Activation of an H$_2$O$_2$-generating NADH Oxidase in Human Lung Fibroblasts by Transforming Growth Factor $\beta_1$*

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The cellular source(s) and mechanisms of generation of reactive oxygen species (ROS) in nonphagocytic cells stimulated by cytokines are unclear. In this study, we demonstrate that transforming growth factor $\beta_1$ (TGF-$\beta_1$, 1 ng/ml) induces the release of H$_2$O$_2$ from human lung fibroblasts within 8 h following exposure to this cytokine. Elevation in H$_2$O$_2$ release peaked at 16 h (% 22 pmol/min/10$^6$ cells) and gradually declined to undetectable levels at 48 h after TGF-$\beta_1$ treatment. NADH consumption by these cells was stimulated by TGF-$\beta_1$ while that of NADPH remained unchanged. NADH oxidase activity as measured by diphenylidodium (DPI)-inhibitable NADH consumption in TGF-$\beta_1$-treated cells followed a time course similar to that of H$_2$O$_2$ release. DPI, an inhibitor of the NADPH oxidase complex of neutrophils and other flavoproteins, also inhibited the TGF-$\beta_1$-induced H$_2$O$_2$ production. Inhibitors of other enzymatic systems involving flavoproteins that may be responsible for the production of H$_2$O$_2$ in these cells, including xanthine oxidase, nitric oxide synthase, and both mitochondrial and microsomal electron transport systems, failed to inhibit TGF-$\beta_1$-induced NADH oxidation and H$_2$O$_2$ production. The delay (>4 h) following TGF-$\beta_1$ exposure along with the inhibition of this process by cycloheximide and actinomycin D suggest the requirement of new protein synthesis for induction of NADH oxidase activity in TGF-$\beta_1$-stimulated fibroblasts.

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1The abbreviations used are: TGF-$\beta_1$, transforming growth factor $\beta_1$; DPI, diphenylidodium; TNF-$\alpha$, tumor necrosis factor $\alpha$; IL-1, interleukin 1; ROS, reactive oxygen species.

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using an extinction coefficient of 43.6 M⁻¹ cm⁻¹.

Measurement of NADH, NADPH Consumption, and Oxidase Activities—Measurements of NADH and NADPH oxidase activities were made using methods similar to those described by Brightman and co-workers (15). Cells were first washed with RPMI medium without phenol red, pH 7.4, and then incubated with NADH (250 μM) or NADPH (250 μM) in the same medium for varying time intervals. The rate of NADH or NADPH consumption was monitored by the decrease in absorbance at λ = 340 nm, using a Hewlett-Packard 8452A diode array spectrophotometer. The absorption extinction coefficient used to calculate the amount of NADH or NADPH consumed was 6.22 m² M⁻¹ cm⁻¹. For measurements of specific oxidase activity, only the DPI-inhibitable rate of consumption of NADH and NADPH was used. This was done by adding DPI (10 μM) 30 min prior to the assays for NADH and NADPH consumption. As shown in Fig. 3, TGF-β1-stimulated NADPH consumption was inhibited by DPI. This "DPI-inhibitable" NADPH consumption was later used to measure NADH oxidase activity at specific time points after exposure to TGF-β1. All measurements were expressed in nanomoles of substrate/min/10⁶ cells.

Cell Counts—Cell counts were performed concurrently with all of the biochemical measurements described. After removal of the extracellular medium for assay, culture dishes were washed with warm physiological saline, incubated with 1.0 ml of trypsin-EDTA for 2–3 min, and rapidly suspended in solution by pipetting. A 0.2-ml aliquot of cell suspension was then diluted for counting in a model 2M Coulter Counter (Coulter Electronics, Hialeah, FL).

Statistical Analysis—Data from various groups were expressed as means ± S.D. Statistical comparisons were made using the Student's t test for unpaired samples. For studies involving more than two groups, two-way analysis of variance was determined using the Scheffe’s test (GB-STAT; Dynamic Microsystems, Silver Spring, MD). Statistical significance in all cases was defined at p < 0.05.

RESULTS

Effect of TGF-β1 on H₂O₂ Release—The rate of extracellular release of H₂O₂ from fibroblasts treated with a single dose of TGF-β1 (1 ng/ml) was measured at regularly timed intervals over a 48-h period. There was no measurable release of H₂O₂ from unstimulated cells. H₂O₂ release by TGF-β1-treated cells was detected at 8 h following exposure, with no demonstrable increase at 4 h (Fig. 1). Measurements at even earlier time points, less than the 4-h exposure time shown (i.e. 1 and 2 h), also failed to demonstrate any H₂O₂ production (results not shown). The peak rate of H₂O₂ release was seen at 16 h following treatment with TGF-β1 prior to a gradual decrease to baseline (undetectable levels) by 48 h.

Effect of TGF-β1 on NADH and NADPH Consumption—The rates of NADH and NADPH consumption by control and TGF-β1-stimulated cells were determined at 16 h of exposure to TGF-β1, corresponding to the peak rate of H₂O₂ release observed (Fig. 1). As shown in Fig. 2, the rate of NADH consumption by TGF-β1-treated cells was 2-fold higher than that of control cells (approximately 0.37–0.40 nmol of NADH/min/10⁶ cells versus 0.19 nmol of NADH/min/10⁶ cells, respectively). The baseline rate of NADPH consumption was almost 4-fold lower (~0.05 nmol of NADPH/min/10⁶ cells) than that of NADH, and there was no significant change in the rate of NADPH consumption by cells treated with TGF-β1.

Effect of DPI in TGF-β1-induced NADH and NADPH Consumption—The effect of the flavoprotein inhibitor, DPI, on the rate of NADH and NADPH was examined. At a dose that has been previously reported to inhibit cellular flavoprotein oxidases (16), DPI (10 μM) inhibited the increase in NADH utilization by TGF-β1-treated cells. In these experiments, DPI was added 30 min prior to the assay for NADH or NADPH consumption, following a 16-h exposure to TGF-β1. There was DPI-inhibitable NADH consumption noted in unstimulated (control) cells as well, although this was about half that of TGF-β1-treated cells (Fig. 3). In contrast, there was no inhibition of NADPH consumption by control or TGF-β1-treated cells by DPI.

We also examined the possibility that NADH oxidation in the extracellular medium was due to NADH-utilizing enzymes released into the medium of TGF-β1-treated cells. Conditioned media from control and TGF-β1-treated cells were assayed for NADH oxidase activity (by measuring DPI-inhibitable NADH consumption) and H₂O₂. Both NADH oxidase activity and H₂O₂ were undetectable in the conditioned media of control and TGF-β1-treated cells. The rates of DPI-noninhibitable NADH consumption were similar in both groups. The requirement for the fibroblast monolayer to be in direct contact with the extracellular medium for detection of NADH oxidase activity and H₂O₂ release suggests that these reactions are taking place in or around the surface of fibroblast plasma membranes. Measurement of NADH oxidase activity cannot, therefore, be accounted for by release of enzymes into the extracellular medium.

Effect of Inhibitors on TGF-β1-induced NADH Consumption and H₂O₂ Release—Although previously reported as a specific inhibitor of NAD(P)H oxidases (16), DPI and other related compounds are now recognized as relatively nonspecific inhib-
TGF-β1 Induced NADH Oxidase Activity

...was detected at 4 h following TGF-β1 treatment, and peak activity was noted at 16 h (Fig. 4). As with H2O2 release, NADH oxidase activity decreased to control levels by 48 h of exposure to TGF-β1.

**DISCUSSION**

Certain cytokines have the ability to stimulate the production of ROS in nonphagocytic cells (10, 12, 19). The source(s) of cytokine-stimulated ROS generation, however, have not been clearly identified. We have previously reported on the stimulatory effect of TGF-β1 on bovine pulmonary artery endothelial cell H2O2 production without being able to identify its source (12). In the present study, we extend this observation to human lung fibroblasts, which, unlike endothelial cells, demonstrate no release of H2O2 extracellularly in the unstimulated state but, similar to endothelial cells, can be induced to generate H2O2 by TGF-β1. Our study suggests that the source of this H2O2 is the 2-electron reduction of O2 via a novel electron transport system most likely localized in fibroblast plasma membranes. This electron transport system involves a flavoprotein which specifically utilizes NADH as the electron donor. Further, the activation of this flavoprotein oxidase by TGF-β1 appears to require new protein synthesis.

NADH and NADPH are ubiquitous dinucleotides (each containing adenine and a nicotinamide ring) that function as carriers of “reducing equivalents” and are used as coenzymes in many cellular oxidation-reduction reactions. They do this by the transfer of hydride ion (H, 2 electrons plus a proton) from the 4-position of the nicotinamide ring. The only structural difference between NADH and NADPH is the presence of an extra phosphate group at the 2-position of the adenine nucleotide moiety of NADPH. Although this extra phosphate group is far from the active redox region of the nicotinamide ring and is of no importance in the hydride ion transfer reaction, it appears to confer specificity for the enzymes to which NADPH can bind as a coenzyme.

Based on our findings of NADH-specific flavoprotein oxidase activity in TGF-β1-treated fibroblasts, we can construct the following simple diagram

\[
\begin{align*}
\text{NADH} & \rightarrow \text{oxidized flavoprotein} \rightarrow H_2O_2 \\
\text{NAD}^+ & \rightarrow \text{reduced flavoprotein} \rightarrow O_2 + H^+ \\
\text{Overall reaction:} & \quad \text{NADH} + O_2 + H^+ \rightarrow \text{NAD}^+ + H_2O_2
\end{align*}
\]

**REACTION 1**

The potential for additional “carriers” of electron transport from NADH (donor) to O2 (acceptor) exists. Moreover, it cannot be determined conclusively from our current experiments if the reduction of O2 to H2O2 involves the intermediate formation of O2- or if this is a direct 2-electron transfer reaction. We have been unable to demonstrate O2- formation in association with TGF-β1-induced NADH oxidase activity using superoxide dismutase-inhibitable ferricytochrome reduction as an assay for O2- release (results not shown). Direct 2-electron reduction of O2 has been reported for flavoprotein oxidases (20). If we assumed no “leakage” of electrons along the electron transport chain from NADH to O2, the overall stoichiometry of NADH:H2O2 would be expected to be 1:1. A number of factors may affect the measurement of the concentrations of these biomolecules under the experimental conditions (using whole cells) of our study. Therefore, the results of this study cannot be used to establish stoichiometric relationships.

Several lines of evidence suggest that the location of the TGF-β1-induced NADH oxidase is most likely at the plasma membrane. Since NADH and NADPH do not cross plasma...
membranes, consumption rates of these biomolecules measured in the extracellular medium are likely to represent oxidation by enzyme(s) on the outer aspect of the membrane. We considered the possibility of NADH oxidation by non-flavoprotein oxidases and dehydrogenases or degradation by nonoxidative pathways and, therefore, specifically measured DPI-inhibitable consumption of NADH. DPI, previously thought to be a specific inhibitor of NADPH oxidase, is now recognized as a relatively nonspecific inhibitor of flavoproteins by direct binding (17). It is unlikely that DPI, in the manner used in our studies, could diffuse through the membrane to inhibit intracellular flavoprotein oxidoreductases. However, we have assessed the contributions of intracellular sources of ROS involving flavoproteins with the use of specific inhibitors of these enzyme systems, and our results support the likelihood that they are not involved in TGF-β1-induced H₂O₂ generation.

TGF-β1-induced NADH oxidase activity appears to require new protein synthesis based on the finding of a lag time (>4 h, <8 h) following exposure to TGF-β1 and its inhibition by cycloheximide and actinomycin D. It is unlikely that this activity is mediated directly by TGF-β1 since this peptide has been shown to bind rapidly to its receptor(s) and is internalized and degraded by lysosomal enzymes within 4 h at 37 °C (21). Moreover, assays for NADH consumption and H₂O₂ release are made after first washing off the medium and without the reintroduction of TGF-β1 in assay solutions. Together, these observations suggest that binding of TGF-β1 to its receptor(s) results in the activation of a signaling cascade that results in the induction of H₂O₂-generating capacity by the cell via an NADH-specific flavoprotein oxidase. It appears likely that some component of this protein complex, required for enzymatic activity, has to be newly synthesized. Our finding of detectable H₂O₂ release in TGF-β1-treated cells in the presence of cycloheximide and actinomycin D, despite an apparent complete inhibition of oxidase activity (by the observed lowering of NADH consumption to control levels, Table I) suggests that the relatively smaller amounts of H₂O₂ measured under these conditions may be unrelated to NADH oxidase activity. H₂O₂ detected under conditions when protein synthesis is inhibited might reflect a relative imbalance of oxidant stress and cellular antioxidant capacity since reduced levels of newly formed superoxide dismutase, catalase, and components of the glutathione redox cycle would be expected under these conditions. Moreover, TGF-β1 alone has been reported to suppress the expression of these antioxidant enzymes in rat hepatocytes (22).

Although the source(s) of cytokine-stimulated ROS have remained largely speculative, some recent studies have attempted to identify the ROS-generating enzymatic systems. Meier et al. (10) have reported on a superoxide-generating NAPDH oxidase in human skin fibroblasts stimulated by TNF-α and IL-1 that was initially thought to be similar to the NADPH of neutrophils (10), but subsequently has been shown to be structurally and genetically distinct (11). There are several important differences between TNF-α- and IL-1-stimulated ROS production in nonphagocytic cells (10, 19) and the TGF-β1-induced H₂O₂ production observed in our studies. First, NADH specificity has not been reported previously. Secondly, the formation of ROS in response to TNF-α and IL-1 is immediate, and there is no apparent requirement for new protein synthesis. Thirdly, we are unable to demonstrate O₂⁻ formation in association with TGF-β1-induced NADH oxidase activity. Finally, we have not observed similar NADH oxidation or H₂O₂ production in intact lung fibroblasts in response to TNF-α and IL-1 using short incubation (immediate activation).

**Table I** Effect of inhibitors on TGF-β1-induced NADH consumption and H₂O₂ release

Exposure to TGF-β1 (1 ng/ml) was for 16 h. Cycloheximide and actinomycin-D were introduced at the same time as TGF-β1. All other inhibitors were added at the end of the 16 h exposure time (i.e. 30 min prior to assays for NADH consumption and H₂O₂ release). Superoxide dismutase was added directly to the reaction mixtures. SOD = Cu,Zn-superoxide dismutase. l-NAME = l-Nω-nitroarginine methyl ester, ND = not detectable. Values are mean ± S.D., n = 4.

| Inhibitor (dose) | NADH consumption | H₂O₂ release |
|-----------------|------------------|--------------|
| Control         | 0.20 ± 0.02      | ND           |
| TGF-β1 (1 ng/ml)| 0.38 ± 0.03      | 22.3 ± 1.7   |
| TGF-β1 + l-NAME (1 mM) | 0.38 ± 0.02 | 21.1 ± 2.1   |
| TGF-β1 + allopurinol (100 µM) | 0.37 ± 0.02 | 21.3 ± 1.6   |
| TGF-β1 + potassium cyanide (1 mM) | 0.39 ± 0.02 | 21.6 ± 2.1   |
| TGF-β1 + methoxyoosphalen (250 µM) | 0.36 ± 0.03 | 21.2 ± 2.2   |
| TGF-β1 + SOD (100 units/ml) | 0.38 ± 0.02 | 21.7 ± 3.3   |
| DPI (10 µM)     | 0.11 ± 0.01      | ND           |
| TGF-β1 + DPI (10 µM) | 0.14 ± 0.01^b  | 2.6 ± 0.5^b  |
| Cycloheximide (1 µg/ml) | 0.19 ± 0.01 | ND           |
| TGF-β1 + cycloheximide (1 µg/ml) | 0.19 ± 0.02^b | 8.6 ± 0.6^b  |
| Actinomycin D (0.05 µg/ml) | 0.19 ± 0.01 | ND           |
| TGF-β1 + actinomycin D (0.05 µg/ml) | 0.18 ± 0.02^b | 3.8 ± 0.5^b  |

^a p < 0.05 versus control.
^b p < 0.05 versus TGF-β1.

![Graph](https://example.com/graph.png)
or longer exposure (induction of activity possibly through new protein synthesis, results not shown).

The presence of a growth factor-responsive (epidermal growth factor, insulin, pituitary extract) NADH oxidase which may function as part of a redox system at the plasma membrane has been proposed by Brightman and co-workers (15). In these studies, NADH oxidase activity was measured by monitoring NADH utilization and O2 consumption, not H2O2 production. In fact, ROS production in association with this activity is apparently lacking.2 Another recent study has demonstrated the stimulation of NADH and NADPH oxidase activity in smooth muscle cells by angiotensin II (23). NADH oxidoreductase activity has also been shown to be a major source of O2 in unstimulated bovine coronary artery endothelium (24). The relationship between the TGF-β1-induced NADH oxidase activity noted in our studies and similar activities reported in these former studies is, at present, unclear.

The best characterized NADPH- or NADH-utilizing flavoprotein oxidase associated with the formation of ROS is that of phagocytes (25). Although there are some obvious similarities such as the involvement of a flavoprotein as part of the functional enzymatic complex and the use of O2 as the electron acceptor, there are also several differences from TGF-β1-induced NADH oxidase activity. The most striking difference is the specificity of the oxidase for NADH (and not NADPH). The kinetics of the formation of ROS by TGF-β1-induced NADH oxidase activity is characterized by the sustained release of low concentrations of H2O2 for several hours from fibroblasts in comparison to the rapid, short-lived release of relatively larger amounts of ROS from the “respiratory burst” of neutrophils. New protein synthesis is not required for the activation of the NADPH oxidase in neutrophils by the stimulants studied.

Although the functional significance of TGF-β1-induced NADH oxidase activity remains speculative at this time, there is increasing recognition of redox-active biomolecules as regulators of cell function (8, 9). The kinetics and lower concentrations of ROS generated by TGF-β1 as well as the lack of evidence for cell toxicity favor the potential for the H2O2 generated to function as a signaling molecule. The lack of under-

2 D. J. Morre, personal communication.
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