Food & Function

Cite this: DOI: 10.1039/c1fo10161g

www.rsc.org/foodfunction

PAPER

An intervention study in obese mice with astaxanthin, a marine carotenoid – effects on insulin signaling and pro-inflammatory cytokines

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Received 14th August 2011, Accepted 27th October 2011
DOI: 10.1039/c1fo10161g

Astaxanthin (ASX), a xanthophyll carotenoid from the marine algae *Hematococcus pluvialis*, has anti-obesity and insulin-sensitivity effects. The specific molecular mechanisms of its actions are not yet established. The present study was designed to investigate the mechanisms underlying the insulin sensitivity effects of ASX in a non-genetic insulin resistant animal model. A group of male Swiss albino mice was divided into two and fed either a starch-based control diet or a high fat-high fructose diet (HFFD). Fifteen days later, mice in each dietary group were divided into two and were treated with either ASX (6 mg kg^{-1} per day) in olive oil or olive oil alone. At the end of 60 days, glucose, insulin and pro-inflammatory cytokines in plasma, lipids and oxidative stress markers in skeletal muscle and adipose tissue were assessed. Further, post-receptor insulin signaling events in skeletal muscle were analyzed. Increased body weight, hyperglycemia, hyperinsulinemia and increased plasma levels of tumor necrosis factor-α and interleukin-6 observed in HFFD-fed mice were significantly improved by ASX addition. ASX treatment also reduced lipid levels and oxidative stress in skeletal muscle and adipose tissue. ASX improved insulin signaling by enhancing the autophosphorylation of insulin receptor-β (IR-β), IRS-1 associated PI3-kinase step, phospho-Akt/Akt ratio and GLUT-4 translocation in skeletal muscle. This study demonstrates for the first time that chronic ASX administration improves insulin sensitivity by activating the post-receptor insulin signaling and by reducing oxidative stress, lipid accumulation and proinflammatory cytokines in obese mice.

1. Introduction

Insulin plays a central role in the maintenance of glucose homeostasis. The metabolic effects of insulin are principally mediated by a distinct signaling pathway called the phosphatidylinositol 3-kinase (PI 3-kinase) pathway which is responsible for glucose uptake, lipogenesis and glycogen synthesis.[1] Insulin exerts these effects by binding and activating the insulin receptor-β which has intrinsic tyrosine kinase activity. This in turn phosphorylates the insulin receptor substrates (IRS-1 and 2) and triggers a chain of events causing the activation of p85PI 3-kinase and a protein kinase called Akt. Activated Akt facilitates the translocation of glucose transporters to the cell membrane enabling glucose entry into the cell.[2] Skeletal muscle and adipose tissue are the major targets of insulin which account for the majority of insulin-mediated glucose uptake in the post-prandial state.[3]

Insulin resistance, the hall mark of type 2 diabetes (T_2D), is defined as the inability of insulin to facilitate glucose uptake and metabolism in the target organs.[4] Insulin resistance and compensatory hyperinsulinemia cluster with obesity, dyslipidemia and hypertension in the metabolic syndrome (MS). MS has emerged as a global epidemic that predisposes to the evolution of T_2D and cardiovascular disease.

Sustained administration of high fat-high fructose diet (HFFD) to normal mice induces insulin resistance together with body weight gain, hyperglycemia, hyperinsulinemia, dyslipidemia and hypertension.[5] It is well known that obesity and insulin resistance frequently co-exist with oxidative stress and increased oxidative stress has been recognized as an early instigator of insulin resistance.[6] Investigations show that HFFD feeding also increases the generation of reactive oxygen species (ROS). Recently, obesity was shown to be associated with increased inflammation and secretion of proinflammatory cytokines, among which tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) interfere with insulin signaling.[7]

Astaxanthin (3,3′-dihydroxy-4,4′-diketo-β-carotene, ASX), a red carotenoid pigment, is predominantly produced by the marine algae *Heamatococcus pluvialis.* Considerable interest on the health promoting effects of ASX has been evident from the literature. For instance, addition of ASX reduces oxidative stress and inflammation in cardiovascular diseases states,[8] restores

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blood pressure in spontaneously hypertensive rats[9] and increases the endurance capacity against exercise-induced fatigue in mice.[10] The anti-obesity effects in high fat diet-fed mice[11] and anti-hyperglycemic effects in db/db mice[12] have also been shown.

ASX improves whole body insulin sensitivity and insulin-stimulated glucose uptake in muscle of insulin resistant animals.[13,14] However, the mechanisms by which ASX improves insulin sensitivity have not been explored. To the best of our knowledge, the effect of ASX on the level of pro-inflammatory cytokines is also unexplored. Therefore, we adapted the HFFD-mice as a model of obesity/dyslipidemia-induced insulin resistance and studied the effect of ASX administration on insulin sensitivity, lipid levels and oxidative stress in skeletal muscle and adipose tissue, TNF-α and IL-6 levels in circulation and post-receptor insulin signaling in skeletal muscle of these mice.

2. Materials and methods

2.1. Animals and experimental design

Adult male *Mus musculus* albino mice of Swiss strain weighing 25–35 g were procured from and maintained in the Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College and Hospital, Annamalai Nagar. They were individually housed under hygienic conditions (22–24 °C) in polypropylene cages under 12 h light/12 h dark cycle. Animal care and experimental procedures were approved by the Institutional Animal Ethical Committee (IAEC), Rajah Muthiah Medical College, Annamalai Nagar. *vide* letter No: 591-160/1999/CPCSEA.

The animals received the standard pellet diet and water *ad libitum*.

After one week of acclimatization, the animals were divided into four groups of 6 mice each and were maintained as follows:

Group 1: CON – Animals received the control diet. Olive oil was administered (0.3 mL kg $^{-1}$ per day orally) from day 16 for the next 45 days.

Group 2: HFFD – Animals received the HFFD. Olive oil was administered (0.3 mL kg^{-1} per day orally) from day 16 for the next 45 days.

Group 3: HFFD + ASX – Animals received the HFFD. ASX was administered (6 mg kg^{-1} per day in olive oil) from day 16 for the next 45 days.

Group 4: CON + ASX – Animals received the control diet. ASX was administered (6 mg kg^{-1} per day in olive oil) from day 16 for the next 45 days.

The high fat-high fructose diet (HFFD) consisted of the following composition, (g per 100 g) 45.0 fructose, 10.0 ground nut oil, 10.0 beef tallow, 22.5 casein, 0.3 DL-methionine, 1.2 vitamin mixture, 5.5 mineral mixture and 5.5 wheat bran. The diet was prepared every day. Fructose provided 39%, fat 40% and casein 21% of the total calories from HFFD. The standard laboratory chow consisted of 60% (w/w) starch, 22.08% (w/w) protein and 4.38% (w/w) fat. Body weight, food intake and fluid intake were measured every 3rd day for the whole experimental period.

The dosage of ASX used is based on the literature[10] and on our previous study.[15]

Fasting blood samples were collected from experimental animals on the 16th day of the experimental period for the measurement of glucose and insulin to confirm the development of insulin resistance.

2.2. Sampling

On the 60th day, mice were fasted overnight before being anaesthetized with ketamine hydrochloride (30 mg kg^{-1}, i.m.) and then killed by decapitation. Blood was collected and plasma was separated by centrifugation (1500 g, 15 min, 37 °C) and used for analysis. The body was then cut open. Epididymal adipose tissue and skeletal muscle were removed quickly and rinsed in ice-cold saline. Epididymal adipose tissue was weighed. Tissue homogenate was prepared in ice-cold 0.1 M Tris-HCl buffer, pH 7.4.

2.3. Plasma parameters

Plasma glucose and insulin were measured using kits obtained from Agappe Diagnostics Pvt Ltd., Kerala and Accubind Microwells, Monobind Inc, CA, USA, respectively. The levels of plasma TNF-α and IL-6 were determined by ELISA using commercial kits obtained from BD Biosciences, San Jose, CA, USA and Koma Biotech, Seoul, South Korea respectively.

2.4. Lipid analysis and oxidative stress markers

Lipids were extracted from epididymal adipose tissue and skeletal muscle and the levels of total cholesterol, triglycerides and free fatty acids were measured. Lipid hydroperoxides (LHP) and protein carbonyl, the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione-*S*-transferase (GST) and the content of vitamins C and E and reduced glutathione (GSH) were analysed. Standard spectrophotometric methods used for analysis are outlined elsewhere.[16]

2.5. Insulin stimulation and muscle processing

At the end of the 60 days, a set of animals ($n = 3$) in each group were injected 15 U kg^{-1} human recombinant insulin intraperitoneally. After 30 min, the animals were sacrificed and the skeletal muscle was removed. Proteins were extracted from the muscle samples of each group of mice. The tissue samples were homogenized in ice-cold homogenization buffer (50 mM Tris, 0.25% SDS, 150 mM NaCl, 1% NP-40, 1 mM EDTA and a protease inhibitor cocktail) and centrifuged at 1500 rpm for 10 min at 4 °C. The supernatant obtained was again centrifuged at 12 000 rpm for 20 min at 4 °C. The resulting supernatant was retained as the total cell lysate and the pellet obtained was resuspended in 0.5 mL of buffer and used as the membrane fraction. The protein content in the cytosolic and membrane fraction was estimated by the method of Lowry *et al.*[17]

2.6. Western blotting

Samples (cytosolic and membrane fraction) containing 75 μg of protein were resolved by 8.0% sodium dodecyl sulphate PAGE, electrotransferred onto nitrocellulose membrane and incubated with anti-Akt antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), phospho-Ser^{473}Akt antibody (Cell Signaling

Technology, Inc, Danvers, MA, USA) or anti-GLUT 4 antibody according to the manufacturer's instructions. The nitrocellulose membranes were then probed with respective secondary antibodies. In order to make sure that protein loading was equal in all samples, blots were probed with anti-β-actin antibody (Sigma). Protein bands were visualized by enhanced chemiluminescence method using the SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific). Quantification of band intensity was performed using AlphaEaseFc software.

2.7. Immunoprecipitation and immunoblotting

Muscle homogenates (500 μg protein) were incubated overnight at 4 °C with antibodies specific to insulin receptor-β (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) or IRS-1 (Cell Signaling Technology, Inc, Danvers, MA, USA) and the immune complexes were captured by adding 50 μl protein-A agarose beads. Immune complexes were pelleted at 4000 *g* for 10 min at 4 °C and washed thrice with the homogenization buffer. The immune complexes were suspended in Laemmli sample buffer and boiled for 5 min. Protein-A CL agarose was removed from the denatured proteins by centrifugation at 4000 *g* for 10 min at 4 °C. The supernatant was subjected to SDS-PAGE (8.0% gel) and electrotransferred onto nitrocellulose membrane. Following transfer, the membranes were probed with phosphotyrosine antibody (Cell Signaling Technology, Inc, Danvers, MA, USA) residues for the detection of tyrosine phosphorylation status of IR-β or p85PI3-kinase antibody for IRS1-p85PI3-kinase association. The immunoblot was stripped of bound antibodies and then re-probed with antibodies specific to insulin receptor-β or IRS-1 to normalize the tyrosine phosphorylation or p85PI3-kinase. Protein band detection and quantification were performed as mentioned earlier.

2.8. Statistical analysis

All the values were expressed as means ± SD of 6 mice from each group and statistically evaluated by one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) for multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. General observations and plasma biochemistry

Food and fluid intake did not differ significantly between the experimental groups (data not shown). Significant increases in body weight and the epididymal fat pad weights in HFFD group were observed as compared to CON (Table 1). The plasma glucose and insulin levels were higher in the HFFD group than CON (Table 1, $p < 0.05$). Furthermore, HFFD feeding caused significant increase in plasma levels of TNF-α and IL-6 (Table 1). The rise in fasting insulin level in HFFD group compared to CON confirms the development of insulin resistance after high calorie feeding. The levels were close to normal in ASX-treated HFFD-fed mice.

3.2. Lipid levels and lipid peroxidation products

The levels of total lipid, TG, FFA and cholesterol in skeletal muscle were significantly increased (by 17%, 19%, 32% and 57%, respectively) and in adipose tissue (by 57%, 77%, 86% and 38%, respectively) in the HFFD-group as compared to CON. ASX treatment restored the lipid levels to near-normal levels (Table 2). HFFD group showed significantly higher levels of LHP and protein carbonyl as compared to CON (Table 2). In HFFD + ASX group, the levels of these variables were significantly lower ($p < 0.05$) as compared to HFFD.

3.3. Enzymatic and non-enzymatic antioxidants status

The activities of SOD, CAT, GPX and GST in skeletal muscle were decreased by 37%, 32%, 22% and 20%, respectively in skeletal muscle and by 52%, 34%, 38% and 30%, respectively in adipose tissue of HFFD as compared to CON (Table 3). In HFFD + ASX, the activities returned back to near-normal. The levels of vitamins C, E and GSH in skeletal muscle and adipose tissue were significantly lower in the HFFD group than in CON (Table 3). In HFFD + ASX, the levels of these non-enzymatic antioxidants were brought to near-normal as compared to untreated HFFD group. No significant differences were observed between CON and CON + ASX groups in the antioxidant status.

3.4. ASX improves insulin signal transduction in the skeletal muscle of HFFD-fed mice

The protein levels of IR-β and IRS-1 were similar between these groups. HFFD feeding markedly reduced insulin-stimulated IR-β tyrosine phosphorylation and IRS-1/PI3K association. However, ASX supplementation caused a significant increase in the tyrosine phosphorylation of IR-β (Fig. 1) and insulin stimulated p85PI3-kinase-IRS-1 association (Fig. 2) compared to HFFD-fed mice. Total Akt did not differ between ASX-untreated and ASX-treated HFFD-fed mice but insulin-stimulated Akt-Ser^{473} phosphorylation (Fig. 3) was significantly higher ($p < 0.05$) in the ASX + HFFD group. Insulin-stimulated GLUT-4 protein level in the membrane fraction (Fig. 4) of skeletal muscle from HFFD-fed mice was reduced to about 43% of that in CON group. On the other hand insulin-stimulated GLUT-4 protein level was higher in ASX + HFFD mice than that of mice fed HFFD alone and reached that observed in CON.

4. Discussion

ASX has been shown previously to lower plasma glucose and insulin levels and improve insulin sensitivity. In our previous study, we found that ASX improved liver function and limited lipid accumulation in liver of HFFD-fed mice without any toxic effects.[15] This study reports its effects on post-receptor insulin signaling events in the skeletal muscle of HFFD-fed mice. The important findings in this study are 1) ASX significantly lowers plasma insulin and glucose in HFFD-fed mice and improves insulin sensitivity and 2) ASX administration to HFFD-fed mice potentiates post-receptor insulin signaling events in skeletal muscle and reduces oxidative stress and inflammation. These

Table 1 Body weight, epididymal fat weight, plasma parameters[a,b]

Parameters	CON	HFFD	HFFD + ASX	CON + ASX
Initial body weight (g)	27.17 ± 0.75	26.50 ± 0.55	26.83 ± 0.75	26.67 ± 0.82
Final body weight (g)	31.33 ± 0.74	36.66 ± 0.74[c]	33.01 ± 0.81[d]	30.88 ± 0.95
Epididymal fat pad weight (g per 100 g b.w.)	1.10 ± 0.06	3.10 ± 0.25[c]	1.40 ± 0.08[d]	0.90 ± 0.05
Glucose (mg dL^{-1})	83.91 ± 4.52	134.81 ± 8.98[c]	98.77 ± 4.74[d]	82.66 ± 4.74
Insulin (μU mL^{-1})	17.42 ± 1.46	30.54 ± 2.63[c]	22.24 ± 1.76[d]	18.67 ± 0.96
TNF-α (pg mL^{-1})	11.63 ± 0.80	38.15 ± 1.89[c]	19.25 ± 0.93[d]	14.41 ± 0.98
IL-6 (pg mL^{-1})	89.89 ± 4.9	194.57 ± 12.5[c]	112.76 ± 6.3[d]	95.92 ± 6.45

[a] Values are means ± SD of 6 mice from each group. [b] CON – control mice; HFFD – high fat-high fructose diet fed mice; HFFD + ASX – high fat-high fructose diet fed mice treated with astaxanthin; CON + ASX – control mice treated with astaxanthin. [c] Significant as compared to CON ($p < 0.05$; ANOVA followed by DMRT). [d] Significant as compared to HFFD ($p < 0.05$; ANOVA followed by DMRT).

results imply that ASX reverses impaired insulin responsiveness in skeletal muscle and acts as an insulin sensitizer in this model.

HFFD consumption caused insulin resistance on the 16th day (data not shown) and at the end of study. Consumption of high fat plus high fructose or sucrose diet accelerates the development of obesity and insulin resistance.[18,19] Previous studies have demonstrated that high fat plus fructose produces defects in proximal steps of insulin signaling and decreases insulin stimulated PI-3kinase activity.[20]

The muscle samples for investigating insulin signaling events were prepared from animals after insulin stimulation since previous studies have noted no changes in the downstream insulin signaling proteins in fructose-fed rats under basal state.[21] ASX treatment increased insulin-stimulated insulin receptor-β tyrosine phosphorylation and IRS-1 associated PI3-kinase expression, p-Akt/Akt ratio and GLUT-4 translocation in skeletal muscle. These data provide strong evidence for blood glucose-lowering and insulin sensitivity effects of ASX.

Oxidative stress in HFFD-fed mice could be due to hyperglycemia since elevated glucose can generate free radicals *via* mechanisms like autooxidation, protein glycation and polyol pathway.[22] ROS can activate protein kinases like c-jun N-terminal protein kinase (JNK), p38 mitogen-activated protein kinases (p38 MAPK) and extracellular regulated kinases $^1/_2$ (ERK $^1/_2$) that phosphorylate the serine/threonine residues of IRS-1 and IRS-2. These kinases downregulate IRS-mediated insulin signaling and contribute to the development of insulin resistance.[23] Administration of HFFD caused insulin resistance in mice as assessed by fasting hyperinsulinemia. Concomitant with this, HFFD also interrupted IRS-PI3K-Akt insulin signaling pathway in skeletal muscle.

Enzymatic antioxidants such as SOD, CAT and GPx are themselves susceptible to free radicals attack. These primary antioxidant enzymes are involved in the direct elimination of free radicals. Decreased levels of vitamins C and E could be either due to increased utilization for trapping free radicals or due to decreased regeneration from their oxidized forms.[23]

Adipose tissue from HFFD-fed mice is fat laden as evident from the lipid levels. Under these conditions, there is a net efflux of lipids from adipose tissue resulting in ectopic fat uptake by skeletal muscle. This accounts for the rise in skeletal muscle lipids. Intramyocellular lipid is a major culprit for the development of insulin resistance mediated by the generation of toxic lipid metabolites such as long chain fatty acids, ceramide and diacylglyerol. There is evidence that these lipotoxic substances could negatively influence insulin sensitivity by activating the stress-sensitive pathways.[24] The reduction of lipid accumulation by ASX suggests that it might act to shift lipid metabolism from

Table 2 Levels of lipids and oxidative stress markers in skeletal muscle and adipose tissue[a]

Parameters	CON	HFFD	HFFD + ASX	CON + ASX
Skeletal muscle				
Total lipid[d]	10.45 ± 0.58	12. 21 ± 0.89[b]	11.10 ± 0.78[c]	10.79 ± 0.61
Cholesterol[d]	2.30 ± 0.19	2.75 ± 0.13[b]	2.52 ± 0.13[c]	2.24 ± 0.17
Triglycerides[d]	4.31 ± 0.34	5.70 ± 0.18[b]	4.78 ± 0.17[c]	4.43 ± 0.22
Free fatty acids[d]	1.28 ± 0.10	2.01 ± 0.16[b]	1.75 ± 0.13[c]	1.39 ± 0.10
LHP[e]	1.46 ± 0.07	2.38 ± 0.17[b]	1.90 ± 0.11[c]	1.60 ± 0.11
Protein carbonyl[e]	0.22 ± 0.02	0.35 ± 0.02[b]	0.26 ± 0.02[c]	0.20 ± 0.01
Adipose tissue				
Total lipid[d]	12.35 ± 0.61	19.39 ± 0.95[b]	13.44 ± 0.76[c]	12.25 ± 0.65
Cholesterol[d]	3.07 ± 0.18	5.46 ± 0.26[b]	3.31 ± 0.17[c]	2.93 ± 0.15
Triglycerides[d]	4.66 ± 0.27	8.69 ± 0.43[b]	5.56 ± 0.23[c]	4.95 ± 0.22
Free fatty acids[d]	3.31 ± 0.17	4.57 ± 0.22[b]	3.55 ± 0.17[c]	3.12 ± 0.22
LHP[e]	1.09 ± 0.07	1.80 ± 0.11[b]	1.29 ± 0.05[c]	1.17 ± 0.06
Protein carbonyl[e]	0.26 ± 0.01	0.34 ± 0.03[b]	0.29 ± 0 02[c]	0.24 ± 0.02

[a] Values are means ± SD of 6 mice from each group. CON – control mice; HFFD – high fat-high fructose diet fed mice; HFFD + ASX – high fat-high fructose diet fed mice treated with astaxanthin; CON + ASX – control mice treated with astaxanthin. [b] Significant as compared to CON ($p < 0.05$; ANOVA followed by DMRT). [c] Significant as compared to HFFD ($p < 0.05$; ANOVA followed by DMRT),LHP-lipid hydroperoxides. [d] mg g^{-1} tissue. [e] nmol mg^{-1} protein.

Table 3 Activities of enzymatic antioxidants and the levels of non-enzymatice antioxidants (μmol mg^{-1} protein) in skeletal muscle and adipose tissue[a,d]

Parameters	CON	HFFD	HFFD + ASX	CON + ASX
Skeletal muscle				
SOD[e(A)]	3.47 ± 0.27	2.17 ± 0.12[b]	2.73 ± 0.24[c]	3.32 ± 0.25
CAT[e(B)]	38.88 ± 3.65	26.30 ± 2.52[b]	31.02 ± 2.65[c]	35.32 ± 2.92
GPX[e(C)]	5.20 ± 0.44	4.03 ± 0.28[b]	4.49 ± 0.20[c]	4.95 ± 0.46
GST[e(D)]	3.64 ± 0.24	2.93 ± 0.20[b]	3.30 ± 0.30[c]	3.70 ± 0.22
GSH	2.44 ± 0.19	1.17 ± 0.13[b]	1.70 ± 1.22[c]	2.26 ± 0.20
Vitamin C	0.60 ± 0.03	0.35 ± 0.02[b]	0.45 ± 0.03[c]	0.56 ± 0.03
Vitamin E	0.68 ± 0.04	0.30 ± 0.02[b]	0.37 ± 0.03[c]	0.63 ± 0.04
Adipose tissue				
SOD[e(A)]	2.85 ± 0.13	1.36 ± 0.09[b]	2.57 ± 0.15[c]	2.76 ± 0.10
CAT[e(B)]	29.38 ± 1.77	19.32 ± 1.25[b]	22.12 ± 1.68[c]	27.52 ± 1.65
GPX[e(C)]	3.62 ± 0.22	2.24 ± 0.14[b]	2.91 ± 0.18[c]	3.43 ± 0.22
GST[e(D)]	2.84 ± 0.11	1.99 ± 0.11[b]	2.22 ± 0.13[c]	2.74 ± 0.15
GSH	1.87 ± 0.09	0.95 ± 0.05[b]	1.35 ± 0.05[c]	1.79 ± 0.08
Vitamin C	0.38 ± 0.02	0.20 ± 0.01[b]	0.31 ± 0.02[c]	0.36 ± 0.02
Vitamin E	0.44 ± 0.03	0.23 ± 0.01[b]	0.36 ± 0.01[c]	0.42 ± 0.02

[a] Values are means ± SD of 6 mice from each group. CON – control mice; HFFD – high fat-high fructose diet fed mice; HFFD + ASX – high fat-high fructose diet fed mice treated with astaxanthin; CON + ASX – control mice treated with astaxanthin. [b] Significant as compared to CON ($p < 0.05$; ANOVA followed by DMRT). [c] Significant as compared to HFFD ($p < 0.05$; ANOVA followed by DMRT). [d] SOD – superoxide dismutase, CAT – catalase, GPX – glutathione peroxidase, GST – glutathione-*S*-transferase, GSH – reduced glutathione. [e] [A] – Amount of enzyme which gave 50% inhibition of nirtobluetetrazolium (NBT) reduction/mg protein; [B] – moles of H_2O_2 utilized/min/mg protein; [C] – μmoles of GSH utilized/min/mg protein; [D] – nmoles of glutathione-1-chloro 2,4-dinitrobenzene (CDNB) conjugate formed/min/mg protein.

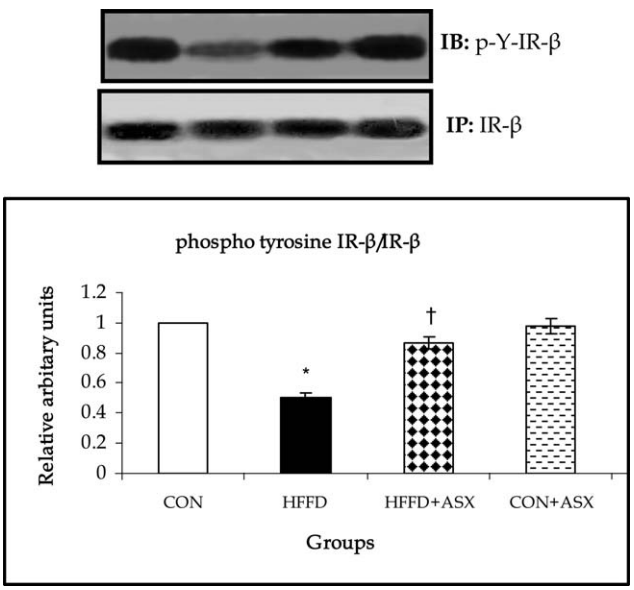

Fig. 1 Effect of ASX on IR-beta tyrosine phosphorylation in skeletal muscle of experimental animals after insulin stimulation. The extent of IR-β tyrosine phosphorylation was assessed by an anti-phospho tyrosine western blot of IR-β immunoprecipitates. Blots were stripped of bound antibodies and re-probed with anti-IR-β antibody to normalize the blots for protein levels. Densitometric quantification of phosphotyrosine IR-β to IR-β level is expressed as relative arbitrary units. Data are expressed as means ± SD for 3 mice. IP – immunoprecipitation; IB – immunoblotting. * $p < 0.05$ Compared to control; † $p < 0.05$ Compared to HFFD.

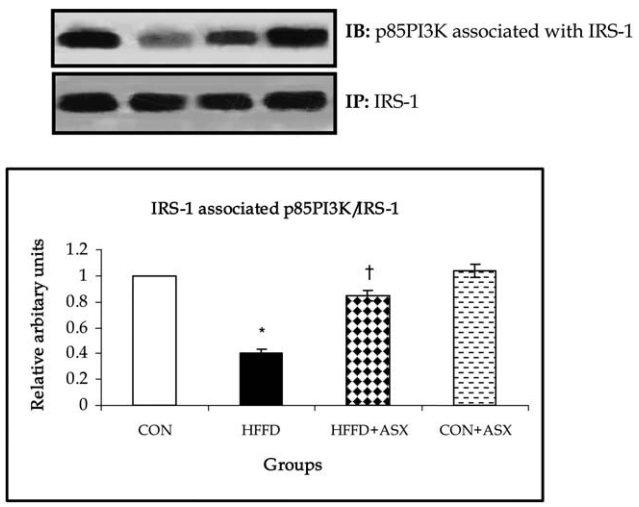

Fig. 2 Effect of ASX on association of p85PI3-kinase with IRS-1 in skeletal muscle of experimental animals after insulin stimulation. The association of the p85PI3-kinase with IRS-1 protein was assessed by an anti-p85PI3-kinase western blot of IRS-1 immunoprecipitates. Blots were stripped and re-probed with anti-IRS-1 antibody to normalize the blots for protein levels. Densitometric quantification of IRS-1 associated p85PI3-kinase to IRS-1 level is expressed as relative arbitrary units. Data are expressed as means ± SD for 3 mice. IP – immunoprecipitation; IB – immunoblotting. * $p < 0.05$ Compared to control; † $p < 0.05$ Compared to HFFD.

synthesis and storage to oxidation and thereby deactivate the stress pathways. ASX has been shown to stimulate the increased utilization of fattyacids as the source of energy in cells by accelerating the oxidation of lipids.[11] Thus ASX negates the lipotoxicity effects of HFFD feeding.

Food intake of mice did not differ significantly between the groups but HFFD-fed mice received more calories than the CON. This has been reported by us in an earlier publication.[15] This suggests that ASX may act by increasing energy expenditure by promoting fat oxidation. One limitation in this study is that energy expenditure and fat oxidation in different groups by indirect calorimetry are not measured. ASX has been shown to

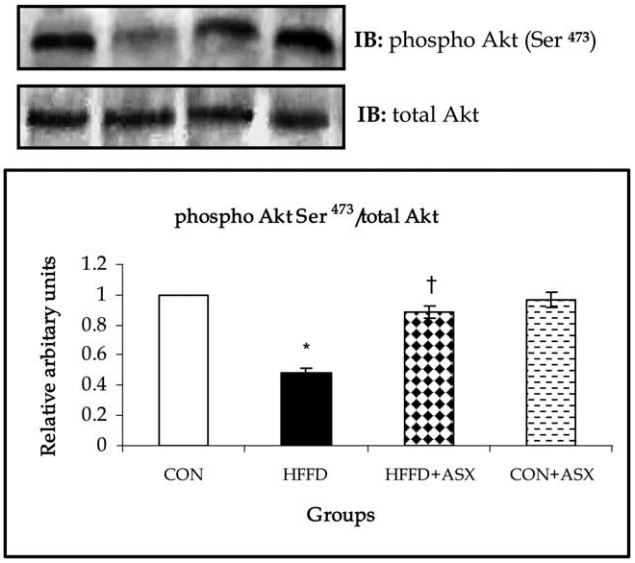

Fig. 3 Effect of ASX on Akt phosphorylation in skeletal muscle of experimental animals after insulin stimulation. For determining the extent of Akt activation, proteins were immunoblotted with phospho-specific anti-Ser^{473} Akt antibody. Blots were stripped and re-probed with anti-Akt antibody to normalize the blots for protein levels. Densitometric quantification of pAktSer^{473} to Akt levels is expressed as relative arbitrary units. Data are expressed as means ± SD for 3 mice. IB – immunoblotting; * $p < 0.05$ Compared to control; † $p < 0.05$ Compared to HFFD.

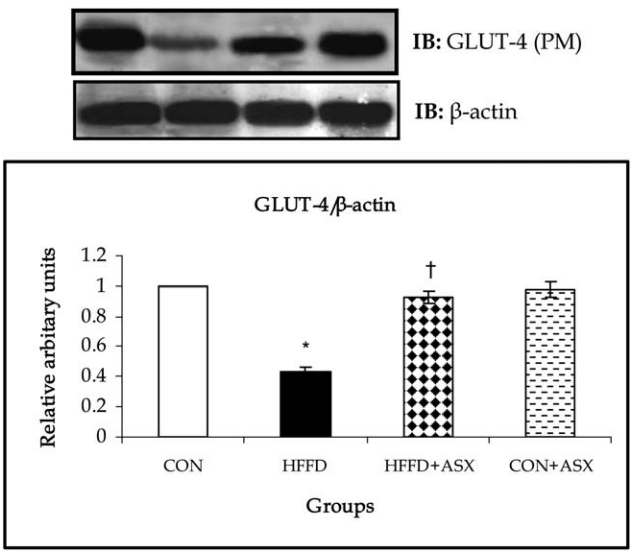

Fig. 4 Effect of ASX on GLUT-4 translocation in skeletal muscle of experimental animals after insulin stimulation. For determining the extent of GLUT-4 translocation to the membrane proteins were immunoblotted with anti-GLUT-4 antibody. Blots were stripped and re-probed with anti-β-actin antibody to normalize the blots for protein levels. Densitometric quantification of GLUT-4 to β-actin levels is expressed as relative arbitrary units. Data are expressed as means ± SD for 3 mice. IB – immunoblotting; PM – plasma membrane * $p < 0.05$ compared to control; † $p < 0.05$ Compared to HFFD.

increase fatty acid catabolism in skeletal muscle during exercise through the upregulation of carnitine palmitoyltransferase-I. Ikeuchi *et al.*[11] also noted that ASX has no effect on food intake but reduces body weight in obese mice possibly by increasing energy expenditure. This explains the mechanism by which ASX promotes weight reduction and increases energy expenditure in HFFD-mice. A weight-matched control group to distinguish the effect of ASX to alter the studied parameters is necessary to address this issue and will be undertaken in future.

Fat accumulation in adipose tissue is associated with chronic low-grade inflammation, which is suggested to be a potential mechanism for obesity-induced insulin resistance.[25] Recent studies suggest that increased circulating FFAs may exert proinflammatory and insulin-desensitizing effects by interacting with toll-like receptors. This interaction turns on several intracellular inflammatory signaling pathways that impose negative effects on insulin signaling cascade.[26] HFFD-fed mice appear to suffer from systemic inflammation as suggested by elevated IL-6 and TNF-α levels. An increase in inflammatory pathways related to insulin resistance in rats fed high fat diets has been suggested.[27]

TNF-α increases serine phosphorylation of both IR-β and IRS-1 and decreases tyrosine phosphorylation. This leads to increased ubiquitination/proteosomal degradation of IRS-1, or decreased ability of IRS-1 to engage the p85 subunit of PI3K leading to insulin resistance. IL-6 upregulates suppressor of cytokine signaling-3 protein (SOCS-3) *via* activation of signal transducer and activator of transcription (STAT) 3. Activated SOCS-3 can bind directly to the insulin receptor, inhibit tyrosine phosphorylation of IRS-1 and -2 and interrupt insulin signaling.[28] A notable decrease in circulating levels of TNF-α and IL-6 in ASX-treated animals suggest that ASX may influence insulin action through its anti-inflammatory activity. Further studies are needed to understand the mechanisms underlying the regulatory effects of the ASX on the expression of these cytokines and inflammatory pathways.

5. Conclusion

The present study demonstrates that ASX administration is associated with improved insulin signaling in skeletal muscle despite the consumption of HFFD possibly by lowering oxidative stress and lipid accumulation. Thus ASX might be a novel approach for the treatment of insulin resistance and associated disorders.

Acknowledgements

This study was financially supported by a project grant from the University Grants Commission, New Delhi, India.

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