Forkhead Transcription Factor FOXO1 Is Regulated by Both a Stimulatory Thyrotropin Receptor Antibody and Insulin-Like Growth Factor-1 in Orbital Fibroblasts from Patients with Graves' Ophthalmopathy

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Background: Activation of thyrotropin receptor (TSHR) and/or insulin-like growth factor (IGF-1) receptor (IGF-1R) enhances HA production and adipogenesis in orbital fibroblasts from patients with Graves' ophthalmopathy (GO) and recapitulates the tissue remodeling characteristic of the orbit in GO. A functional relationship between TSHR and IGF-1R has long been postulated, and recently bidirectional crosstalk between the receptors in GO fibroblasts was demonstrated. Because the transcription factor Forkhead box O-1 (FOXO1) was recently shown to be a critical downstream mediator of TSH and IGF-1 effects on thyrocyte proliferation, studies were designed to determine whether FOXO1 might similarly act as a common mediator of M22, a stimulatory TSHR antibody (TSAb), and IGF-1 in GO orbital fibroblasts.

Methods: *FOXO1* mRNA and protein were measured in orbital tissue specimens derived from normal individuals and patients with GO. In addition, the control of FOXO1 cellular localization was investigated using quantitative Western blotting of fractionated cell lysates from orbital fibroblasts treated with M22 and/or IGF-1 with or without specific TSHR, IGF-1R, or PI3K/AKT1/2 inhibitors.

Results: Significantly lower levels of both *FOXO1* mRNA and protein were found in GO orbital tissue specimens compared with normal orbital tissues ($M = 39\%$, $p = 0.043$; $M = 46.4\%$, $p = 0.028$, respectively). In addition, treatment of GO orbital cultures with M22, IGF-1, or M22 plus IGF-1 increased cytoplasmic FOXO1 compared with control (1.63-fold, $p = 0.008$; 1.68-fold, $p = 0.001$; 1.61-fold, $p \leq 0.001$, respectively) and decreased nuclear FOXO1 ($M = 28\%$, $p = 0.002$; $M = 38\%$, $p \leq 0.001$; $M = 35\%$, $p = 0.007$, respectively). These effects were inhibited by co-treatment with the respective, but not the opposite, receptor antagonist. AKT inhibition of M22 or IGF-1-treated cultures was found to increase nuclear (1.4-fold, $p = 0.026$; 1.3-fold, $p = 0.001$, respectively) and decrease cytoplasmic (24.2%, $p = 0.001$; 36%, $p = 0.004$, respectively) FOXO1 localization.

Conclusions: These data point to FOXO1 as an important mediator of TSAb and IGF-1 action via their cognate receptors in GO orbital fibroblasts. These findings provide a link between the low FOXO1 protein levels demonstrated in GO orbital tissue and the tissue remodeling characteristic of GO, and suggest novel therapy for GO aimed at increasing nuclear expression of FOXO1 in GO target cells.

Introduction

GRAVES' OPHTHALMOPATHY (GO) is a debilitating and potentially sight-threatening ocular autoimmune disease associated with Graves' hyperthyroidism. The orbit in GO is characterized by an increase in the volume of the extraocular muscles and orbital fat tissues. This connective tissue remodeling stems from increased production of hyaluronan (HA) by orbital fibroblasts and the development of new fat cells derived from a subset of these cells (1). In addition,

inflammatory cytokines and chemokines found within the orbital tissues are secreted by infiltrating immune cells, mast cells, as well as the resident orbital fibroblasts. As in Graves' hyperthyroidism, the thyrotropin receptor (TSHR) has been identified as a primary target antigen in GO, and autoantibodies directed against this receptor appear to play a direct role in disease development (2). TSHR activation by TSHR antibodies (TSAb) in cultured orbital fibroblasts results in both increased HA production and enhanced adipogenesis (3–6). Occurring within the orbit, these altered

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cellular processes would lead to tissue changes characteristic of the orbit in GO.

While TSA_b appear to have a direct pathogenic role in GO, it is unclear whether abnormal activation of IGF-1R within the orbit plays a role in the development of GO. However, a functional relationship between the TSHR and insulin-like growth factor-1 receptor (IGF-1R) was suggested in early studies demonstrating synergistic upregulation of cell proliferation and DNA synthesis in thyrocytes following simultaneous activation of both receptors (7–9). More recent studies showed that immunoglobulin G (IgG) isolated from the sera of patients with Graves' disease (GD-IgG), known to contain stimulatory TSA_b, increases production of HA by GO orbital fibroblasts. This effect is attenuated in cells either treated with a specific IGF-1R blocking antibody (1H7) or transiently transfected with a dominant negative mutant IGF-1R (10,11). Similarly, treatment of cells with 1H7 reduces M22-induced activation of both the cAMP/adenylate cyclase and phosphatidylinositol 3-kinase (PI3K)/AKT signaling cascades in GO cells (4).

A more recent study by Kreiger *et al.* found TSH or M22-induced HA secretion to be only partially inhibited in GO orbital fibroblasts treated with linsitinib, an IGF-1R-selective receptor kinase antagonist (12). Conversely, they also demonstrated HA production induced by IGF-1 to be partially inhibited by a small molecular TSHR antagonist, termed C1, while M22-induced HA secretion was completely attenuated by this antagonist. M22 did not stimulate autophosphorylation of the IGF-1R, the initial step in IGF-1R activation, indicating that M22 does not directly activate the IGF-1R. The authors concluded that both the TSHR activated by TSH or M22, and the IGF-1R activated by IGF-1, show bidirectional crosstalk and that M22 activation of the TSHR likely initiates 2 signaling pathways. In that model, activation of the TSHR alone constitutes the major pathway; the secondary pathway is based on TSHR-dependent activation of the IGF-1R.

The aim of the current study was to identify a downstream molecule regulated by activation of both the TSHR and the IGF-1R that might serve as a therapeutic target in GO. The Forkhead box O-1 (FOXO1) transcription factor mediates diverse cell functions, including differentiation, adipogenesis, oxidative stress response, apoptosis, and cell proliferation in many different cell types (13,14). FOXO1 has recently been shown to be a critical downstream mediator of TSH and IGF-1 effects on thyrocyte proliferation by promoting its exclusion from the nucleus in a PI3K/pAKT-dependent fashion (15). It was hypothesized that FOXO1 might similarly function as a common negative regulator of TSA_b and IGF-1R action in GO orbital fibroblasts. Accordingly, the expression of *FOXO1* mRNA and protein in orbital tissues derived from normal individuals and patients with GO was measured. In addition, the regulation of FOXO1 cellular localization was investigated in GO orbital cells cultured in the presence of M22 and/or IGF-1 with and without specific antagonists of TSHR, IGF-1R, or PI3K/pAKT signaling.

Materials and Methods

Cell culture

Orbital adipose tissue specimens were obtained from euthyroid female patients with GO undergoing orbital decom-

pression surgery for severe disease. The tissues were minced and placed directly in plastic culture dishes, allowing pre-adipocyte fibroblasts to adhere and proliferate, as described previously (16). The cells were initially grown in a humidified 5% CO₂ incubator at 37°C in medium 199 containing 20% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, UT), gentamicin (20 µg/mL), and penicillin (100 IU/mL). They were subsequently maintained in an undifferentiated state in 75 mm² flasks in medium 199 containing antibiotics and 10% FBS. This study was approved by the Mayo Clinic Institutional Review Board and was carried out according to institutional policy.

A series of experiments was performed to assess the control of FOXO1 cellular localization in undifferentiated GO orbital fibroblasts treated with the monoclonal TSA_b M22 (100 ng/mL; Kronus, Boise, ID; no. M22-1b) (17) and/or IGF-1 (10 ng/mL; R&D Systems, Minneapolis, MN; no. 291-G1). In some of these studies, a small molecule TSHR antagonist NGCG00229600 (C1; 10 µM) was used that was synthesized by the National Center for Advancing Translational Science and was kindly provided by Dr. Marvin Gershengorn (NIDDK, Bethesda, MD) (18). In addition, these experiments utilized a specific IGF-1R antagonist antibody (1H-7; 5 µg/mL; BD Biosciences, Franklin Lakes, NJ; no.555998), a PI3K inhibitor (LY294002; 50 µM; Cell Signaling Technology, Beverly, MA), and an AKT1/2 inhibitor (AKT-i-VIII [AKTi-1/2]; 10 µM; Calbiochem, Billerica, MA; #124018). In these experiments, confluent cells were pretreated with C1, 1H7, LY294002, or AKT1/2 inhibitor VIII for 30 min followed by 15 min exposure to the experimental conditions.

Real-time reverse transcription polymerase chain reaction analysis of FOXO1 tissue expression

Total RNA was isolated from orbital tissue specimens obtained from GO patients during the course of orbital decompression surgery (*n*=4) or from unaffected individuals undergoing orbital surgery for nonmalignant conditions (*n*=4) using a commercial kit (RNeasy kit; Qiagen, Valencia, CA). These studies were approved by the Mayo Clinic Institutional Review Board and carried out according to institutional policy. A minus reverse transcription reaction was performed on all samples to verify the absence of DNA contamination. Synthesis of cDNA proceeded using 750 ng of total RNA incubated with random hexamers, followed by a 100 µL reverse transcription reaction with 6.25 IU of MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA). Intron-spanning oligonucleotide primers and Taqman probes for *FOXO1* and *GAPDH* were purchased from Applied Biosystems. Conditions used were: 25°C for 10 min, 37°C for 60 min, and 95°C for 5 min. Expression of *GAPDH* was used to correct for differences in quantity of total RNA added to a reaction and to compensate for different levels of inhibition during reverse transcription of RNA and during polymerase chain reaction (PCR). Quantitative PCR reactions were performed in a 96-well optical reaction plate. Amplification reactions contained cDNA equivalent of 5 ng total RNA, 900 nM of the forward and reverse primers, and 250 nM of the probes in a volume of 25 µL using the Universal Taqman 2X PCR mastermix (Applied Biosystems). The reaction mixture without added cDNA was used as no

template control. The thermal cycling conditions used were: 2 min at 50°C for optimal AmpErase UNG activity, 10 min at 95°C to activate Amplitaq Gold DNA Polymerase, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The target genes and *GAPDH* were amplified in separate wells. All reactions were performed in duplicate in the ABI PRISM® 7700 Sequence Detector (Applied Biosystems) and the data pooled. The standard curve method was used to quantify the expression of the various genes and *GAPDH* rRNA in each sample. For each experimental sample, a gene was considered not expressed if amplification was not detected by cycle 40. The normalized results were expressed as the ratio of *FOXO1* to *GAPDH* rRNA.

Protein extraction and Western blotting; FOXO1 cellular localization studies

Lysates were prepared from cell cultures by scraping them from the dishes in Complete Lysis-M buffer (Roche, Indianapolis, IN). Protein was extracted from the lysates using nuclear and cytoplasmic extraction reagents (NE-PER; Thermo Scientific, Rockford, IL). In other studies, total protein was extracted from GO and normal orbital tissue specimens by homogenizing tissues in the same buffer. Protein concentration was determined against BSA standards using Nanodrop 1000 (Thermo Fisher Scientific, Wilmington, DE). Extracts (5–10 µg) were separated using electrophoresis in 4–12% Bis-Tris gels, electrotransferred to polyvinylidene fluoride membranes, and blotted with a primary antibody against the TSHR (Santa Cruz Biotechnology, Santa Cruz, CA; #sc-7816), FOXO1, IGF-1R, tubulin, Sp1, or glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; Cell Signaling Technology; #2880, #3018, #2148, #5931, #2118, respectively) at 1:1000 dilution. The appropriate secondary IgG-horseradish peroxidase-linked conjugate (Cell Signaling Technology, #7074) at 1:2000 dilution was applied, followed by enhanced chemiluminescence detection. In each case, an initial blot was performed omitting the primary antibody to verify the absence of nonspecific binding of the secondary antibody. Findings are expressed both visually by a representative immunoblot and via densitometric analysis of bands that were quantitated using ImageJ 1.47V analysis software and normalized relative to its loading control.

Statistical analyses

The paired *t*-test was used to evaluate differences in means for continuous variables, with values presented as the mean ± standard error of the mean (SEM). Differences between values were considered significant when the *p*-value was <0.05. The Mann–Whitney rank sum test was used to assess differences between groups.

Results

FOXO1 mRNA and total protein levels are reduced in GO orbital tissue specimens

In order to explore the potential importance of FOXO1 in the pathogenesis of GO, both mRNA encoding FOXO1 as well as total (unfractionated) FOXO1 protein levels were measured in orbital tissue specimens (noncultured) obtained from patients with severe GO at the time of orbital decompression surgery (*n*=4) or unaffected normal individuals undergoing orbital surgery for nonmalignant conditions (*n*=4). Significantly lower levels of *FOXO1* mRNA were found in GO orbital tissue specimens compared with normal orbital tissues ($M=37 \pm 9.3\%$, $p=0.043$; $n=4$ each; data not shown). Similarly, Western blotting studies demonstrated significantly lower levels of FOXO1 protein in GO orbital tissues compared with normal orbital tissues ($M=46.4 \pm 8.8\%$, $p=0.028$; Fig. 1)

M22, IGF-1, or M22 plus IGF-1 decrease nuclear and increase cytoplasmic FOXO1 protein levels

It was hypothesized that FOXO1 might function as a common mediator of TSHR and IGF-1R signaling in GO orbital fibroblasts. Accordingly, experiments were performed to determine whether treatment with M22, IGF-1, or a combination of the two agents, with or without C1 or 1H7, might increase the cytoplasmic fraction and/or induce nuclear exclusion of this protein (*n*=9). Quantitation of Western blots showed that treatment of confluent GO orbital cultures for 15 min with M22, IGF-1, or the combination of M22 plus IGF-1 resulted in increased cytoplasmic FOXO1 protein compared with control levels (1.63-fold, $p=0.008$; 1.68-fold, $p=0.001$; 1.61-fold, $p=0.001$, respectively; Fig. 2). In addition, M22, IGF-1, or the combination of M22 plus

FIG. 1. Top, representative Western blot of Forkhead box O-1 (FOXO1) and GAPDH protein in normal and Graves' ophthalmopathy (GO) orbital tissue samples. Bottom, densitometric quantitation showing net intensity of bands in normal (*n*=4) and GO (*n*=4) samples (normalized to GAPDH; **p*<0.05).

FIG. 2. Top, representative Western blot showing nuclear and cytoplasmic fractions of FOXO1 and GAPDH protein in undifferentiated GO orbital fibroblast cultures treated with IGF-1 (10 ng/mL) or M22 (100 ng/mL) with or without C1 (10 μ M) or 1H7 (5 μ g/mL). Bottom, densitometric quantitation showing net intensity of bands (normalized to GAPDH; $n=9$; $*p<0.05$). Top brackets refer to differences in cytoplasmic fractions; bottom brackets refer to nuclear fractions.

IGF-1 treatment were found to decrease nuclear FOXO1 protein compared with control levels ($M=28\%$, $p=0.002$; $M=38\%$, $p=0.001$; $M=35\%$, $p=0.007$, respectively). No synergistic effects were detected following simultaneous activation of both TSHR and IGF-1R.

Neither C1 nor 1H7 alone were found to alter cytoplasmic or nuclear FOXO1 compared with control levels. However, M22 plus C1 decreased cytoplasmic FOXO1 protein to control levels, representing a significant decrease from M22-treated levels ($M=23.5\%$, $p=0.041$). Similarly, treatment with IGF-1 plus 1H7 decreased cytoplasmic FOXO1 protein to control levels, representing a significant decrease from IGF-1-treated levels ($M=34.5\%$, $p=0.006$). Changes in nuclear FOXO1 levels in cultures treated with either M22 plus C-1 or IGF-1 plus 1H-7 did not reach significance. Additionally, 1H7 was found to have no effect on cytoplasmic or nuclear FOXO1 levels in M22-treated cultures, and C1 was found to have no effect on cytoplasmic or nuclear FOXO1 levels in IGF-1-treated cultures.

AKT inhibition of M22 or IGF-1-treated cultures increases nuclear and decreases cytoplasmic FOXO1 protein localization

Other experiments were performed to assess whether PI3K/pAKT signaling is involved in the cytoplasmic increase and nuclear exclusion of the FOXO1 protein induced by M22 or IGF-1. Again both M22 and IGF-1 were found to induce nuclear exclusion of FOXO1 (28.4% decrease, $p=0.026$; 37.9% decrease, $p=0.001$, respectively; Fig. 3) and increase cytoplasmic FOXO1 protein levels (1.29-fold, $p=0.001$; 1.37-fold, $p=0.006$, respectively). Neither the PI3K inhibitor LY294002 nor the AKT1/2 inhibitor VIII alone altered control levels of FOXO1. However, in IGF-1-treated cells, both inhibitors significantly decreased cytoplasmic FOXO1 protein levels (24% inhibition, $p=0.011$; 36% inhibition, $p=0.004$, respectively). In addition, AKT inhibition signifi-

cantly increased nuclear FOXO1 levels (1.3-fold, $p=0.001$), while PI3K inhibition in IGF-1-treated cultures did not significantly impact nuclear FOXO1 levels.

In M22-treated cultures, AKT inhibition decreased cytoplasmic (24.2% decrease, $p=0.001$) and increased nuclear FOXO1 protein levels (1.4-fold, $p=0.026$). Cells cultured with M22 plus LY294002 showed a trend toward decreased cytoplasmic FOXO1 levels (8.5% inhibition, $p=0.074$) and did not significantly impact nuclear FOXO1 protein levels.

Discussion

The proteins of the Forkhead box O (FOXO) family are transcription factors that participate in many and varied cellular processes, depending on the cell type and nature of the stimulus (19). While these proteins have overlapping functions, specialized roles for the various members of the family have been described. In particular, FOXO1, a substrate of AKT, has been shown to be a negative regulator of adipogenesis (20). This is potentially relevant to GO, which is in part characterized by enhanced adipogenesis within the orbital tissues (1). FOXO1 is thought to inhibit adipogenesis by binding to the promoter sites of peroxisome proliferator-activated receptor gamma ($PPAR-\gamma$) (21). This prevents its transcription of $PPAR-\gamma$ required to initiate adipogenesis. In contrast, adipogenesis proceeds following stimulation by insulin/IGF-1, which leads to phosphorylation of FOXO1 by AKT on Thr-24, Ser-256, and Ser-319, and rapid exclusion of FOXO1 from the nucleus. Cytoplasmic FOXO1 is then ubiquitinated and degraded, and thus unable to inhibit adipogenesis. While FOXO1 has been firmly implicated in the negative regulation of adipogenesis, it is unknown whether it is also involved the control of HA synthesis.

It was found that orbital tissues obtained at the time of orbital decompression surgery from patients with GO contain significantly lower levels of FOXO1 mRNA (37% lower) and protein (46% lower) than do orbital tissues from unaffected

FIG. 3. Top, representative Western blot showing nuclear and cytoplasmic fractions of FOXO1 and GAPDH protein in undifferentiated GO orbital fibroblast cultures treated with IGF-1 (10 ng/mL) or M22 (100 ng/mL) with or without an AKT inhibitor (10 μ M) or PI3K inhibitor LY294002 (50 μ M); Bottom, densitometric quantitation of net intensity of bands (normalized to GAPDH; $n=3$; $*p<0.05$). Top brackets refer to differences in cytoplasmic fractions; bottom brackets refer to nuclear fractions.

individuals (Fig. 1). This constitutes the first demonstration of FOXO1 expression in GO and normal orbital tissues. These findings mirror an earlier report demonstrating this protein to be more highly expressed in differentiated rat thyroid cells and human thyroid tissue than in human thyroid tumor-derived cells or surgical thyroid cancer specimens (12). Krieger *et al.* reported that treatment of differentiated thyrocyte cultures with either TSH or forskolin, an agent that constitutively activates adenylyl cyclase, for 24–48 h significantly increased FOXO1 protein levels. In contrast, neither IGF-1 alone nor wortmannin (a PI3K inhibitor) in concert with either IGF-1 or TSH impacted thyrocyte FOXO1 protein levels. Treatment of thyrocytes with bortezomib, a protease inhibitor, inhibited the drop in FOXO1 protein levels seen following treatment with TSH; this agent did not impact IGF-1-treated cells (9). Brenner-Gati *et al.* concluded that the cAMP pathway mediates FOXO1 levels in thyrocytes via degradation by the proteasome. In contrast, in some other tissues, FOXO1 proteasomic degradation appears to be entirely dependent on activation of the PI3K pathway (9). While mechanisms involved in FOXO1 degradation were not investigated, it is possible that high TSAb levels in patients with Graves' disease might act via cAMP (or PI3K/pAKT) to stimulate the proteasome machinery and degrade the FOXO1 protein within the orbit, resulting in the low levels of this protein detected in the GO tissue specimens. In addition, as it was found that both IGF-1 and M22 increase cytosolic levels of FOXO1, thus facilitating its degradation by the proteasome, their presence in GO tissues might contribute to the reduction in FOXO1 expression.

In order to determine whether FOXO1 might function as a common mediator of TSHR and IGF-1R signaling in GO orbital fibroblasts, the potential impact of these agents on the cellular localization of this transcription factor was studied. It was demonstrated that treatment with M22, IGF-1, or a combination of M22 plus IGF-1 significantly increases levels of cytoplasmic FOXO1 protein and also decreases nuclear FOXO1. No synergistic effects were detected following

simultaneous activation of both TSHR and IGF-1R. These findings constitute the first evidence that either TSAb or IGF-1 may act independently in GO orbital fibroblasts to impact cellular functions such as adipogenesis known to be negatively regulated by FOXO1.

The finding that either M22 plus C1 or IGF-1 plus 1H-7 significantly decreased cytoplasmic FOXO1 and trended toward increasing nuclear FOXO1 was expected and is consistent with C1 and 1H-7 being specific antagonists of their target receptors. In contrast, it was found that 1H7 had no effect on cytoplasmic or nuclear FOXO1 levels in M22-treated cultures, and C1 had no effect on cytoplasmic or nuclear FOXO1 levels in IGF-1 treated cultures. In contrast, Krieger *et al.* demonstrated a bidirectional TSHR/IGF-1R crosstalk with partial suppression of HA by C1 or 1H-7 in GO cells stimulated by IGF-1 or M22, respectively, and synergistic HA secretion when stimulated both M22 and IGF-1 (12). The present findings would appear to counter those of Krieger *et al.* regarding TSHR/IGF-1R crosstalk in GO orbital fibroblasts. However, because FOXO1 levels were assessed following only 15 min stimulation with M22 or IGF-1, while HA secretion was measured after 5 days in culture in the study by Krieger *et al.*, it is possible that crosstalk develops over a longer time frame than does the rapid exclusion of FOXO1 from the nucleus. Alternatively, although M22 and IGF-1 stimulate both HA production and the nuclear exclusion of FOXO1 in a pAKT-dependent fashion (see below), it is possible that genes involved in HA production are not transcriptional targets of FOXO1.

The serine/threonine-specific protein kinase AKT is downstream of insulin/IGF-1 signaling and is activated by PI3K through the phosphorylation of insulin receptor substrates-1 and -2. One of the substrates of AKT is FOXO1, which is inactivated by phosphorylation by AKT (13). The current study demonstrates that the cellular localization of FOXO1 in response to either TSHR or IGF-1R activation is AKT-dependent. These findings are similar to those by Zaballos and Santisteban showing AKT regulation of FOXO1 exclusion from the nucleus

in thyrocytes in response to TSH or IGF-1 treatment; simultaneous treatment with both compounds was not reported (15). In those studies, as in the present study, PI3K inhibition by LY290042 was less efficient. In conjunction with previous work in which PI3K/pAKT signaling was demonstrated to be involved in both adipogenesis and HA secretion in GO orbital fibroblasts (3,4), these results are compatible with both cellular processes being regulated by FOXO1 in these cells.

In conclusion, this study provides the first evidence that FOXO1 is involved in the actions of both TSAb and IGF-1 via their cognate receptors in GO orbital fibroblasts. The data do not point toward FOXO1 as a mediator of TSHR/IGF-1R crosstalk in these cells. These findings may provide a link between the low FOXO1 protein levels demonstrated in GO orbital tissue and the tissue remodeling characteristic of GO, and suggest that it may be of interest to develop novel therapies aimed at increasing nuclear expression of FOXO1 in GO fibroblasts. Targeting the TSHR, rather than the ubiquitous IGF-1R or both receptors simultaneously, would be expected to raise FOXO1 levels within the orbit without imparting nonspecific effects in other tissues.

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The authors have nothing to disclose.

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