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Effect of digoxin on the in vitro secretion of renin and angiotensin II/III immunoreactivity by the human adrenal gland

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Cardiac glycosides in man inhibit renin secretion, probably through a direct effect at the renal level (i.e. inhibition of juxtaglomerular cell Na/K ATPase). Since there is evidence that the human adrenal possesses an intrinsic renin-angiotensin system, we investigated the effect of digoxin on the in vitro generation of renin and angiotensin II/III, as well as of aldosterone, by the human adrenal gland. Minced normal adrenal tissues were studied in a superfusion system, measuring in the 15-min superfusate fractions active renin by immunoradiometric assay and angiotensin II/III and aldosterone by radioimmunoassay, respectively. In a first set of four experiments using different concentrations of digoxin in sequence for 45 min periods, digoxin 10^{-5}, but not 10^{-8} and 10^{-6} mol/l, significantly reduced renin and angiotensin II/III output from adrenals, while no change in aldosterone was observed. In a second set of three experiments, the addition of digoxin 10^{-5} mol/l for 120 min caused a sustained reduction of renin and angiotensin II/III, but not of aldosterone. In the final experiment, the decrease of renin and angiotensin II/III during superfusion with digoxin 10^{-5} mol/l was significantly greater than that observed during superfusion with digoxin in the presence of antidigoxin antibodies. Our data indicate that digoxin at high doses reduces renin and angiotensin II/III but not aldosterone secretion by the human adrenal gland. This suggests two different effects of digoxin, probably both mediated by inhibition of the Na/K ATPase activity, on the adrenal renin-angiotensin- and aldosterone-secreting cells.

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Biochemical mechanisms by which cardiac glycosides influence electrolyte transport and renin secretion at the renal level have been explored by several investigators. Infusion of ouabain into the renal artery has been shown to prevent the renin increase induced by ureteral occlusion and aortic clamp as well as by furosemide in animals (1, 2). Short-term administration of digoxin in man results in a marked decrease of plasma renin activity and aldosterone (3, 4). Churchill et al. have proposed that the digitalis inhibitory effect on plasma renin activity (PRA) is mediated by the inhibition of Na/K ATPase, leading to increased intracellular sodium and calcium, in the juxtaglomerular cell's membrane (5, 6). In fact, a competitive antagonist of ouabain at its Na/K ATPase receptor site, canrenoate, has been shown to prevent digoxin-induced renin suppression (7, 8). Besides the blood-borne circulating renin-angiotensin system, there are now several indications for the existence of complete local angiotensin-generating systems in various extrarenal tissues (9, 10). Such systems may regulate cell-to-cell communication and function in an autocrine and/or paracrine mode. All the components of the renin-angiotensin system have been demonstrated in the adrenal of mammalian species, including humans (11, 12). Recently, we provided evidence that the human adrenal gland in vitro generates and releases both renin and angiotensin II/III imunoreactivity, and that locally formed angiotensin may be a paracrine regulator of physiological aldosterone secretion (13). The aim of the present study was to investigate the effect of digoxin on the in vitro generation of renin and angiotensin II/III, as well as of aldosterone, by the human adrenal tissue.

Materials and methods

Eight normal adrenals, including cortex and medulla portions, were obtained surgically from patients undergoing unilateral expanded nephrectomy for kidney cancer. The adrenal tissue specimen contained no tumour at histological examination. None of the patients (5M and 3F aged 43–67 years) had clinical symptoms of adrenal dysfunction. All were on a diet containing 120 to 150 mmol sodium and 60 mmol potassium daily, and had been off any drug for at least three months before the operation.

Superfusion experiments

As previously reported in detail (13), portions (0.3–0.5 g wet weight) of adrenal tissue were plunged in ice-cold Medium 199 (Gibco), containing 3.6 mmol/l K, 25 mmol/l $NaHCO_3$, and 0.1% bovine serum albumine (BSA) (BSA, RIA grade, Sigma Co, St. Louis, MO) at pH 7.4. Samples were finely minced, placed in 1 ml chambers with Bio-Gel P-2 (Bio-Rad, Richmond, CA) used as a support matrix, and superfused at a flow rate of 0.5 ml/min by Medium 199 gassed with $95\%O_2/5\%CO_2$ at 37C°. Fractions corresponding to 15 min superfusion were collected in chilled tubes with an inhibitor solution of 5 mmol/l EDTA and 0.1 mmol/l captopril (kindly provided by ER Squibb, Princeton, NJ), lyophylized and stored frozen until the assay for renin, angiotensin II/III and aldosterone. In a first set of four experiments, similar amounts of the same tissue sample were placed in two chambers and superfused for 60 min of baseline collection period. One of the two chambers was then superfused consecutively for 45 min with three different digoxin (Lanoxin, Wellcome Italia S. p. A., Pomezia-Rome, Italy) concentrations, 10^{-8}, 10^{-6} and 10^{-5} mol/l, by changing to a second reservoir of medium; a 45 min interval period between the first two doses and 30 min after the last dose were left for basal collection of medium alone. Collection of the fractions continued also with medium alone for 210 min in the parallel chamber, being considered as control. In a second set of three experiments, after 60 min of baseline collection one of the two chambers was superfused for 120 min with digoxin at a concentration of 10^{-5} mol/l, and the parallel one (i.e. control) continued with medium alone. In a last experiment, three parallel chambers were used. After 60 min of baseline collection, one chamber was superfused with medium alone (i.e. control), the second one with digoxin 10^{-5} mol/l and the third one with digoxin 10^{-5} mol/l in the presence of 1 g/l digoxin-binding antibodies (Fab fragments Digibind, Wellcome Foundation Ltd, Beckenham Kent, England) (14); this part of the superfusion lasted 75 min. Since 1 mg antibodies block 15 μg digoxin and we used 40 ml medium for the adrenal superfusion, antibodies were approximately twofold in excess of digoxin concentration.

Fractions were also assayed for lactic dehydrogenase (LDH) activity (Sigma test kits) as an index for cell damage. LDH activity in the superfusate fractions did not show significant variations over the time of superfusions, either in control or in digoxin-treated samples.

Radioimmunoassays

Dried samples were redissolved in 2 ml buffer consisting of 0.05 mol/l K_2PO_4, 0.003 mol/l Na_2-EDTA, 0.02% NaN_3, 0.01% Triton X-100, pH 7.4 and 2.5 g/l BSA.

For hormone assay we used the methods previously reported in detail (13). The concentration of active renin was measured by an immunoradiometric assay kit (Diagnostics Pasteur, Marnes La Coquette, France) using two monoclonal anti-human renin antibodies 3E8 and 4G1, as previously described (15). The limit of detection was 2 ng/l (i.e. pg/ml), the intra-assay coefficient of variation (cv) 6%, and the interassay cv 10%. Since the exact molecular weight of active renin is unknown, traditional weight units instead of SI units are used.

Angiotensin II/III was assayed using a double-antibody radioimmunoassay (RIA). The first antibody (Arnel Products Co, Inc, New York, NY) raised in rabbits had 100% cross-reactivity with angiotensin II and all other C-terminal fragments, and less than 0.1% with angiotensin I. The second one was a donkey anti-rabbit antibody coupled to magnetic beads (Amerlex M separation reagents, Amersham International plc, Buckinghamshire, England). The lowest concentration of angiotensin II/III detected was 1 ng/l (i.e. pg/ml). Intra-assay cv was 7%, interassay cv 10%. Since the antibody against angiotensin II shows 100% cross-reactivity with angiotensin III, it is not possible to use a single molecular weight for molar transformation.

Aldosterone was measured by RIA with a commercial kit (ALDOK-^{3}H, Sorin, Italy). Detectability was 20 pmol/l, intra-assay cv 5%, interassay cv 8%.

The antisera for renin, angiotensin II/III and aldosterone used in our immunoassays showed a good specificity, since a cross-reactivity less than 0.05% with digoxin at the doses used in our study was observed. For superfusate samples the results are given as pg or pmol (for aldosterone) $\times$ (0.1 g tissue)$^{-1}$ $\times$ 15 min.

Statistics

In the first set of four superfusion experiments, basal variations (i.e. fractions collected at 1–60, 120–150, 210–240, 300–315 min) and variations during digoxin (i.e. fractions at 75–105, 165–195, 255–285 min) of renin, angiotensin II/III and aldosterone were compared with corresponding control variations using one-way ANOVA.

In the second set of three experiments, the response of renin, angiotensin II/III and aldosterone to administered digoxin or to medium alone was expressed as the percentage change from baseline, with baseline defined as the means of the first four consecutive 15 min fractions. In each experiment, mean $\pm$ SEM of percentage change (N = 8) during digoxin was compared with that obtained in control by one-way ANOVA.

In the final superfusion experiment, the same statistical procedure was also used for comparison of percentage changes (N = 5) during digoxin vs control, and during digoxin vs digoxin in the presence of antidigoxin antibodies. Significance was defined as a p-value less than 0.05.

Results

In the first set of superfusion experiments (Fig. 1), digoxin at a concentration of 10^{-5} mol/l significantly reduced renin (11 ± 1.6 vs 6.2 ± 0.8, 12.5 ± 2.3 vs 5.5 ± 1.3, 11.5 ± 1.7 vs 5 ± 1.4 pg $\times$ 0.1 g tissue^{-1} $\times$ 15 min, $p<0.05$, at 255, 260 and 275 min fraction, respectively) and angiotensin II/III output (33.7 ± 6 vs 11.5 ± 4.4, 29.5 ± 4.6 vs 11.7 ± 3.7, 30 ± 5.3 vs 12.2 ± 2.3 pg $\times$ 0.1 g tissue^{-1} $\times$ 15 min, $p<0.05$, respectively) from adrenal over control levels (N=4); no difference between basal (i.e. time intervals in the absence of digoxin) and corresponding control periods was observed. The response of renin and angiotensin II/III was not modified using digoxin at either 10^{-8} or 10^{-6}

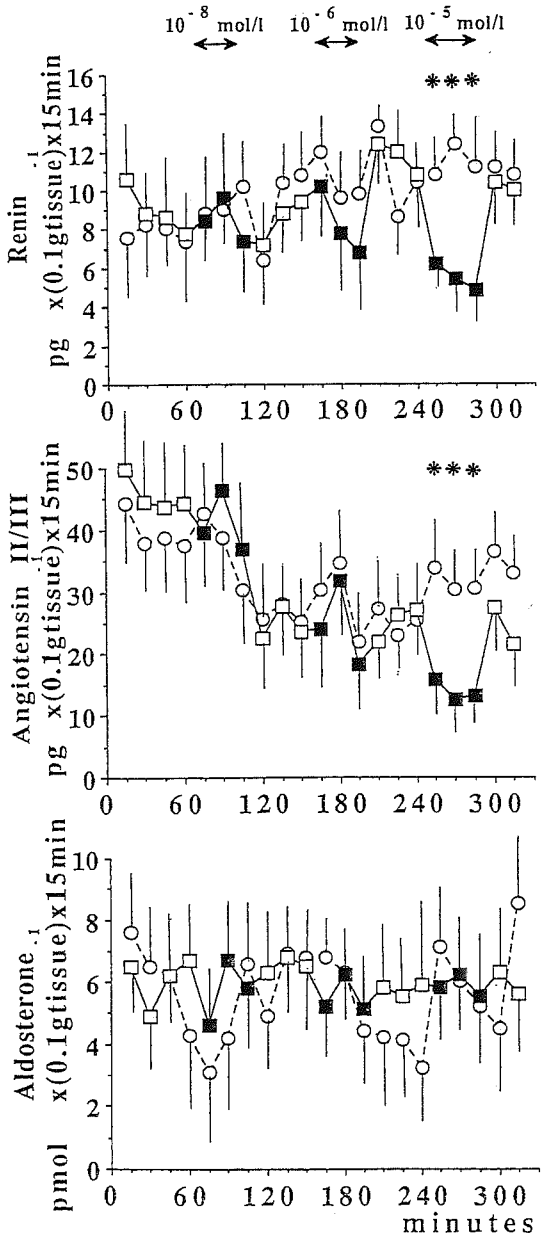

Fig. 1. Renin, angiotensin II/III and aldosterone concentrations during superfusion in vitro of human adrenals (N=4) with three different digoxin concentrations, 10^{-8}, 10^{-6}, 10^{-5} mol/l, in sequence (solid square symbols), in comparison with control (open symbols). Open square symbols represent basal changes. Asterisks (*$p<0.05$) denote significant difference of hormone levels during digoxin over control.

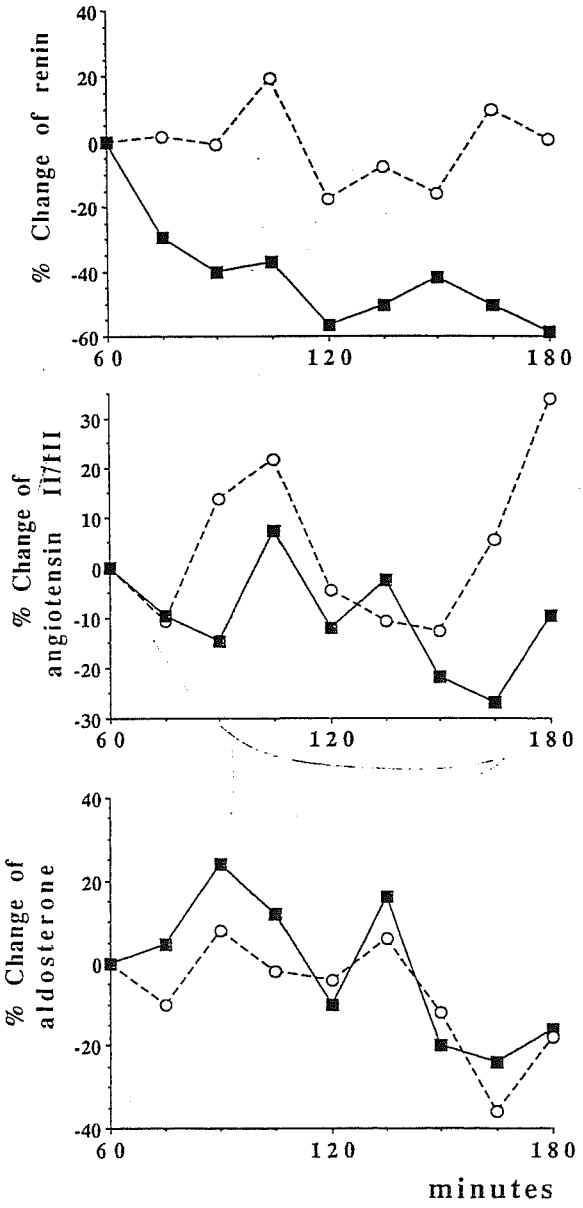

Fig. 2. Response of renin, angiotensin II/III and aldosterone to 10^{-5} mol/l digoxin (solid symbols) in comparison with control (open symbols), during in vitro superfusion of one human adrenal, as a single example of three experiments. The response is depicted as the percentage change from the mean of the first four consecutive 15 min collection fractions. After this 60 min period, eight 15 min fractions were collected from superfusion of tissue in two parallel chambers, one with medium containing digoxin and one with medium alone.

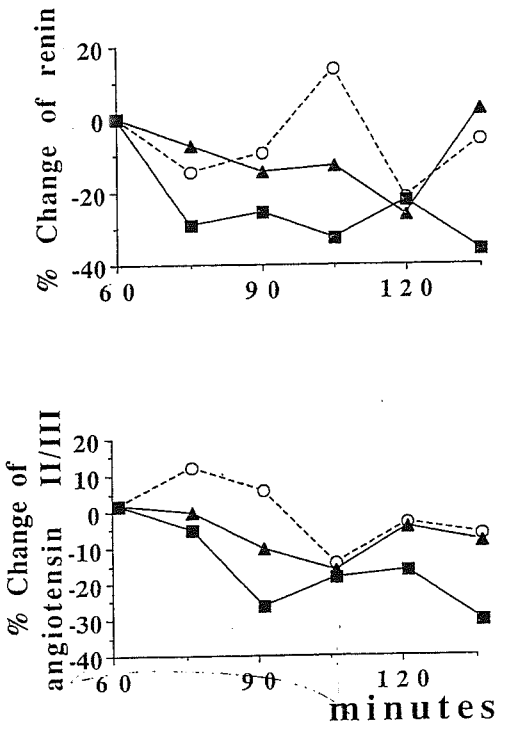

Fig. 3. Response of renin and angiotensin II/III, in comparison with control, during in vitro superfusion of one human adrenal gland. The response is depicted as the percentage change from the mean of the first four consecutive 15 min collection fractions. After this 60 min period, five 15 min fractions were collected from superfusion of three parallel chambers, one with medium alone (open symbols), one with 10^{-5} mol/l digoxin (solid square symbols) and one with 10^{-5} mol/l digoxin in the presence of antidigoxin antibodies (solid triangle symbols).

mol/l, although an insignificant decrease of these substances was observed at the latter dose in some of the experiments. There was no change of aldosterone secretion in response to digoxin at any dose. We previously found that, using the same in vitro experimental model, ACTH at 10^{-7}–10^{-6} mol/l was able to significantly increase aldosterone production from adrenal tissue (unpublished observation).

In all three experiments of the second group (see a single example in Fig. 2), the addition of digoxin 10^{-5} mol/l for 120 min in the superfusion medium caused a sustained reduction of renin and angiotensin II/III released by the adrenals significantly different from control (-61 ± 2 vs $-11\pm4\%$, -18 ± 2 vs $2\pm3\%$, -45 ± 3 vs $-1\pm4\%$, $p<0.01$, and -53 ± 5 vs $32\pm12\%$, -62 ± 6 vs $-35\pm9\%$, -11 ± 3 vs $4\pm5\%$, $p<0.05$, $N=8$, respectively). No difference in percentage change of aldosterone was detected during superfusion with digoxin compared with superfusion with medium alone.

In the final experiment (Fig. 3), percentage decrease of renin and angiotensin II/III during superfusion with digoxin 10^{-5} mol/l was significantly greater than that observed during superfusion with digoxin in the presence of antidigoxin antibodies (-11 ± 4 vs $-29\pm2\%$ and -20 ± 4 vs $-9\pm2\%$, $p<0.05$, $N=5$, respectively). The same concentration of antidigoxin antibodies alone, tested separately, did not show any effect on hormone secretion (data not shown).

Discussion

Our data document that both renin and angiotensin II/III immunoreactivity levels were lowered by digoxin in the perfusate of normal adrenal glands, while aldosterone did not show significant changes. The reduction of renin secretion from adrenal tissue is consistent with previous studies showing an inhibitory effect of ouabain on renin output from incubated rat renal cortical slices (5, 6). In fact, renin secretion has been found to be one of the few systems in which an increase in cellular sodium and calcium, as that due to the ouabain-mediated blockade of Na/K ATPase pump, inhibits a secretory process (16). While renin inhibition in renal studies was obtained at a concentration of 10^{-3} mol/l (17), in our experimental preparations a similar effect was observed using digoxin at a much smaller dose. This could be due to use of the superfusion technique, which permits nutritional fluid to better reach the framework of the cells (18). Inhibition of adrenal renin and angiotensin II/III secretion was not a non-specific effect, since it was mostly reversed by the presence of antidigoxin antiserum. Lack of complete inhibition of the digoxin effect may be related to the biological cross-reactivity of this antibody, shifting its binding to the several steroids produced by the adrenal superfusion.

Decrease of renin and angiotensin II/III in the perfusates was not accompanied by a change in aldosterone levels. Based on the hypothesis that adrenal angiotensin II/III plays a role as a paracrine regulator of aldosterone secretion by human adrenal gland, this result would not be expected. However, it is well known that digitalis glycosides can variously affect aldosterone biosynthesis in animal adrenal tissues (19, 20). The effect is attributed to the direct inhibition of glomerulosa cell membrane bound Na/K ATPase pump, and depends on the dose of drug used, on the extracellular potassium concentrations and on the presence or absence of aldosterone-stimulating agents. At concentrations of ouabain in a range similar to that we used for digoxin, several laboratories have reported a stimulatory effect on aldosterone secretion by isolated rat adrenal glomerulosa cells (21, 22). Although we cannot compare the sensitivity of rats to digitalis glycosides with that of human species, in our whole gland preparation a direct stimulation of aldosterone by glomerulosa portion may also have occurred. However, this would have been counterbalanced by the simultaneous inhibition of adrenal angiotensin-mediated decrease of aldosterone. Alternatively, an insensitivity to 10^{-5} mol/l digoxin, as a result of higher Na/K ATPase activity and/or greater number of

Na/K ATPase active pump sites, might be present in the aldosterone-secreting cells as compared with adrenal renin-angiotensin-secreting cells. In this case, the magnitude of angiotensin II/III decrease should be insufficient to affect the basal aldosterone production. This concept, however, is not supported by our previous observation of a comparable degree of angiotensin II/III reduction accompanied by a clear aldosterone decrease when the adrenal is perfused with an angiotensin converting enzyme inhibitor (13). Finally, our study, using the whole gland, does not permit us to assess the distribution of renin-angiotensin-secreting cells in the different anatomical portions of human adrenal. The amount of renin and angiotensin II/III produced by the fasciculata-medullary region could be greater than that produced by glomerulosa. This could have maintained aldosterone level in spite of the fall in total renin and angiotensin II/III induced by digoxin.

The possibility that digoxin has a similar effect on the adrenals in vivo in humans can only be a matter of speculation. Digoxin has been shown to accumulate over time within the human adrenal glands, as in other tissues, in a ratio 100 to 1 with plasma (23). Therefore, although the digoxin concentation used for superfusion was much higher than that known as the therapeutic plasma range inman, intra-adrenal digoxin concentration reached in our short-term study might not be too far off that occurring in a long-term in vivo situation.

A similar inhibitory effect on other tissue renin-angiotensin systems also needs to be explored. In this respect, the presence of an endogenous digitalis-like factor (24, 25), possibly acting as an inhibitor of vascular wall-generated angiotensin II, may indicate an appropriate negative feedback mechanism for a humoral agent causing vasoconstriction (26).

In conclusion, our results indicate that digoxin at high doses reduces renin and angiotensin II/III but not aldosterone secretion by the human adrenal gland. This suggests two different effects of digoxin, probably both mediated by inhibition of Na/K ATPase activity, on the adrenal renin-angiotensin and aldosterone-secreting cells.

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