Syndromes of Insulin Resistance in the Rat
Inducement by Diet and Amelioration With Benfluorex

LEONARD H. STORLIEN, NICHOLAS D. OAKES, DAVID A. PAN, MASATAKA KUSUNOKI, AND ARTHUR B. JENKINS

Insulin resistance, mainly in skeletal muscle, is linked to a cluster of prevalent diseases including NIDDM, dyslipidemias, hypertension, and cardiovascular disease. To determine if an oversupply of lipid is associated with the development of skeletal muscle insulin resistance, we examined the effect of the hypolipidemic agent benfluorex in dietary models of insulin resistance. Adult, male Wistar rats were divided into six groups and maintained for 4 wk on diets high in complex carbohydrate, fructose or fat, with or without 50 mg • kg−1 • day−1 of benfluorex, given orally. Insulin action was assessed using a hyperinsulinemic (−100 mU/L) euglycemic clamp, with 2-deoxyglucose tracer for individual tissue evaluation, in chronically cannulated conscious animals. Compared with starch feeding, fructose and fat feeding significantly impaired insulin action at the whole-body level (−46% and −41%, respectively, both P < 0.001), as well as in individual skeletal muscles. Fructose feeding increased circulating TGs (by 80%, P < 0.01) but not skeletal muscle TGs; whereas, fat feeding increased skeletal muscle TGs (by 59%, P < 0.01) but not circulating TGs. With benfluorex, however, diet had no effect on circulating and storage TGs; and development of skeletal muscle insulin resistance in the two diet groups was prevented. Feeding fructose but not fat significantly increased mean arterial BP (by 13%, P < 0.05), an effect prevented by benfluorex. These effects support the hypothesis that the development of muscle insulin resistance in these models is linked to local or systemic oversupply of lipid. These diet models—and the parallel effect of benfluorex on insulin resistance, lipids, and hypertension—may prove useful in the search for the mechanisms that underlie the human disorders associated with insulin resistance. Diabetes 42:457–62, 1993

Impairment of insulin action (insulin resistance) has long been recognized as one of the major metabolic abnormalities of NIDDM. In the past few years, a role for insulin resistance, perhaps associated with hyperinsulinemia, has been proposed in a cluster of diseases, including NIDDM, hypertension, dyslipidemias, and cardiovascular disease (1), a list to which we would add central obesity.

Although genetic influence undoubtedly is a strong factor in the predisposition to insulin resistance, life-style factors such as diet and level of physical activity also play a significant role. Several early studies in rats demonstrated insulin resistance in vitro after feeding diets high in fat or simple sugars (2–4). With the adaptation of the euglycemic clamp technique to rats, studies in vivo have confirmed these results (5–8). One of these latter studies (7) also has linked the insulin resistance of fructose feeding to its well-known effect of elevating circulating TGs (9), an important consideration given that fructose intake in western diets is 10–15% of calories—and rising (10). Further, the insulin resistance of fat feeding is linked to increases in skeletal muscle TG storage (8), skeletal muscle being the most important tissue for insulin stimulated glucose disposal. The results of these and other studies in this series have been interpreted within the framework of the glucose/fatty acid cycle of Randle et al. (11,12).

In addition to insulin resistance and hypertriglyceridemia, other features of the "Syndromes of Insulin Resistance" include hypertension and a disposition to obesity via reductions in metabolic rate. Again, hypertension has
SYNDROMES OF INSULIN RESISTANCE IN THE RAT

TABLE 1
Body weight at time of study plus basal and euglycemic clamp plasma glucose and insulin levels

<table>
<thead>
<tr>
<th>Body weight (g)</th>
<th>Plasma glucose (mM)</th>
<th>Plasma insulin (mU/L)</th>
<th>Plasma glucose (mM)</th>
<th>Plasma insulin (mU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>344 ± 8</td>
<td>7.2 ± 0.2</td>
<td>28 ± 3</td>
<td>6.6 ± 0.1</td>
</tr>
<tr>
<td>Starch and benfluorex</td>
<td>323 ± 7</td>
<td>7.1 ± 0.2</td>
<td>25 ± 3</td>
<td>6.6 ± 0.2</td>
</tr>
<tr>
<td>Fat</td>
<td>335 ± 4</td>
<td>7.6 ± 0.1</td>
<td>32 ± 6</td>
<td>6.7 ± 0.1</td>
</tr>
<tr>
<td>Fat and benfluorex</td>
<td>332 ± 5</td>
<td>6.8 ± 0.1</td>
<td>22 ± 4</td>
<td>6.8 ± 0.1</td>
</tr>
<tr>
<td>Fructose</td>
<td>368 ± 7</td>
<td>7.4 ± 0.1</td>
<td>34 ± 5</td>
<td>7.0 ± 0.2</td>
</tr>
<tr>
<td>Fructose and benfluorex</td>
<td>337 ± 6</td>
<td>7.3 ± 0.3</td>
<td>23 ± 2</td>
<td>6.7 ± 0.2</td>
</tr>
</tbody>
</table>

Data are means ± SE, n = 6–7 in all cases. Body weights and basal plasma insulins of the benfluorex-treated groups were lower than nontreated groups by two-way ANOVA (both P < 0.05).

been demonstrated after fructose feeding (13), and there is evidence of reduced metabolic rate associated with insulin resistance that has been induced by fat feeding (5).

The above evidence suggests 1) that increases in dietary fructose or fat in rats may result in useful models of many features of the syndromes of insulin resistance; 2) that increased lipid availability may be central to the symptomatology of the syndromes; and 3) that an agent that lowers TG supply might ameliorate the range of abnormalities that develop with fructose or fat feeding. This study was designed to further characterize the high-fructose and high-fat models, and to investigate the modulating effects of benfluorex, a pharmaceutical agent that lowers lipid levels by reducing hepatic TG output (14).

RESEARCH DESIGN AND METHODS

All experiments were approved by the Garvan Institute Animal House Ethics Committee and comply with the NH and MRC (Australia) guidelines for the "Care and Use of Animals for Research Purposes."

Adult, male Wistar rats (bred in our facility), aged 54–60 days and weighing ~270 g at the beginning of the study, were housed individually in wire cages in a temperature-controlled room (22 ± 1°C) on a 12 h/12 h light/dark cycle (lights on at 0600).

Six groups of rats were fed diets high in starch (starch, 70% of calories), fructose (fructose, 35% of calories; 35% starch), or fat (fat, 59% of calories as safflower oil), with or without benfluorex administration. Diets were matched in terms of protein, fiber, vitamins, and minerals, as described previously (5,7,8). Each rat was given 310 kJ/day, which was approximately equal to their ad libitum intake of laboratory chow. Rats were fed just before the beginning of the dark cycle. A pair-feeding regimen was used to avoid variations in caloric intake. Any spillage was collected and additional diet of equal weight was added to the following day's intake. Diets were prepared fresh every 3–4 days and stored at 4°C. Benfluorex was introduced to the diet on day 2 at a concentration designed to provide 12.5 mg · kg⁻¹ · day⁻¹, and gradually increased to 50 mg · kg⁻¹ · day⁻¹ over a 3-day period.

Euglycemic clamp studies. After 30 ± 1 days on their respective diet, rats were anesthetized with 55 mg/kg sodium pentobarbital (Nembutal, Abbott Laboratories, Sydney, Australia) intraperitoneally and 0.3 mg/kg atropine sulfate (Astra Pharmaceuticals, Sydney) intramuscularly and fitted with chronic carotid and jugular cannulae. Studies were conducted 3–4 days after surgery in unrestrained conscious rats housed in wire cages (25 × 25 cm). This time was sufficient to allow the rats to essentially regain preoperative food intake and body weight (see RESULTS). The studies were performed during the light period, when the rats were observed to be inactive. Euglycemic, hyperinsulinemic clamps were performed on 6-h deprived rats, as described previously (5–8,15). Briefly, a continuous infusion of porcine insulin (Actrapid, Novo Industrie, Denmark) was administered at a dose of 29.4 pmol · kg⁻¹ · min⁻¹ to achieve plasma insulin concentrations in the mid-upper physiological range (see Table 1). This infusion was maintained for ~2 h. The arterial blood glucose concentration was clamped at the basal fasting level using a variable rate-glucose infusion.

Insulin sensitivity within individual tissues in vivo was studied as described previously (5–9). The nonmetabolizable glucose analogue 2,6-(³H)-2-deoxy-D-glucose (2.96 MBq) and D-(U-¹⁴C)-glucose (1.85 MBq) were administered together as an intravenous bolus ~80–90 min after commencement of the insulin infusion. Blood samples for monitoring blood and plasma glucose concentrations and plasma tracer concentrations were obtained at 2, 5, 10, 15, 20, 30, and 45 min after bolus administration. At the completion of the clamp, rats were rapidly anesthetized (Nembutal, 110 mg/kg, intravenously), and the following hindquarter muscles were quickly removed and frozen for subsequent analysis: superficial or white part of the quadriceps (containing mainly fast-twitch glycolytic fibers), deep-red part of the quadriceps (containing mainly fast-twitch oxidative-glycolytic fibers), extensor digitorum longus (containing a mixture of fast-twitch oxidative-glycolytic and glycolytic fibers) (16). The following tissues also were rapidly removed from the trunk of the animal and frozen: diaphragm (a mixture of all three muscle fibers), heart, epididymal, subcutaneous and inguinal white adipose tissues, and interscapular
brown fat. An estimate of tissue glucose metabolic rate (the glucose metabolic index, $R_g'$) was calculated as described previously (15). Blood samples to determine plasma insulin levels (0.4 ml) were taken at the start, before tracer administration, and at sacrifice. The red blood cells of the first two samples were reconstituted in 0.4 ml saline and returned to the animal. The sampling cannula was kept patent with lightly heparinized saline; <1 U of heparin was infused into the animal when the red blood cells were returned.

**Plasma and muscle TG.** Separate groups of rats were anesthetized with 80 mg/kg Nembutal intraperitoneally after a 6-h fast; blood was taken from the cut tip of the tail to determine plasma TG level. A red quadriceps leg muscle also was quickly removed and freeze-clamped to determine tissue TG level.

**BP measurement.** A sample for basal blood glucose was taken from the chronically indwelling carotid catheter in 6 h–deprived rats, as in the clamp studies. The catheter was then attached to a flexible sampling line filled with physiological saline, and the rat was allowed to settle for a minimum of 60 min. A blood sample was taken after the settling period to ensure that blood glucose was at or below the basal level, as an indication that the animal was not stressed. BP measurements were obtained with a pressure transducer (Bentley-Trantec, Irvine, CA). Mean BP was determined by continuously integrating the instantaneous pressure signal obtained from the pressure transducer over 30-sec periods. Integration was achieved using a standard operational amplifier implementation, featuring an analogue integrator with precise electronic components. Integrated analogue signals were captured by a computer-controlled A/D converter. The device was calibrated by simultaneous measurement of a physiological range of static pressures with a mercury manometer. Regression of the integrator output voltage against the manometer readings always produced $r^2 > 0.99$.

**Analytical methods.** Plasma samples for determination of tracer concentration were deproteinized immediately in 5.5% ZnSO$_4$ and saturated Ba(OH)$_2$. Activity of $^{3}H$ and $^{14}C$ in an aliquot of the supernatant were determined by scintillation spectrometry (Beckman, Fullerton, CA), using a quench-corrected (external standard) dual-label-counting program. Blood and plasma glucose concentrations were measured using a 23 AM glucose analyzer (YSI, Yellow Springs, OH). Plasma samples to determine TG and insulin levels were stored at $-20^\circ C$ before analysis.

Plasma TG concentrations were measured on a visible recording spectrophotometer (UV-240 Graphicord, Shimadzu, Kyoto, Japan) using an assay kit (Sigma, 336, St. Louis, MO). The assay uses lipoprotein lipase, glycerol kinase, glycerol-1-phosphate dehydrogenase, and diaphorase to convert TG to formazan, which is measured by colorimetry. Plasma insulin was analyzed by double-antibody RIA, using a rat-insulin standard (Novo, Bagsvaerd, Denmark) for the basal samples and a human insulin standard for the clamp determinations.

Tissue TGs were analyzed using a modified procedure of Denton and Randle (17). Frozen tissue was weighed and extracted with 12 ml of chloroform-methanol (vol:vol 2:1) using a hand-operated ultra-turrax homogenizer (Thyristor Regler TR 50, Janke and Kunkel, Staufen, Germany), then rotated for 4 h at room temperature. Separation of phases was obtained after addition of H$_2$SO$_4$ (2 ml, 1M), with centrifugation at 250 g for 10 min. The upper phase was discarded. The lower phase was washed with another addition of H$_2$SO$_4$ as above, dried with $\sim$100 mg of anhydrous Na$_2$SO$_4$, briefly centrifuged, and filtered. Phospholipids then were removed on silica and the TGs saponified by heating at 65°C for 1 h. A known volume of the extract then was evaporated to dryness using nitrogen, and the residue was assayed for triacylglycerol with a colorimetric enzymatic procedure (as above).

**Statistical analysis.** Statistical comparisons were made using the Statview 512+ statistical package (Abacus Concepts, Berkeley, CA). Unless otherwise noted, group statistical comparisons were by one-way ANOVA and individual comparisons by Fisher post hoc tests, with $P < 0.05$ considered acceptable.

**RESULTS**

Table 1 shows the basal characteristics of the six groups of rats. Benfluorex reduced food intake in the initial week of exposure, resulting in reduced weight gain in the treated groups. During the final 2 wk on the experimental diets, food intake in the treated groups returned to control levels, and body weight gain was similar in all groups. Body weight at the time of study was 6–9% lower in the benfluorex groups. ANOVA did not show an overall difference in plasma glucose levels among groups ($P > 0.05$). Basal insulin levels were 28–34 mU/L in the diet-only groups and significantly lower (11–32%, $P < 0.05$) in the benfluorex groups. Body weight change from operation to study was <15 g for all animals (range, −14 to 4 g, no significant difference among groups).

Food intake was within 10% of preoperative levels in the 2 days before the study (data not shown), with no difference between groups.

Table 1 also includes the relevant clamp data. Insulin infusion during the euglycemic clamp produced similar elevations (to 100–120 μU/L) in insulin levels among groups, whereas plasma glucose was maintained at near basal levels. Figure 1 shows the glucose infusion rate required to maintain euglycemia as the index of whole body insulin action. As described previously, both fat and fructose feeding significantly impaired insulin action ($P < 0.01$), compared with starch-fed controls. Benfluorex in the diet did not alter insulin action in the starch group and only partially ameliorated the decreased insulin resistance associated with high-fat feeding. Fructose-induced impairments in insulin action were prevented by benfluorex.

Table 2 shows the individual tissue $R_g'$ calculated from the accumulation of 2-deoxyglucose phosphate. In skeletal muscle, both fat and fructose feeding impaired insulin action, effects consistently prevented by benfluorex. Both fat and fructose feeding marginally elevated $R_g'$ in heart tissue; and benfluorex lowered $R_g'$ consistently.
produced elevated plasma TG levels; whereas benfluorex largely prevented this effect. Figure 2B shows red quadriceps TG levels. Consistent with our previous findings, a significant elevation of TG was noted in the control fat-fed group but not in the control fructose-fed group. Benfluorex eliminated the TG accumulation in the control fat-fed group but did not affect TG levels in any other group.

Figure 3 shows the effects of diet and benfluorex treatments on BP. Fructose feeding significantly increased mean BP compared with the starch-fed control, but the rise with fat feeding did not reach statistical significance. Benfluorex significantly ameliorated the hypertension of fructose feeding.

**DISCUSSION**

The so-called syndromes of insulin resistance represent a number of disease states that are associated via the common disorders of insulin resistance and hyperinsulinemia. The results of this study show that rats fed diets high in fat and fructose exhibit many of the major pathophysiological characteristics associated with this cluster of diseases, and, therefore, may be useful animal models for their study. Further, the amelioration of many of the symptoms with benfluorex suggests that the common mechanism may involve a disorder in lipid metabolism.

Our previous results have suggested that stored TG is an important factor in skeletal muscle insulin resistance in rats fed high-fat diets (8). Benfluorex blocks both TG accumulation and insulin resistance. Equally important, our previous results indicate that circulating TG is a significant factor in fructose-induced insulin resistance (7). Again, circulating TG is lowered by benfluorex and insulin action is improved. Possible mechanisms linking disturbances in glucose metabolism to lipid availability include the glucose-fatty cycle, which could account for effects on insulin-stimulated glucose oxidation; other data have highlighted effects on glycogen synthesis (18).

The association between hypertension and hypertriglyceridemia, and insulin resistance is not clear. In this study, fructose feeding and the concomitant induction in insulin resistance led to a significant rise in mean BP; fat feeding produced only a tendency toward a rise in pressure. However, Kaufman et al. (19) recently have

**TABLE 2**

Summary of tissue-specific Rg' in $\mu$mol $\cdot$ 100 g$^{-1}$ min$^{-1}$ at euglycemic clamp insulin levels for the six treatment groups

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Starch (Mean ± SE)</th>
<th>Starch and benfluorex (Mean ± SE)</th>
<th>Fat (Mean ± SE)</th>
<th>Fat and benfluorex (Mean ± SE)</th>
<th>Fructose (Mean ± SE)</th>
<th>Fructose and benfluorex (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red quadriceps</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hindlimb</td>
<td>12.0 ± 0.4</td>
<td>11.5 ± 1.5</td>
<td>8.7 ± 1.0$^*$</td>
<td>13.1 ± 1.9</td>
<td>8.2 ± 1.7$^*$</td>
<td>13.1 ± 2.4</td>
</tr>
<tr>
<td>Extensor digitorum longus</td>
<td>7.4 ± 1.1</td>
<td>6.8 ± 0.8</td>
<td>4.9 ± 1.0$^*$</td>
<td>6.3 ± 1.2</td>
<td>3.9 ± 0.5$^*$</td>
<td>7.1 ± 1.0</td>
</tr>
<tr>
<td>White quadriceps</td>
<td>3.8 ± 0.4</td>
<td>3.1 ± 0.5</td>
<td>2.2 ± 0.2$^*$</td>
<td>3.6 ± 0.2</td>
<td>1.9 ± 0.3$^*$</td>
<td>4.8 ± 0.8</td>
</tr>
<tr>
<td>Diaphragm</td>
<td>25.8 ± 1.6</td>
<td>24.2 ± 3.7</td>
<td>16.8 ± 1.5$^*$</td>
<td>22.5 ± 2.6</td>
<td>15.0 ± 2.8$^*$</td>
<td>27.3 ± 1.0</td>
</tr>
<tr>
<td>Heart</td>
<td>46.0 ± 2.5</td>
<td>26.4 ± 4.0</td>
<td>58.2 ± 8.5</td>
<td>40.8 ± 6.2</td>
<td>53.5 ± 6.2</td>
<td>48.4 ± 6.5</td>
</tr>
<tr>
<td>Epididymal WAT</td>
<td>4.3 ± 0.8</td>
<td>3.8 ± 0.7</td>
<td>0.9 ± 0.2$^*$</td>
<td>0.8 ± 0.1</td>
<td>2.0 ± 0.4$^*$</td>
<td>1.0 ± 0.2</td>
</tr>
</tbody>
</table>

Data are means ± SE. n = 5-7 in all cases. Statistical analysis was by one-way ANOVA on each tissue with Newman-Keuls individual post hoc tests. Data for the skeletal muscles are summarized in Fig. 2C.

*P < 0.05, differs from the starch control group and from the same diet with benfluorex.

†P < 0.05, differs from the starch control group but not from the same diet with benfluorex.
shown that a high-fat diet increases BP in rats that are allowed to overeat and in which hyperinsulinemia is observed; again, perhaps suggesting that excess lipid supply may be a common variable. A second factor relates to the potential existence of enhanced stress susceptibility observed in fat- and fructose-fed rats. High-fat feeding has been associated clearly with increased responsiveness to stress (20,21), and certainly acute fructose feeding stresses the metabolism (20). Blood glucose was monitored in this study to ensure that the rats were not stressed by the BP measuring technique, at least to the extent evidenced by this rather crude measure. However, both fat- and fructose-fed rats presented with slightly higher blood glucose levels. Perhaps the mere handling of the animals necessary to attaching cannulae is stressful—an effect that is countered by benfluorex’s demonstrated corticosterone-reducing effects under a variety of conditions (22). Stress susceptibility may be an integral part of the syndromes of insulin resistance and a critical factor leading to the overt disease states (23,24). Although the results of this study did not provide a definitive mechanism, potential models for the study of the interactions between insulin resistance and hypertension were developed.

Benfluorex’s amelioration of insulin resistance in muscle but not white adipose is interesting. This result is closely parallel to the effects of fish oils in a high-fat diet (25). The levels of insulin achieved during the clamp resemble those seen prandially in rats on similar diets (7,26); the net effect of such an amelioration of insulin resistance in muscle, but not fat, should be to partition glucose uptake away from storage as lipid. A common effect found with fish oils and benfluorex is the resultant lowering of TG supply, which occurs through reduction of hepatic TG output (14,27). Although it is not apparent whether this observation has any causal links to the muscle/adipose dichotomy, once again, the net effect should be a reduction in tissue lipid storage.

Given the points made in the previous two paragraphs, it is possible to draw parallels between the present diet models and the genetic models of obesity and insulin resistance, such as the Zucker fatty rat. This latter model has an increased stress susceptibility (28) and a marked channeling of energy away from lean tissue and growth and into adipose tissue. This partitioning of energy is particularly notable when food is restricted (29). In the Zucker rat, research has focused on abnormalities related to control of the hypothalamic-pituitary-adrenal axis (28,30). In addition, the powerful ameliorating effect of the antgliocorticoid RU 38486 on development of obesity recently has been demonstrated (31). As stated above, benfluorex also acts to ameliorate the stress hyper-responsivity observed with the diet models. Evidence certainly exists to support an inhibitory effect by
SYNDROMES OF INSULIN RESISTANCE IN THE RAT

benfluorex on the corticosterone response to either an ethanol or a fructose load; and it has been shown that corticosterone activates phosphatidate phosphohydrolase, which appears to have a central role in controlling hepatic TG production (32). It is tempting to hypothesize that stress susceptibility is central to the etiology of the syndromes of insulin resistance disease cluster, and that benfluorex plays an ameliorating role via modulation of the hypothalamic-pituitary-adrenal stress axis.

ACKNOWLEDGMENTS
This study was supported by the National Health and Medical Research Council (Australia) and Servier Laboratories.

We thank Blackmore’s Laboratories for vitamin mix, Souad Sader and Vicki Theos for expert technical assistance.

The current address of Dr. M. Kusunoki is the 1st Department of Internal Medicine, Aichi Medical University, Aichi, Japan.

REFERENCES