Purinergic receptor X7 is a key modulator of metabolic oxidative stress-mediated autophagy and inflammation in experimental nonalcoholic steatohepatitis

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Das S, Seth RK, Kumar A, Kadiiska MB, Michelotti G, Diehl AM, Chatterjee S. Purinergic receptor X7 is a key modulator of metabolic oxidative stress-mediated autophagy and inflammation in experimental nonalcoholic steatohepatitis. Am J Physiol Gastrointest Liver Physiol 305: G950–G963, 2013. First published October 24, 2013; doi:10.1152/ajpgi.00235.2013.—Recent studies indicate that metabolic oxidative stress, autophagy, and inflammation are hallmarkstes of nonalcoholic steatohepatitis (NASH) progression. However, the molecular mechanisms that link these important events in NASH remain unclear. In this study, we investigated the mechanistic role of purinergic receptor X7 (P2X7) in modulating autophagy and resultant inflammation in NASH in response to metabolic oxidative stress. The study uses two rodent models of NASH. In one of them, a CYP2E1 substrate bromodichloromethane is used to induce metabolic oxidative stress and NASH. Methyl choline-deficient diet feeding is used for the other NASH model. CYP2E1 and P2X7 receptor gene-deleted mice are used to establish their roles in regulating metabolic oxidative stress and autophagy. Autophagy gene expression, protein levels, confocal microscopy-based-immunolocalization of lysosome-associated membrane protein (LAMP2A) and histopathological analysis were performed. CYP2E1-dependent metabolic oxidative stress-induced increases in P2X7 receptor expression and chaperone-mediated autophagy markers LAMP2A and heat shock cognate 70 protein caused depletion of light chain 3 isoform B (LC3B) protein levels. P2X7 receptor gene deletion significantly decreased LAMP2A and inflammatory indicators while significantly increasing LC3B protein levels compared with wild-type mice treated with bromodichloromethane. P2X7 receptor-deleted mice were also protected from NASH pathology as evidenced by decreased inflammation and fibrosis. Our studies establish that P2X7 receptor is a key regulator of autophagy induced by metabolic oxidative stress in NASH, thereby modulating hepatic inflammation. Furthermore, our findings presented here form a basis for P2X7 receptor as a potential therapeutic target in the treatment for NASH.

5,5-dimethyl-1-pyrroline N-oxide-nitrene adducts; lipid peroxidation; tyrosine nitration; cytokines; CYP2E1; light chain 3 isoform B; GABA-A receptor-associated protein

NONALCOHOLIC STEATOHEPATITIS (NASH) is associated with metabolic oxidative stress, often ascribed to enhanced oxidation of membrane lipids, mitochondrial uncoupling of the electron transport chain, and higher cytochrome P450 enzyme activity (18, 29, 30). Metabolic oxidative stress leads to cellular stress and induces cell death mechanisms, which modulate inflammatory microenvironment in NASH (5, 24).

Cell death mechanisms in disease pathogenesis are synonymous with necrosis, autophagy, and apoptosis; however, much attention is recently focused on the various roles of autophagy in cellular processes, inflammation, energy homeostasis, and immunity (8, 35). Despite increased attention on autophagy, the mechanisms that control autophagy in NASH remain unclear. Autophagy is a central eukaryotic process with many cytoplasmic homeostatic roles (8). It is a critical pathway for degradation of intracellular components by lysosomes and has established roles in hepatic lipid metabolism, insulin sensitivity, and cellular injury (2). Autophagy, dependent on the involvement of the cellular components, has been classified into macro, micro, and chaperone-mediated autophagy. It is crucial, however, that these processes rely on formation of autophagosomes, increased levels of light chain 3 isoform B (LC3B), fusion of autophagosome with the lysosome, and degradation of the cellular cargo by lysosomal enzymes (2, 15, 23, 26). Interestingly, a recent investigation by Lin. et al. (21) reports pharmacological promotion of autophagy-alleiliated steatosis and injury in alcoholic and nonalcoholic fatty liver. Given the importance of autophagy in cellular homeostasis and disease pathogenesis, it is also important to study both the inducers and regulators of autophagy in a pathological state like NASH. One of the key components of the inflammatory microenvironment in various disease pathologies is the inotopic purinergic receptors (P2 receptors) that respond to the damage-associated molecular patterns (3, 28). There is an increased focus on a subclass of these receptors (P2X7 receptor), which bind ATP with high affinity and are known to regulate inflammation in early steatohepatitic injury in mice (5). Apart from the binding to ATP, purinergic receptor X7 (P2X7) receptor (P2X7r) has been shown to increase NADPH oxidase activity, increase Kupffer cell- major histocompatibility complex (MHC) class II expression, and regulate inflammation (5). With increased focus on purinergic receptors, inflammasome activation, and autophagy in NASH, we explored the possibility of P2X7r as a key regulator of autophagy and the resultant inflammation in NASH.

The present study utilizes two distinct models of experimental NASH, which have been established to produce pathology of full-blown NASH. The toxin model of NASH utilizes coadministration of high-fat diet and a low-dose environmental...
toxin bromodichloromethane (BDCM) (7, 31). The diet-induced model exposes mice to methyl-choline-deficient (MCD) diet for 8 wk (36, 37). In a first ever report, we show that in both models of NASH there was an increased metabolic oxidative stress, which caused higher expression of P2X7 receptors. Oxidative stress induced both early and late autophagy proteins and was dependent on the presence of P2X7 receptors. P2X7 receptor modulated LC3B protein depletion, an event that is crucial for autophagy. Further absence of P2X7 receptors significantly reduced downstream inflammation activation and NASH pathophysiology. The present study also advances our understanding in considering P2X7r-linked autophagy pathways as potential therapeutic targets in NASH.

MATERIALS AND METHODS

Mouse Model

Pathogen-free, custom diet-induced obesity (DIO) adult male mice with a C57BL/6J background (Jackson Laboratories, Bar Harbor, ME) were used as models of toxin-induced NASH. They were fed with a high-fat diet (60% kcal) from 6 wk to 16 wk. All experiments were conducted at the completion of 16 wk. The animals were housed one in each cage before any experimental use. Mice that contained the deleted P2X7r gene (purinergic receptor X7 knockout) (8) were used as models of toxin-induced NASH. They were fed with a high-fat diet and treated identically to DIO mice. Another set of mice were pathogen-free, adult male with a C57BL/6J background fed with MCD diet and were used as models for diet-induced NASH. They were fed with MCD diet from 8 to 16 wk. Mice that contained the disrupted P2X7r gene were also fed with MCD. Mice had ad libitum access to food and water and were housed in a temperature-controlled room at 23–24°C with a 12-h:12-h light/dark cycle. All animals were treated in strict accordance with the NIH Guide for the Humane Care and Use of Laboratory Animals, and the experiments were approved by the institutional review boards at NIEHS, Duke University, and the University of South Carolina at Columbia.

Induction of Liver Injury in Mice

DIO mice or high-fat-fed gene-specific knockout mice at 16 wk were administered BDCM (2.0 mmol/kg, diluted in olive oil) through the intraperitoneal route. However, DIO mice treated with olive oil (diluent of BDCM) were used as control. After completion of the treatment, mice of all study groups were killed for liver tissue and serum for the further experiments. MCD-diet-fed wild-type or gene-specific knockout mice were killed at 16 wk for liver tissue for experiments. However, methylcholine-sufficient (MCS) diet-fed mice were used as control.

ELISA

Immunoreactivity for 5,5-dimethyl-1-pyrroline N-oxide (DMPO)-nitrore adduct was detected in liver homogenate using standard ELISA (4).

Histopathology. Liver tissue sections from each mouse were fixed in 10% neutral buffered formalin. Formalin-fixed liver sections were stained with hematoxylin and eosin and observed under the light microscope. Collagen content in liver tissue was evaluated using Sirius red-stained liver sections. Each liver section was stained with Picro-Sirius red (Sigma-Aldrich, St. Louis, MO) and counterstained with fast green (Sigma-Aldrich).

Serum ALT, alkaline phosphatase, and albumin levels. Blood from DIO, DIO + BDCM, and P2X7 receptor-deleted mice were collected by cardiac puncture, and serum was separated by standard techniques. The serum alanine aminotransferase (ALT) and alkaline phosphatase were measured at the clinical chemistry core. Albumin was measured using the commercially available kit (Alpha Diagnostic, San Antonio, TX).

Immunohistochemistry. Formalin-fixed, paraffin-embedded liver tissue from all the mice groups were cut into 5-μm-thick tissue sections. Each section was deparaffinized using standard protocol. Briefly, sections were incubated with xylene twice for 3 min, washed with xylene:ethanol (1:1) for 3 min, and rehydrated through a series of ethanol (twice with 100%, 95%, 70%, 50%), twice with distilled water, and finally rinsed twice with PBS (Sigma-Aldrich). Epitope retrieval of deparaffinized sections was carried out using epitope retrieval solution and steamer (IHC-World, Woodgale, MD) following the manufacturer’s protocol. The primary antibodies were 1) anti-4-hydroxynonenal, 2) anti-3-nitrotyrosine, 3) anti-P2X7r, 4) anti-GABA-A receptor-associated protein (GABARAP), 5) antilyssosome-associated membrane protein (LAMP)-2A, 6) anti-MHC II, 7) anti-IL-1β, 8) anti-IFN-γ, and 9) anti-LC3B. Primary antibodies were purchased from Abcam (Cambridge, MA), Millipore (Temecula, CA), and R&D Systems (Minneapolis, MN) and used in 1:250 dilutions. Antibody-specific immunohistochemistry were performed using Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) following manufacturer’s protocols. 3,3’ Diaminobenzidine (Sigma-Aldrich) was used as a chromogen substrate. Sections were counter-stained by Mayer’s hematoxylin (Sigma-Aldrich). Washing with PBS (Sigma-Aldrich) was performed thrice between the steps. Sections were mounted in Simpo mount (GBI Laboratories, Mukilteo, WA) and observed under a ×20 oil objective. Morphometric analysis was done using CellSens Software from Olympus America.

Western Blotting

Tissue (30 mg) from each liver sample was homogenized in 100 μl of RIPA buffer (Sigma-Aldrich) with protease inhibitor (1×) (Pierce, Rockford, IL) using dounce homogenizer. The homogenate was centrifuged, and the supernatant was diluted 1:5 and used for SDS PAGE and subjected to Western blotting. Novex (Invitrogen, Carlsbad, CA) 4–12% bis-tris gradient gel was used for SDS PAGE. Proteins were transferred to nitrocellulose membrane using precut nitrocellulose/filter paper sandwiches (Bio-Rad Laboratories, Hercules, CA) and Trans-Blot Turbo transfer system (Bio-Rad) in case of low molecular weight proteins and using wet transfer module from Invitrogen in case of high molecular weight proteins. A solution of 5% non-fat milk was used for blocking. Primary antibodies against heat shock cognate 70 (Hsc 70), GABARAP, LC3B, LAMP-2A, Caspase-1, β-actin (all from Abcam), high-motility group box protein (HMGB)-1 (Millipore) at recommended dilutions, and compatible horseradish peroxidase-conjugated secondary antibodies were used. Pierce ECL Western Blotting substrate (Thermo Fisher Scientific, Rockford, IL) was used. The blot was developed using BioMax MS Films and cassettes (with intensifying screen, Kodak). The images were subjected to densitometry analysis using Lab Image 2006 Professional 1D gel analysis software from KAPELAN Bioimaging Solutions (Lipzig, Germany).

Quantitative RT-PCR

Gene expression levels in tissue samples were measured by two-step qRT-PCR. Total RNA was isolated from liver tissue by homogenization in TRIzol reagent (Invitrogen) according to the manufacturer’s instructions and purified with the use of RNAeasy mini kit columns (Qiagen, Valencia, CA). Purified RNA (1 μg) was converted to cDNA using iScript cDNA synthesis kit (Bio-Rad) following the manufacturer’s standard protocol. qRT-PCR was performed with the genome-specific primers using SsoAdvanced SYBR Green supermix (Bio-Rad) and CFX96 thermal cycler (Bio-Rad). Threshold Cycle (Ct) values for the selected genes were normalized against 18S (internal control) values in the same sample. Each reaction was carried out in
Table 1. Sequences for the primers used for real-time PCR in 5’ to 3’ orientation

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
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<tr>
<td>CYP2E1</td>
<td>Sense: GGGGACATGCGTCTCCGATGAT</td>
</tr>
<tr>
<td></td>
<td>Antisense: CCGAAGCTTCTCCGATTCGG</td>
</tr>
<tr>
<td>P2X7r</td>
<td>Sense: CGGAATTCCGTCATAGGATGTTT</td>
</tr>
<tr>
<td></td>
<td>Antisense: CCCCACCTCTGGAGCCATTG</td>
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<tr>
<td>GABARAP</td>
<td>Sense: AAGGAAAGACAGGACACGCTGGA</td>
</tr>
<tr>
<td></td>
<td>Antisense: GGATTGCCAAGTGGAGTGGT</td>
</tr>
<tr>
<td>Atg-2A</td>
<td>Sense: AAATCAACAGTGGCTAAGGGG</td>
</tr>
<tr>
<td></td>
<td>Antisense: GAGAAGTGGGCTGATCTGTC</td>
</tr>
<tr>
<td>LC3B</td>
<td>Sense: GCTGTCGAAGAACAACACGAGAC</td>
</tr>
<tr>
<td></td>
<td>Antisense: AGTGAATCTAGTGACCCAGGA</td>
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<tr>
<td>MHC II</td>
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<td></td>
<td>Antisense: CGTGGTGCCGCTCATAATTGTC</td>
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<tr>
<td>LAMP2A</td>
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<tr>
<td></td>
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<tr>
<td>TGF-β</td>
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<tr>
<td></td>
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<tr>
<td>COL-1-α-1</td>
<td>Sense: TGACAGATGGGCTGAGGAGAGAG</td>
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<tr>
<td></td>
<td>Antisense: GGTTGCGCACAGTGGTCTTC</td>
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<tr>
<td>α-SMA</td>
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<td></td>
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<td>Hsc 70</td>
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<td>Caspase-1</td>
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<td></td>
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P2X7, purinergic receptor X7; GABARAP, GABA-A receptor-associated protein; Atg-2A, autophagy-related protein 2A; LC3B, light chain 3 isoform B; MHCII, histocompatibility complex class II; LAMP2A, lysosome-associated membrane protein 2A; SMA, smooth muscle actin; Hsc 70, heat shock cognate 70; HMGB-1, high-mobility group box protein 1.

triplicate for each gene and for each tissue sample. DIO mouse liver sample was used as the control for comparison with all other liver samples in the toxin-induced NASH group, and MCS-diet-fed mouse liver sample was used as control for comparison with all other liver samples of the diet-induced NASH group. The relative fold change was calculated by the 2^{–ΔΔCt} method. The sequences for the primers used for real-time PCR are provided in Table 1.

Confocal Laser Scanning Microscopy

Frozen tissue sections after formalin fixation were analyzed by confocal microscopy using Zeiss LSM 510-UV Meta (Carl Zeiss, Oberkochen, Germany) and a Plan-Neofluor ×40/1.3 objective with different zoom levels. The primary antibody for LAMP-2A was obtained from Abcam; the Cy3-conjugated affinity pure secondary antibody was obtained from Jackson ImmunoResearch (West Grove, PA). The BODIPY dye and DAPI used were supplied by Molecular Probes (Eugene, OR).

Statistical Analyses

All in vivo experiments were repeated three times with three mice per group (N = 3; data from each group of 3 mice were pooled). All in vitro experiments were repeated three times, and the statistical analysis was carried out by ANOVA followed by the Bonferroni post hoc correction for intergroup comparisons. Quantitative data from Western blots as depicted by the relative intensity of the bands were analyzed by performing a Student’s t-test. P < 0.05 was considered statistically significant.

RESULTS

Steatohepatitic Injury in Obese Mice is Associated with Increased Metabolic Oxidative Stress

Metabolic oxidative stress in obesity-induced steatohepatitic injury is caused following increased CYP2E1 activity, mitochondrial uncoupling of the electron transport chain, or toxin metabolism following administration of hepatotoxic drugs (1, 5, 11, 22). We and others (5, 31, 32) have shown that toxin-induced steatohepatitis models produce metabolic oxidative stress. The toxin- and diet-induced steatohepatitic injury models that produce metabolic oxidative stress are relevant to clinical outcomes because patients with NASH show oxidative stress and have been hypothesized to augment disease progression (27). Our results demonstrate that CYP2E1 increases lipid peroxidation and oxidative and nitrosative stress upon steatohepatitic injury. 4-Hydroxypropanal (4-HNE), a marker for lipid peroxidation, was found to be increased in liver tissues from both the toxin (DIO + BDCM) and diet models (MCD-diet-fed mice) (Fig. 1, A, i–ii and iv–v, and C) compared with the respective controls (DIO and MCS diet). DIO mice with deletion of the CYP2E1 gene and administered BDCM showed decreased 4-hydroxypropanal adducts, compared with DIO + BDCM only group (Fig. 1, A, ii–iii, and C). Tyrosine nitration, a marker of oxidative and nitrosative stress, as evidenced by 3-nitrotyrosine immunoreactivity was found to be increased in liver tissues from both the toxin (DIO + BDCM) and diet models (MCD-diet-fed mice) (Fig. 1, B, i–ii and iv–v, and D) compared with the respective controls (DIO and MCS diet). DIO mice with deletion of the CYP2E1 gene and administered BDCM showed decreased 3-nitrotyrosine immunoreactivity, compared with DIO + BDCM only group (Fig. 1, B, ii–iii, and D). To estimate the extent of protein radical formation and corresponding oxidation, DMPO-nitroxide adducts were measured in the liver homogenates of the toxin model of steatohepatitic injury. Results indicated that DMPO-nitroxide adducts were significantly increased in DIO + BDCM group compared with DIO only group (P < 0.05) (Fig. 1E). CYP2E1-
A

(i) 4-HNE DIO
(ii) 4-HNE DIO+BDCM
(iii) 4-HNE CYP2E1 KO
(iv) 4-HNE MCS
(v) 4-HNE MCD

B

(i) 3NTyr DIO
(ii) 3NTyr DIO+BDCM
(iii) 3NTyr CYP2E1 KO
(iv) 3NTyr MCS
(v) 3NTyr MCD

C

4-HNE (% Positive Immunoreactive Cells)

D

3NTyr (% Positive Immunoreactive Cells)

E

DMPO Nitrone Adducts (Relative light units)

F

CYP2E1 (Normalized Expression X Fold DIO)

G

CYP2E1 (Normalized Expression X Fold MCS)

H

CYP2E1/β-Actin Ratio (Arbitrary Units)

I

CYP2E1/β-Actin Ratio (Arbitrary Units)
deleted mice fed with high-fat diet and coadministered BDCM had a significant decrease in the nitrone adducts (Fig. 1E, \( P < 0.05 \)). Hepatic CYP2E1 mRNA expressions showed an increase in DIO+BDCM- (not statistically significant) and MCD-diet-treated mice compared with DIO- or MCS-diet-fed mice (Fig. 1, F and G; \( P < 0.05 \)), suggesting that increased CYP2E1 expression might play a prominent role in the metabolic oxidative stress generation in steatohepatitic injury. The protein levels of CYP2E1 showed a small increase in DIO+BDCM group compared with DIO group (not statistically significant) but was unchanged in the MCS and MCD groups (Fig. 1H). Given the importance of P2X7 receptor in innate immunity, inflammation, cell death, and disease pathogenesis in NASH following its activation, we investigated the role of metabolic oxidative stress in modulating the expression of hepatic P2X7 receptors, those that might amplify inflammation. Gene expression was measured by qRT-PCR. Results indicated that there was a significant increase in P2X7 receptor mRNA expression in DIO+BDCM group and MCD-diet-fed group compared with DIO- and MCS-diet groups (\( P < 0.05 \)) (Fig. 2, A and B). Deletion of the CYP2E1 gene in mice fed with high-fat diet and exposed to BDCM had significant decrease in the P2X7 receptor mRNA expression compared with DIO+BDCM group (\( P < 0.05 \)) (Fig. 2A). Similar observations were found in the P2X7 receptor immunoreactivity in hepatic tissue. DIO+BDCM group and MCD-diet-fed group had higher P2X7 receptor levels compared with DIO-only and MCS-diet groups (Fig. 2C, i–ii and iv–v). Mice that had the deletion of CYP2E1 gene but were coadministered with high-fat diet and BDCM had decreased levels of P2X7 receptors (Fig. 2C, iii). Hepatocytes, Kupffer cells, and liver sinusoidal endothelial cells had increased expression of P2X7 receptor mRNA in the toxin model compared with untreated samples.

**CYP2E1-Mediated Metabolic Oxidative Stress in Steatohepatitic Injury Induces Macrophagophagy and Causes Increased Expression of Chaperone-Mediated Autophagy-Related Proteins**

To study the effect of metabolic oxidative stress on the induction of autophagy-related proteins in steatohepatitic injury, markers of macro and chaperone-mediated autophagy were analyzed. Results indicated that mRNA expressions of early autophagy genes, GABARAP, autophagy-related protein 2A (Atg2A), and LC3B, and components of the lysosomal translocation complex, LAMP2A and Hsc 70, were significantly elevated in DIO+BDCM group and MCD-diet-fed group compared with DIO- and MCS-diet-fed group, respectively (Fig. 3, A and B) (\( P < 0.05 \)). Mice that had a deletion of CYP2E1 gene and were coexposed to high-fat diet and BDCM had a significant decrease in early autophagy markers GABARAP, Atg2A, and LC3B compared with DIO+BDCM group, whereas there was no significant change in the expression pattern of Hsc 70 (Fig. 3A). LAMP2A mRNA levels were decreased in CYP2E1-deleted mice, but the change was not significant (Fig. 3A). Immunoreactivity of early autophagy marker GABARAP (initiator of the autophagy process) and LAMP2A, an important constituent of the lysosomal translocation complex, was increased in livers of steatohepatitic mice in both models of injury (Fig. 3, C and D, i–ii and iv–v). Absence of the CYP2E1 gene caused a decrease in the immunoreactivity of both GABARAP and LAMP2A, suggesting that metabolic oxidative stress and related oxidized proteins caused due to the involvement of CYP2E1 has led to the initiation and sustenance of the autophagy process (Figs. 3, C, iii, and D, iii). Hsc 70, which is responsible for recognizing and mediating substrate targeting for chaperone-mediated autophagy, was not significantly altered in both DIO+BDCM group and MCD-diet group (Fig. 3, E and F) (\( P < 0.05 \)); however, the deletion of the CYP2E1 gene had significantly decreased levels of Hsc 70 in the toxin model (Fig. 3, E and F) (\( P < 0.05 \)), suggesting a possible role of CYP2E1 in regulating the Hsc 70 expressions in this model of steatohepatitic injury.

**P2X7 Receptor Modulates Autophagy Induced by Metabolic Oxidative Stress in Steatohepatitic Injury**

Based on our present findings that show a significant increase in P2X7 receptor gene expression upon increased metabolic oxidative stress in NASH models, we investigated its role in mediating autophagy and modulating proteins in lysosomal translocation complex. mRNA expression of early autophagy-inducing genes, GABARAP, Atg2A, and LC3B, were significantly downregulated in P2X7 receptor gene-deleted mice coadministered with high-fat diet+BDCM and in MCD-diet-fed mice compared with DIO+BDCM- and MCD-diet-fed groups, respectively (Fig. 4, A and B). Lysosomal translocation complex proteins Hsc 70 and LAMP2A, which are significant players in chaperone-mediated autophagy, were downregulated in P2X7 receptor-deleted mice, either treated with toxin BDCM or fed with MCD diet, compared with wild-type counterparts (\( P < 0.05 \)) (Fig. 4, A and B). Western blot analysis of GABARAP revealed a significant decrease in the band intensity, signifying decreased GABARAP protein levels in P2X7 gene-deleted mice treated with both the toxin and MCD diet compared with the wild-type counterparts (\( P < 0.05 \)) (Fig. 4, C and D). Interestingly, P2X7 receptor-deleted mice from both DIO+BDCM- and MCD-diet groups had significantly increased levels of LC3B compared with their wild-type counterparts (\( P < 0.05 \)) (Fig. 4, C and D). In contrary to the P2X7 receptor gene-deleted mice, protein levels of LC3B were decreased in DIO+BDCM- and MCD-diet-fed groups compared with DIO- and MCS-diet-fed groups, suggesting LC3B depletion in NASH (Fig. 4E). Immunohistochemistry of LC3B also showed increased LC3B protein immunoreactivity in P2X7 receptor-deleted liver compared with DIO+BDCM only group (Fig. 4F). LAMP2A protein levels, as studied by immunohistochemistry, showed a decreased immunoreactivity in P2X7 receptor-deleted mice, having been exposed to toxin or MCD diet compared with the wild-type counterparts (Fig. 5A). Because Hsc 70 plays a crucial role in recruiting peptides for the lysosomal translocation process and is a key mediator of late autophagy, Western blot analysis of Hsc 70 was carried out (6, 20). Results showed that P2X7 receptor gene-deleted mice had a significant decrease in Hsc 70 protein levels compared with their wild-type counterparts (\( P < 0.05 \)) (Fig. 5, B and C). The results demonstrate that P2X7 receptor modulates both the early autophagy and the late chaperone-mediated lysosomal translocation processes in steatohepatitic injury.
P2X7 Receptor Is a Key Regulator of LAMP2A Association with the Lysosomal Membrane During Increased Metabolic Oxidative Stress in Steatohepatitic Injury

To show the extent of lysosomal membrane association of LAMP2A, confocal laser scanning microscopy was performed. Results showed that there was an increase (3-fold) in the number of events of lysosomal membrane (green) associations of LAMP2A (red), as evidenced by colocalization spots (yellow) in DIO/H11001 BDCM group compared with DIO only group (Fig. 6A, v–viii). P2X7 receptor-deleted mice that were coadministered with high-fat diet and BDCM had no visible colocalizations in the same numbers of field analyzed (Fig. 6A, ix–xii). MCD mice liver had more LAMP-2A membrane fusions compared with MCS mice as evidenced by confocal microscopy (Fig. 6B). These results suggested that the absence of P2X7 receptor gene significantly affected LAMP2A association with the lysosomal membrane.

Fig. 2. A and B: mRNA expression of purinergic receptor X7 (P2X7) receptor as assessed by quantitative real-time PCR, normalized against DIO and MCS groups. CYP2E1 KO represents liver mRNA expression from CYP2E1 gene-depleted mice fed with high-fat diet (*P < 0.05). C: immunohistochemistry of P2X7 receptor protein from liver slices of DIO, DIO/BDCM, CYP2E1 KO, MCS, and MCD groups. The localization of the receptor-positive staining is shown by arrows.
P2X7 receptor as a therapeutic target in liver fibrosis.
P2X7 Receptor Modulates Autophagy-Linked Inflammation in NASH

To explore the regulation of P2X7 receptor and its downstream effectors in autophagy-linked inflammation in steatohepatitic injury, mRNA expression of caspase-1, IL-1β, TNF-α, and IFN-γ were studied. Results indicated that there was a significant increase (more than 17-fold) of caspase-1, IL-1β (4-fold), TNF-α, and IFN-γ in both DIO/BDCM group and MCD-diet-fed group, compared with DIO- or MCS-diet-fed group (Fig. 7, A and B). Cleaved caspase-1, which is an indicator of active caspase-1, was significantly decreased in P2X7 receptor gene-deleted mice from both DIO/BDCM-and MCD-treated group compared with the wild-type counterparts (Fig. 7, C and D). Immunohistochemistry of IL-1β and IFN-γ showed an increase in the immunoreactivity of both these proteins in livers of DIO/BDCM- and MCD-diet-fed groups compared with only DIO- or MCS-diet-fed groups (Fig. 7, E and F, ii–iv), whereas a decrease was observed in livers...
of mice that contained the deletion of the P2X7 receptor gene (Fig. 7, E and F, iii and vi) (16, 19). Results also indicated that P2X7 gene-deleted mice from both DIO/BDCM-coexposed group had decreased amounts of HMGB-1 protein, a damage-associated molecular pattern linked to inflammation, as analyzed by Western blot (Fig. 8, A and B), compared with their wild-type controls (P < 0.05) although the HMGB-1 protein level was not significantly different in MCD-treated group (Fig. 8, A and B). The decreased expression of HMGB-1, IL-1β, IFN-γ, and cleaved caspase-1 in P2X7 receptor-deleted mice shows that metabolic oxidative stress-induced autophagy and corresponding inflammation are regulated by the P2X7 receptors in steatohepatitic injury.

P2X7 Receptor Gene-Deleted Mice Are Protected from Steatohepatitic Injury and Fibrosis

To ensure that the genetic deletion of P2X7 receptor in mice exposed with either high-fat diet+BDCM or MCD-diet-fed group were protected from symptoms of steatohepatitic injury, mRNA expressions of fibrotic markers α-smooth muscle actin (SMA), TGF-β, and Col-1-α1 were analyzed. Results showed that α-SMA, TGF-β, and Col-1-α1 were significantly decreased in P2X7 receptor gene-deleted mice compared with wild-type mice either fed with high-fat diet and BDCM or MCD (Fig. 8C, P < 0.05). Hematoxylin and eosin stainings of the liver slices of P2X7 receptor-deleted mice administered the toxin and fed with high-fat diet showed decreased hepatocyte necrosis compared with wild-type mice fed with high-fat diet (Fig. 8D). There was a decrease in Picro-Sirius red staining in livers of P2X7 receptor gene-deleted mice compared with the wild-type mice fed with high-fat diet and BDCM, suggesting decreased fibrosis (Fig. 8E). DIO+BDCM mice also had increased serum ALT, alkaline phosphatase, and albumin levels compared with DIO and P2X7 receptor gene-deleted mice.

DISCUSSION

To date, nonalcoholic fatty liver disease and its associated steatohepatitic injury are considered an emerging epidemic in light of the dramatic increase in obesity rates (9, 12). With the progressive nature of NASH and its rising prevalence, there is a significant need for specific and targeted treatments. This is complicated by the fact that there are not many validated therapies for nonalcoholic fatty liver disease other than weight loss, which is well known to have a poor long-term success rate (10). Cellular death pathways and inflammation play crucial roles in NASH pathophysiology, and it is essential that we identify new mediators of NASH pathophysiology that are important regulators of autophagy and inflammatory pathways. Impairment of autophagy that is correlated with hepatic lipid accumulation and obesity has been found to have a significant impact on progression of NASH (2). There is also a perception that agents that augment autophagy can have therapeutic potential in NASH (21). However, no significant research has been in place that links metabolic oxidative stress, autophagy,
and P2X7 receptors and their crosstalk in modulating disease progression in NASH. Targeting a key regulator of each of these components can have benefits in therapy of NASH. This study shows that P2X7 receptor, which is upregulated by metabolic oxidative stress (Fig. 2), serves as a key regulator in modulating the oxidative stress induced-autophagy process (Fig. 3). P2X7 downregulation in CYP2E1 knockout mice, coupled with an earlier report by this group that showed extracellular ATP (ligand for P2X7) release from necrosed hepatocytes (those that had higher 4-HNE staining) in a CCl4-mediated NASH model, correlates oxidative stress, upregulation of P2X7 receptor, and its downstream events (5). The P2X7 receptor might modulate the autophagy process by allowing depletion of LC3B, which is supposedly an early autophagy marker (Fig. 4E), while increasing, albeit in small proportions, Hsc 70 and LAMP2A mRNA levels (Fig. 3, A and B) and allowing LAMP2A association with the lysosomal membrane (Fig. 6). Interestingly, LC3B mRNA levels in NASH models showed a significant increase, whereas the protein levels decreased (Figs. 3, A and B, and 4E). The

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Fig. 6. A: LAMP2A colocalization with lysosomal membrane (×60 zoom 3). Confocal laser scanning image of LAMP2A and membrane lipid (Bodipy stain) from liver sections of DIO, DIO+BDCM, and P2X7 receptor-deleted group coexposed to high-fat diet and BDCM. B: LAMP2A colocalization with lysosomal membrane (×60). Confocal laser scanning image of LAMP2A and membrane lipid (Bodipy stain) from liver sections of MCS, MCD, and P2X7 receptor-deleted group fed with MCD diet. Gray arrows show LAMP2A localizations, whereas white arrows depict colocalizations of LAMP2A with lysosomal membrane depicted by bodipy staining. Number of colocalizing events as shown by overlay of images were counted to reveal the extent of LAMP2A attachment to the lysosomal membrane.
Fig. 7. P2X7 receptor modulates inflammation in NASH. A and B: mRNA expression of proinflammatory mediators, as measured by quantitative real-time PCR in DIO, DIO+BDCM, P2X7 receptor gene-deleted mice, coexposed to high-fat diet and BDCM (toxin), MCS, MCD, and P2X7 receptor gene-deleted mice fed with MCD diet. The expression levels were normalized against DIO in toxin model and MCS in diet model. C: Western blot analysis of cleaved caspase-1 (active caspase-1) and its corresponding band quantification analysis (D) (*P < 0.05). Individual lanes representing DIO+BDCM and P2X7 receptor gene-deleted groups were separated from the parent blot to ensure better visibility. E and F: Immunoreactivity of IL-1β and IFN-γ as analyzed by immunohistochemistry. (i–vi): DIO, DIO+BDCM, P2X7 receptor-deleted group coexposed to high-fat diet and BDCM (toxin), MCS, MCD, and P2X7 receptor-deleted group exposed to MCD diet, respectively.
Fig. 8. A: Western blot analysis of high-motility group box protein (HMGB)-1 from liver homogenates of DIO+BDCM, P2X7 receptor-deleted group coexposed to high-fat diet and BDCM (toxin), MCD, and P2X7 receptor-deleted group exposed to MCD diet, respectively. B: Band quantification analysis following normalization against β-actin (*P < 0.05). C: quantitative real-time PCR analysis of the mRNA expression profiles of fibrosis markers in DIO+BDCM, P2X7 receptor-deleted group coexposed to high-fat diet and BDCM (toxin), MCD, and P2X7 receptor-deleted group exposed to MCD diet, respectively (*P < 0.05). SMA, smooth muscle actin. D: Hematoxylin and eosin staining of liver sections from DIO+BDCM group and P2X7 gene-deleted group coexposed to BDCM and high-fat diet. Areas of necrosis are shown in demarcated gray dashed lines and arrow heads. E: Picro-Sirius red staining for fibrosis in liver sections from DIO+BDCM group and P2X7 gene-deleted group coexposed to BDCM and high-fat diet.
contrasting result might point to a translational level regulation and can be explored further. These events can be crucial mediators of chaperone-mediated autophagy in the hepatic lobe, thus increasing inflammation, in a manner perhaps similar to P2X7 receptor-mediated release of autophagolysosomes/phagolysosomes into the extracellular matrix, causing increase in inflammation (33). The above mechanism of release of phagolysosomes to the extracellular matrix might be speculative for NASH at this point, but this study certainly proves the dependence of depleted LC3B and increased levels of LAMP2A and Hsc 70 on P2X7 receptor in both models of experimental NASH.

A previous study by our group showed that P2X7 receptor was crucial for causing Kupffer cell activation and inflammation following release of ATP from necrosed hepatocytes in CCl4-mediated early steatohepatitic injury (5). In the present study, we show that metabolic oxidative stress, which is associated with NASH, caused an upregulation of P2X7 receptors (Fig. 2). Metabolic oxidative stress also, mainly characterized by oxidatively modified proteins, has been known to induce chaperone-mediated autophagy, increased substrate translocation by Hsc 70 toward the lysosomal membrane, and increased LAMP2A levels (17). Furthermore, P2X7 receptors have been associated with autophagy, disruption of normal lysosomal functions, and release of autophagolysosomes to the extracellular matrix in the microglial cells, causing an increase in inflammation (33). Our present study shows that there was a significant downregulation of both early and late autophagy proteins, including LAMP2A in P2X7 receptor gene-deleted mice. P2X7 receptor-deleted mice also showed decreased release of IL-1β, IFN-γ, and HMGB-1 (damage-associated molecular pattern that contributes to inflammation, Ref. 34), observations that were dependent on significantly lower cleaved caspase-1 protein levels, implying the possible involvement of P2X7 receptors. This study, however, falls short on exploring the exact molecular mechanism of P2X7 receptor involvement and on having a clear answer whether it is the increased calcium ion-mediated change in lysosomal pH, lysosomal functional impairment, or expulsion of the lysophagosome to the extracellular matrix that caused an increase in the inflammation. The present study also establishes a direct correlation between the decreased numbers of LAMP2A-lysosomal membrane associations, reduced inflammation, and decreased NASH pathophysiology in P2X7 receptor gene-deleted mice (Figs. 6, 7, and 8). These observations assume significance because LAMP2A levels and its association with the lysosomal membrane are an important step for chaperone-mediated autophagy, which occurs late in the autophagy process. Our studies showed a depletion of LC3B, a autophagosome protein in NASH pathophysiology (Fig. 4E), but not in LAMP2A, which is contrary to a study by Fortunato et al. (13) that showed decreased LAMP2A levels linked to decreased fusion of the autophagosome with the lysosome, resulting in necrotic cell death and inflammation. This may be due to a different mechanism of a dysfunctional lysosomal function compared with our model that primarily evidenced LC3B depletion. Recent studies also link P2X7 receptor to a dysfunctional lysosome and autophagy protein LC3B (25, 33). In both cases, the fate of the cell and its link to immune activation remain a dysregulated lysosomal compartment and are regulated by ATP-binding P2X7 receptors in the latter study (25).

Interestingly, our study indicates significantly decreased LC3B protein levels in DIO+BDCM- and MCD-diet-fed livers compared with DIO- and MCS-diet-fed groups, whereas absence of P2X7 receptor gene elevates the LC3B protein levels (Fig. 4, C–E). This result is important because it has been shown that depletion of autophagic protein LC3B enhances caspase-1 activation and increases in inflammatory microenvironment (25). We have also found higher caspase-1 activation and levels of inflammatory indicators IL-1β, TNF-α, IFN-γ, and HMGB-1 in DIO+BDCM- and MCD-diet-fed groups, whereas a decrease in these indicators was seen in P2X7 receptor gene-deleted mice (Fig. 7). Taken together, the present study provides the first evidence that P2X7 receptor deletion results in downregulation of autophagy-related proteins, inflammation, and disease pathophysiology in NASH (Figs. 7 and 8).

Having significant evidence that P2X7 receptor is a key regulator of autophagy in NASH, it may be presumed that targeting the P2X7 receptor or the autophagy process in NASH can emerge as a good therapeutic option for the treatment of NASH. It is increasingly becoming clear that there are a series of studies that promote autophagy as a beneficial mechanism in NASH and an equal number of studies that conclude autophagy is central in causing NASH; the present study only predicts the role of P2X7 receptor-mediated defective autophagy as a cause for inflammation in NASH (14). The translational impact of this study will be enhanced with more mechanistic studies in the future involving cell-specific P2X7 receptor knockdowns and functional roles of different autophagy proteins in the presence or absence of the P2X7 receptor.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


