Octreotide: A Somatostatin Analogue, Protects Liver against CCl₄-Induced Liver Injury in Mouse Model

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ABSTRACT

Carbon tetrachloride (CCl₄) is a well known model compound for inducing hepatic injury in experimental animals. The aim of this study was to evaluate the hepatoprotective potential of a somatostatin analogue (octreotide 30 µg kg⁻¹) in mice following oral administration of carbon tetrachloride (CCl₄, 0.5 mL kg⁻¹). Hepatic total thiol was determined colorimetrically by Elman’s reagent. Expression levels of somatostatin receptor-2 and vascular endothelial growth factor were detected by immunoblotting using specific antibodies against SSTR-2 and VEGF. Administration of CCl₄ caused a significant increase in the release of serum Alamine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and total bilirubin. Significantly enhanced hepatic lipid peroxidation and marked depletion of reduced glutathione were observed after CCl₄ intoxication. It was also found that CCl₄ Administration caused over expression of TNF-α mRNA and Vascular Endothelial Growth Factor (VEGF) protein. Octreotide treatment showed hepatic protection by significantly reducing elevated levels of AST and ALT enzymatic activities, lipid peroxidation and total bilirubin which had been raised by acute intoxication of CCl₄ in addition to its ability to increase total thiol. Notably, octreotide significantly reduced expression of the inflammatory marker tumor necrosis factor alpha (TNF-α) mRNA and VEGF protein. Moreover, octreotide treatment was accompanied by marked increase in the expression of somatostatin receptor type-2 (SSTR2). The results of this study indicate that octreotide has potential protective effects against liver injury.

Key words: Lipid peroxidation, TNF-α, VEGF, total bilirubin, alanine transaminase

INTRODUCTION

Somatostatin (SST) is a neuropeptide that exists in two active forms, a 14 amino acid peptide (somatostatin-14) and an amino-terminally extended form (somatostatin-28). In the body, somatostatins are produced mainly by endocrine, gastrointestinal, immune and neuronal cells as well by certain tumors (Dalm et al., 2004). SST regulates a number of immune functions, among others lymphocyte proliferation, immunoglobulin production and the release of pro-inflammatory cytokines (Ten Bokum et al., 2000). Being an unstable peptide with a very short elimination half-life of 2-4 min that is followed by rebound secretion forms the main obstacle for using native somatostatin for therapeutic purposes (Pawlikowski and Melen-Mucha, 2004). On the other hand, Octreotide which is an analogue of somatostatin has a longer elimination half-life of 90-120 min after the administration of a single intravenous dose, with no rebound secretions and fewer side effects (Marbach et al., 1993). Because of the much longer duration of action both in vivo and
in vitro, octreotide represents a more suitable alternative for clinical application or experimental studies than the native hormone. Somatostatin and the octreotide are widely used in the treatment of metastatic neuroendocrine tumors (Leong and Pasiela, 2002), acute pancreatitis (Lowe et al., 2000) and gastrointestinal and pancreatic fistulas (Foster and Lefor, 1996). Octreotide has been used in the treatment of secreting pituitary adenomas, neuroendocrine tumors, variceal bleeding and refractory diarrhea syndromes (Kouroumalis et al., 1998; Lamberts et al., 1996).

The impact of somatostatin and its analogues on many diseases has been thoroughly investigated in many experimental studies. However, their protective effect from liver injury has not been elucidated so far. Therefore, we aimed to investigate the protective effect(s) of octreotide against carbon tetrachloride-induced liver injury in mice model.

MATERIALS AND METHODS

The present study was conducted on March 2010 until April 2011.

Chemicals: Octreotide (Sandostatine® 0.1 mL mL⁻¹) was purchased from Novartis international AG (Basal, Switzerland). RNA extraction kit was purchased from BioFlux, Bioer Technology. RevertAid™ First strand cDNA synthesis kit for RT PCR amplification as well as page Ruler™ prestained protein ladder were purchased from Fermentas. Tag master-high yield for PCR amplification and low range DNA ladder 50-1000 bp linear scale were purchase from Jena Bioscience. Polyvinylidene Fluoride PVDF Amersham Hybond™-P membrane from GE Healthcare was used for protein transfer. Nitroblue Tetrazolium (NBT) and 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP) from Fermentas. All other chemicals and solvents used in this study were of analytical grade and were obtained either from Sigma Chemicals Company or commercial suppliers, unless otherwise mentioned.

Treatment and sampling: In this study we used 35 Male Albino mice (30±5 g b.wt.) were purchased from animal house of Assiut University. Mice were divided into three groups; first group (control group, n = 5) received normal saline in olive oil orally by intragastric gavage. Second group (CCL₄-treated group, n = 15) received a single oral dose of 0.5 mL kg⁻¹ CCL₄ dissolved in olive oil using an intragastric gavage. The third group (Octreotide-treated group, n = 15) received first a single oral dose of 0.5 mL kg⁻¹ CCL₄ in the same way like the second group then followed by subcutaneous injection of 30 µg kg⁻¹ octreotide divided into two doses separated with 12 h-interval. Mice were sacrificed at 12, 24 and 48 h later. Blood samples were collected and livers were excised rapidly and used for RNA preparation or homogenized in homogenization buffer (20 mM Tris, 100 mM NaCl, 1 mM EDTA, 1 µg mL⁻¹ leupeptin, 1 µg Apotinin, 1 µg bezafibrate and 0.5% Triton X100). Protein content of liver homogenate was determined using Buret reagent and BSA as a standard. After adding the protease inhibitor mix, liver homogenate was divided into aliquots and stored at -80°C till use.

Assessment of liver function tests: Blood samples were drawn by puncturing retro-ombital venous sinus, centrifuged and serum was isolated for serological studies. Serum ALT, AST and total bilirubin were estimated using commercially available kits according to the manufacturer’s instructions (Biodiagnostic, Egypt).
Oligonucleotides used for amplifications: NCBI reference sequences: NM_013398.3 and NM_007398.3 were used to design primers for TNF-α and β-actin, respectively. The coding sequences were used to design the primer pairs and the distance between the two primers was 500 bases.

The primer sets as following:

- TNF-α forward: 5'-GTCTCAGGCCCTTCTCTCATCC-3'
- TNF-α reverse: 5'-CAGGTATATGGGCTCATAACC-3'
- Beta actin forward: 5'-GGATGAGGAATCGCTGGCG-3'
- Beta actin reverse: 5'-CTCGTAGATGGGCGACAGTG-3'

RNA preparation and RT-PCR: Total RNA fractions were prepared using total RNA extraction kit from (BioFlux, Bioer Technology Co., Ltd.) according to the instruction manual. The first strand cDNA was synthesized according to the instruction manual of RevertAid™ First strand cDNA synthesis kit (Fermentas) from mouse liver total RNA. The PCR was performed using Tag master/high yield (Jena Bioscience) according to the following conditions: pre-denaturing for 5 min at 94°C, denaturing at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for one min. The amplification was carried out in 28 cycles using a Biometra cycler.

Determination of liver total thiol and lipid peroxidation: Colorimetric determination of liver total thiol using Ellman’s reagent, Thiobarbituric Acid Reactive Substance (TBARS) [equivalent to Malondialdehyde (MDA)] were done according to the methods described by Elliott et al. (1999).

SDS-PAGE and western blotting: Fifty microgram from each sample homogenate were denatured by boiling for 5 min in 2% SDS and 5% 2-mercaptoethanol and loaded into separate lanes of a 12% SDS-PAGE gel. The samples were separated by electrophoresis at 100 volts for 2 h. The separated proteins were electrically transferred onto PVDF membrane using T-77 ECL semi-dry transfer unit from Amersham Biosciences for 2 h. The membrane was blocked in TBS buffer containing 0.05% Tween and 5% non-fat milk for one hour. The membranes were then incubated either with rabbit polyclonal anti SSTR-2 (H-50) or anti VEGF (SANTA CRUZ Biotechnology, INC). Polyclonal goat anti-rabbit or anti-mouse immunoglobulin conjugated to alkaline phosphatase (Sigma-Aldrich, Schelldorf, Germany) diluted 1:5000 in the 10x diluted blocking buffer served as secondary antibody. Detection of proteins bands was done by adding alkaline phosphatase buffer (100 mM tris pH 9.5; 100 mM NaCl; 5 mM MgCl2) containing substrate, 6.6 µL NBT/mL and 3.3 µL BCIP/mL from (stock of 50 mg mL⁻¹ nitroblue tetrazolium NTB and 50 mg mL⁻¹ 5-bromo-4-chloro-3-indolyl phosphate BCIP in 70% formmamide). Color reactions were stopped by rinsing with Stop buffer (10 mM Tris-Cl, pH 6.0, 5 mM EDTA).

Statistical analysis: All data are presented as the Mean±SD. A Student’s unpaired t-test was used for comparison between the two groups. The results were considered statistically significant if the probability was less than at least 5% (p<0.05). Statistical comparisons were performed between CCl4 group and octreotide treated group. Using GraphPad Free Statistic Software.

RESULTS
Assessment of CCl4-induced liver cell injury: We first investigated the released markers of liver cell integrity ALT and AST in the blood stream in the CCl4-treated mice. Oral administration of 0.5 mL CCl4 kg⁻¹ using an intragastric gavage resulted in a highly significant increase in the
Fig. 1(a-b): Effect of octreotide on Serum levels of transaminases: (a) Levels of ALT, (b) levels AST. Transaminases were measured in the serum of control untreated mice received only saline in olive oil, control mice receiving 0.5 mL kg⁻¹ CCl₄ and treated mice receiving 0.5 mL kg⁻¹ CCl₄ plus 30 µg kg⁻¹ octreotide, mice were sacrificed after 12, 24, 48 h. Octreotide treated group showed significant reduction in enzymatic activities in comparison to control group receiving only CCl₄. Data are presented as Mean±SD in each group. (n = 5 mice in each group. ALT, p<0.004, 0.01 and 0.0003, AST p<0.2, 0.006 and 0.0007 after 12, 24 and 48 h, respectively vs CCl₄ group). (ns) non significant, *significant and ***extremely significant.

mean values of ALT (Fig. 1a) and AST (Fig. 1b) levels at all time points in comparison to the control group (received saline in olive oil). In spite, subcutaneous injection of 30 µg kg⁻¹ octreotide did not restore the serum level of ALT and AST to the normal level, however it result in decrease levels of ALT and AST significantly in comparison to the CCl₄ group ALT, p<0.004, 0.01 and 0.0003, AST p<0.2, 0.006 and 0.0007 after 12, 24 and 48 h, respectively versus CCl₄ group).

Malondialdehyde content: Figure 2 shows the MDA equivalent in serum of the different experimental groups. We observed a significant increase in the level of MDA in the CCl₄-treated group compared to the control group (received saline in olive oil), the same results was obtained by Khadr et al. (2007). On the other hand, a significant decrease in the MDA in the octreotide treated group was noted compared to the CCl₄-group p<0.002, 0.0004 and 0.0001 after 12, 24 and 48 h, respectively versus CCl₄ group).

Serum levels of bilirubin: In accordance with previous results, we were able to show that the serum level of Bilirubin in CCl₄ group was found significantly higher than its level in the control group (Hassan et al., 2008). As shown in Fig. 3, we observed a significant decrease in the level of bilirubin in the octreotide treated group compared to the control CCl₄ group. p<0.04, 0.06 and 0.0007 after 12, 24 and 48 h, respectively vs CCl₄ group).

Hepatic total thiols content: Total thiols content of 100 mg of liver homogenate was measured in the different experimental groups. As shown in Fig. 4; a dramatic initial decrease in the level of GSH after CCl₄ treatment was noticed, this observation is similar to data obtained by Gupta et al.
Fig. 2: Effect of octreotide on measurement of lipid per-oxidation. MDA levels were measured in tissue homogenate of liver samples. Groups and treatment details are the same as described in Fig. 1. Octreotide treated group showed extremely significant reduction in lipid peroxidation in comparison to control group receiving only CCl₄. Data are presented as Mean±SD in each group. (n = 5 mice in each group, p<0.002, 0.0004 and 0.0001 after 12, 24 and 48 h, respectively vs CCl₄ group)

Fig. 3: Effect of octreotide on serum levels of bilirubin: levels of bilirubin were measured in the serum. Groups and treatment details are the same as described in Fig. 1. Octreotide treated group showed significant reduction in bilirubin in comparison to control group receiving only CCl₄. Data are presented as Mean±SD in each group. (n = 5 mice in each group, p<0.04, 0.06 and 0.0007 after 12, 24 and 48 h, respectively vs CCl₄ group)
Fig. 4: Effect of octreotide on liver total thiol concentration. Total thiol were measured in tissue of liver homogenate. Groups and treatment details are the same as described in Fig. 1. Octreotide was clearly able to increase levels of total thiol as control untreated mice received distilled water. Data are presented as Mean±SD in each group. (n = 5 mice in each group, p<0.0009, 0.0004 and 0.0005 after 12, 24 and 48 h, respectively vs CCl₄ group).

![Graph showing thiol concentration over time with octreotide treatment]

Fig. 5: Semi-quantitative RT-PCR of inflammatory marker TNF-α. TNF-α mRNA expression levels in mouse livers of control, CCl₄ and Octreotide group was estimated by RT-PCR. Groups and treatment details are the same as described in Fig. 1. PCR products were visualized by ethidium bromide staining in an agarose gel. TNF-α expression was clearly decreased in octreotide treated group vs CCl₄. Actin (housekeeping gene) was used as a positive control for gene expression and to show the integrity of RNA.

In present study, levels of total thiol in the octreotide treated group were gradually increased by time in compared to the CCl₄ group p<0.0009, 0.0004 and 0.0005 after 12, 24 and 48 h, respectively verses CCl₄ group).

**TNF-alpha expression:** We assessed the expression of the TNF-α mRNA using RT-PCR in the different experimental groups. There was a gradual increase in the expression level of TNF-α.
mRNA in the CCl₄-treated group at all time points in comparison to the control. The highest increase was found at 48 h as shown in Fig. 5. Interestingly, octreotide treatment resulted in a significant decrease in the expression level of the TNF-α mRNA when compared to the CCl₄-treated group.

Expression of somatostatin receptor type-II: Using Western blot technique, we determined the level of SSTR2 protein expression that had been synthesized by liver cells in response to CCl₄ treatment alone or with a later octreotide treatment at three different time points (12, 24 and 48 h after treatment) in comparison to the control group. The results exhibited over expression of SSTR2 protein synthesized by liver cells at all time points after CCl₄ treatment while the expression was much higher during the octreotide treatment as shown in Fig. 6a.

Expression of VEGF: The expression level of VEGF protein gradually increased in response to CCl₄ treatment of mice and it was much more expressed at 48 h of the treatment. On the other hand, VEGF expression level was significantly reduced after octreotide treatment in comparison to the CCl₄ group as shown in Fig. 6b.

DISCUSSION

Liver is the major organ that is responsible for metabolism of drugs and toxic chemicals. The exposure to these toxic chemicals like acetaminophen, chloroform, dimethyl nitrosamine and CCl₄ is known to cause hepatic injury. Liver dysfunction is characterized by degeneration, necrosis or apoptosis of hepatocytes. CCl₄-induced hepatic injury is a commonly used experimental model for
evaluating the hepatoprotective or anti-hepatotoxic activity of the drugs (Jamshidzadeh et al., 2005). The disturbance in the transport function of the hepatocytes as a result of hepatic injury causes the leakage of enzymes from cells due to altered permeability of cell membranes (Zhai et al., 2010) which results in raised levels of ALT and AST in the serum. Subcutaneous administration of octreotide remarkably attenuated the increasing tendency of the levels of studied enzymes and subsequently recovered the physiological status towards normalization. The ALT and AST are the most sensitive markers of hepatocellular injury (Patrick-Iwuanyanwu et al., 2007) and their elevation in serum is indicative of cellular leakage and loss of the functional integrity of cell membranes in liver (Rajesh and Latha, 2004). In our study we found a significant increase in levels of ALT and AST in the CCl_4 group, this is in agreement with previous studies carried out by Chavda et al. (2010) and Moundipa et al. (2007). Decline in ALT and AST levels after octreotide administration indicated improvement in cellular integrity and status of hepatic cells.

Lipid peroxidation is a process that is caused by the free radicals which circulate through the body and attack macromolecules like DNA, lipids in membranes and cellular proteins of the body. The antioxidant system present in the body counteracts the damages caused by free radicals (Elliott et al., 1999). As previously reported by many researchers, free radical activity is involved in the pathogenesis of many diseases including heart and cardiovascular system (Kehrer, 1993). Octreotide treatment for CCl_4 treated mice markedly reduced the lipid peroxidation about 50% of their corresponding values in mice which were given CCl_4 only. Moreover, octreotide was able to keep the level of GSH in the octreotide treated group higher than its level in CCl_4 group.

Bilirubin is an important degradation product of hemoglobin and is normally excreted into the bile. In addition, it represents one of the most useful clinical parameters to determine the degree of severity of hepatic necrosis (Klaassen and Watkins, 1984; Sha et al., 2008). Increase in total serum bilirubin concentration after CCl_4 administration might be attributed to the failure of the damaged hepatic parenchyma to normally uptake, conjugate and excrete the produced bilirubin. An important observation following octreotide treatment was the profound decrease in the elevated serum bilirubin level which suggests the possibility of using the compound in the acute cases of jaundice.

It is reported that TNF-α plays an important role in inflammation. Present study clearly shows that octreotide treatment significantly attenuates CCl_4 induced liver inflammation by decreasing TNF-α expression level. Present result in accordance with Zhang et al. (2008) who found that both octreotide and Baicalin had obvious protective effect on the multiple organ injury in severe acute pancreatitis probably by decreasing secretion of TNF-α.

Oral administration of CCl_4 resulted in over expression of SSTR2 in comparison to the control group. On the other hand, octreotide treatment for CCl_4-treated mice induced higher expression of SSTR2 in comparison to the CCl_4-treated group. This may explain the function of the octreotide as hepatoprotective against liver injury.

VEGF plays an essential role in regulating angiogenesis during embryonic development, normal tissue growth, wound healing, as well as numerous pathological processes. VEGF functions as an endothelial cell mitogen (Yoshida et al., 1996), chemotactic agent (Leung et al., 1989) and inducer of vascular permeability (Brkovic and Sireos, 2007).

In contrast to VEGF, Somatostatin analogues exert antiangiogenic actions through a broad-range inhibition of both the synthesis and the effect of angiogenic factors including platelet-derived growth factor, VEGF and fibroblast growth factor-2. These factors are secreted by tumor cells as well as by infiltrating inflammatory cells and their involvement in tumor
progression has been well proven (Ferrara, 1999). Octreotide was reported to be capable of inhibiting the tumor expression level of VEGF as well as VEGF serum level in colorectal cancer patients (Cascini et al., 2001). Present experiments declared that octreotide has an antiangiogenic action through inhibiting the synthesis of VEGF, where octreotide-treated mice showed a much lower expression level of VEGF compared to its expression level in the mice of CCl₄-treated group. According to our knowledge, present study is the first attempt for evaluation of hepatoprotective effect of octreotide against liver injury induced by CCl₄ in mouse model. However, the markers of liver cell injury that we measured are in agreement with other researchers.

In conclusion, treatment with octreotide following CCl₄ treatment significantly ameliorated the enzymatic alterations which resulted from CCl₄-induced liver injury in mice, probably due its anti-inflammatory effects that inhibit CCl₄ free radical derivative formation and prevent cellular damage. Interestingly, it is possible that octreotide possesses strong potentials as an emerging hepatoprotective agent that can keep structural integrity of bio-membranes.

REFERENCES


