# Melatonin restores the basal level of lipid peroxidation in rat tissues exposed to potassium bromate *in vitro*

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## Abstract

**OBJECTIVE:** Potassium bromate ($KBrO_3$) is a prooxidant and carcinogen. Melatonin is a highly effective antioxidant. Indole-3-propionic acid (IPA; indole substance) and propylthiouracil (PTU; antithyroid drug) reveal some antioxidative effects. The aim of the study was to evaluate $KBrO_3$-induced lipid peroxidation (LPO) *in vitro* in tissues collected from control or melatonin-treated rats, and to compare potential preventive effects of melatonin, IPA and PTU.

**MATERIALS AND METHODS:** Kidney, liver and lung homogenates from either control or melatonin-pretreated rats (0.0645 mmol/kg b.w., i.p., twice daily, 10 days) were incubated in the presence of $KBrO_3$ (0.1, 0.5, 1.0, 2.5, 5.0, 10.0 mM). Then, control lung homogenates were incubated with $KBrO_3$ (10.0 mM) together with melatonin (0.01, 0.1, 0.5, 1.0, 5.0, 7.5 mM), or with IPA or with PTU. LPO products (malondialdehyde+4-hydroxyalkenals) were measured spectrophotometrically.

**RESULTS:** Melatonin injections prevented $KBrO_3$-induced LPO in lung homogenates. Melatonin, IPA and PTU, used *in vitro*, reduced $KBrO_3$-induced LPO in control lungs. Unexpectedly, $KBrO_3$ caused a concentration-dependent decrease in LPO in liver and kidney homogenates from control but not from melatonin-treated rats.

**CONCLUSIONS:** Potassium bromate-induced LPO in the rat lung homogenates suggests that the lung may be the target for this carcinogen. An exposure of organisms to melatonin decreases tissue sensitivity to $KBrO_3$-induced damage, possibly by restoring the oxidative balance.

ORIGINAL ARTICLE

**Abbreviations**

| | |
|---|---|
| Br· | - bromine radical |
| BrO | - bromine oxide |
| $BrO^-$ | - oxobromate ion |
| $BrO_2$ | - bromine dioxide |
| $H_2O_2$ | - hydrogen peroxide |
| IPA | - indole-3-propionic acid |
| $KBrO_3$ | - potassium bromate |
| LPO | - lipid peroxidation |
| MDA + 4-HDA | - malondialdehyde + 4-hydroxyalkenals |
| NO· | - nitric oxide |
| ·OH | - hydroxyl radical |
| $ONOO^-$ | - peroxynitrite anion |
| ROS | - reactive oxygen species |
| $O_2^{-}\cdot$ | - superoxide anion radical |
| PTU | - propylthiouracyl |

## INTRODUCTION

Potassium bromate ($KBrO_3$) has been classified as a compound belonging to Group 2B of carcinogens ("a possible human carcinogen") (IARC 1986). $KBrO_3$ has been demonstrated to induce tumors in rat kidneys (Kurokawa *et al.* 1990; Wolf *et al.* 1998). This agent was used as a food additive in flour treatment and as a component of cold-wave hair lotions and is still used in barley processing (FAO/WHO 1979). Since 1992, the usage of $KBrO_3$ in the treatment of flour has no longer been approved (JECFA 1992). Additionally, this chemical compound is still present in the environment as it is formed as an oxyhalide by-product during water ozonation (van Dijk-Looijaard and van Genderen 2000).

Oxidative stress plays an important role in the carcinogenicity of $KBrO_3$ (Sai *et al.* 1991). This agent has been demonstrated to cause oxidative modification of DNA, lipids and proteins (Chipman et., 1998; Murata *et al.* 2001). $KBrO_3$ decreases the activity of glutathione peroxidase – a well-known antioxidative enzyme, and increases formation of the following free radicals and reactive oxygen species (ROS): superoxide anion radical ($O_2^{-}\cdot$), nitric oxide (NO·) and peroxynitrite anion ($ONOO^-$) (Watanabe *et al.* 2002). However, experimental evidence suggests that the most reactive free radical – hydroxyl radical (·OH), and also $O_2^{-}\cdot$, as well as hydrogen peroxide ($H_2O_2$) are not directly involved in the prooxidative effects of $KBrO_3$, and the most well known antioxidative enzymes – superoxide dismutase and catalase – do not protect against oxidative DNA damage, due to this chemical compound (Murata *et al.* 2001). On the other hand, some other bromine compounds, such as oxobromate ions ($BrO^-$), bromine oxides (BrO, $BrO_2$) and bromine radicals (Br·), formed during $KBrO_3$ reduction by sulfhydryl compounds, such as glutathione or cysteine, have been found to be responsible for DNA damage and carcinogenicity of $KBrO_3$. This mechanism may be involved in $KBrO_3$-induced lipid peroxidation (LPO).

Due to the prooxidative action of $KBrO_3$, antioxidants are expected to protect against carcinogenicity caused by this compound. In fact, certain antioxidant substances, like ebselen (Watanabe *et al.* 2002), kolaviron (Farombi *et al.* 2002) and melatonin (El-Sokkary 2000) have been shown to prevent oxidative damage to macromolecules, caused by $KBrO_3$ in kidneys.

Melatonin (N-acetyl-5-methoxytryptamine) – the pineal hormone – effectively neutralizes free radicals and reactive oxygen species (ROS) and favourably influences oxidative enzymes (Reiter *et al.* 2000; Tan *et al.* 2007). It has been found to protect against oxidative damage to macromolecules, caused by different potential carcinogens and prooxidative agents (Buyukokuroglu *et al.* 2007; Karbownik *et al.* 2000a,b; 2001b,c,e; 2005; Karbownik and Lewinski 2003; Reiter *et al.* 2005).

Indole-3-propionic acid (IPA) is an indole substance with the chemical structure similar to that of melatonin, which also reveals antioxidative properties (Karbownik *et al.* 2001a,b,f).

Some antioxidative effects of propylthiouracil (PTU), an antithyroid drug, have also been found (Hicks *et al.* 1992). PTU has been shown to decrease LPO and the formation of NO·, and to increase the activity of superoxide dismutase (Seven *et al.* 2001).

The aim of the study was to evaluate the prooxidative effect of $KBrO_3$ in the rat kidney, liver and lung homogenates, and to compare potential protective effects of melatonin, IPA and PTU against LPO, caused by $KBrO_3$.

## MATERIALS AND METHODS

### *Chemicals*

Melatonin, IPA, PTU, and $KBrO_3$ were purchased from Sigma-Aldrich (St. Louis, MO). The LPO-586 kit for LPO was purchased from Calbiochem (La Jolla, CA). Other chemicals were of analytical grade and came from commercial sources.

### *Animals*

The procedures, used in the study, were approved by the Ethical Committee of the Medical University of Łódź. Rat tissues – kidney, liver and lung – collected from 17 animals of either the control group, previously treated with 0.9% NaCl:ethanol (vol:vol, 10:1) (i.p., 2 times daily, for 10 days) or a group of rats, previously treated with melatonin (0.0645 mmol/kg b.w., i.p., 2 times daily, for 10 days), were frozen on solid $CO_2$ and stored at –80 °C until assay.

### *Incubation of rat tissue homogenates*

Rat tissues were homogenized in ice cold 50 mM Tris-HCl buffer (pH 7.4) (5%, w/v), and then incubated for 30 min at 37 °C in the presence of examined substances.

In Experiment I, homogenates of rat tissues – liver and lung – collected from either the control or the melatonin treated rats, were incubated in the presence of $KBrO_3$, used in the following concentrations: 0.1, 0.5, 1.0, 2.5, 5.0, 10.0 mM. In the case of kidney


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homogenates, the range of examined concentrations additionally comprised 0.01 and 0.001 mM, because of an unexpected $KBrO_3$-induced decrease in LPO, as observed in the pilot study. A concentration-dependent increase in LPO was observed in the control lung homogenates (but not in homogenates of lung from melatonin-treated rats), with statistical significance for the concentration of 10.0 mM. This concentration was selected for the subsequent experiment to induce LPO.

As stimulatory effects of $KBrO_3$ on LPO had been observed exclusively in lung homogenates from the control rats, that tissue was the only one used in the two subsequent experiments for evaluation of the oxidative effect of selected antioxidants.

In Experiment II, in order to determine the effects of melatonin, IPA and PTU on the basal LPO in rat lung homogenates, the homogenates were incubated in the presence of melatonin in the concentrations of 0.01, 0.1, 0.5, 1.0, 5.0 and 7.5 mM, or in the presence of IPA or PTU in the concentrations of 0.01, 0.1, 0.5, 1.0, 5.0, 7.5 and 10.0 mM. The highest used concentration of melatonin was 7.5 mM, due to its limited solubility in ethanol – lower than that of IPA.

In Experiment III, lung homogenates were incubated in the presence of $KBrO_3$ (10.0 mM) in order to induce LPO and, additionally, in the presence of one of the antioxidants (melatonin or IPA or PTU) in the concentrations mentioned above, for evaluation of their potential protective effects.

The reactions were stopped by cooling the samples on ice. Each experiment was run in duplicate and repeated three times.

*Measurement of LPO products*

Concentrations of malondialdehyde + 4-hydroxyalkenals (MDA+4-HDA), as the index of LPO, were measured spectrophotometrically in the rat tissue homogenates. The homogenates were centrifuged at 3,000 × g for 10 min at 4 °C. The supernatant (200 µl) was mixed with 650 µl of a methanol/acetonitrile (1:3, v/v) solution, containing a chromogenic reagent, *N*-methyl-2-phenylindole, and vortexed. After adding 150 µl of methanesulfonic acid (15.4 M), the incubation was carried out at 45 °C for 40 min. The reaction between MDA + 4-HDA and *N*-methyl-2-phenylindole yielded a chromophore, which was spectrophotometrically measured at the absorbance of 586 nm, using a solution of 10 mM 4-hydroxynonenal as standard. The level of LPO is expressed as the amount of MDA + 4-HDA (nmol) per mg protein.

*Measurement of Protein*

Protein was measured, using the method of Bradford (1976), with bovine albumin as standard.

*Statistical Analysis*

The data were statistically analyzed, using unpaired Student's t-test. Results are expressed as means±SEM. Statistical significance was determined at the level of $p<0.05$.

## RESULTS

A tendency towards concentration-dependent increase in LPO was observed in the control lung homogenates after $KBrO_3$ treatment, with statistical significance for the concentration of 10.0 mM (Figure 1a). Thus, the concentration of 10.0 mM was selected for the subsequent experiment to induce LPO in the control lung homogenates. $KBrO_3$ treatment did not increase LPO in the lung homogenates from the melatonin-treated rats in any significant way (Figure 1b).

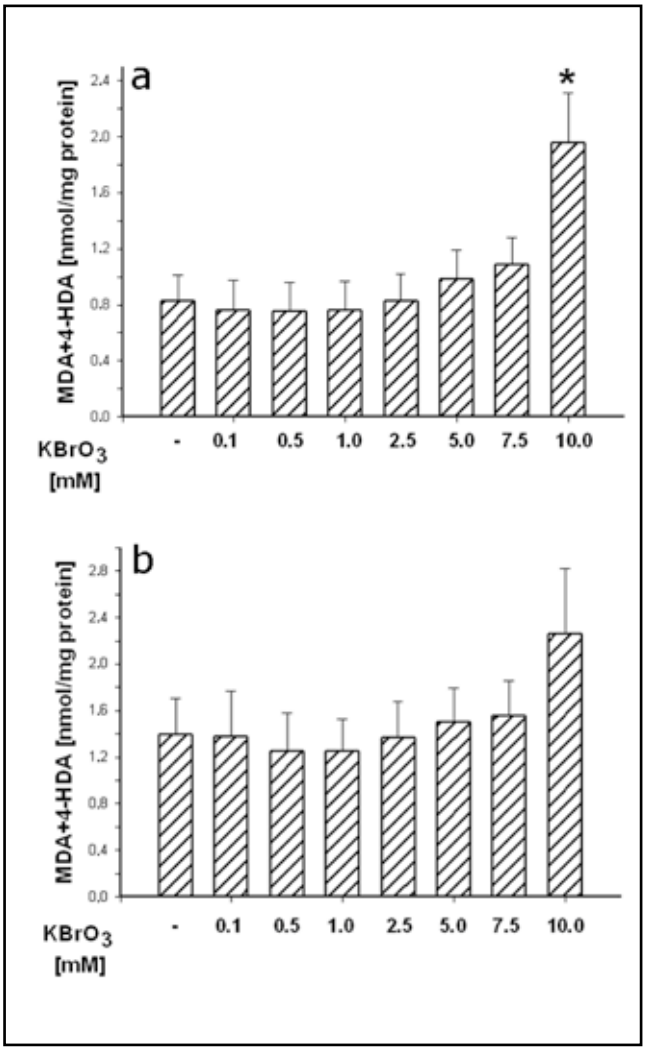


**Fig. 1.** Concentrations of malondialdehyde + 4-hydroxyalkenals (MDA+4-HDA) in homogenates of lungs, collected from the rats that were previously injected with 0.9% NaCl:ethanol (vol:vol, 10:1) (i.p., twice daily, for 10 days) (**a**) or melatonin (0.0645 mmol/kg b.w., i.p., twice daily, for 10 days) (**b**), incubated for 30 min in the presence of potassium bromate ($KBrO_3$) (0.1, 0.5, 1.0, 2.5, 5.0 and 10.0 mM). The results are expressed in nmol MDA+4-HDA per 1 mg protein. The bars represent the mean±SEM of three (3) independent experiments run in duplicates.
* $p<0.05$ vs. controls.

Unexpectedly, in kidney homogenates from both the control and the melatonin-treated rats, $KBrO_3$ caused a concentration-dependent decrease in LPO, with statistical significance for the concentrations of 1.0, 2.5, 5.0 and 10.0 mM in the control group (Figure 2a). Only for the highest applied concentration of 10.0 mM was the statistical significance recorded in the lung homogenates from the melatonin-treated rats (Figure 2b).

In the liver homogenates from the control rats, $KBrO_3$ tended to decrease the basal LPO with the statistical significance found only for the concentration of 10.0 mM (Figure 3a). In liver homogenates from the melatonin-treated rats, $KBrO_3$ did not significantly influence the level of LPO (Figure 3b).

The incubation of lung homogenates for 30 min in the presence of melatonin or PTU did not cause any changes in the level of basal LPO (Figure 4a,b). IPA, in the highest used concentration (10.0 mM), decreased the basal LPO in the lung homogenates (Figure 4c).

Melatonin – in the concentrations of 5.0 and 7.5 mM – significantly reduced $KBrO_3$-related increase in LPO in the control lung homogenates (Figure 5). Also IPA prevented $KBrO_3$-induced LPO but that effect reached statistical significance only for the highest concentration of 10.0 mM (Figure 6). Unexpectedly PTU, in all the used concentrations, reduced $KBrO_3$-induced LPO (Figure 7).

## DISCUSSION

An *in vitro* demonstration of the prooxidative effects of $KBrO_3$ in rat lung suggests that it may be the target organ for this carcinogen, but such an assumption awaits experimental confirmation. The stimulatory

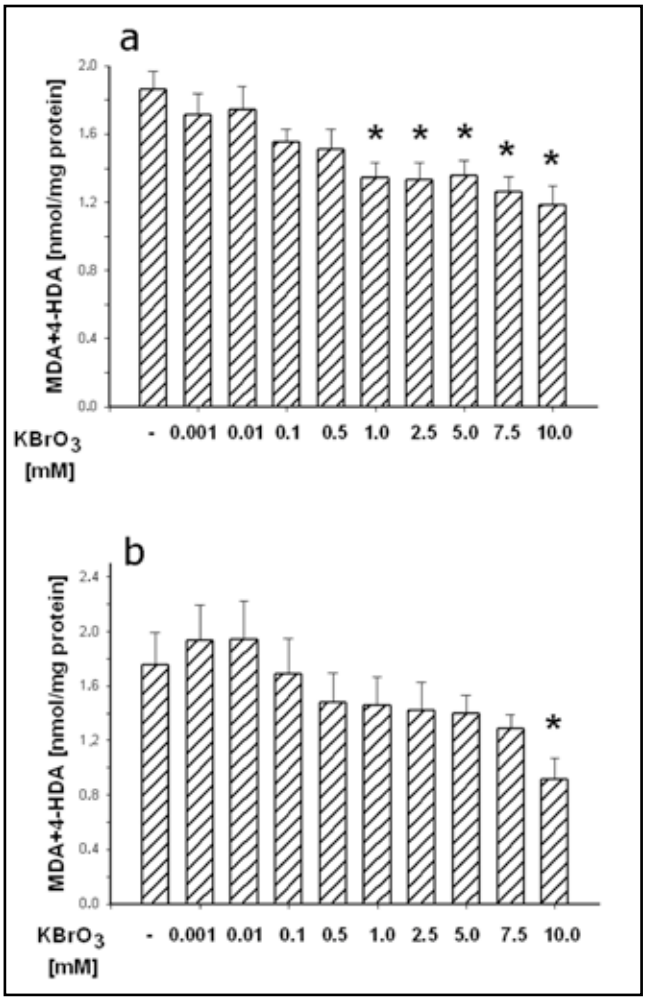


**Fig. 2.** Concentrations of malondialdehyde + 4-hydroxyalkenals (MDA+4-HDA) in kidney homogenates, collected from the rats that were previously injected with 0.9% NaCl:ethanol (vol:vol, 10:1) (i.p., twice daily, for 10 days) (**a**) or melatonin (0.0645 mmol/kg b.w., i.p., twice daily, for 10 days) (**b**), incubated for 30 min in the presence of potassium bromate ($KBrO_3$) (0.001, 0.01, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0 mM). The results are expressed in nmol MDA+4-HDA per 1 mg protein. The bars represent the mean±SEM of three (3) independent experiments run in duplicates. * $p<0.05$ vs. controls.

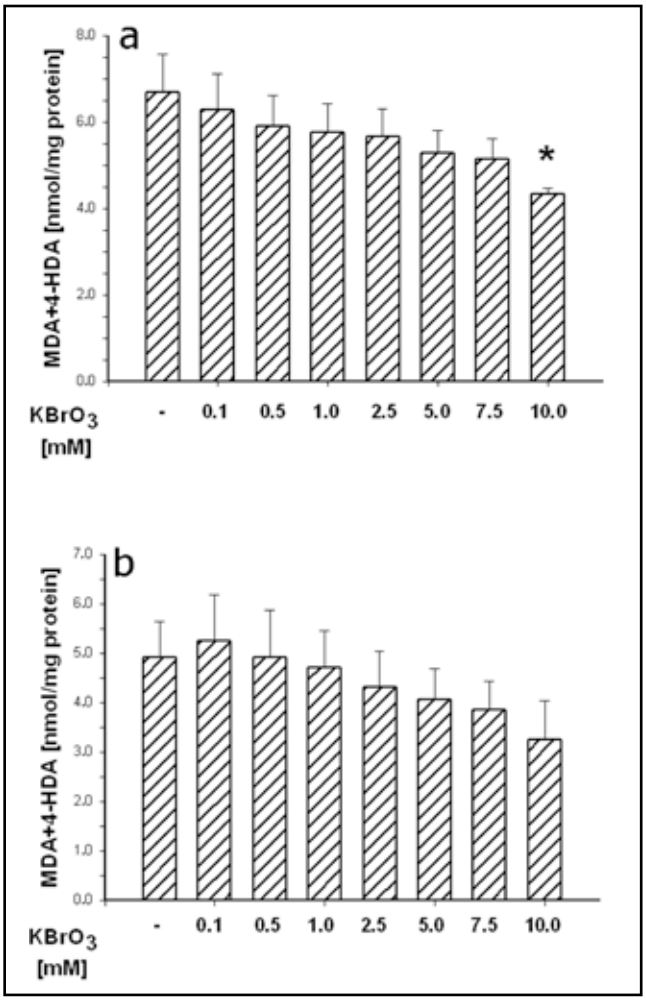


**Fig. 3.** Concentrations of malondialdehyde + 4-hydroxyalkenals (MDA+4-HDA) in liver homogenates, collected from the rats that were previously injected with 0.9% NaCl:ethanol (vol:vol, 10:1) (i.p., twice daily, for 10 days) (**a**) or melatonin (0.0645 mmol/kg b.w., i.p., twice daily, for 10 days) (**b**), incubated for 30 min in the presence of potassium bromate ($KBrO_3$) (0.1, 0.5, 1.0, 2.5, 5.0, 10.0 mM). The results are expressed in nmol MDA+4-HDA per 1 mg protein. The bars represent the mean±SEM of three (3) independent experiments run in duplicates.
* $p<0.05$ vs. controls.


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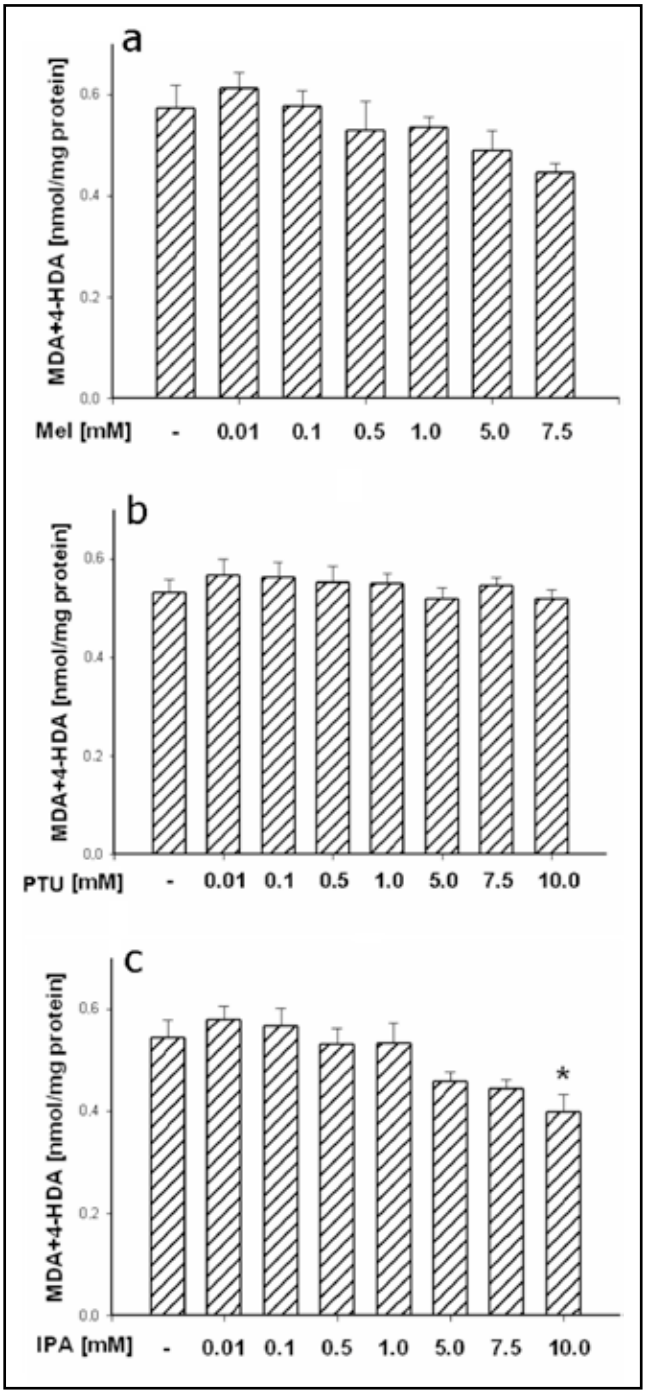


**Fig. 4.** Concentrations of malondialdehyde + 4-hydroxyalkenals (MDA+4-HDA) in rat lung homogenates, incubated for 30 min in the presence of melatonin (0.01, 0.1, 0.5, 1.0, 5.0 and 7.5 mM) (**a**) or propylthiouracil (PTU) (0.01, 0.1, 0.5, 1.0, 5.0, 7.5 and 10.0 mM) (**b**), or indole-3-propionic acid) IPA (0.01, 0.1, 0.5, 1.0, 5.0, 7.5, 10.0 mM) (**c**). The results are expressed in nmol MDA+4-HDA per 1 mg protein. The bars represent the mean±SEM of three (3) independent experiments run in duplicates.
* $p<0.05$ vs. controls.

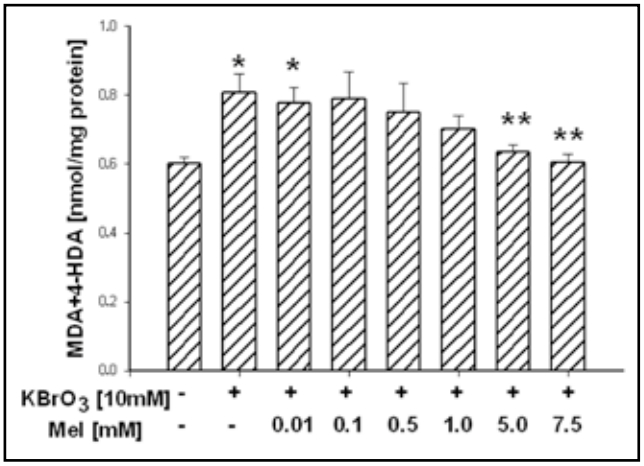


**Fig. 5.** Concentrations of malondialdehyde + 4-hydroxyalkenals (MDA+4-HDA) in rat lung homogenates, incubated for 30 min in the presence of potassium bromate ($KBrO_3$) (10.0 mM) and melatonin (0.01, 0.1, 0.5, 1.0, 5.0 and 7.5 mM). The results are expressed in nmol MDA+4-HDA per 1 mg protein. The bars represent the mean±SEM of three (3) independent experiments run in duplicates. * $p<0.05$ vs. controls; ** $p<0.05$ vs. $KBrO_3$.

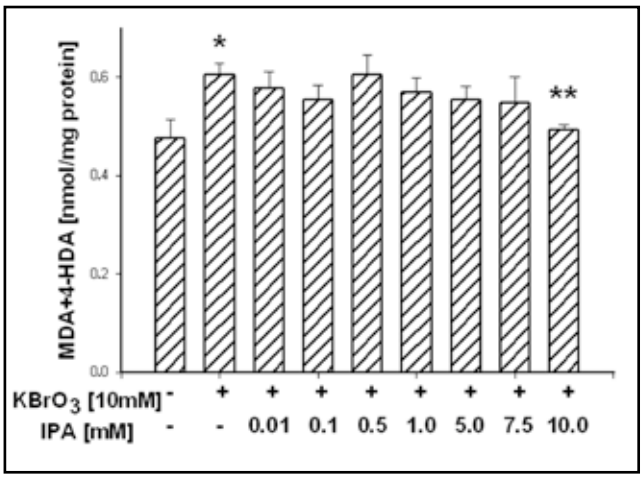


**Fig. 6.** Concentrations of malondialdehyde + 4-hydroxyalkenals (MDA+4-HDA) in rat lung homogenates, incubated for 30 min in the presence of potassium bromate ($KBrO_3$) (10.0 mM) and indole-3-propionic acid (IPA) (0.01, 0.1, 0.5, 1.0, 5.0, 7.5 and 10.0 mM). The results are expressed in nmol MDA+4-HDA per 1 mg protein. The bars represent the mean±SEM of three (3) independent experiments run in duplicates.
* $p<0.05$ vs. controls; ** $p<0.05$ vs. $KBrO_3$.

effect of $KBrO_3$ on LPO in rat lungs is our original observation.

In contrast to the present *in vitro* observation, in our earlier *in vivo* study (Karbownik *et al.* 2006) $KBrO_3$ did not increase LPO in rat lungs, which could then have resulted from the much lower carcinogen concentration in lungs after its i.p. injections, comparing to those obtained in lung homogenates under *in vitro* conditions.

Of great importance is the observation that $KBrO_3$ did not increase LPO in lung homogenates, collected from the rats which were pretreated with melatonin. This confirms that melatonin, when given *in vivo*, has a carry-over effect to the *in vitro* situation (Karbownik *et al.* 2001d). A similar effect of melatonin, given *in vivo*, was found when LPO was induced by Fenton reaction ($Fe^{2+} + H_2O_2$) in rat liver homogenates (Karbownik *et al.* 2000a). Thus, both the present and the earlier results suggest that melatonin, given to living organisms,

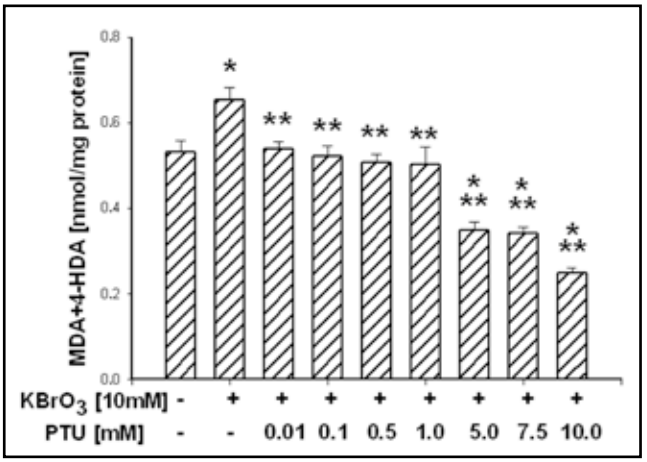


**Fig. 7.** Concentrations of malondialdehyde + 4-hydroxyalkenals (MDA+4-HDA) in rat lung homogenates, incubated for 30 min in the presence of potassium bromate ($KBrO_3$) (10.0 mM) and propylthiouracil (PTU) (0.01, 0.1, 0.5, 1.0, 5.0, 7.5 and 10.0 mM). The results are expressed in nmol MDA+4-HDA per 1 mg protein. The bars represent the mean±SEM of three (3) independent experiments run in duplicates.
* $p<0.05$ vs. controls; ** $p<0.05$ vs. $KBrO_3$.

decreases the sensitivity of different tissues to prooxidative effects of different exo- and/or endogenic factors.

A question arises if melatonin directly revealed the above mentioned protective effects by direct facing $KBrO_3$ (Karbownik and Reiter 2000; Ruiz-Rabelo *et al.* 2007; Vijayalaxmi *et al.* 1996). Due to the rather a short half-life of melatonin (approximately half an hour), no presence of this indole substance, given in pharmacological doses, should be expected in any tissue of the organism at time of tissue collections (in our study – 12 hours after the last melatonin injection). Instead, especially when given chronically, melatonin may change the level of oxidative balance, lowering the susceptibility of tissues to oxidative attacks and this could constitute the mechanism, explaining the protective effect of melatonin as described above.

Although $KBrO_3$ has been demonstrated to induce renal tumors (Kurokawa *et al.* 1990) and LPO (Karbownik *et al.* 2005; 2006) in rats *in vivo*, it was not found in our study to induce LPO in rat kidneys. Conversely, $KBrO_3$ decreased the basal level of LPO in kidney homogenates. Thus, the prooxidative mechanism of $KBrO_3$, involved in its tumorigenic effect, is not confirmed under *in vitro* conditions, at least with relation to oxidative damage to lipids. It does not mean, however, that under *in vitro* conditions $KBrO_3$ reveals any beneficial effects. The basal LPO illustrates the balance between the production and detoxification of reactive oxygen species, which, beside their well-known damaging effects, are also required for physiological processes. Thus, the significant decrease of LPO, following any carcinogen treatment, could disturb the level of oxidative balance and should be treated as an undesirable effect.

A similar effect of $KBrO_3$ was observed in the present study in rat liver homogenates, in which that prooxidant, when used in the highest concentration, decreased the basal level of LPO. The explanation of this phenomenon could be the same as above.

Whereas in both control tissues – the kidney and liver – $KBrO_3$ clearly decreased the basal LPO, such an effect was much weaker in tissues collected from the melatonin treated animals, while in the liver homogenates from melatonin-pretreated rats it completely disappeared. Thus, even in case of decreased LPO, due to external factors, melatonin, given *in vivo*, helps keep oxidative balance by restoring oxidative damage to the physiological level.

Any potential application of the present results in clinical practice requires discussing several points. Each potential antioxidant, used in humans, should be deprived of any side effects. Only such an oxidant could be used in humans, exposed to prooxidants and carcinogenic factors. Melatonin fulfills these requirements because no side effects of melatonin have ever been observed, even when very high pharmacological doses of that indoleamine were used either in humans (Vijayalaxmi *et al.* 1996) or in animals (Karbownik *et al.* 2000b).

Indole-3-propionc acid is an indole substance, possessing similar properties to melatonin. Subtle structural differences between these two molecules determine their different biological effects. In our earlier study, the protective effects of IPA against oxidative damage, caused by $KBrO_3$ given *in vivo*, were proven in such tissues as thyroid gland, kidney and blood serum (Karbownik *et al.* 2005; 2006). IPA also protects against prooxidative effects of some carcinogens under *in vitro* condition (Karbownik *et al.* 2001a). However, the current knowledge on the mechanism of IPA action, on its metabolism and, especially, on its absolute safety is not sufficient to recommend its use in clinical practice.

Among all three examined antioxidants, PTU appeared the most effective against $KBrO_3$-induced LPO in rat lungs. Similarly, in our earlier similar *in vitro* model, with the use of porcine thyroid, PTU was more effective than melatonin and IPA and under *in vivo* conditions, those three antioxidants revealed similar protective effects against $KBrO_3$-induced LPO in rat thyroid (Karbownik *et al.* 2005). However, the protective effect of PTU against $KBrO_3$-induced LPO provides merely some knowledge on the action of this antithyroid drug. Chronic treatment with PTU in euthyroid patients would definitely result in hypothyroidism, which excludes its potential use as exclusively that of an antioxidant. Additionally, serious side effects of PTU, such as agranulocytosis and toxic effects on the liver, are known (Bandyopadhyay *et al.* 2002).

In conclusion, the rat kidney, liver and lung reveal differential sensitivity to $KBrO_3$, while it is only the lung tissue which is susceptible to its prooxidative effects. This suggests that rat lung may be the target organ for $KBrO_3$. Exposure of an organism to melatonin decreases the sensitivity of its tissues to $KBrO_3$-induced oxidative changes (both increased and decreased oxi-


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dative damage), possibly by restoring the oxidative balance. Thus, melatonin could be recommended for its antioxidative and – possibly – cancer preventive effects. The protective effects of IPA and PTU against $KBrO_3$-induced LPO in homogenates of the rat lung have allowed to identify new details of the antioxidative mechanism of the agents in question. As on one hand IPA is not proved for its absolute safety, and – on the other PTU is used in the therapy of hyperthyroidism (causing hypothyroidism, when overdosed, and also other more serious side effects), these two compounds do not deserve for recommendation as regards their possible cancer prevention properties.

## ACKNOWLEDGEMENTS

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