nium sulfate. Rinsed pellets were suspended in 25 µl water. Protein A (20 µl of a 5% wet wt/vol solution) or PEG-6000 (200 µl of a 35% solution) were added to assay tubes followed by vortexing at room temperature. Particular care was required while vortexing the viscous PEG in order to obtain a homogeneous solution. For the protein A and PEG procedures, tubes were incubated at room temperature for 15 min, centrifuged at 1500 × g for 15 min, supernatants aspirated, and pellets suspended in 50 µl water. For all 3 procedures, samples were brought to a final volume of 500 µl with Scintiverse (Fisher) and counted by scintillation spectroscopy (LKB). Standard curves were analyzed by a logit-log transformation program.

Results and conclusions. The assay competition curves for ecdysone, 20-hydroxyecdysone and 3-epi-ecdysone obtained by the 3 procedures (PEG, ammonium sulfate, protein A) using both the micro- and macro-RIA protocols are shown in the figure. As evident from this figure, the 3 precipitating agents had little effect on assay sensitivity or specificity. The amounts of ecdysteroids required to inhibit 50% [^{3}H]-ecdysone binding (I_{50}) for the 3 procedures varied as follows: micro-assay – 0.11–0.13 ng ecdysone/assay, 0.39–0.49 ng 20-hydroxyecdysone/assay, 13.0–19.0 ng 3-epi-ecdysone/assay; macro-assay – 1.3–1.8 ng ecdysone/assay, 4.0–6.2 ng 20-hydroxyecdysone/assay, 120–150 ng 3-epi-ecdysone/assay.

The 3 procedures have been used in our laboratory to determine total ecdysteroid levels in tissues and hemolymph during the metamorphosis of *Manduca sexta*, as well as for the quantification of HPLC fractions of ecdysteroids from larval hemolymph and embryos. Although results were equivalent for the 3 methods, the protein A procedure was easier and more rapid. For example, precipitation did not have to be done in the cold as required with ammonium sulfate, and rapidity of vortexing was not critical. Indeed, one of the pitfalls in obtaining reliable results with ammonium sulfate is the tendency of inexperienced researchers to vary the time and/or intensity of the vortexing step after initial addition of the precipitating agent. Further, with protein A, 1 centrifugation step was sufficient to lower non-specific binding to less than 10% of total counts bound. This reduced the time required for assay termination by 30–50%. The protein A technique is, therefore, recommended highly as an alternative to the standard ammonium sulfate protocol for terminating ecdysteroid RIAs. In addition, it should be an efficient tool for use in vertebrate steroid hormone RIAs as well. To our knowledge, this is the first description of the use of protein A in a steroid hormone RIA but it may have even wider applicability since it also appears to be useful in the juvenile hormone (sesquiterpene) RIA[19].

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Effect of insulin on the synthesis and release of lipid peroxide by cultured hepatocytes isolated from normal and diabetic rats

K. Kosugi[1], Y. Harano, A. Kashiwagi, M. Suzuki and Y. Shigeta

The Third Department of Medicine, Shiga University of Medical Science, Ohtsu, Shiga 520-21 (Japan), 17 May 1983

Summary. Lipid peroxide content in hepatocytes isolated from ketotic diabetic rats was higher than normal, and the release of peroxide into the media was also elevated for the initial 18 h. Insulin suppressed both peroxide release and synthesis by cultured hepatocytes isolated from normal and from diabetic rats.

Lipid peroxide (LPO) in serum has been reported to be elevated in diabetic subjects, especially with macro- or micro-angiopathy[2]. Since LPO has been suggested to play an important role in tissue injury of vascular origin[3,4], elevated LPO in diabetes may therefore play a role in the pathogenesis of diabetic angiopathy[5]. Further, liver has been reported to be a major organ for both the synthesis and removal of LPO[6,7]. Therefore, insulin deficiency may possibly stimulate the formation and release of hepatic LPO in the diabetic. We report here our study of the effect of insulin on LPO production in liver, done in an effort to elucidate the mechanism causing high serum levels of LPO in diabetes. We have assessed LPO synthesis and release by isolated and cultured hepatocytes from normal and diabetic rats. In vitro effects of insulin on LPO synthesis and release by cultured hepatocytes have also been investigated.

Materials and methods. Diabetes was produced in male Sprague-Dawley rats, weighing 250–300 g, by the intravenous injection of streptozotocin, 10 mg/100 g b.wt. Those rats exhib-

Table 1. Effect of insulin and linolenate on LPO release into media from cultured hepatocytes isolated from normal and ketotic diabetic rats (Mean ± SE)

Hours after hepatocyte inoculation[a]		Hepatocytes from normal rats (MDA nmole/mg protein/12 h)	Hepatocytes from diabetic rats	Hepatocytes from normal rats + 0.3 mM linolenate
18-30 h	Control	2.24 ± 0.28 (n = 16)	2.32 ± 0.35 (n = 7)	4.28 ± 0.29 (n = 6)
	Insulin	0.70 ± 0.18[b] (n = 6)	1.05 ± 0.10[c] (n = 6)	2.98 ± 0.44[c] (n = 6)
30-42 h	Control	2.23 ± 0.17	2.50 ± 0.20	4.18 ± 0.37
	Insulin	0.57 ± 0.12[b]	1.04 ± 0.16[b]	2.62 ± 0.46[c]

[a] LPO release into media by cultured hepatocytes with or without insulin supplementation was determined for the 2 successive 12-h periods after the supplementation. The insulin (100 ng/ml) was added to the media at 18 h after cell inoculation. [b] $p < 0.005$ by t-test vs control. [c] $p < 0.05$ by t-test vs control.

iting glycosuria and ketonuria 2 days later were used for the study. They were fed ad libitum on a normal laboratory chow (CLEA-CE II, CLEA, Osaka) until they were anesthetized by a intraperitoneal injection of sodium pentobarbital (5 mg/100 g) and processed for the isolation of hepatocytes. Isolation of hepatocytes was performed by a method previously reported[8]. The isolated cells were washed twice by centrifugation and then suspended in Krebs-Ringer-bicarbonate buffer. Cell viability was more than 95% in hepatocytes isolated from both normal and diabetic rats. For the culture of the cells, 500–600 × 10^4 cells were inoculated in a Falcon dish (100 × 20 mm) containing 8 ml of 17% fetal calf serum, 100 U/ml penicillin G, 50 µg/ml streptomycin and 83% F-10 media. On the second day, 18 h after inoculation, cells were attached to the bottom of the dish with a plating efficiency of 60–70%. Then the medium was changed with hormonal addition. An aliquot of medium was taken for LPO determination after 12 and 24 h. At 24 h, the medium was renewed by aspiration and cells were washed 3 times with ice-cold saline. One ml of 1% SDS was added to the plate and the cells were collected using a rubber policeman.

LPO was determined by Yagi's method[9]. 50 µl of culture media or solubilized cell suspension was mixed with 4.0 ml 0.083N-sulfinic acid and 0.5 ml of 10% phosphotungstic acid, and then centrifuged at 2,000 × g for 10 min. After suspending the precipitate in 1.0 ml of distilled water, 1.0 ml thiobarbituric acid reagent (0.67% thiobarbituric acid-acetic acid 1:1 (V/V)) was added. Then the mixture was incubated for 1 h at 95 °C, cooled to 4 °C in an ice, and mixed with 5 ml of n-butanol. The resulting mixture was centrifuged at 2000 × g for 10 min. The absorbance of an aliquot from the upper butanol layer was measured at 553 nm, excited at 515 nm, by a fluorometer (Shimazu FR510). LPO content is expressed as nmoles of malondialdehyde (MDA). As a standard, MDA formed from 1,1,3,3-tetraethoxypropane was used.

Results. Plasma concentrations of LPO in the diabetic rats (mean ± SE; 13.35 ± 0.93 MDA nmole/ml; $p < 0.001$ (n = 5)) were significantly higher than those of normal rats (6.09 ± 0.31 (n = 6)). The concentration of LPO in hepatocytes isolated from the ketotic diabetic rats (1.41 ± 0.09 MDA nmole/mg protein; $p < 0.05$) increased by 1.3 folds compared with normal rats (1.07 ± 0.11). During the initial 18 h after cell inoculation, the release of LPO into media by hepatocytes from diabetic rats (3.98 ± 0.38 MDA nmole/mg protein/18 h; $p < 0.025$ (n = 16)) was significantly higher than that by normal hepatocytes (3.00 ± 0.11 (n = 7)). After 18 h, cultured normal hepatocytes released a similar amount of LPO per 12 h for the next 24 h (2.0–2.5 MDA nmole/mg/protein/12 h). Cultured hepatocytes isolated from diabetic rats also released LPO linearly (2.0–2.7) (table 1). Thus, after 18 h there was no difference between the quantity released from hepatocytes of normal and diabetic rats.

When insulin (100 ng/ml) was added to the media of the cultured hepatocytes from the normal and ketotic diabetic rats, LPO release was significantly suppressed. When 0.3 mM linolenic acid was added to the media of normal cultured hepatocytes, LPO release almost doubled. In vitro addition of insulin again suppressed LPO release (table 1). 24 h after insulin supplementation, LPO content was measured in the hepatocytes. The hepatocytes treated with insulin contained less LPO than those not treated with insulin (table 2). Since insulin treatment increases protein content, LPO content was also analyzed per cell number. LPO content per 10^6 cells was also significantly lower in insulin-treated hepatocytes (table 2).

Table 2. Effect of insulin on LPO content in normal cultured hepatocytes (Mean ± SE)[a]

LPO content	No insulin (MDA nmole)	Insulin (100 ng/ml) (MDA nmole)
Per mg protein	4.10 ± 0.26 (n = 6)	3.08 ± 0.34[b] (n = 8)
Per 10^6 cells	6.23 ± 0.32 (n = 6)	5.14 ± 0.33[b] (n = 8)

[a] LPO contents of the cells were measured after 42 h of culture and in the absence or presence of insulin for 24 h. [b] $p < 0.05$ by t-test vs hepatocytes without insulin treatment.

Discussion. In the present study, we have examined the effect of insulin on the production of LPO by hepatocytes. Concentrations of LPO were higher in plasmas from acutely ketotic diabetic rats than in those from normal rats. LPO content was also higher in hepatocytes from ketotic diabetic rats. Insulin suppressed the release and production of LPO by cultured hepatocytes. These findings suggest that under condition of insulinopenia, LPO synthesis and release are increased in hepatocytes. A high level of free fatty acid caused by insulin deficiency may also have contributed to the pathogenesis of the high level of serum LPO[11] in the diabetic state, since linolenic acid increases LPO release by the hepatocytes in both the absence and presence of insulin.

Insulin could decrease the production by hepatocytes of LPO by either suppressing its synthesis or enhancing its degradation. Insulin has a beneficial trophic effect on hepatocytes[12]. In insulin deficiency, structural derangement of mitochondria and microsomes[13] has been reported. This may increase free radical formation through abnormal electron transport, thus perhaps causing increased LPO production. Insulin is also thought to suppress LPO synthesis by decreasing free fatty acid concentrations in culture media through an increase in triglyceride synthesis[14]. However, the concentrations of free fatty acid in the media in which insulin was present and absent were the same at the end of incubation (control 0.04 ± 0.02 mM (n = 6) vs Insulin 0.03 ± 0.01 mM (n = 6)). As regards the possible effect of insulin on the degradation of LPO, damaged hepatocytes under condition of insulin deficiency may be unable to remove LPO[6,7] and insulin may activate the LPO-degrading enzyme, glutathione (GSH) peroxidase. Activation of glucose-6-phosphate dehydrogenase by insulin[15] may be linked to the stimulation of GSH peroxidase through GSH reductase.

Thus, we have shown that hepatocytes produce significant amounts of LPO, and that insulin suppresses the formation of LPO and its release by cultured hepatocytes. Therefore, the increased serum concentrations of LPO, often observed in diabetic subjects, may to some degree have their origin in alteration in hepatic metabolism consequent to insulin deficiency.

1 To whom correspondence should be addressed.
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Sex steroids in the rat submaxillary gland during the estrus cycle

E. Paulo[1]

Laboratory of Animal Endocrinology, Institute of Zoology, Jagiellonian University, PL-30-060 Krakow (Poland), 9 June 1982

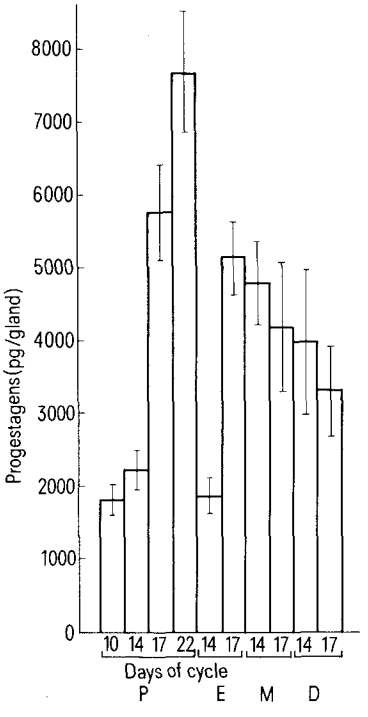
Summary. Relatively high progestagen levels were found in female rat submaxillary glands, with a maximum in the proestrus stage at 22 h and 2 minima; in proestrus between 10 and 14 h, and in estrus at 14 h. Estrogen and androgen concentrations in the gland were undetectable during most of the cycle except in the proestrus stage, when the highest level was determined at 14 h for estrogens and at 17 h for androgens.

The rat submaxillary glands are a source of polypeptide hormones[2-6], and sex steroids as well[7]. Among steroids, relatively high progestagen levels were found in the submaxillary glands of adult females and they still increased further several times during pregnancy[8]. In the present study, sex steroid fluctuations were found in the rat submaxillary gland during the estrus cycle.

Material and methods. Virgin female Wistar rats (130–180 g), maintained under controlled lighting of 12 h light and 12 h darkness, and fed pelleted food and water ad libitum, were used. Vaginal smears were taken every morning and only the rats which showed 3 consecutive 4-day cycles were chosen for the study. Submaxillary glands (5–6 per group) were excised from the rats under ether anesthesia on each day of the cycle at different hours: in proestrus (P) at 10, 14, 17 and 22 h, in estrus (E), in metestrus (M) and in diestrus (D) at 14 and 17 h. The glands were stored at −20 °C until steroid analysis was carried out by radioimmunoassay. Then the glands were homogenized with 2 ml phosphate buffer, and 200 µl of homogenate was extracted with ethyl ether for estrogen and androgen analysis, and with n-hexane for progestagen analysis. All samples were assayed in duplicate.

Estrogens were estimated according to Hotchkiss et al.[9], progestagens were measured according to Abraham et al.[10], androgens were determined according to Dufau et al.[11]. These methods were the same as described in detail previously[8]. The steroids measured are referred to as estrogens, progestagens and androgens because the antiserum used in the estradiol-17β RIA cross-reacted also with estrone (66%) and estriol (2.1%); the antibody used in the progesterone RIA cross-reacted also with pregnenolone (5%), with 20α-hydroxy-pregn-4-en-3-one (1.8%) and with 17α-hydroxy-progesterone (1.1%); the antibody used in the testosterone RIA cross-reacted also with dihydrotestosterone (20.8%), with androstenedione (7.4%) and with dehydroisoandrosterone (3%). The results of the assays are shown graphically as hormone mean values for the whole gland ± SEM.

Results and discussion. Changes in the submaxillary gland progestagen level of 4-day cycle rats are given in the figure. Progestagen concentration in the P stage was lowest at 10 and 14 h (about 2000 pg per gland), considerably increased at 17 h (up to more than 5000 pg per gland) and it reached the maximum at 22 h (above 7000 pg per gland). In the E stage at 14 h a sharp drop of progestagens (to below 2000 pg per gland) was found; this was then followed by a rapid increase in the same stage at 17 h to 5000 pg per gland. In the M and D stages a gradual decrease to about



Progestagen contents of the submaxillary gland of a female rat at different days and hours of the estrus cycle. Each bar is the mean ± SEM per gland for a minimum of 5 duplicate determinations.