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Effect of Growth Hormone and Estrogen Administration on Hepatocyte Alterations in Old Ovariectomized Female Wistar Rats

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Aging could be due to the accumulation of oxidative damage. On the other hand, growth hormone (GH) and estrogen deficiency induce deleterious effects on different tissues, and hormonal replacement could counteract these effects. We have investigated whether GH and estrogen administration modify some parameters related to oxidative stress and inflammation in hepatocytes isolated from old ovariectomized female rats. Twenty-two month-old ovariectomized animals were divided into control rats, rats treated with GH, rats treated with estradiol, and rats treated with GH+estradiol. Two-month-old intact female rats were used as young reference group. Hepatocytes were isolated, cultured, and CO and NO release, ATP, cyclic-guanosyl monophosphate (cGMP), and lipid peroxide (LPO) content of cells, as well as phosphatidylcholine (PC) synthesis, were measured. Hepatocytes isolated from old ovariectomized rats showed a decrease in ATP content and PC synthesis compared to young rats. Age also induced an increase in LPO, NO, CO, and cGMP. Treating old rats with GH significantly increased ATP and reduced CO and cGMP levels. Estradiol administration improved all the parameters that were altered. Co-administration of GH and estrogens induced a more marked effect than estrogens alone only in cGMP content. In conclusion, administration of estrogens to old ovariectomized females seemed to prevent oxidative changes in hepatocytes, whereas the effect of GH is not so evident.

Key Words: Growth hormone; estrogens; menopause; oxidative stress; aging; hepatocyte.

Introduction

Aging is accompanied by several changes in the structure and function of different organs and tissues, including the liver *(1,2)*. Furthermore, aging increases the sensitivity of the liver to different damages, such as anoxia/reoxygenation injury *(3)* or toxicity to drugs *(4)*. According to the "Free radical theory of aging," the physiological decline that occurs with age is, at least in part, due to accumulative damage induced by reactive oxygen (ROS) and nitrogen (RNS) species to cells and molecules *(5–7)*. In fact, an increase with age in the amount of oxidative damage to various macromolecules has been reported *(6)*. On the other hand, some studies have also revealed that age induces an increase in proinflammatory enzymes and molecules *(8)*, suggesting that this process could involve a pro-oxidant and a pro-inflammatory status.

Some of the changes and alterations in metabolism, body composition and organ function that accompany aging have been proposed to be, at least in part, related to the physiological decline in the growth hormone (GH)/insulin-like growth factor-1 (IGF-1) axis that occurs with age *(9,10)*, because some of these changes resemble those found in adult patients with chronic GH deficiency (GHD) and hypophysectomized experimental animals *(10–15)*. Exogenous GH administration induces beneficial effects on lipid profile and different organs and tissues in aged animals and humans *(16–20)*. Moreover, GH administration or IGF-1 overexpression is able to prevent oxidative damage and modulate inflammatory response, as well as to induce antioxidant defenses in some experimental models *(21–23)*. In fact, we have previously shown that rhGH administration to old male and female rats was able to improve some parameters related to the oxidative and inflammatory damage associated with aging in hepatocytes, and that this effect was apparently more marked in males, probably because alterations are also more evident in males than in their age-matched female counterparts *(24)*.

Epidemiological data show that the rate of progression of chronic hepatic disease is faster in men than in women, suggesting a possible protective effect of estrogens on the

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Table 1
Estradiol Plasma Levels, Uterine Weight, IGF-1 Plasma Levels, and IGF-1 Hepatic Content[a]

	Young	Old control	Old GH	Old E	Old GH+E
Plasma estradiol (pg/mL)	37 ± 4.8	23.5 ± 5	32.6 ± 3	525 ± 131$^{\$}$	592 ± 53$^{\$}$
Uterine relative weight (g/100 g)	0.236 ± 0.03	0.077 ± 0.01$^{\&}$	0.070 ± 0.01$^{\&}$	0.226 ± 0.03	0.259 ± 0.03
Uterine absolute weight (g)	0.613 ± 0.07	0.28 ± 0.04$^{\&}$	0.271 ± 0.05$^{\&}$	0.985 ± 0.06	0.983 ± 0.12
Plasma IGF-1 (nmol/mL)	649 ± 52	504 ± 79	1074 ± 69^{+}	364 ± 44*	984 ± 71^{+}
Hepatic IGF-1 (pg/mg tissue)	196 ± 20	111 ± 14$^{\#}$	236 ± 36	139 ± 17$^{\#}$	258 ± 29

[a]Effect of growth hormone and estrogen administration on old ovariectomized female rats. Values are expressed as mean ± SEM. Young intact female rats (Young), old ovariectomized rats (Old control), old ovariectomized rats treated with growth hormone (Old GH), old ovariectomized rats treated with estrogens (Old E), old ovariectomized rats treated with growth hormone and estrogens (Old GH+E). $^{*}p < 0.01$ vs young rats; $^{\#}p < 0.01$ vs young rats and animals treated with GH. $^{\$}p < 0.001$ vs animals not treated with estradiol; $^{\&}p < 0.01$ vs young and estradiol-treated rats; $^{+}p < 0.01$ vs animals not treated with GH.

liver *(25)*. In this sense, both menopause and ovariectomy are known to induce deleterious effects on different organs and systems *(26,27)*. Estrogens have been shown to exert a positive influence on several tissues *(26,28)* and to have antioxidant and anti-inflammatory properties *(29–32)*. In old women, the decline in somatotrophic axis and ovarian function converge. Thus, it seem interesting to study the effect of both hormonal replacements on tissues in which their effects have not been clearly elucidated, such as the liver. We have already described that GH administration was able to improve several parameters related to oxidative and inflammatory damage in hepatocytes isolated from old male rats, and that such effect was not so evident in old intact females *(24)*. Because old female rats maintain a certain production of ovarian estrogens until very late in their lives, this estrogenic production could play a protective role in old female rats as compared with males. Therefore, we decided to investigate the effect of GH and estrogen administration on these same parameters in primary hepatocyte cultures obtained from livers of old female rats that had been ovariectomized at 12 mo of age, that is, rats that have lacked these ovarian estrogens for half their lives as a model of menopause, and using young intact females as a reference group.

Results

Plasma estradiol levels were significantly increased in both groups of estradiol-treated animals as compared to non-treated rats. No significant differences were found among young animals, old controls, and old rats treated with GH (Table 1).

Uterine weight was used as an index of estrogen exposure. As expected, this parameter was significantly reduced in both groups of old untreated and GH-treated ovariectomized animals as compared to young intact and old ovariectomized estradiol-treated rats (Table 1). Uterine relative weight was similar in young intact and both groups of estrogen-treated old animals.

Plasma IGF-1 levels were reduced in old animals when compared with young ones, although this reduction was significant only in the group of old estrogen-treated rats. When rhGH was administered, either alone or with estrogens, a significant increase was observed (Table 1). In the liver, the IGF-1 content was significantly decreased in old control and old estrogen-treated rats when compared with young ones, and rhGH administration was able to significantly increase this parameter when given alone or in combination with estrogens.

Cellular ATP content showed a significant decrease in old ovariectomized animals (Fig. 1) when compared to 2-mo-old females. Treatment with both GH and estrogens attenuated this decrease to a similar extent, showing that treated rats' ATP levels were significantly higher than those of untreated old animals. Co-administration of estrogens and GH treatment did not show any effect different from that of GH or estrogens alone.

As shown in Fig. 2, LPO content of hepatocytes was also affected by age. This parameter was significantly increased in old control ovariectomized rats as compared to young intact females. GH administration was not able to significantly decrease LPO levels, although a tendency to reduction was observed. In contrast, when old animals were treated with estradiol, a significant decrease in LPO levels was observed. When GH and estrogens were administered together, LPO levels were significantly lower than those of old untreated control and GH-treated rats, but differences were not significant when compared to animals treated only with estrogens.

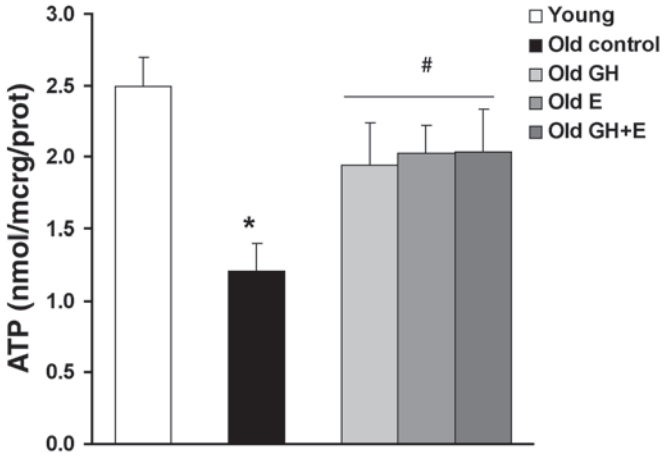

Fig. 1. Adenosyl triphosphate (ATP) content of hepatocytes isolated from female Wistar rats (nmol/μg protein): Effect of growth hormone and estrogen administration on old ovariectomized female rats. Values are expressed as mean ± SEM. Young intact female rats (Young), old ovariectomized rats (Old control), old ovariectomized rats treated with growth hormone (Old GH), old ovariectomized rats treated with estrogens (Old E), and old ovariectomized rats treated with growth hormone and estrogens (Old GH+E). *$p < 0.001$ vs Young; $^{\#}p < 0.01$ vs Old control.

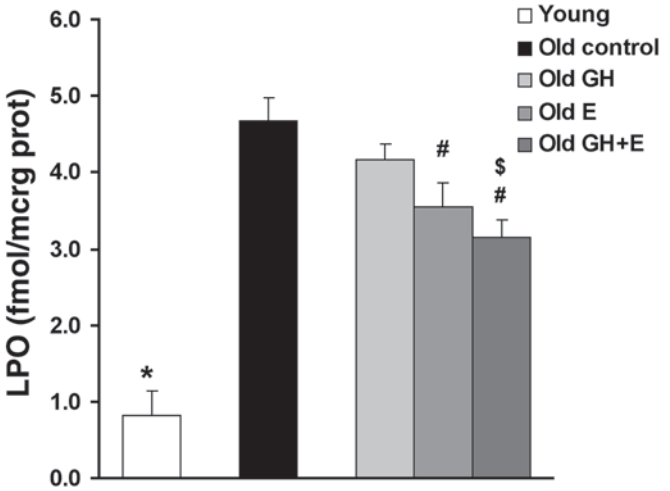

Fig. 2. Lipid peroxide (LPO) content of hepatocytes isolated from female Wistar rats (fmol/μg protein): Effect of growth hormone and estrogen administration on old ovariectomized female rats. Values are expressed as mean ± SEM. Young intact female rats (Young), old ovariectomized rats (Old control), old ovariectomized rats treated with growth hormone (Old GH), old ovariectomized rats treated with estrogens (Old E), and old ovariectomized rats treated with growth hormone and estrogens (Old GH+E). *$p < 0.001$ vs rest of groups; $^{\#}p < 0.05$ vs Old control; $^{\$}p < 0.05$ vs Old GH.

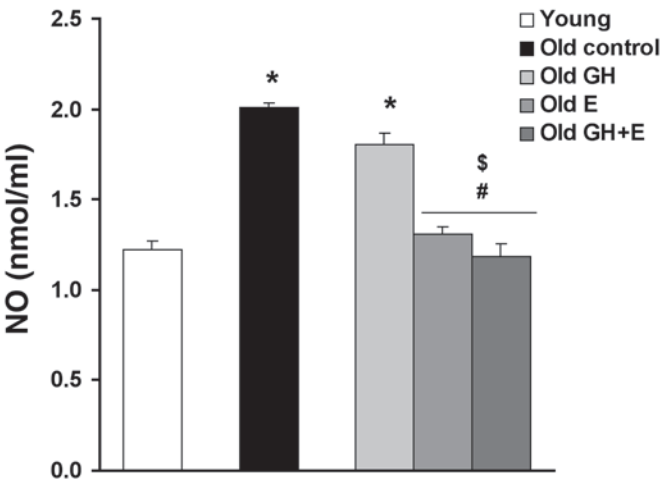

Fig. 3. Nitric oxide (NO) release from hepatocytes isolated from female Wistar rats (nmol/mL): Effect of growth hormone and estrogen administration on old ovariectomized female rats. Values are expressed as mean ± SEM. Young intact female rats (Young), old ovariectomized rats (Old control), old ovariectomized rats treated with growth hormone (Old GH), old ovariectomized rats treated with estrogens (Old E), and old ovariectomized rats treated with growth hormone and estrogens (Old GH+E). *$p < 0.001$ vs Young; $^{\#}p < 0.001$ Old control; $^{\$}p < 0.001$ Old GH.

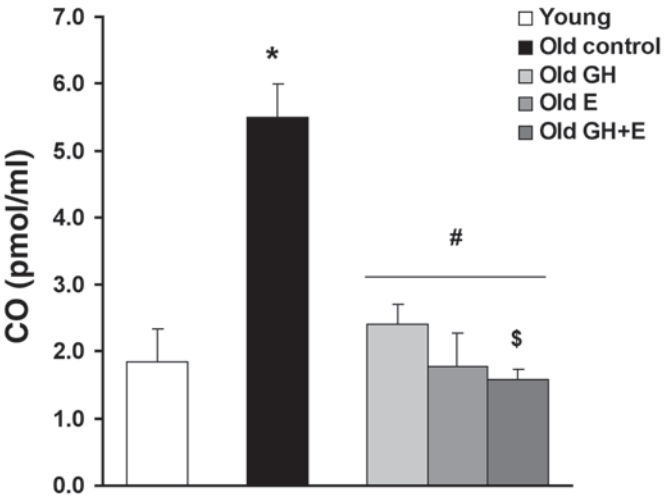

Fig. 4. Carbon monoxide (CO) release from hepatocytes isolated from Wistar rats (pmol/mL): Effect of growth hormone and estrogen administration on old ovariectomized female rats. Values are expressed as mean ± SEM. Young intact female rats (Young), old ovariectomized rats (Old control), old ovariectomized rats treated with growth hormone (Old GH), old ovariectomized rats treated with estrogens (Old E), and old ovariectomized rats treated with growth hormone and estrogens (Old GH+E). *$p < 0.001$ vs Young; $^{\#}p < 0.001$ vs Old control; $^{\$}p < 0.05$ vs Old GH.

NO release to the medium was significantly increased with age in old ovariectomized untreated rats when compared to young intact animals (Fig. 3). GH administration did not significantly reduce NO release, whereas the administration of estradiol was able to reduce this parameter significantly, reaching values similar to those of young rats. Combined treatment with GH and estradiol decreased NO release to a similar extent than estradiol alone.

Figure 4 shows that CO release was significantly more marked in old ovariectomized nontreated female rats than in young intact animals. Both treatments with estradiol and GH were able to normalize CO release in old ovariectomized

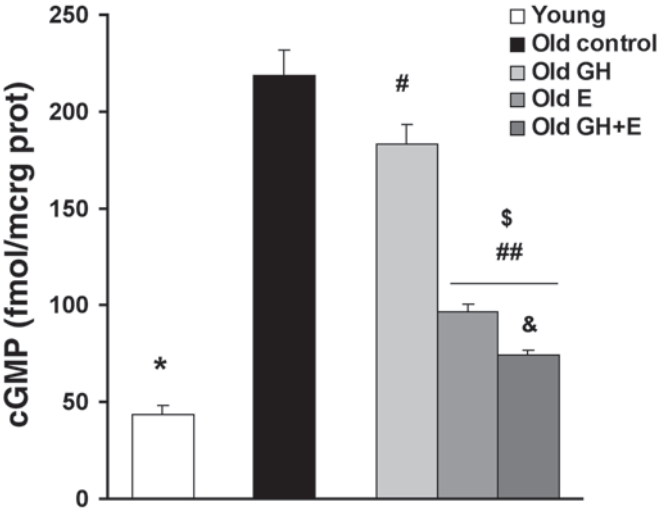

Fig. 5. Cyclic guanosyl monophosphate (cGMP) content of hepatocytes isolated from female Wistar rats (fmol/μg protein): Effect of growth hormone and estrogen administration on old ovariectomized female rats. Values are expressed as mean ± SEM. Young intact female rats (Young), old ovariectomized rats (Old control), old ovariectomized rats treated with growth hormone (Old GH), old ovariectomized rats treated with estrogens (Old E), and old ovariectomized rats treated with growth hormone and estrogens (Old GH+E). $^{*}p < 0.001$ vs rest of groups; $^{\#}p < 0.05$ and $^{\#\#}p < 0.001$ vs Old control; $^{\$}p < 0.01$ vs Old GH; $^{\&}p < 0.05$ vs Old E.

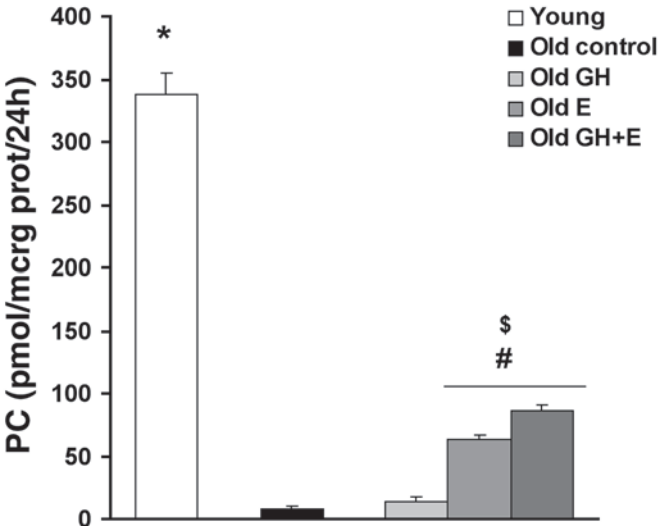

Fig. 6. Phosphatidylcholine (PC) synthesis of hepatocytes isolated from female Wistar rats (pmol/μg protein/24 h): Effect of growth hormone and estrogen administration on old ovariectomized female rats.Values are expressed as mean ± SEM. Young intact female rats (Young), old ovariectomized rats (Old control), old ovariectomized rats treated with growth hormone (Old GH), old ovariectomized rats treated with estrogens (Old E), and old ovariectomized rats treated with growth hormone and estrogens (Old GH+E). $^{*}p < 0.001$ vs rest of groups; $^{\#}p < 0.001$ vs Old control; $^{\$}p < 0.05$ vs Old GH.

rats, obtaining levels similar to those found in 2-mo-old animals. Rats treated with GH and estrogens together showed significantly lower levels than GH-treated rats, but differences compared to animals treated only with estradiol were not significant.

As could be expected, cGMP content of hepatocytes isolated from old rats showed a dramatically age-related significant increase (Fig. 5). Again, both estradiol and GH treatments were able to significantly reduce cGMP content in hepatocytes, although this decrease was more evident in estrogen-treated rats than in GH-treated ones. In this case, co-administration of GH and estradiol further reduced cGMP content, reaching values significantly lower than estradiol-treated animals.

When PC *de novo* synthesis was tested, a marked decrease was found in old untreated rats as compared to young controls (Fig. 6). When the old animals were treated with GH, no significant differences were observed as compared to untreated animals. In contrast, estrogens induced a significant increase in PC synthesis by the hepatocytes. Addition of GH to estradiol did not further increase PC synthesis.

Discussion

In contrast to what happens in women after menopause, female rats maintain a certain estradiol secretion until very late in their lives (around 20–24 mo) *(33,34)*. Thus, to obtain an experimental model of long-term ovarian estrogen deprivation in rats, similar to the situation of old postmenopau-sal women, we considered it convenient to ovariectomize the animals at around 12 mo of age, which is almost half the lifespan of this rat strain. In fact, the ovariectomized adult/old female rat has been considered a proper model for the study of other menopausal alterations, such as osteoporosis *(26,35)*, and we have used a similar menopause model in order to investigate the effect of rhGH on vascular function *(65)*.

On the other hand, it has previously been described that hepatocytes isolated from old intact female rats show changes in some parameters related to oxidative stress as compared to young animals, and these changes have been shown to be even more pronounced in males and old ovariectomized females *(36)*, which underlines the importance of estrogens in preserving the function of different organs and systems. In the present study we investigated the effect of different hormonal replacement therapies on this experimental model of estrogen deprivation.

Plasma estradiol levels were not found to be significantly different between young intact and old ovariectomized control rats. This finding was not surprising, because during most of the estrous cycle (except pro-estrous phase), estradiol concentrations are physiologically low (around 20–30 pg/mL); thus, ovariectomy was not expected to induce a dramatic reduction in estradiol levels compared to

young rats. This finding is similar to those obtained in other studies performed with ovariectomized female rats by our group and others, in which no significant differences in plasma estradiol levels between intact and ovariectomized rats were found *(37,38, 65)*. A better indicator of persistent estrogen action is the relative uterine weight. This parameter more reliably reflected estrogen exposure, because the groups of intact rats or ovariectomized rats treated with estradiol showed weights significantly higher than those of ovariectomized animals not receiving estradiol. The finding that old estradiol-treated rats showed similar relative uterine weights than intact young rats could mean that, although estrogen plasma levels were high in estradiol-treated animals, a nearly physiological substitution dosage was achieved.

The decline in the GH/IGF-1 axis of our animals can be observed through the reduction in plasma levels of IGF-1 in old rats as compared to the young ones. This decrease was only significant in the group of old rats treated with estrogens. However, when hepatic IGF-1 content was measured, a significant reduction was found in all old non-GH-treated groups as compared to young animals. This finding could mean that, although tissular IGF-1 production is significantly decreased with age, this fact is not totally reflected in plasma levels, as previously reported *(20)*. As expected, GH administration induced a significant increase in IGF-1 levels in both liver and plasma, when given either alone or combined with estrogens.

Estrogens have been shown to have antioxidant properties and are also able to prevent peroxidative membrane damage *(29,30,39)*. Moreover, estrogens were able to protect hepatocytes undergoing oxidative stress *(40)* and were able to preserve hepatic integrity and function in several experimental models of liver injury in which oxidative damage was involved *(41,42)*. These findings are in accordance with the present study, in which estrogen administration was able to partially prevent the increase in LPO content of hepatocytes induced by both aging itself and ovariectomy. LPO is a marker of cellular oxidative damage, and it has been reported to increase with aging in different tissues *(4, 43)*. The importance of this phenomenon lies in the fact that after its initiation by free radicals, it becomes a self-perpetuating chain reaction that reduces membrane fluidity and can alter closely situated proteins, which negatively affects membrane functions *(44)*. The present study also showed that GH administration tended to reduce LPO content of cells isolated from old ovariectomized rats. Both GH and IGF-1 administration have previously been shown to reduce oxidative stress and to improve antioxidant defenses in some experimental models in which oxidative damage is involved, such as CCl_4-induced liver cirrhosis *(21)*, thermal injury *(45)*, and in old male rats *(24)*. Transgenic Mini rats, in which GH production is suppressed, showed a higher susceptibility to hepatotoxic agents inducing oxidative damage *(46,47)*. In another transgenic experimental model, IGF-1 overexpression reduced diabetes-induced oxidative stress in cardiomyocytes *(23)*. In humans, it has been demonstrated that patients with GH deficiency showed increased free radical production and oxidative damage, and that GH administration was able to improve this situation *(48)*. Although the differences found in the present study between old ovariectomized control and GH-treated rats were not very evident, the tendency seems to point to a reduction in LPO content of cells.

Mitochondria isolated from brain and liver of female rats exhibited higher antioxidant gene expression and lower oxidative damage than males and ovariectomized females *(49)*, and estrogen administration was able to restore these parameters in ovariectomized females, which points to a protective effect of estrogens against mitochondrial function impairment. These data support the findings of the present study, in which estrogen administration prevented the decrease in ATP content exhibited by old ovariectomized female rats. Several changes in mitochondrial function have been reported to be induced by age, including the alteration of the activity and expression of different mitochondrial enzymes and elements of the mitochondrial electron transport chain, and these facts could lead to a reduction in ATP synthesis and energy supply in old cells *(44,50,51)*. Oxidative damage seems to be involved in this phenomenon, as oxidative stress was able to inhibit mitochondrial respiration *(52)*. In our present study, GH administration was able to prevent the reduction in ATP content in cells isolated from old ovariectomized animals. This finding agreed with previous studies, in which the age-induced reduction in the expression of some components of the mitochondrial respiratory chain was prevented by GH administration *(51)*. Moreover, these results were supported by previous findings of our group, in which GH administration improved ATP content of cells isolated from old male and intact female rats *(24)*. In our study, NO release to the culture medium was found to be increased in hepatocytes isolated from old ovariectomized rats when compared with young animals, and estrogen administration was able to prevent this effect. NO is one mediator involved in the inflammatory responses *(53)* and it can also act as a free radical, either directly or through the peroxynitrites generated by its interaction with superoxide anion (O_2^-) *(54,55)*. With aging, an increase in proinflammatory enzymes and molecules, such as NO, iNOS, and cytokines, has been reported *(8)*, which is in accordance with the results obtained in the present study. Estrogens have been shown to exert anti-inflammatory actions in several experimental models *(32)*. This effect could explain our finding of a decrease in NO release to the medium when old ovariectomized rats were treated with estradiol. GH and IGF-1 could also play a role in the regulation of the inflammatory response, because its administration was able to modulate hepatic acute phase response and cytokine production in experimental animals *(22)* and in humans *(56)*. In our study, a tendency toward a reduction in NO release

was found in old ovariectomized GH-treated rats as compared to untreated animals, although these differences were not significant.

The decrease in oxidative damage and in inflammatory mediator production induced by estrogens could also be the explanation for the reduction in CO release found in old ovariectomized rats treated with estrogens. GH administration was also able to significantly reduce CO release. CO is an endogenously synthesized molecule that shares some of the physiological actions of NO. CO also acts through the activation of guanylate cyclase, like NO, with the subsequent intracellular increase of cGMP *(57)*. The two main endogenous sources of CO are lipid peroxidation and heme metabolism by heme oxygenase (HO) *(57,58)*, and the induction of both pathways has been observed in the presence of oxidative and inflammatory damage *(58,59)*. In fact, the HO-1-CO pathway has been proposed to act as a defense system against oxidative and inflammatory stress, and CO levels are considered an accurate marker of cellular damage *(58,59)*. In our study we have found that CO production is significantly increased in old ovariectomized female rats, similarly to what has been previously described in males *(24)*, and this finding could mean that this pathway was stimulated by the increase in ROS and proinflammatory molecules that occurs with age. Thus, the reduction in CO release found in rats treated with estradiol or GH could indicate that the inflammatory and oxidative stress of the cells have been reduced by these treatments.

The role of NO and CO as signal transduction molecules, regulating sGC activity, is well established *(57,58)*. Both molecules induce the activation of the enzyme, thus increasing cGMP production. Our study showed that cGMP content of hepatic cells isolated from old ovariectomized female rats was significantly higher than that of young ones, in accordance with the increased levels of NO and CO release that were found in the culture medium. Both treatments with estradiol or GH were able to reduce this parameter, as could be expected by the observed decrease in NO and CO release induced by its administration.

The reduction in oxidative damage and NO production could also account for the improvement in PC *de novo* synthesis induced by estradiol administration in old ovariectomized female rats. Phospholipids, and particularly PC, are components of cell membranes that play an essential role in the maintenance of membrane structure and fluidity *(60)*. In our study, a significant decrease in PC synthesis was found in old ovariectomized rats. This could be due to the increase in oxidative stress and proinflammatory molecules associated with aging. This fact has been previously reported in other experimental models, such as type II pneumocytes in culture, which showed a decrease in PC synthesis mediated by NO *(61)*. The decrease in NO release induced by estradiol administration could explain the improvement in PC *de novo* synthesis found in estradiol-treated animals.

We can conclude that hepatocytes isolated from old ovariectomized female rats, used as a model of menopause, exhibited changes in several parameters related to oxidative and inflammatory damage when compared to intact young females. All these alterations showed a significant improvement by estrogen administration. GH treatment was also able to significantly reduce CO release and cGMP content of cells, increasing its ATP levels. Although the rest of the evaluated parameters tended to improve with GH treatment, differences as compared to old ovariectomized untreated animals were not found to be significant. Co-administration of GH and estrogens did not seem to exert any effect significantly different from that of estradiol alone, except in the case of cGMP content.

Materials and Methods

Animals

Twenty-four female Wistar rats 22 mo of age were used in the present study. They had been ovariectomized at 12 mo of age, according to the following procedure: rats were anaesthetized with Equithesin (0.3 mL/100 g weight, ip) and two small incisions (8 mm) were made through the skin and the muscle back walls in parallel with the animal body line. The ovaries were then located and a silk thread was tightly tied around the oviduct, including the ovarian blood vessels, the oviduct was sectioned and the ovary was removed. Muscle wall was then sutured with a synthetic absorbable thread and the skin with metallic clips. The animals were given a standard laboratory rat diet (A.04; Panlab, Barcelona, Spain) and water *ad libitum,* in a light- and temperature-controlled room. Animals were divided into four experimental groups (n = 6 each group): vehicle-treated animals, rats treated with recombinant human growth hormone (rhGH, Serono, Spain; 2 mg/kg/d diluted in sterile saline solution, sc, in two daily injections at 10:00 h and 17:00 h), rats treated with estrogens (estradiol valerianate, Sigma, St. Louis, MO, USA; 125 µg/week, sc, diluted in sunflower oil), and rats treated with rhGH and estrogens at the same dosages. All treatments were performed for 10 wk. Six other intact female rats 2 mo of age were used as a reference group. All the animals received humane care according to the Guidelines for Ethical Care of Experimental Animals of the European Union, and the experimental procedures had been approved by the Animal Care Committee of the School of Medicine of the Complutense Uniersity.

After 10 wk of treatment, rats were sacrificed by decapitation. Young rats were sacrificed in estrous or diestrous-1. Blood and liver were collected and processed as described later. Uterus was also removed and weighed, as an index of estrogen exposure.

Hormone Levels

Trunk blood was collected and centrifuged to obtain plasma, and a piece of liver was homogenized. Liver and

plasma IGF-1 levels were measured as previously described *(62)* by a specific radioimmunoassay (RIA), using reagents kindly provided by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDKD) and a second antibody obtained in our laboratory. Plasma estradiol levels were measured by a RIA kit commercially available (COAT-A-COUNT, DPC, Los Angeles, CA).

Hepatocyte Isolation and Culture

Isolated hepatocytes were prepared as previously described *(63)*. After isolation, hepatocytes were cultured by inoculating 5×10^5 cells in Falcon dishes each containing 5 mL of RPMI 1640 medium (10% fetal calf serum, 100 IU/mL penicillin G, 50 µg gentamicin). The viability of cells was 93 ± 2% (trypan blue exclusion test); 18 to 20 h after inoculation, the cells were attached to the bottom with a plating efficiency of 70% to 80%. The unattached and nonviable cells were removed, media were changed, and cells were cultured for 24 h more. At the end of the culture period, media and cells were separately collected to perform different measurements.

In a parallel experiment, cells were incubated in the presence of 10 m*M* D-[U-^{14}C]glucose (12.5 Ci/mol; Radiochemical Centre, Amersham, Buckinghamshire, UK), for 120 min, and the incorporation of D-[U-^{14}C]glucose into *de novo* synthesized phosphatidylcholine (PC) was measured as previously described *(64)*.

Biochemichal Determinations

Cellular content of adenosyl triphosphate (ATP), lipid peroxides (LPO), and cyclic-guanosyl monophosphate (cGMP) were measured by commercially available kits (Sigma; Camille Biochemical Company, Thousand Oaks, CA; and ^{125}I-RIA Kit, Radiochemical Centre, Amersham, Bucks, England, respectively).

Nitric oxide (NO) release to the medium was measured by the Griess reaction as NO_2 concentration after NO_3 reduction to NO_2. Briefly, samples were deproteinized by the addition of sulfosalicylic acid. They were then incubated for 30 min at 4°C, and subsequently centrifuged for 20 minutes at 12,000*g*. After incubation of the supernatants with *Escherichia coli* NO_3 reductase (37°C, 30 min), 1 mL of Griess reagent (0.1% naphthylenediamine dihydrochloride, 1% sulfanilamide, 2.5% H_3PO_4) was added. The reaction was performed at 22°C for 20 min, and the absorbance at 546 nm was measured, using $NaNO_2$ solution as standard. The measured signal is linear from 1 to 150 µ*M* ($r = 0.994$, $p < 0.001$, $n = 5$), and the detection threshold is approx 2 µ*M*.

To quantify the amount of carbon monoxide (CO) released, the ratio of carboxihemoglobin after hemoglobin addition was measured. Hemoglobin (4 µ*M*) was added to samples and the mixture was allowed to react for 1 min, to ensure maximum binding of CO to hemoglobin. Then, samples were diluted with a solution containing phosphate buffer (0.01 mol/L monobasic potassium phosphate/dibasic potassium phosphate, pH 6.85) containing sodium dithionite, and after 10 min at room temperature, absorbance was measured at 420 and 432 nm against a matched curve containing only buffer.

Protein determination was performed by the Bradford method. The basis of this method is the addition of Coomassie brilliant blue dye to proteins. This union induces a shift in maximum dye absorbance from 465 to 595 nm. Absorbance is measured at 595 nm, comparing to a known standard curve.

Reproducibility within the assay was evaluated in three independent experiments. Each assay was carried out with three replicates. The overall intraassay coefficient of variation has been calculated to be <5%. Assay to assay reproducibility was evaluated in three independent experiments. The overall interassay coefficient of variation has been calculated to be <6%.

Statistical Analysis

Results are expressed as the mean ± SEM, from $n = 6$. The results are presented as the mean ± SEM. Mean comparison was done by the Kuskal–Wallis test followed by a Mann–Whitney test; a confidence level of 95% ($p < 0.05$) was considered significant.

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