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ORIGINAL ARTICLE

Wei-dong Zhang · Masataka Nagao · Takehiko Takatori
Kimiharu Iwadate · Yoshiyuki Itakura · Yoshihiro Yamada
Hirotaro Iwase · Tsuneaki Oono

Immunohistochemical dynamics of leukotoxin (9,10-epoxy-12-octadecenoic acid) in lungs of rats

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Abstract This paper investigates the immunohistochemical dynamics of leukotoxin (9,10-epoxy-12-octadecenoic acid, LTx) in the lungs of rats exposed to hyperoxia with or without paraquat. The rats were treated with 100% oxygen or ambient air for 24, 48, 72 and 96 h in the presence or absence of a low or high dose paraquat (1,1′-dimethyl-4,4′-bipyridinium, PQ) injection. Immunostaining for LTx demonstrated positive reactions in the neutrophils that showed a progressive increase in intensity of staining with time in all groups exposed to 100% oxygen and in the group with high dose PQ, but the positive findings were weak in the group injected with low dose PQ only. We found the positive immunostaining reaction not only in neutrophils but also in alveolar macrophages. This indicates that LTx is produced by alveolar macrophages as well as by neutrophils depending on the treatment period under hyperoxic conditions, suggesting that LTx is an important chemical mediator in pulmonary diseases.

Key words Leukotoxin · 9,10-epoxy-12-octadecenoic acid · Immunohistochemistry · Macrophages

Zusammenfassung
Dieser Artikel untersucht die immunhistochemische Dynamik von Leukotoxin (9,10-Epoxy-12-octadecensäure, LTx) in den Lungen von Ratten, die einer Hyperoxie mit oder ohne Paraquat ausgesetzt waren. Die Ratten wurden behandelt mit 100% Sauerstoff oder Umgebungsluft für 24, 48, 72 und 96 Stunden mit oder ohne Injektion einer niedrigen oder hohen Dose Paraquat (1,1′-Dimethyl-4,4′-bipyridium, PQ). Die Immunfärbung für LTx zeigte positive Reaktionen in den Neutrophilen. Diese zeigten eine zunehmende Verstärkung der Färbungsintensität in Abhängigkeit der Zeit in allen Gruppen mit Exposition von 100% Sauerstoff und in der Gruppe mit hoher Dose PQ, jedoch waren die positiven Befunde in der Gruppe mit Injektion nur niedriger Dose PQ schwach. Wir fanden die positive Immunfärbungsreaktion nicht nur in Neutrophilen, sondern auch in Alveolarmakrophagen. Dies zeigt, daß LTx sowohl von den Alveolarmakrophagen als auch von den Neutrophilen, abhängig von der Behandlungszeitdauer unter hyperoxischen Bedingungen, hergestellt wird, was dafür spricht, daß LTx ein wichtiger chemischer Mediator in Lungenerkrankungen ist.

Schlüsselwörter Leukotoxin · 9,10-Epoxy-12-octadecensäure · Immunhistochemie · Makrophagen

Introduction

9,10-Epoxy-12-octadecenoic acid (Fig. 1), a substance recently discovered in neutrophils, was named leukotoxin (LTx) in 1986 [1, 2]. In the last decade, many studies on LTx have been carried out in the biochemical and physiological fields. LTx was reported to exist in polymorphonuclear leukocytes (PMNs), especially in neutrophils and in extracts of rice plants. It was considered important, because PMNs play a significant role in the anti-inflammatory response and injury of tissues and antifungal effects against a rice blast disease [1, 2, 3–5]. On the other hand, LTx has a cytotoxic activity towards human tumor cells as well as normal ones [6]. Moreover, LTx has been found in burned skin indicating that LTx exerts an uncoupling effect on mitochondrial respiration as a shock factor [7–9].

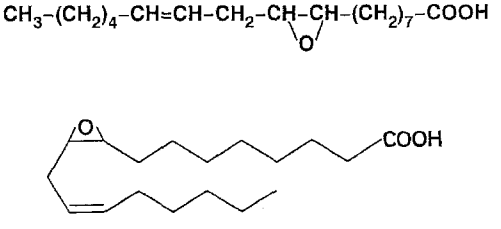

Fig. 1 Structure of leukotoxin (9,10-epoxy-12-octadecenoic acid)

W.-d. Zhang · M. Nagao · T. Takatori (✉) · K. Iwadate
Y. Itakura · Y. Yamada · H. Iwase · T. Oono
Department of Forensic Medicine, Faculty of Medicine,
University of Tokyo, Tokyo 113, Japan

Table 1 Experimental procedure for the 30 rats

Group	*N*	Treatment
1	2	Control group
2	8	PQ iv 3 mg/kg and O_2 exposure for 24, 48, 72, 96 h
3	8	O_2 exposure for 24, 48, 72, 96 h
4	8	PQ iv 3 mg/kg and air exposure for 24, 48, 72, 96 h
5	2	O_2 exposure for 48 h and recovery for 5 days
6	2	PQ iv 50 mg/kg and air exposure until death (ca 12 h)

LTx was also reported to cause thrombosis related to the occurrence of disseminated intravascular coagulation (DIC) and that intravenous administration of LTx depressed the cardiac function of dogs leading to heart failure [10–12]. Therefore, LTx appears to play an important physiological role in all living organisms.

Ozawa et al. [13, 14] reported that LTx was found in PMNs collected from the bronchoalveolar lavage fluid (BALF) of patients suffering from adult respiratory distress syndrome (ARDS) and of rats exposed to pure oxygen and administered the herbicide paraquat (1,1′-dimethyl-4,4′ bipyridinium, PQ) [15]. In the field of forensic pathophysiology, this compound seems to play an important role in conditions such as cardiovascular dysfunction. However, we found no reports in the literature demonstrating the presence and localization of LTx in neutrophils. In a previous study we produced an anti-LTx serum which selectively recognizes LTx [16]. The present paper deals with the immunohistochemical demonstration of the dynamics of LTx in the lungs of rats exposed to pure oxygen and treated with or without PQ.

Materials and methods

An ABC Kit for an immunohistochemical staining was purchased from Biomeda Corp., USA. LTx was synthesized from linoleic acid and purified by thin-layer chromatography as described previously [16]. The monoclonal antibody ED1 was supplied by Serotec, UK. All other reagents were supplied by Wako Pure Chemical Ltd., Japan.

Male Sprague-Dawley rats ($n = 30$), weighing 210 ± 10 g, were divided into 6 groups (Table 1). Group 1 (control, $n = 2$) was were exposed to ambient air until sacrificed. Group 2 ($n = 8$) was intravenously injected with PQ (3 mg/kg) and exposed to pure oxygen for 24, 48, 72, 96 h. Group 3 ($n = 8$) was exposed to pure oxygen for 24, 48, 72, 96 h without any treatment. Group 4 ($n = 8$) was intravenously injected with PQ (3 mg/kg) and exposed to ambient air for 24, 48, 72, 96 h. Group 5 ($n = 2$) was exposed to pure oxygen for 48 h and then to ambient air for 5 days. Group 6 ($n = 2$) was intravenously administered lethal amounts of PQ (50 mg/kg) and exposed to ambient air until death occurred (ca 12 h). The atmosphere in the polystyrene chambers was maintained under oxygen-rich conditions (more than 95% O_2) during all experiments. The room temperature was 20° C–25° C. All rats were provided with standard laboratory food and water *ad lib.*

These experiments were carried out according to the Rules and Regulations of the Animal Research Committee Faculty of Medicine, University of Tokyo, Tokyo, Japan.

The rats were anesthetized with light diethyl ether and perfused via the trachea with 4% paraformaldehyde (PFA, dissolved in 0.1 M phosphate buffer, PB, pH 7.4) for 10 min. The lungs were removed and cut into small blocks. The tissue blocks were fixed in PFA at 4° C overnight, washed in 0.01 M phosphate-buffered saline (PBS, pH 7.4), embedded in paraffin and cut into 2–4 μm sections.

The polyclonal antibody against LTx reported previously was used [16]. Briefly, LTx was synthesized from linoleic acid with peracetic acid, purified by thin-layer chromatography, and conjugated with bovine serum albumin (BSA) by means of the mixed anhydride technique [17]. The LTx-BSA antigen was injected into rabbits, and anti-LTx serum was obtained 4 months later. According to the radioimmunoassay for LTx, a 60-fold dilution of antiserum bound approximately 50% of methylated [^{14}C]-LTx, and unlabeled LTx was detected to at least 5 ng in the radioimmunoassay system. This antiserum had a strong specificity for LTx and little cross-reactivity with the other analogs of LTx tested. Before use of the anti-LTx serum for the immunohistochemical staining, the antiserum was absorbed with acetone powder and the lung sections with BSA overnight at 4° C to remove nonspecific substances.

The ED1 monoclonal antibody was used to confirm alveolar macrophages in lung tissue and is the name of the clone obtained by immunization of BALB/c mice with rat spleen cells [18], which gives a specifically positive reaction in both tissue macrophages and free macrophages (alveolar and peritoneal macrophages). In an immunocytochemical field, the ED1 antibody has often been used to specify the alveolar macrophages in rats by an immunostaining method [19–21].

We selected an avidin-biotin-peroxidase complex method (ABC) for the immunohistochemical staining using anti-LTx serum and ED1 monoclonal antibody. A routine hematoxylin-eosin method was used for contrast staining. The specificity of staining with the LTx antibody was checked by comparing to staining using the anti-LTx serum which was completely absorbed with LTx-BSA. The method for LTx staining in the lung tissue of rats was applied as follows:

1) Deparaffinize in xylene and ethanol.
2) Immerse in 0.5% H_2O_2 (hydrogen peroxide) in PBS for 30 min.
3) Rinse in PBS 5 times for 5 min each.
4) Incubate with normal goat and/or rabbit serum at a 1:10 dilution for 15 min.
5) Incubate with anti-LTx rabbit serum (1:1600 dilution) and/or ED1 monoclonal antibody (1:400 dilution) at 4° C overnight.
6) Rinse in cold PBS 5 times for 10 min each and in PBS 3 times for 5 min each.
7) Incubate with goat anti-rabbit IgG and/or rabbit anti-mouse IgG labeled with biotin (from ABC kit) for 1 h.
8) Rinse in PBS 5 times for 10 min each.
9) Incubate with the ABC complex (peroxidase labeled with avidin, from ABC kit) for 10 min.
10) Rinse in PBS 5 times for 10 min each and in cold 0.05 M Tris-HCl buffer (TB) 2 times for 5 min each.
11) Develop with 0.02% 3,3′-diaminobenzidine (DAB) solution containing 0.065% NaN_3 and 0.003% H_2O_2 in TB at 5° C for 6 min.
12) Stop the development in tap water, rinse in distilled water and incubate with 10% formalin for 10 min.
13) Rinse in distilled water 3 times for 5 min each.
14) Counterstain with Mayer's hematoxylin.
15) Rinse in distilled water, dehydrate and mount.

All procedures were performed at room temperature unless otherwise specified.

Results

Macroscopically, the lungs treated with hyperoxia (regardless of the PQ treatment) showed a dark-purple colouration and the lungs were bigger than the controls. Similar findings were also observed in the lungs of the

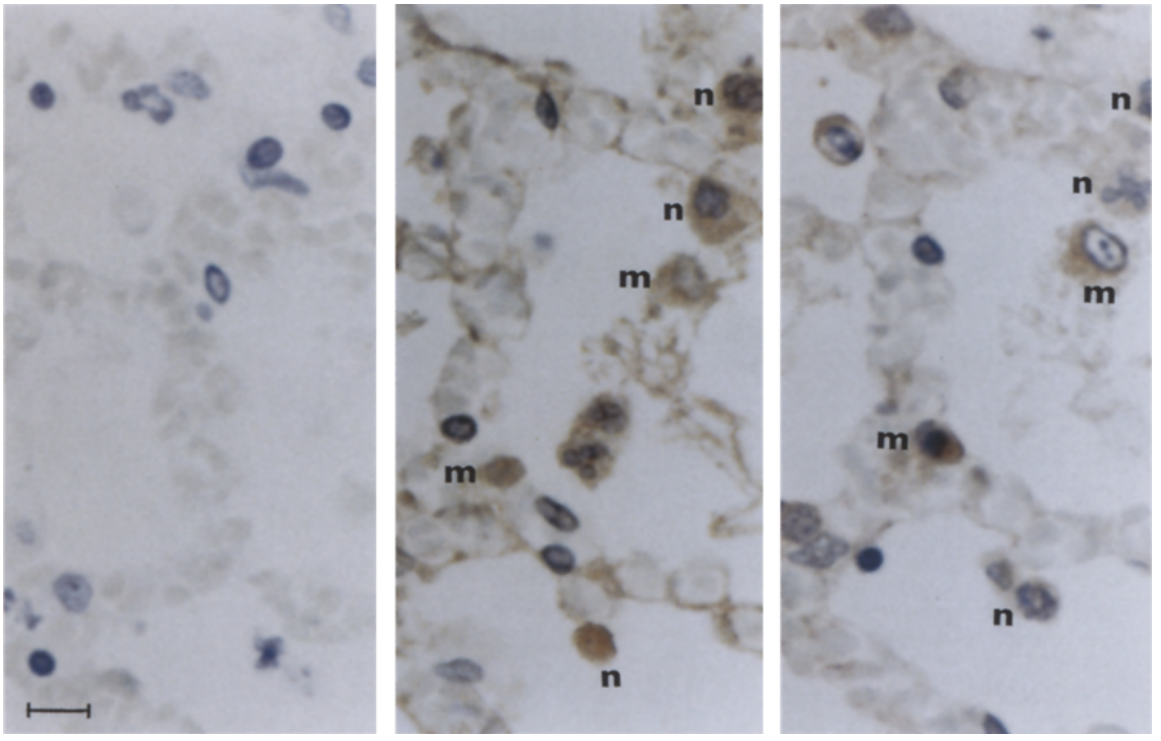

Fig. 2 Positive reactions with anti-LTx serum *(middle)* and anti-ED1 monoclonal antibody *(right)* in alveolar macrophages *(m)* and neutrophils *(n)* of the lungs of rats treated with hyperoxia for 48 h and control *(left)*. ABC staining with DAB. *Bar* = 10 μm

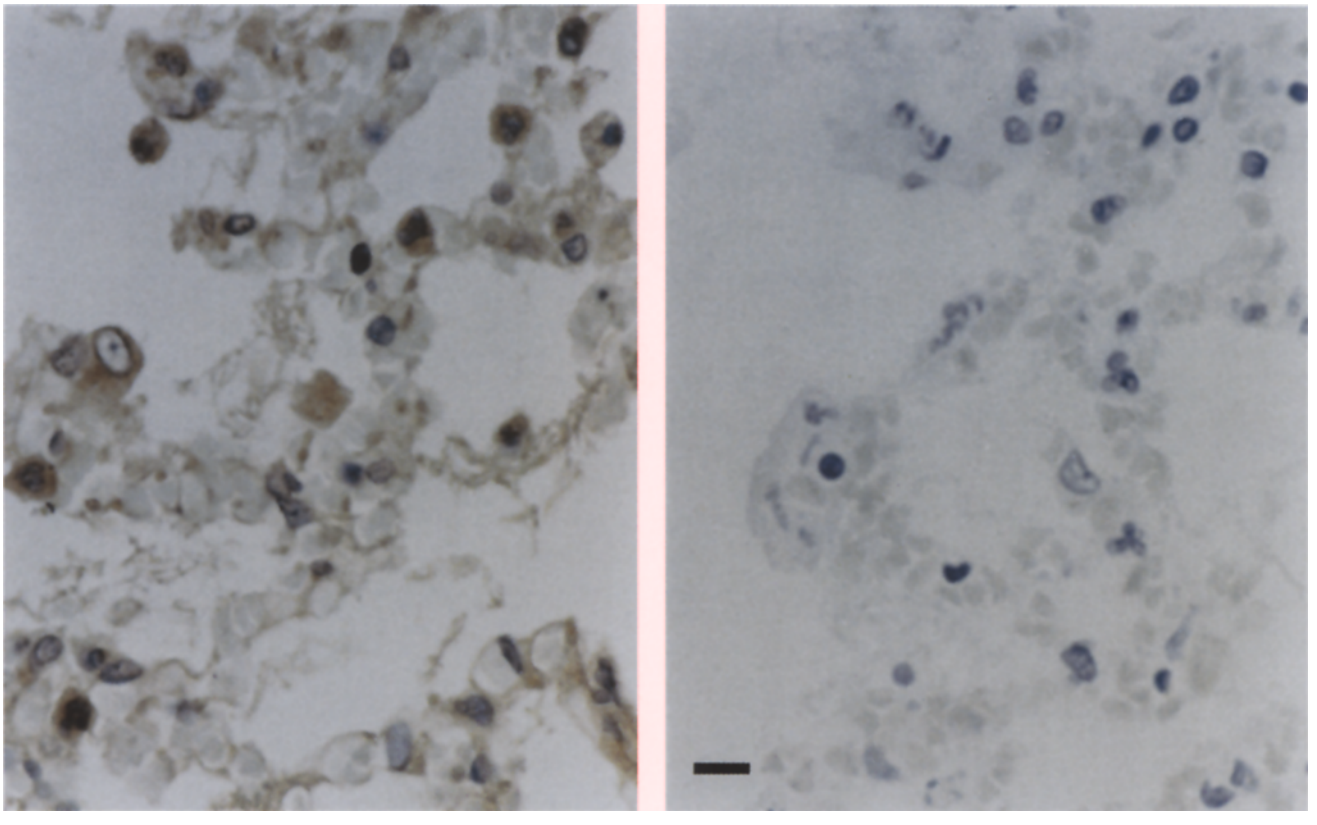

Fig. 3 Positive reaction with anti-LTx serum shown in the lungs of rats treated with hyperoxia for 72 h *(left)* and control *(right)*. ABC staining with DAB. *Bar* = 9 μm

Table 2 Immunohistochemical observations in rats treated with hyperoxia and/or paraquat using anti-LTx serum

[a]Recovered after 5 days; [b]neutrophils; [c]alveolar macrophages; –: no treatment or no positive cells; +: < 9 positive cells/× 66 field; ++: 10 ~ 19 positive cells/× 66 field; +++: > 20 positive cells/× 66 field

	Group															
	1	2				3				4				5	6	
PQ treat. mg/kg	–	3	3	3	3	–	–	–	–	3	3	3	3	–	50	
O_2 treat. Time/h	–	24	48	72	96	24	48	72	96	–	–	–	–	48[a]	–	
NP[b]	–	+	+	++	+++	+	+	++	++	++	+	–	+	+	+++	
AM[c]	+/–	+	++	+	+++	++	+	++	+++	++	+	+	+	++	+++	

rats treated with high dose PQ. Immunohistochemical findings in the lungs of the rats treated as described in the experimental procedure, showed positive reactions with anti-LTx antiserum in neutrophils and alveolar macrophages. The alveolar macrophages were confirmed to be positive by the ED1 antibody, whereas the neutrophils were negative (Fig. 2). In the group treated with hyperoxia for 72 h the lungs showed significantly different findings from the control group (Fig. 3), because in the former group positive reactions were observed with anti-LTx serum as well as inflammatory cell infiltration.

In the groups 2 and 3 treated with hyperoxia regardless of the PQ dose, positive reactions with anti-LTx serum were found in the neutrophils and the intensity of staining increased with time. In group 4 treated with low dose PQ only, this finding was observed to get weaker with time. On the other hand, in group 5, positive reactions in the neutrophils were shown to be weaker than those in the groups 2 and 3. In group 6 treated with the high-dose PQ, strongly positive reactions could be seen in the neutrophils whereas in the group 1 (control group), only weak staining of alveolar macrophages was found. The dynamics of the positive reactions in alveolar macrophages were almost the same as those in the neutrophils (Table 2).

Discussion

Hyperoxic exposure, i.e. oxygen toxicity, has frequently been used as a typical model for acute pulmonary injuries. The lungs have a special structure and function and are therefore always exposed to a variety of stimulants. The free radicals produced in the lungs are one of these and lead to lung injury [22–26]. Under hyperoxic conditions, radicals such as superoxide and/or hydrogen peroxide in the mitochondria of the lungs are 15–20 times higher than those under normal conditions and the production of oxidants from the lungs is 25% or more compared to the normal condition [27]. Moreover, many unsaturated fatty acids in cell membranes react with these radicals leading to cell damage [28]. However, some metabolic substances, including oxidants produced via an arachidonate cascade through the activity of phospholipase A_2 [29], injure capillary endothelial cells and surfactant substances. This leads to pulmonary edema and alveolar instability in a similar way to breathing poisonous gases [26, 30].

The large quantities of neutrophils, which are inflammatory cells, are accumulated in lung tissue by a neutrophil chemotactic factor (NCF) produced by alveolar macrophages and stimulated by some cytokines, proteolytic enzymes (elastase, collagenase, plasminogen activator etc.) and other chemical transmitters leading to tissue damage [31].

Linoleic acid exists in the phospholipid layer of biomembranes similar to arachidonic acid, is oxidized by epoxygenase and converted to LTx [15]. The LTx produced via a linoleate cascade may be of interest since leukotriene produced from the arachidonate cascade is closely related to lung damage and/or its remodeling [15, 32].

In this paper, we have demonstrated the immunohistochemical dynamics of LTx in lungs of rats exposed to hyperoxia. The localization of LTx in neutrophils became stronger with time under the hyperoxic conditions regardless of the level of PQ administration. These findings indicate that the LTx produced from the neutrophils can be confirmed immunohistochemically and that the increasing production of LTx is dependent on the treatment period under hyperoxic conditions. We also demonstrated that a positive reaction with anti-LTx serum in alveolar macrophages occurs in lungs. This is the first report that LTx is found not only in neutrophils but also in alveolar macrophages. This result might be of significant importance in terms of explaining diseases concerning alveolar macrophages, e.g. pulmonary fibrosis. From our results, LTx production could be related to the high dose (50 mg/kg) PQ treatment. The lungs damaged by the high dose PQ administration might be due to the neutrophils or alveolar macrophages that are activated to produce LTx.

The anti-LTx serum was first used for the immunohistochemical investigation in lungs of rats exposed to hyperoxia with or without PQ treatment. As a result, it was found that LTx is produced in alveolar macrophages as well as neutrophils. In the medico-legal field, this finding may play an important role in the pathophysiological explanation of the cell interaction involved in inflammatory responses such as myocardial infarction.

Although the substance, 9,10-epoxy-12-octadecenoic acid, was named leukotoxin, we would like to propose that the name be changed to leukoepoxy acid to avoid confusion with the leukotoxin involved in toxin production from some bacteria as described previously [33–35].

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