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PPARα mediates the effects of the pesticide methyl thiophanate on liver of the lizard *Podarcis sicula*

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Abstract

The majority of environmental pollutants are potential peroxisomal proliferators which include a heterogeneous group of compounds known to determine massive peroxisomal proliferation and hepatocarcinogenesis in rodents. Peroxisomal proliferation is accompanied by the induction of the peroxisomal fatty acid β-oxidation pathway mediated by a class of transcription factors named peroxisome proliferators activated receptors (PPARs). This phenomenon demonstrated also in ectotherm animals after exposition to environmental pollutants may be utilized as biomarker in environmental impact studies. In the present work we have tested the sensitivity to methyl thiophanate (TM) of the lizard *Podarcis sicula* in order to propose a biological model for monitoring the ecotoxicological effects of this pesticide on terrestrial sentinel species. The data obtained demonstrate that exposition to sub-lethal concentrations of TM leads to hepatocellular morphological changes and glycogen depletion, apoptosis, as well as probable peroxisomal proliferation attested by the increase of acyl-CoA oxidase (AOX). This effect seems to be mediated by the concomitant increase of PPARα. On the basis of these results we propose that also in *Podarcis sicula*, as just proposed for aquatic organisms, peroxisomal proliferation and AOX increase may be considered new biomarkers to evaluate pollution by organic compound in terrestrial environments.
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Keywords: Methyl tiophanate; Lizard; Liver; PPARα; AOX; SOD; Catalase; Apoptosis

1. Introduction

Thiophanate methyl (TM) is a thioallophanate compound and a systemic fungicide widely used to control fungal diseases of crops due to its broader range of activity, in comparison with other common fungicides, as well as lower general toxicity (Canton, 1976; Traina et al., 1998). It is metabolised by animals into benzimidazole compounds, including the well-known reproductive toxicant carbendazim. Thiophanate methyl is widely used in agricultural and horticultural practice in Campania region (Italy) and does not cause reproductive and/or developmental toxicity, since even at high doses it does not induce effects on spermatogenesis or on early embryogenesis (Traina et al., 1998). Nevertheless its administration in pregnant CD rats produced histological and histomorphometric alterations in thyroid and adrenals of CD rat pups suggesting that TM may act as a weak endocrine disrupter (Maranghi et al., 2003). Furthermore, its metabolite carbendazim caused histopathological damages in endocrine glands of the rat (Barlas et al., 2002). The possible impact of these pesticides needs to be considered since many of these compounds accumulate due to their persistence in the environment. Moreover, their adverse effects can occur at lower dose levels than those causing tumorigenicity or teratogenicity (Melnick et al., 2002), with long-term consequences on health (Davis, 1993; Foster and McIntyre, 2002).

Pollutants of industrial, agricultural and urban origin as some pesticides are potential peroxisome proliferators, which comprise a heterogeneous group of compounds known for their ability to cause massive proliferation of peroxisomes and liver carcinogenesis in rodents (Peters et al., 1997, 1998).

Peroxisomes are ubiquitous single-membrane limited organelles important in many cellular processes related to lipid metabolism, oxyradicals homeostasis, catabolism of purines and metabolism of amino acids and glyoxylate (Reddy and

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Mannaerts, 1994; Singh, 1997). Peroxisomes possess the unique feature to proliferate in response to various chemical unrelated compounds, named peroxisome proliferators. The peroxisomal proliferation is usually accompanied by induction of all three enzymes of the peroxisomal β-oxidation pathway, namely, acyl-CoA oxidase (AOX), multifunctional enzyme and thiolase (Lazarow and De Duve, 1976; Reddy and Mannaerts, 1994). The response appears to be mediated by peroxisome-proliferator activated receptors (PPARs), members of the nuclear receptor family which comprise different isotypes of the receptor (α, β, and γ) that have been cloned from various species including humans (Schmidt et al., 1992), rodents (Gottlicher et al., 1992; Issemann and Green, 1990), amphibians (Dreyer et al., 1992) and more recently fishes (Batista-Pinto et al., 2005). PPARα mediates the activity of peroxisome proliferators by modulating the expression of a wide variety of genes, including those involved in the peroxisomal β-oxidation of fatty acids (Zhang et al., 1992). Among PPAR subtypes present in various tissues (Lemberger et al., 1996), studies with a PPARα-null mouse have revealed that this isotype alone is critical in the tumor-promoting activity of peroxisome proliferators (Peters et al., 1997, 1998). The peroxisome proliferators-induced tumorigenesis is the result of altered gene expression, such as the growth regulatory genes c-Myc (Belury et al., 1998; Vanden Heuvel, 1999; Vanden Heuvel et al., 1998) or oxidative enzymes as acyl-CoA oxidase (Okamoto et al., 1997), in target cells.

Based on many recent experimental results, it has become evident that ectotherm animals may be threatened by peroxisome proliferators as fish and bivalve mollusc species (Carajavaille et al., 2003; Ibabe et al., 2004), accordingly, peroxisome proliferation could be used as a biomarker of exposure to a variety of pollutants in environmental pollution assessment.

In the present study we tested the sensibility of the lizard *Podarcis sicula* to TM with the aim to develop a biological model for monitoring the ecotoxic effects of this pesticide on sentinel species. In fact the lizards are a significant part of many ecosystems and are important in many food chains. Living in the wild they are threatened or endangered after pollutant exposure and may be proposed as ideal bioindicators for the assessment of environmental contamination impact (Lambert, 1997; Campbell and Campbell, 2000, 2002; Sanchez-Hernandez and Sanchez, 2002; Shen et al., 2005; Holem et al., 2006). Moreover, *Podarcis* is a good model for toxicological experiment since it adapts easily to laboratory conditions and is resistant to diseases and injury from handling practices. Our attention was focused on the liver, an organ which plays a key role in the more important metabolic processes and detoxicant mechanisms.

The aim of the present study was (a) to validate the use of lizards as bioindicators of the environmental contamination, (b) to assess the adverse effects of TM on the liver of lizards, (c) to evaluate if the effects of TM in the lizard are mediated by PPARs.

2. Materials and methods

Thiophanate methyl technical product (CAS no. 23564-05-8, 96.2%) was obtained from SIPCAM (Milano, Italy). Antibodies against polyclonal Caspase-3, polyclonal PPARα were from Santa Cruz (CA, USA); polyclonal anti-catalase was from Rockland (Gilbertsville, PA, USA); peroxidase conjugated anti-rabbit or anti-mouse IgG, polyclonal anti-actin, diaminobenzidine, Nonidet P-40, sodium deoxycholate, sodium dodecyl sulphate, sodium fluoride, tetrasodium pyrophosphate, orthovanadate, ethylenediaminetetraacetic acid, 3,3′-diamino-benzidine (DAB), phenyl-methylsulfonyl fluoride, aprotinin, leupeptin, pepstatin, polyacrylamide were from Sigma Co. (St. Louis, MO). Protein assay kit was from Pierce (Rockford, IL, USA). Polyvinyldifluoride membrane was from Millipore Corporation (Bedford, MA, USA). Nonfat dry milk was from Biorad laboratories (Hercules, CA, USA). Enhanced chemiluminescence kit was from Amersham (Little Chalfont, Buckinghamshire, UK).

2.1. Chronic treatment

Thirty adult males of the lizard *Podarcis sicula* (mean mass 10 g), captured in the field around Benevento and Caserta, Italy, were subdivided into two groups of fifteen animals. One group was used as control, one as experimental group. The animals were housed in 25 × 50 cm glass terraria with the soil covered with leaves and branches of tree to mimic natural habitat at seasonal temperature and photoperiod, fed with *Tenebrio molitor* larvae and slices of fresh tomatoes, watered ad libitum and used after an acclimation period of at least one week. The experiment was performed in the months of February–March. To mimic the treatment procedures for spraying agricultural soils we refer to the concentration of TM used to spray one hectare of field and compare with the measures of aquaria to calculate the TM dose. TM 1.5% in water was used to spray the aquaria, the food and water. TM solution was administered by spraying with low pressure device every one week for one month, 2 h before the lizards were housed in. The mortality of animals after the TM exposure was of 3%.

At the thirtieth day the animals were killed under anaesthesia with ether vapours. The livers were quickly excised and then cut longitudinally into two parts. One was used for Western and immunoblotting, the other was fixed either in Bouin's fluid, in Carnoy, or in 10% phosphate-buffered saline (PBS) formaldehyde on ice for 4–5 h, dehydrated, embedded in paraplast and cut at 5 μm. Sections were stained either with haematoxylin–eosin or for histochemical detection of glycogen content by periodic acid for Schiff reaction (PAS) with or without previous diastase digestion.

2.2. Immunocytochemistry

Serial sections were immunostained by PAP (peroxidase–antiperoxidase) reaction using mammalian polyclonal antibodies (Sternberger, 1974). Polyclonal antibodies against Caspase-3 (1:100), PPARα (1:100), Catalase (1:100) Acyl CoA oxidase (AOX) (1:100), (generous gift of Prof. Singh, Medical University of South Carolina, USA) affinity purified polyclonal super oxide dismutase 1 (SOD1) (1:500 purified by Prof. Cimini), and TGFβ-1 (1:300 Sigma) were used.

The following control procedures were performed to test the specificity of the reagents: (1) omission of the primary antiserum and incubation of the sections either with immune or non-immune serum (1:10 and 1:20); (2) use of reptilian and mammalian livers as positive controls; (3) absorption of the primary antiserum with its specific peptide (10 nmol/mL of optimally diluted antiserum) for 24 h at 4 °C. When specific peptides were used, the staining was abolished.

The immunocytochemical reactions were performed on all the treated animals and six controls.

2.3. Tunel reaction

The Tunel reaction was performed with a DNA fragmentation detection kit (Tdt-FragEL™ — Calbiochem). Positive and negative controls to Tunel reaction were performed in each experiment. Positive control sections were treated with Proteinase K (1:100 in 10 mM Tris pH 8) for 10 min at room temperature and then exposed to Tdt (Terminal deoxynucleotidyl transferase), biotin-labeled and biotin-unlabeled deoxynucleotides. Biotinylated nucleotides were detected using a streptavidin–horseradish (HRP) conjugate: diamino-benzidine reacts with the labeled sample to generate an insoluble colored substrate at the site of DNA fragmentation. For negative controls, Tunel reaction was performed without adding the Tdt enzyme.

The Tunel reaction was performed on five treated specimens that presented more accentuated morphological alterations of nuclei at histological observations and five random controls.

Light microscopic observations were performed using a Zeiss Axioskop microscope equipped for bright field and fluorescence microscopy. Images were captured either onto photographic film (Ilford Pan F) or using a TV camera attached to the microscope.

2.4. Preparation of liver extract

The livers of animals that underwent the dose–response experiment was rinsed with PBS, removed, weighed, minced, pooled and homogenized (10% w/v) using a glass potter homogenizer in lysis buffer (RIPA) (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate, SDS, 100 nM sodium fluoride, 2 mM tetrasodium pyrophosphate, 2 mM orthovanadate and 5 mM ethylenediaminetetaacetic acid, EDTA, in PBS), freshly added with protease inhibitors (1 mM phenyl-methylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin), filtered through three layers of cheese-cloth and centrifuged at 3500 ×*g* at 4 °C for 30 min. The supernatant was collected and stored at −80 °C until further analysis (liver extract). All procedures were carried out at 4 °C.

2.5. Electrophoresis and Western blotting

Protein concentration was determined by the bicincholinic acid protein assay kit. Samples of liver extract (50 μg total proteins each) were run on 10% SDS polyacrylamide gel under denaturing conditions as described by Laemmli (1970). After electrophoresis proteins were transferred onto polyvinyldifluoride membrane according to Towbin et al. (1979). The polyvinyldifluoride membranes were blocked by 5% nonfat dry milk in Tris-buffered saline (TBS: 20 mM Tris–HCl, pH 7.4 containing 150 mM NaCl), overnight at 4 °C. Blots were probed for 2 h at RT with either of the following primary rabbit polyclonal antibodies: anti-PPARα (1:1000), anti-catalase (1:100), anti-SOD1 (1:1000), anti-AOX (1:200), and anti-actin (1:1000). All the antibodies were diluted with TBS containing 0.25% Tween-20 (TTBS) and 5% nonfat dry milk. As secondary antibodies, peroxidase conjugated anti-rabbit or anti-mouse IgG (1:2000) in TTBS containing 5% nonfat dry milk were used for 1 h at RT. Molecular markers were from Sigma.

Immunoreactive bands were visualized by enhanced chemiluminescence, according to the manufacturer's instructions. The relative densities of the immunoreactive bands were determined and normalized with respect to β-actin, using a semiquantitative densitometric analysis (Kodak ID Image Analysis software Rochester, NY, USA).

2.6. Statistical analysis

Statistical significance of paired samples was performed by SPSS software and analyzed by ANOVA test, followed by Scheffe's "post hoc test" analysis. *, $p<0.05$; **, $p<0.001$.

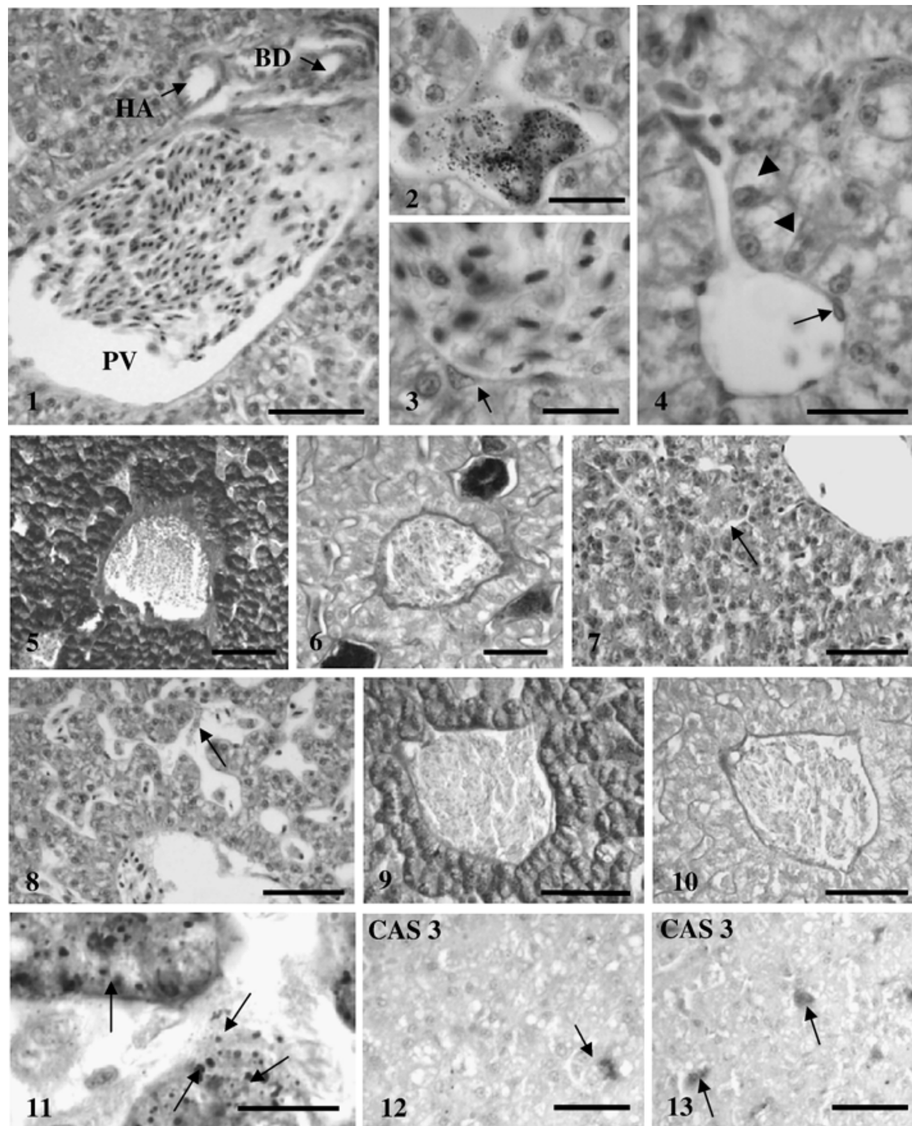
3. Results

3.1. Histology of liver of P. sicula

3.1.1. Control group

The hepatic parenchyma of *Podarcis sicula* consisted of anastomosing tubules formed by hepatocytes arranged predominantly in a two-cell thick wall. The lumina of the tubules formed the bile canaliculi; among the tubules very tortuous sinusoids lied with fenestrated endothelial wall. The tubules connect with ducts near the portal areas in which were present branches of portal vein, of hepatic artery and a bile duct, which constituted the portal triads (Fig. 1). Arterial and venous blood supply emanating from one triad anastomosed into sinusoids. They crossed the parenchyma and delivered the blood into hepatic venules scattered throughout the liver parenchyma.

There are different liver cell types: tall and columnar hepatocytes which formed the wall of tubules: pigmented Kupffer cells, the melanomacrophages, often clustered in groups of 3–4 cells inside the sinusoids (Fig. 2), Ito stellate cells localized in the space between the basal lamina of hepatocytes and fenestrated endothelium, called the space of Disse (Fig. 3), the endothelial cells and finally the blood cells (Fig. 4). Little connective tissue surrounded the portal triads. Bile ducts of superior order ultimately collecting in the hepatic duct outside the liver were surrounded by connective tissue which followed them up to the exit from the liver. The hepatocytes of *Podarcis* were large cells with a round euchromatic nucleus and a prominent nucleolus. The cytoplasm appeared clear with many lipid droplets (Fig. 4). The glycogen content appeared as a large cluster of PAS positive material, not



Figs. 1–13. 1. A particular of liver section of a control lizard showing the portal triad with branches of the portal vein (PT), the hepatic artery (HA, arrow) and the bile ducts (BD, arrow) in the portal area. HE stain. Bar 50 μm. 2. Liver of a control lizard. A cluster of melanomacrophages was present in blood vessels inside the hepatic parenchyma. HE stain. Bar 50 μm. 3. Control lizard. A blood vessel filled with blood cells in the hepatic parenchyma. The arrow points to an Ito cell, localized in the Disse space between the basal lamina of hepatocytes and the fenestrated endothelium. HE stain. Bar 50 μm. 4. Control lizard. A blood vessel in the hepatic parenchyma: the arrow indicates the endothelial cells, the arrowheads point to the vacuoles in the cytoplasm of the hepatocytes. HE stain. Bar 50 μm. 5. Control lizard. Two adjacent sections stained by PAS method without and with previous diastase digestion to show that PAS positive content of hepatocytes was formed of glycogen. Note that glycogen is mainly located above the nucleus of hepatocytes. HE stain. Bar 50 μm. 6. Control lizard. Two adjacent sections stained by PAS method without and with previous diastase digestion to show that PAS positive content of hepatocytes was formed of glycogen. Note that glycogen is mainly located above the nucleus of hepatocytes. HE stain. Bar 50 μm. 7. A liver section of a control specimen showing blood vessels supplying the parenchyma (arrow) in comparison with that of a TM-treated lizard in which the hepatic parenchyma shows particularly dilated sinusoids (arrow). HE stain. Bar 50 μm. 8. A liver section of a control specimen showing blood vessels supplying the parenchyma (arrow) in comparison with that of a TM-treated lizard in which the hepatic parenchyma shows particularly dilated sinusoids (arrow). HE stain. Bar 50 μm. 9. TM-treated lizard. Two adjacent sections stained by PAS technique without and with previous diastase digestion to show the decrease of glycogen content of hepatocytes in comparison with that of control lizards. PAS method. Bar 50 μm. 10. TM-treated lizard. Two adjacent sections stained by PAS technique without and with previous diastase digestion to show the decrease of glycogen content of hepatocytes in comparison with that of control lizards. PAS method. Bar 50 μm. 11. TM-treated lizard. Only PAS positive small granules of glycogen are present in the hepatocytes. Very small granules could be seen in the blood (arrow). PAS technique. Bar 50 μm. 12. Liver section of control and TM-treated lizards, respectively. Rare immunoreactive-cells for caspase-3 (CAS 3) are detected (arrow) in the control liver. A slight increase is present in TM-treated liver (arrows). PAP technique, DAB stain. Bar 100 μm. 13. Liver section of control and TM-treated lizards, respectively. Rare immunoreactive-cells for caspase-3 (CAS 3) are detected (arrow) in the control liver. A slight increase is present in TM-treated liver (arrows). PAP technique, DAB stain. Bar 100 μm.

present in diastase-treated sections, which occupied the apical part of hepatocytes (Figs. 5 and 6). It was not uniformly distributed in the hepatic parenchyma, showing a patched localization.

3.2. TM-exposed lizards

The histology of the liver of TM-exposed lizards compared with that of the control animals showed particularly dilated

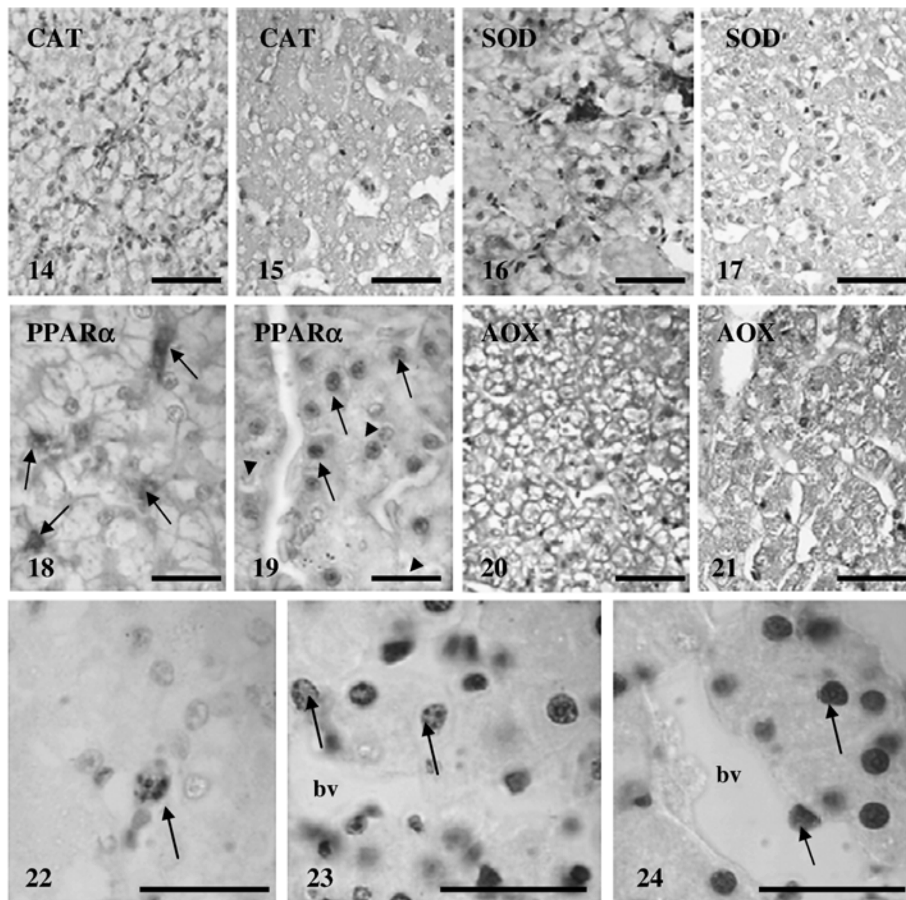
sinusoids (Figs. 7 and 8). The glycogen content of hepatocytes was decreased and fragmented in small granules, as revealed by PAS reaction (Figs. 9 and 10). Very small granules of glycogen could be seen in blood vessels (Fig. 11).

3.3. Immunocytochemistry

Immunoreactivity to caspase–3 was detected in a few hepatocytes and in bile ducts of the controls; in the treated lizards the number of immunoreactive cells slightly increased (Figs. 12 and 13).

A diffuse immunostaining for catalase and for Cu, Zn- and Mn-superoxide dismutases was found in hepatocytes and melanomacrophages of controls, but in the treated lizards the intensity of immunostaining for catalase was only slightly increased while that for SOD1 and 2 appeared weaker than in controls (Figs. 14–17).

Immunoreactivity for PPARα in the control lizards was evident in some nuclei of hepatocytes and in vascular endothelium, while in the treated animals PPARα immunoreactivity was found both in cytoplasm and in most of the nuclei of hepatocytes, in melanomacrophages and in blood cells



Figs. 14–24. 14. Immunoreactivity for catalase in the liver section of control and TM-treated lizards, respectively. The TM-treated liver shows a diffuse, weaker immunoreactivity for catalase than the control liver. PAP technique, DAB stain. Bar 50 μm. 15. Immunoreactivity for catalase in the liver section of control and TM-treated lizards, respectively. The TM-treated liver shows a diffuse, weaker immunoreactivity for catalase than the control liver. PAP technique, DAB stain. Bar 50 μm. 16. Immunoreactivity for SOD1 in the liver section of control and TM-treated lizards, respectively. The TM-treated liver shows a lighter immunoreactivity for SOD1 than the control liver. PAP technique, DAB stain. Bar 50 μm. 17. Immunoreactivity for SOD1 in the liver section of control and TM-treated lizards, respectively. The TM-treated liver shows a lighter immunoreactivity for SOD1 than the control liver. PAP technique, DAB stain. Bar 50 μm. 18. Immunoreactivity for PPARα in the liver section of control and TM-treated lizards respectively. In the control specimen the immunoreactivity for PPARα can be detected in the blood cells only (arrows), while in TM-treated lizards the majority of nuclei are labelled (arrows), few of them remain unlabelled (arrowheads). PAP technique, DAB stain. Bar 50 μm. 19. Immunoreactivity for PPARα in the liver section of control and TM-treated lizards respectively. In the control specimen the immunoreactivity for PPARα can be detected in the blood cells only (arrows), while in TM-treated lizards the majority of nuclei are labelled (arrows), few of them remain unlabelled (arrowheads). PAP technique, DAB stain. Bar 50 μm. 20. Immunoreactivity for AOX in the liver section of control and TM-treated lizards, respectively. The immunoreactivity for AOX is absent in the control liver while in the TM-treated liver it is diffusely present in the hepatocytes. PAP technique, DAB stain. Bar 50 μm. 21. Immunoreactivity for AOX in the liver section of control and TM-treated lizards, respectively. The immunoreactivity for AOX is absent in the control liver while in the TM-treated liver it is diffusely present in the hepatocytes. PAP technique, DAB stain. Bar 50 μm. 22. Rare nuclei Tunel-positive are constantly present in the liver of control lizards (arrow). bv=blood vessel. Tunel reaction. Bar 50 μm. 23. Tunel reaction in TM-treated lizards. Numerous Tunel-positive nuclei (arrows) are localized around blood vessels. Their number is increased in comparison with the controls. bv=blood vessel. Bar 50 μm. 24. Tunel reaction in TM-treated lizards. Numerous Tunel-positive nuclei (arrows) are localized around blood vessels. Their number is increased in comparison with the controls. bv=blood vessel. Bar 50 μm.

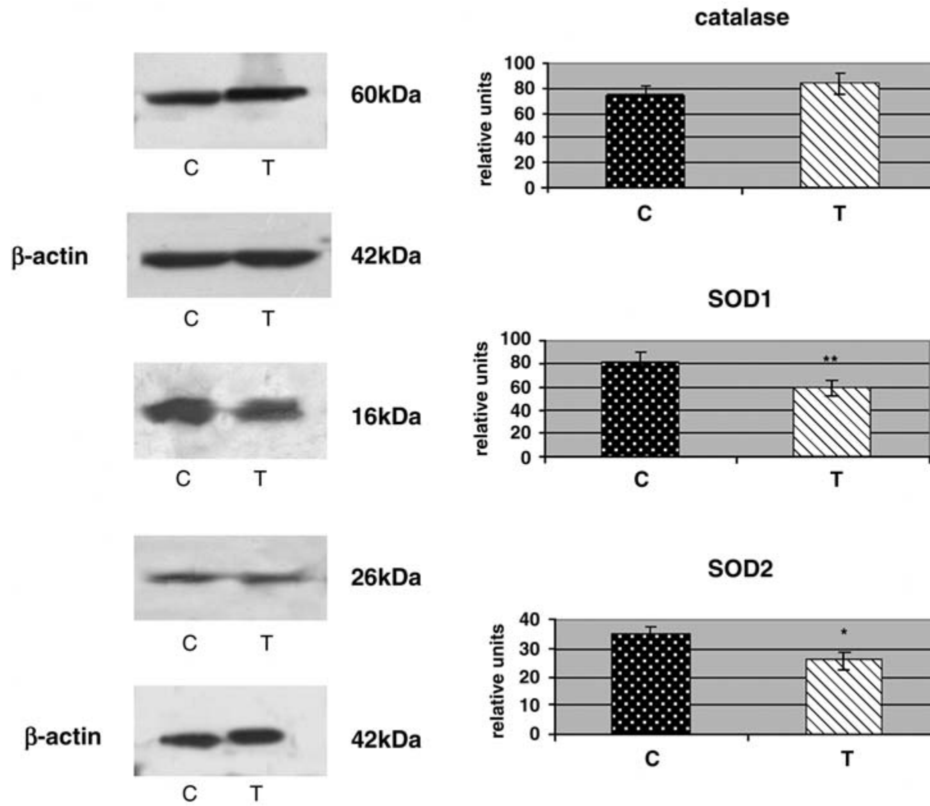


Fig. 25. Western blotting analysis for catalase, SOD2 and SOD1 in control and treated liver homogenates. No significant difference in catalase levels was observed while SOD2 and SOD1 appear significantly decreased in the treated animals. Data are means ± SD of 5 different experiments performed on pooled liver homogenates. An example of Western blotting is shown. *, $p<0.05$; **, $p<0.001$.

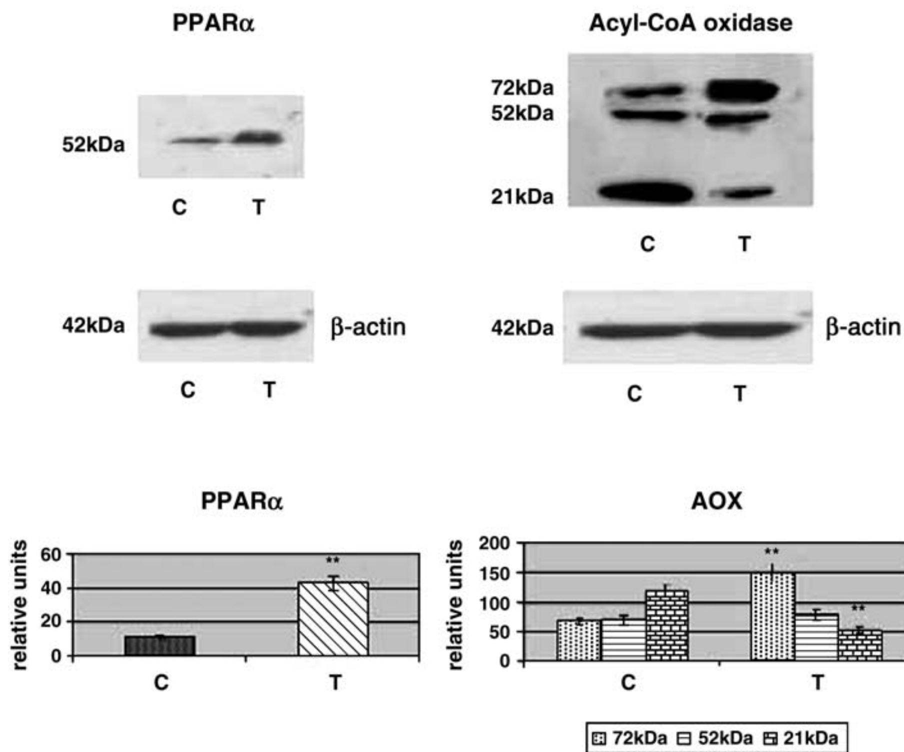


Fig. 26. Western blotting analysis for PPARα and AOX in control and treated liver homogenates. PPARα significantly increased more than fourfold with respect to the control values. AOX showed a distinctive pattern: the 72 kDa full-length form increased significantly after treatment, while the 52 kDa processed form did not significantly vary and the 21 kDa form significantly decreased in the treated lizard with respect to the controls. Data are means ± SD of 5 different experiments performed on pooled liver homogenates. An example of Western blotting is shown. *, $p<0.05$; **, $p<0.001$.

(Figs. 18 and 19). The immunostaining for acyl-CoA oxidase (AOX) was also increased in hepatocytes of TM exposed lizard in comparison with controls (Figs. 20 and 21).

3.4. Tunel reaction

In the control specimens a few nuclei of hepatocytes were positive to Tunel reaction, while in the treated lizards the positivity to Tunel was evident particularly around arborizations of portal vein where endothelial cells and many nuclei of hepatocytes were positively immunostained (Figs. 22–24).

3.5. Western blotting

The Western blotting of liver homogenates from control and treated lizards showed no significant difference in catalase content, whereas SOD2 and SOD1 significantly decreased in the treated animals (Fig. 25). PPARα significantly increased more than fourfold with respect to the control values. AOX showed a distinctive pattern: the 72 kDa full-length form increased significantly after treatment, while the 52 kDa processed form did not significantly vary and the 21 kDa form significantly decreased in the treated lizard with respect to the controls (Fig. 26).

4. Discussion

This study examined the alterations of liver of the lizard *P. sicula* following the TM exposure in conditions similar to those of agricultural and horticultural practice. Several studies examined the susceptibility of reptiles to toxic chemicals (Lambert, 1997; Campbell and Campbell, 2000, 2002; Sanchez-Hernandez and Sanchez, 2002; Shen et al., 2005; Holem et al., 2006) but few data are available on the effects of the fungicides on lizards. Thiophanate methyl is considered a weak endocrine disruptor, showing effects at high dose levels (560 mg/kg bw) in mammals (Maranghi et al., 2003). In fact in rat pups exposed in utero, the fungicide caused histological and histomorphometric alterations in thyroid and adrenals, namely increased karyomegaly and hydropic degeneration in the adrenal cortex and a slight decrease of the relative cortex/medulla area ratio. Mice treated with thiophanate methyl (192 mg/kg bw) showed a twofold increase in adrenal gland weights (Thomas and Schein, 1974). Rats exposed to carbendazim (300 and 600 mg/kg bw per day for 15 weeks), a metabolite of thiophanate methyl and a systemic broad-spectrum fungicide, exhibited an increase in serum T_3 levels and histopathological changes in thyroid and parathyroid glands and degeneration of adrenal tissue (Barlas et al., 2002). In ectotherm vertebrates a recent study on the amphibian *Triturus carnifex* (Capaldo et al., 2006) showed that TM caused alteration in adrenals increasing catecholamine, especially epinephrine, release and decreasing both synthesis and release of corticosterone and aldosterone.

The present study is the first report dealing with the effects of thiophanate methyl on liver morphology and some metabolic activities as well as of peroxisomal enzymes and PPARs in lizards. Our data demonstrated that exposure to sublethal concentration of TM results in hepatocellular changes as it is evident by the enlargement of the sinusoids and by glycogen depletion suggestive of possible adaptative response. Glycogen depletion has been observed in fish Nile Tilapia (*Oreochromis niloticus*) after exposure to fungicide Mancozeb by Figueiredo-Fernandes et al. (2006), and in rats exposed to the PPARα ligand clofibric acid (Cheon et al., 2005) suggesting that PPARα is likely to play a central role in adaptation, whereas enlargement of sinusoids has been described by Selmanoglu et al. (2001) in liver and kidney of rats administered with carbendazim.

In *Podarcis*, TM increases caspase 3 and Tunel positive hepatocytes. It is known that peroxisome proliferators induce hepatocyte cell proliferation and suppress apoptosis leading to tumors only in rodents. In our model, an increase of apoptotic events is observed, suggesting that in this species PPAR activation leads to the activation of different pathways. This finding is in agreement with other reports describing apoptosis induction by peroxisome proliferators (PPs) in mammalian liver (Canuto et al., 1998), breast, stomach and endothelium cancer cells (Elstner et al., 1998; Takahashi et al., 1999; Bishop-Bailey and Hla, 1999). Activation of PPARα and γ induces apoptosis in macrophages activated with TNFα/interferon γ by negative interfering with the anti-apoptotic nuclear factor-kappaB (NF-kB) signalling pathway (Chinetti et al., 1998). In mouse hepatocytes nafenopin, a potent peroxisome proliferator, inhibited bcl-2 and bak, two proteins involved in the control of cell apoptosis (Christensen et al., 1998). The different conclusions about the role of PPs in programmed cell death likely depend upon the PPAR subtype which they activate and/or could reflect differences in signalling pathways of different cell models (Keller et al., 2000). Moreover the increase of ROS level may activate the apoptotic mitochondrial pathway. In fact, immunocytochemical detection of catalase in control and exposed lizards and its semiquantitative assessment by Western blotting demonstrated no significant increase in exposed animals though the toxic effect of fungicide is known to be related to its capacity for catalyzing oxidative reactions (or to increase H_2O_2-producing enzymes), leading to the production of reactive oxygen species (ROS) (Lopes et al., 2001). These highly reactive compounds may also induce tissue alterations and change some physiological responses of fish, leading to oxidative stress (Sies, 1991; Paris-Palacios et al., 2000; Varanka et al., 2001). Moreover, Fimognari et al. (1993) showed that in human peripheral blood lymphocytes treated in vitro with TM the frequency of apoptotic cells was increased in function of the concentration while cellular proliferation was delayed. Regarding SOD1, the cytosolic and peroxisomal form, and SOD2, the mitochondrial form, of O_2^- scavenger enzymes, TM appears to decrease both enzymes as assessed by immunocytochemistry and immunoblotting.

TM may act, like other pesticides, as a potential peroxisomal proliferator. In our experimental model peroxisomal proliferation is suggested by AOX increase as revealed by immunocytochemistry and immunoblotting results.

The AOX increase, a H_2O_2-producing enzyme, is not accompanied by catalase increase, leading to a H_2O_2 imbalance. This fact may lead to oxidative stress not counteracted by an

increase of ROS scavenger enzymes. This result is in agreement with other reports on rodent liver, where a ROS-depending hepatocarcinogenesis has been proposed (Schrader and Fahimi, 2004).

Parallel to the observed AOX increase, a significant increase of PPARα protein, its regulator transcription factor, is observed. It is therefore possible to hypothesize that TM, as other xenobiotics, may activate and increase PPARα leading to the increase of the transcription of its target gene, AOX. Recently, peroxisomal proliferation and AOX have emerged as a sensitive biomarker of exposure to several organic pollutants in mussels (Carajavaille et al., 2003), but in our knowledge up to now, these studies have concerned only aquatic organisms. Our experimental model, *Podarcis sicula*, appears, since its features of sentinel species, to be a useful tool to assess environmental pollution in terrestrial habitats. On the basis of the data presented here we suggest that also in *Podarcis* AOX and PPARα may be proposed as novel markers of organic pollution in terrestrial environments.

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