Smokeless Nicotine Administration Does Not Result in Hypertension or a Deterioration in Glucose Tolerance or Insulin Sensitivity in Juvenile Rats

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We have previously reported that smokeless nicotine resulted in hypertension, but not a deterioration in glucose tolerance or insulin action in young adult male rats. To evaluate the effect of nicotine in juvenile animals, we studied 6-week-old male and female Sprague-Dawley rats and implanted 25-mg nicotine (N) or placebo (P) pellets. Weight gain was controlled by chow restriction in all 4 groups of rats. Males were generally heavier than females, both before and after N or P placement; there was no difference in weight between N and P groups for each sex. Systolic blood pressure, measured noninvasively, increased modestly, but not significantly, after N placement in both male and female rats. Glucose tolerance and insulin action were assessed by an oral glucose tolerance test (OGTT). Areas under the curve (AUC) were calculated for glucose (AUC_{GLU}), insulin (AUC_{INS}), and free fatty acids (FFA) (AUC_{FFA}). Insulin action was calculated by several indices, which have correlated with more invasive studies. None of these metabolic parameters were significantly impacted by nicotine treatment, consistent with our observations in adult male rats. In summary, smokeless nicotine at this dose has no significant effect on observed cardiovascular or metabolic parameters in sexually immature male and female rats.

CIGARETTE SMOKING is a major risk factor for coronary heart disease.[1-3] Cigarette smoking has been associated with exacerbated hypertension, a worsened lipid profile, elevated plasminogen activator concentrations, and a deterioration in insulin action.[4-6] However, cigarette smoke contains, in addition to nicotine, carbon monoxide, and a myriad of other gaseous and particulate agents.[7] Studies in humans of smokeless nicotine (either oral snuff or chewing tobacco) have suggested that the effects of nicotine do not explain all the effects on electrolyte and hormone balance of smoking,[8] and that there is, at most, a modest effect of smokeless tobacco on cardiovascular risk factors.[9] We have previously reported that smokeless nicotine, administered to young adult male rats, resulted in hypertension, but no deterioration in glucose tolerance or insulin action.[10] However, use of smokeless nicotine is increasing worldwide, in young men and women, as well as children.[11-16] Therefore, we focused our attention on a juvenile animal model of smokeless nicotine exposure. We also wished to further investigate the relationship of nicotine per se with other cardiac risk factors, such as glucose tolerance and insulin action, to assess whether juvenile, sexually immature animals were more sensitive to the possible metabolic effects of nicotine than young adult animals. We[17] and others[18] have previously reported the relationship of these cardiac risk factors, and this relationship has been described as "Syndrome X." Our earlier studies documenting that smokeless nicotine caused, in adult rats, a sustained increase in blood pressure, but no deterioration in glucose tolerance,[10] were characterized by the fact that nicotine-exposed animals gained less weight than control animals (unless dietary intake was controlled), and the concern that this variation in weight gain could obscure the metabolic and hemodynamic effects of nicotine. Thus, the current studies were designed to test the hypothesis that smokeless nicotine will cause hypertension and deterioration in glucose tolerance and insulin action in sexually immature animals when weight gain is controlled.

MATERIALS AND METHODS

Experimental Animals

Four-week-old male and female Sprague-Dawley rats (~190 and 160 g, respectively) were purchased from Bantin and Kingman, Fremont, CA. These animals reach sexual maturity at 8 to 10 weeks of age. After a 1-week acclimation to the Animal Research Facility, rats were randomly assigned to receive nicotine (N) or placebo (P) treatment. The protocol was approved by the Department of Veterans Affairs Northern California Health Care System Animal Studies Subcommittee and the Animal Use and Care Committee, UC Davis.

Treatment

We have reported[10] that 25 to 30 mg nicotine pellets resulted in hypertension, and 50-mg pellets resulted in detectable urinary metabolites of nicotine in young adult animals. Thus, to study these younger and smaller animals, we used 25-mg pellets. Animals were randomly assigned to treatment groups. N or P pellets were implanted subcutaneously while animals were anesthetized by sodium pentobarbital, 50 mg/kg intraperitoneally, as described previously.[19] These pellets were purchased from Innovative Research (Toledo, OH) and release drug into the circulation for a period of 3 weeks.

Diet Control

We have previously noted that nicotine treatment of rats in the manner described here resulted in a reduced rate of weight gain, compared with control (roughly 85% of control: 6.1 g/d *v* 7.2 g/d[10]). We, therefore, corrected for this by limiting the daily food intake in both N- and P-treated animals. Animals were provided 20 g each of standard laboratory rodent chow daily and water ad libitum and housed in single cages to ensure consistent food intake. Animals were main-

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Table 1. Biologic Characteristics of Animals at the Time of OGTT

Treatment	Age (wk)	Weight (g)	Systolic BP (mm Hg)	Glucose (mg/dL)	Insulin (ng/mL)	FFA (μEq/mL)
Nicotine M (n = 13)	7.8 ± 0.25	235.5 ± 27.2	79.8 ± 5.7	103.3 ± 16.6	1.01 ± .33	1.61 ± .24
Nicotine F (n = 15)	7.5 ± 0.17	188.5 ± 16.6	79 ± 1.4	130.7 ± 43.5	1.01 ± .39	1.3 ± .42
Placebo M (n = 16)	7.7 ± 0.23	228.5 ± 19.1	75.7 ± 3.5	109.9 ± 15.2	0.96 ± .32	1.7 ± .36
Placebo F (n = 16)	7.5 ± 0.18	190.1 ± 16.3	76 ± 3.5	120.2 ± 29.4	0.97 ± .29	1.31 ± .28
P	<.05, M Nic *v* F Nic, F Nic *v* M Plac	<.001, M *v* F for both treatments	NS	NS	NS	NS

Abbreviations: Nic, nicotine; Plac, placebo; M, male; F, female; NS, not significant; BP, blood pressure; FFA, free fatty acids; OGTT, oral glucose tolerance test.

tained in a 12-hour light/12-hour dark cycle, and weight was monitored regularly to assure adequate weight gain in both experimental groups.

Blood Pressure Measurement

During acclimation and after pellet implantation, the animals' blood pressure was measured noninvasively in duplicate[20] via the tail artery several times weekly using a transducer, amplifier, and chart recorder purchased from IITC, Woodland Hills, CA. We have previously reported our experience with this equipment[21]; we have found reliable blood pressure recording is possible with modest increases in ambient temperature, eg, 28°C, obtained by maintaining the chambers and cages under an incandescent bulb before blood pressure recording.

Metabolic Testing

An oral glucose tolerance test (OGTT) was performed at 2½ weeks after pellet placement. Remaining chow, if any, was withdrawn at 8 AM on the morning of testing. At 1 PM, baseline blood was drawn from a nicked tail vein[22] and glucose given by gavage (1.75 g/kg body weight) using a feeding needle as described previously.[23] Blood was drawn at 30 and 60 minutes. Hemostasis was achieved by application of silver nitrate to minimize blood loss. The possible confounding effect of silver nitrate on biochemical testing was minimized by allowing blood to flow freely on subsequent samples, washing the tail tip clean, or, in some instances, by nicking the tail again. Blood was collected in microfuge tubes on ice and allowed to clot. These tubes were centrifuged in a refrigerated (4°C) centrifuge at 2,000 × *g* for 20 minutes to allow maximum separation of clot from serum, and serum was aspirated and stored at -70°C until assay. All samples from a given animal were assayed on 1 occasion to reduce variability. Glucose and free fatty acids (FFA) were measured by spectrophotometric assays using commercially available kits obtained from Sigma, St Louis, MO and Wako, Richmond, VA, respectively. Insulin was measured by radioimmunoassay using rat insulin as a standard with a kit obtained from Linco, St Louis, MO. As previously reported,[23] this assay has a limit of detection of 0.1 ng/mL, with interassay and intra-assay coefficients of variation of 11.23% and 1.98%, respectively. All measurements were performed in duplicate. These assays have been previously described.[21] For metabolic parameters (eg, glucose, etc), the response to the OGTT was calculated by triangulation as the area under the curve (AUC). In addition, semiquantitative indices of insulin action were calculated. These included the ratio of fasting glucose: insulin (G_0/I_0),[24] the ratio of the AUCs for glucose and insulin, (AUC_{GLUC}/AUC_{INS}),[25] and a derivative of the product of fasting glucose and insulin, ($10{,}000/[(G_0)(I_0)]$).[26] These derived values have been shown elsewhere to correlate with more direct indices of insulin sensitivity, such as those derived from glucose clamp studies.

Statistics

All data are expressed as mean ± SD. Student's *t* test was used for group comparisons, and $P < .05$ was considered significant.[27]

RESULTS

Table 1 shows the biologic characteristics of the animals at the time of OGTT. For each gender, there was no significant difference in weight or age as a function of treatment group. Males were heavier than females ($P < .001$), and N-treated animals, both males and females, had slightly, but not significantly, higher blood pressure. Males were slightly, but not biologically meaningfully, older than female rats. Fasting serum glucose and FFA were slightly, but not significantly, higher in N-treated animals, while serum insulin was lower, but not significantly.

Figure 1 demonstrates the glucose response during the OGTT. While there was an expected postglucose load increase of serum glucose at 30 to 60 minutes, there was no significant difference in serum glucose between N- and P-treated animals. There was no significant difference in glucose AUC as a result of either gender or nicotine treatment (data not shown). The insulin response during the OGTT is shown in Fig 2. Again, despite the apparent postglucose load peak at 30 minutes, there was no significant difference between the N- and P-treated animals. There was no significant difference in insulin AUC as a result of either gender or nicotine treatment (data not shown). Figure 3 demonstrates the serum FFA response to an oral glucose load. Male P-treated rats had slightly, but not significantly, higher FFA values than N-treated animals, while females treated with nicotine had slightly, but not significantly, higher FFA values than P-treated rats. Consequently, there was a significant difference in AUC_{FFA} between male and female P-treated rats ($P < .001$).

Table 2 shows the results for the calculated indices of insulin action. There was no significant difference in these values as a result of either sex or treatment.

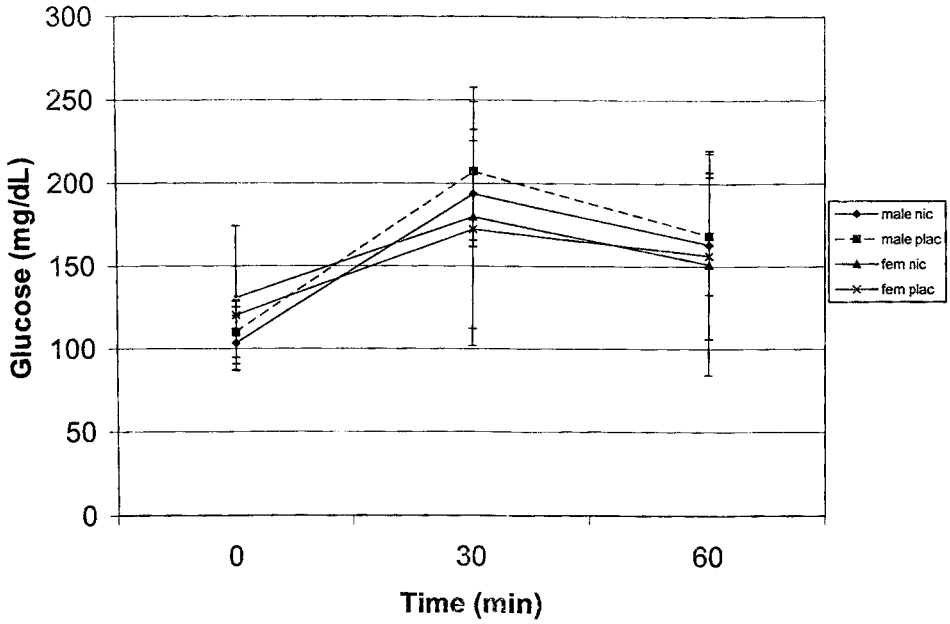

Fig 1. Serum glucose response to OGTT in P- and N-treated rats.

DISCUSSION

While cigarette smoking is uniformly considered to be a major risk factor for coronary artery disease, the mechanism underlying this increased risk is unclear. Cigarette smoking has been associated with insulin resistance,[4] whereas the use of smokeless tobacco has had less clear effects.[9] The effect of nicotine on blood pressure has been similarly obscure; in part this response may depend on use habits: the use of smokeless tobacco (chewing tobacco or snuff) in young healthy adult men reportedly results in a modest cardiovascular alteration, although these may have been regular users,[9] whereas Mundal et al[28] reported that nicotine gum increases blood pressure in nonsmokers. Cigarette smoking in nonsmokers results in an acute increase in blood pressure,[2] while in habituated smokers, cigarette smoking results in a reduction in blood pressure,[29] as does transdermal nicotine,[30] whereas acute withdrawal results in an elevation of blood pressure.[31] On the other hand, habitual smokers may have higher ambulatory blood pressure than nonsmokers.[32,33] Cigarette smoking may be particularly harmful to the elderly,[34] and users of both smoked and smokeless tobaccos have a higher cardiovascular risk than nonsmokers.[35]

In distinction to our previous experience with young adult male rats, we were unable to induce hypertension in the sexually immature animals reported here. The mechanism of the

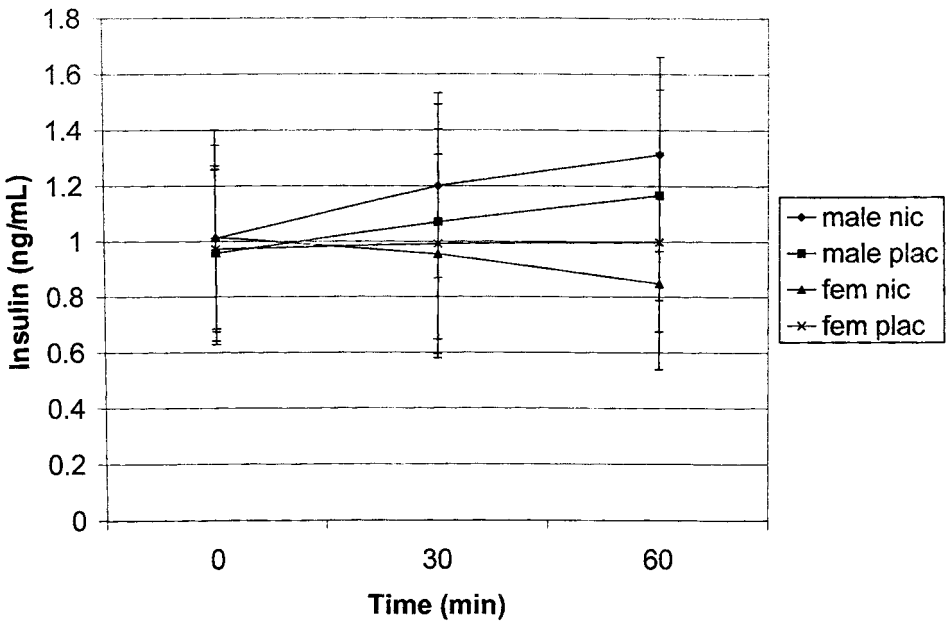

Fig 2. Serum insulin response to OGTT in P- and N-treated rats.

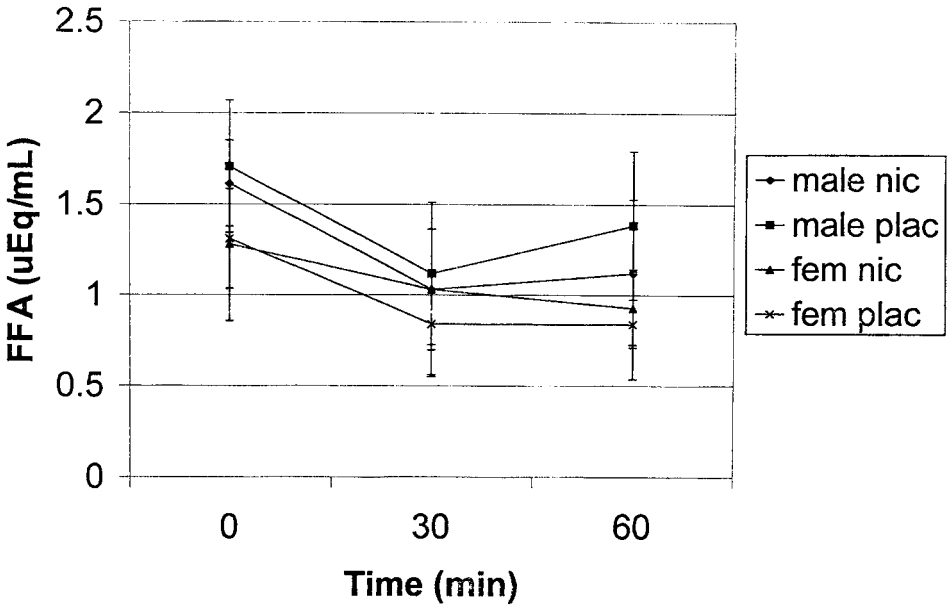

Fig 3. Serum FFA response to OGTT in P- and N-treated rats.

hypertensive response is reportedly due to an activation of the sympathetic nervous system.[2,3,36-38] Reportedly, no changes occur in the renin-angiotensin-aldosterone system,[39] while plasma calcitonin and urinary cortisol are increased in tobacco users.[8] Nicotine is accumulated over time in heart, stomach, muscle, and pancreas and may explain the predisposition of these tissues to pathologic manifestations.[40] The failure to induce hypertension in the young animals studied here suggests at least a permissive role for sex steroids. This suggests that adolescence, an age group characterized by rapid and dramatic increases in sex steroids, might be a particularly sensitive age in terms of the adverse cardiovascular effects of nicotine.

On the other hand, and in keeping with our previous experience with young adult male rats[10] we were unable to report any deterioration in glucose tolerance, when weight gain was controlled, in N- and P-treated rats of both sexes. We had previously reported that an animal model of essential hypertension, the Spontaneously Hypertensive Rat (SHR), was characterized by deterioration in glucose tolerance and insulin action.[41] Our finding of a lack of a metabolic effect after sustained nicotine exposure differs from other investigators, who have reported that cigarette smoking impacts glucose tolerance.[42] Our previous finding of nicotine-induced hypertension without a significant metabolic impact was consistent with previous studies from our laboratory,[23] failing to demonstrate a deterioration in glucose tolerance in other animal models of induced hypertension, as well as human studies showing a lack of association between abnormal glucose tolerance and secondary hypertension.[43,44]

Similarly, we did not demonstrate deterioration in calculated insulin action, assessed indirectly via OGTTs. While this was surprising given some human data suggesting deterioration in insulin action after cigarette smoking, it is consistent with the notion that unlike the situation of essential hypertension, secondary hypertension is not associated with an exacerbation of insulin resistance. Nicotine reportedly impairs insulin secretion in rats,[45] presumably via islet nicotinic receptors[46]; the human data is less clear.[47] The lack of a commensurate deterioration in the insulin response to glucose confirms that there was no deterioration in insulin action. Historically, studies describing insulin resistance resulting from nicotine have been based on cigarette smokers. On the other hand, a 6-week nicotine replacement therapy in a smoking cessation program was associated with a deterioration in insulin sensitivity above the (smoking) baseline, which improved after completion of nicotine replacement.[48] An acute 2-hour infusion of nicotine impaired insulin sensitivity in patients with type 2 diabetes, but not control subjects.[49] Thus, there is some inconsistency regarding the apparent effects of nicotine on insulin sensitivity in humans, which may reflect the underlying clinical conditions (smoking, diabetes), the duration of nicotine exposure (hours,

Table 2. Indices of Insulin Action Derived From OGTT

Group	G_0/I_0	AUC_{GLUC}/AUC_{INS}	$10000/[(I_0)(G_0)]$
Nicotine-male	115.9 ± 49.9	149.3 ± 52.1	104.7 ± 25.9
Nicotine-female	138.9 ± 89.1	166.1 ± 70	88.2 ± 34.3
Placebo-male	123.4 ± 44.7	171.7 ± 63.5	101.5 ± 28.6
Placebo-female	127.7 ± 45.4	159.1 ± 54.7	102.2 ± 47.7
P	NS for gender or treatment	NS for gender or treatment	NS for gender or treatment

Abbreviation: NS, not significant.

weeks), and the frequency of exposure (sustained, single- or repetitive-bolus).

There was no significant effect on fasting FFA resulting from nicotine treatment. While nicotine has been reported to increase fasting FFA[50]; the normal postglucose reduction in FFA is further evidence that insulin action has not been altered appreciably. Because Andersson et al[50] had reported an increase in FFA in humans after systemic nicotine administration, we considered the possibility that a type II error had occurred. We, therefore, calculated the sample size one might need to demonstrate a significant nicotine-mediated increase in FFA. Assuming that one is willing to accept a type I error of 0.05, wishing a probability of 0.8 of detecting a true difference, and considering fasting FFA data for population characteristics, one would need a sample of roughly 55 animals in each group. This suggests that the nicotine effect on FFA is, in rats, small. Our sample size was 13 to 16 rats per group. While this is smaller than the 55 estimated to be needed, the sample size used was comparable to that we previously used to study adult rats exposed to nicotine,[10] as well as studies in SHR.[41] Eliasson et al[51] reported only a modest effect on FFA in humans after smoking in nasal nicotine spray, although others have reported a more significant effect.[50]

Nicotine exposure has reportedly resulted in either weight loss or reduced weight gain, both from the thermogenic effect of tobacco smoke, as well as alteration in metabolic rate.[2,45,52] This effect appears to be mediated by nicotine.[53] Indeed, our preliminary data in rats fed ad libitum showed that nicotine treatment resulted in reduced weight gain.[10] In the current studies, we controlled weight gain by controlling dietary intake. Because food intake was the same between N- and P-treated animals, the implication is that the metabolic rate was the same in the 2 groups. An alternate explanation is that the effect of nicotine was somehow balanced by relative caloric restriction in the P-treated animals resulting in comparable weight gain; this is unlikely because caloric restriction would be expected to result in a reduced metabolic rate.[54] On the other hand, it should be considered that chow-controlled rats may not fully be representative of humans fed ad libitum.

The nicotine dose used in these studies was roughly 10 mg/kg/d. While this might seem high compared with smokeless nicotine doses in humans, Andersson et al[50] infused nicotine at 5 μg/kg/min; this roughly equals 7.2 mg/kg/d. The nicotine concentrations so obtained were felt to be comparable to those attained during moderate smoking. Shoaib and Stolerman[55] have reported that infusing nicotine to rats at 0.72 mg/kg/d yielded plasma nicotine values of 120 ng/mL, similar to those reported in smokers. A similar dose-level relationship was reported in humans chewing nicotine gum: a dose of 0.1 mg/kg yielded a plasma concentration of approximately 10 ng/mL.[56] Furthermore, Murrin et al[57] have reported that subcutaneous nicotine delivery provides plasma nicotine and cotinine levels directly correlated to the delivered dose. Thus, we believe that this model of smokeless nicotine administration mimics the nicotine effects potentially obtained in human smokers, without the confounding features (and other constituents) of smoke.

Our study was a short-term metabolic study. The lack of significant cardiovascular or metabolic impact does not preclude the possibility of adverse long-term health outcomes. Chronic nicotine exposure in rats resulted in changes in cerebral glucose utilization, as well as behavioral changes.[58] In addition, human studies have demonstrated an increased risk of a variety of cancers and other conditions after use of smokeless tobacco.[59,60]

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