:MeOH:H_2O (68:30:4, v/v/v) as polar solvent or with n-hexane:diethylether:glacial acetic acid (80:20:1, v/v/v) as an unpolar solvent system.

Phospholipase C Hydrolysis

Lipid extracts before and after derivatisation with 2,4-dinitrophenylhydrazine were evaporated under a stream of nitrogen. The residue was resuspended in phosphate buffer (20 mM, pH 7.4, containing 2.5 mM Ca^{2+}) and incubated with 20 U phospholipase C for 30 min. at 40°C. After extraction with $CHCl_3$:MeOH (2:1), aliquots of the $CHCl_3$ layer were analyzed by HPLC or TLC.

RESULTS AND DISCUSSION

1. Experimental studies

Plasma incubated in the presence of iron-EDTA complex and high concentrations of H_2O_2, underwent a strong oxidation. The primary peroxidation products of plasma lipids are lipid hydroperoxides, which further decompose under these conditions to form aldehydes derivatizable with 2,4-dinitrophenylhydrazine.

Figure 1 Panel (A) demonstrates a HPLC separation of a derivatized lipid extract obtained from a sample of plasma which was only incubated with buffer (Table 1). Intact PC (monitored at 205 nm) and oxPC-DNPH derivatives (monitored at 370 nm) are clearly resolved from the other major neutral phospholipids such as phosphatidylethanolamine, sphingomyelin and lyso-PC under the described chromatographic conditions. Panel (B) and (C), show HPLC-separations of 2,4-dinitrophenylhydrazine treated lipid extracts from plasma incubated with iron-EDTA and iron-EDTA + H_2O_2, respectively. Compared with both buffer only (A) and iron-EDTA (B) incubated plasma, there is a massive increase of oxidized PC (oxPC-DNPH) as well as simultaneous decrease of intact PC (Panel C). Additionally, incubation with phospholipase C results in complete absence of the oxPC-DNPH derivatives and intact phospholipids as shown in Panel (D).

The results obtained from the different plasma incubation experiments are summarized in Table 1. For comparison in an *in vitro* oxidation experiment we determined TBARS in the lipid extract. After oxidation the TBARS value increased 15-fold (data not shown). The calculation concerning nmol oxPC-DNPH derivatives is based on a calibration curve of soybean oxPC-DNPH derivatives as an external standard and gives a good linear response as shown in Figure 2 with a detection limit of <20 pmol (absolute amount injected). Typical HPLC runs are illustrated in Figure 3 for the determination of native (A) and oxidized (B) standard soybean PC after reaction with 2,4-dinitrophenylhydrazine and solid phase preseparation. The oxPC-DNPH derivatives (monitored at 370 nm) co-elute with unaltered (intact) PC (monitored at 205 nm) with a retention time of 9.8 minutes. The amount of derivatives injected corresponds to 1.1 nmol.

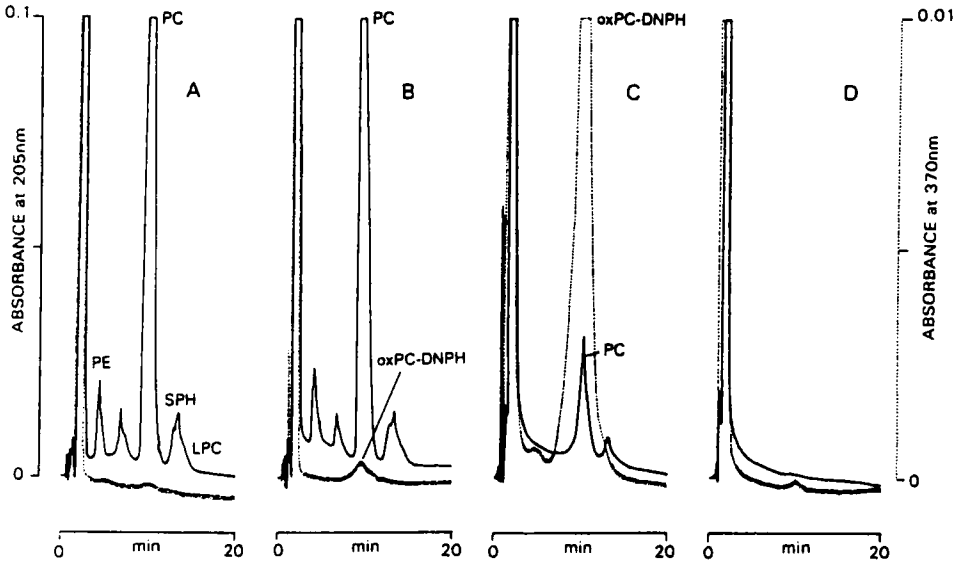

FIGURE 1 Cation-exchange HPLC chromatograms of the *in vitro* plasma oxidation experiments. Neutral plasma phospholipids were obtained from 2,4-dinitrophenylhydrazine treated lipid extract by NH_2-solid phase extraction. (A) HPLC separation of phospholipids from a sample of plasma which was only incubated with buffer (Table 1), (B) and (C) from plasma incubated with iron-EDTA and iron-EDTA + H_2O_2, respectively; (D) same experiment as in (C) additionally incubated with phospholipase C. HPLC conditions and detection as given in Methods. Peaks: PE = phosphatidylethanolamine; PC = phosphatidylcholine; SPH = sphingomyelin; LPC = lyso-PC.

TABLE 1

Plasma PC-bound aldehydes determined as 2,4-dinitrophenylhydrazone derivatives by cation-exchange HPLC of three plasma oxidation experiments. Diluted plasma (1:4.2, with 20 mM phosphate buffer, pH 7.4) was incubated for 2 hrs at 37°C with iron-EDTA complex and H_2O_2 to initiate oxidation. Diluted incubated plasma with and without iron-EDTA complex served as controls. Final concentrations were 2 mM Fe^{2+}; 2.4 mM EDTA and 25 mM H_2O_2 (for more details see Methods)

Conditions	nmol/mg PC	(mol/mol PC)*100
plasma; incubated only	1.1 ± 0.3	0.08 ± 0.02
plasma + iron-EDTA	2.1 ± 0.8	0.16 ± 0.06
plasma + iron-EDTA + H2O2	158.0 ± 11.4	12.34 ± 0.89

Values are given as mean ± SD; n = 3, for each condition

TLC examination revealed comparable chromatographic behaviour using the polar TLC system (described in Methods). The standard oxPC-DNPH derivatives (visible as a yellowish band) and the intact PC nearly co-migrate on the silica plate, giving Rf-values of 0.30 and 0.28, respectively. In contrast strong oxidized but not derivatized standard PC is splitted into two main bands, which migrate in the same TLC system within sphingomyelin (SPH) and the unoxidized PC bands (not shown). In TLC experiments using unpolar solvents the DNPH-derivatives (liberated after digestion of derivatized soybean PC with phospholipase C) migrate to the diglyceride

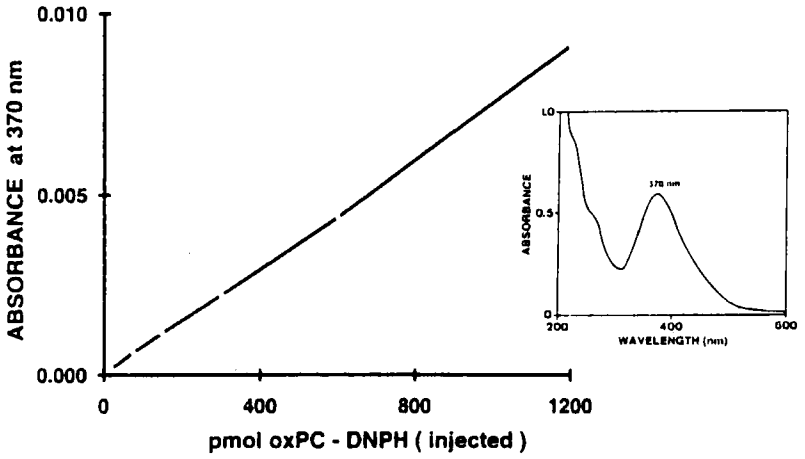

FIGURE 2 Relationship between injected amount of standard soybean oxPC-DNPH derivatives and peak height (expressed as absorbance units). Values are means of duplicate determinations. Insert: Absorption spectrum of the hydrazones of oxidized soybean PC showing a maximum at a wave length of 370 nm. The concentration of the stock solution was calculated on the basis of an average molar extinction coefficient of 25500 for aldehyde-DNPH derivatives.[12, 23]

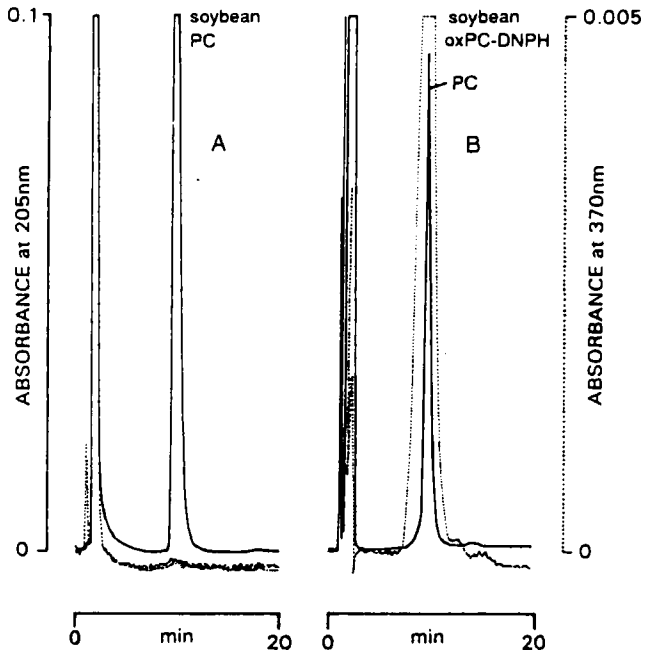

FIGURE 3 HPLC profiles of TLC purified standard soybean phosphatidylcholine before (A) and after (B) exposure to strong oxidation followed by treatment with 2,4-dinitrophenylhydrazine and NH_2 solid phase separation. HPLC conditions and detection as given in Methods.

region as yellowish spots (not shown). Similar results were obtained with oxidized plasma samples after derivatisation and subsequent TLC separation.

2. *PC Bound Aldehydes in Plasma of Burn Patients*

A HPLC chromatogram of a derivatized lipid extract obtained from plasma of a healthy volunteer is shown in Figure 4A. The amount of the derivatives obtained is smaller than 0.8 nmol/ml plasma and is near the detection limit. An analogously processed plasma sample from a burn patient, in which low levels of plasma antioxidants were found[27] revealed a high value of oxPC-DNPH derivatives (Figure 4B). This is an indication of severe oxidation processes as demonstrated in the *in vitro* experiments. Due to the low levels of PC (in part only 30% of control values) measured in plasma of the patients, the oxPC-DNPH concentrations were related to the PC content (see in Methods) of the corresponding sample.

The determined values for oxPC-DNPH derivatives in plasma extracts of eight healthy volunteers were 0.78 ± 0.23 nmol/ml plasma or respectively 0.58 ± 0.18 nmol/mg PC (mean values ● SD). For comparison we have also measured TBARS in 6 of these control samples. The obtained values were 0.53 ± 0.08 nmol/ml plasma. The PC-carbonyl concentration related to PC content in plasma of the healthy volunteers as well as of ten burn patients (samples were taken from day 1 to 4 post burn injury) are illustrated in Table 2. Plasma of some of the patients contained

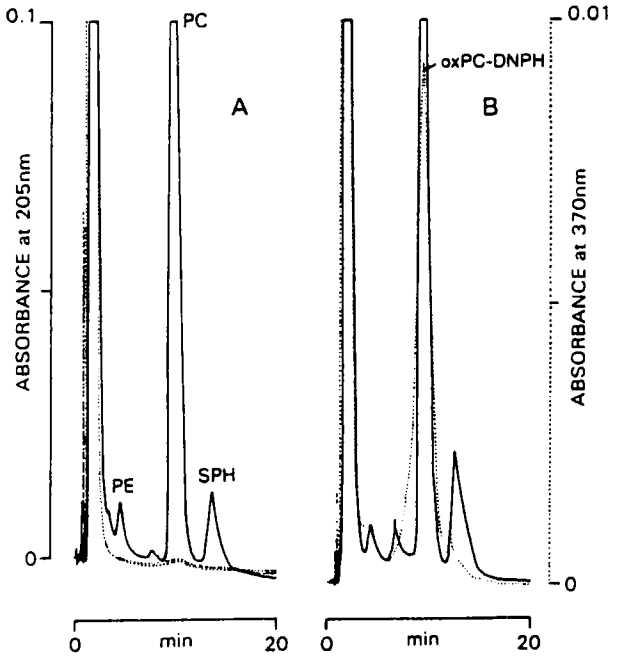

FIGURE 4 HPLC separation of derivatized neutral phospholipids obtained from plasma of a healthy volunteer (A) and of a patient with severe burn injury (B). HPLC conditions and detection as given in Methods; peak abbreviations see Figure 1.

TABLE 2

PC-carbonyl concentrations related to PC content in plasma extracts of eight healthy volunteers (controls) and ten burn patients (P1–P10). Blood samples were taken from day 1 to 4 post burn injury (consecutive blood samples from day 1 to 4 were not available for all patients). High PC-carbonyl concentrations can be detected in plasma of some of the burn patients comparable to those found in plasma after in vitro oxidation

controls	(n = 8; mean ● SD)			0.58 ± 0.18			(range: 0.3–0.78)			
patients	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
day 1	170.2	1.5	90.1	3.0	4.6	45.7	12.9	99.7	58.4	13.1
2	3.6	6.9	48.6	2.1		16.2	10.5	9.6	31.2	21.4
3	4.6	4.7	25.0	2.5		20.4	9.1	8.0	31.9	21.8
4	6.5	5.9			2.7	19.2	5.2	6.8	36.1	14.8

Values are expressed as nmol oxPC-DNPH/mg plasma PC

carbonyl concentrations similar to those found in plasma after *in vitro* incubation (Table 1).

The exact nature of these PC-aldehydes still remains to be investigated. Separation of the aldehydes from the intact PC as well as the separation of the main species of these aldehydes by means of appropriate analytical techniques (i.e. LC-MS, GLC-MS) would be indispensable.

Alternatively, digestion of PC fractions with phospholipase A_2 could be helpful for identification if the residue liberated from sn-2 position can be analyzed. In initial experiments we have tried to digest the PC aldehyde and intact PC fraction with phospholipase A_2. Unfortunately the enzymatic digestion was not complete, but the yield was similar for both, intact PC and PC aldehyde (~50%).

A further still unresolved problem is the precise quantification of the oxPC-DNPH derivatives, due to the lack of authentic standards. Recently, the synthesis and characterization of 5-oxovaleric and 9-oxononanoeic acid esters of cholesterol and the common glycerophospholipids, phosphatidylethanolamine and PC have been reported by Kamido *et al.*[14] These semi-synthetic lipid ester core aldehydes could serve as reliable standards for identification and adequate quantification.

With our approach we have tried to study the qualitative and semiquantitative alteration of sn-diacyl phosphatidylcholine in *in vitro* oxidation systems as well as in plasma of patients with severe burn injuries. Biological relevance may be attributed to oxidatively modified plasma phospholipids containing aldehyde functions attached to the acyl residue. For example, Smiley *et al.* found that oxidatively fragmented PC activates human neutrophils via the platelet activating factor (PAF) receptor[17] and such in the sn-2 residue altered PCs are substrates for human plasma PAF-acetylhydrolase.[25,26] Concerning cytotoxicity the similarity to certain mediators (i.e. PAF) might indicate a pathophysiological role of oxidatively modified phosphatidylcholine containing reactive carbonyls in the sn-acyl residue.

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