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Preclinical report

Decreased cortisol secretion by adrenal glands perfused with the P-glycoprotein inhibitor valspodar and mitotane or doxorubicin

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The aim of this study was to investigate the role of P-glycoprotein (P-gp) in the adrenal gland. It has been presumed that P-gp, rather than being involved in physiological cortisol secretion, plays a role in protecting the adrenacortical cells from xenobiotics. To explore this a study was performed on perfused bovine adrenal glands. Individual experimental groups were perfused with either a selective P-gp blocker (valspodar) alone, with a xenobiotic (mitotane or doxorubicin) alone or with both valspodar and a xenobiotic. The cumulative amounts of cortisol secreted in each individual group were calculated and the two-sample *t*-test was used to compare the mean values of cumulative amounts. The mean value of cortisol secreted from the group of adrenals perfused with the P-gp blocker was not significantly different from that of the control group. Treatment with either mitotane or doxorubicin decreased the amount of cortisol secreted but not significantly when compared to the amount of cortisol secreted in basal conditions. However, treatment with the P-gp blocker valspodar in addition to either mitotane or doxorubicin significantly decreased cortisol secreted compared to the amount of cortisol secreted by the glands treated with either mitotane (p=0.009) or doxorubicin (p=0.017) alone. The regressive changes discovered in all experimental groups were most prominent when valspodar was used with either mitotane or doxorubicin. We found that P-gp blockade increases by xenobiotic (mitotane and doxorubicin)-induced damage of adrenocortical cells, which points to a role of P-gp in the protection of adrenal gland from xenobiotics. [© 2000 Lippincott Williams & Wilkins.]

Key words: Adrenal gland, cortisol, P-glycoprotein, valspodar, xenobiotics.

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Introduction

Today, multidrug resistance (MDR) is the most thoroughly examined mechanism of resistance to cytostatic agents. In the early 1960s a 170 kDa transmembrane glycoprotein was identified; it was named P-glycoprotein (P-gp), and it was established that it actively transports a wide range of structurally unrelated cytostatics out of the cell and is therefore responsible for MDR.[1–3] Later it was established that P-gp also transports various other hydrophobic drugs from the cells. These substances, called MDR modulators, by binding themselves to P-gp or being transported by P-gp, prevent the transport of cytotoxic agents from the cell.[2,4] At this moment, one of the most promising MDR modulators is valspodar, an analog of cyclosporine A. It has no immunosuppressive or nephrotoxic effects and *in vitro* is a 2–10 times more effective P-gp blocker than cyclosporine.[5,6]

P-gp is also present on normal cells of the vertebra, where its function has not been thoroughly explored yet. Based on the tissue distribution and functional studies, it has been proposed that P-gp plays a role in the protection of organs from toxic xenobiotics, by active excretion of these compounds into bile, urine or intestinal lumen and by preventing accumulation in some organs such as the brain.[7–10] Knockout mice with a genetic deficiency in *mdr* genes were healthy and were fertile under laboratory conditions;[11] however, after exposing these animals to various drugs drastic changes in the pharmacokinetics with altered toxicity of the drugs were found and the effect was enhanced by treatment with the MDR modulator valspodar.[12–14] This observation shows that P-gp plays

a vital role in protecting the organism from various toxins from the environment—xenobiotics.

P-gp is also present in the adult human as well as other mammal's adrenal gland cortex[15,16] and the role of P-gp in this gland is still the subject of discussion. It has been established that steroid hormones are substrates for P-gp[17–20] and *in vitro* studies, performed on transepithelial transport systems, revealed that some steroids like aldosterone, dexamethasone and cortisol are transported by P-gp, while others such as progesterone bind to but are not transported by P-gp.[17] Both properties seem to vary according to steroid hydrophobicity and the phosphorylated state of P-gp.[21] According to these *in vitro* studies it was presumed that P-gp is involved in physiological secretion of steroid hormones from the adrenal cortex; however, the *in vivo* research has not confirmed this presumption yet.

Our previous studies conducted in rabbits did not confirm the involvement of P-gp in steroid hormone secretion from the adrenal gland.[22,23] Serum cortisol levels were found to have increased rather than decreased after P-gp blockade, either with cyclosporine A or valspodar. The increased serum cortisol levels observed after valspodar as well as cyclosporine A were most likely caused by the increased corticotrophin secretion from the pituitary gland, since immunophilins were found to be potent stimulators of propiomelanocortin-derived peptides such as adrenocorticotropic hormone (ACTH).[24] Unfortunately, we were not able to measure ACTH in rabbits by means of commercially available kits. On the basis of our previous studies we presumed that adrenal cortex P-gp is not involved in cortisol secretion but in the protection of adrenocortical cells from toxic xenobiotics. To explore this and to exclude the influence of a selective P-gp blocker on the hypothalamic–pituitary–adrenal axis, we performed a study on perfused adrenal glands. Our hypothesis was that P-gp blockade by a highly selective P-gp modulator (valspodar) will allow toxic substances such as mitotane and doxorubicin to cause greater damage to the adrenal cortex.

Materials and methods

Bovine adrenal glands were obtained from a local slaughterhouse. The glands were dissected free of fat and connective tissue, and cannulated via the adrenal vein within 30 min of the death of the animal. After the primary perfusion with Medium 199 (cat. no. 21200; Gibco Life Technologies, Paisley, UK), the organs were transported on ice into the laboratory. It is estimated that no more than 2 h elapsed between the death of an animal and the connection of the organs to the perfusion pump.

Adrenal glands were perfused retrogradely through a vein with Medium 199. $NaHCO_3$ (Merck, Darmstadt, Germany) was used to adjust the pH level of the perfusion fluid to a value of pH 7.4 and 156 mmol/l Na^+. A K^+ concentration of 5.7 mmol/l was obtained by dissolving the solution. The medium was continuously bubbled with pure O_2 and heated to 37°C. Retrograde perfusion was carried out at a rate of 2 ml/min for 180 min. To evoke cortisol secretion β_{1-24}-corticotrophine (Synacthen®; Ciba-Geigy, Basel, Switzerland) at a concentration of 0.41 μg/ml was added to the perfusion fluid.

In the study we had a group of glands (n=6) perfused under basal conditions and five treatment groups of six or 11 glands. Individual treatment groups were perfused with either the P-gp blocker valspodar alone, with a xenobiotic (doxorubicin or mitotane) alone or with both P-gp blocker and a xenobiotic. As xenobiotics we used the agent with the strongest adrenolytic activity, i.e. mitotane (Lysodren®; Bristol-Myers Squibb, Anabolic, Irvine, CA), at a concentration of 833 μg/ml of the perfusate and the cytostatic doxorubicin (Adriablastina®; Farmitalia, Carlo Erba, Italy) at a concentration of 25 μg/ml of the perfusate. For the P-gp blockade, the selective blocker valspodar (PSC833 disolved in 60% Cremophor-EL; Novartis Pharma, Basel, Switzerland) was used at a concentration of 83 μg/ml of the perfusate.

At the end of the perfusion all specimens were collected and immediately fixed in 10% phosphate-buffered formalin and embedded in paraffin. The 4 μm thick sections were stained with hematoxylin & eosin for light microscopic examination.

To analyze the amount of cortisol secreted under basal conditions and under various treatment conditions the perfusate samples were collected every 30 min (=60 ml) for 3 h. The cortisol levels in each sample were measured by radioimmune assay (CORT-CT2; CIS Biointernational, Gif-sur-Yvette, France). The cumulative amounts of cortisol secreted were calculated by multiplying the concentrations with cumulative volumes of the collected perfusate. The results for each experimental group are represented as mean ± SD of the cumulative amount of cortisol secreted (nmol) in a particular time interval. The first 30 min sample was not included in the analysis because of the equilibration of the cortisol secretion under laboratory conditions. The two-sample *t*-test was used to compare means of cumulative amounts. Before applying this test, the *F*-test was used for the evaluation of homoscedascity. Depending on the results of this test, the *t*-test for equal or unequal

variances was performed. Values of $p<0.05$ were considered significant.

Results

The mean values of cortisol secreted in 2.5 h perfusion, under basal and treatment conditions, are presented in Table 1 and Figure 1. The amount of cortisol secreted was not affected by valspodar treatment alone. The mean value of cortisol secreted by six glands treated with valspodar alone was 71.25 nmol (SD=28.42), whereas the mean value of cortisol secreted by the group of six glands under basal conditions was 90.2 nmol (SD=83.02) (p=0.616).

Table 1. Mean values (±SD) of cortisol secreted by perfused bovine adrenal glands during a perfusion period of 2.5 h under basal and treatment conditions

Conditions[a]	Cortisol (nmol)
Basal conditions (n=6)	90.2±83.02
Valspodar (n=6)	71.25±28.42
Mitotane (n=6)	37.03±17.8
Mitotane + valspodar (n=6)	8.55±5.31
Doxorubicin (n=11)	54.49±29.02
Doxorubicin + valspodar (n=11)	28.95±11.25

[a]n= number of glands in each experimental group.

Treatment with either mitotane or doxorubicin resulted in decreased amounts of cortisol secreted—-after mitotane treatment the mean value of cortisol secreted was 37.03 nmol (SD=17.8), whereas after doxorubicin treatment it was 54.49 nmol (SD=29.02). However, the differences did not reach statistical significance when compared to cortisol secreted under basal conditions (p=0.347 and p=0.186, respectively). Treatment with the P-gp blocker valspodar in addition to mitotane or doxorubicin significantly decreased the amount of cortisol secreted. In six glands treated with mitotane and valspodar the mean value of cortisol secreted was 8.55 nmol (SD=5.31), which is significantly less compared to the basal conditions (p=0.061) or to mitotane alone (p=0.009). Eleven glands treated with doxorubicin and valspodar secreted 28.95 nmol (SD=11.25) of cortisol, which is significantly less compared to the 11 glands treated with doxorubicin alone (p=0.017).

To evaluate the dynamics of cortisol secretion, the amounts of cortisol secreted in 30 min time periods were calculated and are presented in Table 2. The amount of cortisol secreted under basal conditions was increasing until the third 30 min period and then gradually decreased. In the mitotane group, the amount of cortisol secreted was initially lower than the amount secreted under basal conditions and gradually decreased with each period. In the mitotane and valspodar group, cortisol secretion was already

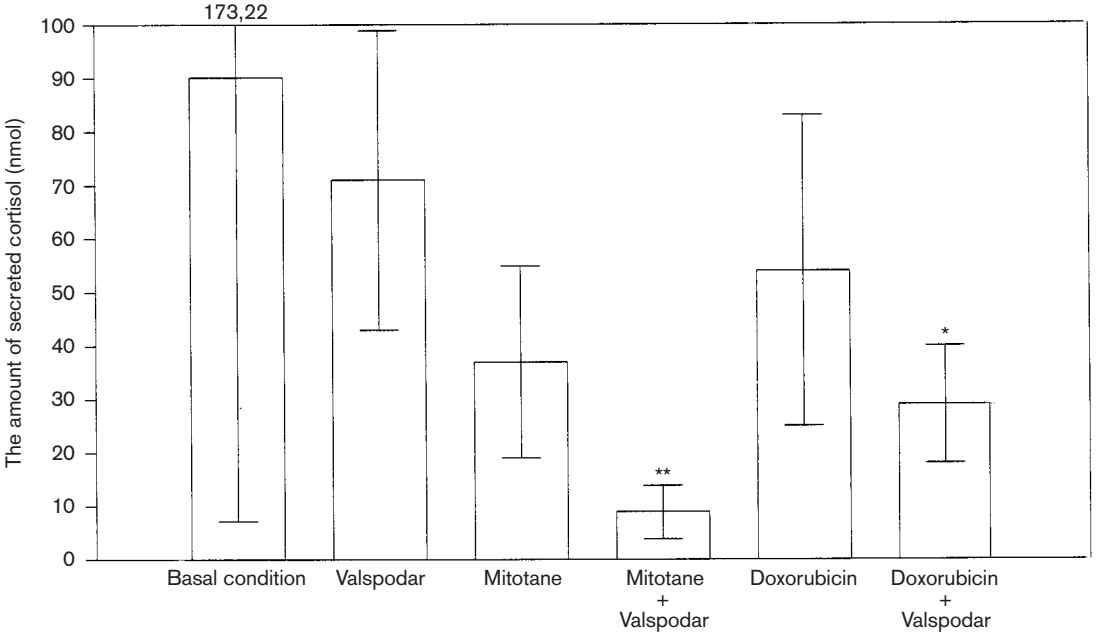

Figure 1. Mean values (±SD) of cortisol secreted by perfused bovine adrenal glands during a perfusion period of 2.5 h under basal and treatment conditions. Statistical analysis was performed by the two-sample t-test. Significance of differences for mitotane alone: $^{**}p$=0.009 and for doxorubicin alone: $^{*}p$=0.017.

extremely low during the first time period and was further decreasing. The amount of cortisol secreted by the mitotane and valspodar group was significantly lower than cortisol secreted by the mitotane group at all time intervals ($p < 0.05$) (Figure 2).

The pattern of cortisol secretion during doxorubicin perfusion was similar to that of mitotane but less impaired. In all 30 min periods the amounts of cortisol secreted from the glands treated with doxorubicin alone were lower than the amounts secreted under basal conditions. The P-gp blockade caused a further decrease in cortisol secretion, which did not become significant until after 90 min of perfusion (Figure 3).

Histopathological changes were discrete; necrotic foci were not found in any of the specimens studied. No morphological changes were discovered in the glands perfused under basal conditions, whereas all other experimental groups showed some degree of regressive changes in the zona fasciculata of the adrenal cortex. Epithelial cells had lost their granulation resulting in the collapse of cells. In addition, most of the cells had condensed and spindle nuclei. These changes were minimal in the group of glands treated with valspodar alone while they were increasingly evident in the groups treated by doxorubicin, mitotane, doxorubicin and valspodar, and mitotane and valspodar, respectively. The most florid changes appeared in the group of glands treated with mitotane and valspodar (Figure 4). Adrenal medulla was not involved.

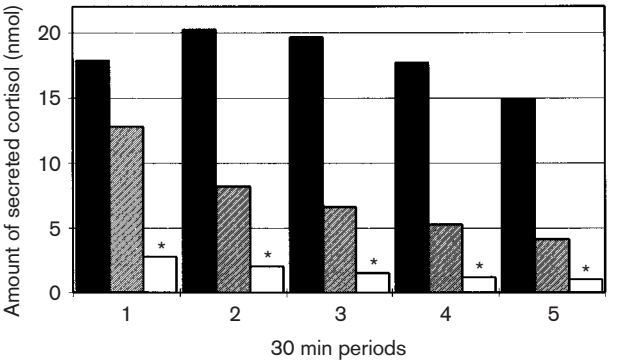

Figure 2. Mean values of cortisol secreted by perfused bovine adrenal glands during different 30 min periods of perfusion under basal conditions (solid bars), mitotane (hatched bars) and mitotane+valspodar treatment (open bars). Statistical analysis was performed by the two-sample *t*-test. Significance of difference for mitotane alone: $^{*}p < 0.05$.

Table 2. Mean values (±SD) of cortisol secreted (nmol) by perfused bovine adrenal glands during 30 min periods of perfusion under basal and treatment conditions

Time periods	Basal conditions	Mitotane	Mitotane + valspodar	Doxorubicin	Doxorubicin + valspodar
1 (30–60 min)	17.85±13.57	12.81±8.08	2.79±1.69	12.15±7.42	9.32±4.47
2 (60–90 min)	20.2±20.18	8.21±5.74	2.01±1.25	11.62±7.39	6.77±2.35
3 (90–120 min)	19.62±19.28	6.61±2.51	1.53±0.98	11.11±6.39	5.5±2.35
4 (120–150 min)	17.66±16.78	5.27±1.43	1.22±0.91	10.45±4.96	4.13±1.88
5 (150–180 min)	14.87±13.93	4.14±1.53	1±0.65	9.16±4.4	3.23±2.17

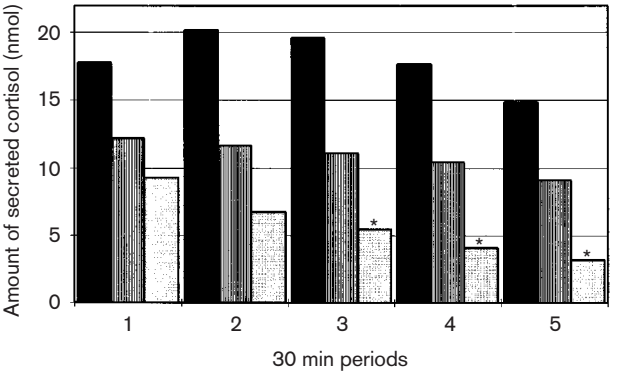

Figure 3. Mean values of cortisol secreted by perfused bovine adrenal glands during different 30 min periods of perfusion under basal conditions (solid bars), doxorubicin (hatched bars) and doxorubicin+valspodar treatment (open bars). Statistical analysis was performed by the two-sample *t*-test. Significance of difference for doxorubicin alone: $^{*}p < 0.05$.

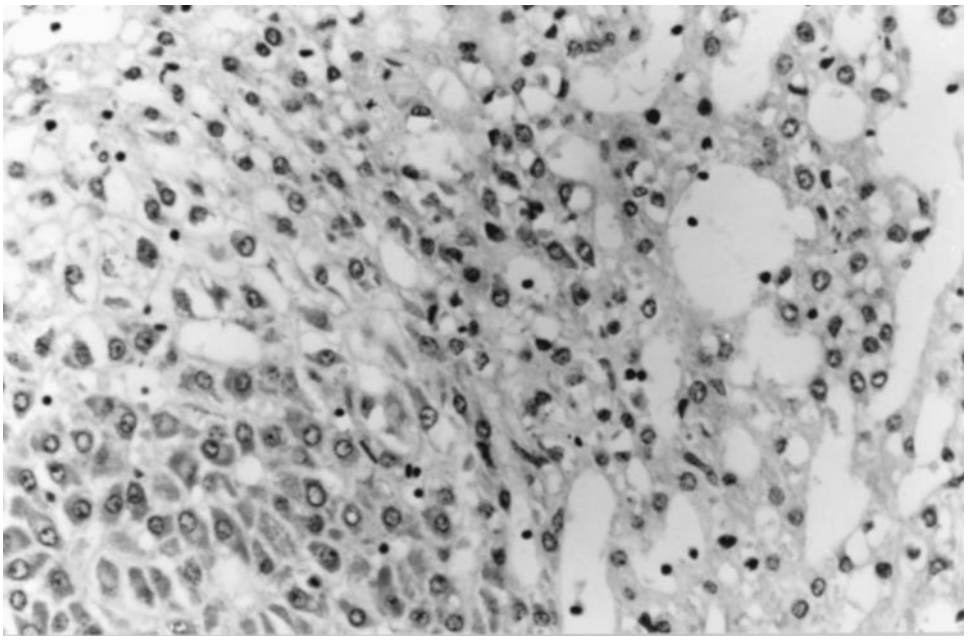

Figure 4. The zona fasciculata (ZF) of the adrenal cortex treated with mitotane and valspodar. Most of the cells are collapsed, degranulated with condensed nuclei. Normal ZF in the upper left corner. (H&E, objective × 40.)

Discussion

Up to now it was presumed that P-gp may play a role in the transport of cortisol from the adrenocortical cells. *In vitro* studies have shown that some steroid hormones, like cortisol, bind to P-gp[17–20] and are also transported by it in transepithelial systems.[17] However, our previous studies using P-gp blockers *in vivo* failed to prove the role of P-gp in cortisol secretion.[22,23] Results of these studies were unclear because of the possible stimulatory effect of P-gp blockers on the hypothalamic–pituitary–adrenal axis. Therefore, we decided to continue our research on isolated organs, thus avoiding the influence of P-gp blockers on the hypothalamus and pituitary gland. We found that P-gp blockade with valspodar did not have any effect on cortisol secretion from the perfused bovine adrenal glands. The mean value of cortisol secreted from the glands treated with valspodar did not differ from the mean value of cortisol secreted by the glands under basal conditions. Our observation that P-gp blockade does not alter cortisol secretion from the adrenal gland is in concordance with our previous observations[22,23] and the observation from Borst *et al.*,[11] who found that knockout mice were healthy and were reproducing normally under laboratory conditions, and confirms our hypothesis that P-gp in the adrenal cortex is not involved in steroid hormone secretion.

We found that the treatment with xenobiotics, mitotane and doxorubicin alone, decreased the amount of cortisol secreted from the perfused adrenal glands compared to the control group but the level of statistical significance was not reached. The reason for this could be due to small samples and large differences in the amounts of cortisol secreted by each individual gland in the control group. The toxic effect of both mitotane as well as doxorubicin increased significantly after applying the P-gp blocker valspodar and reached the level of statistical significance. Therefore, we can conclude that P-gp blockade increases the toxicity of xenobiotics for the adrenal gland.

Before applying the P-gp blocker, the impairment of cortisol secretion was already greater with mitotane than doxorubicin. After the application of valspodar the difference became even larger. We believe that the reason for this is the greater toxicity of mitotane, an oral chemotherapeutic with the highest adrenolythic activity, which is used for the treatment of adrenocorticothropic carcinoma.[25]

By monitoring cortisol secretion in time intervals we found that the adrenal glands perfused by xenobiotics, especially by mitotane, secreted less cortisol than those under basal conditions over all the time, which points to a continous damage of adrenocortical cells. At all time periods the decrease in cortisol secretion was greater when using mitotane compared to

doxorubicin, and the simultaneous application of both P-gp blocker and a xenobiotic resulted in significantly decreased amounts of cortisol secreted in the first 30 min period after valspodar and mitotane treatment, and only in the third 30 min period after valspodar and doxorubicin treatment, which is in concordance with the already mentioned fact that mitotane is a stronger adrenocorticolytic agent.

Regressive changes were observed to some extent in the cortex of all glands; however, these changes were found to be minimal in the group of glands treated with valspodar alone, already mitotane and to some lesser extent also doxorubicin treatment alone caused some changes, while either of them combined with valspodar resulted in major regressive changes. The extent of these changes is in concordance with the alteration of adrenocortical function measured by cortisol secretion.

The results of our study confirm the hypothesis that the function of P-gp in the adrenal gland is the protection of the gland from xenobiotics and can be compared with the results of other studies which showed that the function of P-gp in various other organs such as the brain is the protection from xenobiotics.[7,10] The question remains whether protection from xenobiotics is the only function of P-gp in the adrenal gland. Transport mechanisms of endogenous cortisol have not been clarified yet, but our results exclude that P-gp takes part in them. Considering the results of *in vitro* studies which show that some steroids bind and may even be transported by P-gp,[17,19] we speculate that P-gp in the adrenal cortex may protect the cells from some steroid metabolites. Examination of the protective role of P-gp from endogenic substances should be the matter of future research, which will determine the final role of P-gp in the adrenal gland.

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