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Alteration of Activities of Hepatic Antioxidant Defence Enzymes in Developing Chick Embryos After Glucocorticoid Administration – A Factor to Produce Some Adverse Effects?

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Abstract

Liver tissue is one of the principal targets of glucocorticoids, therefore changes in the balance between hepatic oxidative and reductive capacity may greatly influence adverse effects of glucocorticoid therapy. In this study, effects of glucocorticoid on the activities of hepatic antioxidant defence enzymes were examined by using developing chick embryos.

After the administration of 0·25 μmol hydrocortisone sodium succinate, a typical glucocorticoid, to 15-day-old chick embryos, glutathione peroxidase, glutathione reductase, catalase and superoxide dismutase in the liver generally began to decrease at around 4 h, reaching 60–70% of control levels between 24 and 48 h. These changes were observed much earlier than the elevation of the hepatic thiobarbituric acid reacting substance (TBARS) level which began to increase from 20 h, reaching about six times the control level at 48 h after hydrocortisone administration. Conversely, the elevated TBARS level decreased back to the normal level with the recoveries of these enzyme activities. Furthermore, it was found that the aniline hydroxylase activity, measured as a marker of oxidative activity, began to increase after around 12 h.

These results suggested that TBARS levels were possibly produced by the suppression of antioxidant defence abilities and the significant induction of oxidative activity in the liver by glucocorticoid. As the elevated TBARS in the liver can be distributed to tissues, TBARS will be involved in the occurrence of some of the glucocorticoid-induced adverse effects such as cataract formation.

The appearance of adverse effects of glucocorticoid, including cataract in man, has been recognized as a potential problem in high-dose or long-term therapy (Erill 1991). We have developed a model of glucocorticoid-induced cataract in the developing chick embryo and have found that thiobarbituric acid reacting substance (TBARS), as an indicator of lipid peroxide level, was elevated in the lens (Nishigori et al 1986a), blood (Nishigori et al 1986b) and liver (Nishigori et al 1984) during glucocorticoid treatment. These glucocorticoid-induced phenomena can be effectively prevented by radical scavengers such as ascorbic acid (Nishigori et al 1985, 1986a). Halliwell & Gutteridge (1990) described more than 70 diseases in which reactive oxygen species have been implicated in pathological processes. Thus, it is possible that some of the glucocorticoid-induced adverse effects are initiated by the production of lipid peroxide via elevated oxidative or peroxidative steps in the liver.

The liver of the chick embryo, as well as other animals, is one of the principal target tissues of glucocorticoids (Oikarinen 1987). Accordingly, changes of the hepatic function—balance of oxidative and reductive activities—may greatly influence the occurrence of adverse effects, since the elevation of lipid peroxidation in living cells is associated with serious damage to essential structural proteins and enzymes, and well known as one of the risk factors of various diseases (Halliwell & Gutteridge 1990). Nelson & Ruhmann-Wennhold (1975) reported that the superoxide anion production of rat liver microsomes increased after glucocorticoid administration. Castro et al (1970) also found that glucocorticoids enhanced the activities of hepatic NADPH-cytochrome C reductase and cytochrome P-450 reductase in adrenalectomized rats.

In the present study, we examined the effects of glucocorticoid on the alterations of hepatic radical

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scavenging enzymes and glutathione-related enzymes—superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase—in developing chick embryos. The data obtained suggest the possibility that a high dose of glucocorticoid tends to generate reactive oxygen species in the liver of the developing chick embryo, leading to the production or elevation of lipid peroxides.

Materials and Methods

The study was conducted in accordance with the Declaration of Helsinki and the Care and Use of Animals (DHEW Publication, NIH 80-23).

Materials

Xanthine and xanthine oxidase (from buttermilk) were obtained from Boehringer Mannheim (Mannheim, Germany). Aniline, 1-chloro-2,4-dinitrobenzene, nitro-blue tetrazolium and hydrogen peroxide were obtained from Wako Pure Chemical Industry Ltd (Tokyo, Japan). Bovine serum albumin, NADPH, glutathione, glutathione reductase (type 111, from baker's yeast), oxidized glutathione, sodium azide, and hydrocortisone sodium succinate (hydrocortisone) were purchased from Sigma Chemical Co. (St Louis, MO). Cortexolone was purchased from Aldrich Chemical Co. (Milwaukee, WI). Cortisone sodium succinate and cortexolone sodium succinate were prepared from cortisone and cortexolone, respectively. All the other reagents were of analytical grade.

Treatment of animals

One-day-old fertile White Leghorn eggs were purchased from a local hatchery and incubated in a humidified incubator at 37·5°C. Hydrocortisone (0·25 μmol in 0·2 mL sterilized water) and other steroids were administered to 15-day-old developing chick embryos through a small hole in the air sack. The embryos were further incubated for the indicated time, then the livers were removed and washed with cold isotonic saline. By this procedure, over 90% lenses became cataract 48 h after hydrocortisone treatment and then their opacity disappeared by 96 h (Lee et al 1991, 1995).

Determination of TBARS

Immediately after homogenization of the chick-embryo liver in double-distilled water using a Biotron, TBARS in the homogenate was determined fluorometrically with thiobarbituric acid as described previously (Nishigori et al 1984). Malondialdehyde was used as a standard.

Preparation of hepatic enzyme sample

The pooled livers were rinsed with cold isotonic saline, blotted and weighed. All subsequent steps were carried out at 0–4°C. The livers were homogenized in four volumes of 10 mM phosphate buffer (pH 7·4) containing 0·25 M sucrose, 1·0 mM EDTA, and 0·1% Triton X-10 (Sigma Chemical Co., St Louis, MO) using a Biotron. The homogenate was centrifuged at 12 000 rev min^{-1} for 30 min and the supernatant was used for assays of activities of glutathione reductase (Goldberg & Spooner 1984), glutathione peroxidase (Hosoda & Nakamura 1970), superoxide dismutase (Beauchamp & Friedovich 1971) and catalase (Aebi 1984). All measurements were carried out using a thermostatically controlled Shimazdu UV-2200 spectrophotometer.

Aniline hydroxylase was determined as described previously (Nishigori & Iwatsuru 1982). Briefly, the reaction mixtures containing 0·75 mL of the supernatant and 0·25 mL 20 mM aniline with 12 mM $MgCl_2$ in the buffer were incubated at 37°C for 30 min, then centrifuged at 3000 rev min^{-1} for 5 min. Samples of the supernatant (1·0 mL) were mixed with 1·0 mL 10% (w/v) sodium carbonate in water and with 0·5 mL 4% (v/v) phenol in 0·4 M sodium hydroxide. The mixture was incubated at 37°C for 1 h, and the absorbance was determined at 640 nm. The protein content of enzyme preparations was determined by the method of Lowry et al (1951), using bovine serum albumin as a standard.

Statistics

Student's *t*-test for unpaired observations was used for statistical evaluation of the data. $P < 0.05$ was considered significant.

Results

Elevation of TBARS level by hydrocortisone

The change in TBARS level in the liver after hydrocortisone administration was examined. As shown in Figure 1 (inset), the hepatic TBARS level remained unchanged during normal growth of chick embryos from day 15 (0 h) to day 19 (96 h). However, when 0·25 μmol hydrocortisone was administered to 15-day-old chick embryos the TBARS level increased markedly after about 20 h, reached a value approximately six times that of the control at 48 h after hydrocortisone administration, and then decreased as described in a previous paper (Nishigori et al 1985). Since the adverse effects of hydrocortisone are dose-dependent, the effect of hydrocortisone dosage on the TBARS level was determined at 48 h after hydrocortisone administration. The TBARS level in the liver increased depending on dose from 0·05 μmol/egg to 0·25 μmol/egg.

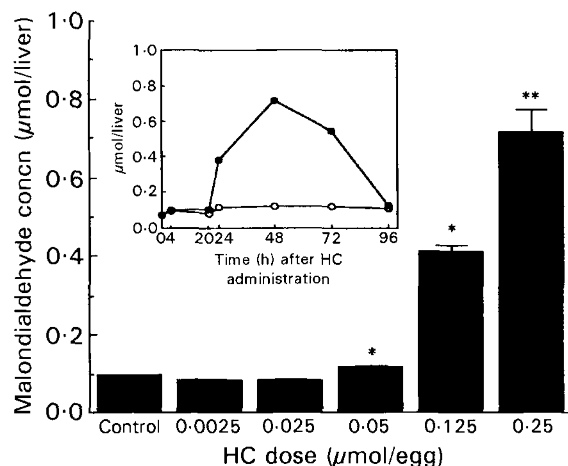

Figure 1. Effect of hydrocortisone succinate sodium (HC) on hepatic TBARS level in developing chick embryo. Hydrocortisone at the indicated dosage was dissolved in 0·2 mL of sterilized water and administered to 15-day-old chick embryos. At 48 h after hydrocortisone administration, TBARS was determined as described in Materials and Methods. Data are expressed as μmol malondialdehyde per liver. Each point represents the mean ± s.e. from five experiments. Significance values are relative to control. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$. Inset: each point represents the mean of two experiments. ○, Control; ●, hydrocortisone-treated.

Enzyme activities related to antioxidant

Table 1 shows the effect of hydrocortisone on glutathione-related enzymes such as glutathione peroxidase and glutathione reductase activities. Glutathione peroxidase catalyses the oxidation of glutathione by hydrogen peroxide and lipid peroxide. The glutathione peroxidase-glutathione system, therefore, is of primary physiological importance in protecting the liver from oxidative attack (Halliwell & Gutteridge 1990). The glutathione peroxidase activity showed a slight increase during normal growth of chick embryos from day 15 (0 h) to day 18 (72 h). However, when 15-day-old chick embryos were administered hydrocortisone, the glutathione peroxidase activity began to decrease from 4 h after administration, falling to 70% of the normal level at 24 h and then increasing. The glutathione reductase activity remained at 25–32 nmol (mg protein)$^{-1}$ min^{-1} during normal growth, but following hydrocortisone administration, it started to decrease after 4 h, fell to 70% of the normal level at 24 h, and then recovered to the normal level at 72 h. These data indicate that glutathione-related defence functions against oxidative stress were impaired after hydrocortisone administration.

The effect of hydrocortisone on superoxide dismutase and catalase activities were also determined (Table 2). The superoxide dismutase and catalase activities remained almost constant during normal growth of chick embryos. At 48 h, the superoxide dismutase and catalase activities were about 70% and 55% of the normal levels, respectively. Catalase showed the largest decrease among the four enzymes investigated.

As described above, it is noteworthy that the activities of the four antioxidant enzymes as well as glutathione declined in 15–20 h before the elevation of TBARS level, and conversely, the elevated TBARS level decreased when the levels of these enzymes recovered.

Aniline hydroxylase activity, as a marker of oxidative activity in the liver, was examined (Figure 2). The hepatic aniline hydroxylase activity showed a slight increase during normal growth of chick embryos from day 15 (0 h) to day 17 (48 h). When hydrocortisone was administered to 15-day-old chick embryos, the level of aniline hydroxylase activity began to increase from 12 h before the onset of TBARS elevation (Figure 1, inset) and reached a value approximately four times that of the control at 30 h after hydrocortisone administration, and decreased thereafter. This result indicated that hydrocortisone induced oxidative activity in the liver.

Table 1. Changes in chick-embryo hepatic glutathione peroxidase and glutathione reductase activities after hydrocortisone administration.

Time after treatment (h)	Glutathione peroxidase (nmol (mg protein)$^{-1}$ min^{-1})		Glutathione reductase (nmol (mg protein)$^{-1}$ min^{-1})	
	Control	Treatment	Control	Treatment
0	20·0 ± 1·2		25·1 ± 1·7	
4	23·3 ± 2·2	17·7 ± 2·3	32·5 ± 2·6	31·4 ± 1·4
24	27·2 ± 3·6	19·4 ± 0·9*	32·8 ± 4·3	24·0 ± 2·8
48	26·8 ± 3·6	20·8 ± 3·1	32·0 ± 4·2	21·8 ± 3·1
72	30·5 ± 1·7	31·1 ± 1·0	32·0 ± 1·8	30·8 ± 1·9

Hydrocortisone succinate sodium (0·25 μmol/egg) was administered to 15-day-old chick embryos (0 h). At the indicated time, the activities in hepatic tissues were determined as described in Materials and Methods. Data represent the mean ± s.e. from five experiments. Significance values are relative to control. $*P < 0{\cdot}05$.

Table 2. Changes in chick-embryo hepatic catalase and superoxide dismutase activities after hydrocortisone administration.

Time after treatment (h)	Catalase (μmol (mg protein)$^{-1}$ min^{-1})		Superoxide dismutase (μmol (mg protein)$^{-1}$ min^{-1})	
	Control	Treatment	Control	Treatment
0	46·0 ± 2·8		27·0 ± 0·5	
4	44·8 ± 2·4	29·4 ± 3·9	31·8 ± 0·8	28·4 ± 1·5
24	42·4 ± 1·8	35·7 ± 1·0	30·9 ± 5·9	26·6 ± 4·2
48	45·4 ± 3·0	26·0 ± 3·0**	29·7 ± 4·5	23·0 ± 1·5
72	47·8 ± 2·6	30·3 ± 2·4*	30·3 ± 0·9	26·9 ± 1·4
96	50·9 ± 1·7	46·0 ± 1·0	30·0 ± 1·1	27·8 ± 0·9

Hydrocortisone succinate sodium (0·25 μmol/egg) was administered to 15-day-old chick embryos (0 h). At the indicated time, the catalase and superoxide dismutase activities in hepatic tissues were determined as described in Materials and Methods. Data represent the mean ± s.e. from five experiments. Significance values are relative to control. **$P < 0·001$ *$P < 0·05$.

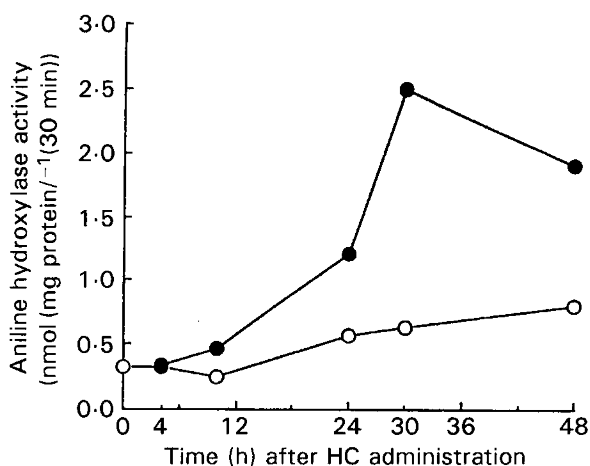

Figure 2. Alteration of hepatic aniline hydroxylase activity in chick embryo. Hydrocortisone (0·25 μmol/egg) was administered to 15-day-old chick embryos (0 h). At the indicated time, aniline hydroxylase activity in hepatic tissues was determined as described in Materials and Methods. ○, Control; ●, hydrocortisone-treated. Each point represents the mean of two experiments.

Table 3 shows the relationship between hydrocortisone doses and aniline hydroxylase activity, TBARS content in the liver and cataract formation at 48 h after hydrocortisone administration. The inducing effect of hydrocortisone on hepatic aniline hydroxylase activity was observed from the dose of 0·025 μmol/egg. However, it seemed that higher doses were required to cause the marked elevation of TBARS level in the liver and cataract formation as one of the adverse effects due to glucocorticoid administration.

It was also found that these effects were induced by biological activities of glucocorticoid. Namely, the administration of 0·25 μmol/egg of the sodium succinate salt of cortisone or cortexolone did not change aniline hydroxylation and had no effect on TBARS elevation and cataract formation.

Discussion

The liver is one of the most active tissues and its changes in metabolic activities greatly alter the blood components to influence other tissues. Although it is said that the value of TBARS represents an indirect indicator of lipid peroxides, the alteration profiles of TBARS production in the liver by hydrocortisone will give some information to clarify the mechanism of glucocorticoid-induced adverse effects. Lipid peroxidation in the liver is controlled by a delicate balance between the availability of substrates and the levels of initiators and suppressors (Halliwell & Gutteridge 1990). Particularly, the antioxidant defence systems in the liver play an important role in regulating the production of lipid peroxide. The present data show that, although the mechanisms are unclear, a high dose of hydrocortisone caused decrease of the hepatic antioxidant defence enzymes such as glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase and of glutathione content (Lee et al 1991). These decreases of the radical scavenging abilities and the elevation of aniline hydroxylation activity preceeded the elevation of TBARS in the liver. It was also demonstrated that glucocorticoid produced superoxide anion in the rat liver and enhanced the activities of enzymes producing excess superoxide anion (Nelson & Ruhmann-Wennhold 1975). As lipid peroxide could be initiated by the enhanced production of oxygen free radicals and the impaired enzymatic and non-enzymatic defences of the liver, it is conceivable that the liver of chick embryo treated with hydrocortisone easily tends to the production of the reactive oxygens and lipid peroxides. Interestingly, these hydrocortisone-induced phenomena recovered to control levels with time; the elevated TBARS decreased with recovery of radical

Table 3. Relationship between hepatic aniline hydroxylase activity and TBARS content in the liver and cataract formation in the lens in developing chick embryos.

Dosage (μmol/egg)		Liver		Lens			
		Aniline hydroxylase(%)	TBARS (%)	Frequency of cataract			
				I	II	III	IV–V
Control		100	100	20	0	0	0
Hydrocortisone	0·0025	118±8	95	20	0	0	0
	0·025	280±27*	87	20	0	0	0
	0·05	N.D.	130	12	4	4	0
	0·25	444±60*	680	2	0	0	18
Cortisone	0·25	124±26	90	20	0	0	0
Cortexolone	0·25	117±13	90	20	0	0	0

The sodium succinate of corticoid at the indicated dosage was dissolved in 0·2 mL sterilized water and administered to 15-day-old chick embryos. At 48 h after corticoid administration, aniline hydroxylase activity in liver was determined as described in Materials and Methods. Data are expressed as average± s.e. from four experiments. TBARS content in liver (Figure 1, Nishigori et al 1984) and classification of cataract in lens (Lee et al 1995) were summarized. Lenses were clear (stage I), had pale (II) or distinct (III) opaque ring between nuclear and cortical region, and almost opaque in nuclear (IV–V). Data were expressed as number of lenses. *Significance values are relative to control, $P < 0·01$. N.D. = not detected.

scavenging enzymes. The hepatic glutathione level in the hydrocortisone-treated chick embryos began to decrease from around 4 h, was about 50% of the matched control at 24 h, and thereafter recovered, returning to control levels 48 h after hydrocortisone administration (Lee et al 1991). Thus, the recoveries of the glutathione peroxidase and glutathione levels in the liver mainly caused the degradation of lipid peroxide, resulting in the decrease of TBARS.

We also demonstrated that the administration of 0·25 μmol/egg of cortisone or cortexolone had no effect on aniline hydroxylation and the elevation of TBARS levels in the liver and cataract formation. It is known that the C-11 positions of cortisone and cortexolone are hardly hydroxylated in chick liver (Moscona & Piddington 1967; Cohen & Kulka 1975), presumably because of lacking, or quite low activy of, 11β-hydroxysteroid dehydrogenase (type 1). Therefore, the phenomena described above could be caused by the biological actions of glucocorticoid since the C-11 β-hydroxyl group of the structure is known to be essential for the biological activities (Moscona & Piddington 1967; Cohen & Kulka 1975). In addition, phenobarbital treatment, which stimulated hepatic hydroxylation activity of aniline in developing chick embryos at a similar rate to hydrocortisone treatment (Nishigori & Iwatsuru 1982), did not elevate TBARS in the liver and produce cataract (unpublished data).

In conclusion, synergistic effects of glucocorticoid produced decrease of the antioxidant defence system, the significant elevation of oxidative activity and lipid peroxides in the liver, possibly leading to the appearance of some adverse effects. Further examination of the changes in the metabolic pattern in liver should help to clarify the mechanism of oxidative stress produced by glucocorticoid.

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