Toxic Effects of a Chlorothiazide-Diazoxide Combination on Adipose Tissue and Kidneys of Intact Rats*

HAROLD P. SETTLE, JR., WILLIAM J. MUNSIE, and JOHN A. OWEN, JR.

Division of Clinical Pharmacology, Department of Internal Medicine, and the Department of Pathology, School of Medicine, University of Virginia, Charlottesville, Virginia

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Summary. In order to explore effects of chlorothiazide (CTZ) and diazoxide (DZX) on rat adipose tissue, as being possibly related to previously reported hyperglycaemia in man, intact rats fed *ad lib* were given daily injections of CTZ (200 mg/kg), DZX (50 mg/kg), or both for 14 days. They were then killed and their fat pads were incubated in buffer with and without insulin (250 μ units/ml). DZX (but not CTZ) and the combination significantly decreased baseline glucose oxidation and incorporation into lipids; response to insulin was significantly impaired by either drug alone and especially by the CTZ-DZX combination. An unexpected effect of the drug combination was severe renal disease with azotaemia and death as early as the fourth day. Histologically, the primary change seemed to be epithelial hyperplasia in the collecting tubules suggestive of the effect of potassium depletion. This in turn produced tubular obstruction with proximal dilatation and focal, acute, interstitial inflammation in both cortex and medulla.

Effets toxiques d'une association chlorothiazide-diazoxide sur le tissu adipeux et les reins de rats intacts

Résumé. Afin d'explorer les effets du chlorothiazide (CTZ) et du diazoxide (DZX) sur le tissu adipeux du rat, comme étant en relation possible avec l'hyperglycémie précédemment constatée chez l'homme, on a administré à des rats intacts, nourris *ad libitum*, des injections quotidiennes de CTZ (200 mg/kg), de DZX (50 mg/kg) ou des deux à la fois pendant 14 jours. Ils étaient alors tués et leur tissu adipeux était incubé dans un tampon avec et sans insuline (250 μ unités/ml). DZX (mais non CTZ) ainsi que l'association des deux substances diminuaient significativement l'oxydation basale du glucose et l'incorporation dans les lipides; la réponse à l'insuline était diminuée significativement par l'une ou l'autre drogue seule, et spécialement par l'association CTZ-DZX. L'association des deux drogues provoqua un effet inattendu, à savoir une atteinte rénale sévère avec azotémie et mort dès le quatrième jour. Histologiquement la modification primaire semble être une hyperplasie épithéliale dans les

tubules collecteurs, suggérant l'effet d'une déplétion de potassium. Ceci provoqua à son tour une obstruction tubulaire avec dilatation proximale et inflammation interstitielle focale aiguë dans le cortex et la moelle.

Toxische Auswirkungen einer Chlorothiazid-Diazoxid Kombination auf Fettgewebe und Nieren von intakten Ratten

Zusammenfassung. Die Wirkung von Chlorothiazid (CTZ) und Diazoxid (DZX) auf Rattenfettgewebe wurde untersucht, insbesondere, um zu klären, ob sich zwischen diesen Effekten und der nach Verabreichung dieser Medikamente schon früher beim Menschen beschriebenen Blutzuckererhöhung Zusammenhänge finden lassen. Dabei erhielten intakte Ratten mit freiem Zugang zur Nahrung während 14 Tagen tägliche Injektionen von 200 mg/kg CTZ, 50 mg/kg DZX oder beide Medikamente zusammen. Dann wurden die Tiere getötet und ihre Nebenhoden-Fettgewebsanhänge mit oder ohne Insulinzusatz (250 μ E/ml) in Puffer inkubiert. DZX (nicht aber CTZ) und ihre Kombination führten zu einer signifikanten Senkung der Basis-Oxydation und des Einbaus von Glucose in Lipide. Das Ansprechen des Gewebes auf Insulin wurde durch jede der beiden Drogen und besonders durch die Kombination von CTZ und DZX signifikant gehemmt. Als unerwartete Folge traten bei den mit der Medikamenten-Kombination behandelten Tieren schwere Nierenerkrankungen mit Urämie und Tod, teilweise schon am 4. Tage ein. Als Primärschädigung schien, wie die histologische Untersuchung ergab, eine Epithel-Hyperplasie der Sammelröhrchen vorzuliegen, was auf die Möglichkeit hindeutet, daß es sich dabei um die Auswirkungen eines schweren Kalium-Mangelzustandes handeln könnte. Weiter kam es infolge dieser Hyperplasie zu einem Verschuß der Tubuli mit proximaler Erweiterung und zu akuten, abszedierenden Entzündungen im Interstitium von Rinde und Mark.

Key-words: Acute pyelonephritis, adipose tissue *in vitro*, azotaemia, chlorothiazide, diazoxide, glucose oxidation, lipid incorporation, potassium depletion.

Chlorothiazide, the prototype of the benzothiadiazine group of drugs, was synthesized in 1957 and soon proved itself a potent diuretic and anti-hypertensive agent. WILKINS (1959) was the first to describe its tendency to produce hyperglycaemia or unmask latent diabetes mellitus. Diazoxide, synthesized in 1961, is a chlorothiazide derivative with anti-hypertensive but

only mildly saluretic properties; its clinical trials also brought reports of hyperglycaemia (DOLLERY et al., 1962), now thought to be due to direct inhibition of insulin secretion (FAJANS et al., 1966; SELTZER and CROUT, 1966).

Only three articles have reported direct effects of these agents on glucose utilization by peripheral tissues. FIELD and MANDELL (1964) found that neither chlorothiazide (16-160 μ g/ml) nor diazoxide (30-300 μ g/ml) altered glucose oxidation by the rat epididymal fat pad *in vitro*, but intraperitoneal injections of chlorothiazide (1 mg/100 g body weight daily for 14

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days) significantly reduced the effect *in vitro* of insulin on adipose tissue but not on diaphragm. BARNETT and WHITNEY (1966) carried out similar studies with different results: chlorothiazide (600 μ g/ml) *in vitro* inhibited both the basal glucose uptake and the insulin response of rat hemidiaphragm but not of adipose tissue; diazoxide in the same concentration inhibited the basal glucose uptake of adipose tissue but not that of hemidiaphragm, and did not depress the insulin response of either tissue. The discrepancy between these effects of diazoxide may be due to dosage differences; the different chlorothiazide effects cannot be readily explained. The present studies were designed to re-investigate the impact of these drugs on adipose tissue and to clarify, if possible, its relationship to the hyperglycaemic effect.

Some time after this study was completed, there appeared the report of JANSEN et al. (1967), showing that a single subcutaneous injection of diazoxide (2–20 mg per 100 g of body weight) acutely raised the blood sugar and impaired the incorporation into adipose tissue fatty acid of labelled glucose given by tube to intact mice. Crystalline insulin (0.25 units per animal) reversed these diazoxide-induced effects, but when given alone did not increase the radioactivity in the adipose tissue despite a marked fall in blood sugar.

Fig. 1. Weight gains of the groups of rats on various treatments

Methods

104 male Wistar rats weighing 125–150 g were randomly divided into four groups assigned to receive fourteen daily i.p. injections: controls received 1.0 ml sterile saline; another group received chlorothiazide, 20 mg per 100 g of body weight; a third group received diazoxide, 5 mg per 100 g of body weight; and the last group received chlorothiazide and diazoxide in the above doses, by separate injections. Chlorothiazide was obtained as commercial sodium salt, Lyovac® Diuril®¹, and made into solution (25 mg/ml

of normal saline) immediately prior to injection. Diazoxide was obtained as Hyperstat® Solution² (15 mg/ml) and injected as such. The animals were given Purina Lab Chow and water *ad lib*, weighed daily, and decapitated on day 15. Plasma urea and glucose were measured using the Technicon Auto-analyzer.

The epididymal fat pads were removed, and one weighed segment (ca. 200 mg) from each rat was incubated in a flask containing 5 ml of Krebs bicarbonate buffer (pH 7.4), glucose 2.5 mg/ml including 2 μ c of glucose-1-¹⁴C, and crystalline beef insulin, 250 μ units/ml; the other segment was incubated in a

Fig. 2. Scatter diagram of terminal values of plasma urea

similar flask containing the identical medium but without insulin. The flasks were agitated for 3 h in a Dubnoff incubator at 37°C. Glucose oxidation to CO₂ was estimated by the method of RENOLD et al. (1960) and SHEPS et al. (1960), modified so that ¹⁴CO₂ was collected in Hyamine³. After incubation the lipids of the fat pad were extracted and their ¹⁴C incorporation measured by the method of BARUCH and CHAIKOFF

¹ Merck Sharp and Dohme.

² Schering Corporation.

³ Rohm and Haas Company.

(1954), modified to substitute heptane for chloroform in the lipid extraction with sodium ethylate. Each sample was counted in a Packard Tricarb counter and results expressed as dpm per μg of adipose tissue

statistical tests such as the Student's *t* seemed inappropriate; differences between treatment groups were analyzed using the Wilcoxon rank sums method (WILCOXON and WILCOX, 1964).

Fig. 3. Section of renal medulla. Kidney was obtained from azotaemic rat after 14 days' treatment with the combination of chlorothiazide and diazoxide. H & E stain, $\times 48$

Fig. 4. Section of renal medulla, from same kidney (different area) as shown in Figure 3, demonstrating tubular plugging and inflammation in interstitium. H & E stain, $\times 300$

nitrogen. An aliquot of each fat pad was analyzed for nitrogen by the micro-Kjeldahl method. The response to insulin was obtained by subtracting the values for the epididymal segment incubated without insulin from the values for the corresponding paired segment incubated with insulin. Because the values obtained did not follow a normal distribution curve, parametric

treatment. There were two types of pathological changes in these kidneys. One was suggestive of potassium depletion as described in rats by OLIVER et al. (1957), and consisted of hyperplasia of the epithelium of the collecting duct, and dilatation of the more proximal tubules. The other was acute pyelonephritis consisting of acute focal interstitial inflamma-

Results

Animals which received these large benzothiadiazine doses usually looked sick for an hour or so after each injection. As shown in Fig. 1, chlorothiazide treatment did not inhibit weight gain (5.0 g/day), which was essentially the same as that in the control group (5.3 g/day); the diazoxide-treated group gained somewhat less (3.8 g/day), and the combination-treated group least of all (1.6 g/day). This latter group suffered a 33% mortality as compared with 4–7% for the other groups.

None of the rats were hyperglycaemic when killed. But many of those treated with the combination had high values for plasma urea (Fig. 2), an unexpected finding which was the stimulus for considerable further study (SETTLE and OWEN, 1968).

Chlorothiazide treatment did not significantly alter the metabolism of glucose by adipose tissue in buffer alone. Diazoxide markedly depressed glucose oxidation by adipose tissue compared with that by adipose tissue of control and chlorothiazide-treated rats; incorporation of the label into lipid was also impaired but to an extent which was significant only in comparison with the controls. The combination of chlorothiazide and diazoxide was only slightly more inhibitory than diazoxide alone. Either drug significantly impaired the expected responses to insulin; the greatest effect occurred in animals receiving the combination treatment (Table 1).

The persistent failure to thrive, the increased mortality, and the azotaemia at time of killing prompted pathological examination of the kidneys of some rats receiving the combina-

tion, involving both cortex and medulla, with occasional small abscesses and many cellular casts of polymorphonuclear leukocytes in the collecting tubules (Fig. 2–4). These changes were first seen on the fourth day of injection.

Discussion

These rather large doses of chlorothiazide and diazoxide were used because in pilot studies smaller amounts had had but little effect on the glucose tolerance of treated rats. The parenteral route of administration was chosen to obviate the technical problems of tube-feeding.

Several factors may have influenced the metabolism of adipose tissue in these animals. None of the treated groups gained weight as rapidly as the controls, but only the group injected with the combination of drugs lagged strikingly (Fig. 1). These rats were obviously sick and probably ate little (food intake was not measured); semi-starvation undoubtedly played an important role in the altered metabolism of adipose tissue, quite independent of drug effects *per se*.

consider the effects of the drugs themselves. Chlorothiazide treatment for two weeks produced a variable and insignificant reduction of baseline glucose metabolism; more striking and quite significant was the impairment of the expected stimulatory effect of

Fig. 5. Section of renal cortex. Acute inflammation in interstitium and microabscess formation. H & E stain, $\times 300$

Table 1. Glucose-1- ^{14}C metabolism by adipose tissue

Treatment Group & No.	dpm/ μg adipose tissue N (average and range)		<i>P</i> values for rank sums <i>T</i> values for treatment group comparisons*		
	Production of $^{14}\text{CO}_2$	Incorporation of ^{14}C into lipid			
A. Baseline utilization in buffer alone					
Control (36)	24.9 (6.8 — 53.7)	28.5 (7.6 — 78.5)	Control vs. N.S.; N.S.	Chlorothiazide vs.	Diazoxide vs.
Chlorothiazide (29)	30.8 (9.0 — 76.7)	22.9 (3.5 — 60.6)			
Diazoxide (24)	17.1 (5.7 — 104.6)	21.9 (5.1 — 89.4)	< 0.01; < 0.05	< 0.01; N.S.	
Combination (15)	15.0 (2.9 — 35.4)	16.1 (2.4 — 40.3)	< 0.01; < 0.01	< 0.01; N.S.	N.S.; N.S.
B. Effect of insulin (250 $\mu\text{units/ml}$) in buffer (Δ effect)					
Control (36)	54.8 (— 11.6 — 170.0)	81.2 (— 32.7 — 330.6)	Control vs. < 0.01; < 0.01	Chlorothiazide vs.	Diazoxide vs.
Chlorothiazide (29)	17.6 (— 14.2 — 64.2)	28.4 (— 26.7 — 119.7)			
Diazoxide (24)	6.6 (— 9.5 — 25.7)	11.3 (— 25.6 — 90.9)	< 0.01; < 0.01	< 0.05; < 0.01	
Combination (15)	5.9 (— 1.9 — 18.6)	12.4 (— 7.5 — 53.8)	< 0.01; < 0.01	< 0.01; < 0.02	N.S.; N.S.

* In each column, the first figure refers to CO_2 production, the second to lipid incorporation.

Since both chlorothiazide and diazoxide solutions are alkaline (pH 9.3 and 11.2, respectively), direct chemical damage to the epididymal fat pads may have resulted from intraperitoneal injection. This explanation seems unlikely in view of the similar findings by Jansen et al. (1967), employing only a single subcutaneous injection. Nevertheless, a possible additive effect cannot be ruled out.

With these reservations, it is now possible to

insulin. Diazoxide treatment, on the other hand, produced a marked and significant lowering of both baseline and insulin-stimulated metabolism, an effect which was only minimally enhanced by the further addition of chlorothiazide. The changes in glucose oxidation closely paralleled those in incorporation of glucose carbon into lipid. The effects of the combination treatment cannot be distinguished from the systemic complications of the nephrotoxicity.

These effects of chlorothiazide confirm the studies reported by FIELD and MANDELL (1964); despite a 20-fold increase in the dose employed, baseline glucose oxidation was not impaired, the response to insulin was blunted, and hyperglycaemia did not occur. The present findings, like those of JANSEN et al. (1967), implicate diazoxide as a much stronger inhibitor of adipose tissue metabolism than might be inferred from the studies *in vitro* of BARNETT and WHITNEY (1966), who found no depression of insulin response by the same dose that depressed basal uptake. This discrepancy suggests that diazoxide injected *in vivo* may act both directly on adipose tissue and indirectly on adipose tissue and/or insulin secretion, the latter actions perhaps mediated via adrenergic release as postulated by TABACHNICK et al. (1964). A concept of multiple sites of action is also compatible with the observed dissociation of effects on blood sugar and adipose tissue. Although acute diazoxide-induced hyperglycaemia occurs consistently (WOLFF et al., 1963; WOLFF and PARMLEY, 1964) and accompanies acute suppression of adipose tissue (JANSEN et al., 1967), the blood sugar was normal in the present study 24 h after the fourteenth diazoxide injection, at a time when both glucose utilization by, and insulin effect on, adipose tissue were markedly impaired.

The effects on renal function and morphology were totally unexpected and unprecedented, although TABACHNICK et al. (1964) had earlier reported a modest azotaemic effect of diazoxide alone in intact mice and dogs. The present experiments revealed little nephrotoxicity except with the combination treatment, which caused striking growth impairment, mortality and renal pathology. The severity of this systemic illness, if it is consistently demonstrable, will always obscure any long-term effects of the drug combination *per se* on the metabolism of adipose tissue. Further experiments to be reported will attempt to elucidate this phenomenon.

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Dr. JOHN A. OWEN, JR.,
Department of Internal Medicine
University of Virginia School of
Medicine
Charlottesville, Virginia, U.S.A.