Am J Physiol Cell Physiol 302: C1531–C1538, 2012.
First published March 7, 2012; doi:10.1152/ajpcell.00052.2012.

Chronic nicotine induces hypoxia inducible factor-2α in perinatal rat adrenal chromaffin cells: role in transcriptional upregulation of K_{ATP} channel subunit Kir6.2

Shaima Salman, Stephen T. Brown, and Colin A. Nurse

Department of Biology, McMaster University, Hamilton, Ontario, Canada

Submitted 14 February 2012; accepted in final form 6 March 2012

Salman S, Brown ST, Nurse CA. Chronic nicotine induces hypoxia inducible factor-2α in perinatal rat adrenal chromaffin cells: role in transcriptional upregulation of K_{ATP} channel subunit Kir6.2. *Am J Physiol Cell Physiol* 302: C1531–C1538, 2012. First published March 7, 2012; doi:10.1152/ajpcell.00052.2012.—Fetal nicotine exposure causes impaired adrenal catecholamine secretion and increased neonatal mortality during acute hypoxic challenges. Both effects are attributable to upregulation of ATP-sensitive K^+ channels (K_{ATP} channels) and can be rescued by pretreatment with the blocker, glibenclamide. Although use of in vitro models of primary and immortalized, fetal-derived rat adrenomedullary chromaffin cells (i.e., MAH cells) demonstrated the involvement of α7 nicotinic ACh receptor (nAChR) stimulation and the transcription factor, HIF-2α, the latter's role was unclear. Using Western blots, we show that chronic nicotine causes a progressive, time-dependent induction of HIF-2α in MAH cells that parallels the upregulation of K_{ATP} channel subunit, Kir6.2. Moreover, a common HIF target, VEGF mRNA, was also upregulated after chronic nicotine. All the above effects were prevented during co-incubation with α-bungarotoxin (100 nM), a specific α7 nAChR blocker, and were absent in HIF-2α-deficient MAH cells. Chromatin immunoprecipitation (ChIP) assays demonstrated binding of HIF-2α to a putative hypoxia response element in Kir6.2 gene promoter. Specificity of this signaling pathway was validated in adrenal glands from pups born to dams exposed to nicotine throughout gestation; the upregulation of both HIF-2α and Kir6.2 was confined to medullary, but not cortical, tissue. This study has uncovered a signaling pathway whereby a nonhypoxic stimulus (nicotine) promotes HIF-2α-mediated transcriptional upregulation of a novel target, Kir6.2 subunit. The data suggest that the HIF pathway may be involved in K_{ATP} channel-mediated neuroprotection during brain ischemia, and in the effects of chronic nicotine on ubiquitous brain α7 nAChR.

nicotine; chromaffin cells; hypoxia-inducible factor-2α; ATP-sensitive potassium channels; vascular endothelial growth factor; ischemia

THE PROPER TRANSITION of the neonate to extrauterine life depends critically on catecholamine (CAT) secretion from adrenomedullary chromaffin cells (AMC), triggered by asphyxial stressors associated with birth, e.g., hypoxia (17, 26). We recently demonstrated that this direct response to hypoxia is blunted in AMCs derived from the offspring of rat dams exposed to nicotine throughout gestation, providing a potential link between cigarette smoke and impaired arousal as occurs in Sudden Infant Death Syndrome (9). The underlying mechanisms were attributable to the upregulation and subsequent opening of K_{ATP} channels (Kir6.2) during acute hypoxia, thereby favoring membrane hyperpolarization and reduced excitability (10). Indeed, in the latter study pretreatment with the K_{ATP} channel blocker glibenclamide reversed the increased neonatal mortality seen in nicotine-exposed pups subjected to acute hypoxic challenges. Complementary studies using isolated neonatal rat AMCs or a fetal-derived immortalized chromaffin cell line (MAH cells), combined with in vitro exposures to 50 μM nicotine lasting 1 wk, demonstrated that the nicotine-induced K_{ATP} channel upregulation involved α-bungarotoxin-sensitive α7 nicotinic ACh receptors (nAChR) (10). Moreover, there was an obligatory requirement for the transcription factor, hypoxia inducible factor (HIF)-2α, because the above effects of nicotine were absent in MAH cells deficient in HIF-2α (10). In these studies, however, the mechanisms underlying the role of HIF-2α were unclear, as there were no obvious changes in HIF-2α levels in MAH cells after 24-h exposure to nicotine, in contrast to its robust induction after exposure to hypoxia (2% O_2) for a similar period (4, 10). Understanding these mechanisms is of broad interest given the importance of K_{ATP} channel function in neuroprotection and neurovascular remodeling following oxygen/glucose deprivation during brain ischemia and stroke (1, 33), and in the physiology of pancreatic beta cells, which becomes dysregulated after fetal nicotine exposure (8).

In the present study, we revisited the role of HIF-2α in K_{ATP} channel regulation after noting a potential hypoxia response element (HRE) or HIF binding site in the promoter region of Kir6.2 gene that encodes one of two dissimilar subunits of the K_{ATP} channel. Each of four channel pore-forming Kir6.2 subunits combines with a regulatory subunit consisting of the sulfonylurea receptor (SUR) to form a functional tetrameric complex (24). We hypothesized that nicotine exposure may cause a time-dependent induction of HIF-2α, which in turn could lead to transcriptional upregulation of Kir6.2 subunit and, consequently, increased functional K_{ATP} channel expression. Indeed, we found that both HIF-2α and Kir6.2 protein displayed a time-dependent, parallel, and progressive upregulation in wild-type and scrambled control MAH cells exposed to chronic nicotine over 1 wk. Notably, this upregulation of HIF-2α and Kir6.2 was absent in a stable HIF-2α-deficient MAH cell line (>90% knockdown) generated using interference RNAi techniques (5), and chromatin immunoprecipitation (ChIP) assays demonstrated binding of HIF- 2α to the promoter region of the Kir6.2 gene. To validate the specificity of the signaling pathway we used an in vivo model of the rat adrenal gland subjected to chronic nicotine exposure during fetal development. In this model, we took advantage of the fact that the adrenal cortex expresses ATP-dependent K^+ channels (37) but, in contrast to the medulla, lacks expression of α-bungarotoxin-sensitive α7 nicotinic AChR (11, 22). Interestingly,

Address for reprint requests and other correspondence: S. Salman, Dept. of Biology, McMaster Univ., 1280 Main St. West, Hamilton, Ontario, Canada L8S 4K1 (e-mail: salmas@univmail.cis.mcmaster.ca).

this in vivo model supported the main conclusions of the present study.

MATERIALS AND METHODS

Cell culture: MAH cells. The *v-myc* immortalized adrenal chromaffin (MAH) cells were incubated in a humidified atmosphere of 95% air-5% CO_2 at 37°C and grown in L-15/CO_2 medium containing 0.6% glucose, 1% penicillin/streptomycin, 10% fetal bovine serum, and 5 μM dexamethasone as previously described (12). Cells were plated on poly-D-lysine- and laminin-coated 35-mm culture dishes, fed every 1–2 days, and routinely passaged every 3–4 days. HIF-2α knockdown (and scrambled control) MAH cells were generated using short hairpin RNAi (shRNA) interference techniques and propagated as a stable cell line as previously described (4).

Preparation of adrenal tissues from nicotine- vs. saline-treated pups. Experiments were approved by the the Animal Research and Ethics Board at McMaster University and performed in accordance with the guidelines of the Canadian Council for Animal Care. The procedures for nicotine vs. saline injections into pregnant Wistar rats were identical to those described in detail in our previous studies (9). Experimental animals were kept at constant temperature (22°C) and lighting (12:12-h light/dark) with free access to water and food. Female Wistar rats were randomly treated with saline (vehicle) or nicotine bitartrate (1 mg·kg body wt^{-1}·day^{-1}) subcutaneously once a day for 2 wk before mating and then throughout the gestation period as previously described (9, 14). Upon delivery, pups were removed and both adrenal glands from each animal were isolated and kept in sterile medium. In the same medium, most of the outer cortex was trimmed and isolated from the central medullary tissue. The enriched cortical and medullary fractions were kept separate for molecular analysis.

Chromatin immunoprecipitation (ChIP) assay. ChIP assay was performed using a standard protocol (Millipore) as previously reported (5). MAH cells were plated on 100-mm dishes and treated with nicotine base (50 μM) for 7 days at a confluency of 75%. This dose of nicotine was previously shown to be subsaturating for nicotinic receptors on primary rat chromaffin cells (EC_{50} ~25 μM; 9); this dose also produced a similar upregulation of K_{ATP} channel expression in MAH cells and in primary neonatal chromaffin cells following in vitro exposure to nicotine (10). Histones were cross-linked to DNA using 1% formaldehyde for 10 min at 37°C. Immunoprecipitation was performed using antibodies against HIF-2α or control IgG. PCR analysis was utilized to detect HIF-2α binding using primers specific for putative HRE on Kir6.2 promoter sequence (forward: 5′-CTGGAAGGAAGCCAGTCTTG-3′; and reverse: 5′-CCTTCTTGTCCCCCTTTCTC-3′), and negative primers downstream from the promoter region (forward: 5′-GCATAAATGTTTCCCACTCC-3′; and reverse: 5′-TAACTGAAGAAGGGCAGGAA-3′) (Table 1). Primers were designed using Gene Fisher (13). Thermal cycling conditions included Platinum *Taq* DNA polymerase activation at 95°C for 2 min, 35 cycles of denaturing at 95°C for 30 s, and annealing and extension at 60°C for 30 s.

Western immunoblot analysis. Nuclear and cytoplasmic extracts obtained from adrenal tissues and MAH cells were loaded and resolved on SDS-PAGE gel and transferred to PVDC membranes as previously described (4). Membranes were incubated with either primary Kir6.2 rabbit polyclonal antibody (1:1,000 dilution; Alomone Labs), HIF-2α rabbit polyclonal antibody (1:1,000 dilution; Novus Biologicals, Littleton, CO), SUR1 rabbit polyclonal antibody (1:5,000 dilution; Millipore), TATA-binding protein rabbit polyclonal antibody (1:2,000 dilution; Santa Cruz, CA), or primary β-actin rabbit monoclonal antibody (1:10,000 dilution) at 4°C overnight.

Quantitative real time-PCR. Quantitative RT-PCR analysis was performed using the Stratagene (Mx3000p) detection system using ABsolute QPCR SYBR Green Mix. Kir6.2 primers (forward: 5′-ACAAGAACATCCGAGAGCA-3′; and reverse: 5′-CTGCACGATCAGAATAAGGA-3′) and VEGF primers (forward: 5′-AATGATGAAGCCCTGGAGTG-3′; and reverse: 5′-AATGCTTTCTCCGCTCTGAA-3′) (15), were designed (see Table 1) using Gene Fisher (13) and specificity was confirmed using BLAST. Thermal cycling conditions included Platinum *Taq* DNA polymerase activation at 95°C for 2 min, 40 cycles of denaturing at 95°C for 3 s, annealing and extension at 60°C for 30 s, followed by routine melting curve analysis. Samples with no template were used as a negative control. Data were compared using the arithmetic equation $2^{-\Delta\Delta CT}$ (19) and mRNA levels were normalized to Lamin A/C expression (primers: forward 5′-GCAGTACAAGAAGGAGCTA-3′ and reverse 5′-CAGCAATTCCTGGTACTCA-3′) as an endogenous reference (5), and expressed as transcript fold change relative to mRNA from untreated control MAH cells.

Drugs. All drugs were purchased from Sigma-Aldrich and applied fresh to the medium every 2 days.

Statistical analysis. Data from experiments were expressed as means ± SE, and statistical analyses were carried out using one-way ANOVA with Dunnett post hoc multiple comparison. Differences were considered significant if $P < 0.05$.

RESULTS

Effects of chronic nicotine on Kir6.2 subunit expression in control and HIF-2α- deficient MAH cells. Consistent with previous reports from this laboratory (10), exposure of control, wild-type (wt) MAH cells to chronic nicotine (50 μM) for 7 days caused a significant upregulation of K_{ATP} channel Kir6.2 subunit at the protein level (Fig. 1*A*). Moreover, this effect was abolished during continuous coincubation with α-bungarotoxin (100 nM), but not hexamethonium (100 μM) (Fig. 1*A*), confirming the involvement of homomeric α7 (but not α3-containing) neuronal nAChR (10). It is known that multiple α- and β-subunits of neuronal nAChR, including α3, α5, α7, β2, and β4, are expressed in neonatal rat chromaffin cells (10, 32) and MAH cells (our unpublished microarray and PCR data). Importantly, this nicotine-induced upregulation of Kir6.2 was dependent on HIF-2α function because it was absent in HIF-2α -deficient

Table 1. *List of primers used for Q RT-PCR and ChIP analyses*

Gene	Forward Primer (5′ to 3′)	Reverse Primer (5′ to 3′)	Accession No.
Q RT-PCR			
Kir6.2	ACAAGAACATCCGAGAGCA	CTGCACGATCAGAATAAGGA	NM_031358
VEGF	AATGATGAAGCCCTGGAGTG	AATGCTTTCTCCGCTCTGAA	NM_001110333
Lamin	GCAGTACAAGAAGGAGCTA	CAGCAATTCCTGGTACTCA	NM_001002016
ChIP			
Kir6.2 (experimental)	CTGGAAGGAAGCCAGTCTTG	CCTTCTTGTCCCCCTTTCTC	NM_031358
Kir6.2 (negative)	GCATAAATGTTTCCCACTCC	TAACTGAAGAAGGGCAGGAA	NM_031358

Q RT-PCR, quantitative real-time PCR; ChIP, chromatin immunoprecipitation.

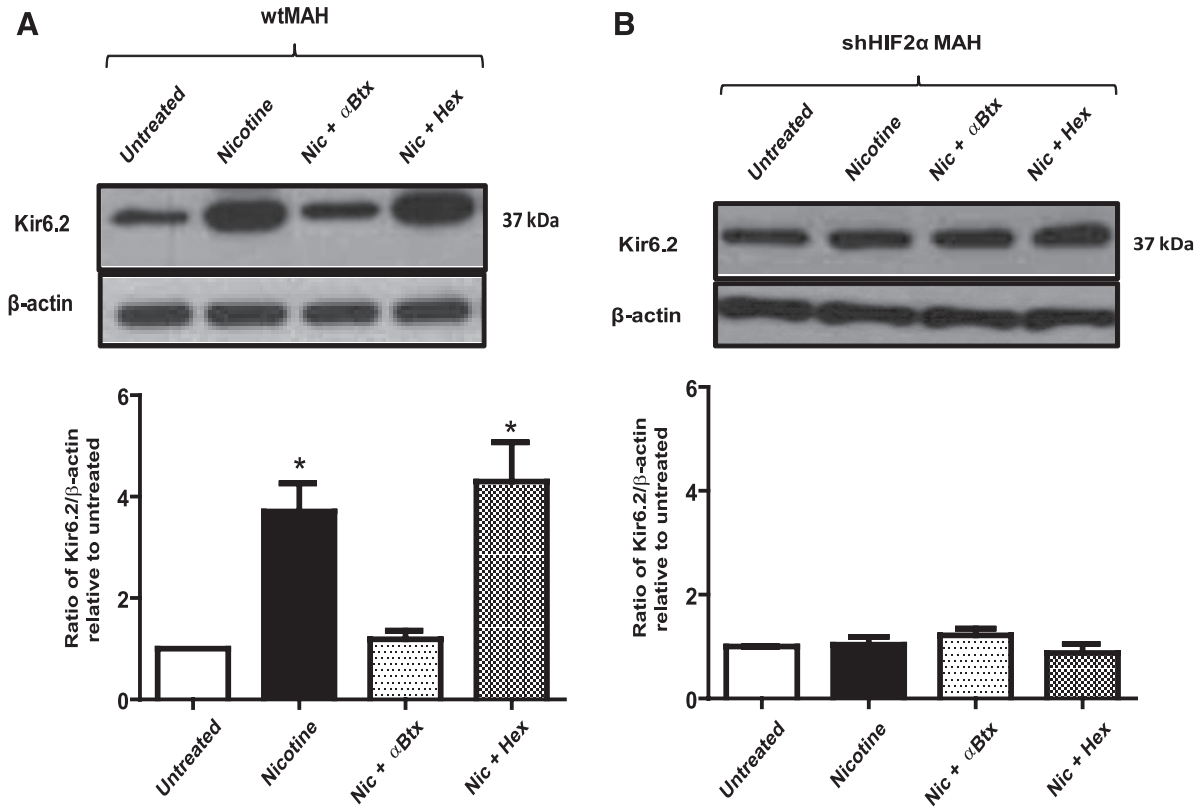

Fig. 1. Effects of chronic nicotine and nicotinic antagonists on Kir6.2 protein expression in *v-myc* immortalized adrenal chromaffin (MAH) cells. *A*: Western blot analysis of Kir6.2 expression (*top*) in wild-type MAH cells (wtMAH) exposed to either nicotine (Nic; 50 μM), nicotine plus α-bungarotoxin (αBtx; 100 nM), or nicotine plus hexamethonium (Hex; 100 μM), for 7 days; densitometric analysis (*bottom*) of relative Kir6.2 protein expression relative to β-actin ($n = 3$). *B*: comparable Western blot analysis of Kir6.2 expression showing lack of nicotine-induced Kir6.2 upregulation in hypoxia-inducible factor (HIF)-2α-deficient (shHIF2α) MAH cells. Results were normalized to β-actin and represented as means ± SE compared with control (untreated). Values are represented as means ± SE of 3 independent experiments (ANOVA; $^*P < 0.05$).

(shHIF2α; >90% knockdown) MAH cells regardless of incubation conditions (Fig. 1*B*).

Our microarray data on MAH cells also revealed a significant upregulation in Kir6.2 transcript after exposure to chronic hypoxia (2% O_2; 24 h), a condition known to cause robust induction of HIF-2α in these cells (4). This raised the question of whether or not chronic nicotine exposure caused induction of HIF-2α in MAH cells as a necessary and sufficient condition for the upregulation of Kir6.2. Because short-term (24 h) exposures of MAH cells to chronic nicotine failed to cause any significant HIF-2α accumulation in our previous study (10), we next investigated whether longer-term exposures were required.

Time-dependent effects of chronic nicotine on HIF-2α accumulation and expression pattern of Kir6.2 and SUR1 subunits of the K_{ATP} channel in MAH cells: role of α7 nAChR. To determine whether or not HIF-2α accumulation in nicotine-treated MAH cells is a time-dependent phenomenon, Western blot analysis was used to probe for HIF-2α at 24 h, 3 days, and 7 days of treatment. As illustrated in Fig. 2A, there was a progressive increase in HIF-2α accumulation that was significant at 3 and 7 days (but not at 24 h) of exposure, with HIF-2α levels being significantly greater at 7 days compared with 3 days (Fig. 2, *A* and *D*).

We next reasoned that if HIF-2α induction was indeed causally related to the nicotine-induced upregulation of Kir6.2, then the kinetics of Kir6.2 expression should follow pari passu that of HIF-2α accumulation. This was indeed the case as illustrated in Fig. 2*B*, *top*, where Kir6.2 protein levels also increased progressively with exposure time and, similar to HIF-2α accumulation, was also significant at 3 and 7 days (but not at 24 h) (Fig. 2, *B* and *D*). Interestingly, the stimulatory effect of chronic nicotine was specific for the Kir6.2 subunit of the K_{ATP} channel because expression of the regulatory SUR1 subunit remained constant (Fig. 2, *C* and *D*).

We also confirmed that, as for Kir6.2 upregulation, the nicotine-induced HIF-2α accumulation at 7 days exposure was dependent on homomeric α7 nAChR because it was prevented during coincubation with the α7 nAChR blocker, α-bungarotoxin (100 nM), but not hexamethonium (100 μM) (Fig. 3*A*). Moreover, as illustrated in Fig. 3*B*, HIF-2α accumulation was markedly suppressed in nicotine-treated HIF-2α-deficient (shHIF2α) MAH cells as expected, although it was robust in nicotine-treated scrambled control (ScCont) cells (Fig. 3*B*). Similarly, exposure to shorter durations of chronic hypoxia (2% O_2; 24 h) caused a robust induction of HIF-2α in control (wtMAH, Fig. 3*A*; ScCont MAH), but not in HIF-2α-deficient (shHIF2α MAH), MAH cells (Fig. 3B).

Chronic nicotine upregulates both Kir6.2 and VEGF mRNA in MAH cells. The observed HIF-2α-dependent upregulation of Kir6.2 protein in MAH cells after chronic exposure to nicotine raised the possibility that this could occur at the transcriptional

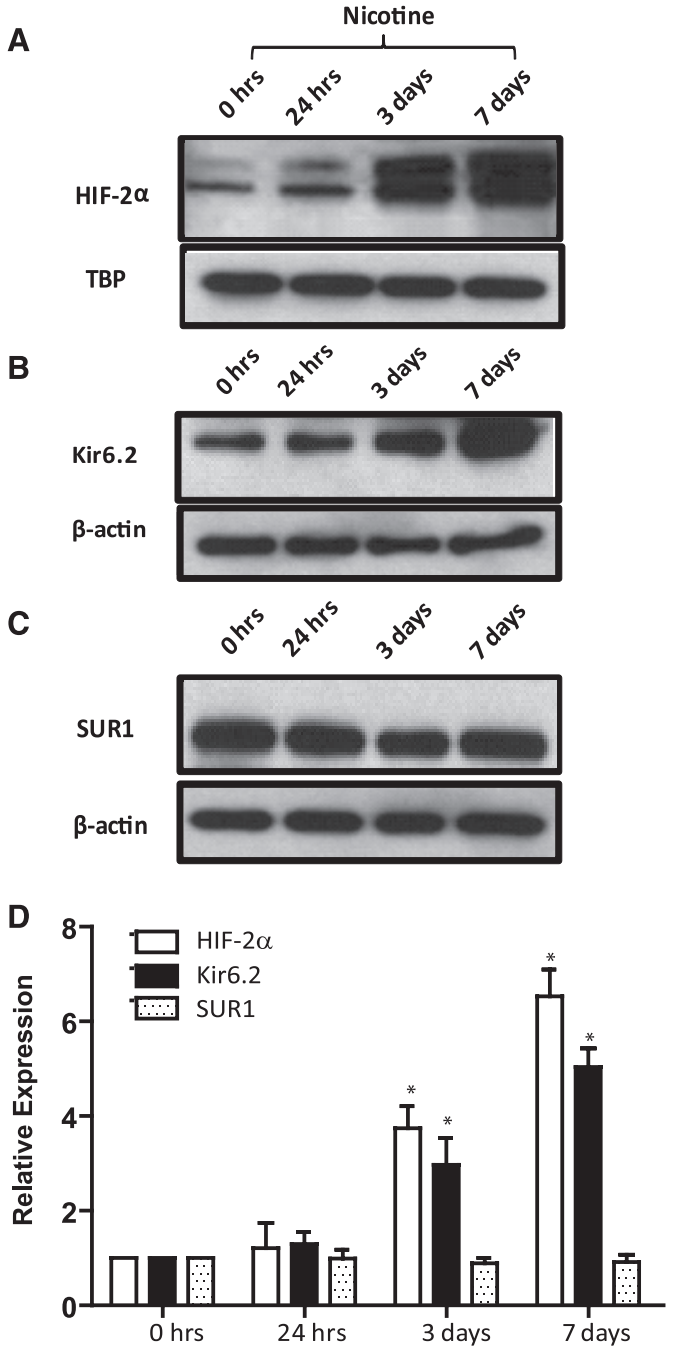

Fig. 2. Time course of the effects of chronic nicotine on Kir6.2, sulfonylurea receptor 1 (SUR1), and HIF-2α expression in MAH cells. Time-dependent HIF-2α (*A*), Kir6.2 (*B*), and SUR1 (*C*) protein expression in MAH cells exposed to nicotine (Nic; 50 μM) for 24 h, 3 days, and 7 days in culture. Densitometric quantitation (*D*) of relative expression data normalized to loading control, i.e., β-actin (for cytoplasmic extracts in the case of Kir6.2 and SUR1) and TATA-binding protein (TBP) (for nuclear extracts in the case of HIF-2α) and plotted as means ± SE compared with control (0 h) of 3 independent experiments (ANOVA; $*P < 0.05$).

level. To test this possibility we probed for Kir6.2 mRNA expression after a 7-day exposure of MAH cells to chronic nicotine. As illustrated in Fig. 4*A*, chronic nicotine caused ~3 fold increase in Kir6.2 mRNA expression as assessed by quantitative real-time PCR analysis. Moreover, this increased expression was prevented during coincubation with the α7

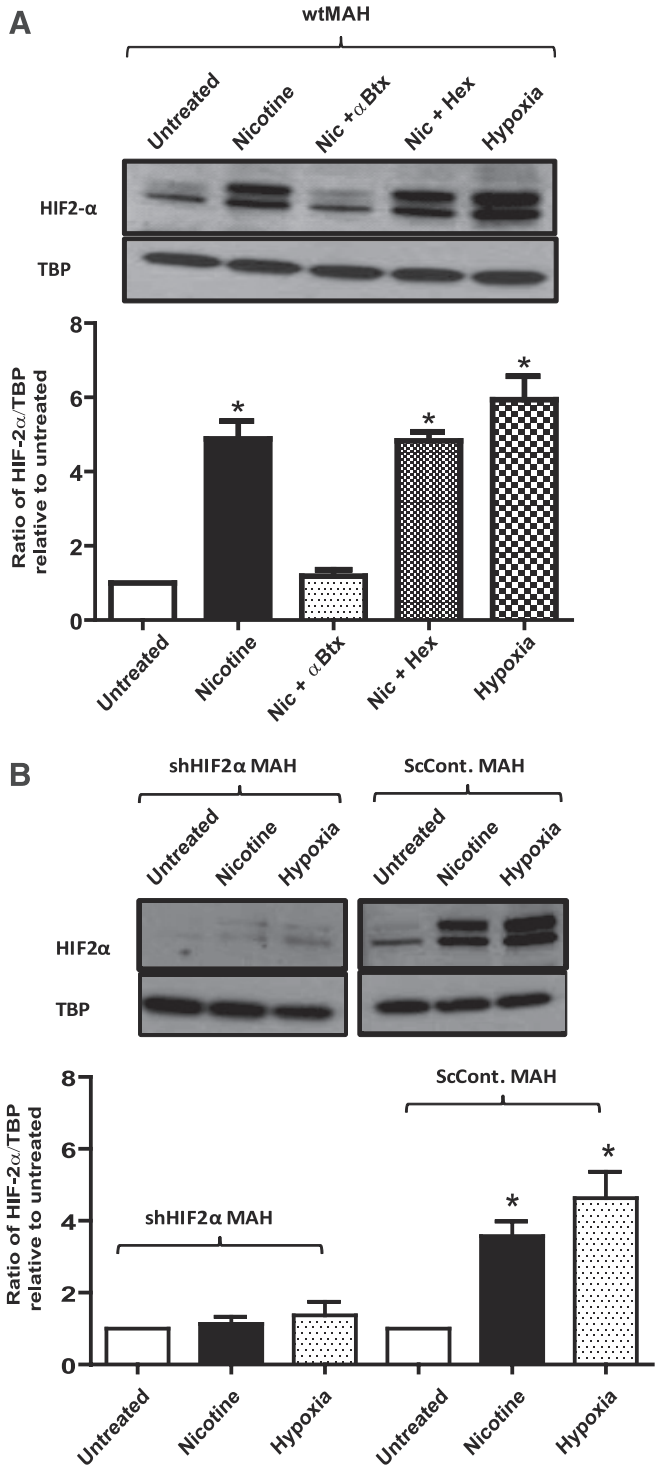

Fig. 3. HIF-2α accumulation in MAH cells exposed to nicotine, nicotine plus nicotinic blockers, or hypoxia. *A*: Western blot analysis of HIF-2α accumulation (*top*) in wtMAH cells exposed to either nicotine (Nic; 50 μM), nicotine plus α-bungarotoxin (αBtx; 100 nM), nicotine plus hexamethonium (Hex; 100 μM) for 7 days, or to chronic hypoxia (2% O_2, 24 h). *B*: Western blot analysis of HIF-2α accumulation in HIF-2α-deficient (shHIF2α) and scrambled control (ScCont) MAH cells exposed to 50 μM nicotine for 7 days. Densitometric analysis of relative HIF-2α accumulation to TATA-binding protein (TBP) is shown in the lower histograms ($n = 3$ independent experiments). Results were normalized to loading control TBP and represented as means ± SE relative to control (untreated) (ANOVA; $*P < 0.05$).

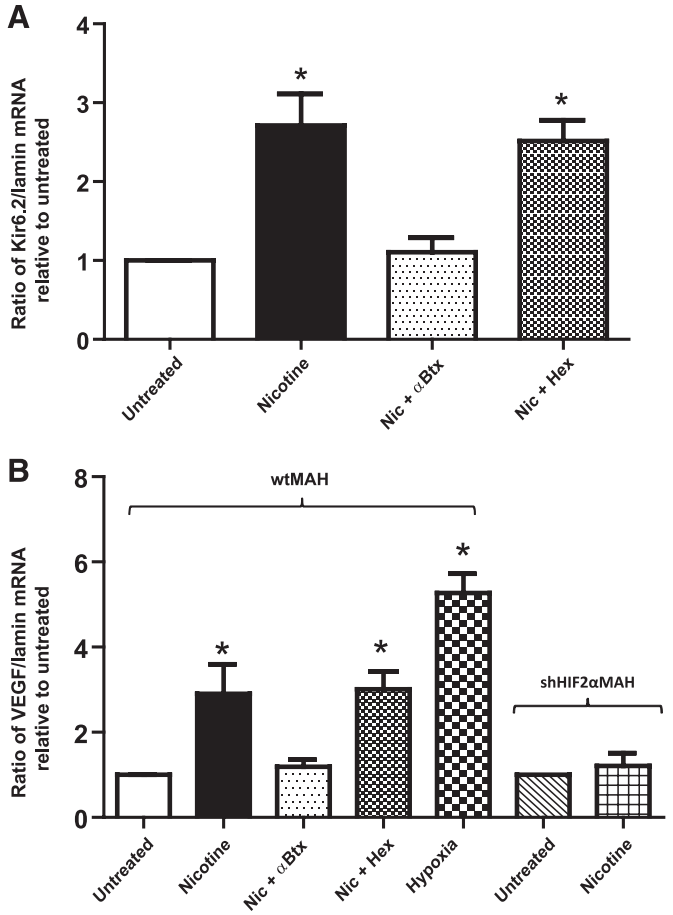

Fig. 4. Effects of chronic nicotine ± nicotinic blockers on Kir6.2 and VEGF mRNA expression in MAH cells. *A*: quantitative RT-PCR analysis of Kir6.2 mRNA levels in wild-type MAH cells (wtMAH) exposed to either nicotine (Nic; 50 μM), nicotine plus α-bungarotoxin (αBtx; 100 nM), or nicotine plus hexamethonium (Hex; 100 μM), for 7 days in culture. Histogram summarizes quantitative RT-PCR data that were normalized to Lamin A/C and plotted as means ± SE compared with control (untreated) of 3 independent experiments (ANOVA; $*P < 0.05$). *B*: similar analysis for VEGF mRNA expression in wild-type (wtMAH) and HIF-2α-deficient (shHIF2α) MAH cells exposed to either nicotine, nicotine plus α-bungarotoxin (αBtx; 100 nM), nicotine plus hexamethonium (Hex; 100 μM) for 7 days, or chronic hypoxia (2% O_2, 24 h). Collected quantitative RT-PCR data for VEGF mRNA expression were normalized to Lamin A/C and presented as means ± SE compared with control (untreated) for 3 independent experiments (ANOVA; $*P < 0.05$); note VEGF mRNA was not upregulated in HIF-2α-deficient cells exposed to nicotine.

nAChR blocker, α-bungarotoxin (100 nM), but not hexamethonium (100 μM) (Fig. 4*A*). These data are consistent with the idea that the effects of chronic nicotine are mediated via HIF-2α-dependent transcriptional upregulation Kir6.2 mRNA, leading to increased expression of K_{ATP} channels at the protein level.

The proposal that chronic nicotine exposure causes HIF-2α induction in MAH cells implies that the transcripts for other well-characterized HIF targets may also be concomitantly upregulated. Our microarray analysis on nicotine-treated MAH cells indicated upregulation of vascular endothelial growth factor (VEGF), a well-known HIF-induced gene. To confirm these data and assess whether the nicotine-induced HIF-2α accumulation was associated with an increase in VEGF mRNA, we used quantitative real-time PCR analysis following a 7-day exposure of MAH cells to chronic nicotine. Indeed, chronic nicotine and as expected chronic hypoxia (2% O_2, 24 h) caused a significant increase in VEGF mRNA in control MAH cells; this effect was absent in HIF-2α-deficient (shHIF2α) MAH cells (Fig. 4*B*). Moreover, coincubation of nicotine with α-bungarotoxin (100 nM), but not with hexamethonium (100 μM), prevented the increase in VEGF mRNA expression (Fig. 4*B*), confirming that the effect was mediated via α7 nAChR. Taken together, these results demonstrate that chronic nicotine acting via α7 nAChR in MAH cells leads to the accumulation of HIF-2α, which in turn mediates upregulation of Kir6.2 mRNA and other common HIF targets such as VEGF mRNA.

ChIP assay reveals binding of HIF-2α to the promoter region of the Kir6.2 gene. Given that the nicotine-induced upregulation of Kir6.2 mRNA in MAH cells was HIF-2α dependent (Fig. 4*A*), it was of interest to determine whether or not Kir6.2 gene expression is directly regulated by HIF-2α. We therefore analyzed the promoter region of the Kir6.2 gene and found a potential hypoxia response element (HRE) (Fig. 5*A*). This site contains the HIF core sequence 5′-GCGTG-3′ spanning nucleotides −1087 to −1083 and the HIF ancillary site 5′-CACAG-3′ spanning nucleotides −1065 to −1061. The region containing both elements is considered the HRE and

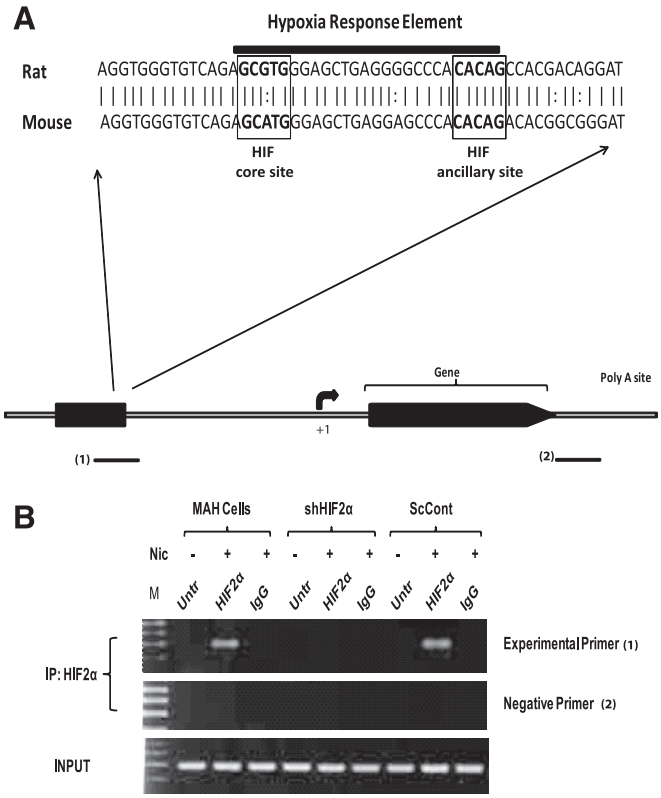

Fig. 5. Hypoxia response element (HRE) in Kir6.2 gene and chromatin immunoprecipitation (ChIP) assay. *A*: putative HRE within promoter region of Kir6.2 gene. *B*: lysates from untreated control (Untr) and nicotine (Nic)-treated wild-type (wt), shHIF2α, and ScCont MAH cells were subjected to ChIP assay using a HIF-2α polyclonal antibody. PCR analysis was performed using a primer pair designed to span the putative HRE (experimental primer) or downstream exon (negative primer). Controls include a ChIP performed using nonspecific IgG monoclonal antibody (IgG) and a starting material control (Input). The gel shown is representative of 3 independent experiments.

seems to be conserved in both the rat and mouse sequence (36) (Fig. 5*A, top*). It is similar in structure to the HRE element found in the promoter of adenosine A2a receptor (A2aR) gene that was recently shown to be regulated by HIF-2α in MAH cells (7). To determine whether or not HIF-2α binds to this region of the Kir6.2 promoter, we performed a chromatin immunoprecipitation (ChIP) assay. Indeed, the ChIP assay on chromatin isolated from nicotine-treated wild-type (wt MAH) and scrambled control (ScCont MAH) cells revealed a prominent band of 288 bp that was conspicuously absent in HIF-2α-deficient (shHIF2α MAH) cells (Fig. 5*B*). In control experiments, no bands were detectable in control IgG immunoprecipitates or in samples that included a pair of primers (negative primer) designed to amplify potential immunocomplexes, should they form downstream of the promoter region. These negative primers failed to amplify DNA, suggesting that there was specific binding of HIF-2α to an upstream site in the promoter region. These findings strongly suggest that HIF-2α binds to the distal promoter region that contains a putative HRE and show that HIF-2α binding to Kir6.2 can be stimulated by a nonhypoxic stimulus, i.e., nicotine.

Effects on chronic nicotine in utero on expression of Kir6.2 and HIF-2α in adrenal medulla vs. cortex. It was of interest to determine whether or not the model proposed above for the chronic effects of nicotine on the MAH cell line could be validated in vivo. To address this, we used an in vivo model where maternal administration of nicotine was previously shown to cause impaired O_2 sensing in chromaffin cells of the affected offspring, attributable to upregulation of K_{ATP} channels (9, 10). Previous studies have demonstrated that K_{ATP} channels are expressed in both the adrenal cortex and medulla (10, 37), whereas α-bungarotoxin-sensitive α7 nicotinic AChRs are expressed only in the medulla (11, 22). This presented a unique opportunity to test the specificity of the proposed pathway by comparing expression patterns in the adrenal cortex vs. medulla of nicotine-exposed pups. As illustrated in Fig. 6, medullary tissue from nicotine-treated pups showed the expected upregulation of both HIF-2α and Kir6.2 protein relative to saline-treated controls. By contrast, there was no difference in Kir6.2 expression (or HIF-2α) in the adrenal cortex between nicotine- and saline-treated pups. These data confirm that a signaling pathway involving α7 nicotinic AChRs is required for the nicotine-induced upregulation of Kir6.2, via HIF-2α.

DISCUSSION

The novel findings of this study are that chronic nicotine exposure leads to a slow, progressive accumulation of HIF-2α in perinatal chromaffin cells via the selective involvement of α7 nAChR, and subsequent transcriptional upregulation of ATP-sensitive K^+ channel subunit, Kir6.2. Although these studies were greatly aided by use a control and HIF-2α-deficient immortalized chromaffin (MAH) cell line, importantly, the main conclusion of the study was validated in an in vivo model. In particular, Western blot analyses of adrenal gland tissues taken from newborn pups exposed to chronic nicotine in utero revealed that upregulation of HIF-2α and Kir6.2 expression also occurred; however, it was confined to the medullary, but not cortical, region. Thus even though K_{ATP} channels are normally expressed in both the adrenal medulla

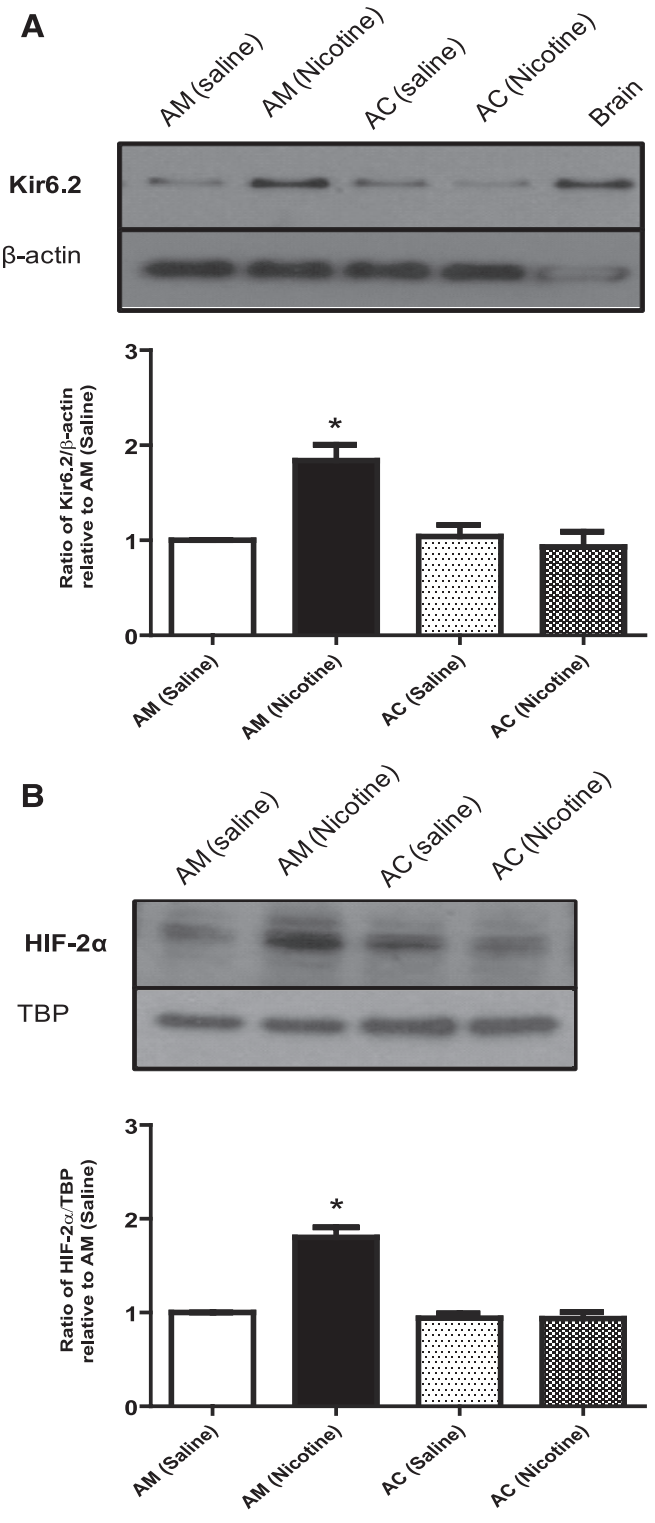

Fig. 6. Effects of in utero chronic nicotine exposure on the K_{ATP} channel subunit (Kir6.2) and HIF-2α expression in neonatal adrenal gland tissues. Western blot analyses of K_{ATP} channel subunit, Kir6.2, expression (*A*) and HIF-2α expression (*B*) in adrenal medulla (AM) and adrenal cortex (AC) of saline- and nicotine-exposed pups. Note increased expression of Kir6.2 subunit in AM but not AC of nicotine exposed pups. Densitometric quantitation (*bottom*) of relative expression data normalized to loading control, i.e., β-actin (in the case of Kir6.2) or TBP (in the case of HIF-2α) and plotted as means ± SE compared with control [AM (saline)] of 3 independent experiments (ANOVA; $*P < 0.05$).

and cortex (10, 37), our failure to observe its upregulation in the cortex is easily explained by the restricted expression of α7 nAChRs to the medulla (11, 22). In addition to providing an additional control using an in vivo model, these data further emphasize the specificity of the signaling pathway leading to K_{ATP} channel upregulation after chronic nicotine.

Our previous studies demonstrated that chronic nicotine in utero and in vitro blunts the acute hypoxic sensitivity of neonatal adrenal chromaffin cells via upregulation of K_{ATP} channels, and involved CaM kinase and PKC signaling pathways (9, 10). Moreover, we demonstrated that in vivo administration of the K_{ATP} channel blocker glibenclamide was able to reverse the increased neonatal mortality seen in nicotine-exposed pups subjected to acute hypoxic challenges (10). The present study helps to provide a mechanistic understanding at the molecular, cellular, and whole animal level of the adverse effects of nicotine exposure in mediating the loss of hypoxia tolerance in the neonate (31). Thus the ability of the K_{ATP} channel blocker glibenclamide to reverse the increased neonatal mortality seen in nicotine-exposed pups subjected to acute hypoxic challenges is explained by the slow accumulation of HIF-2α induced by nicotine acting via α7 nAChR, leading to the transcriptional and functional upregulation of K_{ATP} channels. This in turn causes membrane hyperpolarization and decreased catecholamine secretion during hypoxic challenges.

HIF stabilization and accumulation appears to be a multistep process that involves a number of posttranslational modifications. The signaling mechanisms linking chronic nicotine exposure, α7 nAChRs, and HIF-2α stabilization in MAH cells in the present study remain speculative. MAH cells express several nAChR subunits including α3 (our unpublished microarray data), and the functional pentameric receptors generally show rapid desensitization. However, the functional status of the different nicotinic nAChR subtypes in these cells after chronic nicotine exposure is likely to be more complex and is currently unknown. For example, in studies on the related neuroblastoma SH-SY5Y cell line, chronic nicotine caused upregulation of the numbers of both α7 and α3-containing nAChRs; however, intracellular Ca^{2+} measurements revealed a time-dependent loss of functional α7 nAChR activity in SH-SY5Y cells after ~4 days of exposure (25). Whether a similar loss of α7 nAChR function, and the associated reduction in Ca^{2+} influx, contribute to the delayed HIF-2α stabilization in nicotine-treated MAH cells remains to be determined. In this regard, it is noteworthy that following chronic intermittent hypoxia, increases in intracellular Ca^{2+} appears to degrade HIF-2α in PC-12 cells by activating Ca^{2+}-dependent proteases, i.e., calpains (23). On the contrary, this same treatment leads to increased synthesis, stabilization, and transcriptional activity of HIF-1α in PC 12 cells, via a separate pathway involving PKC and CaM kinase (39). The upregulation of functional K_{ATP} channels in neonatal chromaffin and MAH cells after chronic nicotine also involved the PKC and CaM kinase pathways (10). Thus the obligatory requirement for HIF-2α stabilization raises the possibility that K_{ATP} channel upregulation following chronic nicotine may involve multiple signaling cascades in these cells.

There is precedence for the idea that nicotine exposure can also lead to HIF-1α accumulation, as reported in a previous study on human small cell lung cancers (40). In the latter study, the action of nicotine was also mediated via α7 nAChR and resulted in increased VEGF expression, as observed in the present study. Other proposed signaling pathways such as the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, involved in HIF-1α stabilization (21), may potentially play a role in HIF-2α stabilization in MAH cells; however, this possibility remains to be tested. While the status of HIF-1α protein in our study on HIF-2α-deficient MAH cells remains uncertain, our microarray data suggest that HIF-1α mRNA is relatively unchanged in these mutant cells (our unpublished data). In general, HIF-2α appears to be important in cells of the sympathoadrenal lineage and is the predominant and best-studied HIF in MAH cells as it is stabilized during exposure to either chronic hypoxia, prolyl hydroxylase inhibitors, or iron chelators (4, 5). Irrespective of any potential role of HIF-1α in the present study, our demonstration that HIF-2α binds directly to the promoter region of the Kir6.2 gene in MAH cells strongly suggests that HIF-2α is the key regulator of K_{ATP} channel expression and therefore the mediator of the nicotine-induced loss of hypoxia tolerance in neonatal adrenal chromaffin cells (10, 31). It is noteworthy that there does appear to be a role for HIF-1α in MAH cells exposed to chronic intermittent hypoxia, where several potential HIF-1 targets appear to be regulated (6).

Clinical significance. The demonstration that K_{ATP} channel expression is under transcriptional control by HIF-2α is of general interest given the importance of these channels in neuroprotection during brain ischemia (1, 16), stroke prevention (33), and as protectants against metabolic stress (19). For example, during oxygen/glucose deprivation central neurons respond to the fall in ATP by opening K_{ATP} channels as a protective mechanism that results in the suppression of membrane excitability, and consequently, a reduction in energy demand (1, 16). Such low O_2 ischemic conditions are likely to induce the "master regulator" HIF transcription factor(s) (28, 29), and potentially upregulate K_{ATP} channel expression (as demonstrated herein) as part of a global positive-feedback mechanism to further enhance neuroprotection. K_{ATP} channels also play a central role in diabetes and particularly in the hyperglycemic response of pancreatic beta cells, which also express nicotinic receptors, including α7 nAChR (38). Indeed, maternal nicotine use during pregnancy has been reported to result in mitochondrial dysfunction in pancreatic beta cells attributable to oxidative stress (8), raising the possibility that the HIF pathway was activated under these conditions (39). Finally, the widespread distribution of α7 nAChR in brain adds fuel to the idea that chronic nicotine exposure, e.g., from cigarette smoke, may cause HIF stabilization in several regions of central nervous system. Such stabilization could lead to adaptive responses (e.g., neuroprotection) (2) on the one hand, or maladaptive responses (e.g., disease conditions) on the other, depending on cell type and the particular pattern of HIF expression (29).

ACKNOWLEDGMENTS

We thank Dr. Josef Buttigieg for helpful discussions, Dr. Alison Holloway for assistance with the nicotine injections, and Cathy Vollmer for technical assistance.

GRANTS

This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: S.S., S.T.B., and C.A.N. conception and design of research; S.S. performed experiments; S.T.B. was responsible for the generation of the HIF-2α-deficient cell line; S.S. analyzed data; S.S., S.T.B., and C.A.N. interpreted results of experiments; S.S. prepared figures; S.S. drafted manuscript; S.S., S.T.B., and C.A.N. approved final version of manuscript; S.T.B. and C.A.N. edited and revised manuscript.

REFERENCES

1. **Ballanyi K.** Protective role of neuronal K_{ATP} channels in brain hypoxia. *J Exp Biol* 207: 3201–3212, 2004.
2. **Bencherif M.** Neuronal nicotinic receptors as novel targets for inflammation and neuroprotection: mechanistic considerations and clinical relevance. *J Exp Biol* 207: 3201–3212, 2009.
3. **Birren SJ, Anderson DJ.** A v-myc-immortalized sympathoadrenal progenitor cell line in which neuronal differentiation is initiated by FGF but not NGF. *Neuron* 4: 189–201, 1990.
4. **Brown ST, Nurse CA.** Induction of HIF-2alpha is dependent on mitochondrial O_2 consumption in an O_2-sensitive adrenomedullary chromaffin cell line. *Am J Physiol Cell Physiol* 294: C1305–C1312, 2008.
5. **Brown ST, Kelly KF, Daniel JM, Nurse CA.** Hypoxia inducible factor (HIF)-2α is required for the development of the catecholaminergic phenotype of sympathoadrenal cells. *J Neurochem* 110: 622–630, 2009.
6. **Brown ST, Buttigieg J, Nurse CA.** Divergent roles of reactive oxygen species in the responses of perinatal adrenal chromaffin cells to hypoxic challenges. *Respir Physiol Neurobiol* 174: 252–258, 2010.
7. **Brown ST, Reyes EP, Nurse CA.** Chronic hypoxia upregulates adenosine 2a receptor expression in chromaffin cells via hypoxia inducible factor-2α: role in modulating secretion. *Biochem Biophys Res Commun* 412: 466–472, 2011.
8. **Bruin JE, Petre MA, Raha S, Morrison KM, Gerstein HC, Holloway AC.** Fetal and neonatal nicotine exposure in Wistar rats causes progressive pancreatic mitochondrial damage and beta cell dysfunction. *PLos One* 3: e3371, 2008.
9. **Buttigieg J, Brown S, Zhang M, Lowe M, Holloway AC, Nurse CA.** Chronic nicotine in utero selectively suppresses hypoxic sensitivity in neonatal rat adrenal chromaffin cells. *FASEB J* 22: 1317–1326, 2008.
10. **Buttigieg J, Brown S, Holloway AC, Nurse CA.** Chronic nicotine blunts hypoxic sensitivity in perinatal rat adrenal chromaffin cells via upregulation of K_{ATP} channels: role of α7 nicotinic AChR and hypoxia inducible factor-2α. *J Neurosci* 29: 7137–7147, 2009.
11. **Criado M, Domínguez del Toro E, Carrasco-Serrano C, Smillie FI, Juíz JM, Viniegra S, Ballesta JJ.** Differential expression of a-bungarotoxin-sensitive neuronal nicotinic receptors in adrenergic chromaffin cells: a role for transcription factor Egr-1. *J Neurosci* 17: 6554–6564, 1997.
12. **Fearon IM, Thompson RJ, Samjoo I, Vollmer C, Doering LC, Nurse CA.** O_2-sensitive K^+ channels in immortalised rat chromaffin-cell-derived MAH cells. *J Physiol* 545: 807–818, 2002.
13. **Giegerich R, Meyer F, Schleiermacher C.** GeneFisher–software support for the detection of postulated genes. *Proc Int Conf Intell Syst Mol Biol* 4: 68–77, 1996.
14. **Holloway A, Lim G, Petrik J, Foster W, Morrison K, Gerstein H.** Fetal and neonatal exposure to nicotine in Wistar rats results in increased beta cell apoptosis at birth and postnatal endocrine and metabolic changes associated with type 2 diabetes. *Diabetologia* 48: 2661–2666, 2005.
15. **Huang YF, Yang CH, Huang CC, Ming-Hong Tai MH, Hsu KS.** Pharmacological and genetic accumulation of hypoxia-inducible factor-1α enhances excitatory synaptic transmission in hippocampal neurons through the production of vascular endothelial growth factor. *J Neurosci* 30: 6080–6093, 2010.
16. **Jiang C, Haddad GG.** Modulation of K^+ channels by intercellular ATP in human neocortical neurons. *J Neurophysiol* 77: 39–102, 1997.
17. **Lagercrantz H, Slotkin TA.** The "stress" of being born. *Sci Am* 254: 100–107, 1986.
18. **Lee JW, Park JA, Kim SH, Seo JH, Lim KJ, Jeong JW, Jeong CH, Chun KH, Lee SK, Kwon YG, Kim KW.** Protein kinase C-delta regulates the stability of hypoxia-inducible factor-1 alpha under hypoxia. *Cancer Sci* 98: 1476–1481, 2007.
19. **Livak KJ, Schmittgen TD.** Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C(T)}$ method. *Methods* 25: 402–408, 2001.
20. **Minami K, Miki T, Kadowaki T, Seino S.** Roles of ATP-sensitive K^+ channels as metabolic sensors: studies of Kir6. × null mice. *Diabetes* 53: 176–180, 2004.
21. **Mottet D, Dumont V, Deccache Y, Demazy C, Ninane N, Raes M, Michiels C.** Regulation of hypoxia-inducible factor-1alpha protein level during hypoxic conditions by the phosphatidylinositol 3-kinase/Akt/glycogen synthase kinase 3beta pathway in HepG2 cells. *J Biol Chem* 278: 31277–31285, 2003.
22. **Mousavi M, Hellström-Lindahl E, Guan ZZ, Bednar I, Nordberg A.** Expression of nicotinic acetylcholine receptors in human and rat adrenal medulla. *Life Sci* 70: 577–590, 2001.
23. **Nanduri J, Wang N, Yuan G, Khan SA, Souvannakitti D, Peng YJ, Kumar GK, Garcia JA, Prabhakar NR.** Intermittent hypoxia degrades HIF-2alpha via calpains resulting in oxidative stress: implications for recurrent apnea-induced morbidities. *Proc Natl Acad Sci USA* 106: 1199–1204, 2009.
24. **Nichols CG.** K_{ATP} channels as molecular sensors of cellular metabolism. *Nature*: 440: 470–476, 2006.
25. **Ridley DL, Pakkanen J, Wonnacott S.** Effects of chronic drug treatments on increases in intracellular calcium mediated by nicotinic acetylcholine receptors in SH-SY5Y cells. *Br J Pharmacol* 135: 1051–1059, 2002.
26. **Seidler FJ, Slotkin TA.** Adrenomedullary function in the neonatal rat: responses to acute hypoxia. *J Physiol* 358: 1–16, 1985.
27. **Seidler FJ, Slotkin TA.** Ontogeny of adrenomedullary responses to hypoxia and hypoglycemia: role of splanchnic innervation. *Brain Res Bull* 16: 11–14, 1986.
28. **Semenza GL.** O_2-regulated gene expression: transcriptional control of cardiorespiratory physiology by HIF-1. *J Appl Physiol* 96: 1173–1177, 2004.
29. **Semenza GL.** Regulation of oxygen homeostasis by hypoxia-inducible factor 1. *Physiology* 24: 97–106, 2008.
30. **Slotkin TA, Seidler FJ.** Adrenomedullary catecholamine release in the fetus and newborn: secretory mechanisms and their role in stress and survival. *J Dev Physiol* 10: 1–16, 1988.
31. **Slotkin TA, Lappi SE, McCook EC, Lorber BA, Seidler FJ.** Loss of neonatal hypoxia tolerance after prenatal nicotine exposure: implications for sudden infant death syndrome. *Brain Res Bull* 38: 69–75, 1995.
32. **Souvannakitti D, Kuir B, Yuan G, Pawar A, Kumar GK, Smith C, Fox AP, Prabhakar NR.** Neonatal intermittent hypoxia impairs neuronal nicotinic receptor expression and function in adrenal chromaffin cells. *Am J Physiol Cell Physiol* 299: C381–C388, 2010.
33. **Sun XL, Hu G.** ATP-sensitive potassium channels: a promising target for protecting neurovascular unit function in stroke. *Clin Exp Pharmacol Physiol* 37: 243–252, 2010.
34. **Thompson RJ, Jackson A, Nurse CA.** Developmental loss of hypoxic chemosensitivity in rat adrenomedullary chromaffin cells. *J Physiol* 498: 503–510, 1997.
35. **Thompson RJ, Nurse CA.** Anoxia differentially modulates multiple K^+ currents and depolarizes neonatal rat adrenal chromaffin cells. *J Physiol* 512: 421–434, 1998.
36. **Wenger RH, Stiehl DP, Camenisch G.** Integration of oxygen signaling at the consensus HRE. *Sci STKE* 2005: re12, 2005.
37. **Xu L, Enyeart JJ.** Properties of ATP-dependent K^+ channels in adrenocortical cells. *Am J Physiol Cell Physiol* 280: C199–C215, 2001.
38. **Yoshikawa H, Hellstrom-Lindahl E, Grill V.** Evidence for functional nicotinic receptors on pancreatic β cells. *Metab Clin Expt* 54: 247–254, 2005.
39. **Yuan G, Nanduri J, Khan S, Semenza GL, Prabhakar NR.** Induction of HIF-1α expression by intermittent hypoxia: involvement of NADPH oxidase, Ca^{2+} signaling, prolyl hydroxylases, and mTOR. *J Cell Physiol* 217: 674–685, 2008.
40. **Zhang Q, Tang X, Zhang ZF, Velikina R, Shi S, Le AD.** Nicotine induces hypoxia-inducible factor-1alpha expression in human lung cancer cells via nicotinic acetylcholine receptor-mediated signaling pathways. *Clin Cancer Res* 13: 4686–4694, 2007.