MMSL
Military Medical
Science Letters

Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2012, vol. 81(2), p. 68-75
ISSN 0372-7025
DOI: 10.31482/mmsl.2012.009

ORIGINAL ARTICLE

EFFECT OF SOMAN ON JNK AND P38 MITOGEN ACTIVATED PROTEIN KINASE (MAPK) PATHWAYS

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Received 19th February 2012.
Revised 18th May 2012.
Published 08th June 2012.

Summary

The purpose of our study was to examine an early activation of JNK and p38 mitogen activated protein kinases (MAPK) and their substrate c-Myc after soman poisoning in order to enlighten the pathogenetic mechanism of nerve agent-induced non-specific effects. Male Wistar rats were intramuscularly poisoned by soman (60 $\mu g.kg^{-1}$ - 70% LD_{50}). Samples were taken 4, 24, and 72 hours after poisoning, immunohistochemically stained and phospho-$JNK^{Thr\text{-}183/Tyr\text{-}185}$, phospho-$p38^{Thr180/Tyr182}$, and phospho-c-$Myc^{Thr58/Ser62}$ expressions were measured using a computer Image analysis in apical and cryptal enterocytes of the *colon transversum*. We observed decreased phospho-JNK in apical enterocytes 4 and 24 h after poisoning and increased phospho-JNK in cryptal and apical enterocytes 72 h after intoxication. Phospho-p38 dropped significantly in the apical compartment 72 h after soman poisoning. An activation of c-Myc decreased in both apical and cryptal compartment 4 and 24 h after soman intoxication, while increased in both compartments 72 h after poisoning. Soman poisoning seems to temporarily suppress promitotic pathways of proliferating cryptal cells and causes delayed activation of JNK stress signaling pathway.

Key words: soman; JNK, p38; c-Myc; enterocyte; rat; image analysis

INTRODUCTION

Nerve agents are highly toxic organophosphates representing potential threats to both military and civilian population. The basic mechanism of their toxicity is well known and lies in irreversible binding to and inactivation of acetylcholinesterase (AChE, EC 3.1.1.7), which is associated with accumulation of acetylcholine at the synapses and overstimulation of cholinergic nervous system (1). By contrast, less is known about nerve agent-induced non-specific effects including the influence on non-cholinergic neurotransmitter levels and especially oxidative stress interfering with cellular DNA metabolism and resulting in organophosphate genotoxicity and mutagenicity (2, 3, 4). Oxidative stress and long-term alteration of DNA are considered to contribute to long-term

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toxic effects of nerve agents (4, 5). Therefore, finding the mechanisms of nerve agent-induced non-specific effects might contribute to early diagnosis and complex treatment of nerve agent poisoning.

One of non-specific effects of nerve agents is the stress response after nerve agents exposure. One type of nerve agent-induced cell stress response observed *in vivo* after soman poisoning is the activation and/or alteration of the Mitogen-activated protein kinases (MAPK) (6, 7, 8, 9). MAPK proteins create a superfamily of three distinct kinases ERK (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinase), and p38 that are activated in response to a wide variety of extracellular or intracellular stimuli, including a DNA damage (10, 11, 12). Upon activation, MAPKs translocate into the nucleus, where they phosphorylate transcription factors such as c-Myc, which could be a substrate of ERK, JNK as well as p38 kinase (12). The biological outcome of MAPK action depends on quantitative and qualitative parameteres of stress stimulation and cell type or tissue. In proliferative cells, MAPK signalling supports cell cycle arrest and DNA repair (13, 14). When DNA is successfully repaired, cells restart cell cycle, otherwise (when DNA damage is irreparable) cells undergo apoptosis (15). An increased number of apoptotic cells was observed in stomach, bronchi and lungs in rats poisoned with sublethal doses of soman (16). Finally, remaining cells enhance proliferation to preserve tissue integrity (17). Such biological consequences might have a deleterious impact in non-proliferating and differentiated systems such as central nervous system (CNS) and might be the underlying mechanism of neurological and neuropsychological outcomes detectable months or even years following the recovery of acute organophosphate poisoning (18, 19).

To evaluate effects of soman on stress kinase pathways, we investigated an impact on activation of JNK, p38 and their substrate c-Myc *in vivo*. A model of rat colon enterocytes was chosen for the study, since both undifferentiated and proliferating cells localized at the base of the crypts and differentiated cells at the inner intestinal surface could be recognized.

MATERIAL AND METHODS

Animals

Male Wistar rats aged 12-16 weeks and weighing 250-300 g (Navel, Konarovice, Czech Republic) were kept in an air-conditioned room (22 ± 2 °C and 50 ± 10% relative humidity, with lights from 7.00 to 19.00 hours) and with an allowed access to standard food and tap water ad *libitum*. Before start of the experiment (soman or saline administration), animals spent 15 days of acclimatization in the laboratory vivarium. Handling of experimental animals was done under the supervision of the Ethics Committee of the Faculty of Military Health Sciences in Hradec Kralove (Czech Republic).

Chemicals

Soman (GD; pinacolyl methylphosphonofluoridate) was obtained from Military Technical Institute in Brno (Czech Republic). Its purity (97-98 %) was assayed by acidimetric titration. All other drugs and chemicals of analytical grade were obtained commercially and used without further purification. Soman and saline were administered intramuscularly (*i. m.*) at the volume of 1 ml/kg of body weight.

Procedure

Twenty-four control rats were *i. m.* administered with saline, divided into three groups and killed by cervical dislocation 4, 24 and 72 hours after saline administration, respectively. Twenty-four experimental rats were *i. m.* administered with soman at the dose 60 μg/kg (70% LD_{50}), divided into three groups, and killed by cervical dislocation 4, 24 and 72 hours after the poisoning, respectively.

Histological examination

A central part of *colon transversum* was removed from the rats and carefully fixed with a 10% neutral buffered formalin (Chemapol, Prague, Czech Republic). The samples were subsequently embedded into paraffin (Paramix, Holice, Czech Republic), 5 μm thick tissue sections were cut and immunohistochemical detection of JNK phosphorylation at threonine 183 and tyrosine 185, p38 phosphorylation at threonine 202 and tyrosine 204, and c-Myc phosphorylation at threonine 58 and serine 62 was performed with a standard peroxidase technique. After blocking the endogenous peroxidase activity for 20 min [1.8 ml of 30% hydrogen peroxide (Vitrum, Prague, Czech Republic) in 100 ml methanol (Kulich, Hradec Kralove, Czech Republic)], the tissue sections were incubated for 1 hour with antibodies: rabbit monoclonal anti-phospho-$JNK^{Thr\text{-}183/Tyr\text{-}185}$ diluted 1:50, rabbit monoclonal anti-phospho-$p38^{Thr180/Tyr182}$ diluted 1:50, and mouse polyclonal anti-c-$Myc^{Thr\text{-}58/Ser\text{-}62}$ diluted 1:50 (all from Biotech, Prague, Czech Republic) in phosphate buffered saline (PBS, Sigma-Aldrich, Prague, Czech Republic) pH 7.2 and then washed three times in PBS. All slides were then

incubated with secondary antibodies for 20 min. Ready-to-use biotinylated anti-rabbit secondary antibody (DakoCytomation, Prague, Czech Republic) was used for slides previously incubated with rabbit primary antibodies and biotin-SP-conjugated AffiniPure donkey anti-mouse secondary antibody diluted 1:500 (Spinchem, Plzen, Czech Republic) was used for slides previously incubated with mouse primary antibodies. Excess of secondary antibodies was then washed off with PBS. Subsequently, all slides were incubated with streptavidin horseradish peroxidase (DakoCytomation, Prague, Czech Republic) under the same conditions as the secondary antibody, washed with PBS and finally, 0.05% 3,3´-diaminobenzidinetetrahydrochloride-chromogen solution (Sigma-Aldrich, Prague, Czech Republic) in PBS containing 0.02% hydrogen peroxide was added for 10 min to visualize the antigen-antibody complex *in situ*.

Image Analysis

Stained samples were evaluated using BX-51 microscope (Olympus, Prague, Czech Republic) and computer image analysis ImagePro 5.1. (Media Cybernetics, Bethesda, MD, USA). Ten microscopic fields at a 400fold original magnification were randomly selected from each rat sample. The image analysis was performed separately in two compartments - in apical enterocytes and in enterocytes of lateral sides of crypts in the area of 2250 μm^2 representing 30 – 40 cells per field and compartment. The immuno-reactive structures of inverted RGB (red-green-blue) scale were detected in the range: red 56-255, green 76-255, and blue 94-255, where 0 is white and 255 is black colour. Subsequently, integral optical density (IOD) of viewing fields was measured. The IOD parameter reflects intensity of positivity within the detected area. The scale represents levels from 0 to 2 x 10^5 for the detected area.

Statistical analysis

The Mann-Whitney test was used for the statistical analysis giving mean ± 2 × Standard error of mean (S.E.M.). The differences were considered significant when $p \leq 0.05$.

RESULTS

Phospho-JNK$^{Thr\text{-}183/Tyr\text{-}185}$

In comparison with control animals, phospho-JNK$^{Thr\text{-}183/Tyr\text{-}185}$ was significantly decreased in apical enterocytes 4 and 24 hours after the soman intoxication. The IOD values decreased 5.1- and 2.4-fold, respectively. In the 72h time interval, phospho-JNK$^{Thr\text{-}183/Tyr\text{-}185}$ levels were found significantly higher in both crypts and apical interocytes being 8.7- and 3.3-fold increased, respectively (table 1).

Table 1. Average IOD values of phospho-JNK$^{Thr183/Tyr185}$ per microscopic field ± 2 × S.E.M.

	Integral Optical Density (IOD)		
time (h)	4	24	72
	apical enterocytes		
control	4100 ± 2300	2200 ± 700	1700 ± 800
soman	800 ± 200 [2]	900 ± 300 [2]	5600 ± 3200 [1]
	cryptal enterocytes		
control	300 ± 200	200 ± 100	300 ± 100
soman	100 ± 100	200 ± 200	2600 ± 2000 [1]

Significant differences between control and intoxicated animals: $p \leq 0.05$ - [1]; $p \leq 0.001$ - [2].

Phospho-p38$^{Thr\text{-}180/Tyr\text{-}182}$

When compared with control groups, phospho-p38$^{Thr180/Tyr182}$ was significantly decreased in apical enterocyte compartment 72 hours after the soman poisoning. The IOD value decreased 3.0-fold (table 2).

Phospho-c-Myc$^{Thr\text{-}58/Ser\text{-}62}$

Level of phospho-c-Myc$^{Thr\text{-}58/Ser\text{-}62}$ decreased in both apical and cryptal enterocytes 4 and 24 h after the soman application. In apical cells, IOD values dropped 5.2- and 1.9-fold and in crypts, we observed 1.7- and 1.6-fold decrease, respectively.

In the 72h time interval, the opposite trends could be observed, since IOD values increased 2.1-fold in the apical compartment and 1.4-fold in crypts (table 3).

Table 2. Average IOD values of phospho-p38$^{Thr180/Tyr182}$ per microscopic field ± 2 × S.E.M.

	Integral Optical Density (IOD)		
time (h)	4	24	72
	apical enterocytes		
control	2000 ± 1200	2400 ± 1200	3300 ± 1200
soman	1500 ± 900	1600 ± 400	1100 ± 300 [1]
	cryptal enterocytes		
control	100 ± 100	500 ± 200	200 ± 100
soman	200 ± 200	300 ± 100	400 ± 200

Significant differences between control and intoxicated animals: $p \leq 0.05$ - [1]

Table 3. Average IOD values of phospho-c-Myc $^{Thr-58/Ser-62}$ per microscopic field ± 2 × S.E.M.

	Integral Optical Density (IOD)		
time (h)	4	24	72
	apical enterocytes		
control	14100 ± 3600	12100 ± 3600	8100 ± 2200
soman	2700 ± 800 [2]	6500 ± 3700 [2]	17400 ± 4000 [2]
	cryptal enterocytes		
control	4800 ± 1100	3500 ± 700	4600 ± 800
soman	2800 ± 500 [1]	2200 ± 600 [2]	6300 ± 1200 [1]

Significant differences between control and intoxicated animals: $p \leq 0.05$ - [1]; $p \leq 0.001$ - [2].

DISCUSSION

In this study, we evaluated the effect of soman poisoning on kinases JNK and p38 and transcription factor c-Myc in crypts and apical enterocytes of rat *colon transversum*.

In crypts, we observed decreased phospho-c-Myc 4 (Fig. 1, 2) and 24 h after the soman poisoning. The decreased phospho-c-Myc expression correlates with decreased phospho-Elk-1 measured in the same *in vivo* model (8). Both c-Myc and Elk-1 are substrates of JNK and ERK kinases (12). Since JNK activity was not changed in the 4 (Fig. 3, 4) and 24h time interval, the results indicate that soman poisoning downregulates ERK signalling pathway in crypts early after the intoxication. The biological outcome of decreased phospho-c-Myc expression in crypts early after soman intoxication is not certain. In enterocytes, ERK1/2 signalling supports cell survival and it is required for S-phase entry and proliferation (20, 21), in which c-Myc may participate via a transcriptional regulation of growth stimulating factors such as cyclin D1 and D2 (22, 23). Thus, we may assume that decreased c-Myc activation may contribute to a temporal downregulation of proliferation at the regulatory protein level. Subsequently, increased c-Myc phosphorylation observed in the 72h time interval might serve as a compensatory action for previous inhibition of promitotic pathways. JNK may also actively participate in this compensatory reaction, since the kinase stimulates mitotic activity of intestinal stem cells (24, 25).

In comparison to altered c-Myc and JNK phosphorylation, in crypts, the activation of p38 remained unchanged. Although the physiological role of p38 is not fully understood, we found

phospho-p38 values to be lower in cryptal enterocytes than in apical cells. This indicates that the p38 pathway may participate in the enterocyte differentiation process *in vivo* and is in accordance with *in vitro* experiments linking p38 to differentiation (26, 27, 28). Thus, soman poisoning might not alter p38 signalling in crypts in order not to impair the undifferentiated status of cryptal cells.

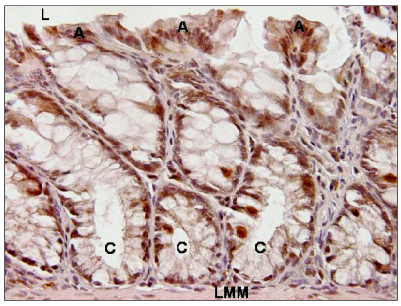

Figure 1. Sample of control (saline, 4 hours after administration) rat *colon transversum* with immunohistochemical detection of phospho-c-Myc$^{Thr\text{-}58/Ser\text{-}62}$ at 400-fold magnification. Phospho-c-Myc$^{Thr\text{-}58/Ser\text{-}62}$ assumed middle strong positivity in apical enterocytes (A) and heterogenous mild positivity in crypts (C). For publication purposes, the sample was counterstained with Harris heamatoxylin. LMM – lamina muscularis mucosae, L – lumen.

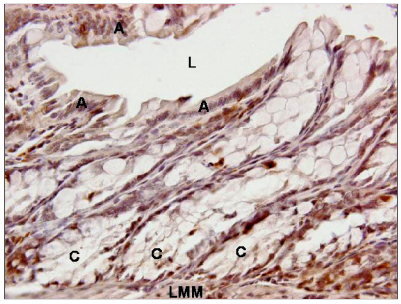

Figure 2. Sample of soman-poisoned (single dose of 70 % LD_{50}) rat *colon transversum* at 400-fold original magnification 4 hours after the poisoning. Soman poisoning decreased phospho-c-Myc$^{Thr\text{-}58/Ser\text{-}62}$ positivity in both apical (A) and cryptal (C) enterocytes. For publication purposes, the sample was counterstained with Harris heamatoxylin. LMM – lamina muscularis mucosae, L – lumen.

NOTE: Despite significant changes in molecular signalling, tissue integrity remained untouched (we did not observe any erosions or necrotic lesions 4, 24, or 72 h after the poisoning).

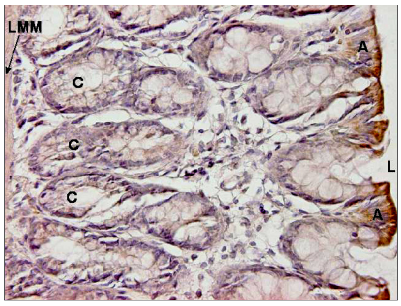

Figure 3. Sample of control (saline, 4 hours after administration) rat *colon transversum* with immunohistochemical detection of phospho-JNK$^{Thr183/Tyr185}$ at 400-fold magnification. In saline treated samples, phospho-JNK$^{Thr183/Tyr185}$ assumed rather cytoplasmatic middle strong positivity in apical enterocytes (A), while in crypts, we observed negative phospho-JNK$^{Thr183/Tyr185}$ expression. For publication purposes, the sample was counterstained with Harris heamatoxylin. LMM – lamina muscularis mucosae, L – lumen.

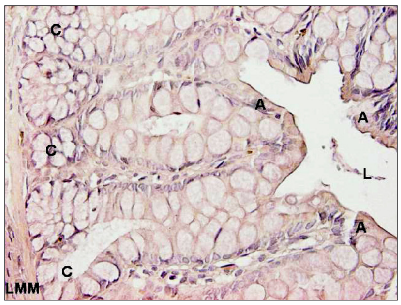

Figure 4. Sample of soman-poisoned (single dose of 70 % LD_{50}) rat *colon transversum* at 400-fold original magnification 4 hours after the poisoning. Soman poisoning decreased apical phospho-$JNK^{Thr183/Tyr185}$ expression, while in crypts (C), phospho-$JNK^{Thr183/Tyr185}$ negativity remained unchanged. For publication purposes, the sample was counterstained with Harris heamatoxylin. LMM – lamina muscularis mucosae, L – lumen.

In differentiated apical cells, the link between JNK/c-Myc signalling pathway and regulation of proliferation is highly unlike. Apidianakis et al. (29) proved that mature intestinal cells are more susceptible to JNK activation and apoptosis than undifferentiated enterocytes. The link between JNK signalling and apoptosis was observed *in vitro* and *in vivo* also by other groups (30, 31, 32). Moreover, c-Myc may participate in the JNK regulated apoptotic process. Ciclitra et al. (1987) observed a progressive increase in c-Myc staining intensity in the villus (apical) enterocytes in small intestinal mucosa of coeliac patients after gluten ingestion (33). Increased c-Myc positivity correlated with the classical coeliac morphological changes including villus atrophy (33), which is at least partially induced by apoptosis (34, 35). Decreased JNK and c-Myc activation observed 4 (Fig. 1 - 4) and 24 h after the soman poisoning might be therefore related to changes in crypts. In order to maintain tissue integrity, it seems that decreased promitotic signalling and possibly reduced cellular input from the cryptal compartment downregulate a natural apoptotic activity of apical cells. In the 72h time interval, in which the promitotic signalling is not blocked [increased phospho-JNK and phospho-c-Myc expression and renewed phospho-Elk-1 levels in crypts (12)], apical cells increase the activity of JNK/c-Myc signalling and are eliminated from the mucosal tissue more rapidly (according to our unpublished data, expression of M30 Cytodeath, a marker of apoptosis, is increased in apical cells 72 h after soman poisoning). In this context, soman poisoning probably suppresses p38 signalling to promote apoptotic process, since p38 seems to protect apical enterocytes against apoptosis *in vivo* (31, 36).

CONCLUSION

Our results show that soman poisoning affects JNK and p38 signalling pathways in time- and differentiation status dependent manner. In undifferentiated cryptal cells, soman intoxication seems to temporarily suppress promitotic pathways during first 24 h after the poisoning, which is followed by increased and probably compensatory activation of JNK/c-Myc signalling in the 72h time interval. In differentiated apical cells, decreased JNK/c-Myc signalling was observed in the 4 and 24 h time interval, while increased JNK/c-Myc activation toghether with decreased p38 MAPK signalling were observed in the 72h time interval.

ACKNOWLEDGEMENTS

We would like to thank Mrs. Šárka Průchová for her skillful technical assistance.

This work was supported by Ministry of Defence of the Czech Republic by the grant FVZ UO – MŠMT SV, č. 907010030281 and the grant „Health Problems of Weapons of Mass Destruction.

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