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# Hypoxia results in an HIF-1-dependent induction of brain-specific aldolase C in lung epithelial cells

Jyh-Chang Jean,[1] Celeste B. Rich,[2] and Martin Joyce-Brady[1]

[1]*The Pulmonary Center and* [2]*Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts*

**Jean, Jyh-Chang, Celeste B. Rich, and Martin Joyce-Brady.**
Hypoxia results in an HIF-1-dependent induction of brain-specific aldolase C in lung epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 291: L950–L956, 2006. First published June 23, 2006; doi:10.1152/ajplung.00087.2006.—Aldolase C (EC 4.1.2.13) is a brain-specific aldolase isoform and a putative target of the transcription factor hypoxia-inducible factor (HIF)-1. We identified aldolase C as a candidate hypoxia-regulated gene in mouse lung epithelial (MLE) cells using differential display. We show that the message accumulates in a robust fashion when MLE cells are exposed to 1% oxygen and is inversely related to oxygen content. Induction in hypoxia is dependent on protein synthesis. We localized a hypoxia-responsive element (HRE) in the aldolase C promoter using a series of deletion and heterologous expression studies. The HRE overlaps with a region of the proximal aldolase C promoter that is also related to its brain-specific expression. The HRE contains an Arnt (HIF-1β) and an HIF-1α site. We show that induction in hypoxia is dependent on the HIF-1 site and that HIF-1α protein is present, by gel-shift assay, within nuclear complexes of MLE cells in hypoxia. Aldolase C mRNA expression is developmentally regulated in the fetal lung, rapidly downregulated in the newborn lung at birth, and inducible in the adult lung when exposed to hypoxia. This pattern of regulation is not seen in the brain. This preservation of this HRE in the promoters of four other species suggests that aldolase C may function as a stress-response gene.

stress-response gene; zebrin II; perinatal; oxygen

OUR LABORATORY IS FOCUSED on the regulation of gene expression in the perinatal lung by oxygen. To identify genes regulated by hypoxia, we used differential display to compare gene expression in mouse lung epithelial (MLE) cells in 1% oxygen vs. 21% oxygen. In this paper, we show that one gene with a very robust pattern of hypoxic induction in these cells was brain-specific aldolase C, an enzyme in the glycolytic pathway (EC 4.1.2.13). Hypoxia-inducible factor (HIF)-1α induces genes for glycolytic enzymes during hypoxia, and the aldolase C and A isoforms have been described as targets of this transcription factor (8, 23). However, the exact mechanism has only been characterized for aldolase A (23). Therefore, we tested the hypothesis that HIF-1 regulates the induction of aldolase C in MLE cells under hypoxic conditions. Herein, we identify a hypoxia-responsive element (HRE) in the aldolase C promoter and define a role for HIF-1α and its cognate DNA binding site in hypoxia induction. We show that this HRE resides in the aldolase C promoter region of other species, suggesting an evolutionarily preserved process. We correlate these findings with in vivo studies comparing aldolase C mRNA expression in the lung vs. the brain during the perinatal period and during exposure of the adult to hypoxia, showing that aldolase C mRNA expression is regulated by oxygen in the lung but not in the brain.

## EXPERIMENTAL PROCEDURES

*Reagents.* Electrophoresis-grade agarose was from International Biotechnologies (New Haven, CT). X-OMAT film for radiography was from Eastman Kodak (Rochester, NY). [α-$^{32}$P]dCTP or [γ-$^{32}$P]dATP, specific activity 800 Ci/mmol, was from ICN (Irvine, CA). The MLE cell line was obtained from American Type Culture Collection and maintained in culture with DMEM plus 10% FCS. Gas cylinders with defined concentrations of oxygen, nitrogen, and carbon dioxide were obtained from Wesco Medical Gases (Bellerica, MA). Modular incubator chambers were from Billips-Rothenburg as described (12). Mice were exposed to hypoxia by enclosing their cages in an air-tight glove bag (9). They were allowed access to food and water ad libitum. Differential display was performed with the Delta differential display kit from Clontech according to the manufacturer's instructions (Palo Alto, CA). The pGL3-basic vector, the pGL3-SV40 promoter vector, the substrate buffer for the luciferase assay, and the gel-shift assay systems were obtained from Promega (Madison, WI). An HIF-1α gel-shift oligonucletotide and antibodies against HIF-1α (sc-8711 and sc-10790) and Ah receptor (AhR; sc-8088) were obtained from Santa Cruz Biotechnology.

*RNA analysis.* Differential display was performed with RNA isolated from MLE cells cultured in 21% oxygen vs. 1% oxygen (hypoxia). Cells were exposed to a mixture of 1% oxygen-5% $CO_2$-balance nitrogen in modular incubator chambers as previously described (12). In some experiments, the cells were exposed to 95% oxygen-5% $CO_2$. RNA was isolated with Trizol and quantified by spectroscopy. Candidate hypoxia-regulated clones from differential display were verified by Northern blot analysis with the use of 10 μg of total RNA (17). Given the highly homologous nature of the coding domains of the aldolase A and C isoforms, a ~600-bp probe was designed and amplified from the 3′ end of aldolase C mRNA (GenBank BC008184) to specifically distinguish the C from the A transcript (PCR upstream primer: TCTCTCAACCTCAAT; downstream primer: AGTACATAGC). Ethidium bromide stain of the 18S ribosomal RNA was used as a control for loading. Autoradiogram signals were quantified by densitometry. All nucleotide sequencing was performed at the DNA Protein Core Facility, Boston University School of Medicine. Sequencing results were compared with those in GenBank by use of the multiple sequence alignment at www.ncbi.nlm.nih.gov.

*Aldolase C promoter, deletion, and mutant constructs.* Organization of the mouse aldolase C promoter was found to be highly similar to the previously characterized human aldolase C promoter in GenBank sequence X07292 (3, 4, 18). Mouse constructs were cloned into the pGL3-basic vector. Preliminary experiments indicated that ~1.2 kb of the upstream region from the mouse aldolase C gene gave a consistent response to hypoxia, which was unaffected by an additional

Address for reprint requests and other correspondence: M. Joyce-Brady, The Pulmonary Center, 715 Albany St., R304, Boston, MA 02118 (e-mail: mjbrady@bu.edu).

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~2 kb of upstream sequences. A series of PCR-derived deletion constructs were generated from this construct. Sequences including the first intron and also ~2 kb of DNA downsteam of the 3′ UTR were also cloned into pGL3-basic vector and analyzed for reactivity in hypoxia. Hypoxia-responsive sequences from aldolase C were cloned into the pGL3 promoter vector. A series of mutated constructs were prepared by site-directed mutagenesis and cloned into the pGL3 promoter vector. Direct sequencing was used to validate the authenticity of each construct before use. Figure 2 summaries the orientation and size of the multiple aldolase C constructs used in this study.

*Transient transfection assay.* This was performed by electroporation with subconfluent cells (10, 12). We used 10 μg of plasmid DNA (8 μg aldolase C-luciferase, 2 μg of CMV-β-galactosidase) in each electroporation experiment. Cells were harvested and assayed for reporter gene activity 40–48 h after transfection. Each transfection experiment was repeated at least three times with the pGL3-basic vector as the negative control and the pGL3-SV40 vector as the positive control. The luciferase assay was performed according to manufacturer guidelines. Cotransfection with CMV-β-galactosidase was used to control for transfection efficiency.

*EMSA.* Nuclear protein extracts were prepared from MLE cells grown in 21% or 1% oxygen according to the protocol outlined by Bohinski et al. (1). Cells were washed and harvested with ice-cold PBS and then sedimented by centrifugation. Cells were lysed by gently vortexing in a buffer of 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.2% NP-40, 1 mM dithiothreitol, and 0.5 mM PMSF. Nuclei were sedimented by centrifugation, resuspended, and incubated on ice in an extraction buffer containing 20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 25% glycerol, 1 mM dithiothreitol, and 0.5 mM PMSF for 10 min and then resedimented by centrifugation at 4°C. The supernatants were assayed for protein content and stored at −80°C until use.

DNA binding assays were performed with the gel-shift assay system from Promega according to the manufacturer's instructions. Double-stranded oligonucleotide probes, spanning the hypoxia-responsive region of the aldolase C promoter (5′-CCAGGGAGT-CACGTAGCTCTG-3′) or the human erythropoietin promoter (5′-TTGCCCTACGTGCTGTCTCAG-3′) (22) were end labeled with [γ-$^{32}$P]dATP and T4 polynucleotide kinase. An unlabeled HIF-1α competitor probe for gel shift was used to demonstrate specificity. For supershift assays, 10 μg of nuclear protein extract were incubated with antibodies against HIF-1α or AhR in gel-shift binding buffer at 23°C for 30 min before addition of probe. Binding buffer consisted of 100 ng/ml of poly(dI-dC)·poly(dI-dC), 10 mM Tris·HCl (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, and 4% glycerol. Radiolabeled oligonucleotide probe (100,000 cpm) was incubated with nuclear extract in a total volume of 10 μl for 30 min. Nuclear extract was omitted as the negative control. DNA-protein complexes were resolved by electrophoresis on a 4% nondenaturing polyacrylamide gel. The gels were dried, and signals were detected by exposure to autoradiography film (19).

*In vivo correlation.* Timed-pregnant and adult mice were housed in the Laboratory and Animal Science Center; animal protocols were approved by the Institutional Utilization and Animal Care Committee at Boston University School of Medicine. Adult mice were exposed to an environment of 7% oxygen-balance nitrogen by enclosing their cages in an airtight glove bag as previously described (9). Oxygen content was monitored continuously during the exposure period. Lung and brain RNA was isolated from late fetal, newborn, and adult mice (10, 17).

*Statistics.* Data from the Northern blot and the transfection experiments are reported as mean and SD from $n = 3$ experiments. The data were compared by ANOVA and Tukey's honestly significant test for post hoc comparison of means using the Statistica software package from StatSoft (Tulsa, OK). $P < 0.05$ was considered significant.

## RESULTS

*Regulation of aldolase C mRNA expression in MLE cells by oxygen.* The candidacy of aldolase C as a hypoxia-responsive mRNA from differential display (J.-C. Jean and M. Joyce-Brady, unpublished observations) was verified by comparing the level of mRNA expression by Northern blot using total RNA harvested from MLE cells exposed to 1% oxygen (hypoxia) vs. 21% oxygen and 95% oxygen, as shown in Fig. 1*A*. Aldolase C mRNA was highly induced in 1% oxygen (31 ± 7.2-fold, $n = 3$; $P < 0.05$) compared with 21% oxygen, and expression was decreased in 95% oxygen (0.4 ± 0.1-fold, $n = 3$; $P < 0.05$), showing a negative correlation of oxygen concentration and level of aldolase C mRNA expression. The time course of induction in 1% oxygen is shown in Fig. 1*B*. Aldolase C mRNA accumulated by 6 h (12-fold, range 7.7 ± 3.7-fold; $P < 0.05$) and peaked at 18 h (39-fold in this blot) and decreased slightly at 36 h, as did MLE cell viability as determined by direct visualization. Addition of cyclohexamide (5 or 100 μM) blocked aldolase C induction at 6 h but less so at 18 h, suggesting the need for protein synthesis early on to achieve induction.

*Localization of hypoxia-responsive sequences with the mouse aldolase C promoter.* An aldolase C construct extending from −1244 to +69 (Fig. 2, *lane A*) exhibited a consistent twofold induction of luciferase activity in 1% oxygen ($P < 0.0002$; Fig. 3*A*). Activities of the pGL3-basic vector (negative control) and the pGL3 promoter vector (positive control for luciferase activity) were unchanged in 1% oxygen vs. 21% oxygen. Analysis of constructs with an additional 2 kb of upstream DNA failed to increase reporter gene activity any further in hypoxia. Hence, a series of 5′ deletion constructs were studied. In Fig. 3*A*, *constructs B* (−859) through *H* (−119) each exhibited a twofold induction of luciferase activity in 1% oxygen ($P < 0.02$). Figure 3*B* shows that *constructs I* (−66) through *L* (−39) also each exhibited an approximately threefold induction of luciferase activity in 1% oxygen ($P = 0.0002$). The basal activity of *construct L* was significantly decreased compared with *I*, and this deletion eliminated an Sp1 site. This lower level of basal activity in 21% oxygen persisted in *constructs M* (−31) and *N* (−21), but induction of luciferase activity in 1% oxygen was now eliminated, suggesting the deletion of hypoxia-responsive sequences between −47 and −21. Additional sequences downstream of the transcription start site in the first intron were examined in *constructs BB* (−859 to +1258) and *CC* (−413 to +1258) to systematically search for any additional hypoxia-responsive sequences in the

**A**    **B**

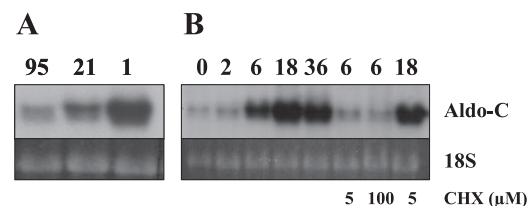95 21 1        0 2 6 18 36 6 6 18



Aldo-C

18S

5 100 5   CHX (μM)

Fig. 1. Dose response and kinetics of aldolase C mRNA induction by hypoxia in mouse lung epithelial (MLE) cells. *A*: total RNA was isolated from MLE cells exposed to 95%, 21%, or 1% oxygen and hybridized with an aldolase C-specific probe in the 3′ region of the mRNA. *B*: time course (units are in hours) of induction of aldolase C (Aldo-C) mRNA in MLE cells exposed to 1% oxygen and effect of cyclohexamide (CHX) on induction. The ethidium bromide-stained 18S band is shown as a loading control for each blot.
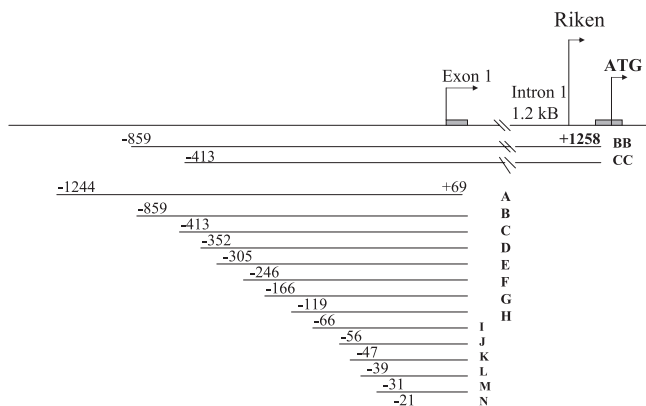
Fig. 2. Schematic of mouse aldolase C gene and DNA constructs. Mouse aldolase C DNA constructs were generated to localize hypoxia-responsive sequences based on a similarity with the human aldolase C gene.

aldolase C gene given this location for the HRE in the human aldolase A gene (23). However, this region in the aldolase C gene did not affect reporter gene expression in hypoxia (Fig. 3A). Lastly, 2-kb of DNA downstream of the 3′-UTR was also examined, but it did not confer any additional hypoxia response either (data not shown).

*Characterization of HIF-1 functional activity.* The region from −47 to −22 of the mouse aldolase C promoter contained

a consensus HIF-1α site, 5′-RCGTG-3′(31), in reverse orientation at −29 to −25, as well as an Arnt (HIF-1β) site (5′-CACGC-3′) upstream at −45 to −41. To confirm that this region was indeed functional in hypoxia, it was cloned upstream of the SV40 luciferase reporter in the absence (Fig 4, *construct A*) and presence (Fig. 4 *construct B*) of nucleotides forming the intact HIF-1 consensus site. There was no difference in reporter gene activity in 1% vs. 21% oxygen for the SV40 promoter vector alone (P in Fig. 4), nor for the constructs with a single (P+A) or a triplet copy (P+AAA) of the aldolase C hypoxia-responsive region in the absence of an intact HIF-1α consensus sequence. In contrast, reporter gene activity in 1% oxygen was induced 10-fold when the aldolase C-responsive region included the intact HIF-1α site (P+B) and 20-fold when two copies of the region were inserted in tandem (P+BB).

Lastly, systematic mutation of the HRE in the aldolase C promoter was examined (14, 23). A series of mutations downstream of the Arnt site were generated within *construct K* as shown in Fig. 5 and were tested for their effect on hypoxic induction in the SV40 promoter. The intact construct K-0 induced activity by 3.4-fold ($P = 0.00015$) in hypoxia. The K-1 mutant disrupted an Sp1 site, which decreased basal activity by 2.4-fold ($P < 0.002$), but hypoxic induction remained intact (3.1-fold; $P = 0.008$). The K-2 and K-3 mutants behaved in an identical fashion to the intact K-0 construct. Mutation of the HIF-1 consensus at the 3′ G alone (5′-RCGT**G**-3′), together with the two adjacent dinucleotides, eliminated hypoxic induction in the K-4 construct, as did mutation of three internal nucleotides (5′-R**CGT**G-3′) within the HIF-1α consensus sites in the K-5 construct. This region in the aldolase C promoter contained a fully functional HIF-1 site.

Analysis of these 26 nucleotides from the mouse aldolase C promoter with the basic local alignment search tool (BLASTN) identified a similarly conversed region in the aldolase C promoters of *Rattus norvegicus* (27), *Homo sapiens* (4), *Pan troglodytes*, and *Xenopus laevis* (33). The HIF-1α site was
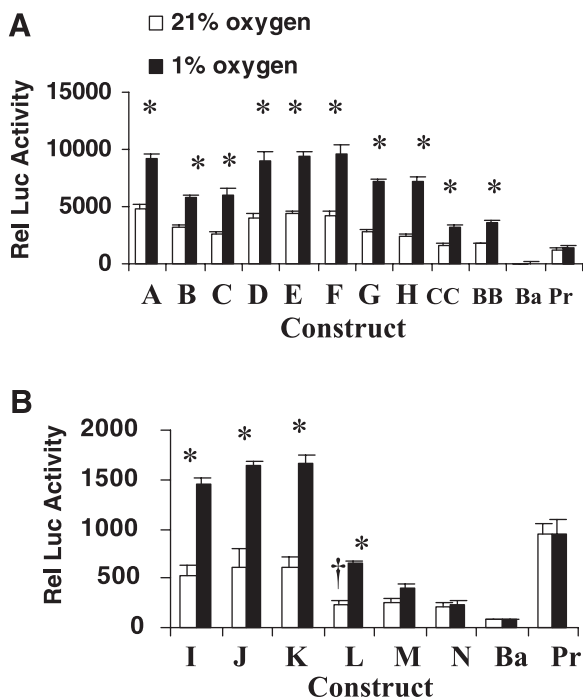


Fig. 3. Hypoxia-responsive element (HRE) localization in the aldolase C promoter. *A*: transient transfections were performed in MLE cells in 21% vs. 1% oxygen using a series of constructs surrounding ~1 kb upstream and downstream of the transcription start site. Data are means and SD from 3 independent experiments. Rel luc, relative luciferase activity. *Significant change in reporter activity for construct in 1% oxygen compared with 21% oxygen. Ba, basic promoter construct as negative control; Pr, SV40 promoter construct as a positive control for luciferase activity. Cells were cotransfected with β-galactosidase to control for transfection efficiency. *B*: analysis of proximal sequences from the mouse aldolase C promoter. *Significant change in reporter activity for construct in 1% vs. 21% oxygen. †Significant decrease in basal activity of promoter construct compared with *construct I*.
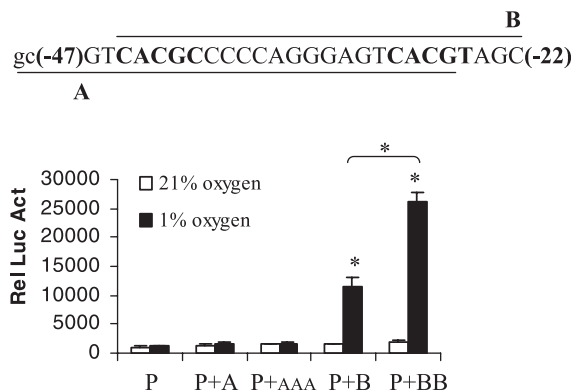


Fig. 4. Functional activity of the hypoxia-responsive region of aldolase C in a heterologous reporter. Transient transfection assays were performed in MLE cells as in Fig. 3. The hypoxia-responsive region of aldolase C was cloned upstream of the SV40 promoter in vector pGL3. Rel luc act, relative luciferase activity. In *construct A*, the hypoxia-inducible factor (HIF)-1 consensus site was interrupted; in *construct B*, it was intact. *Significant increase in reporter gene activity for construct in 1% oxygen vs. 21% oxygen. A and AAA represent 1 and 3 copies of *construct A*. B and BB represent 1 and 2 copies of *construct B*. Asterisk above parenthesis denotes significant increase in activity of *construct B* with 2 copies vs. 1 copy in hypoxia.

K-0  -47GT**CACGC** CCC CAG GGA GTC **ACG** TAGC-22
K-1                  TTT
K-2                        TTT
K-3                              TTT
K-4                                    TAT
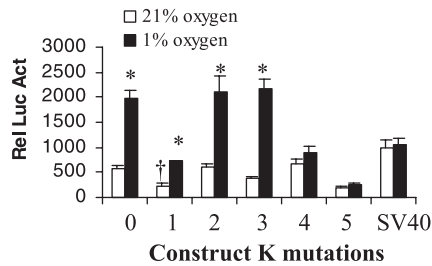K-5                                          TTT



Fig. 5. Mutational analysis of the hypoxia-responsive region of mouse aldolase C. Site-directed mutagenesis was used to mutate the hypoxia-responsive region of aldolase C as depicted at *top*. Transient transfections were performed in MLE cells in 1% vs. 21% oxygen as in Fig. 3. *Significant increase in reporter gene activity for construct in 1% vs. 21%. †Significant decrease in basal activity of promoter construct compared with construct K-0.

preserved in all five species, and the Arnt site (HIF-1$\beta$) was preserved in all but *Xenopus* (Fig. 6*A*).

The aldolase C HRE was partially embedded within a region of the proximal promoter that contained sequences required for brain-specific expression. This overlapping Sp1/Krox site for brain-specific expression also contains an overlapping Arnt/AhR/Sp1 site (Fig. 6*B*).

*EMSA and supershift assay for HIF-1$\alpha$.* EMSA and supershift assays were performed to confirm DNA-protein complex formation in nuclei from cells in 1% vs. 21% oxygen and the presence of HIF-1 protein in the complexes. Results are shown in Fig. 7. No DNA-protein complexes were observed in the presence of aldolase C probe alone (Fig. 7*A*). Complexes were formed when the probe was incubated with nuclear extracts from cells in 1% oxygen, and the band with the slowest migration appeared specific to hypoxia, as the other more rapidly migrating bands were also evident in nuclear extracts from cells in 21% oxygen. Specificity of the hypoxia-inducible band was confirmed by coincubation with cold HIF-1 competitor probe. This gel-shift pattern was similar to that of a human erythropoietin HIF-1 probe used as a positive control (22). In

Fig. 7*B*, no DNA-protein complexes were formed in the presence of aldolase C probe alone. The band with the slowest migration was again specific to hypoxia (C-1%). The intensity of this hypoxia-inducible nuclear complex was eliminated when nuclear extracts were coincubated two different antibodies against HIF-1$\alpha$ (HIF-1 = sc-10790 and HIF-1′ = sc-8711) but persisted when the extracts were coincubated with an antibody against AhR as a control. The antibodies appeared to largely inhibit DNA-protein complex formation because longer exposure showed only faint supershifted bands in the anti-HIF-1$\alpha$ lanes (data not shown).

*Aldolase C mRNA expression in lung and brain.* RNA was isolated from perinatal and adult mice and probed on a Northern blot to compare aldolase C mRNA expression in the lung vs. the brain in vivo (Fig. 8). Aldolase C mRNA was induced in the lung between fetal *day 16* and *18* of gestation (2- to 4-fold, $n = 2$) and then rapidly downregulated by *day 1* after birth so that by *days 2–3* it was barely detectable at all. It remained at this very low level of expression in the adult lung. In contrast, expression was more abundant in the brain; signal was detectable within hours of exposure to film, but the pattern of developmental regulation seen in the lung was absent and the level of expression in the adult brain was more abundant than that in the perinatal brain (increased 4.2 $\pm$ 1.0-fold; $P < 0.05$). However, when adult mice were exposed to an environment of 7% oxygen, lung aldolase C mRNA expression was induced and detectable within 6 h, peaked at 12 h (2.6 $\pm$ 0.8-fold; $P < 0.02$), and was still evident at 24 h, albeit at a lower level of expression than that seen in the fetal lung. In contrast, aldolase C mRNA expression was much more abundant in the adult brain, but no change in the level of expression was observed over the 24-h period of exposure to hypoxia (Fig. 9).

**DISCUSSION**

We have identified and characterized aldolase C as a hypoxia-regulated gene in MLE cells. This induction is mediated in an HIF-1-dependent fashion, confirming previous studies that identified aldolase C as an HIF-1 target gene (8, 23). Herein, we localized a single, functional HRE in the proximal aldolase C promoter and demonstrated the presence of HIF-1$\alpha$ protein within hypoxia-induced DNA-protein nuclear complexes. This HRE is preserved in the aldolase C gene of five

**A**

        Arnt/AhR                    HIF-1
  -47    GT **CACGC** CCCCAG      GGA GT**CACGT**AGCTCTGC GGC   Mm
 -760    GT **CACGC** CCCCAG      GGA GT**CACGT**AGCTCTGC GGC   Rn X07845
  -14    GC **CACGC** CCCCGG A GGA GT**CACGT**AGCTCTGC GAC   Hs NM005165
 -122    GC **CACGC** CCCCGG A GGA GT**CACGT**AGCTCTGC GAC   Pt XM_511798
-1583    GG TATTA  GATCGC  G GCA GT**CACGT**AGCTCTGC    GC Xl D38621

**B**

        **Arnt/AhR/Sp1**              HIF-1
        GT **CACGC** CCCCAG      GGA GT**CACGT**AGCTCTGC GGC
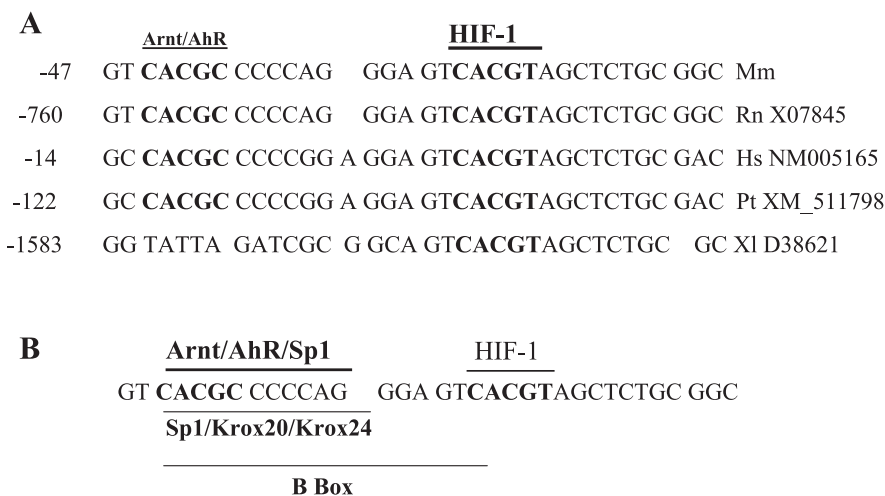        **Sp1/Krox20/Krox24**

              **B Box**

Fig. 6. Comparison of the hypoxia-responsive region in aldolase C gene among species. *A*: BLASTN analysis of the mouse (Mm) hypoxia-responsive region in aldolase C gene (−47 to −22), showing alignment with similar region in aldolase C gene from rat (Rn), human (Hs), chimpanzee (Pt), and frog (X1). Numbers at *left* denote 5′ location of site. Species are listed on the *right* along with the GenBank accession number. The Arnt and the HIF-1 sites are labeled and bolded. *B*: comparison of Arnt and HIF-1 sites in the HRE with brain-specific sites identified in Ref. 20.
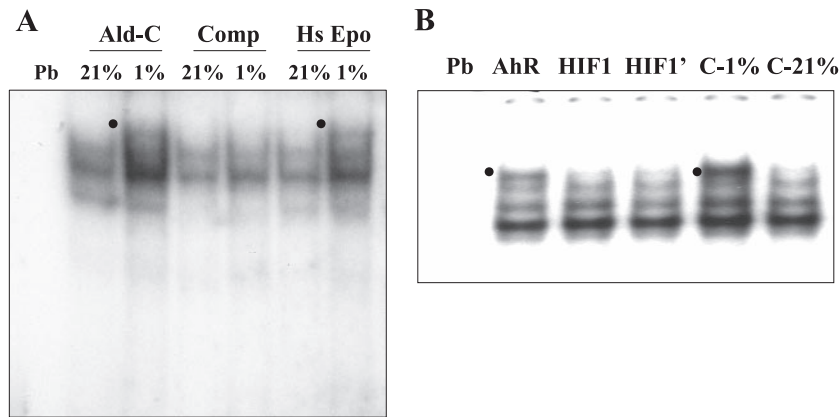
Fig. 7. Gel-shift and supershift assays for HIF-1 protein in nuclear extracts of MLE cells in hypoxia. EMSA and supershift assays were performed with nuclear extracts as described in EXPERIMENTAL PROCEDURES. In A, DNA-protein complexes were absent with aldolase C probe alone (Pb) but present when this probe was incubated with nuclear extracts from cells in 1% oxygen (Ald-C 21% and 1%). The band with the slowest migration (dotted) appeared specific to hypoxia, as the other more rapidly migrating bands were also evident in nuclear extracts from cells in 21% oxygen. Cold probe with the HIF-1α consensus was used in a competition experiment (Comp) to demonstrate specificity of the hypoxia-inducible band. A human erythropoetin HIF-1α probe (Hs Epo; positive control) produced a similar pattern. In B, DNA-protein complexes only formed when aldolase C probe was incubated in the presence of nuclear extracts. Again, the band with the slowest migration (●) was specific to hypoxia (C-1%). This hypoxia-inducible nuclear complex was eliminated when nuclear extracts were coincubated with 2 different antibodies against HIF-1α (HIF1 and HIF1′) but persisted when the extracts were coincubated with an antibody against Ah receptor (AhR) as a control. The antibodies appeared to largely inhibit DNA-protein complex formation because longer exposure showed only faint supershifted bands in the anti-HIF-1α lanes (data not shown).

other species, suggesting a shared ability to activate this gene in response to hypoxia. Developmental regulation of aldolase C mRNA has not been previously demonstrated in fetal mouse lung, although a more limited mRNA expression profile has been shown in the perinatal rat lung (15). The expression of this gene is clearly regulated during the perinatal period. Its basal level of expression in the fetal mouse lung at *day 16* of gestation could be related to the relatively hypoxic state of the fetus, which is the equivalent of ∼3% oxygen, but its upregulation in the fetal lung at *day 18* of gestation is likely independent of oxygen because there is no change in its content during this time. Rather, this induction may reflect expression of aldolase C in a specific subset of lung epithelial cells as a component of a differentiation-related program (12, 13). The rapid downregulation of aldolase C mRNA in the lung after birth could be because of the abrupt rise in oxygen concentration to 21% given that we found an inverse correlation between expression of its transcript and ambient oxygen content. Expression of this mRNA in the adult lung is virtually undetectable by Northern blot. However, it is induced and detectable in the lung within hours when the environmental oxygen content is decreased from 21% to 7%. The level of expression was still

below that of the fetal lung, but the level of oxygen could not be decreased to the fetal level either. To determine the specificity of this hypoxic induction, we showed that aldolase C mRNA expression in the brain, where it is already highly expressed, did not change with this exposure. Our data suggest that aldolase C induction in lung epithelial cells is linked to hypoxia. This contrasts with aldolase C expression in mammary gland epithelial cells, which is induced during pregnancy and lactation by the hormone prolactin (16), and in neuronal cells, where the high level of expression is mediated, at least in part, by tissue-specific enhancer elements (25).

Aldolase C is well characterized as a brain-specific isoform of aldolase and is also known as zebrin II. Aldolase C promoter sequences within a 115-bp region upstream of the transcription start site can drive expression of a reporter gene in a brain-specific fashion (25, 26). This involves, in part, two overlapping Krox-20/Krox-24/Sp1 sites that are functional in a neuronal cell-specific fashion. The HRE that we defined in the present study and located at −47 to −22 is actually embedded within this 115-bp region and located partially within the "B box." This region plays a role in brain-specific expression of aldolase C. Sequences within this B box also form an overlapping AhR/Arnt/Sp1 site in our HRE. This previous investigation (26) noted that mutation of the Sp1 site decreased basal
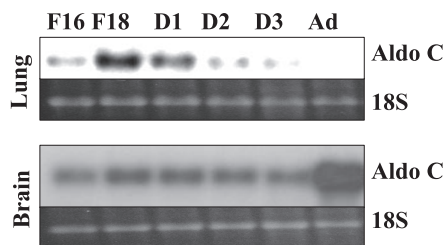


Fig. 8. Expression of aldolase C mRNA in perinatal lung vs. brain. Northern blot was performed with total RNA isolated from lung and brain at fetal *days 16* and *18* day of gestation (F16 and F18) and at newborn *days 1, 2,* and *3* (D1, D2, D3) and as an adult (Ad) and with an aldolase C-specific cDNA probe. The ethidium bromide-stained 18S band is shown as a loading control for each blot.
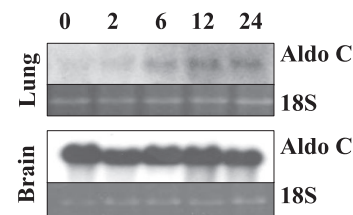


Fig. 9. Expression of aldolase C mRNA in adult lung vs. brain in hypoxia. Northern blot was performed with total RNA isolated from lung and brain of adult mice exposed to 7% oxygen at 0, 2, 6, 12, and 24 h. The ethidium bromide-stained 18S band is shown as a loading control for each blot.

promoter activity in brain cells, and we note in our present study that the same is true for promoter activity in our lung epithelial cells. This confirms a functional role for this Sp1 site in the basal activity of the TATA-less aldolase C promoter. In addition, our aldolase C HRE contains an abundance of brain-related transcription factor binding sites (Krox-20/24, Pax-2/5/8), mammalian stress-related sites (AhR, Arnt, AP-2$\alpha$A/B, AP-1), and general transcription factor sites (Sp1, USF, and GCN4). Brain-specific expression of aldolase C is postulated to require interactions between tissue-specific and the general transcription factor Sp1. Our data suggest that Sp1 affects promoter activity within MLE cells as well (see *construct L* vs. *K* in Fig. 3). In fact, the presence of transcription factor binding sites such as HIF-1, AhR, AP-2$\alpha$, and AP-1 suggests that stress-activated pathways may also converge at this regulatory region to activate aldolase C gene expression in response to environmental stimuli and that this region is preserved in several different species. Further studies will be required to confirm this, but overall it appears that aldolase C can function as a stress-response gene.

There are two other isoforms in addition to aldolase C. Aldolase A is expressed largely in the muscle and aldolase B in the liver (20). The A and C isoforms appear to be more closely related to each other, as shown by nucleotide and protein analyses, than to the B isoform. Indeed, the B isoform functions mainly in gluconeogenesis and fructose metabolism within the liver, whereas both the A and the C isoforms function in glycolysis and are upregulated by exposure of cells to hypoxia (8, 23). Glycolysis is believed to play a central role in cell survival in hypoxia by providing an ongoing source of ATP production, albeit at low levels of efficiency. In this regard, it is not clear why both A and C are induced in hypoxia, unless the two enzymes together can produce more ATP than either one alone. Alternatively, aldolase C may also function beyond glycolysis as a stress-response gene. Aldolase protein associates with the actin cytoskeleton and can provide a local source of ATP production that is important for cytoskeletal integrity (21, 29, 30). In addition, aldolase C has been found in tight association with a plasma membrane oxidoreductase complex in brain cells. The plasma membrane oxidoreductase complex is believed to function as an extracellular membrane redox sensor that induces cellular responses to external oxidant stress. Hypoxia is associated with cellular oxidant stress, and brain cells are quite susceptible to injury in hypoxia (2). Interestingly, hypoxic preconditioning induces tolerance to ischemic injury in the neonatal rat brain by inducing changes in mRNA and protein expression. In the case of the GLUT-1 glucose transporter, both mRNA and protein are induced to increase glucose uptake. In contrast, for several glycolytic enzymes, including aldolase, only protein expression is induced (11). These data regarding the lack of mRNA induction in the brain by hypoxia correlate with our present results but also suggest that aldolase C protein could still be induced in the hypoxic brain at a posttranslational level. Aldolase C may serve a similar function for cells outside of the nervous system.

The HRE that we identified in the aldolase C promoter is clearly a target for HIF-1. This transcription factor functions as a heterodimer composed of the inducible $\alpha$-subunit and the constitutively expressed $\beta$-subunit Arnt (28). Although Arnt is required to mediate hypoxic induction by HIF-1$\alpha$, the Arnt subunit does dimerize with other proteins, most notably the aryl hydrocarbon receptor AhR. The AhR/Arnt pathway is a separate ligand-activated pathway that responds largely to the xenobiotic dioxin via dioxin-responsive elements (5). We used an antibody against AhR as a control in our gel-shift assay. The two different antibodies against HIF-1$\alpha$ were able to completely disrupt formation of the hypoxia-specific nuclear complex, but antibody against AhR did not. The presence of this AhR site suggests that aldolase C may also be inducible by exposure to dioxin.

Interestingly, HIF-1$\alpha$ appears to selectively activate glycolytic genes in hypoxia, including aldolases A and C, as these are not targets of HIF-2$\alpha$ (8). This observation correlates well with our data on the downregulation of aldolase C at birth because HIF-1$\alpha$ expression is also downregulated (7) but HIF-2$\alpha$ is not (6). Recent literature shows that HIF-1$\alpha$ and HIF-2$\alpha$ regulate distinct, as well as common, sets of target genes, despite the two transcription factors sharing similarities in structure, DNA binding, and dimerzation domains (8). Characterizing the *cis*- and *trans*-acting factors that affect the selection of HIF-1$\alpha$ vs. HIF-2$\alpha$ target genes is an active area of research. Identification of HIF-1$\alpha$ target genes is relevant during perinatal lung development because expression of this transcription factor is essential for lung epithelial cell maturation and function and survival at birth (24).

In summary, brain-specific aldolase C is induced by hypoxia in lung epithelial cells via an HIF-1$\alpha$-dependent fashion. Aldolase C may function as part of a stress-response pathway for lung epithelial cell function in hypoxia. Future studies aimed at defining the exact role of this protein in the fetal lung may provide new tools for protecting adult lung epithelial cells from the stress of hypoxia. In addition, further attempts to define unique HIF-1$\alpha$ vs. HIF-2$\alpha$ target genes in the lung could provide new insight into their roles in lung development and in the response of the lung to hypoxia during disease states, such as cystic fibrosis (32) and cancer.

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