Effects of growth hormone and insulin-like growth factor-I on the iron-induced lipid peroxidation in the rat liver and porcine thyroid homogenates

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Submitted: 2009-07-16 Accepted: 2009-08-29 Published online: 2010-08-27

Key words: **growth hormone; insulin-like growth factor-I; iron; lipid peroxidation**

Neuroendocrinol Lett 2010; **31**(4):517–523 **PMID:** 20802456 NEL310410A13

Abstract

OBJECTIVES: Growth hormone (GH) and its tissue mediator, insulin-like growth factor-I (IGF-I), are involved in oxidative processes, lipid peroxidation (LPO) included. Bivalent iron (Fe^{2+}) is frequently used to experimentally induce oxidative damage to macromolecules ($Fe^{2+} + H_2O_2 + H^+ \rightarrow Fe^{3+} + {}^{\bullet}OH + H_2O$). The aim of the study was to evaluate the effect of GH and/or IGF-I on the iron-induced LPO in the rat liver and porcine thyroid homogenates.

METHODS: Rat liver and porcine thyroid homogenates were incubated in presence of GH (100; 10; 1.0; 0.1; 0.01; 0.001; 0.0001 μg/ml) or IGF-I (1000; 100; 10; 1.0; 0.1; 0.01; 0.001; 0.0001 μg/ml) or GH (100 μg/ml) + IGF-I, or $FeSO_4$ + H_2O_2 plus GH and/or IGF-I. The level of LPO was expressed as concentrations of malondialdehyde+4-hydroxyalkenals (MDA+4-HDA) per mg of protein.

RESULTS: GH and/or IGF-I did not change the basal level of oxidative damage to lipids. In the rat liver homogenates, GH did not affect the iron-induced LPO, whereas IGF-I – in the lowest two concentrations – enhanced the process. In porcine thyroid homogenates, GH – in its two lowest concentrations – prevented, whereas in other concentrations, it enhanced the iron-induced LPO. IGF-I, in all used concentrations, enhanced the iron-induced LPO.

CONCLUSION: GH and/or IGF-I may reveal prooxidative effects. This fact does not support their application in the treatment of disorders associated with increased oxidative damage.

Abbreviations:

ANOVA	- one-way analysis of variance
GH	- growth hormone
IGF-I	- insulin-like growth factor-I
LDL	- low-density lipoprotein
LPO	- lipid peroxidation
MDA+4-HDA	- malondialdehyde + 4-hydroxyalkenals
PUFAs	- polyunsaturated fatty acids
ROS	- reactive oxygen species

INTRODUCTION

Reactive oxygen species (ROS), free radicals included, are formed in living organisms under physiological conditions. An overproduction of ROS, due to the action of either external or internal factors, may cause increased damage to macro-

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molecules, lipids included (Dröge 2002; Strosova *et al.* 2008; Valko *et al.* 2007). In turn, the products of oxidative damage to macromolecules may further enhance oxidative stress and damage all the components in the organism. Biological membranes, being a source of polyunsaturated fatty acids (PUFAs), are susceptible to free radical attacks. The action of free radicals on PUFAs results in lipid peroxidation (LPO), which causes structural changes in cellular membranes and impairs the cellular function.

Iron plays a crucial role in physiological processes, being a cofactor for many biological reactions and an essential element for metabolism in different tissues and organs. But – while being in excess – it causes toxic effects, altering cellular integrity and leading to organ dysfunction. Iron manifests its toxicity by a variety of reactions, principally *via* the production of free radicals. Bivalent iron (ferrous, Fe^{2+}), which initiates the Fenton reaction ($Fe^{2+} + H_2O_2 + H^+ \rightarrow Fe^{3+} + {}^{\bullet}OH + H_2O$) – the most basic reaction of oxidative stress, is frequently used to experimentally induce oxidative damage to macromolecules, LPO included (Karbownik *et al.* 2000; 2001; Karbownik & Lewinski 2003a).

Growth hormone (GH) is a polypeptide, which is produced in the anterior pituitary. The growth-promoting and metabolic actions of GH are mediated mainly by insulin-like growth factor-I (IGF-I), derived principally from the liver. It is well known that GH and IGF-I are involved in oxidative processes, especially in LPO, and may also influence antioxidative enzymes. Alterations in GH/IGF-I pathway (e.g., in conditions of GH overproduction or GH deficiency) are tightly linked with the enhanced oxidative stress. In patients with active acromegaly, increased plasma lipid peroxide level (the index of oxidative damage to LDL-cholesterol) was found, that abnormality being directly related to increased GH concentration (Yarman *et al.* 2003). At the same time, increased LPO was also observed in children (Mohn *et al.* 2005) and adults (Kokoszko *et al.* 2006; Ozbey *et al.* 2003; Scacchi *et al.* 2006) with GH deficiency. But, so far, no sufficient data have been available from *in vitro* studies, regarding the relationship between GH/IGF-I and oxidative stress.

The aim of the study was to evaluate the effect of GH and/or IGF-I on the iron-induced LPO in the rat liver and porcine thyroid homogenates.

MATERIAL AND METHODS

The procedures, used in the study, were approved by the Ethical Committee of the Polish Mother's Memorial Hospital – Research Institute.

GH from porcine pituitary, the human recombinant IGF-I expressed in *E. coli*, and ferrous sulphate ($FeSO_4$) 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.

Rat livers were collected from 20 male Wistar rats (the average age of 2 months; weighing about 160 g each). Porcine thyroids were collected from 20 male animals (the average age of 12 months) at a slaughterhouse. Immediately after collection, tissues were frozen on solid CO_2 and stored at –70 °C until assay.

Liver fragments and thyroid tissues were homogenized in ice cold 50 mM Tris-HCl buffer (pH 7.4, 10% or 5%, w/v – in final incubation volume, respectively) and then, incubated for 30 min at 37 °C in presence of the examined substances.

In order to induce LPO, homogenates of rat livers or porcine thyroids were incubated in presence of $FeSO_4$ (15 μM or 40 μM, respectively) + H_2O_2 (0.1 mM or 0.5 mM, respectively) (Karbownik *et al.* 2000; Karbownik & Lewinski 2003a). Additionally, liver or thyroid homogenates were incubated in presence of:

Experiment I: GH (100, 10, 1.0, 0.1, 0.01, 0.001, 0.0001 μg/ml) alone (to check its effect on the basal LPO), or $FeSO_4$ (15 μM or 40 μM, respectively) + H_2O_2 (0.1 mM or 0.5 mM, respectively) + GH (100, 10, 1.0, 0.1, 0.01, 0.001, 0.0001 μg/ml);

Experiment II: IGF-I (1000, 100, 10, 1.0, 0.1, 0.01, 0.001, 0.0001 μg/ml) alone (to check its effect on the basal LPO), or $FeSO_4$ (15 μM or 40 μM, respectively) + H_2O_2 (0.1 mM or 0.5 mM, respectively) + IGF-I (1000, 100, 10, 1.0, 0.1, 0.01, 0.001, 0.0001 μg/ml);

Experiment III: GH (100 μg/ml) + IGF-I (1000, 100, 10, 1.0, 0.1, 0.01, 0.001, 0.0001 μg/ml) (to check their joint effect on the basal LPO), or $FeSO_4$ (15 μM or 40 μM, respectively) + H_2O_2 (0.1 mM or 0.5 mM, respectively) + GH (100 μg/ml) + IGF-I (1000, 100, 10, 1.0, 0.1, 0.01, 0.001, 0.0001 μg/ml).

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

Measurement of LPO products

The concentrations of malondialdehyde + 4-hydroxyalkenals (MDA+4-HDA), as an index of LPO, were measured in liver and thyroid homogenates.

The homogenates were centrifuged at 3000 × g for 10 min at 4 °C. The supernatant 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 addition of 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 4-hydroxynonenal (10 mM) as standard.

The level of LPO was expressed as the amount of MDA+4-HDA (nmol) per mg of protein.

Measurement of protein concentration

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

Statistical analysis

The data were statistically analyzed, using a one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls' test. Statistical significance was determined at the level of $p<0.05$. The results are presented as means ± SEM.

RESULTS

In all the experiments, $FeSO_4$ + H_2O_2, added into the incubation medium, increased the level of LPO (Figures 1–6).

The incubation of rat liver or porcine thyroid homogenates in presence of either GH (used in concentrations of 100, 10, 1.0, 0.1, 0.01, 0.001, 0.0001 μg/ml) or IGF-I (used in concentrations of 1000, 100, 10, 1.0, 0.1, 0.01, 0.001, 0.0001 μg/ml), or GH (100 μg/ml) plus IGF-I (1000, 100, 10, 1.0, 0.1, 0.01, 0.001, 0.0001 μg/ml) did not affect the basal LPO (Figures 1–6).

In rat liver homogenates, GH, used together with $FeSO_4$ + H_2O_2, did not cause any changes in the iron-induced LPO (Figure 1). In turn, IGF-I, whereas in concentrations of 1000, 100, 10, 1.0, 0.1, 0.01 μg/ml did not affect the Fenton reaction-induced LPO, in the lowest used concentrations (0.001 or 0.0001 μg/ml), it even enhanced $FeSO_4$+H_2O_2-induced LPO in rat liver homogenates (Figure 2). The incubation of liver homogenates in presence of $FeSO_4$ (15 μM) + H_2O_2 (0.1 mM) + GH (100 μg/ml) + IGF-I (1000, 100, 10, 1.0, 0.1, 0.01, 0.001, 0.0001 μg/ml), failed to cause any significant changes in $FeSO_4$+H_2O_2-induced LPO (Figure 3).

In porcine thyroid homogenates GH, used in concentrations of 100, 10, 1.0, 0.01 μg/ml, significantly enhanced $FeSO_4$+H_2O_2-induced LPO, whereas in the lowest used concentrations, i.e. 0.001, 0.0001 μg/ml, it completely prevented $FeSO_4$+H_2O_2-induced LPO (Figure 4). IGF-I, in all used concentrations, significantly enhanced $FeSO_4$+H_2O_2-induced LPO. However, the stimulatory effects of the highest (1000, 100, 10, 1.0 μg/ml), as well as, the lowest (0.001, 0.0001 μg/ml) IGF-I concentrations were significantly stronger than those of IGF-I, used in concentrations of 0.1 and 0.01 μg/ml (Figure 5). When the homogenates were incubated in presence of GH (100 μg/ml) plus IGF-I (used in concentrations of 1000, 100, 10, 1.0, 0.1, 0.01, 0.001, 0.0001 μg/ml), they significantly – in all used concentrations of IGF-I – increased $FeSO_4$+H_2O_2-induced LPO (Figure 6).

DISCUSSION

Our study has been the first attempt to evaluate the effects of GH and IGF-I (used alone or together) on the basal and the iron-induced LPO in the liver and thyroid homogenates. The selection of the liver and the thyroid gland for such a study is justified by several factors. The liver is one of the target organs for GH and the main source of IGF-I, being the main mediator of GH tissue effects. The liver plays an important role in detoxifying processes; hepatocytes contain a variety

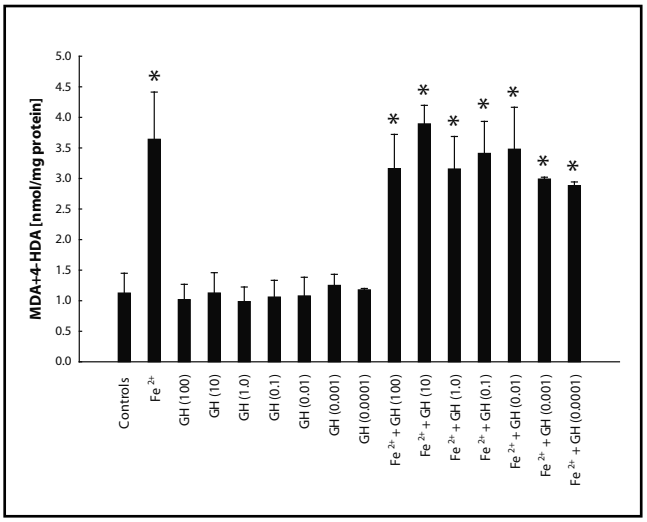

Fig. 1. Concentrations of MDA+4-HDA in rat liver homogenates, incubated for 30 min in presence of $FeSO_4$ (15 μM) + H_2O_2 (0.1 mM), or GH (100, 10, 1.0, 0.1, 0.01, 0.001, 0.0001 μg/ml), or $FeSO_4$ (15 μM) + H_2O_2 (0.1 mM) + GH (100, 10, 1.0, 0.1, 0.01, 0.001, 0.0001 μg/ml). Bars represent means ± SEM of three independent experiments run in duplicates. *$p<0.05$ versus Controls.

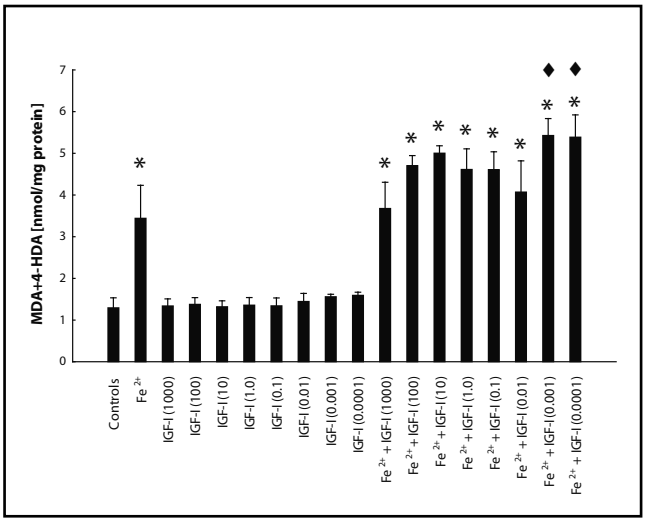

Fig. 2. Concentrations of MDA+4-HDA in rat liver homogenates, incubated for 30 min in presence of $FeSO_4$ (15 μM) + H_2O_2 (0.1 mM), or IGF-I (1000, 100, 10, 1.0, 0.1, 0.01, 0.001, 0.0001 μg/ml), or $FeSO_4$ (15 μM) + H_2O_2 (0.1 mM) + IGF-I (1000, 100, 10, 1.0, 0.1, 0.01, 0.001, 0.0001 μg/ml). Bars represent means ± SEM of three independent experiments run in duplicates. *$p<0.05$ versus Controls; ♦$p<0.05$ versus Fe.

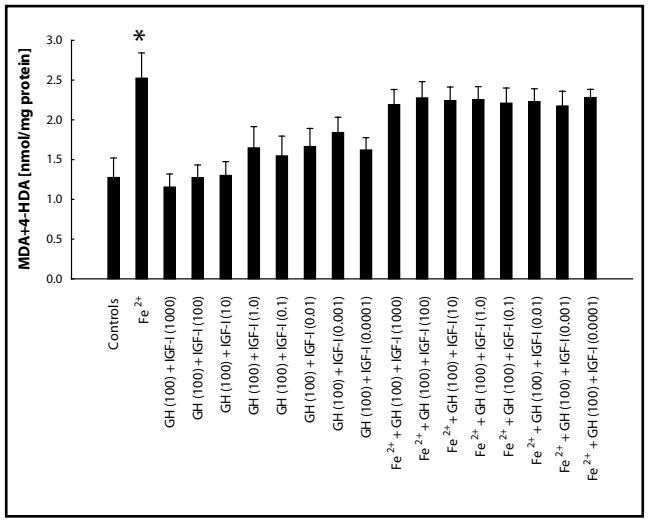

Fig. 3. Concentrations of MDA+4-HDA in rat liver homogenates, incubated for 30 min in presence of $FeSO_4$ (15 μM) + H_2O_2 (0.1 mM), or GH (100 μg/ml) + IGF-I (1000, 100, 10, 1.0, 0.1, 0.01, 0.001, 0.0001 μg/ml), or $FeSO_4$ (15 μM) + H_2O_2 (0.1 mM) + GH (100 μg/ml) + IGF-I (1000, 100, 10, 1.0, 0.1, 0.01, 0.001, 0.0001 μg/ml). Bars represent means ± SEM of three independent experiments run in duplicates. *p<0.05 versus Controls.

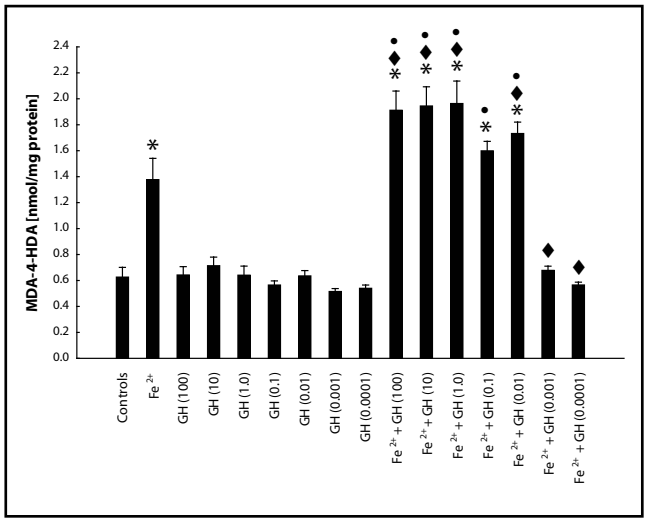

Fig. 4. Concentrations of MDA+4-HDA in porcine thyroid homogenates, incubated for 30 min in presence of $FeSO_4$ (40 μM) + H_2O_2 (0.5 mM), or GH (100, 10, 1.0, 0.1, 0.01, 0.001, 0.0001 μg/ml), or $FeSO_4$ (40 μM) + H_2O_2 (0.5 mM) + GH (100, 10, 1.0, 0.1, 0.01, 0.001, 0.0001 μg/ml). Bars represent means ± SEM of three independent experiments run in duplicates. *p<0.05 versus Controls; ♦p<0.05 versus Fe; ●p<0.05 versus Fe+GH 0.001 or Fe+0.0001.

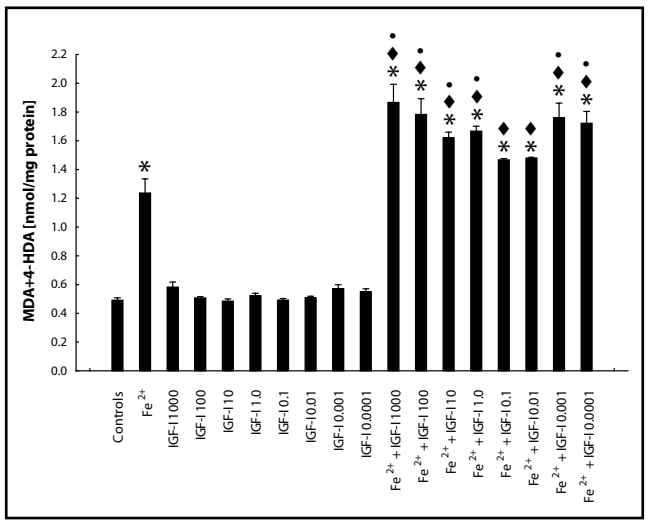

Fig. 5. Concentrations of MDA+4-HDA in porcine thyroid homogenates, incubated for 30 min in presence of $FeSO_4$ (40 μM) + H_2O_2 (0.5 mM), or IGF-I (1000, 100, 10, 1.0, 0.1, 0.01, 0.001, 0.0001 μg/ml), or $FeSO_4$ (40 μM) + H_2O_2 (0.5 mM) + IGF-I (1000, 100, 10, 1.0, 0.1, 0.01, 0.001, 0.0001 μg/ml). Bars represent means ± SEM of three independent experiments run in duplicates. *p<0.05 versus Controls; ♦p<0.05 versus Fe; ●p<0.05 versus Fe+IGF-I 0.1 or versus Fe+IGF-I 0.01.

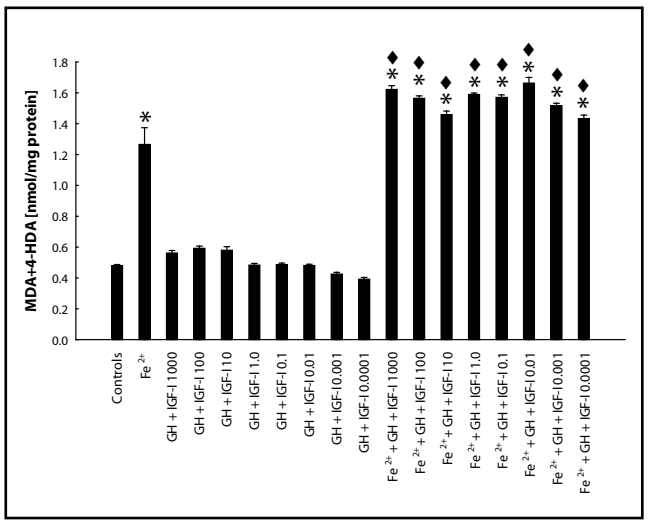

Fig. 6. Concentrations of MDA+4-HDA in porcine thyroid homogenates, incubated for 30 min in presence of $FeSO_4$ (40 μM) + H_2O_2 (0.5 mM), or GH (100 μg/ml) + IGF-I (1000, 100, 10, 1.0, 0.1, 0.01, 0.001, 0.0001 μg/ml), or $FeSO_4$ (40 μM) + H_2O_2 (0.5 mM) + GH (100 μg/ml) + IGF-I (1000, 100, 10, 1.0, 0.1, 0.01, 0.001, 0.0001 μg/ml). Bars represent means ± SEM of three independent experiments run in duplicates. *p<0.05 versus Controls; ♦p<0.05 versus Fe.

of antioxidant enzymatic systems, so they are able to reduce the amount of oxygen free radicals generated during cellular activity (Brown-Borg *et al.* 2002; Hayes & McLellan 1999; Kireev *et al.* 2007; Miyamoto *et al.* 2003). The thyroid gland is an organ in which ROS are involved and even are indispensable for physiological processes (Karbownik & Lewinski 2003b). H_2O_2 is an essential factor for biosynthesis of thyroid hormones. And also, the presence of the antioxidative enzymes (Rhee 1999; Sadani & Nadkarni 1996; Sugawara *et al.* 1988) and antioxidative proteins (peroxiredoxins) (Kim *et al.* 2000) has been well documented in the thyroid gland. GH and IGF-I are important for optimizing thyroid growth and function (thyroid hormone secretion, metabolism and action) (Chen *et al.* 2004; Dumont *et al.* 2003; Fayet & Hovsépian 2004; Lewinski *et al.* 1993).

In our study, the observation of the "lack" of GH and/or IGF-I effects on the basal LPO in rat liver and porcine thyroid, was unexpected, but very desirable. This finding may indicate that GH and IGF-I are worth testing for their potential protective effects against oxidative damage. According to our knowledge, only in one study the effects of GH and IGF-I on the oxidative processes were evaluated under *in vitro* conditions. In this study, GH or IGF-I, applied in concentrations similar to those used in our study, however for a much longer time (24 hours), decreased the activity of antioxidative enzymes in hepatocytes derived from normal, wild-type mice (Brown-Borg *et al.* 2002). However, that decrease in antioxidative enzyme activities (if any) did not result in any increase of LPO in the present study, which could be due either to much shorter time of incubation (1 hour vs. 24 hours) or to direct effects of GH and/or IGF-I on LPO *in vitro*. In turn, in agreement with our results, in only one *in vivo* study did GH not change the basal level of oxidative damage to lipids (expressed as the level of malondialdehyde) in the liver of normal, wild-type mice (Chen *et al.* 2004). In contrast to our findings, in the liver of normal rats treated with GH (single administration) (Youn *et al.* 1998) or IGF-I (a 2-week administration) (Castilla-Cortazar *et al.* 1997), the level of the basal LPO was significantly reduced, compared with untreated healthy controls. This apparent disagreement between the above cited study and the present study may be explained by the fact, that *in vitro* effects can not be directly extrapolated to the *in vivo* conditions.

In the present study neither GH nor IGF-I were able to prevent the iron-induced LPO in rat liver homogenates. The observation of the "lack" of protective effects of GH and IGF-I may support the concept that the liver, as the main target organ for GH-action and the main source of IGF-I, is less susceptible to exogenous administration of these two factors, even when they are used in relatively high concentration. On the other hand, IGF-I, when used in the lowest concentrations (0.001 and 0.0001 μg/ml), increased the iron-induced LPO in liver tissue. These unexpected differences between effects caused by high and low concentrations of IGF-I are difficult to explain. One can suppose that IGF-I, which is always "a factor in place" in the liver, may strongly influence oxidative processes in this tissue by enhancing oxidative damage induced by any pathological factor. However this occurs, when IGF-I is in concentrations close to physiological, slightly exceeding upper normal ranges. It is not excluded that this prooxidative effect of IGF-I results, at least to a certain extent, from the mentioned above inhibitory action on antioxidative enzyme activities in the liver (Brown-Borg *et al.* 2002). In turn, the "lack" of prooxidative effects of IGF-I in high concentrations may result from lower penetration of the factor to cellular membranes due to the mechanical "blockade" but such an explanation is only hypothetical.

In the porcine thyroid, both GH and IGF-I significantly affected the iron-induced LPO. GH, in the lowest used concentrations (0.001 and 0.0001 μg/ml), was able to protect against oxidative damage, caused by the Fenton reaction, thus revealing antioxidative effects; those effects of GH are the only protective effect against the iron-induced LPO, observed in our study. This finding suggests that GH being in concentrations closed to physiological contributes to oxidative balance. It is worth mentioning that also other authors observed protective effects of GH against LPO. For example, GH decreased LPO in rats with thioacetamide-induced liver cirrhosis (Chen *et al.* 2004), in rat liver homogenates after thermal injury (Youn *et al.* 1998), and in the intestine homogenates in septic rats (Huang *et al.* 2002; Jung *et al.* 2003).

In other – higher – used concentrations, we observed prooxidative effect of GH, relying on enhanced oxidative damage to lipids, already caused by iron. Thus, GH, when in excess, contributes to oxidative damage in the thyroid gland, which may result in different pathologies, such as goiter in patients with acromegaly (Kasagi *et al.* 1999) and an increased risk of cancer in acromegalics (Jenkins *et al.* 2006; Matyja *et al.* 2006; Orme et al.1998), although thyroid cancer specifically has not been described with relation to acromegaly. Potential prooxidative properties of GH excess in the thyroid gland were additionally confirmed with even stronger prooxidative effects of IGF-I in the tissue, as observed in the present study.

Consequently to our observations, prooxidative effects of GH and IGF-I have been confirmed in several *in vivo* and *in vitro* studies in animals and in humans. GH and IGF-I excess is associated with increased oxidative stress and early decline in antioxidative enzymes activity in transgenic mice overexpressing GH (Andersson *et al.* 2006; Hauck & Bartke 2001). Also patients with active acromegaly have significantly higher the basal LPO, expressed as a plasma level of malondialdehyde (Yarman *et al.* 2003).

The question arises if GH or IGF-I could be used as an external protective factor, potentially comparable to melatonin (Reiter *et al.* 2008a; 2008b) with regard to their antioxidative action, in the case of disorders other than GH deficiency, associated with increased oxidative stress. Our *in vitro* results do not support such a use of either of the two substances. Also other studies do not favor such a recommendation. GH treatment, administered in patients with critical illnesses, associated with increased oxidative stress (Karbownik-Lewinska *et al.* 2007), appeared to double the mortality and worsen the morbidity (Takala *et al.* 1999). Concerning IGF-I, there is currently unambiguous evidence for an increased risk of cancer, a condition always associated with elevated oxidative stress, in patients with high IGF-I concentrations, even when IGF-I concentrations remain within high-normal ranges (Hankinson *et al.* 1998; Kurek *et al.* 2000).

In conclusion, GH and/or IGF-I may directly contribute to oxidative balance in the liver and the thyroid under physiological conditions but, in the case of induced oxidative stress, they may reveal prooxidative effects, which fact does not support their application in the treatment of disorders, other than GH deficiency, associated with increased oxidative damage.

ACKNOWLEDGEMENTS

The research was supported by a grant from the Ministry of Education of Poland (Project No. 2 P05B 098 30).

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