Title: Chronic Nicotine Induces Hypoxia Inducible Factor-2α in Perinatal Rat Adrenal Chromaffin Cells: Role in Transcriptional Upregulation of $K_{\text{ATP}}$ channel subunit Kir6.2

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Author Contributions: S.S. helped in planning the study, performed all the experiments described in this paper, and wrote the first draft of the manuscript; STB was involved in the planning of the study, data interpretation, and was responsible for the generation of the HIF-2α -deficient cell line; CAN was involved in the planning and design of the experiments, and in the writing of the manuscript.

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Running Head: α7 nicotinic AChR activation upregulates $K_{\text{ATP}}$ channels via HIF-2α

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Abbreviations: CAT, catecholamine; nAChR, nicotinic ACh receptors; MAH, v-myc immortalized adrenal chromaffin; AMC, adrenomedullary chromaffin cells; HIF-2α, Hypoxia inducible factor-2α; shHIF2α MAH, HIF-2α-deficient MAH cells; ScCont, scrambled control; HRE, hypoxia response element; ChIP, chromatin immunoprecipitation; VEGF, vascular endothelial growth factor.
Abstract:

Fetal nicotine exposure causes impaired adrenal catecholamine secretion and increased neonatal mortality during acute hypoxic challenges. Both effects are attributable to upregulation of $K_{\text{ATP}}$ channels and can be rescued by pre-treatment with the blocker, glibenclamide. Though use of \textit{in vitro} models of primary and immortalized, fetal-derived rat adrenomedullary chromaffin cells (i.e. MAH cells) demonstrated the requirements for $\alpha_7$ nAChR stimulation and the transcription factor, HIF-2$\alpha$, the latter’s role was unclear. Using western blots, we show that chronic nicotine causes a progressive, time-dependent induction of HIF-2$\alpha$ in MAH cells that parallels the upregulation of $K_{\text{ATP}}$ 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 $\alpha$-bungarotoxin (100 nM), a specific $\alpha_7$ nAChR blocker, and were absent in HIF-2$\alpha$-deficient MAH cells. ChIP assays demonstrated binding of HIF-2$\alpha$ to a putative HRE 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-2$\alpha$ and Kir6.2 was confined to medullary, but not cortical, tissue. This study has uncovered a
signaling pathway whereby a non-hypoxic 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 $K_{\text{ATP}}$ channel-mediated neuroprotection during brain ischemia, and in the effects of chronic nicotine on ubiquitous brain α7 nAChR.

**Keywords:** nicotine, chromaffin cells, HIF-2α, $K_{\text{ATP}}$ channels, VEGF, ischemia
Introduction

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 $K_{\text{ATP}}$ channels (Kir6.2) during acute hypoxia, thereby favoring membrane hyperpolarization and reduced excitability (10). Indeed, in the latter study pre-treatment with the $K_{\text{ATP}}$ 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 week, demonstrated that the nicotine-induced $K_{\text{ATP}}$ channel upregulation required activation of $\alpha$-bungarotoxin-sensitive $\alpha_7$ 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 24hr exposure to nicotine, in contrast to its robust induction after exposure to hypoxia (2% O₂) 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 remodelling 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 re-visited 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 sulphonylurea 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 week. 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, this in vivo model supported the main conclusions of the present study.

Materials and Methods

Cell culture: v-myc immortalized chromaffin (MAH) cells

The v-myc immortalized adrenal chromaffin (MAH) cells were incubated in a humidified atmosphere of 95% air: 5% CO2 at 37°C and grown in L-15/CO2 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-2a 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 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 weight/d) subcutaneously (s.c.) once a day for two weeks 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 (EC₅₀ ~25 µM; 9); this dose also produced a similar upregulation of Kₐᵥ₃.₄ channel expression in MAH cells following *in vitro* exposure and in primary neonatal chromaffin cells following both *in vitro* and *in utero* 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 HIF responsive element (HRE) on Kir6.2 promoter sequence (forward: 5’ CTG GAA AGC CAG TCT TG 3’; and reverse: 5’ CCT TCT TGT CCC CCT TTC TC 3’), and negative primers downstream from the promoter region (forward: 5’ GCA TAA ATG TTT CCC ACT CC 3’; and reverse: 5’ TAA CTG AAG AAG GGC AGG AA
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 (1:1000 dilution; Alomone Labs Ltd.), HIF-2α rabbit polyclonal antibody (1:1000 dilution; Novus Biologicals, Littleton, CO, USA), SUR1 rabbit polyclonal antibody (1:5000 dilution; Millipore, Canada), TATA-binding protein rabbit polyclonal antibody (1:2000 dilution; Santa Cruz, CA, USA), or primary β-actin rabbit monoclonal antibody (1:10,000 dilution) at 4°C overnight.

**Quantitative Real Time-PCR (Q RT-PCR)**

Quantitative RT-PCR analysis was performed using the Stratagene (Mx3000p) detection system using ABsolute QPCR SYBR Green Mix. Kir6.2 primers (forward: 5’ ACA AGA ACA TCC AGC A 3’ and reverse: 5’ CTG CAC GAT CAG AAT AAG GA
3’) and VEGF primers (forward: 5’
AATGATGAAGCCCTGGAGTG 3’ and reverse: 5’
AATGCTTTCTCCGCTCTGAA 3’ (15), were designed 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) .mRNA levels were
normalized to Lamin A/C expression (primers: forward: 5’-
CAGTACAAGAAGGAGCTA and reverse: 5’
CAGCAATTCTGGTACTCA 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 ± SEM and
statistical analyses were carried out using one-way ANOVA with
Results

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 (w.t.) MAH cells to chronic nicotine (50 µM) for 7 days caused a significant upregulation of K<sub>ATP</sub> channel Kir6.2 subunit at the protein level (Fig. 1A). Moreover, this effect was abolished during continuous co-incubation with α-bungarotoxin (αBtx; 100 nM), but not hexamethonium (Hex; 100 µM) (Fig. 1A), 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, 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 (shHIF2α; >90% knockdown) MAH cells regardless of incubation conditions (Fig. 1B).

Our microarray data on MAH cells also revealed a significant up-regulation in Kir6.2 transcript after exposure to chronic hypoxia
(2% O₂; 24 hr), a condition known to cause robust induction of HIF-2α in these cells (4). This raised the question 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 hr) 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<sub>ATP</sub> 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 hr, 3 days and 7 days of treatment. As illustrated in Fig. 2A (lower panel), there was a progressive increase in HIF-2α accumulation that was significant at 3 and 7 days (but not at 24 hrs) of exposure, with HIF-2α levels being significantly greater at 7 days compared to 3 days (Fig. 2A; histogram).

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. 2B (upper panel) 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 hrs) (Fig. 2B; D). Interestingly, the stimulatory effect of chronic nicotine was specific for the Kir6.2 subunit of the K<sub>ATP</sub> channel because expression of the regulatory SUR1 subunit remained constant (Fig. 2C; 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 co-incubation with the α7 nAChR blocker, α-bungarotoxin (100 nM), but not hexamethonium (100 µM) (Fig. 3A). Moreover, as illustrated in Fig. 3B, HIF-2α accumulation was markedly suppressed in nicotine-treated HIF-2α -deficient (shHIF2α) MAH cells as expected, though it was robust in nicotine-treated scrambled control (ScCont) cells (Fig. 3B). Similarly, exposure to shorter durations of chronic hypoxia (2% O<sub>2</sub>; 24hr) caused a robust induction of HIF-2α in control (wtMAH, Fig. 3A; ScCont MAH), but not in HIF-2α -deficient (shHIF2α MAH), MAH cells (Fig. 3B).
Chronic nicotine upregulates both Kir 6.2 and VEGF mRNA in MAH cells

The observed HIF-2α-dependent upregulation of Kir 6.2 protein in MAH cell after chronic exposure to nicotine raised the possibility that this could occur at the transcriptional 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. 4A, 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 co-incubation with the α7 nAChR blocker, α-bungarotoxin (100 nM), but not hexamethonium (100 µM) (Fig. 4A). These data are consistent with the idea that the effects of chronic nicotine are mediated via HIF-2α-dependent transcriptional upregulation Kir 6.2 mRNA, leading to increased expression of K<sub>ATP</sub> channels at the protein level.

The posit 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% O₂, 24 hr) caused a significant increase in VEGF mRNA in control MAH cells; this effect was absent in HIF-2α-deficient (shHIF2α) MAH cells (Fig. 4B). Moreover, co-incubation of nicotine with α-bungarotoxin (αBtx; 100 nM), but not with hexamethonium (100 μM), prevented the increase in VEGF mRNA expression (Fig. 4B), confirming that the effect was mediated via α7 nAChR. Taken together, these results demonstrate that chronic nicotinic activation of α7 nAChR in MAH cells leads to the accumulation of HIF-2α which in turn mediates upregulation of Kir 6.2 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. 4A), 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) or HIF-binding site (Fig. 5A). 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 seems to be conserved in both the rat and mouse sequence (36) (Fig. 5A, upper). It is similar in structure to the HRE element found in the promoter of adenosine A2α receptor (A2aR) gene that was recently shown to be regulated by HIF-2α in MAH cells (7). In order 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 288bp that was conspicuously absent in HIF-2α-deficient (shHIF2α MAH) cells (Fig. 5B). In control experiments, no bands were detectable in control IgG immunoprecipitates nor 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 non-hypoxic 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 O₂ 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 versus 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 (nor HIF-2α) in the adrenal cortex between nicotine- and saline-treated pups. These data confirm that a signaling pathway involving stimulation of 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 activation of α7 nAChR, and subsequent transcriptional upregulation of ATP-sensitive K⁺ channel subunit, Kir6.2. Though 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 Kₐₕₜₚ channels are normally expressed in both the adrenal medulla 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 Kₐₕₜₚ 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 $K_{\text{ATP}}$ channels, and involved CaM kinase and PKC signaling pathways (9, 10). Moreover, we demonstrated that in vivo administration of the $K_{\text{ATP}}$ 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 $K_{\text{ATP}}$ 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-2$\alpha$ induced by nicotine acting via $\alpha7$ nAChR, leading to the transcriptional and functional upregulation of $K_{\text{ATP}}$ 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 post-translational modifications. The signaling mechanisms linking chronic nicotine exposure, $\alpha7$ nAChRs, and HIF-2$\alpha$ stabilization in MAH cells in the present study remain speculative. MAH cells express several nAChR subunits including $\alpha3$ (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 suggests 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 $K_{\text{ATP}}$ 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 $K_{\text{ATP}}$ 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 $K_{\text{ATP}}$ channel expression (as demonstrated herein) as part of a global positive feedback mechanism to further enhance neuroprotection. $K_{\text{ATP}}$ channels also play a central role in diabetes and particularly, in the hyperglycemic response of pancreatic beta cells which also express nicotinic receptors, including $\alpha_7$ 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 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).

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References


39. Yuan G, Nanduri J, Khan S, Semenza GL, Prabhakar NR. Induction of HIF-1α expression by intermittent hypoxia:

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

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Figure Legends

Figure 1. Effects of chronic nicotine and nicotinic antagonists on Kir6.2 protein expression in 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 HIF-2α-deficient (shHIF2α) MAH cells. Results were normalized to β-actin and represented as mean ± SEM compared with control (untreated).
Values are represented as mean ±SEM of three independent experiments (Anova; *, P < 0.05).

**Figure 2. Time course of the effects of chronic nicotine on Kir6.2, 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 hr, 3 days, and 7 days in culture. Densitometric quantitation (bottom) of relative expression data normalized to loading control, i.e. β-actin (for cytoplasmic extracts in the case of Kir 6.2 and SUR 1) and TATA-Binding Protein (TBP) (for nuclear extracts in the case of HIF-2α) and plotted as mean ± SEM compared with control (0 hours) of three independent experiments (Anova; *, P < 0.05).

**Figure 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% O2, 24 hrs). B) Western blot analysis of HIF-2α accumulation in HIF-2α-deficient (shHIF2α) and scrambled control (ScCont) MAH cells exposed to
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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 mean ± SEM relative to control (untreated) (Anova; *, P < 0.05).

Figure 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 mean ± SEM compared with control (untreated) of three 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 (Hox; 2% O2, 24 hrs). Collected quantitative RT-PCR data for VEGF mRNA expression were normalized to Lamin A/C and presented as mean ± SEM compared with control (untreated) for three independent
experiments (Anova; *, P < 0.05); note VEGF mRNA was not upregulated in HIF-2α-deficient cells exposed to nicotine.

**Figure 5. Hypoxia Response Element 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 non-specific IgG monoclonal antibody (IgG) and a starting material control (Input). The gel shown is representative of three independent experiments.

**Figure 6. Effects of in utero chronic nicotine exposure on the K<sub>ATP</sub> channel subunit (Kir6.2) and HIF-2α expression in neonatal adrenal gland tissues.** Western blot analyses of K<sub>ATP</sub> 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 Kir 6.2) or TATA-Binding Protein (TBP) (in the case of HIF-2α) and plotted as mean ± SEM compared with control (AM (Saline)) of three independent experiments (Anova; *, P < 0.05).
Figure 1. Effects of chronic nicotine and nicotinic antagonists on Kir6.2 protein expression in 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 HIF-2α-deficient (shHIF2α) MAH cells. Results were normalized to β-actin and represented as mean ± SEM compared with control (untreated). Values are represented as mean ±SEM of three independent experiments (Anova; *, P < 0.05).
**Figure 2.** Time course of the effects of chronic nicotine on Kir6.2, 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 hr, 3 days, and 7 days in culture. Densitometric quantitation (bottom) of relative expression data normalized to loading control, i.e. β-actin (for cytoplasmic extracts in the case of Kir 6.2 and SUR 1) and TATA-Binding Protein (TBP) (for nuclear extracts in the case of HIF-2α) and plotted as mean ± SEM compared with control (0 hours) of three independent experiments (Anova; *, P < 0.05).
Figure 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% O2, 24 hrs). 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 mean ± SEM relative to control (untreated) (Anova; *, P < 0.05).
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