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Functional and developmental studies of the peripheral arterial chemoreceptors in rat: Effects of nicotine and possible relation to sudden infant death syndrome

(dopamine type 2 receptors/*in situ* hybridization/tyrosine hydroxylase)

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ABSTRACT **The drive on respiration mediated by the peripheral arterial chemoreceptors was assessed by the hyperoxic test in 3-day-old rat pups. They accounted for 22.5 ± 8.8% during control conditions, but only for 6.9 ± 10.0% after nicotine exposure, an effect counteracted by blockade of peripheral dopamine type 2 receptors (DA2Rs). Furthermore, nicotine reduced dopamine (DA) content and increased the expression of tyrosine hydroxylase (TH) in the carotid bodies, further suggesting that DA mediates the acute effect of nicotine on arterial chemoreceptor function. During postnatal development TH and DA2R mRNA levels in the carotid bodies decreased. Thus, nicotine from smoking may also interfere with the postnatal resetting of the oxygen sensitivity of the peripheral arterial chemoreceptors by increasing carotid body TH mRNA, as well as DA release in this period. Collectively these effects of nicotine on the peripheral arterial chemoreceptors may increase the vulnerability to hypoxic episodes and attenuate the protective chemoreflex response. These mechanisms may underlie the well-known relation between maternal smoking and sudden infant death syndrome.**

Epidemiological studies have shown a dose-dependent relationship between maternal smoking and sudden infant death syndrome (SIDS) (1–6). However, no clear mechanism(s) explaining this relation has been identified. Among numerous compounds present in cigarette smoke, nicotine is of particular interest, since it acts on the peripheral arterial chemoreceptors (7, 8), the most important organs in the defense against hypoxia (9–11). A deficient function of these chemoreceptors has been suggested to be one factor contributing to SIDS (12–14). This suggestion is supported by the findings that carotid body denervation caused unexpected death not only in lambs at 4–5 weeks of age (15, 16) but also in some children with asthma who were "treated" with bilateral carotid body resection (17). Moreover, denervation impaired survival of infant rats (18) and piglets (19). Nicotine is readily transferred in high concentrations to the fetus via the placenta and to the infant by the breast milk (20–23). In fact, a majority (≈75%) of infants who succumbed by SIDS in Stockholm were exposed to nicotine shortly prior to death (24); this should be compared with the fact that only 18% of the mothers in Sweden smoke (25).

High levels of dopamine (DA) have been found in carotid bodies of SIDS victims (13). This may be of relevance, since DA modulates peripheral arterial chemoreceptor activity in most species, including rats (26) and humans (27, 28), and since DA is probably involved in the postnatal resetting of the peripheral arterial chemoreceptors from the low fetal to the high adult sensitivity (29–31). Furthermore, nicotine induces synthesis and release of carotid body DA *in vitro* (8). *In vivo* this effect of nicotine may interfere with the dopaminergic modulation of the hypoxic defense and with the postnatal development of the peripheral arterial chemoreceptors and thus relate smoking to SIDS.

The present investigation was undertaken to study the developmental regulation of carotid body tyrosine hydroxylase (TH) and DA type 2 receptor (DA2R) mRNA expression. In addition, the acute effects of nicotine on peripheral arterial chemoreceptor function and on carotid body biochemistry and gene expression were studied *in vivo* in 3-day-old rat pups with focus on DA and dopaminergic mechanisms.

MATERIALS AND METHODS

Sprague–Dawley rat pups of both sexes (B & K Universal, Stockholm) were studied at embryonic day 21 (E21), at 12 hr after birth, and on postnatal day 1 (P1), P3, and P7. Rats were housed on a 12-hr light/12-hr dark cycle with free access to food and water. Pups were kept with their mothers except when being injected or tested. Drugs and 0.9% NaCl (saline) were given as 50-μl intraperitoneal injections. The experiments were approved by Stockholms norra djurförsöksetiska nämnd (Dnr N178/90 and N159/93).

Normal developmental expression of TH and DA2R mRNAs was studied in carotid bodies of E21, P1, and P7 pups (n = 8–15) by *in situ* hybridization. In a second experiment the effect of postnatal hypoxia on the developmental expression of these mRNAs was studied 12 and 24 hr after birth (n = 5–9). Furthermore, with this technique, the effects of nicotine (0.60 mg/kg) exposure on carotid body TH and DA2R mRNA levels were studied in P3 rats 12 and 24 hr after the injection (n = 5–14). The influence of nicotine on the physiological function of the peripheral arterial chemoreceptors was tested by "physiological chemodenervation" (see below) in P3 rat pups (n = 8). The effect of nicotine on carotid body DA levels after inhibition of catecholamine synthesis with the TH inhibitor α-methyl-*p*-tyrosine (AMPT; ref. 32) was also tested in P3 rat pups (n = 10 or 11).

***In Situ* Hybridization.** After sacrifice the carotid bodies of the rat pups were rapidly dissected out, mounted and frozen in sterile saline on dry ice, sectioned at 14 μm, and thawed onto ProbeOn microscope slides (Fisher Scientific). A synthetic oligonucleotide complementary to nt 1441–1488 of rat TH mRNA (33) and a mixture of two synthetic oligonucleotides complementary to aa 12–26 (34) and 252–267 (35) of rat DA2R mRNA were used. The probes were labeled with [α-[^{35}S]thio]dATP (New England Nuclear) at the 3′ end by terminal deoxynucleotidyltransferase (Amersham) and puri-

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Abbreviations: AMPT, α-methyl-*p*-tyrosine; DA, dopamine; DA2R, DA type 2 receptors; E*n*, embryonic day *n*; P*n*, postnatal day *n*; TH, tyrosine hydroxylase; SIDS, sudden infant death syndrome.

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fied through Nensorb 20 columns (NEN). Hybridization of the nonfixed tissues was performed at 42°C for 16 hr as described (36, 37). After hybridization the sections were rinsed, transferred through distilled water, dehydrated, air-dried, dipped in liquid emulsion (NTB2; Kodak), and exposed for 4 weeks. After exposure the sections were analyzed unstained in a Nikon Microphot-FX microscope, under darkfield conditions (Nikon), and after staining with cresyl violet, under brightfield conditions.

Computerized quantification of levels of TH and DA2R mRNAs in the dipped sections was performed with a Nikon FX microscope and a computerized and digital system as partly described previously (37). The borders of the measuring fields—i.e., over areas of clustered glomus type I cells and/or the whole carotid body that expressed the respective mRNA—were interactively defined. The area (number of pixels) representing specific grains—i.e., silver grains (defined by thresholding)—was related to the area of each measured field, after correction for background activity, whereby a grain density value was achieved. The grain density values were then used to calculate individual means used for statistical evaluation.

Subchronic Exposure to Hypoxia. Three pregnant rats were placed in a Plexiglas chamber with an individual space for each rat at days 17–18 of pregnancy. One day later the O_2 level was reduced to 11–13% and kept at this level throughout the experiment. The CO_2 level was never allowed to exceed 0.1%, and the temperature in the chamber varied between 24.2°C and 26.1°C. All litters were born in the hypoxic environments 2–3 days after the gas switch but were then subjected to different postnatal environments before sacrifice and dissection. One litter was immediately removed to room air and sacrificed after 12 hr, one litter was sacrificed after 12 hr in the hypoxic environment, and one litter was first reared in the hypoxic environment for 12 hr and then allowed to breathe room air for 12 hr, after which the pups were sacrificed. Tissues were handled as described for *in situ* hybridization. The pups were kept with their mothers throughout the experiment.

Body Plethysmography. The respiratory drive mediated by the peripheral arterial chemoreceptors was assessed in unanesthetized rat pups by the hyperoxic test, so-called physiological chemodenervation (38); in this test the relative decrease in ventilation during hyperoxia is assumed to correlate to the influence of the peripheral arterial chemoreceptors on the respiratory drive (30, 31, 38). Pups were placed in a preheated (32–35°C) body plethysmograph, and room air on 100% O_2 were administered (1 liter/min) under normal barometric conditions via a Plexiglas hood placed over the plethysmograph. One hundred percent O_2 yielded >60% O_2 at the pups head 1.5 s after gas switch. Flow into and out of the body plethysmograph produced by breathing was measured with a pneumotachymeter coupled to a pressure transducer (Validyne, North Ridge, CA). Pups were placed in the plethysmograph only when being tested. At all other times the pups were with their mother or together with the littermates.

The hyperoxic test was first performed 5 min after injection of saline. After >30 min of rest the pups received nicotine (0.60 mg/kg; Sigma), and 5 min after that the response to hyperoxia was tested again. More than 1 hr later the pups were injected with domperidone (100 mg/kg; Sigma) and, after another 30 min together with the mother, also with nicotine. Five minutes later the hyperoxic test was performed again. Each pup was tested two to seven times after each treatment and was used as its own control to evaluate differences between means.

High-Performance Liquid Chromatography (HPLC). One hour after administration of AMPT (250 mg/kg; Sigma) the rat pups were injected either with saline or with nicotine (0.60 mg/kg). A third group remained untreated. Pups were sacrificed 20–25 min after the final saline or nicotine injection, and their carotid bodies were rapidly dissected out, transferred to Eppendorf tubes, and immediately frozen and stored at −70°C. The tissues were prepared for HPLC by sonication–homogenization (5 × 15 sec; 23 kHz, ≈40 W; Microson XL-2005, Heat Systems, Farmingdale, NY) in 0.1 M perchloric-acid (Sigma) with internal standard (1 pmol of α-methyl-DA hydrochloride; Sigma). The samples were then centrifuged at 1000 × *g* for 15 min at 4°C and treated with 30 ml of 0.01 M sodium metabisulfite (Sigma) and 25 mg of aluminum oxide (Sigma), 500 ml of 1 M Tris buffer in 2% EDTA (pH 8.65), and 200 ml of 0.1 M perchloric acid with 1% sodium metabisulfite and centrifuged as described above. The supernatants were analyzed by HPLC (BAS PM-4, Bioanalytical Systems, West Lafayette, IN, and CMA/200, Carnegie Medicine, Stockholm) with a Nucleosil 5SA column (Phenomene, Torrance, NY) and electrode detection (BAS LC-48, Bioanalytical Systems). The HPLC analysis was controlled by a Toshiba T1000 computer and CMA/100 version 1.39 software (Carnegie Medicine).

Statistics. Statistical analysis of respiratory responses and of TH and mRNA levels was made by analysis of variance; $P < 0.05$ was taken as significant.

RESULTS

Developmental Expression and Regulation of TH and DA2R mRNA in the Carotid Bodies. By *in situ* hybridization histochemistry, expression of TH and DA2R mRNAs was demonstrated in carotid bodies from prenatal and early postnatal rat pups (Figs. 1 and 2). The levels of TH and DA2R mRNA were high in the fetal carotid bodies but decreased markedly ($P < 0.01$) to 34.3 ± 10.3% and 40.5 ± 8.0% on P1, respectively, and to 18.4 ± 4.5% and 26.7 ± 6.5% on P7, respectively, of the fetal

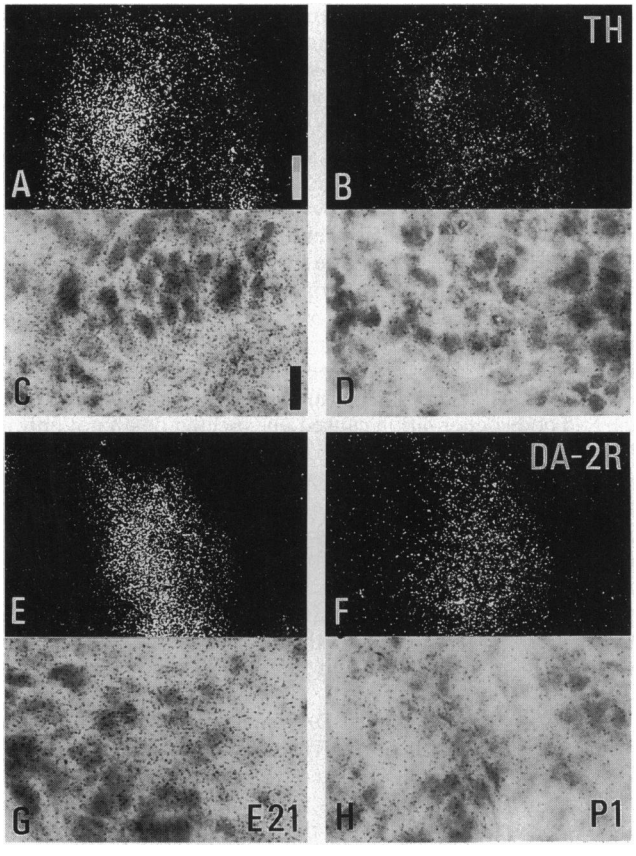

FIG. 1. Darkfield (*A, B, E,* and *F*) and brightfield (*C, D, G,* and *H*) micrographs of carotid bodies of rat pups on E21 (*A, C, E,* and *G*) and on P1 (*B, D, F,* and *H*) hybridized *in situ* with synthetic oligonucleotide probes complementary to mRNA encoding TH (*A, B, C,* and *D*) and DA2R (*E, F, G,* and *H*). [Bars = 90 μm (*A, B, E,* and *F*) and 40 μm (*C, D, G,* and *H*)].

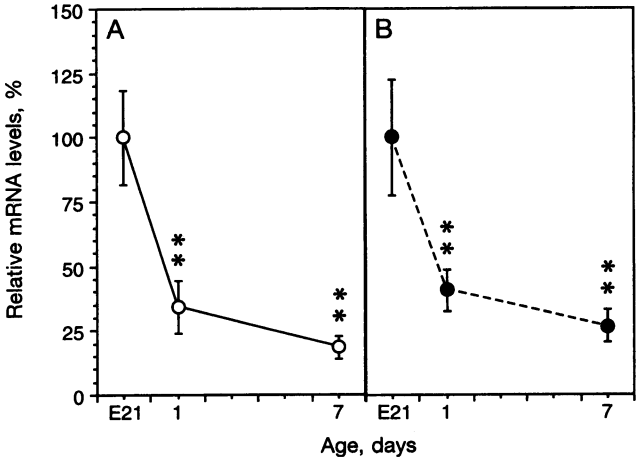

FIG. 2. Levels of TH (*A*) and DA2R (*B*) mRNA in carotid bodies of rat pups on E21, P1, and P7. The levels of TH and DA2R mRNA in pups at E21 were set to 100% and used for relative comparison. The levels of both mRNAs were high on E21 but decreased dramatically ($P < 0.01$; **) by P1 and were still low on P7 ($P < 0.01$; **).

levels (TH, 100 ± 18.2%; DA2R, 100 ± 22.5%) (Figs. 1 and 2). When pups were born and reared 12 hr in 11–13% O_2 (12 hypo), the levels of TH mRNA were 2-fold higher (214 ± 55.0%; $P < 0.01$) than in the pups born and reared in room air for 12 hr (12 norm; 100 ± 23.9%). When the pups were born and reared in hypoxia for 12 hr and subsequently allowed to breathe room air for 12 hr (12+12), the TH mRNA decreased to similar levels found in the 12 norm group (87.9 ± 25.8%) (Fig. 3*A*). The levels of DA2R mRNA in carotid bodies of pups born and reared for 12 hr in the different environments were low and not different from each other. However, the 12 norm value was higher than the 12+12 value ($P < 0.05$) (Fig. 3*A*).

Effects of Nicotine on Peripheral Arterial Chemoreceptor Function. The analysis of respiratory responses in P3 rats revealed that <1 min after the nicotine injection the animals appeared "aroused"—i.e., were physically active—and the hyperoxic chemoreflex seemed to be increased. However, this period was too short to allow reliable and repetitive recordings for statistical analysis, and the experimental and repetitive recordings were performed during a 7-min period starting 5 min after the nicotine/saline injection.

The hyperoxic test led to a 22.5 ± 8.8% (mean ± SD) decrease in minute-ventilation during control conditions (Fig. 4). Five minutes after administration of nicotine the ventilatory response to the hyperoxic test had dropped to 6.9 ± 10.0% (Fig. 4; $P < 0.05$). This effect of nicotine was counteracted by pretreatment with the peripheral DA2R antagonist domperidone. Thus, after combined treatment with domperidone and nicotine the response was 30.8 ± 5.2%, which is significantly ($P < 0.05$) different from that seen after nicotine alone (Fig. 4). The ventilatory responses to the hyperoxic test were paralleled by the test-induced changes in respiratory rate (control, 17.4 ± 9.5%; nicotine, 5.4 ± 5.8%; and domperidone nicotine, 16.8 ± 2.3%; see Fig. 4). Basal ventilation was equal in all groups.

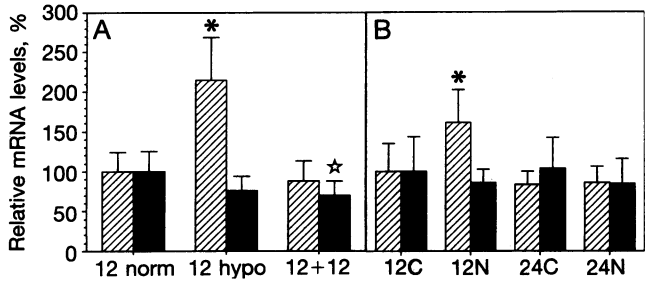

FIG. 3. (*A*) Levels of TH (hatched bars) and DA2R (filled bars) mRNA in carotid bodies in newborn rat pups after 12 hr of exposure to room air (12 norm) or 11–13% O_2 (12 hypo) and after exposure to 12 hr of hypoxia followed by 12 hr of room air (12+12). TH mRNA levels were significantly ($P < 0.05$; *) higher in the 12 hypo group than in the 12 norm and the 12+12 groups, and the levels of DA2R mRNA were lower ($P < 0.05$; ☆) in the 12+12 group than in the 12 norm group. (*B*) Carotid body levels of TH mRNA increased ($P < 0.05$; *) 12 hr after injection with nicotine (12N) in the P3 rat pups compared with animals injected with saline (12C). The levels of DA2R mRNA in the carotid bodies did not change following this treatment.

Effects of Nicotine on Carotid Body DA Content. The effects of nicotine on the release (turnover) of carotid body DA were tested 1 hr after inhibition of catecholamine synthesis by AMPT. The DA content in the carotid bodies was significantly reduced 20–25 min after nicotine administration (1.39 ± 1.78 pmol per pair; $P < 0.01$), as compared with saline controls (3.01 ± 1.78 pmol per pair), which had the same DA levels as rat pups that had not received any drugs (2.90 ± 0.40 pmol per pair).

Effects of Nicotine on Carotid Body Levels of TH and DA2R mRNAs. In carotid bodies of P3 rats, TH mRNA but not DA2R mRNA increased significantly (12N; 161.8 ± 40.4%; $P < 0.05$) 12 hr after injection of nicotine (0.60 mg/kg), as compared with pups injected with saline (12C; 100 ± 34.9%). No effect was observed on TH or DA2R mRNA levels 24 hr after injection (24C and 24C; Fig. 3*B*).

DISCUSSION

Relatively high levels of DA (39, 40) and expression of TH mRNA (41) and DA2R protein (42) and its mRNA (43, 44) have previously been demonstrated in carotid bodies of adult animals and assumed to modulate the hypoxic drive—e.g., in high-altitude acclimatization (45, 46). Using *in situ* hybridization histochemistry, we here demonstrate expression of TH and DA2R mRNAs in carotid bodies from prenatal and early postnatal rat pups, providing evidence for a developmental role of DA. Interestingly, the levels of both mRNAs were high in the fetal carotid bodies but then decreased markedly at P1 and P7. The postnatal drop in carotid body dopaminergic activity, assumed to lead to resetting in O_2 sensitivity of the peripheral arterial chemoreceptors (30, 31), is thus paralleled by a decrease in the levels of mRNAs encoding both the enzyme (TH) synthesizing the transmitter (DA) and the receptor protein (DA2R). In fact, this dramatic decrease in TH and DA2R mRNA may represent a key event in this process.

TH mRNA levels in carotid bodies of rat pups born and reared in a hypoxic environment for 12 hr after birth (i.e.,

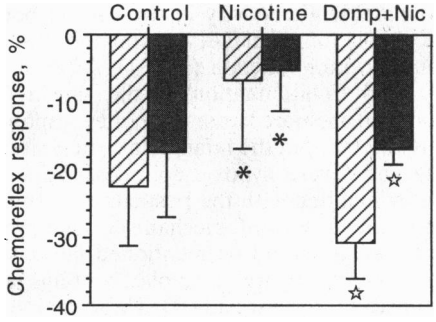

FIG. 4. Respiratory responses to the hyperoxic test in the control situation, after nicotine injection, and after combined treatment with domperidone and nicotine (Domp+Nic). Both ventilation (hatched bars) and breathing frequency (filled bars) were significantly less reduced during hyperoxia after pretest administration of nicotine, as compared with the control ($P < 0.05$; *). This inhibition was blocked by pretreatment with the peripheral DA2R antagonist domperidone ($P < 0.05$ relative responses after nicotine exposure; ☆).

mimicking the low partial pressure of O_2 present *in utero*) were >2-fold higher than in the 12-hr-old normoxic controls. Carotid body TH mRNA is upregulated by hypoxia (41) in adult animals and, thus, it is possible that the high fetal levels of TH mRNA in the carotid bodies are sustained by the relative low O_2 levels *in utero* and that the developmental downregulation of TH mRNA is triggered by the increase in O_2 after birth. As suggested earlier for regulation of TH mRNA levels in adult carotid bodies (see ref. 47), the developmental changes in TH mRNA levels may be due to changes in TH gene expression and/or in TH mRNA stability. In contrast, expression of the DA2R mRNA did not seem to be affected by the O_2 levels.

We found that nicotine caused release of (intrinsic) carotid body DA, as well as an increase in expression of carotid body TH mRNA in P3 pups, and this DA may attenuate the response of the peripheral arterial chemoreceptors via action on peripheral DA2Rs (48). However, immediately (<1 min) after the nicotine injection, we noted an acute stimulatory effect by nicotine. This is in agreement with a previous report on adult animals (7). This stimulatory action of nicotine then either disappeared or was possibly overridden by the nicotine-induced dopaminergic inhibition of the peripheral arterial chemoreceptors.

Three-day-old rat pups were chosen in this experiment because at this stage the dramatic birth-related changes in DA turnover (30, 31) have subsided, but the chemoreceptor function is still not fully mature (30, 31). The dose of nicotine administrated in the present study has been used previously to achieve significant levels of nicotine without toxic effects both in adult and in young rats.

Attenuation of the hypoxic defense could affect the ability to overcome an apnea–hypoxic episode. This may increase the risk for SIDS—e.g., by interference with other chemoreflexes such as vagal laryngeal chemoreflexes (49, 50), a situation that, in fact, has been suggested to relate to SIDS (49, 51–54). Corroborating this idea is the finding that some infants who have suffered from chronic lung disease, a group of children that are at high risk of dying of SIDS, show a depressed hyperoxic response (55).

The infant of a smoking mother has not only been continuously exposed to high concentrations of nicotine *in utero* via the placenta (21, 22) but is also exposed to nicotine after birth via the breast milk (20, 23). Therefore, it is possible that nicotine from smoking, in addition to acutely attenuating the hypoxic drive, may also interfere with the normal postnatal resetting of the peripheral arterial chemoreceptors in humans (56). Based on our observations, this interference may be dual, by inducing release of DA within the carotid body (see ref. 8) as well as by increasing TH mRNA expression (DA synthesis). This occurs during a critical developmental period when a decreased dopaminergic activity in the carotid bodies is assumed to be crucial for normal development of the peripheral arterial chemoreceptor function (30, 31). An insufficient resetting may lead to attenuation of the sensitivity of the peripheral arterial chemoreceptors; i.e., they respond at lower oxygen levels and may put the infant in a critical situation with risk for long and severe hypoxic episodes. We suggest that interference by nicotine with the postnatal resetting of these receptors may be a second mechanism relating maternal smoking to SIDS. It should be mentioned, however, that in addition to nicotine, cigarette smoke contains also other potent substances such as tar and CO. These compounds were not studied here but may well affect the respiratory control, especially in the infant and may influence the development and function of the peripheral arterial chemoreceptors by mechanisms in addition to the ones found here.

In conclusion, our findings suggest, as summarized in Fig. 5, that nicotine (from smoking) induces release and synthesis of carotid body DA which acts on local DA2Rs, leading to inhibition of the hypoxic drive. The present data also suggest

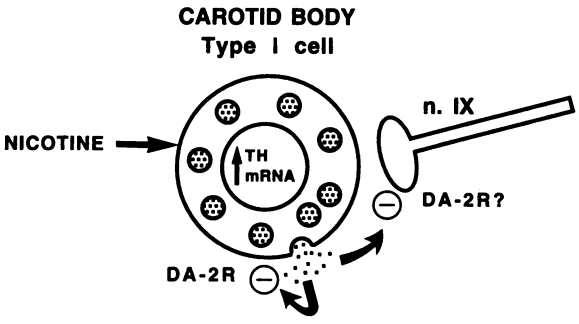

FIG. 5. Schematic illustration of possible effects of nicotine on the peripheral arterial chemoreceptors in type I cells in the carotid bodies. Nicotine increases TH mRNA synthesis and DA release in the carotid body. This may lead to an increased dopaminergic inhibition of the peripheral arterial chemoreceptors via DA2Rs, localized either on the type I cells (autoinhibition) or on the sinus nerve afferents of the glossopharyngeal nerve (n, XI), and to interference with the postnatal development of the peripheral arterial chemoreceptors (see *Discussion*).

that nicotine may interfere with the postnatal resetting of these receptors. Thus, two possible mechanisms are proposed by which nicotine from smoking may interfere with the first line of defense against hypoxia (9–11) and may lead to SIDS, either alone or by interference with other reflexes (49, 51–54).

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