Biochemical and Biophysical Research Communications 412 (2011) 466–472

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Chronic hypoxia upregulates adenosine 2a receptor expression in chromaffin cells via hypoxia inducible factor-2α: Role in modulating secretion

Stephen T. Brown, Edison P. Reyes [1], Colin A. Nurse *

Department of Biology, McMaster University, Hamilton, Ontario, Canada L8S 4K1

ARTICLE INFO

Article history:
Received 28 July 2011
Available online 5 August 2011

Keywords:
Hypoxia
HIF-2α
Chromaffin-derived MAH cells
Catecholamines
Adenosine
Amperometry
Intracellular Ca^{2+}

ABSTRACT

Catecholamine (CAT) release from chromaffin tissue plays an essential role in the fetus which develops in a low O_2 environment (hypoxia). To address molecular mechanisms regulating CAT secretion in low O_2, we exposed a fetal chromaffin-derived cell line (MAH cells) to chronic hypoxia (CHox; 2% O_2, 24 h) and assessed gene expression using microarrays, quantitative RT-PCR, and western blot. CHox caused a dramatic ~12× upregulation of adenosine A2a receptor (A2aR) mRNA, an effect critically dependent upon hypoxia-inducible factor (HIF)-2α which bound the promoter of the A2aR gene. In amperometric studies, acute hypoxia and high K^+ (30 mM) evoked quantal CAT secretion that was enhanced after CHox, and further potentiated during simultaneous A2aR activation by adenosine. A2aR activation also enhanced stimulus-induced rise in intracellular Ca^{2+} in control, but not HIF-2α-deficient, MAH cells. Thus, A2aR, adenosine, and HIF-2α are key contributors to the potentiation of CAT secretion in developing chromaffin cells during chronic hypoxia.

© 2011 Elsevier Inc. All rights reserved.

1. Introduction

Chronic low O_2 (hypoxia) is an important physiological stressor *in utero* that is required for proper fetal development [1–3]. In addition, circulating catecholamines (CAT) help mediate fetal survival by maintaining O_2 homeostasis [3]. Thus CAT-deficient mice arising from null mutations in the biosynthetic enzyme tyrosine hydroxylase [4], or the transcription factor EPAS1 (also known as hypoxia-inducible factor-2α; HIF-2α), die around embryonic day (E) 13.5–14.5 [5], because of their failure to produce norepinephrine which critically maintains cardiac function during fetal hypoxia [3]. The induction of HIF transcription factors during fetal hypoxia helps promote hematopoiesis, chondrogenesis, and angiogenesis [6–8], and recent evidence suggests that HIF-2α is also required for the development of other CAT-biosynthetic enzymes [9]. At birth, hypoxia, as a consequence of uterine contractions and neonatal apneas, elicits a critical CAT surge from adrenomedullary chromaffin cells (AMC) that aids in the preparation of the lungs for air breathing and the maintenance of cardiac conductance [10,11]. Thus, from around mid-gestation to the early postnatal period, there is an inter-dependence between O_2 tension and catecholaminergic functions in sympathoadrenal cells that is crucial for survival.

Despite their physiological importance, the cellular and molecular mechanisms regulating catecholaminergic functions in chromaffin tissue during fetal development are poorly understood. To mimic the low PO_2 microenvironment, several investigators have exposed the chromaffin-derived PC12 cell line to chronic hypoxia *in vitro* in order to investigate changes in gene expression [12–15]. However, because this cell line was derived from *adult* rat adrenal medulla, it is arguable whether it truly recapitulates the developmental status of fetal chromaffin cells. Nonetheless in one such study, exposure of PC 12 cells to chronic hypoxia augmented hypoxia-evoked CAT secretion as a result of increased frequency and size of quantal events [14]. On the other hand, exposure of primary *adult* rat AMC cells to chronic hypoxia caused upregulation of α_{1H} T-type Ca^{2+} channels and low-threshold CAT secretion, without alteration in the quantal events [16]. These data contrast with the observation that after several days exposure of neonates to chronic hypoxia, acute hypoxia-evoked CAT secretion is attenuated [17].

In order to preserve the embryonic status, the present study utilized an immortalized chromaffin cell line (MAH cells) derived from *fetal* E14 rat adrenal medulla [18]. Importantly, this line has retained several characteristics of its fetal sympathoadrenal (SA) progenitor, and expresses similar electrophysiological properties to fetal and neonatal primary AMC [19]. Interestingly, we observed a dramatic HIF-2α-dependent upregulation of adenosine 2a receptor (A2aR) expression in MAH cells after chronic hypoxia. We hypothesized that these receptors may participate in stressor-evoked CAT secretion via autocrine or paracrine feedback pathways, and indeed

* Corresponding author. Address: Department of Biology, McMaster University, 1280 Main St. West, Hamilton, Ontario, Canada L8S 4K1. Fax: +1 905 522 6066.
E-mail address: nursec@mcmaster.ca (C.A. Nurse).
[1] Present address: Facultad de Medicina, Clínica Alemana-Universidad del Desarrollo, Santiago, Chile.

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.07.122

found that A2aR activation regulated Ca^{2+} signaling and CAT secretion in these cells, especially after chronic hypoxia.

2. Materials and methods

2.1. Cell culture

Immortalized MAH cells were passaged and grown as previously described [18,20]. HIF-2α knock-down and scrambled control MAH cells were generated and grown in a humidified atmosphere of 95% air-5% CO_2 as previously described [9].

2.2. Microarray protocol

Five micrograms of total RNA were used to prepare biotin-labeled complementary RNA (cRNA) which was hybridized to Rat genome 230 v2.0 arrays by Affymetrix (Santa Clara, CA, USA). One-cycle target labeling assay was used to generate cDNA in a reverse-transcription reaction with SuperScript II (Invitrogen, Carlsbad, CA, USA) enzyme, which was then subsequently purified and *in vitro* transcribed to biotin-labeled RNA using T7 RNA polymerase. Following the IVT reaction, RNA was fragmented and hybridized onto the GeneChips for 16 h at 45 °C. Procedures that involved washing, staining with streptavidin–phycoerythrin (SAPE) and scanning were done in accordance with the WS004 protocol in the Command Console software. After scanning, raw fluorescence data (cel files) were converted to normalized expression indices using the PLIER algorithm in Genespring software (Agilent Technologies, La Jolla, CA, USA). Arrays were done in triplicate and differential expression analysis was done on filtered probes (expression above the average background) using one-way ANOVA with a *p*-value multiple testing correction threshold set to 0.05.

2.3. Western blots

Nuclear protein was extracted from cells as previously described [9,20], and total cell lysate extracted using RIPA buffer (Tris 50 mM; NaCl 150 mM; SDS 0.1%; Sodium Deoxycholate 0.5%; Triton X 100 1% and protease inhibitors). Protein was run on 8% SDS polyacrylamide gel and transferred to PVDF membrane. Blots were incubated in either 1:1000 dilution of HIF-2α rabbit polyclonal antibody (Novus Biologicals, Littleton, CO), 1:2000 dilution of Tata-Binding-Protein rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), 1:1000 adenosine A2aR antibody (Millipore, USA) at 4 °C overnight. Western blots were performed at least 3 times and a representative blot is shown in the figs.

2.4. Reverse transcription

RNA from MAH cell cultures was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. RNA was quantified in an Eppendorf Biophotometer and 500 ng were treated with DNase I (Invitrogen, Carlsbad, CA, USA) to remove any contaminating DNA. Reverse transcription was carried out on 100 ng of DNase- treated RNA using Superscript III (Invitrogen) and random primers (100 ng). A no RT control was also run to test for the presence of DNA contamination (data not shown).

2.5. Quantitative PCR

QPCR was carried out using the Absolute QPCR SYBR Green Mix (Abgene, Epson, UK) and a Stratagene MX3000P (La Jolla, CA, USA) machine. Analysis was done using the Stratagene MX3000p software using the ΔΔCT method. Gene-specific primers were designed using GeneFisher [21], and synthesized by a local facility (MOBIX, McMaster University). The following primers were used and listed as gene amplified, sequence (forward, reverse), and annealing temperature: Lamin A/C: 5′-GCAGTACAAGAAGGAGCTA-3′ and 5′-CAGCAATTCCTGGTACTCA-3′, 55 °C; A2aR: 5′-TAGTTAGCCCTCCCAGGGACA-3′ and 5′-TGTACACCGAGGAGCCCATG-3′, 55 °C. PCR products were sequenced (at MOBIX) using an ABI Prism automated Sequencer (with T7 polymerase). The sequencing results were analyzed by BLAST and the sequences were matched to the Rattus norvegicus Lamin (GenBank accession number NM_001002016) and A2aR (GenBank accession number NM_053294.3).

2.6. Chromatin immunoprecipitation (ChIP) assay

MAH cells were plated into 100 mm dishes and grown to 75% confluence before addition of 1% formaldehyde to the medium for 10 min at 37 °C. ChIP protocol was followed as previously described [9]. PCR was performed with primers that amplify a region in the adenosine A2a receptor promoter containing the putative hypoxia response elements (HRE); forward [5′-AGG AGT CAC GGT TTC ACA GGC-3′] and reverse [5′-TCC ACA AGG CAT CAG GC TG-3′]) or downstream site (∼4 kb downstream); forward [5′-CTC TCT TCC ATC CAC CGT TGC-3′] and reverse [5′-ATG CCA AAC ACG GAA CCC A-3′] by standard PCR.

2.7. Confocal immunofluorescence

Procedures for immunofluorescence labelling of MAH cells were similar to those described in previous studies from this laboratory [19,22]. The primary antibodies were: anti-tyrosine hydroxylase (1:1000; Millipore, Bellerica, MA, USA) and anti-adenosine A2a receptor (1:400; Millipore). Secondary antibody, conjugated with FITC or Texas red (Jackson Labs, Bar Harbour, ME, USA), was diluted in phosphate – buffered saline (1:50) and incubated for 1 h at room temperature. Positive immunofluorescence was visualized using a Zeiss Laser Scanning (Confocal) System LSM 510 equipped with argon (488 nm) and helium–neon lasers (543, 633 nm). Confocal data acquisition, digital image manipulation and presentation were performed using Zeiss LSM Image Browser and ImageJ (version 1.421, NIH, USA), together with Adobe Photoshop CS3 Extended (version 10.0.1) and Adobe Illustrator CS3 (13.0.1).

2.8. Carbon fiber amperometry

Catecholamine secretion from MAH cells was monitored using carbon fiber amperometry as previously described [22]. The culture was perfused under gravity with Hepes-buffered extracellular solution containing (mM): NaCl, 135; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 2; glucose, 10; and HEPES, 10 (pH 7.4) at 37 °C. High K^+ (30 mM) solutions were used after equimolar substitution for NaCl. Solutions were made hypoxic (PO_2 ∼20 mm Hg) by bubbling with N_2 gas. Data acquisition and analysis were carried out as previously described (Buttigieg et al. 2008b). Quantal events smaller than 2.58 x S.D. of the mean base line noise were excluded from the analysis (99% C.I.), and spike frequency was calculated as the number of spike events/min.

2.9. Intracellular Ca^{2+} measurements

Intracellular [Ca^{2+}] was monitored in MAH cells using fura-2 fluorescence. Experimental procedures, including data acquisition and analysis, were identical to those described for these cells in previous studies from this laboratory [22,23].

2.10. Statistical analysis

Results were expressed as mean ± SEM, and statistical comparisons were done using ANOVA and nonparametric tests (Mann–Whitney U) as appropriate.

3. Results

3.1. Chronic hypoxia upregulates adenosine A2a receptor expression via HIF-2α

As we reported previously [20], exposure to chronic hypoxia (Chox; 2% O_2, 24 h) caused a robust induction of HIF-2α protein in *wild type* (wt) and scrambled control (scCont) MAH cells, but not in a stable MAH cell line bearing >90% knockdown of HIF-2α mRNA (shHIF2α) (Fig. 1A). As a first step towards identifying potential genes regulated by CHox in a HIF-2α-dependent manner we used DNA microarrays (Affymetrix Rat 230 v2.0 whole genome array) on samples obtained from wt and shHIF2α MAH cells. From the array, the upregulation of the adenosine A2a receptor (A2aR) mRNA by CHox was robust and, unexpectedly, this was critically dependent on HIF-2α. The microarray and quantitative RT-PCR (QPCR) data indicated that after exposure to CHox A2aR mRNA was upregulated ~13 and ~9.5fold respectively in *wt*MAH cells (Table 1; Fig. 1B). These data are consistent with previous studies on PC12 cells showing increased expression of A2aR during CHox [13]. However, in contrast to the latter study, where no direct role of HIF was found, the upregulation of A2aR mRNA and protein during CHox was completely abolished in HIF-2α -deficient (shHIF2α) MAH cells (Table 1; Fig. 1B and C). In scrambled control experiments, scContMAH cells showed the expected upregulation of A2aR mRNA and protein after CHox (Fig. 1B and C). To confirm at the protein level that A2aR is expressed at the membrane we used immunocytochemistry. As illustrated in Fig. 1D, positive (and sometimes punctate) A2aR immunofluorescence could be demonstrated with confocal microscopy in tyrosine hydroxylase (TH)-positive MAH cells.

3.2. HIF-2α interaction with adenosine A2a receptor promoter

Because the upregulation of A2aR mRNA in MAH cells during CHox was dependent on HIF-2α, the promoter region of the A2a receptor gene (*adora2a*) was examined for possible HIF-2α binding sites or hypoxia response elements (HREs). The promoter region of *adora2a* contains a putative HRE ~34 bp upstream of the start of exon 1 (**GCGTG**GACTTGAAGCGAC**CACGT**) which is conserved in both the rat and mouse sequence [24] (Fig. 2A). To test whether or not HIF-2α binds to this region of the *adora2a* promoter in intact cells, chromatin immunoprecipitation (ChIP) assays were done on shHIF2α and scControl MAH cells exposed to normoxia (Nox), CHox, and desferrioxamine (DFX), an iron chelator that induces HIF-2α under normoxia [20]. DNA was purified from the immunoprecipitate complex probed with HIF-2α antibodies, and PCR was performed using primers specific for the promoter region and a downstream region (exon 2) of the *adora2a* gene. As illustrated in Fig. 2B, PCR of the promoter region yielded positive bands in scCont MAH cells exposed to CHox or DFX (100 μM). On the other hand, no positive bands were detected in HIF-2α -deficient (shHIF2α) MAH cells regardless of whether they were exposed to CHox or DFX (Fig. 2B). In control experiments, the primers used

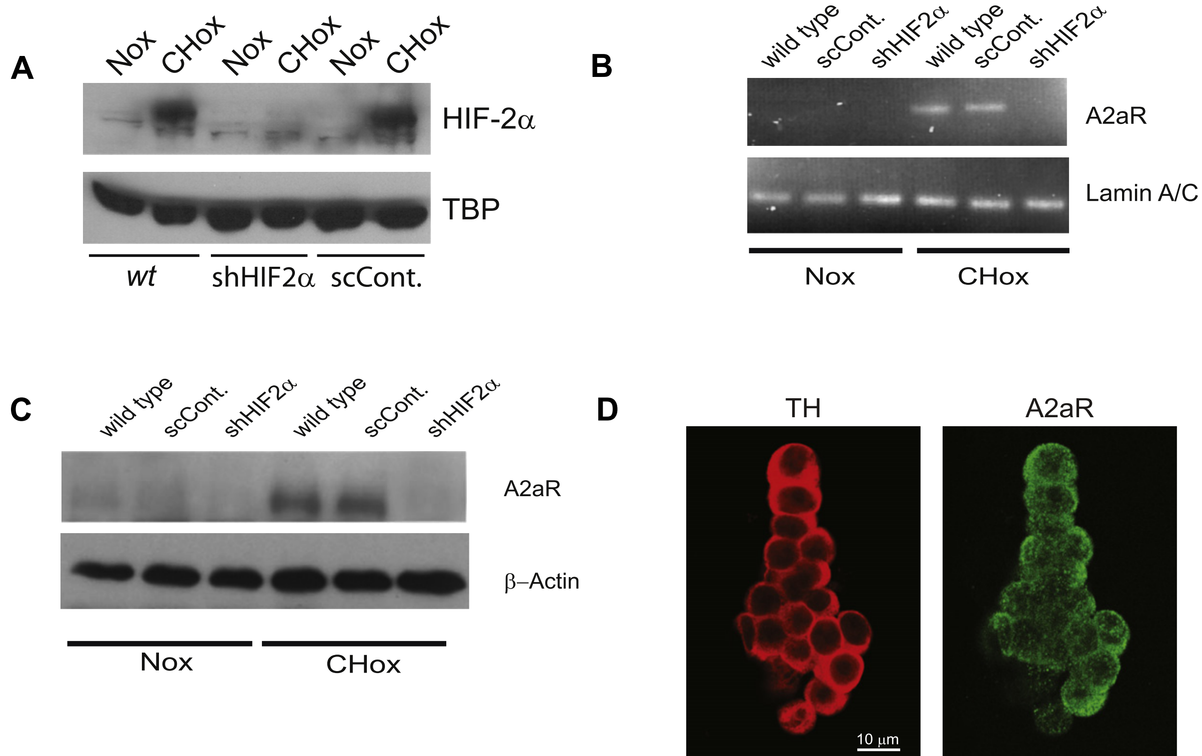

Fig. 1. Expression of HIF-2α and adenosine 2a receptor (A2aR) in control and mutant chromaffin cells. (A) Western blot on nuclear protein for HIF-2α (top panel) in wtMAH, scCont and shHIF2α MAH cells grown under normoxia (Nox; 21% O_2) and chronic hypoxia (CHox; 2% O_2, 4 h), with TBP as internal loading control (bottom panel). (B) RT-PCR on RNA from wtMAH, scCont, and shHIF2α MAH cells grown under Nox (21% O_2) or CHox (2% O_2, 24 h). Primers were specific for the 5′UTR of the A2aR mRNA (top panel); Lamin A/C was used as an internal control (bottom panel). (C) Western blot on total protein for A2aR (top panel) in wtMAH, scCont or shHIF2α MAH cells grown under Nox (21% O_2) vs CHox (2% O_2, 24 h), with β-actin as an internal loading control (bottom panel). (D) Immunofluorescence detection of A2aR (green) and tyrosine hydroxylase (TH; red) in same microscopic field of (normoxic) MAH cells. Scale bar represents 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Differential expression of adenosine A2a receptor in wtMAH and HIF-2α-deficient (shHIF2α) MAH cells during chronic hypoxia. Fold change represents difference in expression from normoxic (21% O_2, 24 h) wtMAH and shHIF2α MAH cells compared to chronically hypoxic (2% O_2, 24 h) wtMAH and shHIF2α MAH cells. Microarray analyses were performed in triplicate and validated by quantitative RT-PCR (n = 3).

	wtMAH cells				shHIF2α MAH cells			
	Microarray		RT-PCR		Microarray		RT-PCR	
	Fold change	*p*-value	Fold change	*p*-value	Fold change	*p*-value	Fold change	*p*-value
Adenosine A2a receptor	13.08	0.0031	9.53	0.021	1.08	0.214	−1.25	0.265

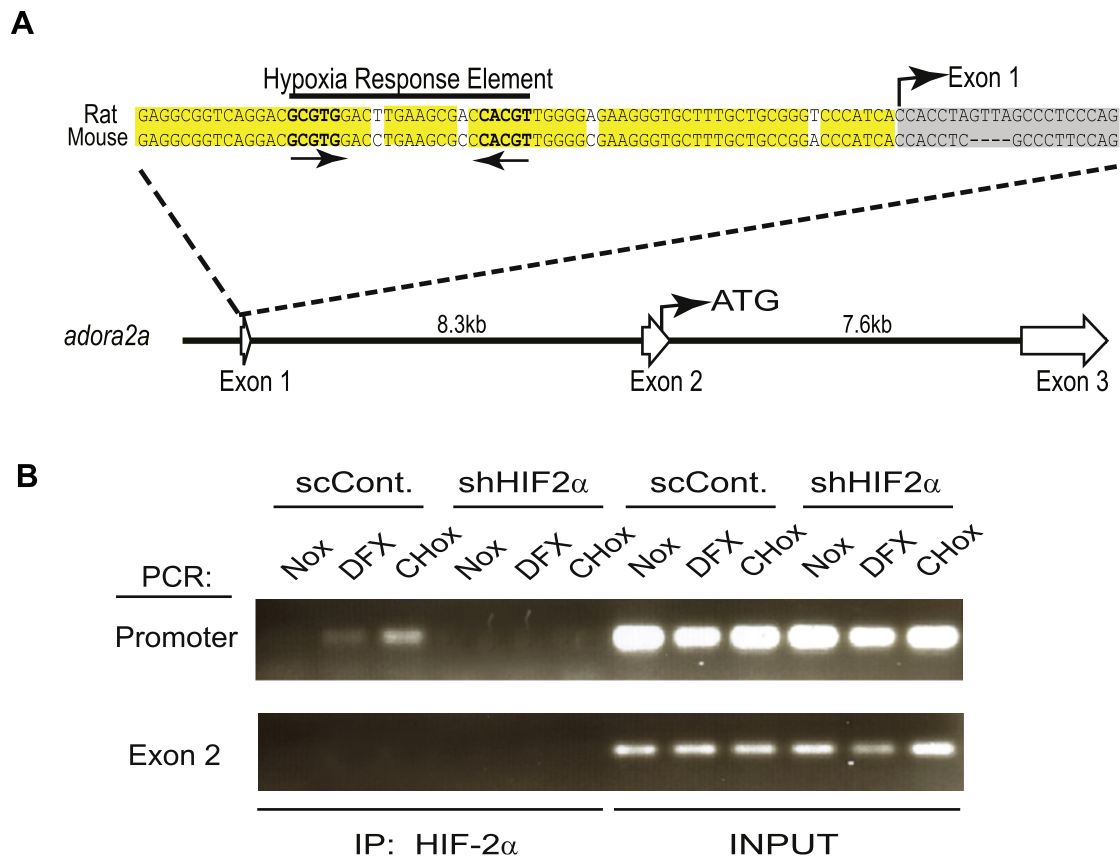

Fig. 2. Putative hypoxia response Element (HRE) in the rat and mouse adora2a gene. (A) Schematic diagram of rat and mouse HRE, located ~34 bp upstream of exon 1. (B) Chromatin Immunoprecipitation (ChIP) of the adora2a gene using HIF-2α antibody. Cell lysate from normoxic, chronically hypoxic (2% O_2; 24 h), and desferrioxamine-treated (DFX, 100 μM) scCont and HIF2α-deficient (shHIF2α) MAH cells was precipitated with rabbit HIF-2α polyclonal antibody. The associated DNA was amplified by PCR using primers specific for the promoter region or exon2 of the rat adora2a gene.

successfully amplified their targets in the input samples (Fig. 2B; input). These data indicate that HIF-2α specifically associates with the promoter region of the *adora2a* gene.

3.3. Adenosine enhances acute hypoxia- and high K^+-evoked catecholamine secretion from MAH cells via adenosine A2a receptors: effects of chronic hypoxia

In this study quantal catecholamine (CAT) secretory events recorded from MAH cells in response to acute hypoxia and high K^+ resembled those reported in previous studies from this laboratory (Fig. 3A) (Buttigieg et al. 2008a). However, when these stimuli were combined with exogenous adenosine (ADO; 5 nM), both mean quantal charge Q (Fig. 3B and E) and quantal event frequency (Fig. 3C and F) were significantly increased. Consequently, there was a marked potentiation in total CAT secretion, reflected by cumulative charge (Fig. 3D and G). Consistent with the upregulation of A2aR in MAH cells grown under CHox (Table 1), stimulus-evoked CAT secretion was greatly potentiated in these CHox cells exposed to 5 nM ADO (Fig. 3B–G). Though cumulative basal secretion was significantly elevated ~2× after Chox, the effect of ADO in enhancing this secretion appeared significant only under normoxia (data not shown).

3.4. Effects of chronic hypoxia on intracellular Ca^{2+} signalling in control and HIF-2α deficient (shHIF2α) MAH cells

Because upregulation of A2aR in chronically hypoxic MAH cells was HIF-2α-dependent, we anticipated that the facilitatory effects of ADO on secretion would be blunted in HIF-2α-deficient (shHIF2α) cells. The greatly diminished synthesis of catecholamines in shHIF2α MAH cells [9] precluded the use of amperometry for these studies. We therefore monitored stimulus-evoked changes in intra-

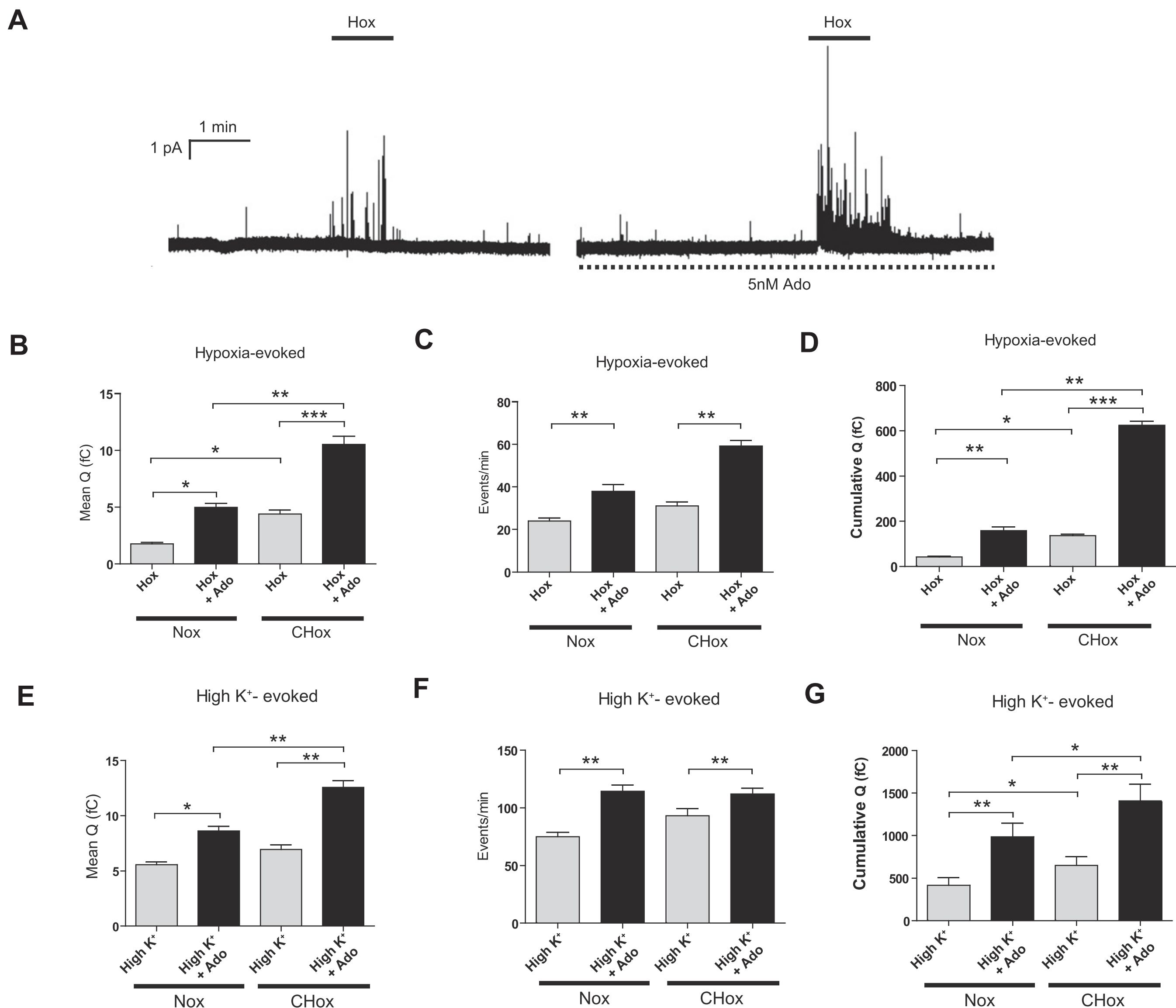

Fig. 3. Effects of adenosine on evoked catecholamine release from MAH cells grown under normoxia (Nox; 21% O_2) vs chronic hypoxia (Chox; 2% O_2, 24 h). Representative traces of quantal catecholamine (CAT) secretion from the *same* normoxic MAH cell exposed to acute hypoxia before (left trace) and during (right trace) adenosine (Ado; 5 nM) is shown in A; note Ado potentiation of hypoxia-evoked CAT secretion (right). Release parameters, i.e. mean quantal charge Q (B and E), event frequency (C and F), and cumulative charge (D and G), were obtained for Nox and Chox cells before and during exposure to adenosine (Ado; 5 nM). CAT secretion was evoked by acute hypoxia (PO_2 ~20 mm Hg) in (B), (C), and (D), and by the depolarizing stimulus high K^+ in E, F, and G. Significant differences are as follows: *indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$. Data are represented as mean ± SEM, where $n = 24$ cells.

cellular Ca^{2+} $[Ca]_i$ levels using Fura-2 spectrofluorimetry as previously described (Fig. 4A and B) [22,23]. Chronic hypoxia caused a significant increase in basal $[Ca]_i$ levels in all MAH cells by approximately 30 nM (58 vs 90 nM, data not shown), though there were no significant differences among wild type (wt), scrambled scControl, and shHIF2α cells (data not shown). As illustrated in Fig. 4C and D, in wild type and scControl MAH cells grown under Nox (21% O_2) and CHox (2% O_2; 24 h), high K^+ evoked a significant rise in $[Ca]_i$ that was potentiated by high concentrations of ADO (50 μM), sufficient to activate both A2aR and A2bR [25,26]; lower concentrations of ADO (5 nM), similar to those used in the amperometric studies reported above were ineffective for reasons that are unclear (data not shown). By contrast, in HIF-2α-deficient (shHIF2α) cells, high concentrations of ADO (50 μM) failed to affect intracellular Ca^{2+} signals evoked by high K^+ even after CHox (Fig. 4E). These functional data are consistent with QPCR data showing the failure of CHox to upregulate A2aR expression in HIF-2α-deficient MAH cells (Table 1). Also, these data suggest that there may be no compensatory increased expression of low affinity A2bR in HIF-2α-deficient MAH cells.

4. Discussion

This study has provided new insight into potential cellular and molecular mechanisms regulating catecholamine secretion in developing adrenomedullary chromaffin cells (AMC) during chronic hypoxia (CHox). Such mechanisms are of interest because the physiological intrauterine environment is constitutively hypoxic (vascular $PO_2 < 30$ mm Hg) and this condition, together with the availability of circulating catecholamines, is critical for proper development (Iwamoto et al. 1989; Bulterys et al. 1990; Ream

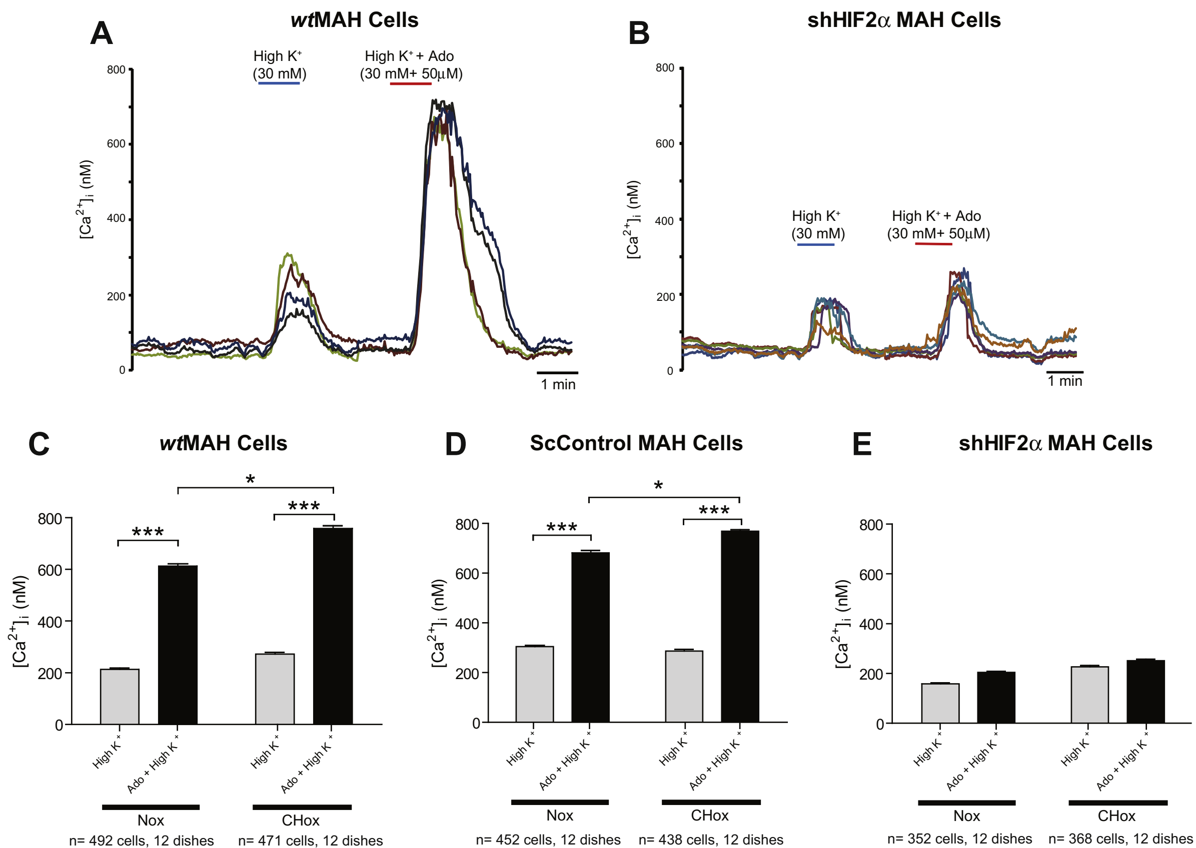

Fig. 4. Effects of adenosine on high K+-evoked intracellular [Ca^{2+}] in MAH cells cultured under normoxia (Nox; 21% O_2) vs chronic hypoxia (CHox; 2% O_2, 24 h). Representative traces of intracellular Ca^{2+} signals in control (wtMAH) and HIF2α- deficient (shHIF2α MAH) cells are shown in (A) and (B). Mean data for intracellular [Ca^{2+}] during exposure to high K+ (30 mM) in the presence or absence of adenosine (Ado; 50 μM) in wt (C), scrambled control (scCont) (D), and HIF2α-deficient (shHIF2α) (E), MAH cells. *Indicates $p < 0.05$, ** indicates <0.01 and *** indicates $p < 0.001$. Data are represented as mean ± SEM.

et al. 2008). Using a combination of molecular tools, we found that CHox caused a dramatic upregulation of adenosine 2a receptor (A2aR) expression in a fetal-derived, chromaffin (MAH) cell model. Significantly, we showed that this upregulation was HIF-2α-dependent, and that exogenous adenosine potentiated catecholamine secretion, especially after chronic hypoxia.

4.1. HIF-2α binds to the promoter of A2aR gene

Though A2aR was previously shown to be upregulated in PC12 cells during chronic hypoxia, no HIF binding site was identified in the rat A2aR gene (*adora2a*) promoter region, and therefore no direct link between HIF transcription factor(s) and *adora2a* was found [13]. By contrast, our data indicated that the upregulation of A2aR gene expression and function during chronic hypoxia was critically dependent on HIF-2α. Interestingly, in a recent study chronic hypoxia increased the expression of A2aR in human endothelial cells via HIF-2α, however, the effect was not observed in mouse endothelial cells [27]. The apparent discrepancy between these data and our study is explained by the fact that the former studies focused on the area immediately upstream of the coding region in the *adora2a* gene. However, it is now known that the *adora2a* mRNA has a large 5′ untranslated region (UTR) encoded by another upstream exon (GeneID 25369). Moreover, it appears that A2aR can have many 5′-UTR splice variants with profound effects on the translation of mRNA and consequently receptor expression [28]. The primers used in our RT-PCR reactions targeted this exon and it was found that the A2aR isoform containing a larger 5′ UTR was upregulated in both *wild type* and scrambled control, but not in HIF-2α-deficient, MAH cells. Examination of the area upstream of this region in the *adora2a* gene revealed a putative HRE (**GCG TG**G ACT TGA AGC GAC **CAC GT**) and chromatin immunoprecipitation assays showed that HIF-2α bound to this region, providing direct evidence for a role of HIF-2α in the hypoxic regulation of the adenosine A2aR gene in rat cells.

In summary, we have identified a critical role for HIF-2α in the upregulation of A2aR in fetal-derived chromaffin cells during chronic hypoxia. We propose that autocrine–paracrine activation of these A2aR by released adenosine may be physiologically important for enhancing catecholamine secretion, necessary for proper fetal development. It may also play a role during the transition to extrauterine life, where a surge in catecholamine secretion in response to asphyxial stressors at birth is critically important in the preparation of the lungs for air breathing [11].

Acknowledgments

This work was supported by operating grants from the Canadian Institutes of Health Research (CIHR; MOP 12037) and the Heart and Stroke Foundation (HSF) of Ontario (#T-5819) to CAN. STB was supported by a Focus on Stroke Award from HSF of Canada, Canadian Stroke Network, CIHR and Astrazeneca Canada Inc. We thank Cathy Vollmer for expert technical assistance.

References

[1] C.A. Jansen, E.J. Krane, A.L. Thomas, N.F. Beck, K.C. Lowe, P. Joyce, M. Parr, P.W. Nathanielsz, Continuous variability of fetal PO_2 in the chronically catheterized fetal sheep, Am. J. Obstet. Gynecol. 134 (1979) 776–783.

[2] A.J. Giaccia, M.C. Simon, R. Johnson, The biology of hypoxia: the role of oxygen sensing in development, normal function and disease, Genes Dev. 18 (2004) 2183–2194.
[3] M.A. Ream, R. Chandra, M. Peavey, A.M. Ray, S. Roffler-Tarlov, H.G. Kim, W.C. Wetsel, H.A. Rockman, D.M. Chikaraishi, High oxygen prevents fetal lethality due to lack of catecholamines, Am J Physiol. Regul. Integr. Comp. Physiol. 295 (2008) R942–R953.
[4] Q.Y. Zhou, C.J. Quaife, R.D. Palmiter, Targeted disruption of the tyrosine hydroxylase gene reveals that catecholamines are required for mouse fetal development, Nature 374 (1995) 640–643.
[5] H. Tian, R.E. Hammer, A.M. Matsumoto, D.W. Russell, S.L. McKnight, The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development, Genes Dev. 12 (1998) 3320–3324.
[6] G.L. Semenza, O_2-regulated gene expression: transcriptional control of cardiorespiratory physiology by HIF-1, J. Appl. Physiol. 96 (2004) 1173–1177. Discussion 1170–1172.
[7] E. Schipani, H.E. Ryan, S. Didrickson, T. Kobayashi, M. Knight, R.S. Johnson, Hypoxia in cartilage: HIF-1alpha is essential for chondrocyte growth arrest and survival, Genes Dev. 15 (2001) 2865–2876.
[8] R.J. Tomanek, A. Ratajska, G.T. Kitten, X. Yue, A. Sandra, Vascular endothelial growth factor expression coincides with coronary vasculogenesis and angiogenesis, Dev Dyn. 215 (1999) 54–61.
[9] S.T. Brown, K.F. Kelly, J.M. Daniel, C.A. Nurse, Hypoxia inducible factor (HIF)-2 alpha is required for the development of the catecholaminergic phenotype of sympathoadrenal cell, J. Neurochem. 110 (2009) 622–630.
[10] T.A. Slotkin, F.J. Seidler, Adrenomedullary catecholamine release in the fetus and newborn: secretory mechanisms and their role in stress and survival, J. Dev. Physiol. 10 (1988) 1–16.
[11] H. Lagercrantz, T.A. Slotkin, The "stress" of being born, Sci. Am. 254 (1986) 100–107.
[12] M.F. Czyzyk-Krzeska, W.R. Paulding, J.E. Beresh, S.L. Kroll, Post-transcriptional regulation of tyrosine hydroxylase gene expression by oxygen in PC12 cells, Kidney Int. 51 (1997) 585–590.
[13] S. Kobayashi, D.E. Millhorn, Stimulation of expression for the adenosine A2A receptor gene by hypoxia in PC12 cells, A potential role in cell protection, J. Biol. Chem. 274 (1999) 20358–20365.
[14] S.C. Taylor, C. Peers, Chronic hypoxia enhances the secretory response of rat phaeochromocytoma cells to acute hypoxia, J. Physiol. 514 (Pt 2) (1999) 483–491.
[15] K.A. Seta, Z. Spicer, Y. Yuan, G. Lu, D.E. Millhorn, Responding to hypoxia: lessons from a model cell line, STKE 2002, Science (2002) RE11.
[16] V. Carabelli, A. Marcantoni, V. Comunanza, A. de Luca, J. Diaz, R. Borges, E. Carbone, Chronic hypoxia up-regulates alpha1H T-type channels and low-threshold catecholamine secretion in rat chromaffin cells, J. Physiol. 584 (2007) 149–165.
[17] D. Souvannakitti, G.K. Kumar, A. Fox, N.R. Prabhakar, Neonatal intermittent hypoxia leads to long-lasting facilitation of acute hypoxia-evoked catecholamine secretion from rat chromaffin cells, J Neurophysiol 101 (2009) 2837–2846.
[18] S.J. Birren, D.J. Anderson, A v-myc-immortalized sympathoadrenal progenitor cell line in which neuronal differentiation is initiated by FGF but not NGF, Neuron 4 (1990) 189–201.
[19] I.M. Fearon, R.J. Thompson, I. Samjoo, C. Vollmer, L.C. Doering, C.A. Nurse, O_2-sensitive K^+ channels in immortalised rat chromaffin-cell-derived MAH cells, J. Physiol. 545 (2002) 807–818.
[20] S.T. Brown, C.A. Nurse, 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 (2008) C1305–C1312.
[21] R. Giegerich, F. Meyer, C. Schleiermacher, GeneFisher–software support for the detection of postulated genes, Proc. Int. Conf. Intell. Syst. Mol. Biol. 4 (1996) 68–77.
[22] J. Buttigieg, S. Brown, M. Zhang, M. Lowe, A.C. Holloway, C.A. Nurse, Chronic nicotine in utero selectively suppresses hypoxic sensitivity in neonatal rat adrenal chromaffin cells, FASEB J. 22 (2008) 1317–1326.
[23] N.A. Piskuric, S.T. Brown, M. Zhang, C.A. Nurse, Glucosensing in an immortalized adrenomedullary chromaffin cell line: role of ATP-sensitive K(+) channels, Neurosci. Lett. (2008).
[24] R.H. Wenger, D.P. Stiehl, G. Camenisch, Integration of oxygen signaling at the consensus HRE, Sci. STKE (2005) re12.
[25] B.B. Fredholm, I.J. AP, K.A. Jacobson, K.N. Klotz, J. Linden, International Union of Pharmacology XXV, Nomenclature and classification of adenosine receptors, Pharmacol. Rev. 53 (2001) 527–552.
[26] B.B. Fredholm, M.P. Abbracchio, G. Burnstock, J.W. Daly, T.K. Harden, K.A. Jacobson, P. Leff, M. Williams, Nomenclature and classification of purinoceptors, Pharmacol. Rev. 46 (1994) 143–156.
[27] A. Ahmad, S. Ahmad, L. Glover, S.M. Miller, J.M. Shannon, X. Guo, W.A. Franklin, J.P. Bridges, J.B. Schaack, S.P. Colgan, C.W. White, Adenosine A2A receptor is a unique angiogenic target of HIF-2alpha in pulmonary endothelial cells, Proc. Natl. Acad. Sci. USA 106 (2009) 10684–10689.
[28] S. Kreth, C. Ledderose, I. Kaufmann, G. Groeger, M. Thiel, Differential expression of 5′-UTR splice variants of the adenosine A2A receptor gene in human granulocytes: identification, characterization and functional impact on activation, FASEB J. 22 (2008) 3276–3286.