Histone deacetylase 9 plays a role in the anti-fibrogenic effect of astaxanthin in hepatic stellate cells

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Abbreviations: α -SMA, α -smooth muscle actin; ASTX, astaxanthin; CollA1, procollagen type I, alpha 1; ECM, extracellular matrix; HAT, histone acetyltransferase; HDAC, histone deacetylase; HSC, hepatic stellate cell; aHSC, activated HSC; iHSC, inactivated HSC; qHSC, quiescent HSC; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; MEF2, myocyte enhancer factor 2; PBC, primary biliary cirrhosis; qRT-PCR, quantitative realtime PCR; TGF β 1, transforming growth factor β 1.

Abstract

Activation of hepatic stellate cells (HSCs) is critical for liver fibrosis development. Previously, we showed that astaxanthin (ASTX), a xanthophyll carotenoid, has anti-fibrogenic effects in LX-2 cells, a human HSC cell line. We sought to determine the effect of ASTX on HSC activation, and to identify molecular mediators that are critically involved in the processes. ASTX prevented the activation of mouse primary HSCs, as evidenced by attenuated induction of procollagen type I α 1. In human primary HSCs, ASTX also inhibited transforming growth factor β 1 (TGF β 1)-induced fibrogenic gene expression. Among 11 classical histone deacetylases (HDACs), difference in HDAC9 mRNA levels between quiescent and activated HSCs was most evident while ASTX significantly decreased the expression of HDAC9 and its transcriptional regulator myocyte enhancer factor 2 (MEF2). ASTX decreased HDAC9 protein as well. In the activated HSCs, ASTX significantly reduced mRNA of HDAC9 and MEF2. Human primary biliary cirrhosis livers showed significantly higher HDAC9 mRNA and protein levels than normal livers, and other liver pathologies also exhibited induced HDAC9 expression. HDAC9 knockdown in LX-2 cells decreased TGF β 1-induced fibrogenic gene expression. In conclusion, ASTX inhibits HSC activation and facilitates HSC inactivation, which is attributable to its inhibitory action on HDAC9 expression.

Keywords: Astaxanthin; Carotenoids; Liver fibrosis; Hepatic stellate cells; Histone deacetylases; HDAC9

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common form of chronic liver diseases in the developed countries [1]. Fatty liver is susceptible to oxidative stress and inflammation, resulting in the progression of simple steatosis to non-alcoholic steatohepatitis (NASH) [2]. In response to oxidative stress, hepatocyte death and inflammation, the liver initiates a wound-healing response by producing extracellular matrix (ECM) and excessive ECM deposition leads to liver fibrosis [3]. Although liver fibrosis is a reversible process, it can slowly advance to cirrhosis, which is considered irreversible and disrupts normal liver functions [4].

Hepatic stellate cells (HSCs) are primarily responsible for ECM production and fibrosis development in the liver [5]. In the normal liver, quiescent HSCs (qHSCs) are present in the Space of Disse and contain cytoplasmic lipid droplets mainly consisting of retinyl esters [6]. In response to injury, qHSCs transdifferentiate to myofibroblast-like cells, which highly express α -smooth muscle actin (α -SMA), a myofibroblast marker, and ECM proteins such as procollagen type I α 1 (COL1A1). The activated HSCs (aHSCs) are highly proliferative and migrate to the sites of injury. Recent studies have suggested that HSC activation is a reversible process [7]. When stimulants, such as oxidants or fibrogenic cytokines, are removed, aHSCs are either eliminated through apoptosis or reverted to an inactive phenotype, i.e., inactivated HSCs (iHSCs) [7]. iHSCs share common features with qHSCs but differ in their sensitivity to fibrogenic insults.

Studies have suggested that histone acetylation as an epigenetic mode of action is involved in HSC activation and liver fibrosis [8]. Pan-histone deacetylases (HDACs) inhibitors, such as trichostatin A and valproic acid, are known to be potent inhibitors of HSC activation both in vitro [9] and in vivo [10]. Inhibitors specific for class II HDACs also play important roles in HSC activation [11]. Furthermore, HDAC inhibitors have been shown to ameliorate fibrosis not

only in the liver but in other organs such as the kidney [12] and the heart [13]. However, there has been limited understanding of HDAC isoform-specific roles in the pathogenesis of fibrosis.

Astaxanthin (ASTX) is a xanthophyll carotenoid abundant in red marine animals, such as salmon and shrimp [14]. Studies have shown that ASTX prevented the development of NASH and liver fibrosis in vivo [15, 16]. However, underlying mechanisms of action for the anti-fibrotic effects of ASTX remain unclear. We previously demonstrated that ASTX prevents fibrogenic responses and reactive oxygen species (ROS) accumulation induced by transforming growth factor β 1 (TGF β 1), a fibrogenic cytokine, in LX-2 cells, a human HSC cell line [17]. In another study, we also reported that ASTX not only inhibits the activation of qHSCs, but also reverts aHSCs to iHSCs [18]. However, molecular mechanisms underlying the anti-fibrogenic effect of ASTX have not been understood. In the present study, we used both mouse and human primary HSCs to explore the underlying mechanism for the anti-fibrogenic effect of ASTX and if the findings were substantiated in human livers with various pathologies.

2. Materials and methods

2.1 Cell culture

Mouse primary HSCs were isolated from C57BL/6J mice using a pronase/collagenase digestion method as we previously described [17]. The cells were then cultured on untreated petri dishes (BD Falcon, Franklin Lakes, NJ), which is a well-established condition for in vitro HSC activation [19]. The cells were maintained in a low-glucose DMEM supplemented with 10% FBS, 4 mM L-glutamine, and P/S (100 U/100 μ g per mL). Human primary HSCs (Zen-Bio, Research Triangle Park, NC) were maintained in a Human Stellate Growth Medium (Zen-Bio, Research Triangle Park, NC) and seeded on a collagen-coated plate (Sarstedt, Newton, NC). LX-

2 cells, kindly provided by Dr. Scott Friedman at the Icahn School of Medicine at Mount Sinai (New York, New York), were maintained in a low-glucose DMEM supplemented with 2% FBS, 4 mM L-glutamine, and P/S (100 U/100 µg per mL). All cells were cultured in a 37°C humidified cell culture chamber with 5% CO₂. Cell culture supplies were purchased from HyClone unless stated otherwise (Thermo Scientific, Logan, UT).

2.2 ASTX treatment

Pure ASTX powder, kindly provided by Fuji Chemical Industry Co., Ltd. (Toyama, Japan), was prepared in DMSO as we described [17]. After plated on an uncoated dish, mouse qHSCs were incubated with 25 µM ASTX during activation. Medium containing ASTX was replenished daily and DMSO vehicle control was always run in parallel. Human primary HSCs were treated with 25 µM ASTX for 24 h, after which they were activated by 3 ng/mL of TGFβ1 (Peprotech, Rocky Hill, NJ) for 24 h.

2.3 Human liver specimens

Normal human liver specimens and human livers with primary biliary cirrhosis (PBC), alcohol cirrhosis (AC), NASH and non-fatty NASH were obtained through the Liver Tissue Cell Distribution System (Minneapolis, MN), which was funded by NIH Contract # HHSN276201200017C.

2.4 Quantitative realtime PCR (qRT-PCR)

Total RNA extraction, cDNA synthesis, and qRT-PCR were conducted using a Bio-Rad CFX96 Real-Time system (Bio-Rad, Hercules, CA) as previously described [20, 21]. Primer sequences will be available upon request.

2.5 Western blot analysis

Whole cell lysates and human tissue protein extracts were prepared and Western blot analysis were performed as we described [21]. The following antibodies were used: α -SMA (Sigma, St. Louis, MO), HDAC3 (Santa Cruz Biotechnology, Santa Cruz, CA; Abcam, Cambridge, MA), HDAC4 (Santa Cruz Biotechnology; Cell Signaling Technology, Inc., Danvers, MA), HDAC9 (Abcam; Novus Biologicals, Littleton, CO), and COL1A1 (Sigma). β -tubulin (Santa Cruz) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Santa Cruz) was used as a loading control.

2.6 Transfection of small interfering RNA (siRNA)

LX-2 cells were transfected with either Silencer Negative Control scrambled siRNA (Invitrogen, Grand Island, NY) or siGENOMETM Human HDAC9 siRNA SMART pool (Dharmacon, Lafayette, CO) for 24 h as previously described [17]. Subsequently, the cells were pretreated with 25 μ M ASTX for 12 h, and then activated by 3 ng/mL TGF β 1 for 12 h.

2.7 Statistical analysis

One-way analysis of variance with Newman Keul's pairwise comparison, or unpaired t-test was used to determine significant differences between groups by GraphPad Prism6

(GraphPad Software, La Jolla, CA). P values less than 0.05 were considered significant and all values were presented as mean $\pm$ SEM.

3. Results

3.1 *ASTX inhibited the activation of primary HSCs*

We firstly determined if ASTX has an inhibitory effect on the activation of both mouse and human primary HSCs. The activation of primary mouse qHSCs was conducted by culturing fresh-isolated HSCs from C57BL/6J mice on plastic substratum for 2 or 6 days while ASTX was added from day 0, day 2 or day 4 until harvest (day 6). The mRNA expression of COL1A1, a fibrogenic marker, was markedly induced during activation, whereas ASTX significantly decreased its level at day 6, regardless of treatment duration (**Figure 1A**). In human primary HSCs, TGF β 1 significantly increased α -SMA and COL1A1 mRNA levels, but ASTX attenuated both basal and TGF β 1-induced expression of the genes (**Figure 1B**).

3.2 *ASTX repressed the expression of HDAC9 and myocyte enhancer factor 2 (MEF2) in HSCs*

HDACs are a class of enzymes that remove acetyl groups from lysine residues in histones [22]. HDACs, particularly class II HDACs, have been suggested to play a critical role in the activation of HSCs [11]. To gain insight into a potential role of HDACs in the modulation of HSC activation by ASTX, alterations in mRNA expression of all 11 classical HDACs were firstly examined during the activation of primary mouse HSCs. While there was a significant, but slight, increase in HDAC3 mRNA, HDAC9 mRNA was increased by ~10-fold in aHSCs compared with qHSCs (**Figure 2A**). HDAC9 protein was markedly increased in aHSCs compared with qHSCs in consistent with the change in its mRNA levels (**Figure 2B**). HDAC3 and 4 protein

levels were also much higher in aHSCs than qHSCs, although their mRNA levels were either increased or remained unchanged during activation. ASTX, however, significantly attenuated the HDAC9 induction during the activation of qHSCs (**Figure 2C**). When ASTX was added at day 4 for 2 d during HSC activation, it decreased HDAC3 and 4 protein levels by ~50% and HDAC9 by ~70%, with HDAC2 minimally altered (**Figure 2D**). We further examined the expression of MEF2, a transcription factor known to induce HDAC9 expression [23]. MEF2a, 2c and 2d mRNA levels were significantly higher in aHSCs than qHSCs, and ASTX significantly attenuated their induction (**Figure 2E**). MEF2b mRNA was not detected in qHSCs and aHSCs (data not shown). Our previous study demonstrated that ASTX facilitates the inactivation of aHSCs [18]. ASTX significantly repressed the mRNA levels of HDAC9 and MEF2 isoforms when aHSCs were incubated with ASTX for 2 d (**Figure 3A & B**).

3.3 HDAC9 expression was significantly higher in diseased human livers than normal livers

PBC is a chronic liver disease that causes destruction of intrahepatic bile ducts. In the PBC human livers, mRNA levels of α -SMA, COL1A1, TGF β 1 and HDAC9 were markedly higher (**Figure 4A**) with concomitant increases in MEF2b and 2c (**Figure 4B**) than normal human livers. HDAC3 mRNA was significantly lower in PBC livers than normal liver specimens while no difference was noted with HDAC4 between human normal and PBC livers. The protein levels of HDAC9, α -SMA, and Collagen I were also markedly increased in human PBC livers (**Figure 4C**). In contrast, HDAC3 protein was noticeably less in PBC livers but large individual variations of HDAC4 protein were observed in both normal and PBC livers. In addition, HDAC9 mRNA expression was also significantly higher in human liver specimens with AC, NASH and non-fatty NASH, in parallel with the elevation of α -SMA and COL1A1 mRNA levels (**Figure**

4D). However, differences in HDAC3 mRNA levels in those pathological conditions were modest.

3.4 HDAC9 knockdown attenuated TGF β 1-induced fibrogenic gene expression in LX-2 cells

As we previously showed [17], TGF β 1 significantly increased the expression of α -SMA, COL1A1, and TGF β 1 in LX-2 cells (**Figure 5A**). ASTX not only repressed the basal levels of α -SMA and COL1A1 expression, but also attenuated their expression induced by TGF β 1 (**Figure 5A**). To determine if HDAC9 mediates TGF β 1-induced fibrogenic response, HDAC9 was knocked down in LX-2 cells by ~70-80%. Expression of α -SMA, COL1A1 and TGF β 1 was markedly lower in the LX-2 cells transfected with HDAC9 siRNA than in controls (**Figure 5B**).

4. Discussion

Fibrosis is a key feature of NASH [24]. Although fibrogenesis is a necessary process for wound-healing, dysregulation of fibrogenic activity of HSCs can lead to liver fibrosis [19, 25]. We previously demonstrated that ASTX has anti-fibrogenic effects in LX-2 cells [17]. Furthermore, we have also observed in mouse primary HSC that ASTX not only prevents the activation of qHSCs but also facilitates the reversion of aHSCs to an inactivated state [18]. In the present study, we found that the novel effects of ASTX are, at least partly, attributable to its inhibitory action on the expression of HDAC9 and its known transcriptional regulator MEF2. Importantly, using human liver specimens with various pathologies, we also demonstrated that HDAC9 may play a critical role in human liver diseases, although the effect of ASTX on fibrosis is yet to be determined in human livers.

In a healthy liver, qHSCs store retinoids in the cytosol and maintain an equilibrium between ECM production and degradation [26]. Upon liver injury, HSCs transdifferentiate to myofibroblasts with high expression of α -SMA and ECM proteins. When qHSCs are plated on an uncoated plastic surface, they undergo “spontaneous activation” and are fully activated within 7 days with similar characteristics of HSCs shown in vivo during liver injury [19]. In the previous study, we observed that ASTX inhibited this activation process, as demonstrated by the attenuated loss of lipid droplets and expression of fibrogenic markers [17, 18]. Also, ASTX has been shown to inhibit TGF β 1-induced proliferation and transdifferentiation of A549 and MRC-5 lung cell lines by stimulating apoptosis [27]. In support of in vitro evidence for the anti-fibrogenic effects of ASTX, in vivo studies have demonstrated that ASTX prevents fibrosis. In bleomycin-injured lung in rats, ASTX reduced collagen accumulation and the number of α -SMA-positive cells [28]. Shen et al. [15] demonstrated that ASTX prevents liver fibrosis induced by either CCL4 or bile-duct ligation in mice by inhibiting nuclear factor κ B and TGF β 1 expression and down-regulating autophagy. A recent study elegantly showed that ASTX not only inhibits but also reverses hepatic inflammation and fibrosis in a lipotoxic NASH mouse model [16]. Importantly, there were some improvements of NASH in NASH subjects after 24 weeks of ASTX supplementation in this study. As such, in vitro and in vivo evidence exists to support the anti-fibrotic effect of ASTX. A large-scale human clinical study is necessary to examine the possibility of the use of ASTX as an anti-fibrotic agent.

Epigenetic modifications, particularly histone acetylation, have been shown to play crucial roles in HSC activation and liver fibrosis [8]. Pan-HDAC inhibitors, such as trichostatin A and valproic acid, are known to be potent inhibitors of HSC activation both in vitro [9] and in vivo [10]. Using inhibitors specific for class II HDACs, studies have shown that class II HDACs

play important roles in HSC activation [11]. N-hydroxy-7-(2-naphthylthio) heptanamide, a HDAC inhibitor, suppressed the protein expression of TGF β 1, α -SMA and COL1A1 in platelet-derived growth factor (PDGF)-stimulated mouse primary HSCs [29]. MS-275, a selective class I HDAC inhibitor, attenuated renal fibrosis by reducing protein levels of α -SMA, collagen I and fibronectin in mice [12]. MPT0E014, a HDAC inhibitor, improved cardiac fibrosis in rats with heart failure by decreasing the migration and proliferation of cardiac fibroblasts [13]. In the present study, HDAC9 mRNA and protein levels in aHSCs were significantly higher than qHSCs while ASTX decreased HDAC9 mRNA and protein levels by ~70-80%. The data suggest that ASTX decreased HDAC9 expression at the transcriptional levels. It has been shown that MEF2a, 2c and 2d are induced during HSC activation and MEF2 knockdown down-regulates α -SMA and COL1A1 expression in primary rat HSCs [30]. We observed in this study that the expression of MEF2a, 2c and 2d was elevated during HSC activation but their induction was markedly attenuated by ASTX in parallel with repressed HDAC9 expression. Of importance is that the expression of both HDAC9 mRNA and protein were markedly increased in PBC human livers. Likewise, the expression of MEF2b and 2c was also increased relative to normal livers. The induction of HDAC9 was also observed in human livers with AC and NASH, suggesting that HDAC9 is presumed to be involved in common pathological pathways in the liver. Furthermore, when HDAC9 was knocked down in LX-2 cells by ~70% using siRNA, the expression of TGF β 1-induced α -SMA, COL1A1 and TGF β 1 was significantly attenuated, supporting that HDAC9 may play a critical role in HSC activation. Therefore, our data suggest that ASTX may inhibit MEF2 expression or transcriptional activity, consequently repressing HDAC9 expression and HSC activation. Future study should be warranted to define the role of HDAC9 in the development of liver diseases and to identify its upstream regulators and downstream effectors.

In contrast to HDAC9, which is presumed to be regulated at the transcriptional level during HSC activation and by ASTX, HDAC3 and HDAC4 are likely regulated at the post-transcriptional level as their protein levels were much higher in aHSCs than in qHSCs although their mRNA levels were minimally changed during activation. Interestingly, ASTX was able to decrease HDAC3 and 4 protein levels by ~50% in HSCs. Class I HDACs (e.g., HDAC3) are known to play a role in HSC activation [31]. Also, Mannaerts et al. [11] suggested that HDAC4 plays a role in HSC activation based on decreased expression of lysyl oxidase, but not Col1A1, α -SMA and Col3A1 expression, upon HDAC4 knockdown in mouse primary HSCs. HDAC4 knockdown inhibited TGF β 1-induced α -SMA expression in normal human lung fibroblasts [32] and HDAC4 was shown to be critical for transdifferentiation of human skin fibroblasts [33]. Therefore, it is tempting to speculate that HDAC3 and 4 may have a negative impact on the development of NASH and liver fibrosis. However, our analysis with human liver specimens of various pathologies argues otherwise. First, in contrast with HDAC9, whose expression was significantly higher in all of tested diseased liver samples than normal human livers, HDAC3 mRNA levels were modestly different among normal and diseased liver samples. Second, HDAC9 protein was markedly higher in PBC human livers than normal human liver specimens (almost non-detectable). However, HDAC3 protein levels were lower in PBC livers while HDAC4 protein did not show any pattern of changes between normal and PBC livers. Considering the liver has a mixture of various cell types, which cell type(s) are responsible for the HDAC expression changes are a matter of future investigation. Further study is warranted to evaluate how ASTX represses HDAC3, HDAC4 and HDAC9 and the contribution of the repression to its anti-fibrogenic activity.

In conclusion, our findings suggest that the role of ASTX in the inhibition of qHSC activation and reversion of aHSCs may be mediated via the pathways regulated by MEF2/HDAC9 axis. Although further work is needed to elucidate the underlying mechanism in detail, the present study provides novel evidence that ASTX is anti-fibrogenic and inhibits HSC activation possibly via epigenetic modes of action. Further clinical studies are warranted to explore its potential use of ASTX as a preventive and/or therapeutic agent against for liver fibrosis and other liver diseases.

Author's contribution

Y. Yang, M. Bae, Y. Park, Y. Lee, T.X. Pham, and S. Rudraiah conducted experiments; J. Manautou and S.I. Koo contributed to data interpretation and manuscript preparation; and J. Lee had primary responsibilities for study design, data interpretation and manuscript preparation.

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Figure legends

Figure 1. ASTX inhibited HSC activation in mouse and human primary HSCs. (A) Mouse primary HSCs were activated for 6 d in the absence or presence of 25 μ M ASTX from day 0, day 2 or day 4 for COL1A1 mRNA measurement. n = 9. Bars with a different letter within the same day are significantly different ($P < 0.05$). (B) Human primary HSCs pre-incubated with 25 μ M ASTX for 24 h were exposed to TGF β 1 (3 ng/ml) for additional 24 h. qRT-PCR for α -SMA and COL1A1. n = 3. Bars with a different letter are significantly different ($P < 0.05$).

Figure 2. ASTX modulated HDAC expression during HSC activation in mouse primary HSCs. (A) qRT-PCR at day 2 and day 6 of HSC activation. n = 4. *, $P < 0.05$. (B) Protein expression of HDAC isoforms at day 2 (qHSCs) and at day 6 (aHSCs). (C) HDAC9 mRNA abundance in HSCs treated with or without 25 μ M ASTX for an indicated day was measured at day 2, 4 and 6 as shown in Figure 1 left panel. n = 9. Bars with a different letter within the same day are significantly different ($P < 0.05$). (D) Protein expression at day 6 in controls and cells treated with ASTX at day 4 for 2 d. (E) qRT-PCR for MEF2 isoforms at day 2, at day 6, and at day 6 of HSCs treated with ASTX for 2 d from day 4. n = 6. Bars with a different letter are significantly different for each gene ($P < 0.05$).

Figure 3. ASTX decreased the expression of HDAC9 and MEF2 isoforms in activated mouse primary HSCs. Mouse primary HSCs were activated for 6 days and subsequently 25 μ M ASTX was added to the activated HSCs for 2 days. qRT-PCR analysis was performed to measure mRNA of HDAC9 (A) and MEF2 isoforms (B). n=6. *, $P < 0.05$.

Figure 4. HDAC9 expression is induced in human liver with several liver diseases. qRT-PCR for fibrogenic genes and HDAC9 (**A**) and MEF2 isoforms (**B**) were conducted with normal and PBC human livers. Mean $\pm$ SEM. n = 9 (normal) or 7 (PBC). (**C**) A representative blot for protein. (**D**) α -SMA and COL1A1 and HDAC9 mRNA levels were determined in normal human livers and liver specimens with AC, PBC, NASH and non-fatty NASH by qRT-PCR. n = 15 (normal, AC, PBC and NASH) or 13 (non-fatty NASH). Bars with a different letter are significantly different for each gene ($P < 0.05$)

Figure 5. HDAC9 knockdown attenuated TGF β 1-response in LX-2 cells. (**A**) LX-2 cells were pretreated with 25 μ M ASTX for 12 h, and subsequently stimulated by 3 ng/mL TGF β 1 for 12 h for qRT-PCR analysis. n = 3. Bars with a different letter are significantly different within the same gene ($P < 0.05$). (**B**) LX-2 cells were transfected with either Scramble control or HDAC9 siRNA for 24 h. Then, the cells were pretreated with ASTX for 12 h, after which they were stimulated by 3 ng/mL TGF β 1 for 12 h for qRT-PCR analysis. n = 3. Bars with a different letter within the same gene are significantly different ($P < 0.05$).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

A

B