Chronic opioids regulate K_{ATP} channel subunit Kir6.2 and carbonic anhydrase I and II expression in rat adrenal chromaffin cells via HIF-2α and protein kinase A

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CO2/H^+ (acid hypercapnia) on AMCs, leading to membrane depolarization, voltage-gated Ca^{2+} entry, and CAT secretion (23, 25, 34). These direct chemosensing mechanisms are suppressed postnatally, in parallel with the development of splanchnic innervation (after the first postnatal week in the rat), and return following denervation of adult AMCs (19, 23, 25, 31, 32, 34). Thus, it is plausible that neurochemicals released from the splanchnic nerve during innervation activate signaling cascades that lead to the suppression of hypoxia and hypercapnia chemosensitivity. In recent tests of this hypothesis, we considered the potential involvement of nicotinic cholinergic and opioid receptor signaling pathways (29), given that acetylcholine (ACH) and opiate peptides are among the presynaptic neurochemicals released from the splanchnic nerve (12, 13, 17). Interestingly, we found that exposure of neonatal rat AMCs to chronic nicotine suppressed only hypoxia chemosensitivity (6), whereas exposure to μ- and/or δ-opioid agonists led to the suppression of both hypoxia and hypercapnia sensitivity (30). In both instances, the suppression of hypoxia sensitivity was attributable to the increased expression of functional ATP-sensitive K^+ (K_{ATP}) channels, which induce membrane hyperpolarization and decreased excitability during hypoxia (5, 30). On the other hand, the suppression of hypercapnia sensitivity in opioid-treated AMCs was associated with decreased expression of the CO_{2}-hydrating enzymes carbonic anhydrase (CA) I and II (30).

In the present study, we were interested in the intracellular signaling mechanisms leading to the blunting of O_2 and CO_{2} chemosensitivity in opioid-treated chromaffin cells, and therefore focused on factors regulating K_{ATP} channel and CA expression. As a first step, we considered the potential role of the transcription factor hypoxia-inducible factor (HIF)-2α, because: 1) HIF-2α stabilization in nicotine-treated AMCs mediated the transcriptional upregulation of K_{ATP} channel subunit Kir6.2 (28); and 2) the HIF pathway has previously been implicated in the signaling cascade activated in neuroblastoma cells during chronic opioid exposure (8). To facilitate these studies, we used a fetal-derived, immortalized rat chromaffin cell line, i.e., MAH cells, that is sensitive to both hypoxia and hypercapnia (7, 9) and express Kir6.2 and CAII mRNA and protein (5, 27, 28). Moreover, the availability of a stable HIF-2α-deficient (>90% knockeddown) MAH cell line (shMAH) allowed us to investigate the role of HIF-2α in the opioid signaling cascade. Because protein kinases [e.g., protein kinase C (PKC) and calmodulin kinase (CaMK)] were implicated in the upregulation of Kir6.2 in nicotine-treated chromaffin cells (5), we also investigated their potential involvement in opioid
receptor signaling in MAH cells using pharmacological inhibitors. Finally, to validate the predictions of this in vitro model, we used a physiologically relevant in vivo model where pregnant dams received daily injections of morphine just before and throughout gestation. The expression patterns of HIF-2α, Kir6.2, and CAI and -II were then compared in chromaffin tissues from the adrenal glands of neonates born to morphine-vs. saline-exposed dams. This in vivo model was of additional interest because the use of opiates during pregnancy whether for drug abuse (e.g., heroin) or replacement therapy (e.g., methadone and naltrexone) has been linked to a number of negative pregnancy outcomes associated with increased fetal and neonatal mortality, as well as the incidence of sudden infant death syndrome (SIDS) (4).

MATERIALS AND METHODS

Cell Culture

v-myc immortalized chromaffin cells. The v-myc immortalized rat chromaffin cell line (MAH) was grown in L-15/CO2 medium containing 0.6% glucose, 1% penicillin/streptomycin, 10% fetal bovine serum, and 5 μM dexamethasone, as previously described (9). A stable HIF-2α-deficient MAH cell line (shMAH), generated using interference RNAi techniques (2), was used in some experiments and grown under similar conditions. All cultures were incubated in a humidified atmosphere of 95% air-5% CO2 at 37°C for varying periods up to ~7 days in vitro. Cells were fed every 1–2 days and routinely passaged every 3–4 days when cell density reached ~70% confluency. When passing cells, medium was removed, and cells were detached using 0.25% trypsin-EDTA. Suspended cells were pelleted by centrifugation, and the pellet was resuspended in pre-warmed medium. Cells were then plated on 35-mm culture dishes coated with poly-D-lysine and laminin.

Adrenal Gland Tissues

All animal experiments were approved by the Animal Research Ethics Board at McMaster University, in accordance with the guidelines of the Canadian Council for Animal Care. Nulliparous 200- to 250-g female Wistar rats (Harlan, Indianapolis, IN) were maintained in a standard protocol provided by a ChIP assay kit (Millipore) as previously described (28); Briefly, MAH cells were plated on 100-mm dishes (Corning) at a confluency of ~0.75 × 10^6 cells/dish and treated with opioid agonists for 7 days in culture. Following chronic treatments, the cultures were treated with 1% formaldehyde for 10 min at 37°C to cross-link histones to DNA. Medium was removed, and cells were washed with ice-cold PBS (pH 7.4) containing protease inhibitors, scraped, and pelleted at 2,000 rpm for 4 min at 4°C. The pellet was then resuspended in 200 μl SDS lysis buffer. Cross-linked DNA was sheared and pelleted at 13,000 rpm for 10 min at 4°C. The lysate supernatant was diluted 10X using the ChIP

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<th>Table 1. Effects of chronic morphine exposure on pregnancy outcomes</th>
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<td><strong>Pregnancy Outcomes</strong></td>
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<td>Proportion of SGA pups, %</td>
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<td>Proportion of dams delivering stillborns, %</td>
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Values are means ± SE; n, no. of animals. SGA, small for gestational age; F, female; M, male. *P < 0.05.

Quantitative Real-Time PCR

Quantitative real-time PCR (QPCR) analysis was performed using the Stratagene (Mx3000p) detection system and Absolute QPCR SYBR Green Mix. Primers were designed using Gene Fisher, 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, and 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 quantitatively using the arithmetic equation 2^(-ΔΔCt) (20); mRNA levels were normalized to Lamin A/C expression and expressed as transcript fold change relative to mRNA from untreated control MAH cells. Each experiment was repeated three to four times. Primers used were as follows: Kir6.2 subunit, forward: 5′-ACA AGA ACA TCC GAG AGC A-3′, reverse: 5′-CTG CAC CAT GAT CAA AAG GA-3′ (accession no. NM_008428); CAIL, forward: 5′-CCG ACA TCC TTC TGT GGA-3′, reverse: 5′-GGG CAG TGG TCA GAG AGC CA-3′ (accession no. NM_010602); vascular endothelial growth factor (VEGF), forward: 5′-ATG TGA AGC CCC CTG GAG G-3′, reverse: 5′-AAT GCT TCC CTT TGC CTA CAA-3′ (14); and lamin, forward: 5′-GAC TCT ACA AGA GAG AAG GCT-3′, reverse: 5′-GAA CTG AAT CCT CTG GAA-3′ (accession no. NM_008084).

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) assay was performed using a standard protocol provided by a ChIP assay kit (Millipore) as previously described (2, 28). Briefly, MAH cells were plated on 100-mm dishes (Corning) at a confluency of ~0.75 × 10^6 cells/dish and treated with opioid agonists for 7 days in culture. Following chronic treatments, the cultures were treated with 1% formaldehyde for 10 min at 37°C to cross-link histones to DNA. Medium was removed, and cells were washed with ice-cold PBS (pH 7.4) containing protease inhibitors, scraped, and pelleted at 2,000 rpm for 4 min at 4°C. The pellet was then resuspended in 200 μl SDS lysis buffer. Cross-linked DNA was sheared and pelleted at 13,000 rpm for 10 min at 4°C. The lysate supernatant was diluted 10X using the ChIP
dilution buffer, and a small portion (1%) was kept for DNA quantification and used as an input control. For immunoprecipitation, lysates were precleared by the addition of protein A agarose/salmon sperm DNA (50% slurry) and maintained under constant agitation for 30 min at 4°C. Samples were incubated with rabbit polyclonal antibody against HIF-2α (Novus Biologicals) overnight at 4°C with constant rotation. For negative controls, the antibody was omitted from the samples. Following immunoprecipitation, agarose beads were pelleted and washed, and the DNA was eluted and reverse cross-linked by adding 5 M NaCl and heating at 65°C for 4 h. DNA was recovered by phenol/chloroform extraction and ethanol precipitation. PCR analysis was used to detect HIF-2α binding using primers specific for a putative hypoxia-responsive element (HRE) present in KCNJ11 (Kir6.2 subunit) sequence (forward: 5′-CGG ACT CTC AGA GCA GTG TA-3′; and reverse 5′-GCA GAC TCT GAC AGT GCC TTT-3′). PCR products were sequenced, and the sequencing results were further matched to Kir6.2 gene by a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Western Blot

MAH cell cultures and adrenal gland tissues were lysed so as to obtain cytoplasmic and nuclear fractions as previously described (3). Protein samples were boiled at 95–100°C for 5 min. Total protein concentration was determined using Bradford assay (1:5 dilution reagent and 1 mg/ml BSA). Samples were loaded and resolved on 10% SDS-PAGE and transferred onto PVDC membranes. Membranes were then washed and incubated with either primary rabbit polyclonal antibody against Kir6.2 (catalog no. APC-020; 1:1,000 dilution; Alomone Labs, Jerusalem, Israel), rabbit polyclonal anti-human CAI antibody (catalog no. Ab112522; 1:1,000 dilution; Abcam, Cambridge, MA), sheep polyclonal anti-human CAII antibody (catalog no. AHP 206; 1:1,000 dilution; AbD Serotec, Kidlington, UK), HIF-1α mouse monoclonal antibody (catalog no. NB 100–105; 1:1,000 dilution; Novus Biologicals, Littleton, CO), or HIF-2α rabbit polyclonal antibody (catalog no. NB 100–122; 1:1,000 dilution; Novus Biologicals), primary rabbit monoclonal β-actin antibody as loading control for cytoplasmic extracts (1:10,000 dilution; Millipore, Billerica, MA), or primary rabbit polyclonal TATA-binding protein antibody as a loading control for nuclear extracts (1:25,000 dilution; Santa Cruz) at 4°C overnight. Membranes were then washed in PBS and incubated in a goat anti-rabbit horseradish peroxidase-linked secondary antibody (1:10,000 dilution; Jackson Labs, Bar Harbor, ME) for 1 h at room temperature. Immunoreactions were visualized using ECL and exposed to XAR film.

Detection of Dopamine and Norepinephrine Release by ELISA

To determine dopamine (DA) and norepinephrine (NE) basal release, medium was removed from the cultures and replaced with 500 μl of extracellular (normoxic) solution containing (in mM): 110 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 glucose, 12 sucrose, and 24 NaHCO3, bubbled with 5% CO2 at pH 7.4, for 15 min. For hypoxic stimulation, the extracellular solution was replaced with an equal volume of the same solution previously bubbled with N2 gas, and cells were then placed in a hypoxic incubator (2% O2; 5% CO2) for 15 min at 37°C. For isohydric hypercapnia exposure (10% CO2; pH 7.4), the extracellular bicarbonate concentration was increased to 48 mM (equimolar substitution with NaCl) so as to maintain the pH at 7.4, and cells were placed in an incubator set at 10% CO2 for 15 min at 37°C. Following hypoxic and hypercapnic stimulation, the extracellular medium was collected for determination of DA and NE release. In addition, DA and NE stores were determined after removal of the “release” medium as follows. MAH cells were gently washed with 1× PBS and lysed in 0.1 N HCl. Cells were then sonicated at 50% power with two sets of 10-s pulses. Release and store samples were then analyzed for DA and NE content using ELISA (Rocky Mountain Diagnostics) as outlined in the manufacturer’s protocol.

Drugs

All drugs were purchased from Sigma-Aldrich (St. Louis, MO), except morphine sulfate, which was purchased from Medisca Pharmaceutique. In cell culture studies, fresh drugs were added to the growth medium every 2 days. For chronic opioid treatments, a combination of the μ-opioid agonist [d-Arg2,Leu5]dermorphin-(1–4)-amide (DALDA) and the δ-opioid agonist [d-Pen2,5,P-CI-Phe8] encephalin (DPPDE) was used at a concentration of 2 μM each. In some experiments, naloxone hydrochloride dehydrate (2 μM) was used as a general opioid antagonist and added at the same time as the agonists. Protein kinase inhibitors used in this study include: 2 μM of H-89 [protein kinase A (PKA) inhibitor], 2 μM of GF-109203X (PKC inhibitor), and 3 μM of KN-62 (CaMK inhibitor).

Data Analysis

Statistical analyses were performed using GraphPad Prism (version 4.0; GraphPad). Molecular data were normalized to loading control, and results were compared using one-way ANOVA followed by Tukey’s post hoc multiple-comparison test and expressed as means ± SE. For pregnancy outcomes, statistical analyses were performed using Student’s t-test (SigmaStat, version 2.03; SPSS, Chicago, IL) by comparing the morphine-injected group with the control saline-injected group. Fisher’s exact test (n = 0.05) was used when categorical variables were compared. Littermates were used as an experimental unit, and values are presented as means ± SE. Asterisk indicates P < 0.05.

RESULTS

In the in vitro experiments reported below, all opioid exposures were performed on immortalized chromaffin (MAH) cell cultures that were incubated with combined μ- and δ-opioid agonists, i.e., DALDA and DPPDE, respectively, at a concentration of 2 μM each. The duration of the exposure period varied from 24 h to 7 days as indicated in the text. Similar to primary neonatal rat AMCs (30), MAH cells express both μ- and δ-opioid receptors as exemplified in the immunocytochemical experiments shown in Fig. 1 (n = 3). In addition, microarray data from our laboratory suggest that MAH cells express μ- and δ-opioid, but not κ-opioid, receptors (data not shown). Thus, MAH cells represent a simple surrogate model to study signaling pathways activated by μ- and δ-opioid receptors in chromaffin cells.

Effects of Chronic Opioid Exposure on KATP Channel Expression in MAH Cells

In our recent study, chronic exposure of primary neonatal rat AMCs to combined μ- (DALDA)- and δ (DPPDE)-opioid agonists (2 μM) for ~7 days in vitro caused an increased expression of the KATP channel subunit, Kir6.2, as determined by Western blot analysis (30). Using both QPCR and Western blotting, we first investigated whether upregulation of Kir6.2 subunit also occurs in MAH cells exposed for ~7 days to medium containing combined opioids. As illustrated in Fig. 2A, the transcript level of Kir6.2 subunit was significantly increased (>2-fold) in opioid-treated MAH cells compared with untreated controls. Moreover, this enhanced Kir6.2 expression was mediated via opioid receptor signaling pathways because it was prevented in MAH cells exposed to combined opioids plus the general opioid receptor blocker naloxone (2 μM; Fig. 2A). Western blot analysis also confirmed the upregulation of Kir6.2 expression at the protein level in opioid-treated MAH.
In other cell types, e.g., rat AMCs and immortalized MAH cells, chronic nicotine exposure caused stabilization of the transcription factor HIF-2α (8). It was therefore of interest to investigate whether or not the opioid-mediated upregulation of Kir6.2 subunit in MAH cells was HIF-2α dependent. To test this, we used Western blots to compare Kir6.2 expression in a HIF-2α-deficient (shHIF-2α MAH) cell line to wt MAH cells. As illustrated in Fig. 3A, there was a robust accumulation of Kir6.2 in MAH cells exposed to chronic opioids for ~7 days in vitro, and this effect was abolished during coincubation with the opioid receptor blocker naltrexone (2 μM).

**Time-Dependent Effects of Chronic Opioids on HIF-2α Accumulation and Kir6.2 Subunit Expression in MAH Cells**

In a previous study, we found that chronic nicotine exposure caused a parallel, progressive, and time-dependent accumulation of HIF-2α and Kir6.2 subunit expression in MAH cells (28). To test whether a similar pattern occurs during opioid exposure, we monitored HIF-2α and Kir6.2 subunit expression in opioid-treated MAH cells at 0 h, 24 h, 3 days, and 7 days in nuclear and cytoplasmic extracts. As illustrated in Fig. 3, B and C, Western blot analysis revealed a slow progressive increase in HIF-2α accumulation that occurred in parallel with the increase in Kir6.2 subunit over the 7-day treatment period. Both increases were significant at exposure periods of 3 and 7 days but not at 24 h (Fig. 3C); by contrast, exposure of MAH cells to chronic hypoxia (2% O2) normally results in a robust HIF-2α accumulation at 24 h (data not shown) (3, 28). This expression pattern of HIF-2α and Kir6.2 subunit is reminiscent of that seen in MAH cells during exposure to chronic nicotine over a similar time period (28).

**HIF-2α Binds to the Promoter Region of Kir6.2 Gene in Opioid-Treated Cells**

The promoter region of the Kir6.2 gene in both rats and mice contains a putative HRE, where the HIF core site (GCCGTG) spans nucleotides ~1087 to ~1083 and the HIF ancillary site (CACAG) spans nucleotides ~1065 to ~1061 (Fig. 4A, top). Using a ChIP assay, we previously demonstrated that HIF-2α bound to the promoter region of Kir6.2 gene, following its stabilization in MAH cells exposed to chronic nicotine (28). To test whether opioid receptor signaling promotes binding of HIF-2α to the promoter region of Kir6.2 gene, we performed ChIP assays on control and HIF-2α-deficient MAH cells exposed to chronic opioids for ~7 days. Indeed, we found that, under these conditions, HIF-2α bound to the Kir6.2 promoter region in wt and ScCont MAH cells but not in shHIF-2α MAH cells, as illustrated in Fig. 4A, bottom. These data complement those demonstrating that HIF-2α deficiency prevents upregulation of Kir6.2 in opioid-treated MAH cells (Fig. 2, C and D) and imply that the mechanism of HIF-2α action most likely involves increased expression of Kir6.2 at the transcriptional level.

To confirm transcriptional activity of HIF-2α in opioid-treated MAH cells, we measured mRNA expression levels of a common HIF-2α target gene, VEGF, using QPCR analysis.
Data indicate that VEGF mRNA is significantly increased in opioid-treated MAH cells (Fig. 4B).

**Chronic Opioid Exposure Downregulates the Expression of CAII in MAH Cells: Role of HIF-2α**

Sensitivity of neonatal rat AMCs to high CO₂ (hypercapnia) is dependent on the activity of the CO₂-hydrating enzymes CAI and -II (23). We previously showed that exposure of primary CAII in MAH Cells: Role of HIF-2α

Sensitivity of neonatal rat AMCs to high CO₂ (hypercapnia) is dependent on the activity of the CO₂-hydrating enzymes CAI and -II (23). We previously showed that exposure of primary neonatal rat AMC to chronic opioids blunts hypercapnia sensitivity, in association with the downregulation of CAI and CAII (30). Although MAH cells express only CAII, they show CO₂ sensitivity similar to primary neonatal AMC (7), thereby providing a model for investigating opioid-mediated blunting of CO₂ sensing. Indeed, exposure of MAH cells to chronic opioids for ~7 days resulted in a significant reduction in CAII transcript as determined by QPCR; furthermore, this reduction was prevented during continuous co-incubation with the opioid receptor blocker naloxone (2 μM; Fig. 5A). Western blot analysis revealed that chronic opioids also caused a naloxone-sensitive downregulation of CAII at the protein level, as illustrated in Fig. 5B.

Because chronic opioids stabilize HIF-2α in MAH cells (Fig. 3), we wondered whether or not the opioid-mediated downregulation of CAII expression was HIF-2α dependent. To test this, we used Western blot analysis to compare CAII expression in shHIF-2α MAH cells following exposure to chronic opioids for ~7 days. As shown in Fig. 6, A and B, the opioid-mediated downregulation of CAII relative to β-actin was present in wt MAH and transected ScCont MAH cells but was absent in shHIF-2α MAH cells. These results demonstrate that HIF-2α is required for the opioid-mediated downregulation of CAII in MAH cells.

**Central Role of PKA in Mediating the Downstream Effects of Opioid Receptor Signaling in MAH Cells**

Various protein kinases are known to be activated during opioid receptor signaling (10). We therefore used pharmacological inhibitors to probe for the potential involvement of three common kinases, i.e., PKC, CaMK, and PKA, in mediating the opposing effects of chronic opioid exposure on Kir6.2 and CAII expression in MAH cells. As illustrated in Fig. 7A, Western blot analysis revealed that inhibition of PKA by H-89 (2 μM) prevented the opioid-induced upregulation of Kir6.2 evident in MAH cells following chronic opioid exposure. By contrast, inhibition of PKC and CaMK by GF-109203X (2 μM) and KN-62 (3 μM), respectively, had no effect on the upregulation of Kir6.2 protein in comparable experiments (Fig. 7A). Likewise PKA, but not PKC or CaMK, inhibition prevented the opioid-induced downregulation of CAII evident in MAH cells following chronic opioid exposure (Fig. 7B). These data suggest a central role of PKA in the opioid receptor signaling pathway, leading to the regulation of key proteins.
involved in the blunting of O₂ and CO₂ chemosensitivity in chromaffin cells.

Given that both PKA activity (Fig. 7, A and B) and HIF-2α accumulation (Figs. 2 and 6) are necessary for the regulation of Kir6.2 and CAII in opioid-treated MAH cells, the question arises whether HIF-2α accumulation is dependent on PKA activity. To address this, we investigated whether the opioid-induced HIF-2α accumulation could occur in the presence of PKA inhibition. As illustrated in Fig. 7C, inhibition of PKA by H-89 did not prevent the opioid-induced HIF-2α accumulation in MAH cells. Furthermore, inhibition of PKC by KN-62 and CaMK by GF-109203X was similarly ineffective (Fig. 7C). Taken together, these data suggest that the regulation of Kir6.2 and CAII in opioid-treated MAH cells requires the separate actions of PKA and HIF-2α.

Chronic Opioids Blunt Hypoxia and Hypercapnia Chemosensing in MAH Cells as Monitored by DA and NE Release

In regards to CAT biosynthesis, MAH cells synthesize predominantly DA and NE because they lack expression of the epinephrine-producing enzyme phenylethanolamine N-methyl-

![Fig. 3. Effects of chronic opioid exposure on Kir6.2 and HIF-2α expression in MAH cells.](image)

![Fig. 4. Chromatin immunoprecipitation (ChIP) assay demonstrating binding of HIF-2α to the promoter region of Kir6.2 gene in opioid-treated MAH cells.](image)
Effects of Chronic Maternal Morphine Exposure on Kir6.2, CAI, CAII, and HIF-2α Expression in the Adrenal Glands of Affected Neonates

To validate the main conclusions from our in vitro model in a physiologically relevant in vivo system, we administered morphine or saline (control) to pregnant Wistar rats throughout gestation. We then compared the expression pattern of Kir6.2, CAI, CAII, and HIF-1α, and HIF-2α in separate “enriched” tissue fractions of the AM and AC, obtained from the adrenal glands of the offspring at birth. Before tissue collection, it was evident that morphine administration had an adverse effect on the well-being and viability of the offspring, as revealed by measures of several parameters indicated in Table 1. As illustrated in Fig. 9, A–C, Western blot analysis revealed there was a significant increase in expression of Kir6.2 and HIF-2α, and a decrease in expression of CAI and CAII, in the medullary-rich regions of glands taken from morphine-exposed pups relative to saline-exposed controls; by contrast, expression of HIF-1α was unchanged. Interestingly, this change in expression pattern for Kir6.2, HIF-2α, and CAII was similar to that seen in opioid-treated MAH cells in vitro. On the other hand, the changes in expression pattern seen in the medullary region were not replicated in cortical-rich regions of glands. Notably, CAI and CAII expression was robust in the medullary-rich regions of glands taken from morphine-exposed pups relative to saline-exposed controls; by contrast, expression of HIF-1α was unchanged. Interestingly, this change in expression pattern for Kir6.2, HIF-2α, and CAII was similar to that seen in opioid-treated MAH cells in vitro. On the other hand, the changes in expression pattern seen in the medullary region were not replicated in cortical-rich regions of glands. Notably, CAI and CAII expression was robust in the medullary-rich regions of glands taken from morphine-exposed pups relative to saline-exposed controls; by contrast, expression of HIF-1α was unchanged. 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to the medullary chromaffin cells, which are the predominant cell type expressing opioid receptors in the adrenal gland (30).

**DISCUSSION**

In a recent study, we reported that chronic exposure of neonatal rat AMCs to μ- and δ-opioid receptor agonists in vitro blunts hypoxia and hypercapnia chemosensitivity via upregulation of K\textsubscript{ATP} channels and downregulation of CAI and CAII, respectively (30). The goals of the present study were to obtain a more complete understanding of the underlying molecular mechanisms and signaling pathways using an in vitro model, and to validate key aspects of these molecular mechanisms in a relevant in vivo model. Using an immortalized, fetal-derived rat chromaffin (MAH) cell line, we found that chronic opioid exposure led to increased expression of Kir6.2 subunit of the K\textsubscript{ATP} channel, and decreased expression of CAII, the only one

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Fig. 7. Effects of protein kinase inhibitors on expression of Kir6.2, CAII, and HIF-2α in MAH cells following chronic opioid exposure. **A**: Western blot analysis showing the effects of protein kinase inhibition on Kir6.2 expression in opioid-treated MAH cells. Inhibitors of protein kinase A (PKA) (H-89) (2 μM), but not inhibitors of protein kinase C (GF-109203X) (2 μM) or calmodulin (CaM) kinase (KN-62) (3 μM), prevented the upregulation of Kir6.2 in opioid-treated cells. Densitometric analysis of Kir6.2 subunit expression normalized to β-actin is shown in histogram (right). A similar protocol was used to study the effects of protein kinase inhibitors on CAII expression relative to β-actin (**B**), and HIF-2α accumulation relative to TBP (**C**), in opioid-treated MAH cells. Note the PKA inhibitor (H-89) prevented the opioid-induced downregulation of CAII expression, but not HIF-2α accumulation, in MAH cells. Data are expressed as means ± SE for three independent experiments for each group, *P < 0.05.
of the CA isoforms expressed in MAH cells. Both effects were mediated via PKA, and were dependent on the transcription factor HIF-2α, because they were abrogated in HIF-2α-deficient MAH cells. Strong support for this schema was obtained from an in vivo model where adrenal tissue from pups born to morphine-exposed dams was probed for Kir6.2, CAI, CAII, and HIF-2α expression. Interestingly, compared with saline-treated controls, adrenal tissue from morphine-exposed pups showed increased expression of Kir6.2 and HIF-2α and decreased expression of both CAI and CAII.

Fig. 8. Effects of chronic opioids on hypoxia- and hypercapnia-evoked dopamine (DA) and norepinephrine (NE) release from MAH cells. ELISA was used to measure DA and NE release, and data were expressed as a ratio of evoked relative to basal release. Chemostimulation with hypoxia (2% O2; 15 min) (A) and isohydric hypercapnia (10% CO2; pH 7.4) (B) caused a significant increase in DA and NE release in control (untreated) MAH cells. Following exposure of MAH cells to chronic opioids for ~1 wk in vitro, the effect of hypoxia and hypercapnia on DA and NE release was significantly reduced. Data are represented as means ± SE, where n = 5 for A and 4 for B (*P < 0.05 and **P < 0.01).

Fig. 9. Effects of maternal morphine injections on the expression of KATP channel subunit (Kir6.2), carbonic anhydrase I (CAI) and CAII enzymes, and HIFs in adrenal gland tissues of affected neonates. Western blot analyses of KATP channel subunit Kir6.2 (A), CAI and -II (B), and HIF-1α and -2α (C) expression in adrenal medulla (AM) and adrenal cortex (AC) of saline- and morphine-exposed rat pups. Note the increased Kir6.2 subunit and reduced CAI and CAII expression (relative to β-actin) in AM, but not AC, of morphine-exposed pups. Also, note the selective increase in HIF-2α, but not HIF-1α, accumulation (relative to TBP) in AM of morphine-exposed pups as shown in C. Data are expressed as means ± SE for three independent experiments for each group, *P < 0.05.
CHRONIC OPIOIDS REGULATE K\textsubscript{ATP} CHANNEL AND CAI AND CAII EXPRESSION

Role of HIF-2\(\alpha\) in Opioid-Induced Regulation of Kir6.2 and CAII Expression

One key factor involved in gene regulation in MAH cells following exposure to chronic \(\mu\)- and \(\delta\)-opioids was the transcription factor HIF-2\(\alpha\). Whereas the opioid-induced regulation of Kir6.2 and CAII was observed in wt and transfected scrambled control MAH cells, it was absent in HIF-2\(\alpha\)-deficient MAH cells. Moreover, following opioid exposure, there was a gradual increase in HIF-2\(\alpha\) accumulation that occurred along a time course similar to the increase in Kir6.2 expression in MAH cells. The importance of HIF-2\(\alpha\) in this signaling pathway was further demonstrated using a ChIP assay, which revealed the direct binding of HIF-2\(\alpha\) to the promoter region of the Kir6.2 gene in opioid-treated MAH cells. These data suggest a molecular explanation for the increased expression of Kir6.2 subunit and increased K\textsubscript{ATP} current density previously reported in primary neonatal rat AMCs exposed to chronic opioids in vitro (28). In the latter study, the increased expression of K\textsubscript{ATP} channels accounted largely for the opioid-mediated blunting of hypoxia sensitivity, which could be restored by simply adding the K\textsubscript{ATP} channel blocker glibenclamide to the extracellular solution. Thus, the ability of chronic opioid exposure to blunt hypoxia sensing in chromaffin cells appears to be dependent on the induction of HIF-2\(\alpha\), leading to the transcriptional upregulation of Kir6.2 and increased expression of functional K\textsubscript{ATP} channels. The opening of these K\textsubscript{ATP} channels in response to a fall in ATP concentration during hypoxia favors membrane hyperpolarization, and therefore a decreased sensitivity to the hypoxic stimulus.

Evidence that a nonhypoxic stimulus such as opioid exposure could lead to HIF induction has been obtained in a previous study on human SH-SY5Y neuroblastoma cells (8). These authors reported that chronic exposure of SH-SY5Y cells to \(\mu\)- and \(\delta\)-opioid receptor agonists led to the induction of HIF-1\(\alpha\) protein and an increase in HIF-1 target gene expression. Whereas these data demonstrated a link between HIF and the biological effects of chronic opioid exposure, there are notable differences when compared with the present study. In particular, HIF-2\(\alpha\) protein (and not HIF-1\(\alpha\)) was the major HIF involved in the regulation of Kir6.2 and CAII in MAH cells, and, moreover, its induction was not apparent at 24 h exposure. By contrast, in SH-SY5Y cells, the induction of HIF-1\(\alpha\) protein was apparent as early as 8 h and peaked at 24 h exposure (8). Although the fate of HIF-1\(\alpha\) in MAH cells is unknown in the present study, it is noteworthy that, in the adrenal tissue of morphine-exposed pups, we found that HIF-2\(\alpha\) was elevated (relative to saline-exposed controls), whereas HIF-1\(\alpha\) remained unchanged. Thus, it still remains plausible that, at earlier time points after opioid exposure, other target genes, specific to HIF-1, may be regulated in chromaffin cells; however, this idea remains to be tested. Alternatively, the opioid-induced regulation of HIFs may occur via the same opioid receptors but in a cell-type-specific manner, depending on the intracellular signaling pathways. In this regard, chronic exposure of different cells to another nonhypoxic stimulus may lead to the differential regulation of HIF-1\(\alpha\) and HIF-2\(\alpha\). For example, activation of nicotinic ACh receptor signaling pathways by exposure to chronic nicotine led to HIF-1\(\alpha\) accumulation in human small cell lung cancers (42). On the other hand, a similar exposure led to HIF-2\(\alpha\) accumulation in MAH cells (28), although there was increased expression of the general HIF target gene, VEGF, in both studies.

Role of PKA in Opioid-Induced Regulation of Kir6.2 Subunit and CAII Expression

Using inhibitors of three common protein kinases, we found that PKA (but not PKC or CaMK) activation was necessary for the opioid-induced regulation of Kir6.2 subunit and CAII expression in MAH cells. Because HIF-2\(\alpha\) induction was also necessary for this regulation, the question arose whether the requirement for PKA activation occurred upstream or downstream of HIF-2\(\alpha\) induction. Our results showed that PKA inhibition did not significantly affect HIF-2\(\alpha\) accumulation in opioid-treated MAH cells, suggesting that PKA activation was required downstream of (or in parallel with) HIF-2\(\alpha\) accumulation. It remains to be determined whether PKA activation during opioid-induced signaling is involved in the transcriptional or posttranslational modification of Kir6.2 subunit and CAII expression. In a recent report, activation of \(\mu\)- and \(\delta\)-opioid receptors in SH-SY5Y neuroblastoma cells led to HIF-1\(\alpha\) accumulation via the phosphatidylinositol 3-kinase/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling pathway (8). It is tempting to speculate that at least part of the opioid receptor signaling in MAH cells is mediated via activation of this pathway, given the evidence that HIF-2\(\alpha\) expression may also be regulated via a distinct mTOR complex and Akt isoform, as shown in renal carcinoma cells (35). However, further experiments are required to test this idea.

Effects of Chronic Morphine In Utero on Expression of Kir6.2, CAII, and HIF-2\(\alpha\) in AM vs. AC of Affected Neonates

It was of interest to test the predictions of the in vitro opioid-treated MAH cell model in a physiologically relevant in...
vivo system. To this end, both medullary and cortical tissues from the adrenal glands of rat pups exposed to chronic morphine in utero were examined for the expression pattern of Kir6.2, CAI and -II, HIF-2α, and HIF-1α. The rat adrenal gland expresses all three types of opioid receptors, and these are restricted to the chromaffin cells of the medulla (30, 37). Interestingly, even though Kir6.2 is expressed in both AC and AM (5, 39), there was a selective upregulation of Kir6.2 protein in the medullary region of morphine-exposed pups compared with saline-exposed controls. This is the expected result if an intact opioid receptor signaling pathway is required for Kir6.2 upregulation and argues against a nonspecific action of morphine following injections in vivo. Moreover, even though the MAH cell model allowed us to test only for the regulation of CAII following opioid exposure, morphine-exposed pups showed downregulation of both CAI and CAII expression. Not surprisingly, this downregulation was again confined to the medullary tissue, the only region of the gland where the two CAs are known to be expressed (23).

Similar to Kir6.2 subunit expression, we detected HIF-2α protein in both medullary and cortical tissues of the adrenal gland. However, chronic morphine exposure led to a selective accumulation of HIF-2α in the medullary tissue only. This result not only validated our in vitro MAH cell model but, once again, emphasized the requirement for an intact opioid receptor signaling pathway for HIF-2α induction, given that the cortical cells do not express µ- and δ-opioid receptors (30). Additionally, the effects of chronic morphine on HIF accumulation were restricted to HIF-2α, for though HIF-1α was expressed in the medulla, its levels were not significantly affected. Generally, the importance of HIF-2α function is thought to be tissue specific, and HIF-2α appears to play a major role in cells of the sympathoadrenal lineage (1–3). Although the two transcription factors are paralogs, they seem to play distinct target-specific roles even when expressed in the same tissue. In fact, in studies using partially deficient HIF-1α and HIF-2α mice, a mutual antagonism between HIF-1α and HIF-2α was found to regulate intracellular redox status and hypoxia sensitivity of carotid body glomus cells and AMCs (40). Using the MAH cell model, we demonstrated that chronic opioids led to HIF-2α accumulation and that HIF-2α deficiency prevented the opioid-induced upregulation of Kir6.2 and downregulation of CAII. These observations suggest that the increased HIF-2α accumulation seen in morphine-exposed neonatal medullary tissue likely led to the transcriptional upregulation of Kir6.2 and downregulation of CAII (and probably CAI as well) in the tissue.

Clinical Significance

While our studies contributed to a general understanding of the role of opioid receptor signaling in the developmental regulation of hypoxia and hypercapnia sensitivity in chromaffin cells, they also have clinical significance. Prenatal exposure of pregnant mothers to opiates (e.g., heroin and related illicit drugs) has been linked to higher rates of stillbirths and SIDS that are often characterized by impaired arousal during asphyxial challenges (4, 16). Indeed, in the present study, we noted that chronic morphine exposure in utero was associated with decreased litter size, and an increase in the percentage of dams delivering stillborns (Table 1), as reported in previous studies (41). In addition, infants born small for gestational age have been proposed to have higher risk of becoming victims to SIDS (11). In this regard, morphine exposure in utero also resulted in a significantly higher proportion of offspring that were growth restricted (i.e., body wt <2 SDs below the average weight of control offspring; Table 1). A recent study reported that use of opiates by pregnant mothers has climbed and that the number of neonates born addicted to painkiller medication (e.g., opioid pain relievers) has tripled in the past decades due to the rise in use and abuse of these drugs (26). Because adrenal CAT release in response to perinatal asphyxia is important for proper arousal, the adverse effects of chronic opioid exposure on the chemosensing properties of chromaffin cells may well contribute to the increased morbidity of drug-exposed neonates. If so, further studies on these chromaffin cells should aid in the development of therapeutic strategies that could benefit the offspring of women who use opiates during pregnancy, whether for pain management or drug abuse.

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DISCLOSURES

The authors declare no conflict of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS

Author contributions: S.S., A.C.H., and C.A.N. conception and design of research; S.S. and A.C.H. performed experiments; S.S. analyzed data; S.S., A.C.H., and C.A.N. interpreted results of experiments; S.S. prepared figures; S.S. and C.A.N. drafted manuscript; S.S., A.C.H., and C.A.N. edited and revised manuscript; S.S., A.C.H., and C.A.N. approved final version of manuscript.

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