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Expression of β-Adrenergic Receptor Subtypes in Proliferative, Involuted, and Propranolol-Responsive Infantile Hemangiomas

James D. Phillips, MD; Haihong Zhang, PhD; Ting Wei, PhD; Gresham T. Richter, MD

IMPORTANCE Propranolol hydrochloride has become the primary medical treatment for problematic infantile hemangioma; however, the expression of propranolol's target receptors during growth, involution, and treatment of hemangioma remains unclear.

OBJECTIVE To measure and compare the expression of β_1-, β_2-, and β_3-adrenergic receptors (ADBR1, ADBR2, and ADBR3, respectively) in proliferative (n = 10), involuted (n = 11), and propranolol-responsive (n = 12) hemangioma tissue.

DESIGN, SETTING, AND PARTICIPANTS Infantile hemangioma specimens were harvested for molecular investigation. Messenger RNA (mRNA) expression of the *ADBR1*, *ADBR2*, and *ADBR3* genes was detected by real-time polymerase chain reaction. Protein level expression was measured by Western blot and standardized with densitometry. A total of 33 specimens were collected from patients in a tertiary pediatric hospital who underwent excision of problematic hemangiomas. This study was conducted from January 18, 2011, to September 24, 2013, and data analysis was performed from February 25, 2015, to June 25, 2016.

RESULTS Of the 33 patients included, 21 were female (64%). The mean (SD) patient age at the time of excision was 7 (2.5) months for the proliferative group lesions, 23.5 (10) months for the involuted group, and 16 (10) months for the propranolol group. The mean level of ADBR1 mRNA expression was significantly higher in proliferative hemangioma than in propranolol-responsive hemangioma (1.05 [0.56] vs 0.52 [0.36]; $P = .01$; 95% CI, 0.12-0.94). There was no difference in ADBR2 expression among the groups. Protein expression of ADBR3 was significantly higher in involuted (0.64 [0.12] vs 0.26 [0.04]; $P < .01$; 95% CI, 0.26-0.49) and propranolol-responsive hemangioma (0.66 [0.31] vs 0.26 [0.04]; $P = .01$; 95% CI, 0.16-0.68) compared with proliferative hemangioma.

CONCLUSIONS AND RELEVANCE These data demonstrate the variable expression of ADBR subtypes among infantile hemangiomas during growth, involution, and response to treatment. These findings may have clinical implications regarding the use of selective vs nonselective β-blockade.

LEVEL OF EVIDENCE 2.

Author Affiliations: Division of Pediatric Otolaryngology and Center for the Investigation of Congenital Aberrancies of Vascular Development, Department of Otolaryngology–Head and Neck Surgery, University of Arkansas for the Medical Sciences, Little Rock (Phillips, Zhang, Wei, Richter); Department of Otolaryngology Head and Neck Surgery, Arkansas Children's Hospital, Little Rock (Phillips, Zhang, Wei, Richter).

Corresponding Author: James D. Phillips, MD, Department of Otolaryngology Head and Neck Surgery, Arkansas Children's Hospital, One Children's Way, Slot 836, Little Rock, AR 72202 (jim.david.phillips@gmail.com).

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Infantile hemangioma (IH) is the most common vascular anomaly in children, affecting 10% of all infants in the first year of life.[1] Infantile hemangiomas tend to have a characteristic cycle of growth with 3 distinct phases: proliferation, quiescence, and involution.[2] Although the natural course of IH may be self-limited, it is estimated that up to 40% of lesions will require intervention owing to comorbidity from bleeding, ulceration, disfigurement, obstruction of vision or airway, or high-output cardiac failure.[3]

After initial reports[4-8] of the beneficial effect of propranolol hydrochloride on IH, this β-blocker has quickly become the primary treatment for these vascular tumors. However, the molecular mechanism by which propranolol affects IH remains unclear. A recent review by Ji et al[9] provides a well-organized summary of the leading theories and supportive research. Advances in understanding the details of β-adrenergic signaling have indicated that multiple signaling cascades are affected by β-adrenergic inhibition. Downstream alteration of the expression of endothelial nitric oxide synthase protein and vascular endothelial growth factor has been implicated in the propranolol-induced inhibition of vasculogenesis in IH.[10,11]

Chisholm et al[12] investigated the presence of β_2- and β_3-adrenergic receptors (ADBR2 and ADBR3, respectively) as well as phosphorylated ADBR2 via immunohistochemical staining of hemangiomas, hemangioendotheliomas, and various other vascular tumors. The investigators found that ADBR2 and ADBR3 were both present in IH during proliferation and involution. Phosphorylated ADBR2 was present in involuted IH tissue but not during the proliferation phase. According to the authors, the significance of this finding is unknown but was speculated to correlate with the involution process. Also using immunohistochemistry, Boucek et al[13] demonstrated that that β_1-adrenergic receptor (ADBR1) and ADBR2 were both present in IH pericytes and endothelial cells as well as in congenital hemangiomas, which are typically nonresponsive to propranolol treatment. These findings led to the conclusion that propranolol responsiveness was not determined by a qualitative difference in expression of ADBR. We have sought to further the investigation of β-adrenergic receptors in IH by conducting a quantitative analysis of messenger RNA (mRNA) and protein expression of ADBR1, ADBR2, and ADBR3 for 3 clinical stages of these tumors: proliferation, involution, and response to treatment with propranolol.

Key Points

Question What is the relative β-adrenergic receptor subtype expression profile for infantile hemangioma in proliferation, involution, and response to propranolol treatment?

Findings In this case-control study of 33 specimens of infantile hemangioma, the level of ADBR1 messenger RNA and protein expression was higher in proliferative hemangioma. Protein expression of ADBR3 was greater in involuted and propranolol-responsive hemangiomas.

Meaning These data demonstrate a variable β-adrenergic receptor expression profile for infantile hemangioma in various stages, providing insight into pathogenesis and potential treatment options.

Methods

Infantile hemangioma specimens were obtained from the surgical resection of problematic lesions in the proliferative and involution phases of growth as well as from surgical resection of lesions that had been treated with propranolol for 1 month or more. All resections were performed at Arkansas Children's Hospital from 2011 to 2012. The tissue was preserved at −80°C until further analysis by real-time polymerase chain reaction (PCR) and Western blot. This study was conducted from January 18, 2011, to September 24, 2013, and data analysis was performed from February 25, 2015, to June 25, 2016. The study was approved by the institutional review board at Arkansas Children's Hospital, and written informed consent was obtained from the responsible caregiver of each of the participants. Data were deidentified for the purposes of testing and analysis.

Real-time PCR

Approximately 30 mg of tissue was used for RNA isolation (RNeasy Plus Kit; Qiagen). All RNA samples were then converted into complementary DNA (TaqMan Reverse Transcription Reagents Kit; Life Technologies) using random primers according to the manufacturer's instructions, and real-time PCR amplifications were performed (7900HT System; Applied Biosystems). The thermal cycling conditions were 1 cycle of 50°C for 2 minutes, 1 cycle of 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. The total reaction volume of 10 µL contained 5 ng of complementary DNA template, 5 µL of 2 × PCR Master Mix (Life Technologies), and 0.5 µL of 20 × primers and probe of target genes or endogenous control assay mix. The target genes were *ADBR1* (OMIM 109630) (Hs02330048_s1; Life Technologies), *ADBR2* (OMIM 109690) (Hs00240532_s1; Life Technologies), and *ADBR3* (OMIM 109691) (Hs00609046_m1; Life Technologies). The eukaryotic 18s ribosomal RNA (Hs99999901_s1; Life Technologies) was used as the endogenous control. The comparative (Ct) method was used to determine relative quantification. The amplification amount of all target genes was normalized against that of 18s ribosomal RNA.

Western Blot

Total proteins were extracted from approximately 50 mg of tissue using tissue protein extraction reagent (T-PER; Thermo Scientific) added with a protease inhibitor cocktail (Halt Protease Inhibitor Cocktail; Thermo Scientific) and EDTA (1 mM). Protein concentrations were measured using a bicinchoninic acid protein assay kit (Thermo Scientific). Total protein (30 µg) was loaded onto protein gels (NuPAGE Novex 4%-12% Bis-Tris Protein gels; Invitrogen) for electrophoresis under reducing conditions and transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked with 5% nonfat milk in phosphate-buffered saline and Tween for 1 hour at room temperature, followed by incubation with primary antibodies at 4°C overnight. The primary antibodies used were rabbit antihuman ADBR1 (1:500; Santa Cruz), rabbit antihuman ADBR2 (1:1000; Abcam), goat antihuman ADBR3 (1:500;

Table. Patient Characteristics

IH Stage	Age at Excision, Mean (SD), mo	Site of Excision, No. of Patients		
		Head and Neck	Trunk and Abdomen	Extremities
Proliferative	7 (2.5)	6	3	1
Involuted	23.5 (10)	7	3	1
Propranolol treated	16 (10)	11	1	0

Abbreviation: IH, infantile hemangioma.

Santa Cruz), and rabbit antihuman glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1000; Cell Signaling Technology). Detection of GAPDH was used as the loading control. After washing with phosphate-buffered saline and Tween (Thermo Scientific) 3 times, membranes were reacted with either horseradish peroxidase–conjugated antirabbit or goat secondary antibodies (1:2000; Santa Cruz) for 1 hour at room temperature. Blots were developed (Novex ECL Chemiluminescent Substrate Reagent Kit; Invitrogen) for 1 minute in the dark and exposed on x-ray film (Thermo Scientific). Total protein concentrations were measured by using densitometry with GAPDH as the loading control.

Statistical Analysis

Statistical analysis for differences in mean amplification (PCR) and protein densitometry (Western blot) was performed with a 2-tailed, unpaired *t* test, with $P < .05$ considered statistically significant. The mean values in PCR were normalized against the 18s ribosomal RNA control. GraphPad Software (QuickCalcs 2016; GraphPad Software Inc) was used to calculate means, SDs, and 95% CIs.

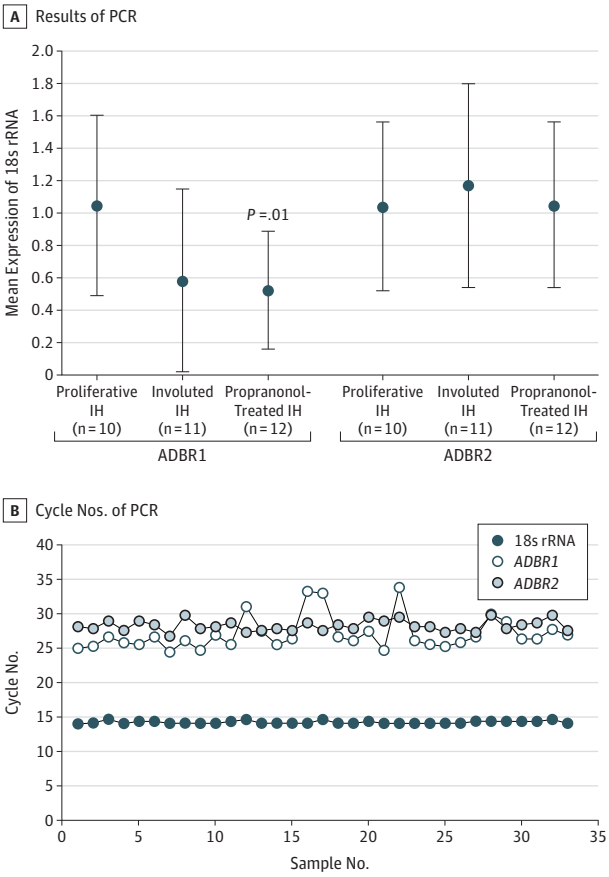
Results

A total of 33 specimens were collected from patients who underwent excision of problematic IH. Ten samples were obtained from lesions in the proliferative phase of growth, 11 were from lesions in involution, and 12 were from lesions that had been treated with propranolol and exhibited some response to treatment as indicated by a decrease in size and erythema. Of the 33 patients included, 21 were female (64%). The mean (SD) patient age at the time of excision was 7 (2.5) months, 23.5 (10) months, and 16 (10) months for each of the respective groups (**Table**). The head and neck were the most common sites of excision. For patients who were treated with propranolol, the duration of treatment ranged from 1 to 15 months before excision. Five patients (15%) underwent excision after propranolol therapy because of a protuberant fibrofatty residuum, 2 patients (6%) experienced regrowth when attempting to wean from propranolol, 2 patients (6%) experienced continued bleeding and ulceration of the lesion while receiving propranolol, 2 patients (6%) did not tolerate the medication, and 1 patient (3%) with IHs at multiple sites underwent excision of some of the smaller lesions owing to parental preference.

Detection of mRNA Expression Using Real-time PCR

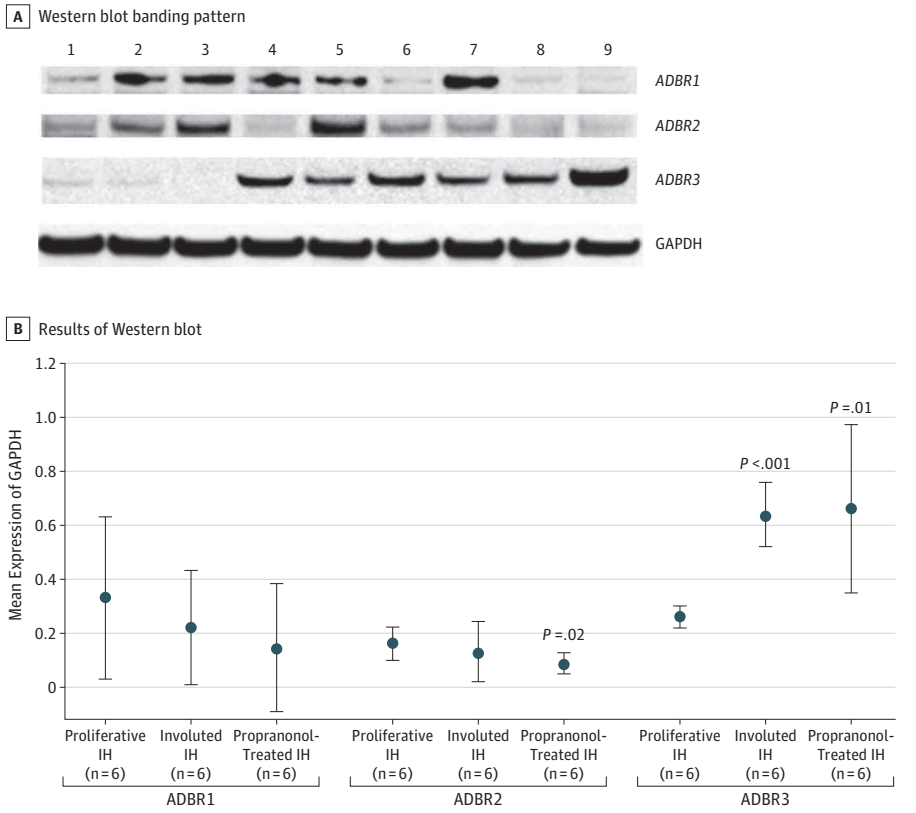
Ten proliferating, 11 involuted, and 12 propranolol-responsive IH specimens were used for real-time PCR to detect mRNA expression of each of the subtypes of β-adrenergic receptors. The mean (SD) expression levels of ADBR1 mRNA in proliferating IH were significantly higher than in propranolol-responsive IH (1.05 [0.56] vs 0.52 [0.36]; $P = .01$; 95% CI, 0.12-0.94). Mean expression of ADBR1 mRNA in the involuted group was also lower compared with the proliferative group, although this difference did not reach statistical significance (1.05

Figure 1. Real-time Polymerase Chain Reaction (PCR) Results of β-Adrenergic Receptors in Infantile Hemangioma (IH)



A, RNA samples from 10 patients with proliferating IH, 11 with involuting IH, and 12 with propranolol-responsive IH were reverse transcripted into complementary DNA and subsequently used for real-time PCR. The messenger RNA expression of the 18s ribosomal RNA was run in parallel as an internal control. The comparative (Ct) method was used to determine relative quantification. The amplification amount of all target genes was normalized against that of 18s ribosomal RNA. The levels of ADBR1 messenger RNA in patients with proliferating IH were significantly higher than in the other 2 groups, but this difference was statistically significant only for propranolol-treated IH. Error bars indicate SD. B, Cycle number data for ADBRs in real-time PCR. Infantile hemangiomas were proliferative in patients 1 to 10, involuted in patients 11 to 21, and propranolol treated in patients 22 to 33.

Figure 2. Results of β-Adrenergic Receptor (ADBR) Protein Expression in Infantile Hemangioma (IH)



A, Representative pictures of protein expression of ADBRs. Lanes 1 to 3, proliferating stage; lanes 4 to 6, involuted stage; and lanes 7 to 9, propranolol responsive. B, Total protein concentrations were measured with densitometry and compared with glyceraldehyde 3-phosphate dehydrogenase (GADPH) as the loading control. Mean ADBR1 protein expression was significantly elevated in proliferating IH compared with involuted IH and with propranolol-treated IH. Error bars indicate SD.

[0.56] vs 0.58 [0.56]; P = .07; 95% CI, −0.04 to 0.98) (**Figure 1**). Mean expression levels of ADBR2 mRNA showed no significant differences in the proliferating and involuted groups (1.04 [0.52] vs 1.17 [0.63]; P = .61; 95% CI, −0.40 to 0.66) or between the proliferating and propranolol-responsive groups (1.04 [0.52] vs 1.05 [0.51]; P = .96; 95% CI, −0.45 to 0.47) (Figure 1). There was no significant amplification of ADBR3 mRNA in any of the 3 groups.

Detection of Protein Expression of β-Adrenergic Receptors Using Western Blot Analysis

Six proliferating, 6 involuted, and 6 propranolol-treated IH specimens were used to detect protein expression of each of the subtypes of β-adrenergic receptors. Mean (SD) ADBR1 protein expression was not significantly different in proliferating IH compared with involuted IH (0.34 [0.30] vs 0.22 [0.21]; P = .47; 95% CI, −0.22 to 0.45) or in propranolol-responsive IH (0.34 [0.30] vs 0.15 [0.24]; P = .26; 95% CI, −0.16 to 0.54) (**Figure 2**). Although involuted ADBR2 mean protein expression was lower than the proliferative specimens, this difference did not reach statistical significance (0.17 [0.06] vs 0.13 [0.11]; P = .51; 95% CI, −0.08 to 0.16). However, propranolol-treated ADBR2 protein expression was significantly lower than expression in the proliferative specimens (0.17 [0.06] vs 0.09 [0.04]; P = .02; 95% CI, 0.02-0.14). Although there was not a distinct amplification of ADBR3 mRNA in any of the groups, ADBR3 protein expression was significantly higher in involuted (0.64 [0.12] vs 0.26 [0.04]; P < .01; 95% CI, 0.26-0.49) and propranolol-responsive (0.66 [0.31] vs 0.26 [0.04]; P = .01; 95% CI, 0.16-0.68) IH compared with proliferative IH.

Discussion

To our knowledge, this investigation represents the first to perform real-time PCR and Western blot analysis on IH in 3 different stages of clinical progression: proliferative, involuting, and responsive to propranolol treatment. Protein and mRNA expression levels for ADBR1 and ADBR2 were consistent in each group. However, although ADBR3 protein was detected in involuted and propranolol-treated IH specimens, there was no distinct ADBR3 mRNA expression in any of the groups, a finding similar to that of previous reports.[14]

Our results indicate differences between ADBR subtype mRNA and protein expression when comparing proliferative IH with either involuted IH or propranolol-responsive IH. Group comparisons showed that ADBR1 mRNA expression tended to be greater in proliferative IH, suggesting the down-regulation of ADBR1 expression as IH progresses into involution, whether by a time-dependent or pharmacologic mechanism. Our findings of a significantly greater amount of ADBR1 expression in proliferating IH compared with treated IH raises the possibility that propranolol's action on ADBR1 is an important component of its molecular mechanism.

The ADBR3 protein was elevated in involuted and propranolol-treated IH. With ADBR3 protein known to be nearly exclusively expressed in adipose tissue,[15] increased expression in treatment-responsive and involuted IH is predictable as the tissue transitions from tangled whorls of vascular endothelial cells to a fatty residuum. However, the timing and mechanism by which ADBR3 protein arises in IH tissue remains elusive. Our study sample included patients with a wide range of exposure to propranolol (1-13.5 months). It would perhaps be instructive to analyze tissue at staged resections after the initiation of propranolol therapy to determine whether the expression patterns shift over time.

As a nonselective β-blocker, propranolol has the potential for adverse effects from its blockade of ADBR2. Inhibition of ADBR2 receptors in the liver may result in hypoglycemia through decreased glycogenolysis and gluconeogenesis.[9] In addition, there exists concern for bronchial hyperreactivity due to blockage of pulmonary ADBR2, which may be present in as many as 8% of patients.[4] For this reason, there has been interest in conducting efficacy trials of an ADBR1-selective drug, such as atenolol. Hemangioma-derived endothelial cell proliferation has been demonstrated[16] to be abolished by both a β_1-selective antagonist as well as a β_2-selective antagonist, although not to the same degree. The administration of vascular endothelial growth factor 2 and extracellular signal-related kinase inhibitors demonstrated a similar effect. Another report[17] found equivalent, dose-dependent inhibition of vasculogenesis by nonselective ADBR blockade, ADBR1 blockade, and ADBR2 blockade. These effects were reversed by the administration of a nitric oxide donor. Early reports[18,19] from a comparison with a historical propranolol-treated group and a small randomized clinical trial indicate comparable clinical results with the use of atenolol.

Delineation of the presence or absence of ADBR subtype expression may prove useful in the investigation of why some IHs are unresponsive to propranolol therapy. Previous reports[20] have shown little histopathologic difference between responders and nonresponders, demonstrating similar immunohistochemical staining for ADBR1 and ADBR2; however, in these studies, the subtypes were not quantified by mRNA and protein expression. The patients in our study did not include any individuals who failed to show a response to propranolol therapy, but this outcome may be an area of future interest.

Potential limitations to the study include the small sample size, although, to our knowledge, this study represents the largest number of IH samples subjected to PCR and Western blot analysis to date. In addition, because the evolution of IH is age dependent, the molecular changes in the propranolol-treated group may be confounded by the fact that 58% of these patients were older than 1 year. However, if only the data from patients younger than 1 year are used, the differences in protein expression remain significant. Another consideration is that 2 patients who experienced regrowth after stopping the medication were included in the propranolol group. It is possible that these patients in effect reverted to a proliferative state in terms of their molecular signature. Finally, an immunohistochemical evaluation of the specimens would be of value to provide insight into which cell types are expressing ADBRs. The pathological endothelial cells of hemangioma tissue would be of primary concern, and it is possible that differing levels of ADBR expression of stromal cells within the samples could affect mRNA and protein expression analysis.

Conclusions

These data demonstrate a difference in the expression of ADBR subtypes in IH between various stages of clinical progression, with indications that ADBR1 is elevated in proliferative lesions but downregulated either by treatment or by the self-limiting nature of IH. In contrast, ADBR3 protein expression, although not mRNA, was elevated in involuting and treatment-responsive specimens compared with proliferative IH, which is consistent with the evolution to a more fatty tissue type in the involuted and treatment-responsive specimens. These findings may have clinical implications regarding the use of selective vs nonselective β-blockade.

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Concept and design: Zhang, Richter.
Acquisition, analysis, or interpretation of data: All authors.
Drafting of the manuscript: Phillips, Zhang, Richter.
Critical revision of the manuscript for important intellectual content: Phillips, Wei, Richter.
Statistical analysis: Phillips, Zhang.
Obtaining funding: Richter.
Administrative, technical, or material support: Zhang, Wei, Richter.
Study supervision: Richter.

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