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endothelium will serve to attenuate the overall oxidative burden. Thus, the integrative effect of IGF signaling would be maintenance and repair of the soma with the concomitant attenuation of an increased oxidative burden by the vascular endothelium.

To test elements of this hypothesis, we examined IGF-1’s effects on dermal fibroblasts, representing nonspecialized somatic cells of the skin, and human umbilical vein endothelial cells (HUVECs), representing the vascular endothelium.

METHODS

Cell Cultures

Human dermal fibroblasts from five independent donors (GM05388, GM05399, GM05658, GM08398, and GM09503) were obtained from Coriell Cell Repositories (Coriell Institute, Camden, NJ). Fibroblasts were cultured in high-glucose Dulbecco’s Modified Eagle Medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin. HUVEC were isolated from umbilical veins of five healthy newborns at the University of Medicine and Dentistry of New Jersey-University Hospital, Newark, NJ, as described (21). Endothelial cells were cultured in gelatin-coated flasks with Endothelial Growth Medium-2 (EGM-2) medium (Lonza, Basel, Switzerland). Cells were maintained at 37°C with 5% CO₂ and passaged at 80%–90% confluence. All cell strains used for experiments were below passage 10 in culture.

Treatments

Cell cultures at 80%–90% confluence were washed twice with PBS and serum-starved overnight in high-glucose DMEM/1% penicillin–streptomycin (fibroblasts) or in endothelial basal medium-2 supplemented with 1% penicillin–streptomycin (HUVECs). Serum-free starvation media contained no growth factors or binding proteins. Cells were then treated with physiologic concentrations (0–100 ng/mL; [22]) of recombinant human IGF-1 (provided by Dr. P. Cohen) in starvation media and collected at 6 hours for transcript expression assays or at 24 hours for protein and activity assays. All experiments were performed with fibroblast and HUVEC cultures between passages 4–9. Five independent donor strains of fibroblasts and HUVECs were used in each experiment.

Quantitative Real-Time PCR

Total RNA was isolated from adherent cells in 60-mm culture dishes using an RNeasy Mini kit (Qiagen, Valencia, CA) according to manufacturer’s instructions. Genomic DNA was removed by treatment with DNase I (Promega, Madison, WI). One microgram RNA was reverse-transcribed using qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD). Antioxidant enzyme transcripts were measured by custom-designed TaqMan assays (Applied Biosystems, Carlsbad, CA) for catalase (Hs00937394), glutathione peroxidase (GPX)-1 (Hs00829989), superoxide dismutase (SOD)-1 (Hs00166575), SOD2 (Hs00167309), and eNOS (Hs01575469); transcript levels were normalized to GAPDH endogenous control (Hs00403636). All reactions were performed in the 7300 Real-Time PCR system (Applied Biosystems). Conditions were as follows: one cycle of 45°C for 3 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Relative mRNA expression was calculated using the ΔCt method, with normalization to the values for GAPDH.

Western Immunoblot Analysis

Following lysis in a buffer containing 0.5% NP-40, cell lysates were prepared by resuspending in equal volumes of sodium dodecyl sulfate loading buffer. Samples were denatured, and proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes and probed with antibodies to catalase, GPX1, and SOD2 (Abcam, Cambridge, MA); SOD1 (Santa Cruz Biotechnology, Santa Cruz, CA); and phospho-eNOS (Cell Signaling Technology, Danvers, MA). Blots were stripped and reprobed with antibodies to β-actin and/or α-tubulin loading controls (Cell Signaling Technology). Immunoreactive protein complexes were visualized with enhanced chemiluminescence reagents (GE Healthcare, Piscataway, NJ). Band intensities were quantified by densitometry and normalized to intensities of loading controls using Labworks software (PerkinElmer, Waltham, MA).

Enzyme Activity Assays

Enzyme activities in supernatants were assayed with kits from Cayman Chemical (Ann Arbor, MI) according to manufacturer’s instructions. In brief, the activity assays of catalase, SOD, and GPX are given below.

Catalase.—Cells were sonicated in a 50 mM potassium phosphate pH 7.0, 1 mM EDTA buffer. Following high-speed centrifugation (>10,000g), supernatants were collected and stored at −80°C. In the assay, H₂O₂ not neutralized by catalase was quantified by an oxidative coupling reaction that released dye detectable at 520 nm (23).

Superoxide dismutase.—Cells were sonicated in a buffer composed of 20 mM HEPES pH 7.2, 1 mM ethylene glycol tetraacetic acid, 210 mM mannitol, and 70 mM sucrose. Following centrifugation at 1,500g, xanthine oxidase was added to supernatants to generate superoxide radical; active SOD in samples reduced fluorescence detectable at 450 nm (24).

Glutathione peroxidase.—Cells were sonicated in a buffer composed of 10 mM Tris pH 7.5, 5 mM EDTA, and 1 mM dithiothreitol. Following high-speed centrifugation,
supernatants were combined with glutathione, glutathione reductase, and NADPH; NADPH oxidation paralleled GPX1 activity and was detectable at 340 nm (25).

Total Antioxidative Capacity Assay
Antioxidative capacity (AOC) was measured with dihydrodorhodamine (DHR 123; Invitrogen, Carlsbad, CA) as an ROS indicator (26). Cells were serum-starved overnight and treated with 0–100 ng/mL IGF-1 for 24 hours. Thirty micromolar DHR was added to cells along with 100 μM H₂O₂ as an oxidative challenge. After 30 minutes, cells were collected by trypsinization and analyzed by flow cytometry, as described (27). Ten thousand cellular events were collected per sample, and mean fluorescence intensity of oxidized rhodamine dye was determined in each IGF-1 treatment condition. AOC was determined by calculating the ratio of DHR-oxidized mean fluorescence intensity of cells unchallenged versus challenged with H₂O₂; a larger ratio corresponded to greater AOC.

Statistical Analysis
Because results from the five independent donor strains within each cell type (fibroblast and HUVEC) were expected to be correlated, repeated measures analysis was performed for each marker using a compound symmetry correlation structure (the same correlation between any two measures within a strain). For transcript and protein levels, differences in marker values from IGF dose of 0 ng/mL were calculated, and a linear model was then fit to these differences using IGF-1 dose on the log₁₀ scale as the independent variable for expression levels. To determine if there were differences in the trend of the IGF-1 dose between cell types, we tested for an interaction. We estimated the slopes for each cell type and tested whether the slope was significantly different from zero. Because activity levels were measured at only two IGF-1 doses (0 and 75 ng/mL), we estimated the mean differences within and between cell types and tested whether the differences were significantly different from zero. Because sample sizes were small, and tests of normality would be underpowered, we did not test for normality of errors. We used robust standard errors for all testing. The analysis was performed using SAS 9.1 (SAS Institute, Cary, NC). Testing was two sided and at the 5% significance level.

RESULTS
IGF-1 Differentially Regulates Antioxidant Enzyme Expression in Fibroblasts and HUVECs
We began by examining the influence of IGF-1 on transcription and expression of key enzymatic antioxidants that neutralize ROS within the cell. Studies in lower organisms (28) and some mammalian cells (12) have demonstrated that IGF-1 phosphorylates FOXO transcription factors, preventing the transactivation and upregulation of genes encoding SOD and catalase. We examined the effects of IGF-1 on copper/zinc SOD (SOD1), manganese SOD (SOD2), catalase, and GPX (GPX1) in fibroblasts and HUVECs. Cell strains from five independent donors were used in each experiment to account for human donor variability. We treated serum-deprived cells with physiologic concentrations of recombinant human IGF-1 (0–100 ng/mL; [22]) for 6 hours and performed quantitative real-time PCR with primers designed to measure expression levels of alternatively spliced transcripts for each enzyme. We observed a dose-dependent decrease of SOD1, SOD2, CAT, and GPX1 transcripts in fibroblasts in response to IGF-1 treatment (Figure 1), an effect that was statistically significant for SOD1 expression (p = 0.25). In contrast, IGF-1 upregulated the expression of these enzymatic antioxidants in HUVECs, with a dose-dependent effect that was significant in catalase and SOD1 expression (p = 0.01 and p = 0.03, respectively). The difference between IGF-1 signaling and consequent influence on antioxidative enzyme transcripts in the two cell types was evidenced by the divergent directions of its effects: In HUVECs, the effect was largely positive (ie upregulation) for all enzymes, whereas in fibroblasts, the effect was largely negative. The differences in response (slopes) of fibroblasts versus HUVECs to IGF-1 were statistically significant for catalase (p = 0.03) and SOD1 (p = 0.003) expressions.

Given the differences in oxidative enzyme transcripts in these cells in response to IGF-1, we next examined protein expression by Western blot analysis 24 hours after treatment (Figure 2A–F). Here, we observed that in the fibroblasts, IGF-1 had a suppressive effect on the expressions of catalase (p = 0.01) and SOD1 (p = 0.03) protein expressions (though some cell strains had increased SOD1 expression when treated with 1 ng/mL IGF-1, repeated measures analysis for dose–response to IGF-1 across all five strains was significantly negative). The trend was in the opposite direction in the HUVECs, where IGF-1 increased catalase, SOD2, and GPX1 protein expressions, although the effect was significant only for GPX1 (p = 0.08). The differences in responses (slopes) between fibroblasts and HUVECs to IGF-1 signaling were significant in GPX1 expression (p = 0.02).

IGF-1 Diminishes SOD Activity and Antioxidative Capacity of Fibroblasts
Having observed differences between IGF-1–mediated changes of antioxidant enzyme expression in fibroblasts versus HUVECs, we next assayed the activities of these enzymes in cell-free assays. We exposed cell strains to 0 or 75 ng/mL IGF-1 and measured total SOD, catalase, and GPX1 activities in supernatants of sonicated cells, normalizing values to total protein content. Following the trend of
SOD transcripts and protein, we observed a significant decrease in total SOD activity of fibroblasts in response to IGF-1 treatment \( (p = .001) \). In contrast, SOD activity in HUVECs was unaffected by exposure to IGF-1; thus, changes in SOD activity in response to IGF-1 treatment differed significantly between the two cell types \( (p = .001; \text{Figure } 2G) \). With regard to catalase and GPX1 activities, there was considerable scatter of the data in the five strains of each cell type (Supplementary Table 1). Although catalase activity appeared to diminish in response to IGF-1 not only in fibroblasts but also HUVECs, these effects were not statistically significant.

We then examined the cumulative AOC by the overall ROS neutralization in a cell-based system. We used dihydrorhodamine (DHR) 123, a ROS indicator that is irreversibly oxidized and converted to fluorescent rhodamine when exposed to intracellular reactive oxygen intermediates (29). Following acute hydrogen peroxide exposure, fibroblasts treated with IGF-1 displayed lower capacity to neutralize ROS, as indicated in Figure 3 by a dose-dependent decrease in the ratio of rhodamine fluorescence in baseline versus H\(_2\)O\(_2\)-challenged cells (negative effect; \( p = .031 \) for the trend). In contrast, the AOC in HUVECs was stable in response to increasing doses of IGF-1, indicating that the oxidative defenses in these cells were not impaired by IGF-1 treatment (Figure 3). Fibroblasts and HUVECs differed significantly in their dynamic abilities to neutralize ROS after preexposure to IGF-1 \( (p = .028) \).

**IGF-1 Upregulates Active eNOS in HUVECs**

We next examined additional pathways through which IGF-1 could modify the oxidative status of somatic cells, thereby explaining further how low IGF-1 levels might affect human health. Akt, a downstream mediator of IIS, phosphorylates and activates eNOS, which catalyzes the production of nitric oxide, a key antiproliferative and under certain circumstances an antioxidant agent in the vasculature (19,30–32). We thus examined eNOS transcription by real-time PCR and measured phosphorylated eNOS levels as a marker of activation in fibroblasts and HUVECs in response to IGF-1 treatment. Although eNOS is primarily expressed in the endothelium, there have been reports of its basal expression and activity in dermal fibroblasts (33). We detected high eNOS expression in all HUVEC cell strains, though there was no correlation with IGF-1 dose (Figure 4A). In contrast, eNOS levels were detectable in only two of the five fibroblast cell strains studied and at levels significantly below those seen in HUVECs. Total eNOS protein in fibroblasts and HUVECs was also not affected by IGF-1 treatment (data not shown). Although eNOS expression was not upregulated in response to IGF-1 in either cell type, we

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**Figure 1.** Insulin-like growth factor 1 (IGF-1) effect on transcription of antioxidant enzymes. Catalase (A), glutathione peroxidase (B), and superoxide dismutase (C-D) transcripts were measured by real-time quantitative PCR. Mean ± SEM of the change (Δ) in relative transcript expression upon IGF-1 treatment is plotted; experiments were performed in duplicate in five cell strains of serum-starved fibroblasts and human umbilical vein endothelial cells between passages 4–9. Effects indicate slopes obtained from linear models of log10 IGF-1 dose versus change in relative transcript expression; \( * p < .05, **p < .01 \) for trend (exact \( p \) values delineated in text).

**Figure 2.** Antioxidant enzyme levels and activities in response to insulin-like growth factor 1 (IGF-1) treatment. Mean ± SEM of Δ-actin and β-tubulin loading controls, and the change (Δ) in relative transcript expression upon IGF-1 treatment is plotted; experiments were performed in duplicate in five cell strains of serum-starved fibroblasts and human umbilical vein endothelial cells between passages 4–9. Effects indicate slopes obtained from linear models of log10 IGF-1 dose versus change in relative transcript expression; \( * p < .05, **p < .01 \) for trend (exact \( p \) values delineated in text).
Figure 2. Antioxidant enzyme levels and activities in response to insulin-like growth factor 1 (IGF-1) treatment. A–F, Protein levels were determined by Western immunoblot analysis. Integrated optical density (IOD) of each enzyme band was normalized to β-actin and α-tubulin loading controls, and the change (Δ) in relative IOD upon IGF-1 treatment is plotted. Shown are catalase (A), glutathione peroxidase (B), and superoxide dismutase (SOD; C–D); mean ± SEM of responses from five strains of each cell type after serum starvation are plotted. Effects indicate slopes obtained from linear models of log_{10} IGF-1 dose versus change in relative IOD; *p < .05, **p < .01 for trend (exact p values delineated in text). E–F, Western blots of antioxidant enzymes and loading controls from a representative fibroblast strain (GM05399, panel E) and human umbilical vein endothelial cell (HUVEC) strain (newborn donor #2, panel F). G, Total SOD activity (units per mL) in supernatants from five strains of fibroblasts and HUVECs after overnight serum starvation was normalized to total protein levels in milligrams; mean ± SEM of change (Δ) in activity upon 24 hour IGF-1 treatment (75 ng/mL) is plotted. Experiments were performed in duplicate in each cell strain, and mean changes were tested for significance (**p = .001).
observed a dose-dependent increase in phospho-eNOS levels in HUVECs (ANOVA p < .001, Figure 4B–C); levels (where detectable) were unchanged in fibroblasts, further suggesting that IGF-1 signals through different mechanisms in these cells to affect changes in ROS neutralization and oxidative capacity.

**Discussion**

The findings of this study might reconcile an apparent paradox: In lower organisms, reduced IIS consistently results in increased life span (34), whereas in humans, low IGF-1 levels are associated with increased mortality and morbidity from aging-related diseases, including atherosclerosis and diabetes (14–16,35). We tested the effect of physiologic concentrations of IGF-1 on fibroblasts as a model of lower organisms and HUVECs as a model of the specialized human vascular endothelium.

We observed largely divergent responses to IGF-1’s effect on the expression of key antioxidant enzymes in these two cell types. In fibroblasts, IGF-1 downregulated SOD and catalase transcripts and protein expression in a manner consistent with that observed in lower organisms (36,37), resulting in an overall negative/suppressive effect on antioxidant capacity. In HUVECs, however, IGF-1 exerted an overall positive effect on the antioxidant capacity by increasing catalase and SOD1 transcripts as well as GPX1 protein expression. We were unable to demonstrate similar trends in the enzymatic activities of catalase and GPX1, but we did observe that in response to IGF-1, total SOD activity decreased in fibroblasts but was undiminished in HUVECs, supporting the notion that SOD inhibition is the driving force in IGF-1–mediated downregulation of AOC in fibroblasts. Of note, antioxidant enzymes interact in a complementary fashion in vivo in response to the physiological state of the cell to tightly regulate ROS neutralization; in vitro activity assays performed in cell-free extracts hardly reflect the interaction of these enzymes within the cell in determining the final enzymatic activities of individual antioxidants.

Aging is characterized by a progressive accumulation of deleterious changes within the cells, which compromise normal function. In the vasculature, increased production of ROS decreases nitric oxide bioavailability and results in inflammation, vasoconstriction, and atherogenesis (32); thus, mechanisms to counteract the oxidative burden in the
endothelium are crucial for successful vascular aging (38). eNOS catalyzes the production of nitric oxide, directly opposing endothelial dysfunction. In this work, we confirmed reports of insulin/IGF-1-stimulated eNOS activation in vascular cells (20,39) by demonstrating that IGF-1 upregulated active (phosphorylated) eNOS in HUVECs; though contrary to previous studies (20,40), we did not see an increase in eNOS expression in response to IGF-1. Notably, a similar trend was not observed in fibroblasts, further indicating that IGF-1 signals that impact oxidative status differ between the two cell types.

IGF-1’s prooxidant effects in lower organisms are mediated through members of the FOXO family, which transcriptionally regulate antioxidant enzymes (12,28). Several recent studies have described FOXO3A genotypes that are associated with longevity in humans (41–43); thus, modulation of FOXO activity by IIS could account for some of the IGF-1’s effects on oxidative status in somatic cells. However, little is known about whether the IGF-1–mediated eNOS response affects the oxidative status of the cell due to intrinsic eNOS activity alone or also by mediating the response of antioxidant enzymes. This concept merits further examination.

Our findings support the following model of IGF-1 signaling, oxidative stress, and human longevity. On the one hand, the IGF-1 pathway is crucial for growth, development, and somatic maintenance, which is essential for long-lived mammals. On the other hand, it simultaneously downregulates antioxidant enzymes, increasing oxidative stress, as occurs in lower organisms. These are the findings displayed by the skin fibroblast. However, in the vascular endothelium, a systemic tissue that interacts with elements at both sides of the vasculature throughout the body (and modeled by the HUVEC), the growth effect of IGF-1 is not accompanied by increased oxidative stress. This phenomenon might stem from the lack of an inhibitory effect of IGF-1 on antioxidant enzymes and the concomitant activation of eNOS. Such a dichotomous action of IGF-1 would best serve humans by facilitating the maintenance/repair potential of IGF-1 while minimizing its injurious effect, particularly in the vasculature. Such a paradigm would explain the association of low circulating IGF-1 with cardiovascular disease and diabetes (14–16,35). Given current findings, it would be interesting to explore the impact of IGF-1 on the oxidative status in cells other than fibroblasts and endothelial cells (e.g., epithelial cells, neurons, muscle, etc.). Of note, IGF-1 diminishes antioxidant defenses in vitro in murine hepatocytes (44) as it does in human fibroblasts.

In summary, the seemingly conflicting roles that IIS plays in determining longevity of model organisms versus humans might now be put into proper context. Worms and flies, which are primarily postmitotic, do not require IGF-1 for proliferative repair; thus, dampened IIS enhances antioxidant defenses through unopposed FOXO activity and confers longevity. The short-lived mouse invests more energy in reproduction and less in somatic repair and might depend on IGF-1 to a lesser extent than long-lived mammals such as humans. Moreover, mice primarily die of cancer (45); thus, low IGF-1 levels decrease proliferation and might lessen the risk for neoplasia. In this context, it is noteworthy that while fibroblasts of GH/IGF-1-deficient Ames mice are markedly resistant to multiple forms of cellular stress in vitro (10,11), these mice (46) (and similarly Lewis dwarf rats; [47,48]) have decreased antioxidant expression and increased oxidative stress in their vasculature, suggesting that were these mammals to have a longer life span, the tradeoff of decreased cancer incidence would be eclipsed by increased mortality from cardiovascular diseases due to low circulating IGF-1. In fact, adults with deficiencies in GH and IGF-1 have lower rates of death from cancer (49) but elevated markers of endothelial dysfunction (50). Clearly, further research is needed to gain an insight into the complex IGF-1–mediated metabolic response in different human cell types and at the systemic level.

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**Supplementary Material**

Supplementary material can be found at: [http://biomedgerontology.oxfordjournals.org/](http://biomedgerontology.oxfordjournals.org/)

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**References**