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ORIGINAL ARTICLE

α-Methylspermidine protects against carbon tetrachloride-induced hepatic and pancreatic damage

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Abstract The role of polyamines in carbon tetrachloride (CCl_4)-induced organ injury was studied in syngenic rats and transgenic rats with activated polyamine catabolism. In syngenic rats, administration of CCl_4 resulted in the induction of hepatic spermidine/spermine N^1-acetyltransferase (SSAT), accumulation of putrescine, reduction in spermine level and appearance of moderate hepatic injury within 24 h. Upon treatment with CCl_4, transgenic rats overexpressing SSAT displayed induction of both hepatic and pancreatic SSAT, with subsequent accumulation of putrescine and decrease of both spermidine and spermine pools. Administration of CCl_4 in SSAT transgenic rats induced not only massive hepatic injury, but also severe acute necrotizing pancreatitis. Pretreatment of the animals with catabolically stable functional polyamine mimetic, α-methylspermidine (MeSpd) prevented pancreatic and hepatic injury in SSAT rats and markedly reduced liver damage in syngenic animals. As assessed by immunostaining of proliferating cell nuclear antigen, MeSpd increased the amount of regenerating hepatocytes in both genotypes. These results show that CCl_4 induces hepatic and pancreatic polyamine catabolism, and the extent of organ damage correlates with the degree of polyamine depletion. Furthermore, MeSpd protects against CCl_4-induced hepatic and pancreatic damage and promotes tissue regeneration.

Keywords Carbon tetrachloride · Spermidine/spermine N^1-acetyltransferase · Pancreatitis · Hepatotoxicity · Regeneration · Polyamines · Polyamine analogues · Liver cirrhosis

Abbreviations

CCl_4	Carbon tetrachloride
DFMO	α-Difluoromethylornithine
IL	Interleukin
ICAM-1	Intracellular cell adhesion molecule-1
Me_2Spm	α,ω-Bismethylspermine
MeSpd	α-Methylspermidine
ODC	Ornithine decarboxylase
SSAT	Spermidine/spermine N^1-acetyltransferase
TNF-α	Tumor necrosis factor-α

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Introduction

The polyamines, spermidine and spermine are multifunctional organic cations important for cell proliferation, differentiation and cell signaling (Thomas and Thomas 2001). They interact with cellular anionic sites, such as DNA, RNA and phospholipids and modulate chromatin conformation, gene expression and membrane stability. In addition, they can be covalently bound to proteins. Many studies indicate that a certain amount of polyamines are needed to preserve tissue functions and structural integrity.

In regenerating liver, polyamine levels increase due to the induction of their biosynthetic enzyme, ornithine decarboxylase (ODC) (Luk 1986). Depletion of hepatic polyamines using irreversible ODC inhibitor, α-difluoromethylornithine (DFMO) (Luk 1986) or transgene-mediated induction of the catabolic enzyme spermidine/spermine N^1-acetyltransferase (SSAT) delays liver regeneration after partial hepatectomy (Alhonen et al. 2002). In the pancreas, induction of SSAT and depletion of spermidine triggers development of acute pancreatitis in SSAT transgenic rats (Alhonen et al. 2000) and in rats with L-arginine- or cerulein-induced pancreatitis (Hyvönen et al. 2006). Similarly, DFMO treatment reduces tissue regeneration after cerulein-induced pancreatitis (Jurkowska et al. 1997). The causal relationship between polyamine depletion and tissue destruction is supported by the findings that administration of catabolically stable polyamine mimetic, α-methylspermidine (MeSpd), prevents the onset of pancreatitis and restores the hepatic regenerative capacity in SSAT transgenic rats (Räsänen et al. 2002).

Cirrhosis is the endstage of chronic liver injury caused by viral hepatitis or alcohol abuse, and organ transplantation remains as the only treatment option for cirrhosis. The disease is characterized by replacement of liver tissue by fibrous scar and the appearance of regenerative nodules. There is continuous deposition of extracellular matrix resulting from increased collagen synthesis and insufficient breakdown, and impaired capability of liver regeneration in comparison with the normal liver (Andiran et al. 2000). In animal experiments, carbon tetrachloride (CCl_4) is commonly being used as a hepatotoxic, cirrhotic and carcinogenic model compound (Manibusan et al. 2007). Acute CCl_4 insult results in centrilobular necrosis, inflammation and fibrosis, and chronic exposure leads to cirrhosis within 8–12 weeks. Interestingly, previous studies indicate that CCl_4 affects polyamine metabolism in rat liver, by inducing both SSAT and ODC, with subsequent reduction in spermidine and spermine levels (Matsui et al. 1981). Because MeSpd was able to restore the regenerative capacity after hepatectomy of SSAT transgenic rats, the present work was carried out to investigate whether MeSpd elicits protective effect on CCl_4-induced organ injury in syngenic and SSAT transgenic rats.

Materials and methods

Animals

Two months old female Wistar rats were used, three to five animals/group. The generation of transgenic rats overexpressing SSAT under the control of mouse metallothionein I promoter has been described (Alhonen et al. 2000). MeSpd was synthesized as described in (Grigorenko et al. 2005). MeSpd (50 mg/kg in saline) was injected intraperitoneally 20 and 4 h before the administration of CCl_4 (1 ml/kg i.p. diluted 1:2 in corn oil, Sigma). Control animals received corn oil/saline only. Animals were killed 24 or 48 h after administration of CCl_4. Animals were kept in a room with a daily cycle of 12 h light/dark period. They had free access to water and standard diet. The animal experiments were approved by the Animal Care and Use Committee of the University of Kuopio and the Provincial Government.

Histological analyses and PCNA staining

Formalin-fixed tissue specimens were embedded in paraffin, cut into 5-μm thick slices and stained with hematoxylin and eosin. Immunohistochemistry of proliferating cell nuclear antigen (PCNA) was carried out as described earlier (Alhonen et al. 2002).

Determination of SSAT activity and polyamine levels

Spermidine/spermine N^1-acetyltransferase and polyamine levels were determined using published methods (Hyvönen et al. 1992; Bernacki et al. 1992).

Statistical analysis

Data are presented as average ± SD. One-way ANOVA with Tuckey's post hoc test was performed with the aid of software package, GraphPad Prism version 4.03. *, **, *** refer to P values of < 0.05, < 0.01 and < 0.001, respectively, when compared with untreated control.

Results

Liver damage

Table 1 shows hepatic SSAT activity and polyamine levels in syngenic and SSAT transgenic rats at 24 and 48 h after the injection of CCl_4. CCl_4-induced SSAT in both genotypes and led to the accumulation of putrescine and in SSAT rats also to appearance of N^1-acetylspermidine (N^1-AcSpd). Interestingly, CCl_4 decreased spermine level and increased spermidine level in syngenic rats, whereas in transgenic rats mainly spermidine was decreased. However, transgenic rats displayed already without any treatments markedly lower spermine levels and higher level of putrescine when compared with syngenic rats. MeSpd accumulated in physiologically relevant levels in the livers of both genotypes, transgenic rats showing higher accumulation.

Table 1 Effect of MeSpd pretreatment and CCl_4 on hepatic polyamine metabolism in syngenic and SSAT transgenic rats

	SSAT (pmol/10 min/mg)	Pu (pmol/mg tissue)	Spd (pmol/mg tissue)	Sp (pmol/mg tissue)	N^1-AcSpd (pmol/mg tissue)	MeSpd (pmol/mg tissue)
Syngenic						
Control	n.d.	n.d.	861 ± 235	833 ± 55	n.d.	
CCl_4 24 h	<5	106 ± 23	1,159 ± 186	590 ± 74	n.d.	
MeSpd + CCl_4 24 h	<5	219 ± 29	1,155 ± 219	528 ± 172	n.d.	627 ± 272
CCl_4 48 h	<5	64 ± 56	1,399 ± 141	521 ± 137	n.d.	
MeSpd + CCl_4 48 h	<5	68 ± 59	1,331 ± 217	484 ± 50	n.d.	485 ± 345
Transgenic						
Control	12 ± 1	1,431 ± 231	986 ± 80	109 ± 36	35 ± 30	
CCl_4 24 h	42 ± 31	2,134 ± 1,592	571 ± 502	65 ± 41	41 ± 35	
MeSpd + CCl_4 24 h	60 ± 12	1,439 ± 380	326 ± 23	41 ± 4	<20	1,254 ± 128
CCl_4 48 h	48 ± 36	3,156 ± 1,970	849 ± 300	109 ± 19	50 ± 21	
MeSpd + CCl_4 48 h	30 ± 7	1,148 ± 303	808 ± 154	129 ± 23	<20	863 ± 20

The rats were injected with MeSpd (50 mg/kg i.p. in saline) 20 + 4 h before the injection of CCl_4 (1 ml/kg i.p. in corn oil) and killed 24 or 48 later. Data are mean ± SD, $n = 3$–5

n.d. not detectable, *Pu* putrescine, *Spd* spermidine, *Sp* spermine, *N^1-AcSpd* N^1-acetylspermidine

Histological examination of the livers of CCl_4-treated syngenic rats showed centrilobular necrosis at 24 h (Fig. 1b), with microvesicular steatosis appearing at 48 h (Fig. 2b) after administration of the compound. CCl_4 induced more severe hepatic injury to SSAT transgenic rats than syngenic rats (Figs. 1e, 2e), with macrovesicular steatosis, lobular inflammation and deterioration of the parenchyma. Pretreatment with MeSpd protected both syngenic and transgenic rats against CCl_4-induced liver damage (Figs. 1c, f, 2c, f). No significant fibrosis was present at 48 h.

Liver regeneration was measured by calculating the percentage of PCNA-positive hepatocytes. PCNA started to slowly increase at 24 h in syngenic rats, and markedly increased at 48 h in both syngenic and transgenic rats (Fig. 3). However, transgenic rats displayed significantly lower PCNA in both time points than syngenic rats (24 h $P < 0.05$, 48 h $P < 0.001$). At 24 h after CCl_4 administration, PCNA was actually decreased in transgenic rats, indicating that polyamine depletion reduced liver regeneration. MeSpd administration prior to CCl_4 increased PCNA in both genotypes and both time points.

Pancreatic damage

As shown in Table 2, CCl_4 treatment induced SSAT in the pancreases of syngenic and SSAT transgenic rats, with subsequent accumulation of putrescine and N^1-AcSpd. Transgenic rats displayed higher reduction in pancreatic spermidine and spermine levels than syngenic rats. The amount of MeSpd accumulating in the pancreas was similar to that of liver.

Syngenic rats treated with CCl_4, with or without MeSpd, did not show any changes in pancreatic histology in 24 or 48 h (Figs. 1h–i, 2h–i). In contrast, transgenic rats developed severe acute pancreatitis, as assessed by appearance of edema within 24 h (Fig. 1k) and massive necrosis within 48 h (Fig. 2k). Some pancreases also showed small areas of fat necrosis (not shown). Prior administration of MeSpd almost completely prevented the onset of the disease, as assessed by tissue histology (Figs. 1l, 2l). None of the pancreases showed measurable PCNA staining (not shown).

Discussion

We and others have shown that several factors that deplete pancreatic spermidine level low enough cause the development of acute pancreatitis (Alhonen et al. 2000; Hyvönen et al. 2006; Räsänen et al. 2003; Herzig et al. 2005; Jin et al. 2008). Here, we present new evidence that further strengthens this view. Administration of CCl_4 did not cause pancreatitis in syngenic rats, since pancreatic spermidine/spermine pools were not decreased low enough to cause pancreatitis. In SSAT transgenic rats, spermidine levels were markedly reduced, leading to the development of severe necrotizing pancreatitis within 48 h. Moreover, prior administration of methylated spermidine analog could substitute for spermidine and prevent the development of

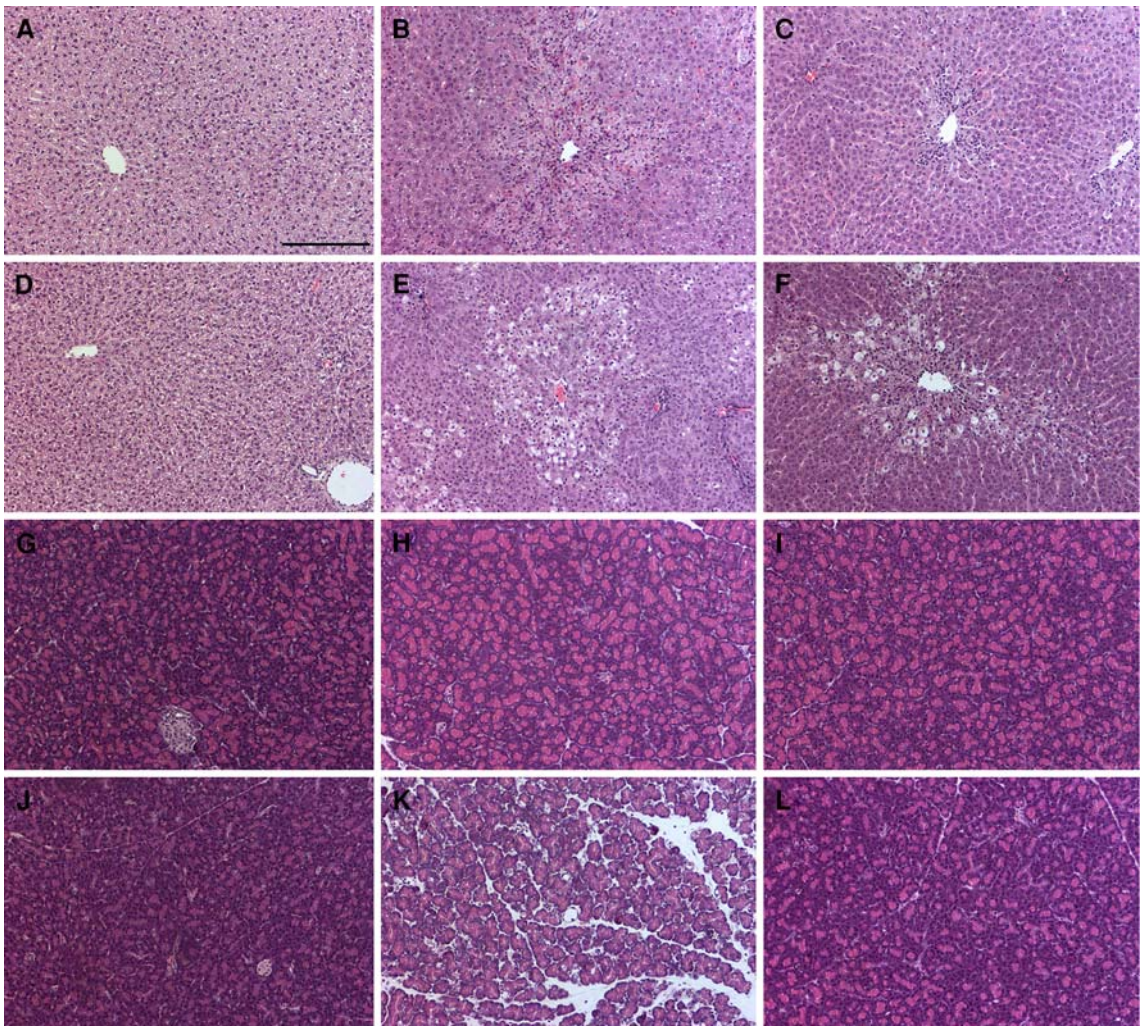

Fig. 1 Effect of CCl_4 (24 h) and MeSpd pretreatment on hepatic (**a**–**f**) and pancreatic (**g**–**l**) histology. Syngenic animals (**a**–**c**, **g**–**i**) and SSAT transgenic animals (**d**–**f**, **j**–**l**). Control (**a**, **d**, **g**, **j**), CCl_4-treated (**b**, **e**, **h**, **k**), and MeSpd + CCl_4 treated (**c**, **f**, **i**, **l**). The rats were injected with MeSpd (50 mg/kg i.p. in saline) or saline 20 + 4 h before the injection of CCl_4 (1 ml/kg i.p. in corn oil) or corn oil only and killed 24 h later. Tissue samples were stained with hematoxylin and eosin. Original magnifications ×100, *scale bar* 200 μm

pancreatitis. This result is in line with our previous publications where zinc-induced pancreatitis in SSAT transgenic rats could be prevented by pretreatment with MeSpd (Räsänen et al. 2002). All these findings indicate that polyamines are essential for the integrity and function of pancreas and, therefore, polyamines must be maintained above certain critical level.

Not only pancreas, but also liver seems to require polyamines for preserving its structural integrity and functions. We previously demonstrated that polyamine depletion delays rat liver regeneration after partial hepatectomy, and that prior MeSpd administration restores the regenerative capacity to normal level (Räsänen et al. 2002). CCl_4 is a commonly used agent to induce either acute liver injury, or cirrhosis or carcinoma when administered at low dose for several weeks. The present data show that CCl_4 induced hepatic SSAT, decreased spermidine and/or spermine pool and caused liver damage which was more extensive in transgenic rats than their syngenic littermates. This is explained by the fact that CCl_4 led to more pronounced reduction in polyamine pools in SSAT transgenic than in syngenic rats. Interestingly, CCl_4 decreased spermidine in transgenic rats, but spermine in syngenic rats. However, both genotypes developed liver injury, and moreover, it was preventable by prior MeSpd administration. Analysis of liver PCNA labeling index indicated that MeSpd facilitated liver regeneration already within 48 h after CCl_4 administration. Because hepatocytes' life span is about 5 months, efficient continuous regeneration is essential for functioning liver. Regeneration also repairs

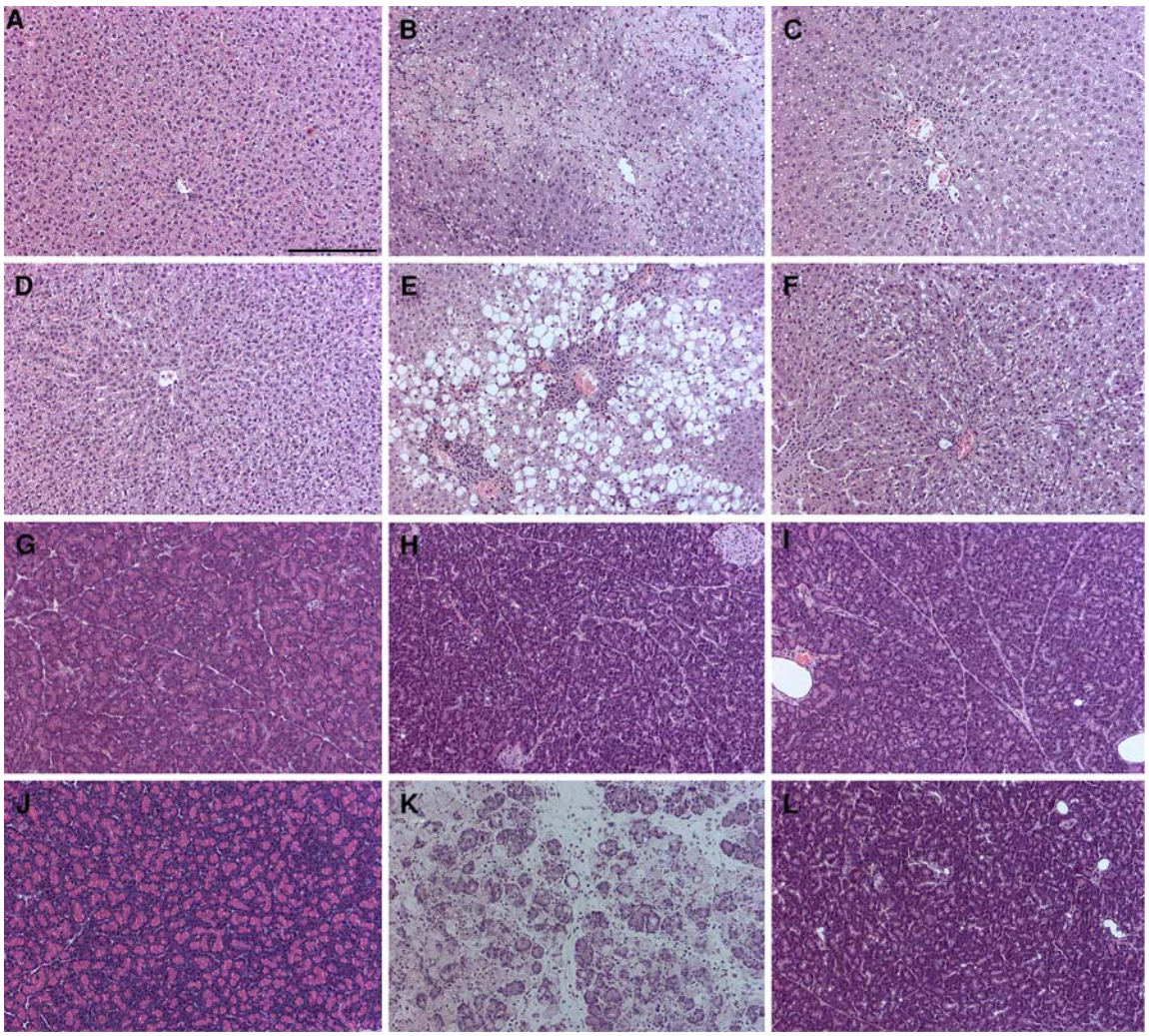

Fig. 2 Effect of CCl_4 (48 h) and MeSpd pretreatment on hepatic (**a**–**f**) and pancreatic (**g**–**l**) histology. Syngenic animals (**a**–**c**, **g**–**i**) and SSAT transgenic animals (**d**–**f**, **j**–**l**). Control (**a**, **d**, **g**, **j**), CCl_4-treated (**b**, **e**, **h**, **k**), and MeSpd + CCl_4-treated (**c**, **f**, **i**, **l**). The rats were injected with MeSpd (50 mg/kg i.p. in saline) or saline 20 + 4 h before the injection of CCl_4 (1 ml/kg i.p. in corn oil) or corn oil only and killed 48 h later. Tissue samples were stained with hematoxylin and eosin. Original magnifications ×100, *scale bar* 200 μm

injuries caused by acute insults. In chronic liver injury, there is abnormal accumulation of extracellular matrix and impaired regeneration. Liver cirrhosis is the advanced stage of fibrosis, and is responsible for significant morbidity and mortality worldwide. Therefore, due to its regenerative and protective effects, MeSpd could be a potential therapeutic for hepatitis and possibly for liver cirrhosis.

The CCl_4-induced liver damage is caused by the metabolism of the drug via CYP2E1, yielding highly reactive trichloromethylradicals within the membrane of the endoplasmic reticulum (Manibusan et al. 2007). These compounds consume reduced glutathione, and are capable of binding covalently to cellular macromolecules and membrane phospholipids, causing membrane destruction. Natural antioxidants, such as glutathione, are capable of quenching these deleterious free radicals. Although the degradation of polyamines forms toxic aldehydes and hydrogen peroxide as by product (Seiler 2004; Agostinelli et al. 2004), intact polyamines have been shown to possess antioxidant properties (Chattopadhyay et al. 2003; Sava et al. 2006; Rider et al. 2007). An elegant study conducted by Ha et al. showed that spermine directly quenches hydroxyl radicals (Ha et al. 1998). Because polyamines are positively charged with flexible backbone, they can also bind to and interact with various negatively charged phospholipids, such as those present in organelle membranes (Schuber 1989). Chen and others found that L-cysteine and L-methionine protect isolated rat hepatocytes from CCl_4-induced hepatotoxicity, and suggested that the protective effect is mediated by polyamines (Chen et al.

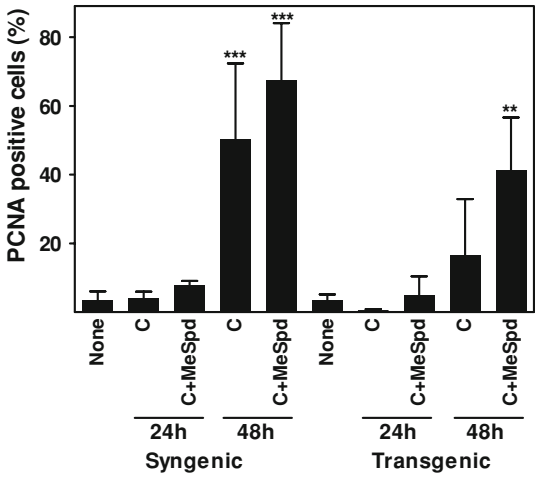

Fig. 3 Effect of MeSpd pretreatment and CCl_4 on the amount of PCNA-positive hepatocytes. The rats were injected with MeSpd (50 mg/kg i.p. in saline) or saline 20 + 4 h before the injection of CCl_4 (1 ml/kg i.p. in corn oil) or corn oil only and killed 24 or 48 h later. Liver sections were immunostained with PCNA antibody and the relative amount of PCNA-positive cells in 25 mm^2 area was calculated. The results are mean ± SD, $n = 3$–5. *C* CCl_4

2000). Recently, polyamines' protective role against lipid peroxidation was revealed in *Trypanosoma cruzi* (Hernandez et al. 2006). In SSAT rats with zinc-induced pancreatitis, lipid peroxidation could be ameliorated by pretreatment with stable spermine analog, α,ω-bismethylspermine (Me_2Spm) (Merentie et al. 2007). In our previous studies investigating the pathogenesis of pancreatitis, it was found that MeSpd pretreatment prevented the degranulation of zymogen granules and translocation of cathepsin B to the cytoplasm (Hyvönen 2007; Hyvönen et al. 2007). Thus, depletion of polyamines from organelle membranes may sensitize membranes to oxidative stress, while MeSpd can substitute for the natural polyamines without being degraded as it is not a substrate for SSAT.

Another known protective effect of polyamines on tissue damage is the suppression of inflammatory reaction (Zhang et al. 1997, 2000). Spermine has been shown to restrain macrophages and suppress the production of inflammatory cytokines. During CCl_4-induced liver injury, Kupffer cells become activated and secrete a variety of cytokines and adhesion molecules, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6 and intracellular cell adhesion molecule-1 (ICAM-1) (Ramadori et al. 2008). We previously reported that Me_2Spm pretreatment could prevent the induction of TNF-α in SSAT transgenic rats with zinc-induced pancreatitis (Merentie et al. 2007), and our recent findings indicate that MeSpd reduces serum TNF-α level also when administered after the induction of pancreatitis (Hyvönen et al. 2009). Thus, polyamines have multiple beneficial effects, having anti-inflammatory and antioxidant properties and preserving structural integrity.

Taken together, the present results further emphasize the importance of proper polyamine homeostasis for hepatic and pancreatic function, tissue healing and structural integrity. Based on the previous and present results, further studies focusing on the therapeutic potential of MeSpd against hepatotoxins, liver cirrhosis as well as pancreatitis are warranted.

Table 2 Effect of MeSpd pretreatment and CCl_4 on pancreatic polyamine metabolism in syngenic and SSAT transgenic rats

	SSAT (pmol/10 min/mg)	Pu (pmol/mg tissue)	Spd (pmol/mg tissue)	Sp (pmol/mg tissue)	N^1-AcSpd (pmol/mg tissue)	MeSpd (pmol/mg tissue)
Syngenic						
Control	<5	n.d.	4,087 ± 357	591 ± 95	n.d.	
CCl_4 24 h	<5	864 ± 122	2,648 ± 186	482 ± 19	<20	
MeSpd + CCl_4 24 h	7 ± 4	784 ± 148	2,332 ± 502	512 ± 83	<20	854 ± 132
CCl_4 48 h	6 ± 2	350 ± 87	3,952 ± 1,275	593 ± 139	<20	
MeSpd + CCl_4 48 h	8 ± 1	128 ± 17	3,141 ± 122	489 ± 29	<20	502 ± 244
Transgenic						
Control	38 ± 12	4,530 ± 2,033	3,745 ± 491	402 ± 100	74 ± 26	
CCl_4 24 h	117 ± 50	15,576 ± 3,251	737 ± 616	159 ± 108	134 ± 54	
MeSpd + CCl_4 24 h	142 ± 13	7,882 ± 2,019	624 ± 97	142 ± 7	52 ± 14	1,243 ± 129
CCl_4 48 h	147 ± 19	3,389 ± 1,838	564 ± 314	123 ± 46	54 ± 32	
MeSpd + CCl_4 48 h	51 ± 7	2,796 ± 783	1,270 ± 266	169 ± 29	21 ± 12	1,059 ± 471

The rats were injected with MeSpd (50 mg/kg i.p. in saline) 20 + 4 h before the injection of CCl_4 (1 ml/kg i.p. in corn oil) and killed 24 or 48 later. Data are mean ± S.D., $n = 3$–5

n.d. not detectable, *Pu* putrescine, *Spd* spermidine, *Sp* spermine, *N^1-AcSpd* *N*-acetylspermidine

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