Effect of astaxanthin on hepatocellular injury following ischemia/reperfusion

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A R T I C L E   I N F O

Article info
Article history:
Received 1 September 2009
Received in revised form 3 November 2009
Accepted 3 November 2009
Available online xxx

Keywords:
Astaxanthin
Liver
Ischemia–reperfusion
Oxidative stress

A B S T R A C T

This study investigated the effect of astaxanthin (ASX; 3,3-dihydroxybeta, beta-carotene-4,4-dione), a water-dispersible synthetic carotenoid, on liver ischemia–reperfusion (IR) injury. Astaxanthin (5 mg/kg/day) or olive oil was administered to rats via intragastric intubation for 14 consecutive days before the induction of hepatic IR. On the 15th day, blood vessels supplying the median and left lateral hepatic lobes were occluded with an arterial clamp for 60 min, followed by 60 min reperfusion. At the end of the experimental period, blood samples were obtained from the right ventricle to determine plasma alanine aminotransferase (ALT) and xanthine oxidase (XO) activities and animals were sacrificed to obtain samples of nonischemic and postischemic liver tissue. The effects of ASX on IR injury were evaluated by assessing hepatic ultrastructure via transmission electron microscopy and by histopathological scoring. Hepatic conversion of xanthine dehydrogenase (XDH) to XO, total GSH and protein carbonyl levels were also measured as markers of oxidative stress. Expression of NOS2 was determined by immunohistochemistry and Western blot analysis while nitrate/nitrite levels were measured via spectral analysis. Total histopathological scoring of cellular damage was significantly decreased in hepatic IR injury following ASX treatment. Electron microscopy of postischemic tissue demonstrated parenchymal cell damage, swelling of mitochondria, disarrangement of rough endoplasmatic reticulum which was also partially reduced by ASX treatment. Astaxanthine treatment significantly decreased hepatic conversion of XDH to XO and tissue protein carbonyl levels following IR injury. The current results suggest that the mechanisms of action by which ASX reduces IR damage may include antioxidant protection against oxidative injury.

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1. Introduction

Ischemia–reperfusion injury of the liver is an important clinical problem in many clinical conditions such as liver transplantation, hepatic surgery for tumor excision, trauma and hepatic failure after hemorrhagic shock (Lemasters and Thurman, 1997; Olthoff, 2001). Partial or, mostly, total interruption of hepatic flow is often necessary when liver surgery is performed. This interruption of blood flow is termed as “warm ischemia” and upon revascularization, when molecular oxygen is reintroduced, the organ undergoes a process called “reperfusion injury” that causes deterioration of organ function (Hasselgren, 1987). Although the mechanisms by which organ damage occurs in I/R injury are incompletely understood, it has been suggested that reperfusion of the liver following ischemia, triggers the activation of kupffer cells causing oxygen free radical formation, production of tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) (McCord, 1985; Adkinson et al., 1986; Carden and Granger, 2000). Elevated levels of the pro-inflammatory cytokines TNF-α and IL-1 promote PMN recruitment and activation which also generates reactive oxygen species (ROS) and leads to the release of proteases (Colletti et al., 1996; Jaeschke et al., 1990). Although increased expression of NOS2 and elevated tissue nitrite (NO2−) and nitrate (NO3−) occurs in animal models of I/R (Isobe et al., 2000) formation of ROS can scavenge NO produced by the sinusoidal endothelial cells and lead to vasodilation and narrowing of the sinusoidal lumen (Aslan and Freeman, 2002).

Reports showing that antioxidant deficiency exacerbate I/R injury and the beneficial outcome of pharmacological interventions such as superoxide dismutase, catalase, vitamin E, or desferrioxamine, support ROS as a major pathophysiological component of I/R (Atalla et al., 1985; Marubayashi et al., 1986; Drugs et al., 1991).

Astaxanthin is a naturally occurring carotenoid pigment and is a powerful biological antioxidant (Palozza and Krinsky, 1992). It

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Activities and materials of this study have been designed and performed in accordance with the recommendations of the Animal Care and Use Committee of the University of XYZ. All procedures were performed under a protocol approved by the Institutional Animal Care and Use Committee. The experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

2. Materials and Methods

2.1. Animals

All experimental protocols conducted on rats were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at ABC University Medical School. Male Wistar rats weighing 250-450 g were housed in stainless steel cages and provided food and water ad libitum. Animals were maintained at 22 ± 1 °C at all times. Rats were randomly divided into two groups of 12 animals each which received olive oil or 5 mg/kg/day ASX (3,3-dihydroxybeta, beta-carotene-4,4-dione; betadine, a complete midline incision was made. The portal vein was exposed and ischemia was performed according to a method previously described (Jaeschke et al., 2001). Because of its polar end groups, ASX spans the cell membrane bilayer allowing it to sit near the lipid/water interface, where free radical attack first occurs and contributes to cell membrane mechanical strength (Palozza and Krinsky, 1992). Astaxanthin stabilizes free radicals by adding them to its long double-bond chain rather than donating an atom or electron to the radical. Consequently, it can resist chain reactions that occur when a fatty acid is oxidized, thus allowing it to scavenge or quench longer than antioxidants that cannot stop this chain reaction (Kurashige et al., 1990).

The liver has effective mechanisms for inactivating and then excreting foreign substances through biotransformation. These functions can lead to significant release of free radicals and oxidation byproducts and therefore it is important to have mechanisms that protect liver cells against oxidative damage. It has been shown that astaxanthin is much more effective than vitamin E in protecting mitochondria from rat liver cells against lipid peroxidation (Guerin et al., 2003; Kurashige et al., 1990).

Previous studies on animal models of IR-induced myocardial injury have shown a linear correlation between the plasma concentrations of astaxanthin and the extent of infarct size reduction (Cross and Lockwood, 2004, 2005). Based on this information, the current experimental protocol was designed to determine whether the tissue protective effect of ASX could be observed in a rat model of liver IR injury. We aimed to determine whether ASX, a potent antioxidant, had any effect on plasma levels of liver enzymes, tissue markers of oxidative stress and histopathologic alterations in an experimental rat model of hepatic IR.

2.2. Rat model of hepatic ischemia–reperfusion injury

Rats were fasted 12 h before surgery, but allowed to drink tap water ad libitum. Rats were anesthetized intraperitoneally with a mixture of ketamine (25 mg/kg, Richter Pharma AG, Wels, Austria) and xylazine hydrochloride (8 mg/kg, Abaxis International B.V., Woerden, Holland). A model of lobar (70%) hepatic warm ischemia was performed according to a method previously described (Jaeschke et al., 1990; Karaman et al., 2006). After shifting and dissecting the abdomen with betadine, a complete midline incision was made. The portal vein was exposed and vessels supplying the median and left lateral hepatic lobes were clamped for 60 min. Reperfusion followed for 60 min via removal of the microvascular clip. The caudal and right lobes retained an intact portal and arterial blood flow, in addition to venous outflow. These lobes served as control and also prevented intestinal congestion. The abdomen was kept closed throughout the experimental period and body temperature was maintained by placing rats under warm lamp. Blood samples were obtained before and after the experiment, from the tail vein and the right ventricle, respectively. At the end of the experimental period, liver was perfused with 0.9% NaCl injected from the left ventricle en route for inferior vena cava. Tissue samples obtained from the left and median lobes of the liver accounted for I/R while diseased right lateral and caudate lobes served as nonischemic group. Obtained liver tissues were either flash frozen in liquid nitrogen and stored at −70 °C or fixed for histological evaluation.

2.3. XO/XDH and ALT enzyme-activity measurements

All tissues were weighed and homogenized in ice-cold 50 mmol/L sodium phosphate buffer (pH 7.4). Homogenates were centrifuged (40,000 × g for 30 min at 4 °C), and supernatants were stored at −80 °C. XO and XD activity were determined with a fluorometric assay described previously (Beckman et al., 1989; Aliciguzel et al., 2003). This assay is based on the conversion of xanthine to the fluorescent product isoxanthopterin and is performed with and without methylene blue to determine XO/XDH activity and XOD activity, respectively. Enzyme activities are calculated on the basis of the linear increase in fluorescence. Plasma alanine aminotransferase activity was measured on an automated analyzer (Roche/Hitachi Diagnostic Systems).

2.4. Histopathological evaluation of liver sections

Paraffin sections stained with hematoxylin and eosin were evaluated by two pathologist blinded to the experimental condition. 20 high-power fields (HPF, 200 ×) were evaluated in all sections for congestion, intracellular edema and necrosis as previously described (Yilmaz et al., 2004). Congestion and intracellular edema was scored as follows: 0 = none, 1 = present in zone III, 2 = present in zones II–III, 3 = present in zones I–II–III. Necrosis was scored as follows: 0 = none, 1 = single or focal necrosis, 2 = submassive necrosis, 3 = massive necrosis + infarction. Total histopathological score was obtained by summation of all scores given to each parameter.

2.5. Immunohistochemical staining

Liver tissues were fixed in 10% buffered formalin solution, washed in phosphate buffered saline (pH 7.4), embedded in paraffin and cut into 4-μm sections. Peroxidase staining was performed as previously described (Aslan et al., 2007).

Briefly, sections were deparaffinized, rehydrated and washed with Tris buffered saline. Endogenous peroxidase activity was blocked by incubating tissue sections with 3% hydrogen peroxide for 5 min prior to application of the primary antibody. Primary antibody incubations were at 25 °C for 60 min using rabbit polyclonal anti-NOS2 (1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA). After sections were washed they were immunostained with an avidin–biotin complex kit (Dako, Carpinteria, CA) followed by hematoxylin counterstaining. Negative controls were performed by replacing the primary antibody with nonimmune serum followed by immunoperoxidase staining. Presence of a red-brown colored end-product in the cytoplasm was indicative of positive staining. Counterstaining with hematoxylin resulted in a pale to dark blue coloration of cell nuclei. All stained tissue sections were visualized via light microscopy (Olympus BX81, Tokyo, Japan). To obtain a quantitative standard for NOS2 immunostaining within the different experimental groups morphometric analysis was performed as previously described (Yicel et al., 2005). The immunostaining scores, obtained according to both the percentage and intensity of positive stained cells, were statistically analyzed by Sigma Stat (version 2.03) software for windows. The percentage of the positive stained cells was scored as follows: 0 = less than 5% of the cells/high powered field (HPF, 40 ×) are stained, 1 = 5% to less than 10% of cells/HPF are stained, 2 = 10% to less than 50% of cells/HPF are stained, 3 = more than 50% of cells/HPF are stained. The intensity of staining within each counted cell was also scored as follows: 0 = none, 1 = weak staining (pale red-brown), 2 = moderate staining (red-brown), 3 = strong staining (dark red-brown). A final immunostaining score was obtained for all sections by adding the two scores.

2.6. Transmission electron microscopy

Samples were prepared for transmission electron microscopy (TEM) as previously described (Yicel et al., 2006). Tissues were prefixed by immersion in 2.2% glutaraldehyde in 0.1 mol/L (pH 7.4) Sorenson's phosphate buffer for 4 h and post-fixed in 1% osmium tetroxide in 0.1 mol/L (pH 7.2) sodium phosphate buffer. The samples were dehydrated in graded ethyl alcohol series and embedded in Araldite CY212. Ultrathin sections (70 nm) were prepared (Leica Ultratuct UCT) and contrasted with uranyl acetate and lead citrate for examination by transmission electron microscopy (LEO 906E, Oberkochen, Germany).
### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>IR</th>
<th>ASX</th>
<th>ASX + IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver XO (mU/g protein)</td>
<td>16.8 ± 2.2 (n=8)</td>
<td>22.2 ± 0.86(^a) (n=10)</td>
<td>6.4 ± 0.73(^b) (n=10)</td>
<td>10.3 ± 0.64(^c) (n=10)</td>
</tr>
<tr>
<td>Liver XDH (mU/g protein)</td>
<td>24.2 ± 0.8(^d) (n=8)</td>
<td>12.3 ± 0.5 (n=10)</td>
<td>21.8 ± 0.5(^e) (n=10)</td>
<td>12.2 ± 0.4 (^f) (n=10)</td>
</tr>
<tr>
<td>Liver XDH:O (mU/g protein)</td>
<td>1.6 ± 0.2 (^g) (n=8)</td>
<td>0.5 ± 0.03 (n=10)</td>
<td>3.8 ± 0.3(^h) (n=10)</td>
<td>1.2 ± 0.1(^i) (n=10)</td>
</tr>
<tr>
<td>Plasma XO (mU/mL)</td>
<td>43.4 ± 2.8 (n=8)</td>
<td>4607.6 ± 502(^j) (n=8)</td>
<td>49.9 ± 2.9 (n=8)</td>
<td>4263.5 ± 727(^k) (n=8)</td>
</tr>
<tr>
<td>Plasma ALT (mU/mL)</td>
<td>43.4 ± 2.8 (n=8)</td>
<td>4607.6 ± 502(^j) (n=8)</td>
<td>49.9 ± 2.9 (n=8)</td>
<td>4263.5 ± 727(^k) (n=8)</td>
</tr>
</tbody>
</table>

\(^a\) p<0.001 vs. ASX and ASX + IR.
\(^b\) p=0.014 vs. control.
\(^c\) p<0.01 vs. control.
\(^d\) p<0.001 vs. IR and ASX + IR.
\(^e\) p<0.05 vs. IR.
\(^f\) p<0.05 vs. IR and ASX + IR.
\(^g\) p<0.05 vs. ASX and control.
\(^h\) p<0.05 vs. ASX.

### 2.7. SDS-PAGE and Western blot analysis

Liver tissue was homogenized in 2 mL ice-cold homogenizing buffer (50 mM KH\(_2\)PO\(_4\), 80 \(\mu\)M leupeptin Sigma–Aldrich, Steinheim, Germany), 2.1 mM Pefabloc SC (SERVA, Heidelberg, Germany), 1 mM phenylmethylsulfonyl fluoride (Sigma–Aldrich), 1/800 g/mL aprotinin (SERVA; pH 7.4). Homogenates were centrifuged (40,000 \(\times\) g, 30 min, 4\(^\circ\)C) and supernatants were stored at −80 \(^\circ\)C until analyzed. Western blot analysis was performed as previously described (Aslan and Canatan, 2008). Briefly, tissue proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. A rabbit polyclonal antibody against NOS2 (1:800 dilution; BD Transduction Laboratories, San Jose, CA) was used for immunoblot analysis. Western blot analysis was performed as previously described (Aslan and Canatan, 2008). Briefly, tissue proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. A rabbit polyclonal antibody against NOS2 (1:800 dilution; BD Transduction Laboratories, San Jose, CA) was used for immunoblot analysis. Proteins separated by SDS-PAGE were also visualized by Electro-Blue Staining solution proteins were washed, separated by SDS-PAGE, and visualized by GelCode Coomassie Blue stain reagent (Pierce Chemical Company, Rockford, IL).

### 2.8. Measurement of tissue protein carbonyl content

Protein-bound carbonyls were measured via a protein carbonyl assay kit (Cat. #1005020 Cayman Chemical, Ann Arbor, MI). The utilized method was based on the covalent reaction of the carbonylated protein side chain with 2,4-dinitrophenylhydrazine (DNPH) and detection of the produced protein-hydrazone at an absorbance of 370 nm. The results were calculated using the extinction coefficient of 22 mM\(^-1\) cm\(^-1\) for aliphatic hydrazones and were expressed as nmol/mg protein.

### 2.9. Measurement of tissue GSH content

Total GSH levels were measured by a commercially available GSH assay kit (Cat. #703002. Cayman Chemical, Ann Arbor, MI). Retina harvested from enucleated globes was homogenized in ice-cold phosphate buffer (50 mM K\(_2\)HPO\(_4\), containing 1 mM EDTA, pH 7). Homogenates were centrifuged (10,000 \(\times\) g, 15 min, 4\(^\circ\)C) and supernatants were deproteinated in 10% metaphosphoric acid (Sigma–Aldrich, Steinheim, Switzerland). The GSSG was reduced to GSH by GSH reductase in the presence of NADPH. The GSH content was determined by measurement of NADPH oxidation using a spectrophotometer.

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Fig. 2. Hematoxylin and eosin staining of liver sections. Hepatic photomicrographs of representative rat are shown from each of the four groups. ASX, astaxanthin; IR, ischemia–reperfusion; CV, central vein. Bar 200 \(\mu\)m.
Table 2
Histopathological scores of liver sections. Values are mean ± SD. Statistical analysis was performed by Kruskal–Wallis one-way analysis of variance on ranks and all pairwise multiple comparison procedures were by Dunn’s method.

<table>
<thead>
<tr>
<th>Group</th>
<th>Congestion (n=6)</th>
<th>Intracellular edema (n=6)</th>
<th>Necrosis (n=6)</th>
<th>Total score (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.17 ± 0.41</td>
<td>0.17 ± 0.41</td>
<td>0</td>
<td>0.33 ± 0.52</td>
</tr>
<tr>
<td>IR</td>
<td>2.5 ± 0.5*</td>
<td>2.0 ± 0.6*</td>
<td>1.67 ± 0.5*</td>
<td>6.16 ± 0.75*</td>
</tr>
<tr>
<td>ASX</td>
<td>0.17 ± 0.41</td>
<td>0</td>
<td>0.17 ± 0.41</td>
<td>0.33 ± 0.52</td>
</tr>
<tr>
<td>ASX + IR</td>
<td>1.33 ± 1.5</td>
<td>1.7 ± 0.8*</td>
<td>0.8 ± 0.4</td>
<td>3.8 ± 1.7***</td>
</tr>
</tbody>
</table>

* p < 0.05; compared to ASX and control.
** p < 0.05; compared to IR alone.

Table 3
Liver protein carbonyl, GSH and nitrite/nitrate levels. All values are mean ± SEM. Statistical analysis was by one-way analysis of variance with all pairwise multiple comparison procedures (Tukey test).

<table>
<thead>
<tr>
<th>Protein carbonyl</th>
<th>GSH</th>
<th>Nitrite and nitrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=6 (nmol/mg protein)</td>
<td>n=10 (nmol/mg protein)</td>
<td>n=7–11 (μM)</td>
</tr>
<tr>
<td>Control</td>
<td>5.2 ± 1.3</td>
<td>4.8 ± 0.4</td>
</tr>
<tr>
<td>IR</td>
<td>49.9 ± 4.4*</td>
<td>2.7 ± 0.2*</td>
</tr>
<tr>
<td>ASX</td>
<td>5.7 ± 0.8</td>
<td>4.8 ± 0.4</td>
</tr>
<tr>
<td>ASX + IR</td>
<td>16.5 ± 1.8**</td>
<td>3.5 ± 0.3**</td>
</tr>
</tbody>
</table>

* p < 0.001 compared to control, ASX and ASX + IR.
** p < 0.05 compared to control and ASX.
# p < 0.001 vs. control and ASX.
## p ≤ 0.01 vs. ASX and control.

assay cocktail of the kit containing DTNB (5,5′-dithiobis-2-nitrobenzoic acid, Ellman’s reagent), glucose-6-phosphate dehydrogenase, GSH reductase, NADP+ and glucose-6-phosphate. The sulfhydryl group of GSH reacts with DTNB to give a yellow colored 5-thio-2-nitrobenzoic acid (TNB) which is measured at an absorbance of 405 nm. The values of total GSH for each sample were calculated from their respective slopes using a GSH standard curve.

2.10. Nitrite and nitrate assay

Nitrate and nitrate assay was carried out as previously described (Aslan et al., 2006). Briefly, samples were transferred to an ultrafiltration unit and centrifuged through a 10-kDa molecular mass cut-off filter (Amicon, Millipore Corporation, Bedford, MA) for 1 h to remove protein. Analyses were performed in duplicate via the Greiss reaction using a colorometric assay kit (Calbiochem, Darmstadt, Germany). Protein concentrations were measured at 595 nm by a modified Bradford assay using Coomassie Plus reagent with bovine serum albumin as a standard (Pierce Chemical Company, Rockford, IL).

3. Results

3.1. ALT, XO and XDH enzyme activity

Plasma ALT and XO levels were significantly increased in all IR groups. Treatment of ASX significantly decreased liver XO levels under basal conditions and increased XDH/XO ratio when com-

Fig. 3. Transmission electron micrographs of liver from all experimental groups. ASX, astaxanthin; IR, ischemia–reperfusion; M, mitochondria; N, nucleus; ER, endoplasmatic reticulum. Bar 2 μm.
Fig. 4. (A) Immunostaining of NOS2 in the liver. Hepatic photomicrographs of representative rat are shown from each of the four groups. ASX, astaxanthin; IR, ischemia–reperfusion; CV, central vein. Bar 200 μm. (B) Quantitation of NOS2 in the liver. Values are mean ± SD. The differences in the immunostaining score among the different groups were analyzed via Kruskal–Wallis one-way analysis of variance on ranks and all pairwise multiple comparisons were performed by Dunn’s method. *p < 0.05 compared to control and ASX.

pared to both control and IR groups. XDH/XO ratio was greater in I/R livers treated with ASX as compared to the non-treated IR group (Table 1).

3.2. Histological analysis

Congestion, intracellular edema and necrosis were significantly greater (*p < 0.05) in IR and ASX + IR groups as compared to ASX and control (Fig. 1, Table 2). Although congestion, intracellular edema and necrosis showed no significant difference among IR groups treated or non-treated with ASX, the total histopathological score was significantly decreased (**p < 0.05) in ASX treated IR vs. IR alone (Fig. 1, Table 2).

Electron microscopy of posts ischemic tissue also demonstrated cell damage, swelling of mitochondria, disarrangement of rough endoplasmatic reticulum which was also partially reduced by astaxanthine treatment (Fig. 2).

3.3. Protein carbonyl, GSH and nitrite/nitrate levels

Ischemia/reperfusion caused a significant increase in liver protein carbonyl levels in both ASX treated and non-treated groups.
ASX treatment significantly reduced levels of protein carbonyl formation in IR (Table 3). Ischemia/reperfusion significantly decreased GSH levels and caused a significant increase in liver nitrate/nitrite levels in both ASX treated and non-treated groups (Table 3). ASX treatment had no significant effect on GSH and nitrate/nitrite levels in IR.

3.4. Nitric oxide synthase-2 expression

Fig. 3A shows hepatic photomicrographs representative from each of the four groups. Increased NOS2 staining was localized to the pericentral hepatocytes in both IR groups and not evident in the control and ASX treated groups (Table 3). ASX treatment had no significant effect on GSH and nitrate/nitrite levels in IR.

4. Discussion

This study examined the effect of ASX treatment on liver injury resulting from I/R. Astaxanthin was dissolved in olive oil and administered at a dose 5 mg/kg/day via oral gavage for 14 consecutive days before the induction of hepatic I/R. Plasma appearance and tissue accumulation of ASX was studied previously in rodents after single- and multiple-dose regimens (Showalter et al., 2004). One time dosing at 500 mg/kg resulted in significant appearance of ASX in plasma (0.2 mg/L) and liver (0.9 mg/L) (Fig. 5).

ASX treatment showed no significant effect on plasma XO and ALT activity following liver I/R injury, however it did decrease basal levels of hepatic XO activity and increased liver XDH/XO ratio in I/R (Table 1). Considering that XOR-specific activity in the liver is much greater than in plasma (Kooij et al., 1992), a small amount of enzyme released from splanchic tissues can cause a significant increase in circulating plasma levels of the enzyme (Aslan et al., 2001).

Xanthine dehydrogenase (XDH) is a molybdenopterin-containing flavoprotein that oxidizes hypoxanthine to xanthine, and xanthine to uric acid. It has two identical subunits containing FAD, molybdenum, and Fe-S clusters that facilitate electron transfer from substrate to the electron acceptor NAD+ (Enroth et al., 2000). Xanthine dehydrogenase can be converted to XO by either proteolytic cleavage of the amino terminus or more rapidly by thiol oxidation leading to intramolecular disulfide formation (Enroth et al., 2000; Amaya et al., 1990; McKelvey et al., 1988). Only the dehydrogenase form of the enzyme can reduce NAD+ and form NADH and hydrogen peroxide (H2O2) production. Treatment of ASX under basal conditions significantly decreased liver XO levels and increased XDH/XO ratio when compared to control (Table 1). Likewise, treatment of ASX prior to IR injury increased XDH/XO ratio when compared to non-treated IR livers. This effect of ASX is likely due to the prevention of thiol oxidation which will lead to a decrease in intramolecular disulfide formation. Prevention of intramolecular disulfide formation within XDH will decrease conversion of the enzyme to XO and increase the XDH/XO ratio as seen in this study.

ASX treatment leads to a slight protection in I/R livers as reflected by significantly lower total histopathological score (Fig. 1). As stated previously, the total histopathological score was obtained by summation of all scores given to intracellular edema, congestion and necrosis. Although histopathological evaluation revealed that intracellular edema, congestion and necrosis in IR livers treated with ASX was greater than control, these findings were much more severe in non-treated liver tissue that underwent IR. This result is reflected in the total score which is significantly lower in ASX treated livers that underwent IR as compared to non-treated IR livers.

Treatment with ASX significantly decreased protein carbonyl formation following I/R injury (Table 3). Protein carbonyl formation is a widely utilized marker for protein oxidation (Stadtman and Oliver, 1991). Carboxyls, formed following reactive oxygen species-mediated oxidation of sugar and membrane lipids, are able to form adducts commonly known as CO-proteins (proteins bearing carbonyl groups) with structural proteins, causing alterations in their biological activity (Shacter, 2000). Reactive carbonyl groups on proteins can also be formed by direct oxidation of protein side-chains (Reznick and Packer, 1994). Astaxanthin is a potent scavenger of oxygen radicals and inhibits the oxidation of proteins (Naguib, 2000; Palozza and Krinsky, 1992).

As stated in the introduction, neutrophil-sinusoidal endothelium adherence observed during liver IR injury is, in part, a response to reactive oxygen radicals, as well as being a response to cytokines with chemotactic properties. Activated neutrophils can secrete several enzymes, such as MPO and elastase, which are indirectly involved in tissue injury (Weiss, 1989). Treatment of ASX significantly decreased liver XO levels under basal conditions and increased XDH/XO ratio in both control and IG groups. ASX treatment also significantly reduced hepatic levels of protein carbonyl formation following IR injury. The effect of ASX treatment on decreased XO activity will diminish a major source of hepatic O2− and H2O2 production during I/R and alleviate tissue destruction in response to reactive oxygen radical formation as observed via histopathological evaluation herein.

Increased expression of NOS2 and elevated tissue nitrite (NO3−) and nitrate (NO2−) levels observed herein (Figs. 3 and 4 and Table 3), are in agreement with previous animal models of liver I/R injury (Isobe et al., 2000). In many cases, NOS2 inhibitors significantly
improve organ function in tissue I/R injury (Isobe et al., 1999). Similarly, I/R-induced tissue injury is attenuated in the liver of NOS2−/− mice (Lee et al., 2001).

Although ASX had no significant effect on NOS2 expression and NO2−/NO3− levels, it still may provide some protection during I/R injury by scavenging ROS which can directly react with NO, leading to the formation of secondary species capable of oxidation and nitration reactions (Aslan et al., 2003).

Based on reported data it is concluded that ASX treatment can offer limited protection in liver I/R injury by reducing oxidant-induced protein carbonyl formation and conversion of XDH to XO. The observed effect of ASX on liver enzymes, and oxidative stress was also reflected by a minor protection against histopathologic alterations.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

This study was supported by a grant (No: 2007.04.0103.002) from Akdeniz University Research Foundation.

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