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Inhibition of intestinal ascorbic acid uptake by lipopolysaccharide is mediated via transcriptional mechanism(s)

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Abstract

Ascorbic acid (AA) accumulation in intestinal epithelial cells is an active transport process mainly mediated by two sodium-dependent vitamin C transporters (SVCT-1 and SVCT-2). To date, little is known about the effect of gut microbiota generated lipopolysaccharide (LPS) on intestinal absorption of water-soluble vitamins. Therefore, the objective of this study was to investigate the effects of bacterially-derived LPS on AA homeostasis in enterocytes using Caco-2 cells, mouse intestine and intestinal enteroids models. Pre-treating Caco-2 cells and mice with LPS led to a significant decrease in carrier-mediated AA uptake. This inhibition was associated with a significant reduction in SVCT-1 and SVCT-2 protein, mRNA, and hnRNA expression. Furthermore, pre-treating enteroids with LPS also led to a marked decrease in SVCT-1 and SVCT-2 protein and mRNA expression. Inhibition of SVCT-1 and SVCT-2 occurred at least in part at the transcriptional level as promoter activity of *SLC23A1* and *SLC23A2* was attenuated following LPS treatment. Subsequently, we examined the protein and mRNA expression levels of HNF1α and Sp1 transcription factors, which are needed for basal *SLC23A1* and *SLC23A2* promoter activity, and found that they were significantly decreased in the LPS treated Caco-2 cells and mouse jejunum; this was reflected on level of the observed reduction in the interaction of these transcription factors with their respective promoters in Caco-2 cells treated with LPS. Our findings indicate that LPS inhibits intestinal carrier- mediated AA uptake by down regulating the expression of both vitamin C transporters and transcriptional regulation of *SLC23A1* and *SLC23A2* genes.

Graphical abstract

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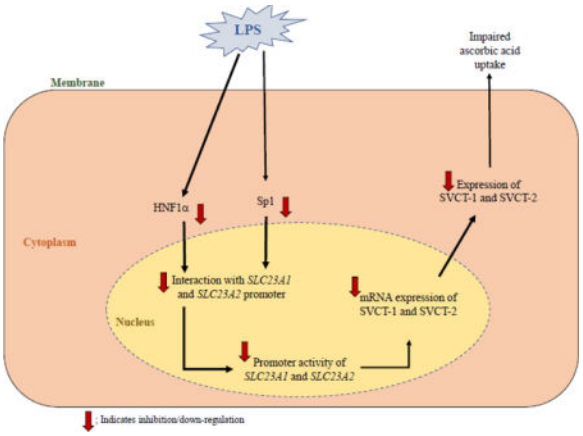
Conflict of interest

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



Keywords

SVCT-1; SVCT-2; transport; uptake; organoids; Vitamin C

1. Introduction

Vitamin C is a potent antioxidant which serves as a cofactor for many important enzymatic reactions [1]. Humans have lost the capability to synthesize vitamin C *de novo*, and instead obtain this nutrient from dietary sources via intestinal absorption. While lack of access to AA-rich foods and malnutrition can lead to dietary vitamin C deficiency, there is also evidence that deficiency can also develop in the condition where absorption and handling of this nutrient is impaired such as in the elderly, smokers and alcoholics [2–5]. There is also evidence indicating that AA is not only critical for normal functioning of various enzymes and proteins, but that optimal vitamin C body homeostasis provides protection against conditions such as hepatic and cardiovascular diseases, cancer, cataract formation and osteoporosis [6–9]. The latter is deemed to be due to the antioxidant properties of vitamin C which can counteract the deleterious impact of reactive oxygen species and oxidative stress [10, 11] which are commonly found in these conditions. Vitamin C deficiency has been implicated as a contributory factor in the pathogenesis of intestinal disorders, including inflammatory bowel diseases (IBD) [10–13].

Intestinal absorption of vitamin C occurs via a Na^+ dependent carrier-mediated process [14–17]. Vitamin C exists in two forms: the reduced ascorbic acid (AA) form which is transported via the sodium-dependent vitamin C transporter-1 and -2 (SVCT-1 and SVCT-2, the products of the *SLC23A1* and *SLC23A2* genes, respectively [18–21]) and the oxidized dehydroascorbic acid (DHA) form which is transported via glucose transporters (GLUT1, GLUT3 and GLUT4 [22]). The SVCT-1 and SVCT-2 are expressed at apical and basolateral membrane domains, respectively, in intestinal epithelial cells [14, 23, 24], and the two human and mouse transporters share significant similarities at the amino acid level [25].

Lipopolysaccharide (LPS) acts as a potent activator of the inflammatory response in the intestine, which contains high levels of LPS from gut microbiota and pathogens such as *Salmonella* [26–29]. Intestinal LPS binds to Toll-like receptors (primarily TLR4) [28, 30],

leading to an activation of various inflammatory signaling pathways (NF-κB and p38 MAPK [31, 32]) and production of pro-inflammatory cytokines [33, 34]. LPS levels are elevated in the blood of patients with IBD and necrotizing enterocolitis (NEC), as well as in those infected with *Salmonella* [27, 29, 35–38]. LPS may also be detectable in the plasma of septic patients [39–41], where it is believed to play a key role in systemic inflammatory response syndrome. While there is some evidence indicating that sepsis can be associated with depletion of AA [42–44], the mechanisms responsible for these findings have not been fully elucidated. In this regard, LPS has been shown to affect intestinal transport of many other nutrients [45–48] which indicates a potential role for LPS in AA deficiency in sepsis via interference with its intestinal absorption; however, the effect of LPS on intestinal AA uptake and hSVCT function has not been examined. As a potential explanation for these observations, we hypothesized that LPS exposure affects the AA uptake in intestinal epithelial cells. Consequently, in this study we have evaluated the impact of LPS on the vitamin C transport system using several different experimental models for AA accumulation including human intestinal Caco-2 cells (in vitro), primary tissue from LPS exposed mice (in vivo) and intestinal enteroids (ex vivo). Results from the in vitro and in vivo model consistently reveal a significant inhibition in AA uptake following exposure to LPS. The attenuated transport of AA was accompanied by a significant reduction in SVCT-1 and SVCT-2, protein, mRNA, hnRNA expression and reduced promoter activity of *SLC23A1* and *SLC23A2* genes. Levels of the transcription factors hepatocyte nuclear factor 1 (HNF1α) and specificity protein 1 (Sp1), as well as the interaction of these transcription factors with their respective *SLC23A1* and *SLC23A2* promoters were also markedly decreased in LPS-treated Caco-2 cells compared to controls. Collectively, these findings demonstrate that the LPS inhibits intestinal AA uptake and this effect is mediated, at least in part, via transcriptional repression of the *SLC23A1* and *SLC23A2* genes.

2. Materials and Methods

2.1. Materials

^{14}C-Ascorbic acid (specific activity 10mCi/mmol; radiochemical purity > 99%) was purchased from American Radiolabeled Chemicals/PerkinElmer Inc., (St. Louis, MO/ Boston. MA). Reconstituted aqueous LPS (*E. coli* 0111:B4) solution was obtained from Sigma (St. Louis, MO). The anti-SVCT-1, SVCT-2, HNF1α, Sp1 and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-rabbit IRDye-800 and anti-mouse IRDye-680 secondary antibodies were obtained from LI-COR Biosciences (Lincoln, NE). All molecular biology grade chemicals and reagents were purchased from commercial vendors. Primers used in this study were synthesized by Sigma Genosys (Woodlands, TX).

2.2. Culturing of Caco-2 cells

Confluent monolayers of human-derived intestinal epithelial Caco-2 cells (derived from a 72 years-old adult male) were used in these experiments (ATCC, Manassas, VA). Caco-2 cells were maintained in EMEM (Gibco) medium (supplemented with 10% fetal bovine serum (FBS) and antibiotics) at 37°C in a CO_2 incubator. Caco-2 cell monolayers (3–4 days post confluence; differentiated) were used to examine the effect of LPS on ^{14}C-AA uptake and

resolve SVCT-1 and SVCT-2 protein, mRNA and hnRNA expression levels. For the latter experiments cells were serum starved (to synchronize the cell cycle to obtain relatively homogenous population of cells) overnight and then treated with 50 μg/ml LPS (a concentration that is relevant to the endotoxin level in the gut) in EMEM medium supplemented with 0.5% FBS.

2.3. Generation of mouse enteroids and LPS treatment

We have prepared intestinal enteroids from 12 weeks old male mice by following the established procedure of Sato et al., with some modifications [49, 50]. Mice were euthanized, the proximal jejunum was removed and then washed with ice cold PBS and inverted, with the inner lining of the intestinal tract facing out. The unwanted villus portion was removed and the remaining tissue cut into approximately 5 mm fragments, which were subjected to a series of PBS washes. Then, EDTA (2.5mM) solution was added and the tissue placed in a high speed rocker for 30 min. The EDTA supernatant was then removed and replaced with ice-cold PBS. The sample was vortexed and the supernatant discarded ('fraction1'). This step was repeated twice (to collect fractions '2' and '3') and these fractions were collected and passed through a 70 μm nylon filter (Fisher Scientific, Hampton, NH) to enrich crypt cells. Each fraction was centrifuged (200g, 5 min, 4°C) to yield a pellet ideally comprised of crypt cells. To culture the crypt cells, the pellet was resuspended in matrigel (Corning, Tewksbury, MA). The crypt cell-matrigel mixture from a single fraction was then evenly distributed in a 48-well tissue culture plate, and left to harden for 20 min at 37°C CO_2 incubator. The matrigel buttons were then submerged in 250 μl of enteroid medium, which contains a cocktail of growth factors as described before [49, 50]. The enteroid were treated with LPS (50 μg/ml) for 48 h (based on previously published studies on impact of LPS on other transporters) and used for determining the levels of SVCT-1 and SVCT-2 protein and mRNA expression. The animal protocol used in this study was approved by the Animal Care and Use Committee (IACUC), VA Medical Center, Long Beach, CA.

2.4. Ascorbic acid uptake

After 48 h of LPS exposure, confluent monolayers of Caco-2 cells were used to perform ^{14}C-AA uptake as described previously [16, 51]. Briefly, cells were incubated (3 min) in Krebs-Ringer (KR) buffer at 37°C in presence of ^{14}C-AA (32μM) and processed for radioactivity accumulation [16, 51]. For *in vivo* studies, 12-week old male C57BL/6 mice were used (Jackson Laboratory, Bar Harbor, ME). The adult mice were administered a single dose of LPS [0.005g/kg body weight, IP (Intraperitoneally)] followed by examination (48 h later) of AA uptake and molecular biology studies. Jejunal sheets were prepared as described previously [16, 52, 53]. The jejunal portion of the intestine was removed immediately after euthanization of the animals and equal pieces (1cm) of jejunal sheets were incubated (5 min) in K-R (pH 7.4) buffer containing ^{14}C-AA (32μM) in the presence and absence of Na^+. The samples were then washed in cold KR buffer and radioactive content was measured [16, 52].

2.5. Real-time PCR analysis

Total RNA was isolated from Caco-2 cells, mouse jejunum tissue and organoids treated with LPS and parallel controls using TRIzol reagent (Life Technologies, Carlsbad, CA). RNA samples were reverse transcribed (RT) to cDNA utilizing i-Script kit (Bio-Rad, CA). RT products were then used for quantitative real-time PCR (qRT-PCR) analysis using gene specific primers (Table 1) in a CFX96 real-time i-cycler (Bio-Rad) as described before [16, 51, 52]. The relative expression of different mRNA was normalized relative to β-actin and quantified using a relative relationship method (Bio-Rad) [54].

2.6. Heterogeneous nuclear RNA (hnRNA) analysis

Total RNA isolated from Caco-2 cells and mouse jejunum treated with LPS and controls were used to perform qRT-PCR with *SLC23A1* and *SLC23A2* gene specific hnRNA primers [16, 51, 55]. RNA samples were treated with DNase I (Invitrogen, Carlsbad, CA) and reverse transcribed using i-Script kit (Bio-Rad). qRT-PCR was performed using the *SLC23A1* and *SLC23A2* gene specific hnRNA primers (Table 1) and a negative control was run in the absence of cDNA template to determine the specific amplification. The qRT-PCR data was normalized to β-actin and calculated as described above [54].

2.7. Cell transfection and promoter assay

Caco-2 cells were grown on 12 well plates and co-transfected in the presence of Lipofectamine 2000 (3 μl/well) (Invitrogen) with either human full-length *SLC23A1* or *SLC23A2* (3μg plasmid DNA/well), 5′-regulatory region constructs [56, 57], and *Renilla* Luciferase-thymidine Kinase (pRL-TK, 100 ng/well; Promega). After 24 h of transfection, cells were treated with LPS (50μg/ml) for a further 48 h and subsequently lysed and both *firefly* and *Renilla* luciferase activity measured using a 20/20 luminometer [56, 57].

2.8. Western blot analysis

For western blot analysis, cultured Caco-2 cells, mouse jejunal tissues, and organoids were homogenized in RIPA buffer (Sigma) in the presence of a complete protease inhibitor cocktail (Roche, Nutley, NJ). Sixty micrograms of total protein (60μg) was loaded into an individual lane of a 4–12% mini gel (Invitrogen) and transferred to PVDF membrane. After transfer, the membrane was probed with primary antibodies [hSVCT-1 (1:1000 dilution; Abgent, CA: Cat # AP12718a), mSVCT-1 (Cat # Sc 9921), hSVCT-2 (Cat # Sc 30114), mSVCT-2 (Cat # Sc 9926), HNF1α (Cat # Sc 6547) and Sp1 (Cat # Sc 14027) 1:200 dilutions; Santa Cruz biotechnology Inc.]. The specificity of the antibodies has been previously determined from protein samples isolated from various in vitro and in vivo preparations [16, 51, 58–60]. Anti-β-actin mouse monoclonal antibody (in 1:3000 dilution; Santa Cruz Biotechnology, Cat # Sc 47778) was used as an internal control. The corresponding secondary antibodies (LI-COR Biosciences) in 1:30,000 dilutions were used as described previously [16, 51]. Relative protein expression was quantified by normalizing the signal intensity against β-actin using application software in the Odyssey Infrared imaging suite (version 3, LI-COR Biosciences).

2.9.Chromatin immunoprecipitation assay (ChIP) and qPCR

Caco-2 cells (4×10^6 cells) were serum starved overnight and used to performed ChIP assay using the Simple ChIP enzymatic chromatin IP kit (Cell Signaling, Inc., Danvers, MA) as described previously [16, 51]. Briefly, chromatin was cross-linked with 1% formaldehyde and the reaction stopped by addition of glycine solution and ice-cold PBS containing protease inhibitor cocktail (PIC). Next cells were scraped and centrifuged (1500 rpm, 5 min) and re-suspended in ice-cold buffer A containing DTT and PIC. Nuclei were prepared and chromatin was digested with micrococcal nuclease, after which samples were sonicated to shear DNA into fragments followed by centrifugation (10,000 rpm, 10 min). Subsequently, 5 μg of digested chromatin isolated from control and LPS treated cells were incubated (16 h, 4°C) with 2 μg of either HNF1α or Sp1 specific antibodies (Santa Cruz Biotechnology). Finally, the immunoprecipitated DNA complex was subjected to DNA purification and qPCR analysis performed using *SLC23A1* and *SLC23A2* promoter specific primers (Table 1) to amplify fragments (−150 to +5; −97 to +42, respectively) relative to transcriptional start site (relative to TSS as +1).

2.10. Statistical Analysis

Uptake data represent means ± SE of multiple separate experimental determinations and are expressed as a percentage of simultaneously performed controls. Student's t-test was used for statistical analysis with $P < 0.05$ considered statistically significant. Protein, mRNA, hnRNA, promoter and ChIP analysis experiments were determined from at least three independent sample preparations.

3. Results

3.1. Effect of LPS on intestinal AA uptake

First, we examined the effect of treating Caco-2 cell monolayers with LPS (50 μg/ml) on carrier-mediated ^{14}C-AA uptake. Exposure of the cells to LPS led to a significant ($P < 0.01$) inhibition in AA uptake compared to simultaneously performed experiments in unexposed Caco-2 cells (Fig. 1A). A similar effect was seen following in vivo exposure of mice to LPS. Carrier-mediated AA uptake by intestinal (jejunum) sheets assessed 48 h after LPS injection (0.005g/kg body weight, IP) resolved a significant ($P < 0.05$) inhibition in carrier-mediated AA uptake compared to controls (Fig. 1B).

3.2. Effect of LPS on molecular aspects of vitamin C uptake by Caco-2 cells, mouse native jejunum and mouse intestinal enteroids

To investigate the basis of the observed inhibition in carrier-mediated AA uptake after in vitro or in vivo exposure to LPS, expression of SVCT-1 and SVCT-2 protein was examined. Total cellular SVCT-1 and SVCT-2 protein levels were determined by western blotting using cell homogenates isolated from Caco-2 cells and native mouse jejunum, respectively. These results revealed significantly decreased expression levels of hSVCT-1, hSVCT-2, mSVCT-1 and mSVCT-2 proteins ($P < 0.01$ for hSVCT-1 and $P < 0.05$ for hSVCT-2, mSVCT-1 and mSVCT-2) in LPS-treated Caco-2 cells as well as LPS-treated mouse jejunum compared to unexposed controls (Fig. 2A, B, C & D). As a third experimental model, ex vivo mouse

intestinal organoids were generated as described before [49, 50] (Fig. 2E); their validation was done as described [49, 50]. Again, results showed that both mSVCT-1 and mSVCT-2 protein expression levels were significantly ($P < 0.05$ and $P < 0.01$, respectively) decreased in LPS exposed enteroids (50μg/ml for 48 h) compared with controls (Fig. 2 F & G). We also examined the effect of LPS on pro- inflammatory cytokines IL-1β and TNFα in these mouse intestinal enteroids to confirm that our findings were due to LPS-mediated activation of the inflammatory pathways. Levels of both IL-1β and TNFα mRNA expression were significantly ($P < 0.01$ for IL-1 β and $P < 0.05$ for TNFα) increased in LPS exposed enteroids compared to their respective controls (0.99 ± 0.01 and 1.94 ± 0.18 for IL-1 β; and 0.9 ± 0.01 and 1.4 ± 0.2 for TNFα, in controls and LPS treated enteroids, respectively, arbitrary units).

To further investigate the mechanism responsible for the observed LPS-dependent inhibition of AA accumulation, we examined the effect of LPS on mRNA levels of hSVCT-1, hSVCT-2, mSVCT-1 and mSVCT-2 in Caco-2 cells and mouse jejunum. The results showed a significant decrease in the levels of expression of hSVCT-1, hSVCT-2, mSVCT-1 and mSVCT-2 mRNA in both Caco-2 cells and mouse jejunum after LPS exposure compared to their respective controls (Fig. 3A, B, C & D). Similarly, mSVCT-1 and mSVCT-2 mRNA expression levels were also significantly decreased in LPS exposed organoids compared to controls (Fig. 3E & F). These results suggest LPS-triggered transcriptional regulation of the *SLC23A1* and *SLC23A2* genes. Levels of SVCT-1 and SVCT-2 hnRNA, reflecting the rate of transcription of each gene were assessed using real-time PCR. Results showed significantly lower levels of expression of the hSVCT-1, hSVCT-2, mSVCT-1 and mSVCT-2 hnRNA ($P < 0.01$ for hSVCT-1, mSVCT-1 and mSVCT-2; $P < 0.05$ for hSVCT-2) in LPS treated groups compared to their controls (Fig. 4A, B, C & D).

Next, the effect of LPS on the activity of the full-length *SLC23A1* and *SLC23A2* promoters was examined. Reporter gene activity driven by either promoter was significantly ($P < 0.01$) reduced in LPS treated Caco-2 cells compared to controls (Fig. 5A & B). Previous studies have shown that the transcription factors HNF1α and Sp1 are essential for the basal activity of the *SLC23A1* and *SLC23A2* promoters, respectively [57, 61, 62]. It is also known that these transcription factors are suppressed in the setting of intestinal inflammation [63]. Therefore, we examined the effect of LPS exposure on HNF1α and Sp1 expression. Western blot analysis showed a significant reduction in HNF1α and Sp1 protein levels in Caco-2 cells (Fig. 6A & B) and mouse jejunum (Fig. 6C & D). Real-time PCR data showed a significant inhibition of HNF1α and Sp1 mRNA expression in Caco-2 cells (98.50 ± 8.6 and 43.3 ± 6.5 for HNF1α and 99.6 ± 0.81 and 53.80 ± 16.3 for Sp1; control and LPS treated, % relative to control, $P < 0.01$ for both) and mouse jejunum (100.33 ± 24.39 and 23.0 ± 9.8 for HNF1α and 100.2 ± 14.1 and 42.2 ± 10.5 for Sp1; control and LPS treated, % relative to control, $P < 0.05$ for both). In addition to the overall changes noted in transcription factor expression, interaction of HNF1α and Sp1 with the *SLC23A1* (−150 to +5) and *SLC23A2* (-97 to +42) promoter regions was determined using a ChIP assay. Indeed, the interaction of HNF1α and Sp1 to the *SLC23A1* and *SLC23A2* promoter, respectively, was significantly ($P < 0.01$ for both) reduced in LPS-treated Caco-2 cells compared to control cells (Fig. 7A & B). Collectively, these findings in both Caco-2 cells and mouse jejunum suggest that the

decreased AA uptake in LPS treatment can be attributed to the lower level of transcription of both *SLC23A1* and *SLC23A2* genes.

4. Discussion

Systemic inflammation, as indicated by the presence of circulating LPS levels in human plasma, is linked to a considerable increase in risk of morbidity and mortality [35, 40]. Levels of LPS can be significantly increased in the blood of IBD and necrotizing enterocolitis (NEC) patients as well as those infected with *Salmonella* [27, 29, 35–38]. LPS may also be elevated in the plasma of septic patients with systemic inflammatory response syndrome [39–41]. Interestingly there is also evidence that patients with sepsis are more likely to have decreased levels of AA in their blood, which can lead to overt or indicate subclinical vitamin C deficiency [42–44]. Accordingly, there are also studies which have shown that administering vitamin C significantly decreased the severity of the LPS induced inflammation and sepsis [39]. While LPS has been shown to affect intestinal transport of many substrates [45–48], little is known about its effect on intestinal AA uptake and the molecular mechanism(s) involved in LPS-mediated alteration of vitamin C transport. Thus, we sought to determine the impact of LPS on the vitamin C transport system using Caco-2 cells (in vitro), mice (in vivo) and intestinal enteroids (ex vivo) as models as well as appropriate molecular approaches.

Our in vitro and in vivo experiments showed a significantly lower carrier-mediated intestinal AA uptake with exposure to LPS when compared to controls. This inhibition was associated with markedly decreased levels of SVCT-1 and SVCT-2 protein, mRNA and hnRNA expression. Similarly, the SVCT-1 and SVCT-2 protein and mRNA expression levels were also markedly decreased in LPS exposed intestinal enteroids. In addition, the *SLC23A1* and *SLC23A2* promoter activities were significantly decreased in LPS treated Caco-2 cells. Together these findings suggest that the lower level of AA uptake in LPS treated Caco-2 cells and mice intestine is, at least partially, mediated at the level of transcription of both *SLC23A1* and *SLC23A2* genes.

In accordance with our finding, there are previous studies which have also shown that LPS causes a down regulation in the expression of a variety of transporters in various tissues [45–47, 63, 64]. The effect of LPS was mediated at the transcriptional level and mediated via an effect on the level of expression of transcription factor(s) needed to drive the expression of the specific gene [63–67]. Recent studies have shown that the nuclear factor HNF1α and Sp1 play an important role in driving the basal activity of the *SLC23A1* and *SLC23A2* promoters, respectively [57, 61, 62] and these nuclear factors also play a role in transporter regulation during inflammation [63]. In this study, we found markedly decreased levels of expression of both HNF1α and Sp1 nuclear factors protein and mRNA in LPS exposed Caco-2 cells and mice intestine compared to their respective controls. This was reflected on level of the observed reduction in the interaction of these transcription factors (HNF1α and Sp1) with their respective promoters in LPS exposed Caco-2 cells. This is consistent with prior studies suggesting that LPS degrades Sp1 and additionally that the observed reduced promoter interaction may also be due to Sp1 dephosphorylation [68]. Collectively, these findings show that LPS inhibits intestinal carrier-mediated AA uptake by down regulating

the expression of *SLC23A1* and *SLC23A2* transcription via mechanism(s) that inhibit the level of expression and function of both HNF1α and Sp1 transcription factors.

These important findings are especially intriguing given the recent data from Marik et. al. which showed that in patients with sepsis, treatment with intravenous (IV) AA as part of a "vitamin C protocol", resulted in a significant improvement of clinical outcomes [69]. While there are studies confirming sepsis-associated vitamin C deficiency which would explain why administration of IV AA was associated with improved outcomes, the underlying causes of this deficiency have not been described. Therefore, our findings provide the potential mechanisms by which sepsis may induce vitamin C deficiency by reducing its absorption. Furthermore, the molecular pathways identified which mediate the down regulation of vitamin C transport system can be used as potential targets for pharmacologic therapy in the future. In this way, rather than infusing patients with large quantities of vitamin C, a treatment which can be associated with an increased risk of oxalate production and systemic oxalosis [70], the pathogenic pathways which lead to inhibition of vitamin C transport can be corrected thereby addressing the underlying cause of the problem rather than its symptoms. In addition, the clinical observations that patients with LPS-induced septicemia have lower circulating levels of AA can be at least partly explained by inhibition of AA transport and down regulation of the vitamin C transport system. Future studies will need to focus on the potential clinical relevance of the findings described in this study. Furthermore, additional preclinical studies are needed to further elucidate the mechanisms responsible for LPS-induced vitamin C transport deficiency and the potential methods that can be utilized to correct them.

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Highlights

- LPS inhibits intestinal ascorbic acid uptake.
- LPS down regulates SVCT-1 and SVCT-2 expression.
- LPS reduced expression of nuclear factors HNF1α and SP1.
- These factors affect *SLC23A1* and *SLC23A2* promoter activity on LPS treatment.

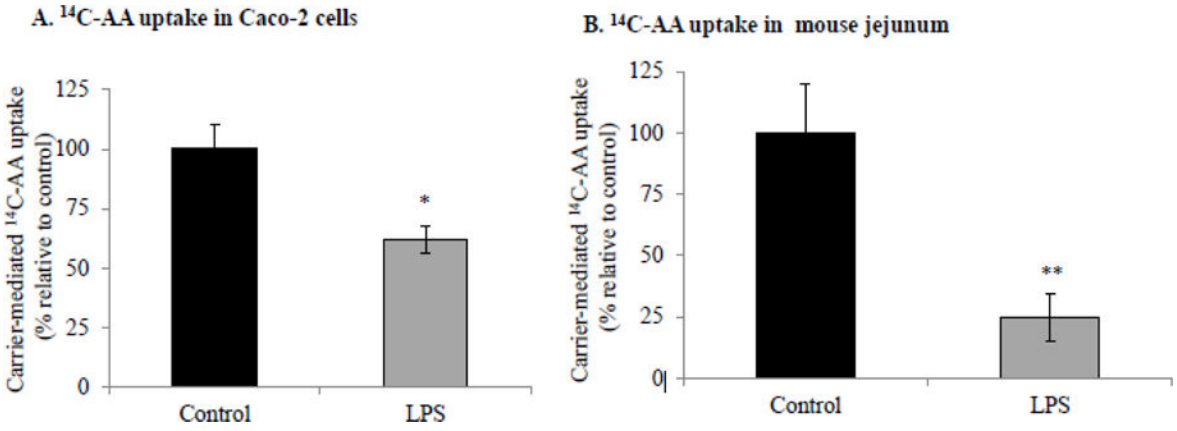

Figure 1. Effect of LPS on AA uptake by Caco-2 cells and native mouse jejunum tissue

A. Confluent Caco-2 cells were serum-starved overnight followed by treatment with LPS (50 μg/ml) for 48 h. AA uptake was measured as described in "Methods". Data are means ± SE of at least three independent experiments with different passages of cells (*$P < 0.01$). **B.** Male C57BL/6 mice (12 weeks old) were injected with LPS (0.005g/kg body weight, IP) and AA uptake was performed using mouse jejunal tissue as described in "Methods". Data are means ± SE of at least three sets of mice (**$P < 0.05$).

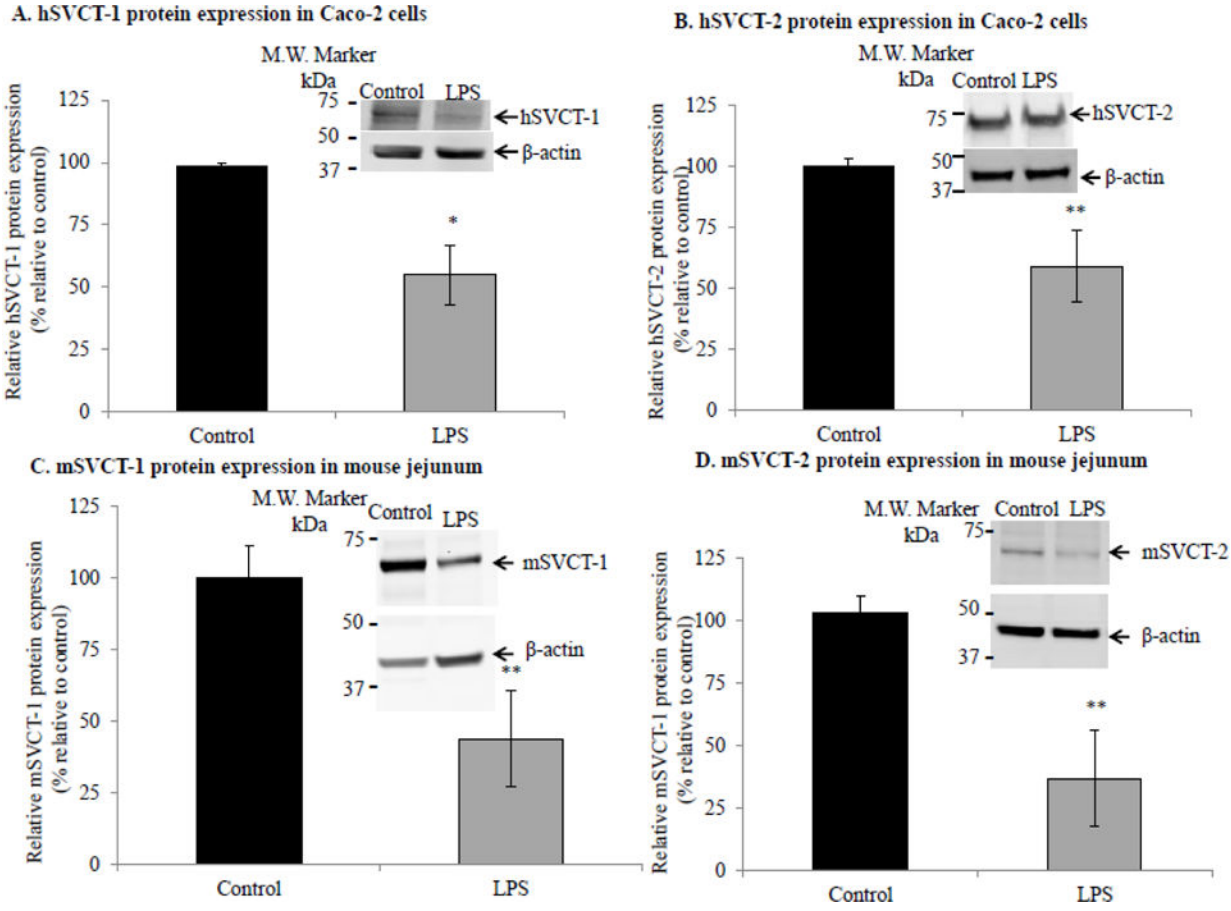

Figure 2. Effect of LPS on the level of expression of SVCT-1 and SVCT-2 protein in intestinal epithelial Caco-2 cells, native mouse jejunum tissue and enteroids

A&B. Caco-2 cells were pre-treated with LPS and after 48 h, the total protein was isolated and subjected to western blotting to determine hSVCT-1 and hSVCT-2 protein expression levels, respectively as described in “Methods”. Data are means ± SE of at least three independent experiments (*P < 0.01; **P < 0.05). **C&D.** Total protein was isolated from mouse jejunum tissue of LPS-treated mice and subjected to western blotting to determine mSVCT-1 and mSVCT-2 protein expression levels, respectively as described in “Methods”. Data are means ± SE of at least three sets of mice (**P < 0.05). **E.** Images of (1–7 days old) mouse intestinal enteroids. **F&G.** Enteroids were pretreated with LPS (50 μg/ml) for 48 h and the level of expression of mSVCT-1 and mSVCT-2 protein were determined as described in “Methods”. Data are means ± SE of at least three separate experiments (*P < 0.01; **P < 0.05).

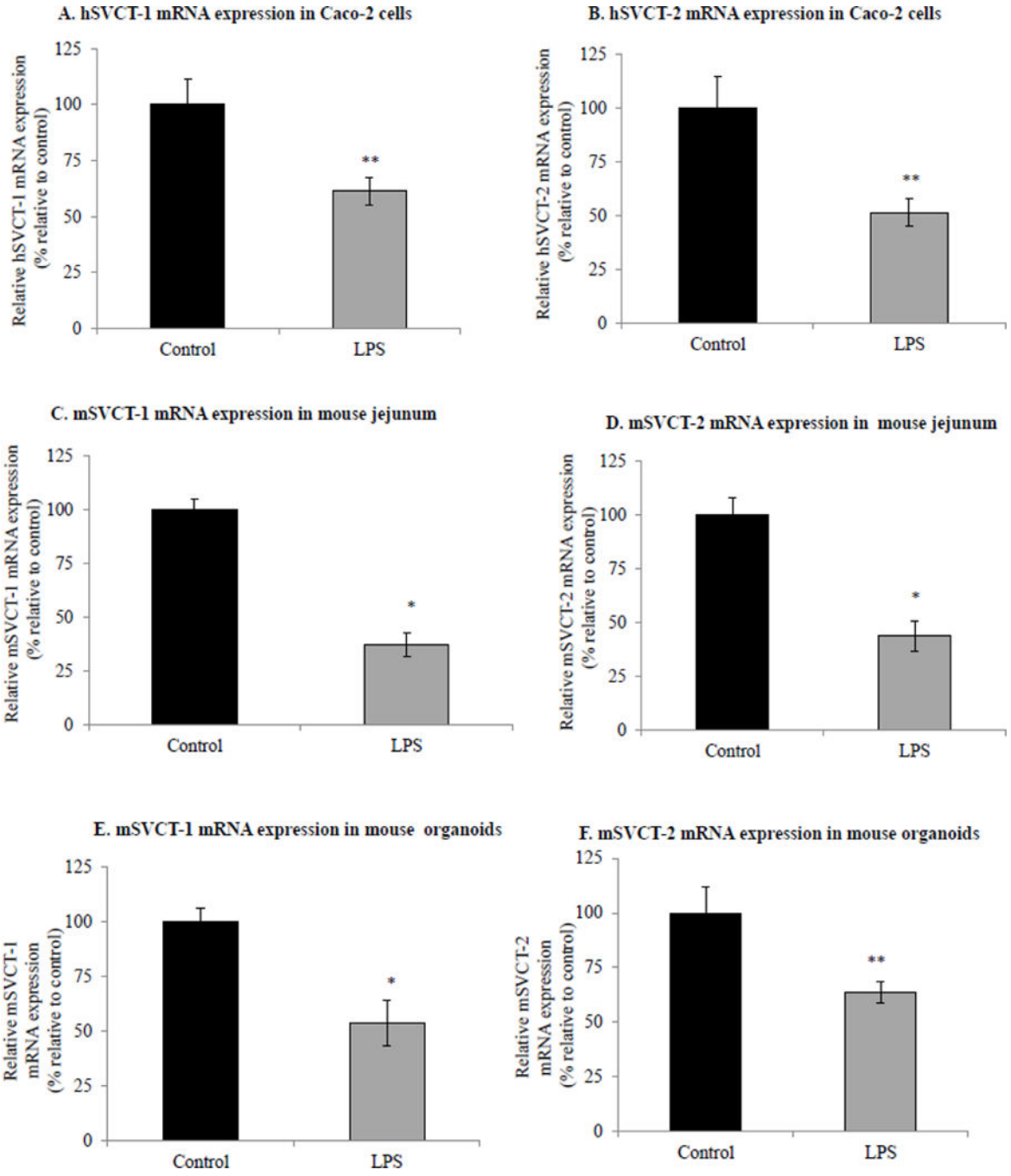

Figure 3. Effect of LPS on the level of expression of SVCT-1 and SVCT-2 mRNA in Caco-2 cells, mouse jejunum tissue and enteroids

A&B. hSVCT-1 and hSVCT-2 mRNA expression levels were determined using total RNA isolated from LPS treated Caco-2 cells and real-time PCR as described in “Methods”. Data are the means ± SE of at least three independent experiments (** $P < 0.05$). **C&D.** mSVCT-1 and mSVCT-2 mRNA expression levels were determined using total RNA isolated from LPS injected mouse jejunum as described in “Methods”. Data are means ± SE of at least three sets of mice (*$P < 0.01$). **E&F.** Real-time PCR for mSVCT-1 and mSVCT-2

in LPS treated and untreated enteroids were performed using gene specific primers as described in “Methods”. Data represents the means ± SE of at least three separate experiments. (*P < 0.01; **P < 0.05).

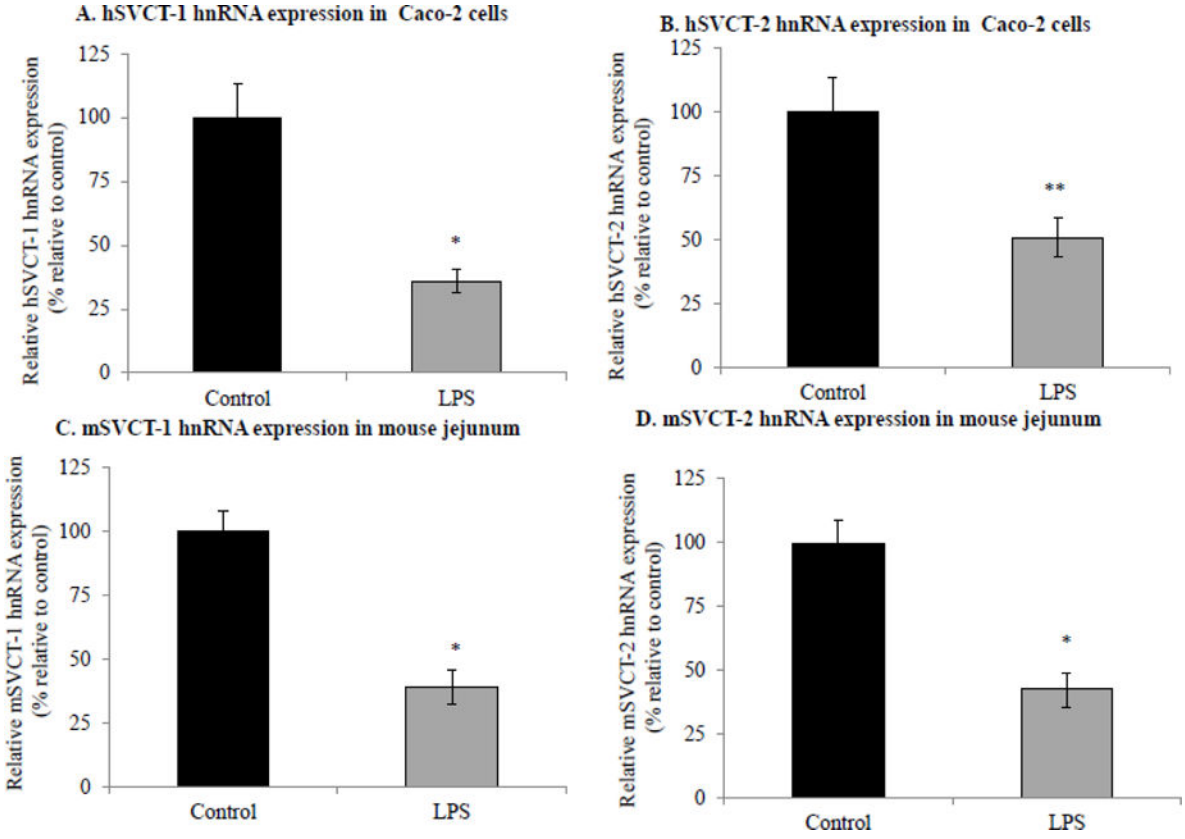

Figure 4. Effect of LPS on heterogenous nuclear RNA (hnRNA) expression of SVCT-1 and SVCT-2

A&B. Real-time-PCR for hSVCT-1 (*SLC23A1*) and hSVCT-2 (*SLC23A2*) in the LPS treated and untreated Caco-2 cells were performed using gene-specific primers as described in "Methods". Data represent the means ± SE of three separate experiments (*P < 0.01; **P < 0.5). **C&D.** Real-time-PCR for mSVCT-1 (*Slc23a1*) and mSVCT-2 (*Slc23a2*) in the LPS injected and control mouse jejunum tissues were performed using gene-specific primers as described in "Methods". Data represents the means ± SE of three separate experiments involving three sets of mice (*P < 0.01).

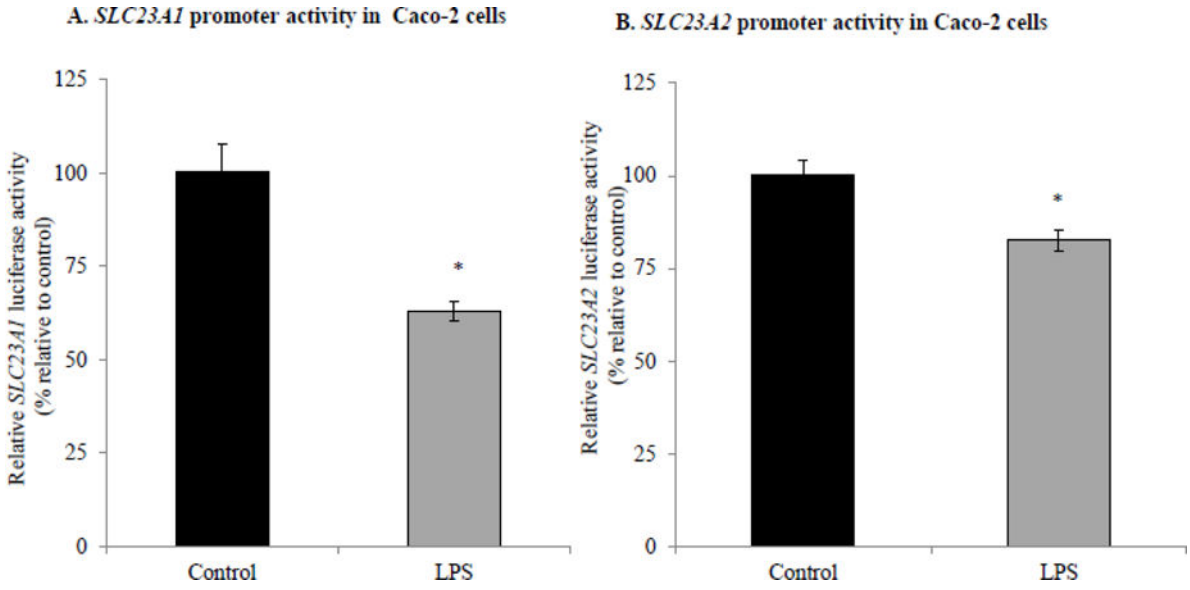

Figure 5. Effect of exposure of Caco-2 cells to LPS on activity of *SLC23A1* and *SLC23A2* promoters

SLC23A1 (**A**) and *SLC23A2* (**B**) promoter activities were determined in LPS treated and untreated Caco-2 cells. The relative *firefly* luciferase activity was normalized by *Renilla* luciferase activity, and the values are expressed as fold over basic. Data are means ± SE of at least four separate experiments (*P < 0.01).

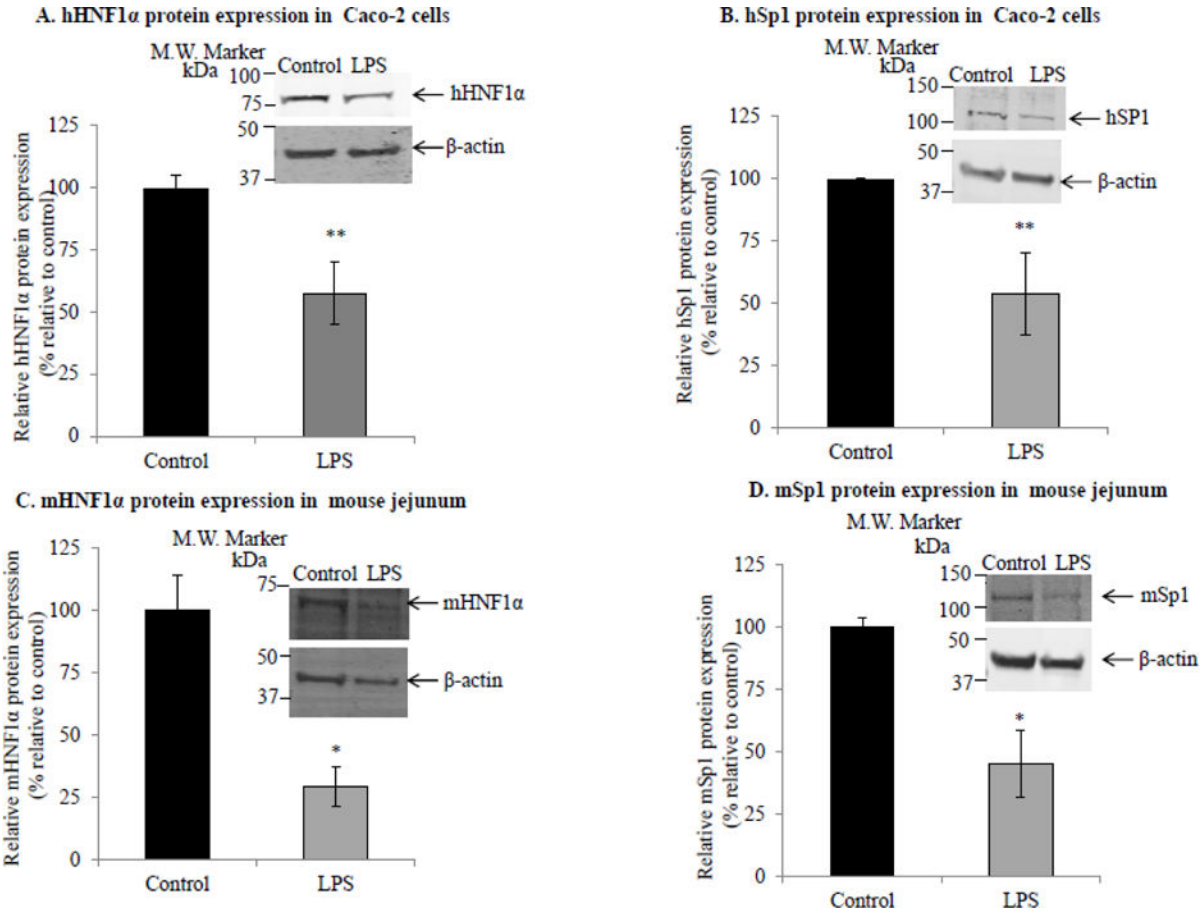

Figure 6. Effect of LPS on the level of expression of HNF1α and Sp1 protein in Caco-2 cells and native mouse jejunum tissue

A&B. Caco-2 cells were pre-treated with LPS and western blotting was performed using hHNF1α and hSp1 specific antibodies as described in "Methods". Data are means ± SE of at least three independent experiments (**P < 0.05). **C&D.** Mouse jejunum tissue total protein was isolated from LPS treated and their control animals, and western blotting was performed using mHNF1α and mSp1specific antibodies as described in "Methods". Data are means ± SE of at least three independent experiments (*P < 0.01).

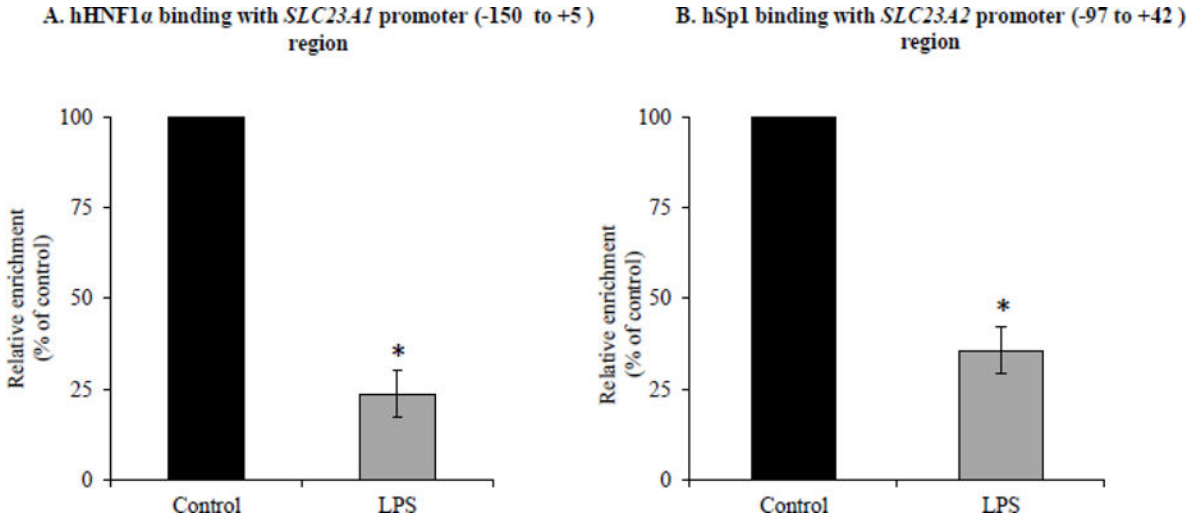

Figure 7. Effect of LPS on the nuclear factors HNF1α and Sp1 binding with *SLC23A1* (−150 to +5) and *SLC23A2* (−97 to +42) promoter regions in Caco-2 cells

The HNF1α **(A)** and Sp1 **(B)** binding with *SLC23A1* (−150 to +5) and *SLC23A2* (−97 to +42) promoter region, respectively were examined in the formaldehyde cross-linked chromatin from LPS treated and control Caco-2 cells followed by immune-precipitation with anti-HNF1α and Sp1 antibodies. The DNA was purified from the immune-precipitated complex and qPCR was performed as described in “Methods”. Data (means ± SE) were normalized relative to input DNA and presented as percentage of relative enrichment (*P < 0.01).

Table 1

Combination of primers used to amplify coding region of the respective genes by RT-qPCR

Gene name	Forward and Reverse Primers (5′-3′)
Real-time PCR	
hSVCT-1	TCATCCTCTTCTCCCAGTACCT; AGAGCAGCCACACGGTCAT
hSVCT-2	TCTTTGTGCTTGGATTTTCGAT; ACGTTCAACACTTGATCGATTC
hHNF1	TGGTGAGAGTATGGAAGACC; AAGGAGGTGCTGGTTCAG
hSp1	CCATACCCCTTAACCCCG; GAATTTTCACTAATGTTTCCCACC
hTBP	TATAATCCCAAGCGGTTTGC; GCTGGAAAACCCAACTTCTG
hβ-actin	CATCCTGCGTCTGGACCT; TAATGTCACGCACGATTTCC
mSVCT-1	CAGCAGGGACTTCCACCA; CCACACAGGTGAAGATGGTA
mSVCT-2	AACGGCAGAGCTGTTGGA; GAAAATCGTCAGCATGGCAA
mHNF1	GCCCCTTCATGGCAACCA; CTCTCCCAGGCCAACGT
mSp1	TATGTTGTGGCTGCTACC; TGTGGGATTACTTGATACTGAA
mTBP	TGACTCCTGGAATTCCCATC; TGTGTGGGTTGCTGAGATGT
mTNFα	CATCTTCTCAAAATTCGAGTGACAA; TGGGAGTAGACAAGGTACAACCC
mIL-1β	CTCTCCAGCCAAGCTTCCTTGTGC; GCTCTCATCAGGACAGCCCAGGT
mβ-actin	ATCCTCTTCCTCCCTGGA; TTCATGGATGCCACAGGA
hnRNA primers	
hSVCT-1	TGGAGACGGAGTTTTGCT; GAGGCTAAGGTGGGAG
hSVCT-2	CCTCCTCCTCAGATCCTTCC; AAGATCCAGGGAGAGGGAAA
hβ-actin	TTCCTGGGTGAGTGGAG; GGACTCCATGCCTGAGAG
mSVCT-1	GCTTCCAGGCTCTAGATGGT; GGGCAAAATCTTCGTTGGGT
mSVCT-2	ACTCTTGTCCATGGCTCTGG; GGGCAAAATCTTCGTTGGGT
mβ-actin	AGATGACCCAGGTCAGTATC; GAGCAGAAACTGCAAAGAT
ChIP assay primers	
hSVCT-1	GCTTGGGTGGCTTTTACTGTCC; ATGACTTGACAAAGGCCAAGGA
hSVCT-2	GGGCGGGGAGGGAGGTG; TGCCCGCTGCAGCCTCCG