Immunohistochemical distribution of isoenzymes of glutathione transferase in adult rat adrenal gland before and after hypophysectomy

Louise MANKOWITZ,*† Joseph W. DEPIERRE,* Bengt MANNERVIK‡ and Hans-Arne HANSSON§

*Unit for Biochemical Toxicology, Department of Biochemistry, Wallenberg Laboratory, Stockholm University, S-106 91 Stockholm, Sweden, †Department of Biochemistry, Uppsala University, Biomedical Center, Box 576, S-751 23 Uppsala, Sweden, and §Department of Histology, University of Göteborg, P.O. Box 33031, S-400 33 Göteborg, Sweden

The distribution of glutathione transferase subunits 1, 2, 3, 4, 7 and 8 in the different cells of the female and male rat adrenal and the effects of hypophysectomy on these isoenzymes were studied using immunohistochemical methods. All these glutathione transferase subunits, with the exception of subunit 1, were present in the adrenal. Each subunit showed, however, its own characteristic distribution pattern. After hypophysectomy, increased staining for these isoenzymes was generally observed, and this effect was also cell-specific. Staining for subunit 2 increased in intensity in the zona fasciculata and reticularis after hypophysectomy, whereas a decrease was observed in the zona glomerulosa. Staining for subunit 8 was increased in the borderline between the capsule and zona glomerulosa, as well as in medullary chromaffin cells after hypophysectomy. The Mu subunits 3 and 4 increased markedly in fascicular and reticular cells after hypophysectomy and staining for subunit 3 was also increased in the medullary cells. A slight, but more general, increase was observed for subunit 7. We conclude from these experiments that the increases in glutathione transferase subunits observed in the rat adrenal after hypophysectomy are due to increased protein synthesis and/or increased protein stability and not to a selective destruction of cells lacking, or with low levels of, the isoenzymes.

INTRODUCTION

The adrenal gland consists of two distinct parts, the medulla and the cortex, with different functions and regulation. The chromaffin cells of the adrenal medulla are responsible for secretion of adrenaline and noradrenaline, while the cortical cells produce steroid hormones. The adrenal cortex can be divided into three different zones of cells. The outermost zone, called the zona glomerulosa, is involved in mineralocorticoid synthesis, while the two inner zones, zona fasciculata and zona reticularis, are the producers of glucocorticoids and, to a lesser extent, sex hormones [1–3]. The synthesis of steroid hormones, as well as several other processes in the adrenal, is regulated by adrenocorticotrophic hormone (ACTH) released by the pituitary [4–6].

The adrenal gland is relatively rich in enzymes involved in detoxication processes and defence against oxygen toxicity, e.g. cytochrome *P*-450 enzymes and glutathione transferases [7,8]. The glutathione transferases are a family of related enzymes that catalyse the conjugation of glutathione to an electrophilic site on a second substrate and are thus involved in the metabolism of both numerous xenobiotics and certain endogenous molecules [9,10]. A possible role for these enzymes in the protection of cells against endogenous products of lipid peroxidation has been postulated [11,12]. In cellular defences the peroxidase activity of glutathione transferases and their capacity to act as binding and transport proteins may also be of great importance [13–15].

The glutathione transferases are divided into three different classes, i.e. Alpha, Mu and Pi, on the basis of their physiological, chemical and immunological properties [16]. At least eight different subunits have been characterized in the rat cytosol, subunits 1, 2 and 8 belonging to class Alpha, subunits 3, 4, 6 and 9 belonging to class Mu and subunit 7 belonging to class Pi. The

cytosolic glutathione transferases are dimers of subunits within the same class [10]. All of these subunits, with the exception of subunit 1 and, possibly subunit 9, are present in the rat adrenal gland [6,17]. Although these proteins can be induced by xenobiotics such as 3-methylcholanthrene, phenobarbital and *trans*-stilbene oxide in the liver, such induction does not seem to occur in the adrenal [7,5]. Hormonal regulation of glutathione transferases in several organs, including the adrenal, is, however, known to occur [18–20]. In a previous study we have shown increased levels of several of these isoenzymes in the rat adrenal in response to hypophysectomy [6]. Subsequent ACTH administration down-regulates these enzyme levels and activities again. Using primary cultures of rat adrenal cells we have demonstrated that this down-regulating effect of ACTH is mediated by cyclic AMP, the common second messenger of ACTH-regulated processes [21].

When studying different processes in the adrenal, it is of importance to keep the heterogeneity of this gland firmly in mind. It is difficult to separate the different regions, because of the small size of this organ in rats, and the availability of material is seriously limited. Therefore, in previous studies on rat glutathione transferases, cytosol or primary cell cultures derived from whole organs have been used. Consequently, the distribution of individual glutathione transferase isoenzymes among the different cell types remains unknown. In this investigation we have used immunohistochemical staining of tissue sections with antibodies directed towards different glutathione transferase subunits in order to map the cellular distribution of these enzymes in rat adrenal and to elucidate whether the up-regulation of several glutathione transferase isoenzymes, which occurs in response to hypophysectomy, is cell-specific.

Abbreviations used: PBS, phosphate-buffered saline; TBS, Tris-buffered saline; ACTH, adrenocorticotrophic hormone.

† To whom correspondence should be addressed.

MATERIALS AND METHODS

Animals and treatment

Female and male Sprague–Dawley rats (175–200 g body weight), untreated, hypophysectomized and sham-operated, were purchased from Møllegaard Breeding Center (Skensved, Denmark). The animals had free access to food and water. The light was on between 06:00 h and 18:00 h. All animals were killed between 08:00 and 10:00 h.

Two groups of animals were studied: (a) rats analysed 4 weeks after hypophysectomy and (b) rats analysed 4 weeks after sham surgery. Each group contained six rats and the experiments were performed three times with female and twice with male rats.

Preparation of tissue for immunohistochemistry

Anaesthetized rats were perfused transcardially via the left ventricle with phosphate-buffered saline (PBS; 50 mM-sodium/potassium phosphate/0.9% NaCl, pH 7.2), followed by buffered formalin [freshly prepared by alkaline hydrolysis of para-formaldehyde to give a final concentration of 4% (w/v)]. The adrenals were dissected and fixed for a total of 4 h and thereafter rinsed in Tris-buffered saline (TBS; 50 mM-Tris/HCl/0.9% NaCl, pH 7.2) containing 7.5% (w/v) sucrose overnight at 4 °C. Sections (thickness 5–8 µm) were prepared with the aid of a cryostat microtome, allowed to adhere to gelatin-coated glass slides, briefly dried and then processed for immunohistochemistry.

Antibodies

Rat glutathione transferases 1–1, 2–2, 8–8, 3–3, 4–4 and the human Pi were purified and antibodies raised in rabbits as described previously [16].

Immunohistochemical procedures

The sections were pretreated with 2% (v/v) normal goat serum and 5% (w/v) fat-free milk in TBS for 30 min. All antisera were used at a dilution of 1:400, except for affinity-purified anti-(glutathione transferase 3–3) antibodies, which were used at a concentration of 25 µg/ml. The antibodies were applied overnight at 4 °C. Thereafter, the sections were extensively rinsed, incubated with anti-rabbit antibodies labelled with fluorescein (diluted 1:50) (Amersham International, Amersham, Bucks, U.K.) or Texas Red (Dakopatts, Stockholm, Sweden), rinsed and finally mounted in buffered glycerol containing antifading substances according to Johnson & Noguera Araujo [22], before analysis in a fluorescence microscope equipped for epi-illumination and with appropriate filters.

Validation of the immunohistochemical procedure

Several experiments were performed in order to verify the specificity of the immunohistochemical reactions. The different antibody preparations were absorbed with either their homologous antigens or with other purified glutathione transferases. The antigens were added at a final concentration of 10 µg/ml to diluted antibodies in TBS containing 1 mg of BSA/ml and 0.04% (w/v) sodium azide, and the mixture was left overnight at 4 °C. Before use, precipitates were removed by centrifugation (12000 g; 5 min). Non-specific binding of the fluorochrome conjugate was checked for by excluding the primary antibodies from the incubation. Furthermore, in other control experiments normal rabbit preimmune serum was used in place of specific primary antibodies. In all these cases no staining was observed.

Morphometry

Anaesthetized rats were fixed by transcardial perfusion with buffered 2.5% purified glutaraldehyde, after an initial rinsing

with PBS. The adrenals were dissected, postfixed in buffered osmium tetroxide, dehydrated in a graded series of ethanol and then embedded in Epon. Sections (1 µm thick) were cut, stained, mounted and then examined by light microscopy. Morphometric measurements were made with the aid of IBAS I+II apparatus (Zeiss–Kontron, Oberkochen, Germany). The areas of the adrenal cortex and medulla were measured on central sections.

RESULTS

Morphometry

The mean area of the adrenal cortex was reduced from the normal value of $12.0 \pm 1.9 \text{ mm}^2$ to $4.89 \pm 0.81 \text{ mm}^2$ 4 weeks after hypophysectomy, i.e. by 59%. The adrenal medulla was concomitantly reduced by 31%, i.e. from $1.59 \pm 0.39 \text{ mm}^2$ to $1.10 \pm 0.59 \text{ mm}^2$ (Fig. 1). Four weeks after hypophysectomy the weight of the adrenal and the amount of cytosolic protein was decreased to 25% of the normal value. A concomitant 3-fold decrease in DNA content was observed, indicating a decrease in the total number of cells (results not shown). This decrease of the adrenal after hypophysectomy is a well-known phenomenon [23].

Immunohistochemistry

In the Figures from the immunohistochemical experiments, the stained regions are the light-coloured ones. Most experiments were performed on female rats, showing the most marked differences in the staining patterns. For subunits 2, 3, 4 and 7 there was no obvious difference between the female and male adrenals. However, differences could be documented for subunit 8 with regard to the cellular patterns in the adrenal cortex before and after hypophysectomy. Therefore only glutathione transferase subunit 8 is described and illustrated for both female and male rats.

Subunit 1. There was no subunit 1 immunoreactivity in the adrenal gland, either normally or 4 weeks after hypophysectomy.

Subunit 2. Every cell in the adrenal cortex showed low to moderate cytoplasmic subunit 2 immunoreactivity, with the exception of the cells in the glomerular zone, many of which were

Fig. 1. Light micrographs of adrenal glands from a normal (a) and a hypophysectomized (b) female rat 4 weeks after the surgery

The capsule is marked by arrowheads and the border between the reticular zone (zr) and the medulla (M) by a broken line. zg, glomerular zone; zf, fascicular zone. Bar = 200 µm.

Fig. 2. Micrographs of female rat adrenals, processed for demonstration of glutathione transferase subunit 2 immunoreactivity

The immunostained section in (a) and (c) comes from a control rat and the section in (b) and (d) from a rat 4 weeks after hypophysectomy. Adrenal capsule is indicated by an arrowhead and intensely stained cells in the glomerular zone (zg) by an arrow. M, medulla. Bar = 200 μ m for (a) and (b) and 50 μ m for (c) and (d).

non-reactive (Fig. 2a). Intensely stained cells were recognized at the border between the glomerular and fascicular zones (Figs. 2a and 2c).

Most adrenal cortical cells showed increased, i.e. moderate, subunit 2 immunoreactivity (Figs. 2b and 2d) 4 weeks after hypophysectomy. A striking feature was the many non-reactive cells in the widened glomerular zone (Figs. 2b and 2d).

Subunit 8. Subunit 8 immunoreactivity prevailed in slender cells in the adrenal capsule, a feature that was less prominent in the female (Figs. 3a and 3c) than in the male (Figs. 3g and 3i) rat.

Faint staining was evident in some of the cells in the outer half of the fascicular zone in the normal female (Figs. 3a and 3c) and in the glomerular zone in male rats (Figs. 3g and 3i). Hypophysectomy increased both the frequency and the intensity of subunit 8 immunoreactivity in the capsular cells and in glomerular zone cells, both in the female (Figs. 3b and 3d) and the male (Figs. 3h and 3k) rat. There was an increased frequency of randomly distributed, intensely stained cells in the medulla of hypophysectomized female (Figs. 3e and 3f) and male (Figs. 3l and 3m) rats.

Subunit 3. Faint cytoplasmic subunit 3 immunoreactivity was evident in most cells in the normal female adrenal cortex (Figs. 4a and 4c), increasing markedly at 4 weeks after hypophysectomy (Figs. 4b and 4d). A striking feature was the distinct staining of cells at the border between the glomerular and fascicular zones in the normal rat (Fig. 4c) and that such cells could also be recognized in the same region of the widened glomerular zone after hypophysectomy (Fig. 4d). Normal adrenal medullary cells showed subunit 3 immunoreactivity (Figs. 4a and 4e), which also increased in intensity after hypophysectomy (Figs. 4b and 4f). In contrast, the vascular cells between the reactive chromaffin cells were negative.

Subunit 4. Cytoplasmic subunit 4 immunoreactivity was evident in cells in the capsule, as well as at low intensities in cells in the inner half of the adrenal cortex of the normal female rat (Figs. 5a and 5c). Hypophysectomy caused after 4 weeks a strong increase in subunit 4 immunoreactivity in the cells in the shrunken fascicular zones, while the cells in the widened glomerular zone remained largely non-reactive (Figs. 5b and 5d). Only rare medullary cells were stained.

Subunit 7. Cytoplasmic faint subunit 7 immunoreactivity was evident in most cortical cells, most distinctly in the glomerular zone (Figs. 6a and 6c). Hypophysectomy increased the frequency of staining of glomerular cells (Figs. 6b and 6d). Concomitantly, there was a prominent increase of intensely stained cells in the reticular zone (Figs. 6b and 6f), as compared with what could be visualized in the normal female rat (Figs. 6a and 6e). A weak staining of fibroblasts was observed in the medulla before and after hypophysectomy (Figs. 6a and 6b).

DISCUSSION

Each glutathione transferase subunit showed its own characteristic distribution pattern in the adrenal gland in normal as well as in hypophysectomized rats. These results further support our conclusion from the specificity tests, i.e. that there was no significant cross-reactivity between the different antisera. A common feature was that hypophysectomy caused dramatic changes in the levels of all the subunits examined, except for the non-detectable subunit 1. This is in good agreement with our earlier conclusions based on immunoblotting of cytosol from whole rat adrenals before and after hypophysectomy, except in

Fig. 4. Micrographs of female rat adrenals, processed for demonstration of glutathione transferase subunit 3 immunoreactivity

The immunostained section in (a), (c) and (e) comes from a control rat and the section in (b), (d) and (f) from a rat 4 weeks after hypophysectomy. Demarcation lines in (e) and (f) indicate the border between the cortical reticular zone (to the right) and the medulla (M). The capsule is marked by arrowheads, and intensely stained cells (c and d) along the border between the glomerular and fascicular zones is marked by arrows. Bar = 200 μm for (a) and (b) and 50 μm for (c)–(f).

Fig. 5. Micrographs of female rat adrenals, processed for demonstration of glutathione transferase subunit 4 immunoreactivity

The immunostained section in (a) and (c) comes from a control rat and the section in (b) and (d) from a rat 4 weeks after hypophysectomy. The capsule is marked by arrowheads. M, medulla. Bar = 200 μm for (a) and (b) and 50 μm for (c) and (d).

Fig. 3. Micrographs of female (a–f) and male (g–m) rat adrenals, processed for demonstration of glutathione transferase subunit 8 immunoreactivity

The immunostained section in (a), (c) and (e) comes from a female control rat, the section in (b), (d) and (f) from a female rat 4 weeks after hypophysectomy, the section in (g), (i) and (l) from a male control rat and the section in (h), (k) and (m) from a male rat 4 weeks after hypophysectomy. The capsule is marked by an arrowhead. M, medulla; zg, zona glomerulosa. Bar = 200 μm for (a), (b), (g) and (h) and 50 μm for (c)–(f) and (i)–(m). There is no part (j).

Fig. 6. Micrographs of female rat adrenals processed for demonstration of glutathione transferase subunit 7 immunoreactivity

The immunostained section in (a), (c) and (e) comes from a control rat and the section in (b), (d) and (f) from a rat 4 weeks after hypophysectomy. Demarcation lines in (e) and (f) indicate the border between the cortical reticular zone (zr) and the medulla (M). The arrowhead marks the capsule. zg, zona glomerulosa. Bar = 200 μ m for (a) and (b) and 50 μ m for (c)–(f).

the case of subunit 2, where no increase was observed earlier. A possible explanation for this apparent discrepancy is that subunit 2 may not be localized exclusively in the cytosol.

The class Alpha subunits 2 and 8 both showed faint to moderate immunoreactivity in the fascicular and reticular zones (Figs. 2 and 3). In addition, cells with high immunoreactivity towards these subunits were enriched along the border between the glomerular and fascicular zones. However, upon closer examination marked differences could be observed, even for the class Alpha subunits. Most cells in the glomerular zone lacked subunit 2 immunoreactivity after hypophysectomy (Fig. 2), while in contrast there was an increased immunoreactivity of subunit 8 in the glomerular zone (Fig. 3d).

On the other hand, hypophysectomy caused more intense staining for the class Mu subunits 3 and 4 in the fascicular and reticular zones (Figs. 4 and 5), while only minor alterations were induced in the glomerular zone. The class Pi subunit 7 showed a more generalized increase after hypophysectomy.

The slender smooth muscle cells in the capsule of normal adrenal gland showed prominent immunoreactivity for subunits 2, 8, 4 and 7. Distinct changes in these patterns of immunoreactivity were observed 4 weeks after hypophysectomy. The two prevailing class Alpha subunits showed opposite patterns, i.e. the staining intensity for subunit 2 decreased (Fig. 2), while that for subunit 8 increased (Fig. 3). On the other hand, the staining pattern for the class Mu subunit 4 did not change after hypophysectomy. These observations stress the importance of examining each cell type, since each one obviously has its own pattern of glutathione transferases and of changes caused by hypophysectomy.

The chromaffin cells in the adrenal medulla showed prominent increases in their immunoreactivities for subunits 8 and 3, belonging to classes Alpha and Mu respectively, after hypophysectomy. For the first one of these, there was an increase in the frequency of equally intensely stained chromaffin cells. In contrast, all chromaffin cells showed subunit 3 immunoreactivity both in the control animals and after hypophysectomy, although at much higher intensities in the latter case. These patterns were

not sex-dependent, i.e. they were the same for both female and male rats.

For most of the glutathione transferase subunits there were quantitative, but no obvious qualitative, sex-related differences with regards to the effects of hypophysectomy. The qualitative patterns could, however, differ between sexes, as reflected by, e.g., the patterns of subunit 8 immunoreactivity. In the normal female rat, very few cells in the glomerular zone showed subunit 8 immunoreactivity (Figs. 3a and 3c), while in the male rat most cells in the glomerular zone were distinctly positive (Figs. 3g and 3i). Hypophysectomy rendered virtually every cell subunit-8-immunoreactive in the male (Figs. 3h and 3k), but only a small fraction in the female rat (Figs. 3b and 3d).

The adrenal glutathione transferases all appeared to be cytoplasmic, on the basis of the immunohistochemical techniques employed here. However, the method employed does not have sufficient resolution to exclude the possibility that some immunoreactivity may be associated with the plasma membrane. There was no nuclear staining in the cells of the adrenal in the case of any of the subunits examined. In contrast, nuclear staining is often encountered in other tissues and organs. The reason for this tissue-related difference in the apparent subcellular distributions of the glutathione transferases is not known.

In conclusion, the present results demonstrate that the patterns of glutathione transferase subunits in the various types of cells in the rat adrenal gland are unique and that the effects of hypophysectomy on these individual isoenzymes are also cell-specific, although, in general, increases in staining intensity were observed. In addition, these results clearly demonstrate that the increases in several glutathione transferase subunits observed after hypophysectomy are due to new synthesis of protein and/or increased protein stability and not to a selective destruction of cells lacking or demonstrating low levels of the isoenzymes. In another study (L. Mankowitz, J. W. DePierre, B. Mannervik & H.-A. Hansson, unpublished work) we have observed an increase in mRNA levels for subunit 4 in the rat adrenal 4 weeks after hypophysectomy, indicating increased transcription and/or increased mRNA stability. The functional explanation(s) for

these distributions of glutathione transferase isoenzymes among the different types of adrenal cells and for the differential responses to hypophysectomy are not known. Such phenomena are, however, likely to reflect differential endogenous roles of these enzymes, rather than different requirements for xenobiotic-metabolizing capacity.

This work was supported by the Swedish Cancer Society, the Swedish Natural Science Research Council and the Swedish Medical Research Council. We thank Stina Dyberg and Elisabeth Norström for their technical assistance.

REFERENCES

1. Tyrell, J. B. & Forsham, P. H. (1987) in *Basic and Clinical Endocrinology* (Greenspan, F. S. & Forsham, P. H., eds.), pp. 272–309, Lange Medical Publications, Los Altos, CA
2. Biglieri, E. G. & Kater, C. E. (1987) in *Basic and Clinical Endocrinology* (Greenspan, F. S. & Forsham, P. H., eds.), pp. 310–325, Lange Medical Publications, Los Altos, CA
3. Goldfien, A. (1987) in *Basic and Clinical Endocrinology* (Greenspan, F. S. & Forsham, P. H., eds.), pp. 326–344, Lange Medical Publications, Los Altos, CA
4. Waterman, M. R. & Simpson, E. R. (1987) *Rev. Biochem. Toxicol.* **8**, 257–287
5. Guenther, T. M., Nebert, D. W. & Menard, R. H. (1979) *Mol. Pharmacol.* **15**, 719–728
6. Mankowitz, L., Castro, V. M., Mannervik, B., Rydström, J. & DePierre, J. W. (1990) *Biochem. J.* **265**, 147–154
7. Bengtsson, M., Montelius, J., Mankowitz, L. & Rydström, J. (1983) *Biochem. Pharmacol.* **32**, 129–136
8. Hornsby, P. J. & Crivello, J. F. (1983) *Mol. Cell. Endocrinol.* **30**, 123–147
9. Mannervik, B. (1985) *Adv. Enzymol. Relat. Areas Mol. Biol.* **57**, 357–417
10. Mannervik, B. & Danielsson, U. H. (1988) *CRC Crit. Rev. Biochem.* **23**, 281–334
11. Ketterer, B., Meyer, D. J., Taylor, J. B., Pemble, S., Coles, B. & Fraser, G. (1989) in *Glutathione S-Transferases and Drug Resistance* (Hayes, J. D., Pickett, C. B. & Mantle, T. J., eds.), pp. 97–109, Taylor and Francis, London
12. Mannervik, B. (1987) *Chem. Scr.* **27A**, 121–123
13. Ketterer, B., Tan, K. H., Meyer, D. J. & Coles, B. (1987) in *Glutathione Transferases and Carcinogenesis* (Mantle, T. J., Pickett, C. B. & Hayes, J. D., eds.), pp. 194–163, Taylor and Francis, London
14. Tan, K. H., Meyer, D. J., Coles, B. & Ketterer, B. (1986) *FEBS Lett.* **207**, 231–233
15. Listowsky, I., Abramovitz, M., Homma, H. & Niitsu, Y. (1988) *Drug Metab. Rev.* **19**, 305–318
16. Mannervik, B., Ålin, P., Guthenberg, C., Jensson, H., Tahir, M. K., Warholm, M. & Jörnvall, H. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7202–7206
17. Ketterer, B. (1986) *Xenobiotica* **16**, 957–973
18. Igarashi, T. & Satoh, T. (1989) *Drug Metab. Drug Interact.* **7**, 191–212
19. Kaplowitz, N., Clifton, G., Kuhlenkamp, J. & Wallin, J. D. (1976) *Biochem. J.* **158**, 243–248
20. Lamartiniere, C. A. (1981) *Biochem. J.* **198**, 211–217
21. Mankowitz, L., Rydström, J. & DePierre, J. W. (1991) *Eur. J. Biochem.*, in the press
22. Johnson, G. D. & Nogueira Araujo, G. M. (1981) *J. Immunol. Methods* **43**, 349–350
23. Idelman, S. (1970) *Int. Rev. Cytol.* **27**, 181–281

Received 17 April 1991/14 June 1991; accepted 18 June 1991