Supplementary Methods

Effects of Insulin and Analogues on Carcinogen-induced Mammary Tumours in High Fat-fed Rats

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Materials and Methods

Animals

Three-week-old female Sprague-Dawley rats were purchased from Charles River (Saint-Constant, QC, Canada), and maintained under a 12 h light/dark cycle with free access to water and chow in a temperature (21 °C) and humidity (40–60%)-controlled room within a specific pathogen free facility in the Department of Comparative Medicine (DCM) at the University of Toronto. They were housed in microisolator cages (2 rats per cage) with corn cob bedding. Environmental enrichment was provided according to DCM. After 1 week acclimatisation the diet was switched from a standard rodent chow (Teklad Global 2018, Teklad, Madison, WI, USA) to a high-fat diet (modified AIN-76A Purified Rodent Diet-high-fat and low-fiber; Dyets Inc., Bethlehem, PA, USA). The fat component of the high-fat diet consisted of 44.2% lard, 9.6% coconut oil, and 46.2% corn oil, and accounted for 40% of total calories. To avoid unnecessary pain and/or distress, we defined endpoints as follows: weight loss exceeding 20 % of normal body weight; ulcerated tumour; tumour mass compromising normal behaviour, ambulation, food and water intake; tumour size exceeding 2.5 cm; persistent anorexia and dehydration that cannot be alleviated. Less than 5% of rats showed one of these endpoints, and were euthanized by anaesthetic overdose (ketamine/xylazine/acepromazine cocktail: 555, 12, and 2 µmol kg⁻¹, resp). Additional rats were enrolled to make up n=30 per group.
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**Cell culture**

MCF-7 cells were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured with Dulbecco's modified eagle medium (Life Technologies, Burlington, Ontario Canada) containing 10% fetal bovine serum (Sigma-Aldrich, Oakville, Ontario, Canada). Cells at approximately 80 to 90% confluence were washed 3 times with ice cold phosphate buffered saline and then lysed by radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technology, Danvers, MA, USA) containing inhibitors on ice. The lysates were centrifuged at 12,000 rpm for 30 min at 4 °C, and the supernatant was used for Western blot.

**Western blot analysis**

Western blots were performed as previously described [1]. Approximately 100 mg of frozen mammary tumor was homogenized in RIPA buffer (Cell Signaling Technology) containing inhibitors (0.3 μmol/l aprotinin, 4.7 μmol/l leupeptin, 1.5 μmol/l pepstatin A, 1 mM NaF, 100 nM okadaic acid, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine). The lysates were centrifuged at 12,000rpm for 30 min at 4 °C and the supernatant was used for Western blot. Briefly, after denaturation by boiling with 1% sodium dodecyl sulfate and 5% 2-mercaptoethanol, 40 μg of the protein was loaded onto 10% bisacrylamide gel for electrophoresis, and then transferred to polyvinylidene fluoride membrane. The membrane was incubated overnight with antibody for phospho-Ser473-Akt (Cell Signaling Technology, 9271, raised in rabbit, RRID: AB_329825, 1:1000), Akt (Cell Signaling Technology, 9272, raised in rabbit, RRID: AB_329827, 1:2000), insulin receptor-β (Santa Cruz, Dallas, TX, USA, sc-711, raised in rabbit, RRID: AB_631835, 1:750), or insulin like growth factor-1 receptor β (Santa Cruz, sc-713, raised in rabbit, RRID: AB_671792, 1:250), except for 1 h incubation for phospho-Thr202/Tyr204-extracellular signal-regulated
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kinase 1/2 (Cell Signaling Technology, 4377, raised in rabbit, RRID: AB_331775, 1:2000), ERK1/2 (Cell Signaling Technology, 9102, raised in rabbit, RRID: AB_330744, 1:3000), or α-tubulin (Santa Cruz, sc-58666, raised in mouse, RRID: AB_632570, 1:2000). Secondary anti-mouse (Santa Cruz, sc-2318, raised in donkey, RRID: AB_641171, 1:2000) and anti-rabbit (Santa Cruz, sc-2031, raised in goat, RRID: AB_631737, 1:2000) IgG antibodies conjugated to horse radish peroxidase and chemiluminescence system (SuperSigna West Pico, Thermo Scientific, Waltham, MA, USA) were used for detection. Antibodies were validated in previous studies by our group [2-4]. The obtained immune-blot bands were quantified using Image J software (U. S. National Institutes of Health, Bethesda, MA, USA).

Hyperinsulinaemic-euglycaemic clamp

To verify that the high-fat diet induced insulin resistance, in separate groups of 9 wk old rats fed normal fat or high-fat diet for 5 wks, hyperinsulinaemic-euglycaemic clamp was conducted as described previously [1]. Four wk old female Sprague-Dawley rats were randomly assigned to standard rodent chow or high-fat diet feeding. At the age of 9 wk, the rats underwent cannulation of both the carotid artery and the jugular vein under general anaesthesia using isoflurane [1]. Sterile technique was used to minimise surgical complications. After the cannulation, rats were housed in individual cages under careful observation. Analgesic (buprenorphine, 0.11 μmol kg⁻¹) were given during surgery and at least every 12 h after surgery for 48 h. There was no experimental dropout because of surgery complications such as stroke, surgical site infection, or massive weight loss (20% from baseline). All rats were deemed healthy by the DCM before and after the cannulation. Three days after the cannulation, insulin solution (Humulin R, Eli Lilly Canada, Toronto, Ontario, Canada) and glucose solution were infused into overnight-fasted rats through the jugular vein for 2 h). Insulin infusion was fixed at the rate of 5 mU kg⁻¹ min⁻¹, and glucose infusion rate
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(GINF) was varied according to plasma glucose readings in order to maintain plasma glucose levels at baseline. Blood samples were taken every 5 min through the artery catheter starting from 30 min before the initiation of the insulin and glucose infusion, and plasma glucose levels were measured using GM 9D analyser (Analox Technologies, Huntington Beach, CA USA). By the hyperinsulinaemic-euglycaemic clamp technique, the average glucose infusion rate necessary to counteract the hypoglycaemic effect of insulin at steady state (i.e., during the last 30 min of the clamp) indicates whole body insulin sensitivity.

Kinetics study

Nine weeks old female Sprague-Dawley rats fed high-fat diet for 5 wk received cannulation of the carotid artery and after a recovery period for 3 days, underwent injection of analogues with timed blood sampling. There was no experimental dropout in this set of rats. On day 1 of the kinetics study subcutaneous glargine injection was initiated. The dose of glargine was started at 5 U kg\(^{-1}\) and raised by 5 U kg\(^{-1}\) every 2 days. On the day when 15 U kg\(^{-1}\) of glargine was given, blood samples were collected through the artery catheter before and after injection (1, 4, 9, and 24 h). After glargine injection, saline, human insulin, and detemir were injected subsequent to a washout-period of one day after each injection day, and blood samples were collected in the same way. The samples were used for the determination of plasma concentrations of unmodified human insulin, insulin analogues and their metabolites by liquid chromatography coupled to ion mobility mass spectrometry [5]. According to the manufacturer’s data sheet, the concentrations of glargine, NPH, and detemir were converted to Unit (U) as follows: insulin 1 U is determined as 0.0364 mg of glargine, 0.0347 mg of NPH, and 0.142 mg of detemir.
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References


