Tumour necrosis factor α activates a p22^{phox}-based NADH oxidase in vascular smooth muscle

Gilles W. DE KEULENAER, R. Wayne ALEXANDER, Masuko USHIO-FUKAI, Nobukazu ISHIZAKA and Kathy K. GRIENDLING[1]
Division of Cardiology, Emory University School of Medicine, 1639 Pierce Drive, Atlanta, GA 30322, U.S.A.

Increasing experimental evidence suggests that non-phagocytic cells express a potent superoxide ($O_2^{-\bullet}$)-producing NADH oxidase that might be related to the phagocytic NADPH oxidase. Here we show that the cytokine tumour necrosis factor α (TNF-α) activates, in a time- and dose-dependent manner, a $O_2^{-\bullet}$-producing NADH oxidase in cultured rat aortic smooth-muscle cells. Dose–response experiments for NADH showed an upward shift of the curve for TNF-α-treated cells, suggesting that TNF-α increased the amount of available enzyme. Using the anti-sense transfection technique, we further demonstrate that the molecular identity of this oxidase includes p22^{phox} (the α subunit of cytochrome b_{558} and part of the electron transfer component of the phagocytic NADPH oxidase), which we recently cloned from a rat vascular smooth-muscle cell cDNA library. In addition, prolonged treatment with TNF-α increased p22^{phox} mRNA expression without affecting p22^{phox} mRNA stability, and only when transcriptional activity was intact. These findings identify a p22^{phox}-containing NADH oxidase as a source for cytokine-induced free radical production in vascular smooth-muscle cells and clarify some of the mechanisms involved in the regulation of vascular oxidase activity.

INTRODUCTION

Growing experimental evidence suggests that non-phagocytic cells express an NADH-driven superoxide ($O_2^{-\bullet}$)-producing enzyme that differs from known mitochondrial oxidoreductases. Recent studies by Mohazzab et al. [1] and by Rajagopalan et al. [2] have shown that this enzyme is the most important source of $O_2^{-\bullet}$ in intact arteries, producing far more $O_2^{-\bullet}$ than arachidonic acid-metabolizing enzymes, xanthine oxidase or mitochondrial sources. It has been shown that the non-phagocytic NADH oxidase activity is membrane-associated and that oxidase activity can be inhibited by flavin-protein inhibitors such as diphenylene iodinium [3]. The precise molecular structure of this oxidase is, however, still uncertain.

We have recently shown that the vascular NADH oxidase seems to be structurally related to, but not identical with, the potent phagocytic NADPH oxidase [4]. p22^{phox}, the small subunit of the cytochrome b_{558} of the phagocytic NADPH oxidase, is expressed in vascular cells [5]; vascular smooth-muscle cells (VSMCs) stably transfected with anti-sense p22^{phox} cDNA contain decreased amounts of inducible NADH oxidase activity [4]. So far only the cardiovascular hormone angiotensin II has been demonstrated to activate this oxidase. Its functional implications are, however, important because NADH oxidase activation and expression seem to lead to endothelial dysfunction and to be mechanistically related to the elevated blood pressure that accompanies angiotensin II infusion into rats [2,6,7].

Vascular oxidative stress also contributes to the pathogenesis of atherosclerosis [8]. One cardinal feature of early atherogenesis is the increase in expression and secretion of cytokines [9], which are potent activators of oxygen free-radical production in numerous cell types [10,11]. However, the molecular source of these free radicals is unknown. Because the NADH oxidase represents a potentially important contributor to control of the oxidative state of the vessel wall, we hypothesized that the VSMC NADH oxidase might be regulated by cytokines. We present evidence that a p22^{phox}-based, NADH-driven oxidase is activated by tumour necrosis factor α (TNF-α), and demonstrate that this enzyme is regulated at multiple levels. These studies have important implications for understanding the potential mechanism of action of cytokines in vascular cells.

EXPERIMENTAL

Cell culture

VSMCs were isolated from rat thoracic aorta by enzymic digestion as described previously [12]. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) calf serum, 2 mM glutamine, 100 i.u./ml penicillin and 100 μM streptomycin, passaged twice a week by harvesting with trypsin/EDTA, and seeded into 80 cm^2 flasks. For experiments, cells between passage 6 and 20 were seeded into 100 mm dishes, fed on alternate days and used at confluence.

In some experiments we used VSMCs that had been stably transfected with anti-sense p22^{phox} cDNA. In these cells p22^{phox} mRNA expression is completely inhibited and expression of the cytochrome component of the oxidase is abolished [4].

NADH oxidase assay

NADH oxidase activity was measured with a lucigenin assay as described previously [3]. Briefly, control cultures and cultures that had been exposed to TNF-α were washed and lysed in buffer containing protease inhibitors [20 mM monobasic potassium phosphate (pH 7.0)/1 mM EGTA/10 μg/ml aprotinin/0.5 μg/ml leupeptin/0.7 μg/ml pepstatin/0.5 mM PMSF]. The cell suspension was then subjected to Dounce homogenization

Abbreviations used: p22^{phox}, 22 kDa subunit of the phagocytic oxidase; TNF-α, tumour necrosis factor α; VSMC, vascular smooth-muscle cell.
[1] To whom correspondence should be addressed.

(100 times, on ice), and the homogenate was stored on ice until use. Protein content was measured in an aliquot of the homogenate by the Lowry method [13]. NADH oxidase activity was measured with lucigenin, which is specific for $O_2^{-\bullet}$, and the validity of the assay was confirmed in separate experiments with cytochrome *c* as the detector [14]. The assay was performed in a 50 mM phosphate buffer, pH 7.0, containing 1 mM EGTA, 150 mM sucrose, 500 μM lucigenin as the detector and NADH as the substrate (final volume 0.9 ml). NADH was used at a final concentration of 100 μM unless otherwise specified. The reaction was started by the addition of 100 μl of homogenate (50–150 μg of protein). Photon emission was measured every 15 s for 10 min in a luminometer. There was no measurable activity in the absence of NADH. A buffer blank (less than 5% of the cell signal) was subtracted from each reading before transformation of the data by comparison with a standard curve generated with xanthine/xanthine oxidase. In some experiments, inhibitors (diphenylene iodinium, KCN, Tiron, allopurinol or N^{ω}-nitro-L-arginine methyl ester) were added to samples 10 min before readings.

Northern blot analysis

Total RNA was isolated with TRI reagent. RNA (12 μg) was separated on denaturing formaldehyde/1% (w/v) agarose gels, transferred to Nytran membranes by overnight upward capillary blotting with 10 × SSC (where SSC is 0.15 M NaCl/0.015 M sodium citrate) and immobilized by UV cross-linking as described previously [15]. Consistency of total RNA loading between samples was controlled by densitometric analysis of 28 S RNA UV fluorescence in the presence of ethidium bromide. The full-length rat $p22^{phox}$ cDNA was labelled with a random-primer labelling kit (Prime It II) and [^{32}P]dCTP. Blots were prehybridized for at least 2 h and hybridized overnight at 42 °C in 1 M NaCl/50 mM Tris/HCl (pH 7.4)/5 × Denhardt's solution/50% (v/v) formamide/0.5% SDS/100 μg/ml sheared and denatured salmon sperm DNA. Denhardt's solution was omitted during hybridization. After hybridization the blots were washed three times in 1 × SSC and 1% (w/v) SDS at 50 °C. The blots were autoradiographed with Hyperfilm-MP at −80 °C and the relative density of each band was determined by laser densitometry. Staining of the 28 S band by ethidium bromide after transfer to the membrane was used for normalization.

Calculations and statistical analysis

NADH oxidase enzyme activity was quantified by calculating the mean amount of $O_2^{-\bullet}$ produced/mg of protein during 1 min. Enzyme activity was linear over a wide range of protein content (less than 10 μg to 200 μg) within one sample. Enzyme activities are presented as means ± S.E.M. Overall statistical significance was assessed by analysis of variance on untransformed data, followed by comparison of group averages by contrast analysis, using the SuperANOVA statistical program (Abacus Concepts, Berkeley, CA, U.S.A.). $P < 0.05$ was considered to be statistically significant.

Materials

All chemicals were of analytical grade or better. BSA and PMSF were from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Soybean trypsin inhibitor, glutamine, penicillin, streptomycin, calf serum and trypsin/EDTA were purchased from Gibco (Grand Island, NY, U.S.A.). TRI reagent was purchased from the Molecular Research Center (Cincinnati, OH, U.S.A.). Denhardt's solution was purchased from 5-Prime, 3-Prime (West Chester, PA, U.S.A.). Common buffer salts were obtained from Fisher (Pittsburgh, PA, U.S.A.). All other chemicals and reagents, including Dulbecco's modified Eagle's medium containing 25 mM Hepes, 4.5 g/l glucose and calf serum, were from Sigma (St. Louis, MO, U.S.A.). [^{32}P]dCTP was purchased from DuPont–NEN (Boston, MA, U.S.A.). Nytran membranes were purchased from Schleicher and Schuell (Keene, NH, U.S.A.) and Biospin columns from Bio-Rad (New York, NY, U.S.A.). Prime-It II probe labelling kits were purchased from Amersham Life Sciences (Arlington Heights, IL, U.S.A.). Diphenylene iodinium was purchased from Toronto Research Chemicals (Downsview, Ontario, Canada). Human TNF-α was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.).

RESULTS

Stimulation of the NADH oxidase with TNF-α

The effect of TNF-α on VSMC NADH oxidase activity is shown in Figures 1 and 2. NADH-dependent $O_2^{-\bullet}$ production by VSMC homogenates was increased in a dose- and time-dependent manner by treatment of the cells with TNF-α. An increase in oxidase activity was detectable at 25 i.u./ml and reached 2.3 ± 0.2-fold over control at 400 i.u./ml. This increase was observed after 30 min treatment with TNF-α and was sustained for at least 24 h.

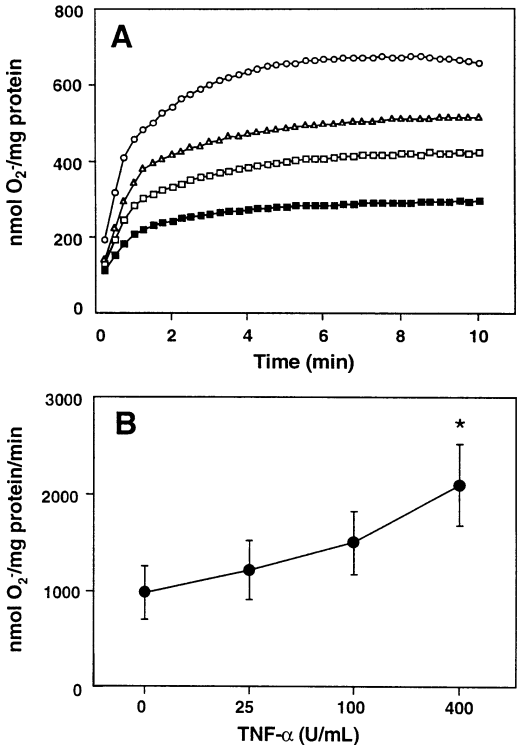

Figure 1 Dose-dependence of NADH oxidase activation by TNF-α in VSMC

(**A**) Graph from a representative experiment showing the effect of TNF-α on NADH-mediated $O_2^{-\bullet}$ production in VSMC homogenates. Cells were exposed to medium alone (■) or to medium with TNF-α at 25 (□), 100 (▲) or 400 (○) i.u./ml for 1 h. Cells were washed, homogenized and assayed for NADH (100 μM)-driven $O_2^{-\bullet}$ production as described in the Materials and methods section. (**B**) Graph showing the dose-dependent increase in NADH oxidase activity induced by TNF-α. Values are calculated means ± S.E.M. for three experiments. Significance: $^*P < 0.05$.

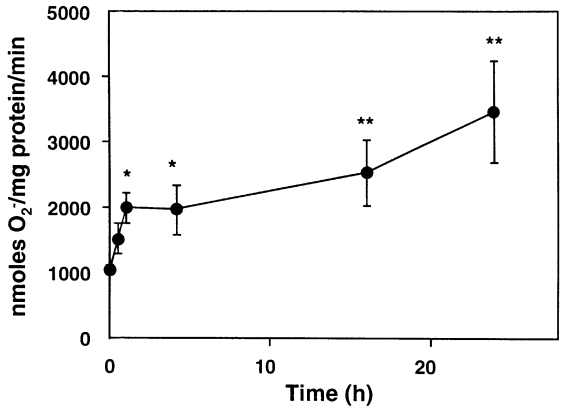

Figure 2 Time course of TNF-α-mediated NADH oxidase activation in VSMCs

VSMCs were exposed to medium alone or to medium with TNF-α (400 i.u./ml) and harvested at the indicated time points. Cells were washed, homogenized and assayed for NADH (100 μM)-driven $O_2^{-\bullet}$ production as described in the Experimental section. Values are calculated as means $\pm$ S.E.M. for at least five experiments at each time point. Significance: $^*P < 0.05$; $^{**}P < 0.001$.

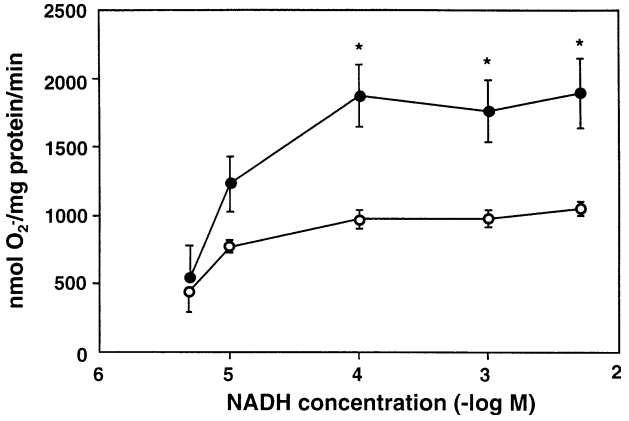

Figure 3 Substrate concentration curve for the vascular NADH oxidase

The effect of TNF-α on NADH-mediated $O_2^{-\bullet}$ production at different concentrations of NADH. VSMCs were exposed to medium alone (○) or to medium with TNF-α (400 i.u./ml) for 1 h (●). Cells were washed, homogenized and assayed for NADH-driven $O_2^{-\bullet}$ production as described in the Experimental section. Each sample was assayed five times at various concentrations of NADH (from 5 μM to 5 mM). Values are calculated as means $\pm$ S.E.M. for three experiments. Significance: $^*P < 0.05$.

At each time point, TNF-α-stimulated NADH oxidase activity was insensitive to KCN (1 mM), N^{ω}-nitro-L-arginine methyl ester (100 μM) and allopurinol (100 μM), indicating that $O_2^{-\bullet}$ production was not derived from mitochondrial electron transport, nitric oxide synthase or xanthine oxidase respectively. NADH oxidase activity was, however, dose-dependently decreased by 10 mM Tiron and the flavin protein inhibitor diphenylene iodinium (80–320 μM) (results not shown), verifying that the active oxygen species is $O_2^{-\bullet}$ and suggesting that the TNF-α-sensitive oxidase is a flavin-containing enzyme.

To determine whether the increase in $O_2^{-\bullet}$ production was due to enhanced activity of existing enzyme or to recruitment or expression of additional enzyme, we constructed a substrate concentration curve. The concentration of NADH was varied from 5 μM to 1 mM. Figure 3 shows that in both untreated and TNF-α-treated (400 i.u./ml, 1 h) cells, NADH oxidase activity was saturated at 100 μmol NADH, and that the plateau of the dose–response curve in TNF-α-treated cells was shifted upwards. Similar results were obtained after 24 h of treatment (results not shown). This suggests that a 1 or 24 h treatment with TNF-α recruits or increases the synthesis of additional oxidase units.

Table 1 Effect of TNF-α on NADH-mediated $O_2^{-\bullet}$ production in VSMCs stably transfected with anti-sense p22^{phox}

VSMCs were exposed to medium alone (control) or to medium with TNF-α (400 i.u./ml) for 1 h. Cells were washed, homogenized and assayed for NADH-driven $O_2^{-\bullet}$ production as described in the Materials and methods section. Values are calculated as means $\pm$ S.E.M. for five experiments. Significance: $^*P < 0.05$; n.s., not significant.

	$O_2^{-\bullet}$ production (nmol/min per mg)	
Incubation	Vector	Anti-sense
Control	3254 $\pm$ 351	3497 $\pm$ 767
TNF	5674 $\pm$ 651*	4185 $\pm$ 1010 (n.s.)

Role of p22^{phox} in TNF-α-induced NADH oxidase activity

NADH-mediated $O_2^{-\bullet}$ production reflects the activity of one or more NADH oxidases. One of these oxidases is a p22^{phox}-containing enzyme, which is activated by angiotensin II [4]. To determine whether the TNF-α-sensitive oxidase is p22^{phox}-based, we used VSMCs that had been transfected with anti-sense p22^{phox} or with vector alone. As outlined in the Experimental section and described in [4], endogenous p22^{phox} mRNA was clearly expressed in cells transfected with vector alone, but was completely absent from the p22^{phox} anti-sense-transfected cell line, leading to a decrease in the content of the cytochrome to a spectrophotometrically undetectable amount. Table 1 shows the effect of TNF-α (400 i.u./ml, 1 h) on NADH oxidase activity in vector-transfected and p22^{phox} anti-sense-transfected cells. At the baseline, NADH oxidase activity was identical in vector-transfected and p22^{phox} anti-sense-transfected cells. After treatment with TNF-α, however, NADH oxidase activity increased to 184 $\pm$ 31 % of control ($P < 0.05$) in vector-transfected cells, whereas TNF-α had no significant effect in anti-sense-transfected cells (124 $\pm$ 13 % of control, $P = 0.52$). This inability of TNF-α to increase $O_2^{-\bullet}$ in p22^{phox} anti-sense-transfected cells did not result from decreased TNF-α receptor expression or coupling, because TNF-α increased MCP-l mRNA expression to an identical extent in both vector-transfected and anti-sense p22^{phox}-transfected cells (results not shown).

Effect of TNF-α on p22^{phox} mRNA

The results presented in Figure 3 suggest that the increase in NADH oxidase activity in response to TNF-α results from an increase in the amount of oxidase expressed. We therefore investigated the ability of TNF-α to regulate p22^{phox} mRNA expression. As shown in Figure 4, TNF-α (400 i.u./ml, 3–24 h) increased p22^{phox} mRNA expression up to 1.91 $\pm$ 0.5-fold after 24 h incubation. This effect seemed to be dependent on increased transcription, because the transcriptional inhibitor 5,6-dichlorobenzimidazole riboside (30 μM, 5 min preincubation) completely inhibited TNF-α-induced p22^{phox} mRNA expression (results not shown) and NADH oxidase activity at 24 h (113 $\pm$ 19 % control).

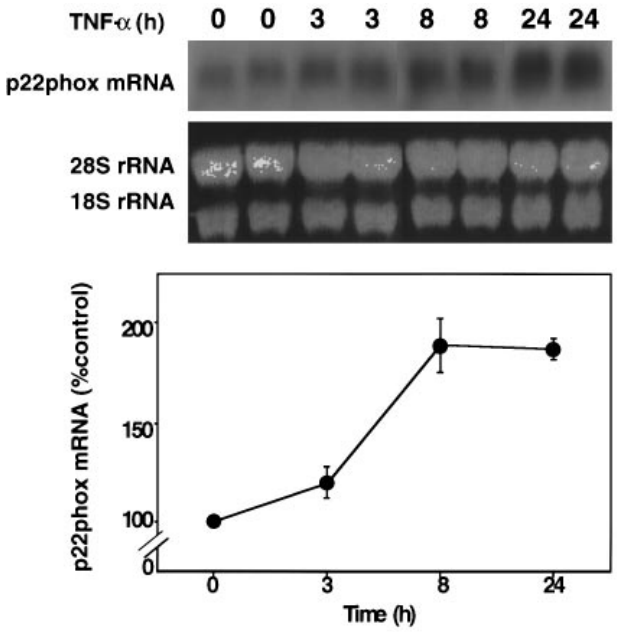

Figure 4 Effect of TNF-α on p22^{phox} mRNA expression

Representative Northern analysis (upper panel) and graph (lower panel) showing the time-dependent effect of TNF-α on p22^{phox} mRNA expression. VSMCs were exposed to medium alone or to medium with TNF-α (400 i.u./ml) and harvested at the indicated time points. RNA was extracted and prepared for Northern analysis as described in the Experimental section. The relative density of each band was determined by laser densitometry and normalized to the 28 S band stained by ethidium bromide. Normalized data are given in the lower panel. Increased p22^{phox} expression after prolonged exposure to TNF-α (more than 8 h) was observed in four independent experiments.

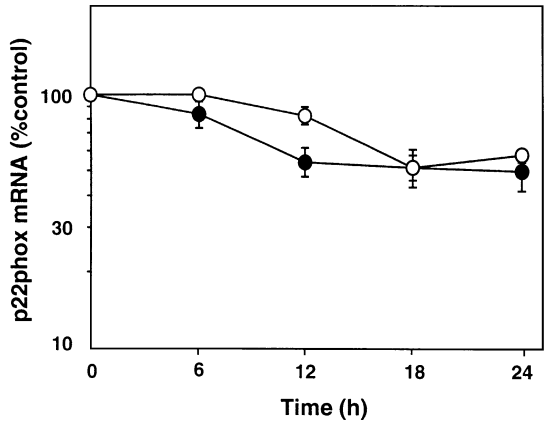

Figure 5 Effect of TNF-α on p22^{phox} mRNA stability

The effect of TNF-α on p22^{phox} mRNA stability. VSMCs were exposed to medium alone or to medium containing TNF-α (400 i.u./ml) for 24 h. The transcription inhibitor 5,6-dichlorobenzimidazole riboside (30 μM) was added and cells were harvested at the indicated time points. RNA was extracted and prepared for Northern analysis as described in the Experimental section. The relative density of each band was determined by laser densitometry and normalized to the 28 S band stained by ethidium bromide. Values are calculated as means ± S.E.M. for two to five experiments.

Furthermore the stability of p22^{phox} mRNA was not significantly affected by TNF-α (Figure 5), although a trend for a slight increase in stability existed. The half-life of p22^{phox} mRNA was approx. 20 h in control cells and 25 h ($P = 0.077$) after TNF-α treatment.

DISCUSSION

The present study demonstrates that TNF-α increases NADH-mediated $O_2^{-\bullet}$ production in cultured VSMCs. This activation was accompanied by an upward shift of the substrate–activity curve, suggesting the recruitment or synthesis of additional oxidase units. In addition we showed that the NADH oxidase mediating TNF-α-stimulated oxidase activity is p22^{phox}-based, as indicated by experiments with cells stably transfected with antisense p22^{phox} cDNA. As expected from the shift in the substrate–activity curve, TNF-α increased p22^{phox} mRNA levels, most probably at the transcriptional level. Thus we have identified in smooth muscle a cytokine-sensitive enzyme that can alter the redox state of the cell.

TNF-α induces the production of oxygen free radicals in numerous cell types, including smooth-muscle-like cells [10,16]. In tumour and transformed cells, TNF-α-stimulated free radical production has been explained by alterations in mitochondrial electron transport and is proposed to be involved in TNF-α-induced cytotoxicity [17]. The origin of TNF-α-induced free radical production in vascular cells is, however, unknown. This study provides the first direct evidence that TNF-α in VSMCs activates an $O_2^{-\bullet}$-producing NADH oxidase system.

The vascular NADH oxidase is a potent membrane-associated extramitochondrial oxidase that uses NADH and to a much smaller extent NADPH as substrates for electron transfer to molecular oxygen [1–3]. Interestingly, previous studies have shown that a component of the vascular oxidase system shares some similarities with the phagocytic NADPH oxidase in that both contain flavin-binding regions that are probably important in the transfer of electrons [3]. In phagocytes the electron transfer element of the NADPH oxidase is cytochrome b_{558}, which includes p22^{phox} as one of its subunits [18]. It has been suggested that, in VSMCs, NADH-dependent $O_2^{-\bullet}$ production might come from a microsomal electron transport chain containing a cytochrome b_{558}-like oxidase and an NADH-dependent reductase of cytochrome b_5 [19]. We have recently shown that VSMCs contain a p22^{phox}-based NADH oxidase that is activated by angiotensin II [4], and the current results demonstrate that TNF-α stimulates the same enzyme. The mechanisms by which TNF-α activates this NADH oxidase, however, seem to differ from those utilized by angiotensin II. Angiotensin II does not cause a rapid increase in oxidase activity [3], nor does it alter p22^{phox} mRNA expression (T. Fukui and K. K. Griendling, unpublished work).

Both the rapid and sustained effects of TNF-α are apparently due to an increase in the amount of active oxidase (the plateau of the dose–response curve for NADH in Figure 3 is shifted upwards in TNF-α-treated cells) rather than to a change in the affinity of the oxidase for NADH (the curve is not shifted to the left). Increases in the amount of active enzyme might result from post-translational activation or assembly of existing protein, increased translation of existing mRNA, or increased mRNA transcription *de novo*. Given that the complete molecular identity of the vascular oxidase is still unknown, at this point it is difficult to distinguish between these possibilities. In this regard, however, we observed that TNF-α increased p22^{phox} mRNA expression, especially after prolonged treatment. This delayed increase in p22^{phox} mRNA expression seemed to result from enhanced transcription *de novo* rather than from alterations in p22^{phox} mRNA stability (Figure 5). Experiments with and without the transcription inhibitor 5,6-dichlorobenzimidazole

riboside indicated that preserved transcriptional activity was necessary to stimulate the NADH oxidase by 24 h. Together these findings suggest that increased $p22^{phox}$ mRNA transcription *de novo* might be required for prolonged activation of the oxidase by TNF-α.

Despite similarities between the vascular and the phagocytic oxidase, significant functional and structural differences exist. First, various investigators have reported that the vascular oxidase prefers NADH to NADPH as a substrate for its activity and that its output is much lower than the phagocytic oxidase [1–3]. Secondly, we and others have been unable to detect the 91 kDa β-subunit ($gp91^{phox}$) of the phagocytic cytochrome b_{558} ([20], and T. Fukui and K. K. Griendling, unpublished work); the role of the cytosolic components of the phagocytic oxidase ($p47^{phox}$, $p67^{phox}$ and Rac-1 or Rac-2) remains to be determined in VSMCs. Thirdly, the peak of the absorbance spectrum of the cytochrome component of the vascular oxidase is shifted slightly to the left (approx. 553 nm) [4]. Lastly, knock-out of $p22^{phox}$ in vascular cells does not affect baseline NADH-mediated $O_2^{-\cdot}$ production, but only abrogates agonist-stimulated oxidase activity (Table 1), suggesting that the $p22^{phox}$-containing oxidase is an inducible protein that has little constitutive activity in untreated cells.

In summary, TNF-α induces a rapid and sustained activation of a $O_2^{-\cdot}$-producing, $p22^{phox}$-based NADH oxidase in VSMCs. This response probably results from an increased availability of active enzyme, which after prolonged exposure to TNF-α might include enhanced $p22^{phox}$ mRNA expression. These findings further our understanding of the regulation of the vascular NADH oxidase and might provide insights into the signalling mechanisms recruited by cytokines in vascular cells.

We thank Dr. Bernard Lassègue, Dr. A. Maziar Zafari and Dr. W. Robert Taylor; Marjorie Akers for technical assistance and helpful discussions; Barbara Merchant-Bailey for editorial assistance; and Carolyn Morris for secretarial assistance. This work was supported by NIH grants HL48667 and HL58863 and a fellowship from the Belgian American Educational Foundation (to G.D.K.).

Received 4 July 1997/5 September 1997; accepted 19 September 1997

REFERENCES

1 Mohazzab-H., K. M. and Wolin, M. S. (1994) Am. J. Physiol. **267**, L815–L822
2 Rajagopalan, S., Kurz, S., Münzel, T., Tarpey, M., Freeman, B. A., Griendling, K. K. and Harrison, D. G. (1996) J. Clin. Invest. **97**, 1916–1923
3 Griendling, K. K., Minieri, C. A., Ollerenshaw, J. D. and Alexander, R. W. (1994) Circ. Res. **74**, 1141–1148
4 Ushio-Fukai, M., Zafari, A. M., Fukui, T., Ishizaka, N. and Griendling, K. K. (1996) J. Biol. Chem. **271**, 23317–23321
5 Fukui, T., Lassegue, B., Kai, H., Alexander, R. W. and Griendling, K. K. (1995) Biochim. Biophys. Acta **1231**, 215–219
6 Fukui, T., Ishizaka, N., Rajagopalan, S., Laursen, J. B., Capers, IV, Q., Taylor, W. R., Harrison, D. G., de Leon, H., Wilcox, J. N. and Griendling, K. K. (1997) Circ. Res. **80**, 45–51
7 Bech-Laursen, J., Rajagopalan, S., Galis, Z., Tarpey, M., Freeman, B. A. and Harrison, D. G. (1997) Circulation **95**, 588–593
8 Alexander, R. W. (1995) Hypertension **25**, 155–161
9 Rus, H. G., Niculescu, F. and Vlaicu, R. (1991) Atherosclerosis **89**, 247–254
10 Radeke, H., Meier, B., Topley, N., Floge, J., Habermehl, G. and Resch, K. (1990) Kidney Int. **37**, 767–775
11 Meier, B., Radeke, H. H., Selle, S., Younes, M., Sies, H., Resch, K. and Habermehl, G. G. (1989) Biochem. J. **263**, 539–545
12 Griendling, K. K., Taubman, M. B., Akers, M., Mendlowitz, M. and Alexander, R. W. (1991) J. Biol. Chem. **266**, 15498–15504
13 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
14 Bellavite, P., Corso, F., Dusi, S., Grzeskowiak, M., Della-Bianca, V. and Rossi, F. (1988) J. Biol. Chem. **263**, 8210–8214
15 Lassègue, B., Alexander, R. W., Nickenig, G., Clark, M., Murphy, T. J. and Griendling, K. K. (1995) Mol. Pharmacol. **48**, 601–609
16 Meier, B., Cross, A. R., Hancock, J. T., Kaup, F. J. and Jones, O. T. G. (1991) Biochem. J. **275**, 241–245
17 Schulze-Osthoff, K., Beyaert, R., Vandevoorde, V., Haegeman, G. and Fiers, W. (1993) EMBO J. **12**, 3095–3104
18 Dinauer, M. C., Pierce, E. A., Erickson, R. W., Muhlebach, T. J., Messner, H., Orkin, S. H., Seger, R. A. and Curnutte, J. T. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 11231–11235
19 Mohazzab-H., K. M., Kaminski, P. M. and Wolin, M. S. (1994) Am. J. Physiol. **266**, H2568–H2572
20 Jones, S. A., Hancock, J. T., Jones, O. T. G., Neubauer, A. and Topley, N. (1995) J. Am. Soc. Nephrol. **5**, 1483–1491