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IDH1 Regulates Phospholipid Metabolism in Developing Astrocytes

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Abstract

Isocitrate dehydrogenase 1 (IDH1) is an evolutionarily conserved enzyme that catalyzes the interconversion of isocitrate to α-ketoglutarate with the concomitant reduction of $NADP^+$ to NADPH. IDH1 has previously been shown to participate in lipid biosynthesis in various tissues such as the liver and adipose tissue. We examined the potential role of IDH1 in phospholipid metabolism in the brain. Here we show that IDH1 is highly expressed in the brain and astrocytes during embryonic development and the postnatal period and subsequently declines in adulthood. Silencing of IDH1 expression using siRNA in astrocytes isolated from E18.5 mouse cortices led to increased incorporation of [^{3}H]-palmitate into the phosphatidylcholines (PCs) and decreased the incorporation of [^{3}H]-palmitate into sphingomyelin and the phosphatidylethanolamines (PEs). In pulse-chase experiments, knock-down of IDH1 expression impaired the turnover of PCs and decreased the synthesis of PEs. The decrease in [^{3}H]-palmitate incorporation into PEs when IDH1 was knocked-down in astrocytes was not due to impairments within the CDP-ethanolamine pathway or in the rate of decarboxylation of phosphatidylserine (PS). In conclusion, our results reveal a role for IDH1 in the synthesis/turnover of phospholipids in developing astrocytes and highlight the lipid alterations resulting from the loss of wild-type IDH1 activity.

Keywords

phospholipid; IDH1; embryo; astrocyte; brain

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Introduction

IDH1 is an evolutionarily conserved enzyme that catalyzes the interconversion of isocitrate to α-ketoglutarate with the concomitant reduction of $NADP^+$ to NADPH [1, 2]. In mammalian cells, IDH1 is localized mainly within the cytoplasm although small but significant amounts of IDH1 have been found within peroxisomes [3].

Recent interest in IDH1 function within the brain and astrocytes has stemmed from the discovery of mutations in the *IDH1* gene in a large portion (>70%) of human low grade gliomas (WHO grades II and III) and secondary glioblastomas (WHO grade IV) that have progressed from the lower grade lesions [4-7]. In gliomas, mutations in IDH1 frequently occur within the catalytic domain of the protein where arginine at position 132 is commonly replaced by histidine ($IDH1^{R132H}$) [4]. Mutation of arginine 132 abolishes the wild-type activity of the enzyme and in the case of $IDH1^{R132H}$, a novel enzymatic activity is gained where $IDH1^{R132H}$ converts α-ketoglutarate to 2-hydroxyglutarate (2-HG) and consumes NADPH in this process [4, 8]. Clinical studies have repeatedly shown that patients whose tumours express mutant $IDH1^{R132H}$ survive significantly longer compared to patients whose tumours express wild-type IDH1 [4, 9, 10].

The function of IDH1 within the brain or astrocytes is not completely known. Early reports have hypothesized a role for IDH1 in cytoplasmic and peroxisomal lipid biosynthesis [11, 12]. Mice engineered to overexpress IDH1 within the liver and adipose tissue were obese compared to wild-type litter mates and had fatty livers and higher levels of plasma triacylglycerols and cholesterol [13]. Several recent reports have shown that IDH1 can contribute to *de novo* lipogenesis in tumour cells by generating acetyl-CoA via the conversion of α-ketoglutarate to isocitrate [14, 15, 16, 17].

The brain is a lipid rich organ with lipids comprising approximately 50% of the brain's dry weight [18]. Compared to other organs in the body, the brain has the second highest lipid content next to adipose tissue [18]. In the present study, we examined the role of IDH1 in lipid metabolism in the brain and astrocytes during embryonic development with a focus on the phospholipids. Phospholipids are key constituents of cellular membranes and serve as downstream mediators in signal transduction pathways [19]. Our study focused on the metabolism of phospholipids during the embryonic period, a time of active lipid synthesis for the growth and development of the brain [20].

Material and Methods

Isolation of Mouse Embryonic Astrocytes

Astrocyte enriched glial fractions were isolated from embryonic day (E) 15.5, E18.5 and adult (3 month old) Balb/c mouse brains as described previously [21]. Brains obtained from 3 month old mice were first minced and digested with papain. Isolated cells were grown in Astrocyte Medium (ScienCell) and cultured up to 7-10 days. The presence of astrocytes was confirmed by the expression of glial fibrillary acidic protein (GFAP) determined by confocal microscopy (Supplementary Figure 1) and real-time quantitative PCR (data not shown). GFAP positive cells comprised >80% of the cell population.

Immunoblotting

Cells and tissue were lysed in lysis buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 1% Igepal, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate and protease inhibitors). Total protein was quantified using the BCA Protein Assay Kit (Pierce). Equal amounts of protein were resolved by SDS-PAGE and immunoblotted using either anti-IDH1 (Santa Cruz Biotechnology), anti-IDH2 (Abcam) or anti-GAPDH (glyceraldehydes-3-phosphate dehydrogenase) (Cell Signaling) antibodies followed by horseradish peroxidise (HRP)-conjugated secondary antibodies (BioRad). Proteins were visualized by enhanced chemiluminescence.

Real Time Quantitative PCR

Total RNA was extracted using TRIzol-chloroform (Life Technologies) and purified using the RNeasy Kit (Qiagen). RNA (2μg) was transcribed to cDNA (Applied Biosystems) and real-time quantitative PCR was performed using the Applied Biosystems StepOnePlus Real-Time PCR System. The following primer sequences were used for *Idh1*: 5′AGGTTCTGTGGTGGAGATGC3′ and 5′TCTGCAGCATCTTTGGTGAC3′, *Idh2*: 5′GGCTGTCAAGTGTGCCACAATC3′ and 5′TTGGCTCTCTGAAGACGGTTCC3′.

Silencing of IDH1 Expression and Thin-Layer Chromatography of Lipids

The expression of IDH1 was knocked-down in astrocytes by electroporation of the cells with 100nM siRNA (OriGene) using the Neon Transfection System (100μl Tip, Life Technologies) with the following settings: voltage 1425, pulse width 30 and pulse number 1. After 48 h, the cells were incubated with either [9, 10-^{3}H]-palmitic acid (2μCi/ml), [^{3}H]-serine (5μCi/ml) or [1, 2-^{14}C]-ethanolamine hydrochloride (0.2μCi/ml) for various times at 37°C. The cells were washed with phosphate buffered saline (PBS), trypsinized and centrifuged. Cell pellets were resuspended in 1.5 ml methanol:chloroform (2:1) and incubated at room temperature for 20 min. Chloroform (0.5ml) was subsequently added followed by 1M NaCl (0.5ml). The mixture was centrifuged at 1000 x *g* for 5 min and the lower lipid phase removed. All radiochemicals were purchased from Moravek Biochemicals. The lipid extracts were resolved by thin layer chromatography (TLC) using Silica Gel H plates (250μm, Analtech). The plates were first washed with chloroform:methanol (2:1) and activated by heating for 1 h at 100°C. The lipid extracts were spotted on the plates and separated in developing solvent consisting of chloroform:methanol: 2-propanol:0.25% KCl:triethylamine (30:9:25:6:18). Phospholipid classes were visualized under UV light after spraying with 0.1% 8-anilino-1-naphthalene sulfonic acid and identified by comparison to authenticated standards (Avanti). Bands corresponding to sphingomyelin, PC, PS, phosphatidylinositol (PI) and PE were scraped from the plates and the amount of radioactivity incorporated was determined by scintillation counting.

Statistical Analysis

The data was analyzed using the Student's t-test or a one-way analysis of variance followed by Tukey's HSD test. Statistical significance was defined as $p < 0.05$.

Results

IDH1 Expression and Tissue Distribution

The expression of IDH1 was examined during embryonic development and in adulthood. In the brain, the expression of IDH1 was maximal during the embryonic period and then declined in adulthood (Figure 1A and B) [22]. The expression of IDH1 appeared to be highest between E13.5-E18.5 and then declined at 2 months of age (Figure 1A and B). IDH1 expression within astrocytes isolated from E15.5 and E18.5 embryonic brains and postnatal day 2 brains also showed higher expression of IDH1 compared to astrocytes isolated from adult (3 month old) mouse brains and whole adult brain tissue (Figure 2). IDH2, the mitochondrial $NADP^+$ dependent isocitrate dehydrogenase also shown to be mutated in a subset of gliomas [5] showed the same expression pattern as IDH1 (Figures 1 and 2).

In adult mice, high levels of IDH1 were present in the liver, adipose tissue and mammary gland, tissues that exhibit high levels of lipid biosynthesis (Supplementary Figure 2). Similar results were observed in rat and human tissues (data not shown). In contrast to IDH1, high levels of IDH2 were noted in the heart and skeletal muscle (Supplementary Figure 3).

IDH1 Regulates Phospholipid Metabolism in Embryonic Astrocytes

To determine whether IDH1 activity participates in the metabolism of phospholipids in developing astrocytes, we examined phospholipid synthesis in embryonic astrocytes where the expression of IDH1 was reduced using siRNA. Astrocytes were isolated from wild-type E18.5 mouse cortices and transfected with IDH1 siRNA for 48 h. Under our experimental conditions, IDH1 protein expression was reduced by approximately 70% (Figure 3A). Following the reduction of IDH1 expression, the cells were incubated with [^{3}H]-palmitic acid, a 16-carbon saturated fatty acid, for various times and the incorporation of [^{3}H]-palmitate into cellular phospholipids was evaluated by TLC and scintillation counting.

When lipids were extracted from E18.5 astrocytes and separated by TLC, the most abundant phospholipid was PC (data not shown). Likewise, when astrocytes were incubated with [^{3}H]-palmitate for 6 h, the majority (50-75%) of the radioactivity taken up by astrocytes was incorporated into PCs. Less (10-30%) [^{3}H]-palmitate was incorporated into sphingomyelin and PEs while <10% was incorporated into PS and phosphatidylinositol (PI) (Figure 3B). Upon knock-down of IDH1 expression, the incorporation of [^{3}H]-palmitate into PCs was increased by 15-20% ($p < 0.05$) (Figure 3B). In contrast, the incorporation of [^{3}H]-palmitate into sphingomyelin and PEs was decreased in cells in which IDH1 expression was knocked-down ($p < 0.05$) (Figure 3B). When astrocytes were incubated with [^{3}H]-palmitate for 3 h, the incorporation of radioactivity into the phospholipid classes was similar to that seen following 6 h of incubation (data not shown). At longer incubations (>6 h) there appeared to be a redistribution of [^{3}H]-palmitate between the PCs and PEs. To examine the redistribution of [^{3}H]-palmitate and to determine whether the changes in PCs and PEs were due to either changes in synthesis or degradation, we performed pulse-chase experiments in which astrocytes were first incubated with [^{3}H]-palmitate for 3 h at 37° C. The cells were then washed and incubated in regular (non-radioactive) growth medium. At various times thereafter, lipids were extracted from the cells and analyzed by TLC. Following the removal

of radioactive medium, [^{3}H]-palmitate incorporated into PCs gradually declined from 68% to 51% after 36 h while the incorporation of [^{3}H]-palmitate into PEs increased from 11% to 29% (Figure 3C). In astrocytes where the expression of IDH1 was knocked down by siRNA, the degradation of PCs was impaired compared to control cells (Figure 3D). When the PEs were examined, the incorporation of [^{3}H]-palmitate into PEs was decreased in cells where IDH1 expression was knocked-down (Figure 3E).

In mammalian cells, the two known routes of PE synthesis are via the decarboxylation of PS and by the CDP-ethanolamine pathway [23]. To determine whether IDH1 regulates PE synthesis via the decarboxylation of PS or through the CDP-ethanolamine pathway, astrocytes were incubated with either [^{3}H]-serine or [^{14}C]-ethanolamine respectively for 6 h at 37°C. When cells were incubated with [^{3}H]-serine, the incorporation of radioactivity into the various phospholipid classes was not statistically different between the control and IDH1 knock-down cells (Figure 4A). When cells were incubated with [^{14}C]-ethanolamine, the incorporation of [^{14}C]-ethanolamine into PEs was significantly higher in cells where IDH1 expression was knocked-down compared to control cells ($p < 0.05$) (Figure 4B). Similar results were obtained when cells were incubated with [^{14}C]-ethanolamine for either 3 h or 12 h. These observations suggest that when IDH1 activity is reduced, flux through the CDP-ethanolamine pathway increases.

Discussion

Here we show that IDH1 regulates phospholipid metabolism in developing astrocytes where IDH1 activity is required for the turnover of PCs and for the synthesis of PEs. The PCs and PEs are the most abundant phospholipids in mammalian tissues comprising up to 50% and 30% respectively, of the total phospholipids [19]. During development, cell number increases and also cell differentiation and maturation occurs. Phospholipids form the plasma membrane and the membranes of intracellular organelles to support cell proliferation. As astrocytes mature, cell size and morphological complexity also increases [24] necessitating a larger plasma membrane surface area. For example, astrocytes isolated from the E15.5 mouse cortex were smaller compared to astrocytes isolated from adult cortices (unpublished observations). IDH1 activity may be required to support both astrocyte proliferation and maturation. Consistent with this hypothesis, mice expressing a mutant form of IDH1 in the brain (in both neurons and glia) die during embryonic development and present with massive hemorrhaging and cell death by E15.5 [25]. In addition to participating in the synthesis/turnover of phospholipids, IDH1 activity protects cells against oxidative stress via the generation of NADPH [26]. Oxidative stress could lead to lipid peroxidation and disturbances in membrane organization resulting from the oxidation of lipids within cellular membranes [26, 27].

During the preparation of our manuscript, Esmaeili *et al*. [28] showed that tumours expressing IDH1^{R132H} had reduced levels of PEs and increased levels of PCs. An increase in PCs and decrease in PEs could alter the function and biophysical properties of cellular membranes [29]. For example, a reduction in cellular and mitochondrial PE content altered mitochondrial morphology and impaired oxidative phosphorylation and cell growth [30].

Knock-down of IDH1 expression in E18.5 astrocytes increased the incorporation of [^{14}C]-ethanolamine into PEs suggesting enhanced flux through the CDP-ethanolamine pathway. The higher incorporation of [^{14}C]-ethanolamine into PEs in the IDH1 knock-down cells might be the result of compensatory mechanisms to increase the synthesis of PEs. Alternatively, IDH1 activity might exert an inhibitory effect on the CDP-ethanolamine pathway. In preliminary experiments, we found that mRNAs encoding enzymes involved in the CDP-ethanolamine pathway such as ethanolamine kinase, CTP:phosphoethanolamine cytidylyltransferase (PCYT2) and ethanolaminephosphotransferase 1 (EPT1) were more highly expressed in adult brains than in embryonic brains (Supplementary Figure 4). These observations further support the hypothesis that IDH1 does not modulate PE synthesis via the CDP-ethanolamine pathway.

Conclusions

We have identified the phospholipids as a class of lipids modulated by IDH1 in developing astrocytes. The robust expression of IDH1 during embryonic development and the postnatal period might be necessary for the optimal synthesis of lipids that would be required to support the growth of the brain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

IDH1	isocitrate dehydrogenase 1
2-HG	2-hydroxyglutarate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
TLC	thin layer chromatography
PE	phosphatidylethanolamine
PC	phosphatidylcholine
PS	phosphatidylserine
PI	phosphatidylinositol

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- IDH1 expression is highest in astrocytes during embryonic development.
- In astrocytes, IDH1 modulates phospholipid metabolism.
- IDH1 activity is required for the turnover of phosphatidylcholines.
- IDH1 participates in the synthesis of phosphatidylethanolamines.

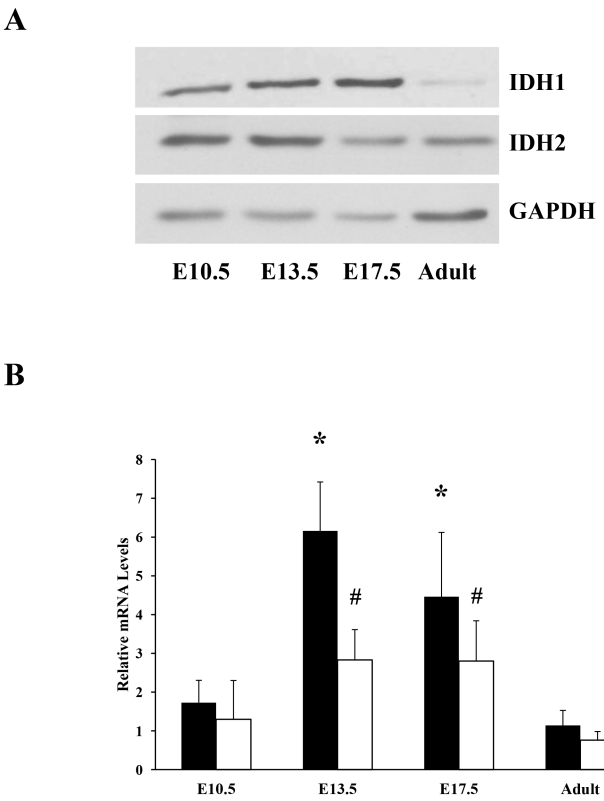


Figure 1.

IDH1 and IDH2 are highly expressed in the brain during embryonic development. Mouse brains were harvested at E10.5, E13.5, E17.5 and 2 months of age (adult). (A) Brain tissue was homogenized and equal amounts of protein were resolved by SDS-PAGE followed by Western blotting using antibodies recognizing the indicated proteins. (B) Total RNA was extracted from (A) and transcribed to cDNA. *Idh1* and *Idh2* mRNA levels were quantified by real-time PCR. The value obtained for the adult brain was set to 1. All values are expressed relative to the adult brain. Results represent the mean ± SD of n=-2-4 mice. *Idh1* ▪, *Idh2* □. $^{*,\#}$ indicates $p < 0.05$ when compared to the adult brain.

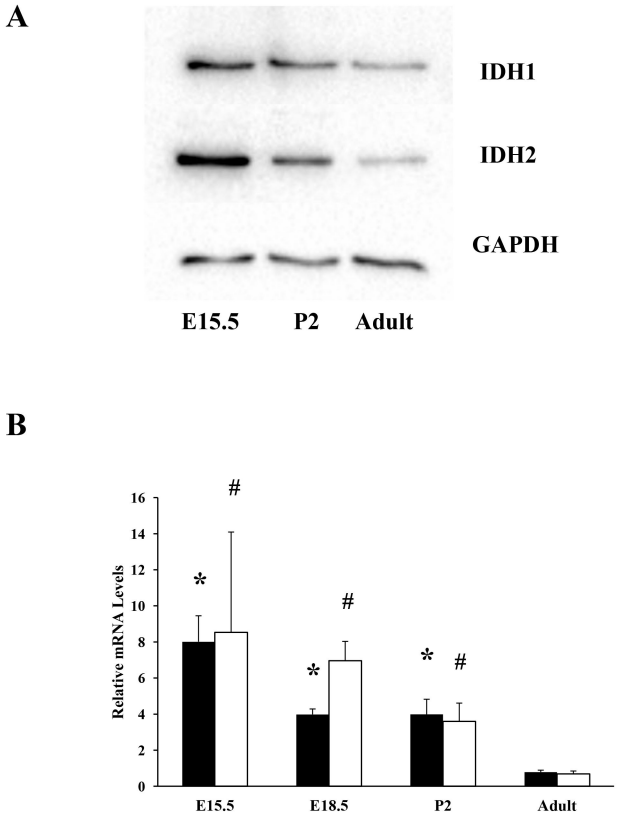
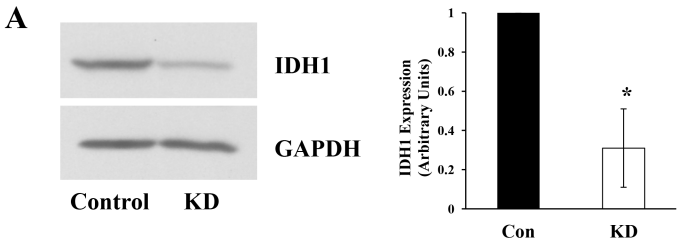
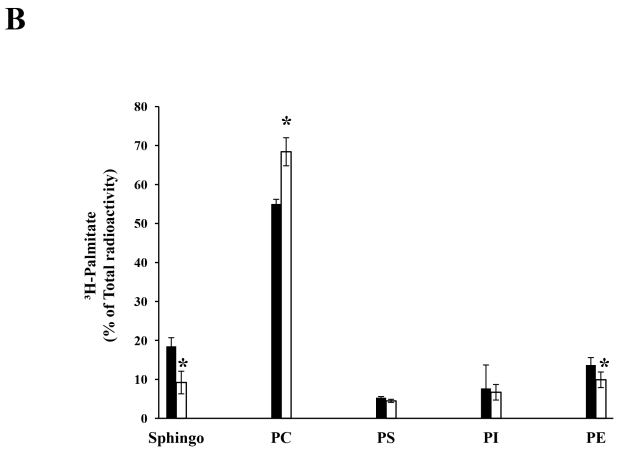


Figure 2.

IDH1 and IDH2 are highly expressed in embryonic astrocytes. Astrocytes were isolated from mouse brains at days E15.5, E18.5, postnatal day 2 (P2) and adulthood (3 months old). (A) Cells were lysed and equal amounts of protein were resolved by SDS-PAGE followed by Western blotting using antibodies recognizing either, IDH1, IDH2 or GAPDH. The membranes were imaged using the BioRad ChemiDoc Imaging System. (B) Total RNA was extracted from cells and transcribed to cDNA. *Idh1* and *Idh2* mRNA levels were quantified by real-time PCR. For each gene, the value obtained from whole adult brain tissue was set to 1. All values are expressed relative to the adult brain. Results represent the mean ± SD of n=2-3 mice. *Idh1* ■, *Idh2* □. $^{*,\#}$ indicates $p < 0.05$ when compared to the adult brain.





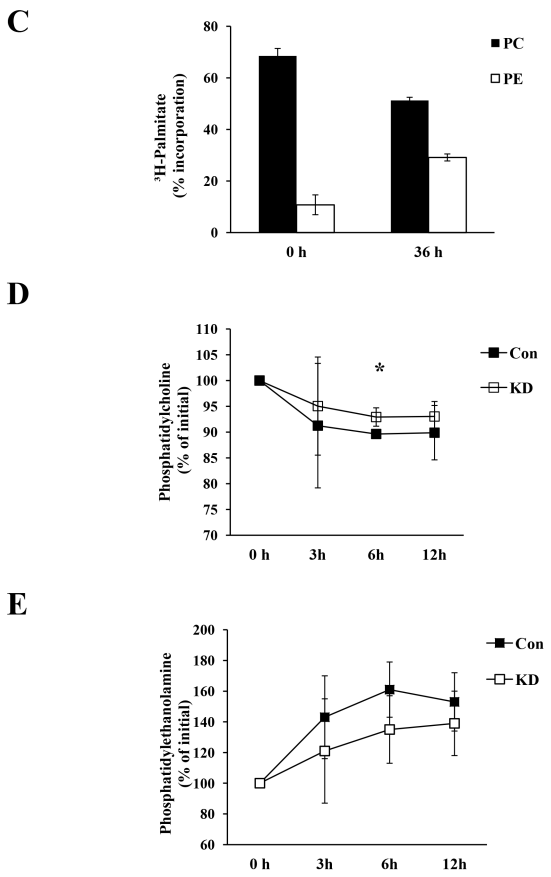


Figure 3.

Knock-down of IDH1 expression in embryonic astrocytes alters phospholipid metabolism. Astrocytes were isolated from E18.5 mouse cortices and electroporated with either IDH1 or control (scrambled) siRNA. After transfection, (A, *Left Panel*) cells were lysed and equal amounts of protein resolved by SDS-PAGE followed by Western blotting using anti-IDH1 or anti-GAPDH antibodies. (A, *Right Panel*) The expression of IDH1 was quantified by densitometry in control and IDH1 knock-down cells. The control was assigned a value of 1. IDH1 expression following knock-down is expressed relative to the control. Results represent the mean±SD from n=4 experiments. (B) Cells were incubated for 6 h at 37°C with [^{3}H]-palmitate. The incorporation of [^{3}H]-palmitate into various phospholipid classes was determined. *Sphingo* sphingomyelin. Control ▪, IDH1 knock-down □. (C, D, E) Astrocytes were incubated with [^{3}H]-palmitate for 3 h at 37°C (represented as t = 0 h). The cells were washed and incubated in regular growth medium. At the indicated times, lipids were extracted from cells and the amount of radioactivity incorporated into PCs and PEs determined. Results represent means ± SD from 3-4 experiments. * Indicates statistical significance $p < 0.05$ (Control versus KD). *Con* Control, *KD* knock-down.

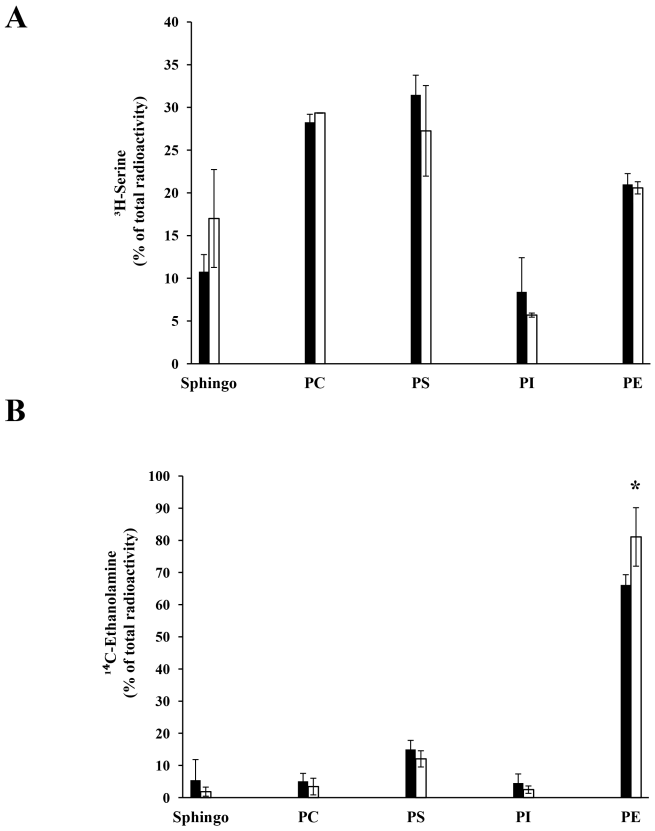


Figure 4.

Silencing of IDH1 expression in embryonic astrocytes increases the flux through the CDP-ethanolamine pathway. Astrocytes were isolated from E18.5 mouse cortices and electroporated with either IDH1 or control (scrambled) siRNA. After transfection, the cells were incubated with either (A) [^{3}H]-L-serine (5μCi/ml) or (B) [^{14}C]-ethanolamine (0.2μCi/ml) for 6 h at 37°C. Total lipids were extracted and resolved by thin layer chromatography. *Sphingo* sphingomyelin. Control **.**, IDH1 knock-down (KD) □. Results represent means ± SD from 2-5 experiments. * Indicates statistical significance $p < 0.05$ (Control versus KD).