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Oxidized phospholipid-induced inflammation is mediated by Toll-like receptor 2

Alexandra Kadl^{1,2}, Poonam R. Sharma^{1,2}, Wenshu Chen^{1,2}, Rachana Agrawal^{1,2}, Akshaya K. Meher^{1,2}, Swetha Rudraiah^{1,2}, Nathaniel Grubbs^{1,2}, Rahul Sharma³, and Norbert Leitinger^{1,2}

¹Robert M. Berne Cardiovascular Research Center, University of Virginia, Charlottesville, VA 22908, USA

²Department of Pharmacology, University of Virginia, Charlottesville, VA 22908, USA

³Department of Medicine, Center for Immunity, Inflammation and Regenerative Medicine, University of Virginia, Charlottesville, VA 22908, USA

Abstract

Oxidative tissue damage is a hallmark of many chronic inflammatory diseases. However, the precise mechanisms linking oxidative changes to inflammatory reactions remain unclear. Herein we show that Toll-like receptor 2 (TLR2) translates oxidative tissue damage into inflammatory responses by mediating the effects of oxidized phospholipids.

Intraperitoneal injection of oxidized 1-palmitoyl-2-arachidonyl-*sn*-3-glycero-phosphorylcholine (OxPAPC) resulted in upregulation of inflammatory genes in wild-type, but not in TLR2^{-/-} mice. *In vitro*, OxPAPC induced TLR2 (but not TLR4)-dependent inflammatory gene expression and JNK and p38 signaling in macrophages. Induction of TLR2-dependent gene expression required reducible functional groups on *sn*-2 acyl chains of oxidized phospholipids, as well as serum co-factors. Finally, TLR2^{-/-} mice were protected against carbontetrachloride-induced oxidative tissue damage and inflammation, which was accompanied by accumulation of oxidized phospholipids in livers.

Together, our findings demonstrate that TLR2 mediates cellular responses to oxidative tissue damage and they provide new insights into how oxidative stress is linked to acute and chronic inflammation.

INTRODUCTION

Oxidative stress is a major cause for tissue damage that accompanies the development of chronic inflammation in metabolic and autoimmune diseases and in various forms of cancer. However, it is not clear how oxidative tissue damage is sensed by cells and ultimately translated into inflammatory reactions. It was proposed that oxidatively modified molecules activate pattern recognition receptors of the innate immune system, thereby triggering inflammatory responses. In support of this hypothesis, it was shown that deficiency of Toll-like receptor (TLR) 2 in mice was protective in various settings of non-infectious

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Correspondence to: Norbert Leitinger, PhD: nl2q@virginia.edu.

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inflammatory disease models including atherosclerosis, reperfusion injury, and diabetes [1–4]. Oxidative tissue damage is a prominent feature in these diseases, however the exact nature of endogenously formed oxidized “danger signals” has not been elucidated. Thus, the rationale for this study was that oxidative modification of phospholipids would lead to the formation of danger signals that could activate TLR-dependent responses.

Oxidized phospholipids derived from oxidation of lipoproteins or from apoptotic cell membranes were shown to accumulate in atherosclerotic lesions and at other sites of chronic inflammation, where they are believed to contribute to disease progression [5]. We and others have shown that oxidized phospholipids induce inflammatory responses in macrophages, endothelial and smooth muscle cells, [6] as well as *in vivo* in the vascular wall [7]. We have recently shown that oxidized phospholipids induce specific macrophage recruitment in the air-pouch model of inflammation, by a mechanism involving CCR2 [8]. On the other hand, oxidized phospholipids induced expression of the anti-inflammatory heme oxygenase-1 (HO-1) independently of inflammatory signaling pathways [9,10].

Apart from pathogen-derived ligands, TLR2 and TLR4 also sense endogenous molecules derived from damaged cells and matrix components. For example, TLR4 recognizes heat shock protein (Hsp) 60, Hsp 70 and fibrinogen, while TLR2 recognizes Hsp 70, hyaluronan, and versican, as most recently shown [11]. Fatty acids have been implied as the endogenous ligands that activate TLR4 and TLR2 in settings of metabolic inflammation [3]. First indications that oxidized phospholipids resemble structures that can be recognized by TLRs came from our studies showing that these lipids compete with bacterial ligands to induce TLR-dependent signaling [12,13]. However, whether TLRs can sense oxidative tissue damage has not been demonstrated. In this study, we tested the hypothesis that accumulation of oxidized phospholipids creates a microenvironment that causes “sterile” TLR2-dependent chronic inflammation.

MATERIALS AND METHODS

Mice

Wild-type control mice (C57BL/6J) and age-matched TLR2^{-/-} mice (*B6.129-Tlr2tm1Kir/J*, backcrossed for 10 generations), TLR4^{-/-} and CD14^{-/-} mice were obtained from Jackson Laboratories (Bar Harbor, ME).

Administration of carbontetrachloride (CCl₄)

12–14 week old female mice were used. For acute CCl₄-induced liver damage, a single dose of 4.0 ml/kg of body weight (1:10 v/v in olive oil) was administered by intraperitoneal injection. Control mice were injected with olive oil only. Mice were sacrificed after the required time of treatment and livers were excised and snap frozen in liquid nitrogen for RNA extraction or processing for histochemistry. All animal experiments were approved by the Animal Care and Use Committee of the University of Virginia.

Histology and immunohistochemistry

Tissues were fixed for 24 hrs in 10% paraformaldehyde at 4°C, dehydrated, imbedded in paraffin and cut into 5 µm thick sections. Tissue sections were mounted on glass slides, deparaffinized, rehydrated, antigen retrieved, stained, dehydrated and stabilized with mounting medium. To detect oxidized phospholipids, the sections were stained with EO6 antibodies (kindly provided by Dr. Joseph Witztum, UCSD).

Generation of bone-marrow-derived macrophages (BMDM)

Bone marrow was isolated from femurs and tibias from female C57BL/6J wild type or TLR2^{-/-} or CD14^{-/-} mice and BMDM were cultured as described previously [10].

RNA isolation and quantitative RT-PCR

qRT-PCR was performed after 3 hours of stimulation of macrophages as described previously [10] using SYBR Green ER[™] qPCR SuperMix reagent (Invitrogen) and MyiQ single color real time PCR detection system from Bio-Rad. Porphobilinogen deaminase (PBGD) or β -2-microglobulin (β -2M) were used for normalization. PCR efficiency was determined for each primer pair from dilution series of a typical sample cDNA. Quantification of target gene expression was performed using a mathematical model by Pfaffl [14].

Western blotting

Signaling pathways induced by oxidized phospholipids or LTA in wild type or TLR2^{-/-} BMDM were analyzed by Western blotting using antibodies against phosphorylated and non-phosphorylated ERK1/2, p38, JNK 1 and 2 and I κ B α (Cell signaling technology) [15]. BMDM from wt or TLR2^{-/-} mice were cultured in medium with 2% serum overnight. Then, macrophages were incubated in medium containing 50 μ g/ml OxPAPC for indicated times. After Western blotting, images were scanned and quantified using Li-Cor Odyssey Infrared Imaging System version 3.0.

Oxidized phospholipid preparation and analysis

Phospholipids PAPC, PGPC, POVPC, DMPC as well as lyso-PC were purchased from Avanti Polar Lipids, Al. OxPAPC was produced by exposure of dry, unoxidized PAPC to air. The extent of oxidation was monitored by positive ion electrospray mass spectrometry (ESI-MS) as described previously [16] [10] using a Finnigan LCQ classic, connected with an HP HPLC 1100 series. Preparative thin layer chromatography for separation of long and short-chain oxidized phospholipids was performed as previously described [17], using SilicaG plates and Chloroform:Methanol:Water (CHCl₃/CH₃OH/H₂O, 10:5:1, v/v/v) as solvent. Lipids were extracted from the plate using CHCl₃/CH₃OH/H₂O (65:25:4, v/v/v), dried under a stream of argon and analyzed by mass spectrometry. For comparison of biological activities, equivalent amounts of isolated fractions and the parental mixture were added to cells.

Statistical analysis

was performed using one-way ANOVA or Student's t-test. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Oxidized phospholipids induce inflammatory gene expression via TLR2 in vivo and in vitro

As a model for oxidatively modified phospholipids that accumulate in chronically inflamed tissue *in vivo*, we used *in vitro*-oxidized 1-palmitoyl-2-arachidonyl-*sn*-3-phosphorylcholine (OxPAPC) [16]. To test whether oxidized phospholipids induce inflammatory gene expression via TLR2, we injected OxPAPC into the peritoneum of wild type (wt) or TLR2^{-/-} mice. After 6 hours, peritoneal lavage cells were harvested, RNA extracted and inflammatory gene expression was analyzed by qRT-PCR. Intraperitoneal injection of OxPAPC caused upregulation of COX-2 and IL-1 β expression in peritoneal cells derived from wt, but not from TLR2^{-/-} mice (Fig. 1A, B). In contrast, expression of HO-1 was induced by OxPAPC in wt and TLR2^{-/-} mice (Fig. 1C). We have previously shown that

induction of HO-1 expression by OxPAPC is mediated by TLR2-independent mechanisms [10] and thus we will use HO-1 expression as control readout throughout the manuscript.

To confirm a role for TLR2 in oxidized phospholipid-induced inflammatory gene expression, we cultured macrophages from the bone marrow (BMDM) of wt and TLR2^{-/-} mice. Treatment with OxPAPC of wt, but not TLR2-deficient BMDM resulted in expression of inflammatory genes Mip1 α and KC (Fig. 1D, E) and COX-2 (Fig. 4A). Expression of HO-1 was induced by OxPAPC in wt and TLR2-deficient BMDM (Figs. 1F, 4B). OxPAPC induced the expression of Mip1 α and KC also in BMDM derived from TLR4^{-/-} mice (Suppl. Fig. 1), while lipopolysaccharide (LPS) had no effect, confirming deficiency of TLR4. These data demonstrate that the effect of OxPAPC on inflammatory gene expression involves TLR2, but is independent of TLR4.

TLR2 is required for OxPAPC-induced JNK and p38 phosphorylation in macrophages

Treatment with OxPAPC resulted in JNK and p38 activation in wt but not in TLR2-deficient BMDM (Fig. 2). Phosphorylation of ERK1/2 was not induced by OxPAPC in BMDM. The TLR2 agonist lipoteichoic acid (LTA) induced all three MAP kinase pathways in wt, but not in TLR2^{-/-} cells. Interestingly, treatment of BMDM with OxPAPC did not result in activation of the NF κ B pathway, as illustrated by a lack of degradation of I κ B α while LTA induced degradation of I κ B α in wt, but not in TLR2^{-/-} BMDM (Suppl. Fig. 2). These data demonstrate that oxidized phospholipids activate the JNK and p38 stress pathways in macrophages by a mechanism that involves TLR2.

Recognition by TLR2 requires reducible functional groups on long chain oxidized phospholipids

To investigate the structural requirements of oxidized phospholipids to induce TLR2-dependent gene expression, OxPAPC was either chemically modified or fractionated into short and long chain oxidized phospholipids (Suppl. Fig. 3), as previously reported [17]. Wild-type or TLR2^{-/-} BMDM were treated with different fractions or individual molecules that are present in OxPAPC and expression of COX-2 was analyzed by real time RT-PCR. OxPAPC, but not PAPC, induced TLR2-dependent upregulation of COX-2 expression (Fig. 3A). Reduction of functional groups by NaBH₄ [16,18,19] abolished the capacity of OxPAPC to induce COX-2 expression. Furthermore, the biological activity resided in the "long chain" fraction (m/z > 782), which was confirmed by a lack of activity of the "short chain" fraction as well as of the individual "short chain" oxidized phospholipids POVPC, PGPC or lysoPC. While the TLR4 ligand LPS induced COX-2 expression in both wild type and TLR2^{-/-} BMDM, the TLR2 ligand LTA induced COX-2 expression only in wt cells (Fig. 3A), confirming deficiency of TLR2. In the same experiment, HO-1 expression was upregulated by OxPAPC in a TLR2-independent manner (Fig. 3B). Biological activity for HO-1 induction was contained in the long chain fraction, as well as in PGPC, as reported previously [20]. Neither LPS nor LTA induced expression of HO-1 mRNA in BMDM (Fig. 3B). Together, these results show that the presence of TLR2 is required to induce inflammatory gene expression by oxidized phospholipids, which is dependent on reducible functional groups on the arachidonate moiety of the modified phospholipid.

Oxidized phospholipids require serum soluble factors to induce TLR2-dependent inflammatory gene expression

Some bacterial-derived as well as endogenously formed ligands were shown to require CD14 for recognition by TLR2 [21,22]. Previously, we and others have shown that oxidized phospholipids bind to CD14 [12,23]. Therefore we examined whether membrane bound and/or soluble CD14 was required for activation of TLR2-dependent gene expression by

oxidized phospholipids. Using BMDM isolated from CD14^{-/-} mice, we demonstrate that induction of COX-2 and IL1 β expression by OxPAPC or the long-chain fraction of OxPAPC was independent of membrane bound CD14 (Fig. 4). Moreover, in the absence of serum, induction of COX-2 and IL1 β expression by OxPAPC or the long-chain fraction was abrogated. Addition of recombinant CD14 was not sufficient to restore effects of oxidized phospholipids on COX-2 expression (Suppl. Fig. 4). However, in the presence of serum, OxPAPC-induced COX-2 and IL1 β expression in CD14^{-/-} cells was restored (Fig. 4). In contrast, OxPAPC-induced expression of HO-1 was independent of serum or membrane CD14. Together, our data demonstrate that soluble serum cofactors are required for mediating effects of oxidized phospholipids by TLR2.

Absence of TLR2 protects against hepatic tissue damage and inflammation induced by oxidative stress

To examine the hypothesis that TLR2 senses oxidative tissue damage, we treated wt and TLR2^{-/-} mice with carbontetrachloride (CCl₄), which is known to induce marked oxidative liver injury [24]. It has been previously reported that oxidized phospholipids mediate tissue damage in livers of CCl₄-treated mice, since overexpression of platelet activating factor acetylhydrolase (PAF-AH), an enzyme that degrades oxidized phospholipids, resulted in protection against CCl₄ induced hepatic injury [24]. Indeed, immunohistochemistry demonstrated that CCl₄ treatment resulted in formation and accumulation of oxidized phospholipids in liver tissue from wt as well as TLR2^{-/-} (Fig. 5A) mice.

Treatment of wild type mice with CCl₄ for either 6 or 24 hours drastically increased hepatic damage as evidenced by increases in serum alanine aminotransferase (ALT). Interestingly, serum ALT levels were significantly lower in TLR2^{-/-} mice, indicating a role for TLR2 in mediating hepatic oxidative tissue damage (Fig. 5B). Furthermore, expression of inflammatory cytokines IL-1 β , TNF α and Gro α , which was induced in livers of CCl₄ treated wt mice, was significantly reduced in TLR2^{-/-} mice. In contrast, expression of HO-1 was upregulated in livers of wt and TLR2^{-/-} mice upon CCl₄ treatment (Fig. 5C). Absence of TLR2 did not affect kidney function after CCl₄ treatment, as there were no significant differences in blood urea nitrogen (BUN) or creatinine levels between wt and TLR2^{-/-} mice (data not shown).

DISCUSSION

The mechanisms that link oxidative tissue damage to activation of the innate immune system are not well understood. Here we tested the hypothesis that oxidative modification of phospholipids leads to the formation of endogenous “danger signals” that trigger an inflammatory response via pattern recognition receptors. Consistent with this model are our findings that oxidation products of the abundant phospholipid PAPC activated TLR2-dependent inflammatory gene expression *in vivo* after peritoneal injection in mice and *in vitro* in bone marrow-derived macrophages (BMDM), as well as previous studies that have shown that oxidized phospholipids induce TLR2 or TLR4-dependent immune responses [25,26]. Although we used OxPAPC as a model in this study, it is possible that other lipid oxidation products cause similar TLR2-dependent effects.

To further understand the mechanisms of biological activity of oxidized phospholipids it is essential to examine structural requirements for their TLR2-inducing activities. Previous studies have revealed that structurally related oxidized phospholipids have different biological activities which were profoundly influenced by the functional groups at the *sn*-2 position [27–29]. A recent study demonstrated carboxyalkylpyrroles, end products of lipid oxidation, to be recognized by TLR2 [30]. We show here that activation of TLR2 by

oxidized phospholipids requires reducible functional groups on non-fragmented *sn*-2 acyl chains.

The complexity of TLR2 ligand recognition is illustrated by the fact that heterodimerization of TLR2 with either TLR1 or TLR6, as well as with CD36, was required for recognition of gram positive bacterial patterns [31–34]. Short chain oxidized phospholipids that contain an ((γ -hydroxy(or oxo)- α,β -unsaturated carbonyl group are ligands for CD36 [29]. Recently, oxidized phospholipids were shown to induce free radical species production via CD36 and TLR2 [35]. We cannot rule out a contribution of CD36 in OxPAPC-induced TLR2-dependent gene expression, however, since our data show the biologic activity to be contained in the long chain fraction of OxPAPC we conclude that the majority of the effects is mediated independently of CD36.

Some TLR2 ligands were shown to require accessory proteins for recognition [21] [22]. Among TLR accessory proteins in serum, CD14 and LPS binding protein (LBP) are of particular interest because we and others have previously shown their interaction with oxidized phospholipids [12,23,36,37]. CD14 is a glycosphosphatidylinositol (GPI)-anchored protein expressed on macrophages and neutrophils, but also exists in soluble form without GPI in serum. Soluble CD14 facilitates the transfer of LPS to TLR4/MD2, and also of microbial ligands to TLR2 receptor complexes [38,39]. Here we show, using BMDM from CD14^{-/-} mice, that inflammatory gene expression induced by oxidized phospholipids does not require membrane-bound CD14, but was dependent on the presence of serum. Moreover, addition of recombinant CD14 was not sufficient to restore TLR2-dependent effects of oxidized phospholipids on inflammatory gene expression. The requirement for serum indicates that other soluble accessory proteins are necessary for activation of TLR2 by oxidized phospholipids.

Compared to conventional TLR2 ligands, the effect of oxidized phospholipids on inflammatory gene expression is significantly weaker. Previous findings that oxidized phospholipids potentially inhibit conventional ligand-induced TLR2 activation point to the possibility that oxidized phospholipids are weak agonists and at the same time strong antagonists of TLR-dependent inflammatory pathways. This scenario is consistent with recent findings by Oskolkova et al, demonstrating a stronger antagonistic effect on TLR signaling by oxidized phospholipids [40].

HO-1 expression is induced by oxidized phospholipids via mechanisms independent of TLR2 [10,41], involving the redox-regulated transcription factor Nrf2. While various compounds in OxPAPC may induce Nrf2-dependent gene expression by activating NADPH-oxidase [42,43], PGPC was shown to activate PKC-dependent signaling [44] that by itself may activate Nrf2 [45]. Other receptors that have been implied to mediate effects of oxidized phospholipids are the VEGF receptor 2 [46], S1P1 receptor [47], prostaglandin E2 receptor 2 [48], or the PAF-receptor [49,50].

To examine the hypothesis that TLR2 is a sensor for oxidative tissue damage *in vivo*, we used a model of carbontetrachloride (CCl₄)-induced oxidative liver injury. It was previously shown that detrimental effects in this model were mediated by oxidized phospholipids, since overexpression of PAF-AH, an enzyme that degrades oxidized phospholipids was protective [24]. Our results show that after application of CCl₄, oxidized phospholipids accumulated in livers of wt as well as TLR2^{-/-} mice. However, hepatic tissue damage and inflammatory gene expression were significantly reduced in TLR2^{-/-} mice compared to wt mice, demonstrating a significant role for TLR2 in translating oxidative changes into inflammatory reactions.

Identification of mechanisms that link oxidative stress and inflammation may lead to novel therapeutic approaches for chronic inflammatory metabolic diseases. We show for the first time that oxidative tissue damage can be translated into an inflammatory response via recognition of oxidized phospholipids by the pattern recognition receptor TLR2, thus providing evidence for a role of the innate immune system in recognition of an oxidatively modulated microenvironment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- How is inflammation linked to oxidative tissue damage?
- Oxidized phospholipids (OxPL) induced TLR2- dependent gene expression in macrophages.
- OxPL induced inflammation in wild-type, but not in TLR2 KO mice.
- TLR2 KO mice were protected against CCl4-induced oxidative tissue damage.
- We conclude that TLR2 senses oxidative tissue damage by recognizing OxPL.

Figure 1. Oxidized phospholipids induce inflammatory gene expression via TLR2 *in vivo* and in cultured bone marrow-derived macrophages

OxPAPC (250 μg) was injected into the peritoneum of either wt or TLR2^{-/-} mice, and cells were isolated after 6 hours by lavage. qPCR for COX-2 (A), IL-1β (B) and HO-1 (C) was performed on mRNA isolated from peritoneal cells (n=3 mice for each condition). **p*<0.05, compared to saline control. Bone marrow-derived macrophages were cultured from wt or TLR2^{-/-} mice and treated with OxPAPC (25 or 50 mg/ml) for 3 hours. Messenger RNA was isolated and qPCR performed for Mip-2 (D), KC (E), and HO-1 (F) (n=4). **p*<0.05, compared to control.

Figure 2. Oxidized phospholipids induce Jnk and p38 phosphorylation via TLR2 in macrophages
 BMDM from wt or TLR2^{-/-} mice were treated with 50 µg/ml OxPAPC for indicated times. Cell lysates were analyzed by SDS-PAGE and Western blotting with anti-phospho JNK, anti-phospho p38, anti-phospho and total ERK antibodies. Images were scanned and quantified using Li-Cor Odyssey Infrared Imaging System.

Figure 3. Activation of inflammatory gene expression via TLR2 requires reducible functional groups on long-chain oxidized phospholipids
 BMDM from wt or TLR2^{-/-} mice were stimulated with 10μg/ml PAPC, 50μg/ml OxPAPC, 10μg/ml LysoPC, 50μg/ml NaBH₄⁻ reduced OxPAPC, 10μg/ml POVPC, 10μg/ml PGPC, 1μg/ml LPS, or 1μg/ml LTA. Isolated short chain fraction and long chain fraction were added in amounts equivalent to 50μg/ml OxPAPC. RNA was isolated after 3 hours, and COX-2 (A) or HO-1 (B) expression was analyzed using qRT-PCR. For reduction, OxPAPC was incubated with sodium borohydride (NaBH₄, 20 μg/ml) in a 0.1M borate buffer (pH 8.0) or borate buffer alone (sham) for 15min at room temperature and then extracted as previously described [16]. The data (n=4) represent mean±SD. **p*<0.05.

Figure 4. Recognition of oxidized phospholipids via TLR2 requires serum accessory proteins but not membrane-bound CD14

Bone marrow-derived macrophages from wt or CD14^{-/-} mice were treated with 10 μ g/ml PAPC, 50 μ g/ml OxPAPC and long chain fraction of OxPAPC for 3 hours either in serum-free or 2% serum-containing medium. RNA was isolated after 3 hours, and COX-2, IL1 β and HO-1 expression was analyzed using qPCR. (n=3); results are shown as mean $\pm$ SD.

* $p < 0.05$.

Figure 5. TLR2 deficiency protects against oxidative tissue damage-induced inflammation (A) Wild-type or TLR2^{-/-} mice were treated with olive oil (control) or carbon-tetrachloride (CCl₄) and immunohistochemistry on livers was performed using the E06 antibody. (B) CCl₄ treatment of wt mice for 6 or 24 hours drastically increased serum ALT levels, which were significantly reduced in TLR2^{-/-} mice. (C) After 6 hours of CCl₄ treatment, inflammatory gene expression in livers of wt and TLR2^{-/-} mice was examined by qRT-PCR. n=5 for each timepoint, **p*<0.05.