Reactive oxygen species stimulate insulin-like growth factor I synthesis in vascular smooth muscle cells

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Abstract

**Objectives:** The objective was to study potential regulation of insulin-like growth factor I (IGF I), its binding proteins, and the IGF I receptor by reactive oxygen species in vascular smooth muscle cells. **Methods:** We used cultured rat aortic smooth muscle cells exposed to xanthine (100 μM) and xanthine oxidase (5 μU/ml) or H₂O₂ (200 μM) and measured IGF I mRNA levels by solution hybridization/RNase protection assays, IGF I protein levels by radioimmunoassay, and IGF binding proteins by Western ligand blotting. Additionally, we measured the effect of anti-IGF I antiserum on xanthine/xanthine oxidase- and H₂O₂-stimulated [³H]thymidine incorporation. **Results:** Xanthine/xanthine oxidase and H₂O₂ stimulated increases in IGF I mRNA and protein levels and reduced IGF binding protein-4 levels in conditioned medium. The effect of xanthine/xanthine oxidase was inhibited by the scavengers superoxide dismutase and catalase. Xanthine/xanthine oxidase- and H₂O₂-stimulated DNA synthesis was completely inhibited by a neutralizing anti-IGF I antiserum. **Conclusion:** Reactive oxygen species increased vascular smooth muscle cell synthesis of IGF I and reduced levels of the inhibitory IGF binding protein-4. Furthermore, reactive oxygen species-induced DNA synthesis was inhibited by an anti-IGF I antiserum. These findings suggest that the autocrine IGF I system plays an important role in vascular smooth muscle cell growth responses to reactive oxygen species. Furthermore, the findings have important implications for understanding biological responses to changes in redox state.

**Keywords:** Insulin-like growth factor I; Free radicals; Gene expression; Insulin-like growth factor I receptor; Smooth muscle cell proliferation; Rat, vascular smooth muscle cells

1. Introduction

Reactive oxygen species (ROS), such as superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radicals (·OH), are produced by a variety of different cells and are increasingly thought to have important physiological and pathophysiological effects (reviewed in Ref. [1]). Thus, polymorphonuclear leukocytes (PMNs) and mononuclear cells produce ROS during the respiratory burst that accompanies phagocytosis, and this production is thought to be important in the inflammatory response (reviewed in Ref. [2]). The major generator of ROS in the PMN is the plasmalemmal NADPH oxidase [3], although other enzymes, notably lipoxygenase and cyclo-oxygenase, may play a role [1]. Other cell types have been shown to produce ROS under a variety of stimuli, such as endothelial cells [4], mesangial cells [5], leukemic cells [6], vascular smooth muscle cells (VSMC) [7], and others. Recently, there has been a growing interest in the potential role of ROS in the pathogenesis of cardiovascular disease in general, and more specifically, in the development of atherosclerosis [8], hypertensive vascular disease [9], and in ischemia-reperfusion injury [10]. For instance, there is now good evidence that the diminished endothelium-dependent vasorelaxation linked to hypercholesterolemic states is mediated by increased superoxide anion production and resultant quenching of nitric oxide (NO), the primary component of endothelium-derived relaxing factor (EDRF) [11].

Endothelial cells produce ROS in response to a variety of cytokines [4] and endothelial redox state may regulate expression of adhesion molecules and thus be instrumental in facilitating monocyte accumulation in the subintima [12]. VSMC may respond to ROS with increased DNA
synthesis and cell turnover [13]. Recently, the vasoconstrictor and trophic factor angiotensin II has been shown to stimulate VSMC formation of superoxide anion via membrane NADH and NADPH oxidases [14]. Inhibition of oxidase activity blunts angiotensin-II-induced VSMC protein synthesis, consistent with the possibility that ROS may function as second messengers. Indeed, growth factors have been shown to stimulate the NADH oxidase [15].

The control of VSMC growth is complex and controlled by a variety of growth factors, including, but not limited to, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), angiotensin II, thrombin and insulin-like growth factor I (IGF I) (reviewed in Refs. [16,17]). Recently, the VSMC IGF I autocrine axis has been shown to play a critical role in growth responses of VSMC to multiple agonists (reviewed in Ref. [18]). Thus, neutralization of IGF I using specific antibodies or downregulation of the IGF I receptor using 'antisense' methodologies blocks growth responses to PDGF [19], angiotensin II [20,21], and thrombin [22]. Because of the increasing evidence that ROS may play an important role in the disordered vascular tone and increased vascular growth responses present in disease states, we postulated that ROS may regulate the VSMC IGF I autocrine system. Our findings demonstrate regulation of both IGF I and its binding proteins by ROS and suggest that this autocrine system mediates ROS-induced increases in cell growth.

2. Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

2.1. Cell culture

VSMC were isolated from rat thoracic aorta, as described previously by Alexander et al. [23]. Cells were grown in medium containing equal parts of Dulbecco’s modified Eagle’s medium (Gibco Laboratories, Grand Island, NY) and Ham’s F-12 (Gibco Laboratories), supplemented with 10% calf serum, 2 mM glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Cells were passaged by harvesting with trypsin-verse and seeding at a 1:8 ratio in 75 cm² flasks. For experiments, cells between passage levels 3 and 15 were seeded into 100 mm dishes or 24- or 48-well cluster dishes. At 80% confluence, cells were quiesced for 48 h in serum-free medium containing antibiotics, glutamine, insulin (0.5 μM), transferrin (5 μg/ml), and ascorbate (0.2 mM) (hereafter designated as ‘serum-free medium’). For experiments, quiescent cells were exposed to xanthine (100 μM) and xanthine oxidase (5 μU/ml) or to 200 μM H₂O₂ for 0–24 h. Because xanthine and xanthine oxidase generate ROS only siently, we replaced these agonists hourly for 6 h. Conditioned medium was harvested for determination of IGF I and IGF binding protein (IGFBP) levels, and total RNA was harvested for measurement of IGF I mRNA levels. For some experiments, cells were exposed with or without 200 U/ml superoxide dismutase (SOD) or 100 U/ml catalase, and then stimulated with xanthine/xanthine oxidase.

2.2. IGF I mRNA levels

To determine the effects of ROS on IGF I mRNA levels, quiescent VSMC were exposed to xanthine and xanthine oxidase or to H₂O₂ for various times. Total RNA was extracted from the cells using the TRI-reagent method (Molecular Research Center, Inc.). RNA was quantitated by spectrophotometry and solution hybridization/RNase protection assays were performed as described previously [20]. In brief, 30 μg of total RNA was hybridized to a [32P]UTP-labeled antisense riboprobe generated by T7 polymerase transcription of a linearized plasmid containing the rat IGF I exon 4 and adjacent intron sequences. The plasmid was kindly provided by Dr. P. Rottwein (Washington University School of Medicine, St. Louis, MO). RNA was co-hybridized using a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) riboprobe [24]. After RNase digestion, samples were proteinase-K-treated, phenol-extracted, ethanol-precipitated and analyzed on a denaturing polyacrylamide urea sequencing gel. Protected IGF I and GAPDH RNA fragments are 182 and 133 bp, respectively. Control hybridizations with 30 μg tRNA give no protected bands. Autoradiograms were exposed for 1–3 d, and protected bands were quantitated by two-dimensional laser densitometry.

2.3. IGF I radioimmunoassay

Specific IGF I immunoreactivity of cell-conditioned medium was determined as described previously in our laboratory [25]. In brief, medium was dialyzed, lyophilized, resuspended in 1 M acetic acid, 0.025 M NaCl and chromatographed using Bio-Gel P-30 polycrylamide columns (Bio-Rad Laboratories, Hercules, CA). IGF I fractions were assayed using a polyclonal anti-IGF I rabbit antisemur kindly provided by Dr. L. Underwood and Dr. J.J. Van Wyk through the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases. Standard curves were generated using human recombinant IGF I (Ciba-Geigy, Suffern, NY).

2.4. Radioligand binding assays

To determine the effects of ROS on IGF I receptors, quiescent VSMC in 24-well plates were exposed to xanthine and xanthine oxidase or to H₂O₂ for 6 or 24 h.
Binding assays were performed as previously described [26]. Briefly, cells were incubated with 0.1 nM \(^{125}\)I-IGF I and 0–0.1 μM unlabeled IGF I for 90 min at room temperature. Cells were washed in ice-cold binding buffer and solubilized in 2N NaOH before counting. All assays were performed in duplicate for each experimental point and data were analyzed using the LIGAND program.

2.5. \(^{3}\)H/Thymidine incorporation

For these experiments, VSMC were quiesced in serum-free medium with 0.1% calf serum for 48 h and then exposed to serum-free medium with or without xanthine/xanthine oxidase or H\(_2\)O\(_2\) for 24 h, in the presence of 1μCi/ml \(^{3}\)H/thymidine. To determine the effect of anti-IGF I antiserum on ROS-induced DNA synthesis, experiments were performed in which cells were exposed with or without agonists and with or without a 1:500 dilution of normal rabbit serum or anti-IGF I antiserum for 24 h. Cells were washed 3 times with ice-cold PBS, incubated on ice for 15 min with 10% trichloroacetic acid, and after 2 washes in ice-cold 95% ethanol, radioactivity was extracted with 0.4N NaOH for assay by liquid scintillation spectrometry. All experiments were performed in triplicate.

2.6. Western ligand blotting

Conditioned medium from quiescent VSMC exposed to xanthine and xanthine oxidase or to H\(_2\)O\(_2\) was acidified with 2N acetic acid (1:1, v/v) for 30 min at room temperature, concentrated using Centricon-10 columns, lyophilized, and resuspended in equal volumes of sodium phosphate buffer, pH 6.5. Western ligand blotting was then performed as previously described [27]. Briefly, identical aliquots of resuspended medium from control or agonist-exposed cells were subjected to non-reducing 12% SDS-PAGE and electrotransferred to 0.45 μm nitrocellulose. Membranes were blocked and then probed with 4 \times 10^{5} cpm/ml of \(^{125}\)I-IGF I for 16–18 h. Rinsed membranes were dried before autoradiography. Exposure times varied from 1–7 d. Bands were sized using molecular weight markers (Bio-Rad Laboratories, Hercules, CA).

2.7. Statistical analysis

Data are expressed as mean ± s.e. Analysis of repeated measures was performed by ANOVA and comparisons between groups was performed using a protected t-test.

3. Results

3.1. Effects of ROS on IGF I expression

Exposure of VSMC to 100 μM xanthine and 5 μU/ml xanthine oxidase caused a rapid increase in IGF I mRNA levels with a peak ~1.5–2.0-fold increase at 3 h (P < 0.01), a gradual reduction thereafter, and a smaller increase at 24 h (Fig. 1). To determine whether changes in IGF I mRNA levels were reflected in changes in secreted IGF I protein, we measured total IGF I levels in conditioned medium from cells exposed to xanthine/xanthine oxidase. As shown in Fig. 2, there was a sustained increase in IGF I release from cells exposed to xanthine/xanthine oxidase (P < 0.01). The increase averaged 56, 40, 39, and 92%
over control at 3, 6, 12, and 24 h, respectively. Xanthine and xanthine oxidase generate a variety of ROS [28]. To determine the contribution of superoxide anion and of H₂O₂ to the observed increase in IGF I release, we performed additional experiments and exposed cells to xanthine/xanthine oxidase for 24 h in the presence or absence of 200 U/ml SOD or 100 U/ml catalase. SOD and catalase inhibited the ability of xanthine and xanthine oxidase to increase IGF I release: serum-free medium 7.65 ± 0.74 ng/dish, xanthine/xanthine oxidase 10.96 ± 0.76 ng/dish, 43% increase, P < 0.01; SOD 8.8 ± 0.47 ng/dish, SOD + xanthine/xanthine oxidase 8.9 ± 0.54 ng/dish, P = NS; catalase 8.8 ± 0.35 ng/dish, catalase + xanthine/xanthine oxidase 9.49 ± 0.74 ng/dish, P = NS (n = 6).

To determine whether H₂O₂ itself could increase IGF I release, we performed experiments using quiescent VSMC exposed to 200 μM H₂O₂ for 0–24 h. As shown in Fig. 3, H₂O₂ also caused an increase in IGF I mRNA levels (P < 0.05). Measurements of total IGF I in conditioned medium from H₂O₂-treated cells indicated an initial increase at 3 and 6 h (51 and 26% increase, respectively, P < 0.05 compared with serum-free medium) with a small but significant reduction in IGF I at 24 h (18% decrease, P < 0.01, Fig. 4). To verify that H₂O₂ was not affecting cell viability at 24 h, we performed cell counts of trypan-blue-excluding cells. Total cell number and the percentage of trypan-blue-excluding cells at 24 h was not different in H₂O₂-treated cells (93%), compared with control (94%).

### 3.2. Effect of ROS on IGF I receptors

To assess the effect of ROS on IGF I receptors, homologous displacement binding experiments were performed. As shown in Fig. 5, neither xanthine/xanthine oxidase nor H₂O₂ caused large changes in IGF I receptor number at 6 h or 24 h. It is, however, noteworthy, that xanthine/xanthine oxidase caused an approximate 30% increase in IGF I receptor number at 24 h (P = 0.11), which may be a biologically relevant effect. Likewise, H₂O₂ caused a small 17% increase in IGF I receptor number at 24 h (P = 0.21). Measurements of IGF I receptor binding-activity indicated that neither xanthine/xanthine oxidase nor H₂O₂ significantly altered this parameter: Kd control 1.56 ± 0.20 nM; Kd xanthine/xanthine oxidase 2.18 ± 0.32 nM, Kd H₂O₂ 2.03 ± 0.47 nM, mean ± s.e. of results from experiments performed at 6 and 24 h (n = 10).
3.3. Effect of ROS on IGFBPs

To measure potential effects of ROS on IGFBPs, we performed Western ligand blotting of conditioned medium from cells exposed to xanthine/xanthine oxidase or to H₂O₂. As we have previously shown, the main IGFBPs secreted by VSMC are a 28 and 24 kDa form of IGFBP-4 [27]. Trace amounts of IGFBP-3 and a ∼ 32 kDa IGFBP (likely IGFBP-2) are also secreted. As shown in Fig. 6, xanthine/xanthine oxidase significantly reduced IGFBP-4 levels at 24 h (21 ± 8.7% decrease, mean ± s.e., n = 5, P < 0.05). H₂O₂ caused an even more marked reduction in IGFBP-4 levels at 24 h (45 ± 9% decrease, mean ± s.e., n = 7, P < 0.01).

3.4. Effect of anti-IGF I anti-serum on ROS-induced DNA synthesis

To determine the potential requirement for IGF I in ROS-induced DNA synthesis, we measured the effect of xanthine/xanthine oxidase and H₂O₂ on [³H]thyminide incorporation in the presence of anti-IGF I antiserum. As shown in Fig. 7, xanthine/xanthine oxidase and H₂O₂ caused approximate 1.5- to 2.0-fold increases in DNA synthesis at 24 h (P < 0.01 in both cases). Normal rabbit serum (1:500 dilution) caused a small increase in basal DNA synthesis, and both xanthine/xanthine oxidase and H₂O₂ caused an additional significant increase in [³H]thyminide incorporation (P < 0.01 in both cases). However, in the presence of anti-IGF I antiserum, neither xanthine/xanthine oxidase nor H₂O₂ significantly stimulated DNA synthesis. Anti-IGF I antiserum alone caused a small (∼18%) decrease in [³H]thyminide incorporation basally, as would be expected in quiescent cells.

4. Discussion

Our findings clearly establish that ROS regulate synthesis of IGF I by VSMC. Thus, xanthine/xanthine oxidase increased IGF I mRNA levels and approximately doubled total IGF I secreted from VSMC over a 24 h period. The increase in IGF I mRNA appeared bimodal with an initial peak at 3 h and a further increase at 24 h. The reason for this pattern is unclear, but it could be related to the fact that xanthine and xanthine oxidase were replaced hourly for 6 h in order to provide a more sustained generation of ROS. Interestingly, xanthine/xanthine oxidase also caused a significant decrease in levels of the inhibitory IGF binding protein, IGFBP-4. Because IGFBP-4 inhibits IGF I growth effects on VSMC [29], it is possible that the reduction in IGFBP-4 could serve to further increase availability of free IGF I. The effect of xanthine/xanthine oxidase on IGF I receptor expression was small, with a non-statistically significant 30% increase in IGF I receptor number at 24 h. We have previously shown that IGF I downregulates its receptor on VSMC [26], and thus it is possible that the ability of xanthine/xanthine oxidase to upregulate IGF I receptors was blunted by the increased IGF I release. It is noteworthy, however, that IGF I receptor density on VSMC is tightly regulated [18]. Thus, 1.5-2-fold increases in IGF I receptor density play a determining role in mitogenic responses to thrombin [22] and to angiotensin II [21]. Furthermore, 50% reductions in IGF I receptor density on VSMC have a profound antiproliferative effect, inhibiting growth responses to 10% serum [30]. It is thus possible that the small increase in IGF I receptor density stimulated by xanthine/xanthine oxidase is biologically relevant.

To provide insights into mechanisms whereby xanthine/xanthine oxidase increases IGF I secretion, we performed experiments using the H₂O₂ scavenger, catalase, and SOD. Both of these scavengers inhibited the ability of xanthine/xanthine oxidase to stimulate IGF I release. This finding is consistent with the participation of both superoxide anion and H₂O₂ in the induction of IGF I synthesis by VSMC. While a detailed study of the chemistry involved in this effect is beyond the scope of this report, a clearly attractive hypothesis is that the ability of xanthine/xanthine

![Fig. 6. Reactive oxygen species reduce levels of IGFBP-4. Quiescent VSMC were exposed without (control, Ctrl) or with H₂O₂ or xanthine/xanthine oxidase (X/XO) for 24 h. Conditioned medium was acidified, concentrated, and subjected to non-reducing 12% SDS-PAGE. Western ligand blotting was performed as described in Section 2. Arrows indicate the two forms of IGFBP-4.](https://academic.oup.com/cardiovascres/article-abstract/33/1/216/296371)
oxidase to stimulate IGF I production is mediated by a superoxide-driven Fenton reaction (reviewed in Ref. [31]). Thus, a combination of the dismutation of superoxide anion and the Fenton reaction in the presence of iron ions would lead to the formation of hydroxyl radicals. Hydroxyl radicals are highly reactive and could initiate a signaling cascade leading to the growth effects that we observed. One could speculate that one of the mechanisms whereby hydroxyl radicals could exert their effects would be through peroxidation of membrane lipids. It is noteworthy in this regard that both SOD and catalase have been shown to inhibit peroxidation of arachidonic acid stimulated by xanthine/xanthine oxidase [32]. It is also noteworthy that xanthine-oxidase-stimulated proliferation of human fibroblasts is prevented by SOD and catalase [33].

In order to determine whether \( \text{H}_2\text{O}_2 \) itself could increase IGF I synthesis, we measured IGF I mRNA and protein levels following exposure to 200 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) for a 24 h period. \( \text{H}_2\text{O}_2 \) caused a transient increase in IGF I mRNA levels, correlated with increased total IGF I levels at 3 and 6 h. At 24 h total IGF I was slightly but significantly decreased. The transient increase in IGF I mRNA and protein levels stimulated by \( \text{H}_2\text{O}_2 \) contrasts with the more sustained induction by xanthine/xanthine oxidase. However, as noted previously, xanthine and xanthine oxidase were replaced hourly for 6 h, perhaps explaining this more sustained induction. Alternatively, because xanthine and xanthine oxidase generate a variety of ROS, the signaling pathway mediating their effect could be different from that utilized by \( \text{H}_2\text{O}_2 \). To determine whether the small reduction in IGF I levels at 24 h could be offset by a parallel decrease in IGF binding proteins, we performed Western ligand blotting. Our findings indicate that \( \text{H}_2\text{O}_2 \) caused a marked reduction in IGFBP-4 levels at 24 h, consistent with the possibility that free IGF I was actually increased at this time point. The ability of xanthine/xanthine oxidase and \( \text{H}_2\text{O}_2 \) to reduce IGFBP-4 levels could be related to direct oxygen radical attack on this protein. Alternatively, ROS could produce changes in IGFBP-4 synthesis, or activate the IGFBP-4 protease that is an important regulator of IGFBP-4 levels [34].

In order to determine a potential requirement of the IGF I autocrine system in ROS-driven DNA synthesis, we exposed cells to \( \text{H}_2\text{O}_2 \) and xanthine/xanthine oxidase in the presence or absence of a neutralizing anti-IGF I serum, and measured \(^{3}\text{H}\)thymidine incorporation. As shown in Fig. 7, xanthine/xanthine oxidase and \( \text{H}_2\text{O}_2 \) caused significant increases in DNA synthesis that were blocked by anti-IGF I antiserum. This finding demonstrates that an active IGF I signaling axis is required for ROS-induced DNA synthesis and suggests that the ability of ROS to increase IGF I release may play an important role in their mitogenic effects. It is interesting to note that \( \text{H}_2\text{O}_2 \) appeared to be more mitogenic for VSMC than xanthine/xanthine oxidase, although its effect to stimulate total IGF I release appeared smaller and certainly more transient. A possible explanation for this could be that \( \text{H}_2\text{O}_2 \), via its greater ability to reduce IGFBP-4 secretion, caused a larger increase in free IGF I levels.

In summary, we have shown that ROS increase IGF I synthesis in VSMC and reduce levels of IGFBP-4 in VSMC-conditioned medium. ROS-induced DNA synthesis is inhibited by an anti-IGF I antiserum, suggesting that the IGF I autocrine system is an important mediator of mitogenic responses to ROS. Our findings could have important implications for understanding physiological and pathophysiological effects of ROS in biological systems.

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