**SHORT COMMUNICATION**

**Insulin does not promote rat mammary carcinogenesis**

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Indirect evidence from both epidemiological studies and animal experiments suggests that insulin may promote breast cancer development. In this study, we directly tested for a promoting effect of insulin on mammary carcinogenesis in Sprague–Dawley rats. Fifty day-old female rats received an i.p. injection of 37.5 mg/kg methyl-nitrosourea (MNU). Five days later, the animals were randomized into two groups. One group received insulin injections five times/week until the time of death, while the other control group received similar injections of normal saline. Over the course of 26 weeks following MNU treatment, the mammary tumour incidence in the insulin-treated group did not differ significantly from the saline-treated controls. Furthermore, the number of tumours per tumour-bearing rat did not differ between groups. Our results demonstrate that insulin is not a promoter of mammary carcinogenesis in this model.

In 1983, Seely and Horrobin (1) proposed that the positive relationship between breast cancer rates and sugar intake seen in international studies might be explained by insulin secretion. They hypothesized that excessive stimulation of insulin release following high consumption of sugars leads to a hormonal environment that predisposes to mammary tumour development. Kazer (2) and Stoll (3) have proposed that insulin resistance may be a risk factor for breast cancer. In support of these proposals, a recent epidemiological study suggests that hyperinsulinaemia with insulin resistance is, indeed, a significant risk factor (4). Furthermore, diets high in fat, energy and carbohydrates, together with abdominal adiposity, lead to insulin resistance, impaired glucose tolerance and compensatory increases in the levels of insulin (5,6). Similar risk factors are associated with breast cancer development (7,8). Since, in many cases, hyperinsulinaemia and insulin resistance precede the development of type II diabetes (9), we might anticipate that type II diabetes would be associated with breast cancer development. However, the few epidemiological studies that have been done have found no such association (10–12).

In animal studies, administration of insulin or glucose to rats bearing mammary tumours has been shown to produce a significant increase in tumour growth, with a greater growth response when the treatments were combined (13,14). Destruction of pancreatic β-cells by alloxan (15) or streptozotocin (16) in rats bearing mammary tumours resulted in significant tumour regression. Insulin administration to rats with streptozotocin-induced diabetes reactivated tumour growth (16). Interestingly, Heuson and Legros (15) also reported that induction of alloxan diabetes after carcinogen administration completely prevented tumour formation. This effect, however, may have been caused by the considerable loss of body weight in the diabetic animals, since caloric restriction is known to inhibit markedly rat mammary carcinogenesis (17). In animals, high fat diets produce insulin resistance (18) and also promote mammary carcinogenesis (19). Finally, a role for insulin in mammary tumour development is suggested by a number of studies in which insulin has been shown to promote the growth of rat mammary carcinoma cells in vitro (20–27).

In view of the indirect evidence from both epidemiological studies and animal experiments suggesting that insulin may have an effect on breast cancer development, we have investigated whether administration of insulin promotes mammary cancer in rats. A recent study has shown that insulin administration promotes colon tumour development in rats initiated with azoxymethane (28). In that study, insulin was administered exogenously in a manner that simulated the high levels of insulin that occur after boluses of rapidly absorbed carbohydrates in animals resistant to insulin. Here, we report the effects of the same protocol of insulin administration on female Sprague–Dawley rats in which mammary cancer was initiated with methyl-nitrosourea (MNU*).

Sixty female Sprague–Dawley rats (43 days old), purchased from Charles River Laboratories (St Constant, Quebec), were housed at 22 ± 2°C at 50% humidity with a 12-h light:dark cycle, the dark cycle extending from noon until midnight. Tap water and pelleted AIN-93M diet (Dyets, Bethlehem, PA) were provided ad libitum throughout the experiment. At 50 days of age, the animals received an i.p. injection of 37.5 mg/kg MNU (Sigma Chemical Co., St Louis, MO) dissolved in 0.05% acetic acid in normal saline and used within 30 min of preparation (29). Five days after carcinogen administration, they were randomized into two groups. One group then received s.c. insulin (Iletin II NPH insulin isophane pork, Eli Lilly, Scarborough, Ontario) injections (0.2 ml, diluted in normal saline) five times/week (Monday through Friday) between 10 a.m. and 11 a.m. The control group received similar injections of normal saline. NPH is a medium-acting insulin that peaks ~4–5 h post-injection, returning to basal values within about 8 h (30). During the first 5 days of injections, insulin dosage was gradually increased from 5 IU/kg for the first 2 injection days to 10 IU/kg for the next 2 days to 15 IU/kg for the remainder of the experiment as described by Tran et al. (28). The insulin dosage was recalculated after each weekly body weight measurement.

Blood samples were collected from the retro-orbital sinuses of rats under halothane anesthesia. Ten weeks after MNU administration, samples were collected from 10 animals/group 4 h after insulin or saline injections. Serum insulin was measured using an I125-RIA kit (ICN Pharmaceuticals, Costa Mesa, CA) and blood glucose was measured using a glucometer (Miles Canada, Etobicoke, Ontario).

*Abbreviation: MNU, methyl-nitrosourea.

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The rats were palpated 3 weeks following MNU administration and weekly thereafter. The location of each tumour was recorded. Moribund animals, those with tumours >20 mm in diameter or those remaining 26 weeks after MNU administration, were killed and the tumours excised. A tumour from each of the tumour-bearing, insulin-treated rats was fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin for histopathological examination.

Body weights, serum insulin, blood glucose, tumour latency, tumour multiplicity and tumour doubling time were compared in the two groups by Student’s t-test. The rates of tumour appearance were analysed by the Mantel–Haenszel procedure (31), and final tumour incidences were analysed by the $\chi^2$ test. All values are given as means ± SE unless indicated otherwise.

Throughout the experiment, there was no significant difference in the mean body weights between the control and insulin-treated groups (Figure 1). To assess the effects of the insulin injections, we measured serum insulin and blood glucose levels in animals 10 weeks following carcinogen administration. As expected, 4 h after injection serum insulin levels were significantly higher in the insulin-treated group than in the saline-treated controls (275.1 ± 45.2 versus 37.7 ± 3.9 µIU (n = 10), respectively; $P < 0.001$), and blood glucose levels were significantly lower in the insulin-treated group than in the saline-treated group (4.6 ± 0.8 versus 7.4 ± 1.0 mmol/l (n = 10), respectively; $P < 0.001$).

The cumulative tumour incidences for the two groups of rats are shown in Figure 2. Neither the rate of tumour appearance nor the final tumour incidence in the insulin-treated group differed significantly from those appearing in the saline-treated controls. Mammary tumours induced by treating 50-day-old control rats with MNU have been consistently shown by others (29,32) to be adenocarcinomas. Histopathological examination of tumours selected at random from each of the tumour-bearing, insulin-treated animals showed that the insulin treatment did not change the tumour type. The number of tumours per tumour-bearing rat did not differ between groups [2.3 ± 0.3 (n = 23) versus 2.3 ± 0.3 (n = 24) for insulin- and saline-treated groups, respectively]. In addition to the mammary tumours, we found only one other tumour in the insulin-treated rats (a lymphoma) and one other in the controls (a lipoma).

In this study of the possible promoting effects of insulin on breast cancer development, we chose to use the well-characterized model of mammary carcinogenesis in Sprague–Dawley rats initiated with MNU at 50 days of age (31–33). The pathogenesis of these tumours, and the dependence of tumourigenesis on hormonal and dietary factors closely resembles human breast cancer development (34). In order to be able to observe a promoting effect of insulin, we chose an MNU dose of 37.5 mg/kg body weight. This is somewhat lower than the more widely used dose of 50 mg/kg that rapidly induces tumours in a high percentage of animals (29,32). Since insulin administration has been reported to promote colon tumour development in rats (28), we decided to follow the same protocol in order to determine whether insulin promotes mammary tumour development in a similar manner. This protocol involves daily insulin injections that simulate the high levels of blood insulin that occur after animals resistant to insulin ingest boluses of rapidly absorbed carbohydrates.

As reported in the colon study with male Fisher rats (28), we found that insulin given 5 days a week at a dose of 15 IU/kg body weight to Sprague–Dawley rats led to increased levels of serum insulin and reduced levels of blood glucose, but had no effect on body weights. In contrast to the results of Tran et al. (28) in the colon, however, the insulin injections in our experiments on mammary tumourigenesis did not significantly change the rate of tumour appearance, the final tumour incidence or the number of tumours per tumour-bearing animal compared to saline-treated controls. Since mammary tumours in rats are strongly sex hormone-dependent for both induction and growth (35), we reasoned that a possible promoting effect of insulin may have been offset by a change in hormone levels. This seems unlikely, however, since the length of the estrous cycle of the insulin-treated group was not different from the control group (data not shown).

It is of interest that rat colon tumours seem to be promoted by insulin while mammary tumours are not. The difference in response of these two tissues to elevated serum insulin might be caused by differences in the levels of insulin and insulin-like growth factor 1 receptors in preneoplastic cells. Insulin can act as a growth factor via these receptors (36) and over
expression of one or both of the receptors in preneoplastic cells during hyper-insulinaemia could provide a selective growth advantage to these cells.

Another possible mechanism that might explain the difference in response of the two tissues to insulin involves the ras gene family. The signal transduction pathway that is involved in regulation of gene expression and mitogenicity by insulin is mediated by ras (37). Thirty to 66% of rat colon tumours initiated by 1,2-dimethylhydrazine or azoxymethane contain Ki-ras mutations (38–40), while 80–90% of rat mammary tumours initiated by MNU contain Ha-ras mutations (41,42). Oncogenic ras genes, however, are active constitutively and may be insensitive to regulatory factors such as insulin that activate normal ras function (36). It is possible, therefore, that the majority of preneoplastic mammary cells that contain an oncogenic Ha-ras allele are insensitive to the effects of insulin, while those preneoplastic colonocytes that do not contain an oncogenic Ki-ras allele are promoted by insulin. Human breast cancers rarely contain ras oncogenes (43), though 60–70% of human breast carcinomas exhibit over expression of the c-Ha-
ras gene (44–47). Thus, although insulin does not promote rat mammary carcinogenesis, further studies of the possible role of hyperinsulinaemia in human breast cancer, as well as the mechanism of the promoting effect of insulin in rat colon carcinogenesis are merited.

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