Journal of Cellular Biochemistry 68:339–354 (1998)

Thiol Redox Modulation of Tumor Necrosis Factor-α Responsiveness in Cultured AIDS-Related Kaposi's Sarcoma Cells

S.R. Mallery,[1]* D.J. Landwehr,[1] G.M. Ness,[1] Y.M. Clark,[1] and C.M. Hohl[2]

[1]Departments of Oral Surgery and Pathology, Colleges of Dentistry and Medicine, Ohio State University, Columbus, Ohio 43210
[2]Medical Biochemistry, Colleges of Dentistry and Medicine, Ohio State University, Columbus, Ohio 43210

Abstract Both clinical and experimental evidence indicates that AIDS-related Kaposi's sarcoma (AIDS-KS) has a multifactorial pathogenesis with factors such as HIV viral load, latent virus induction, and opportunistic infections contributing to disease progression. However, a consistent feature that unites these apparently diverse putative etiologic agents is sustained serum elevations of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α). While virtually every cell responds to TNF-α with gene activation, the extent of TNF-α-mediated cellular signaling is regulated by a delicate balance between signal activation and signal arresting events. Reactive oxygen intermediates (ROI), which are generated as a consequence of TNF-α membrane interaction, are part of this TNF-α-initiated cellular activation cascade. Previous studies in our laboratory have shown that AIDS-KS cells possess impaired oxygen intermediate scavenging capacities, thereby establishing conditions permissive for the intracellular retention of ROI. In this study, we used cellular capacity to upregulate the cytoprotective enzyme superoxide dismutase (SOD) to address the extent of cellular response to TNF-α. Concurrent with the SOD analyses, nucleotide profiles were obtained to assess cellular bioenergetic responses during TNF-α challenge. Proliferative growth levels of mitochondrial (Mn)SOD activities showed an activity spectrum ranging from lowest activity in AIDS-KS cells, to intermediate levels in matched, nonlesional cells from the AIDS-KS donors, to highest activities in HIV^- normal fibroblasts. In contrast, following TNF-α challenge, the AIDS-KS and KS donor nonlesional cells showed a 11.89- and 5.86-fold respective increase in MnSOD activity, while the normal fibroblasts demonstrated a 1.35-fold decrease. Subsequent thiol redox modulation studies showed that only the normal fibroblast cultures showed a potentiation of TNF-α-mediated MnSOD upregulation following GSH depletion. In addition, provision of the GSH precursor, N-acetylcysteine during TNF-α challenge only diminished MnSOD activity and mitochondrial compartmentalization in the AIDS-KS cells, a finding that likely reflects the lower levels of reduced thiols in this cellular population. Our data, which show that a perturbation in their cellular thiol redox status accentuates AIDS-KS cellular responsiveness to TNF-α, suggest a biochemical rationale for the recognized TNF-α AIDS-KS clinical correlation. J. Cell. Biochem. 68:339–354, 1998.

Key words: glutathione; reactive oxygen intermediates; HIV; signal transduction; cytokines; redox state

Clinical and experimental evidence both suggest that acquired immunodeficiency syndrome-related Kaposi's sarcoma (AIDS-KS) has a multifactorial etiology with factors such as latent virus induction, sustained inflammation, and aberrant cytokine production contributing to disease progression [10,18,30,32]. Further, the milieu in which AIDS-KS arises is paradoxical due to the profound immunostimulation coinciding with significant immunosuppression [10,18,30,32]. Although AIDS patients develop a $CD4^+$ lymphopenia, there is generally a sparing effect on their neutrophils and macrophages [8]. Consequently, the host phagocytic cellular response remains intact [8]. Activated macrophages are the primary source for tumor necrosis factor-α (TNF-α), a cytokine maintained to be integral in AIDS progression [15,17,30]. Serum TNF-α levels, which are initially increased by human immunodeficiency virus-positive (HIV^+) seroconversion, are fur-

Contract grant sponsor: National Institutes of Health; Contract grant number: NHLB R01 48547; Contract grant number: DE RO1 12183; Contract grant number: CA UO1 66531.

*Correspondence to: S.R. Mallery, Departments of Oral Surgery and Pathology, Colleges of Dentistry and Medicine, Ohio State University, Columbus, Ohio 43210-1241.

Received 12 August 1997; Accepted 29 September 1997

ther augmented by opportunistic infections that arise, like AIDS-KS, during the latter stages of AIDS [8,18,30].

Since most cells constitutively express both the 55- and 75-kDa tumor necrosis factor-α (TNF-α) receptors, much of the modulation of the extent of the TNF-α cellular response is conducted at the signal transduction level [15,43]. Upon TNF-α receptor interaction, a signal transduction cascade is initiated through activation of phospholipases C and A2, as well as neutral sphingomyelinases [9,15,16]. Reactive oxygen intermediates (ROI) are generated as a direct consequence of TNF-α membrane interaction (NAD(P)H oxidase release of $O_2^{\cdot-}$) and subsequently act as secondary messengers in TNF-α signal transduction [15,20]. Provided efficient initial signal transduction occurs, TNF-α can subsequently promote the downstream activation of both the mitogen activated protein kinase (MAP kinase) cascade as well as the multifaceted transcriptional activator, nuclear factor κB (NF-κB) [15,40]. Notably, both processes are highly effective at instigating overall cellular activation [6,7].

A diminished cellular capacity to degrade ROI would provide conditions favorable for optimal TNF-α responsiveness and subsequently facilitate AIDS-KS disease progression [14,20,42,44]. Intracellular conditions that either increase the levels of ROI, or decrease intracellular thiol levels, promote the activation of NF-κB [1,38]. Because of its regulatory role in gene induction, one of the many results of NF-κB activation would be enhanced cellular cytokine expression, creating the opportunity for autologous growth loops [4,7]. Additional studies, which are supported by clinical trials using antioxidants, have shown that increases in the intracellular reduced thiol content inhibit TNF-α-mediated initiation of HIV replication [19,36,36].

Several biochemical interactions depict how the cellular glutathione (GSH) redox status is closely associated with ROI-mediated signal transduction [20,42,44]. First, the intracellular redox state (buffered by GSH) is coupled to the oxidation state of cysteine residues in proteins by thiol disulfide interchange [28,42]. Because high levels of intracellular free thiols (primarily GSH) can function as ROI scavengers, high cellular levels of GSH decrease the opportunity for ROI to act as secondary messengers [20,42]. Also, all protein tyrosine phosphatases (e.g., CD 45, the enzyme that downregulates the MAP kinase cascade) are dependent on intact, reduced thiol groups [14,44,51]. Finally, high levels of intracellular reduced thiols may influence protein folding and enzyme activation, thereby inhibiting activation of protein kinases that phosphorylate inhibitory factor κB (IκB) and subsequently activate NF-κB [42,45]

Previous studies in our laboratory have shown that AIDS-KS cells possess impaired oxygen intermediate scavenging capacities, which establishes a condition permissive for the intracellular retention of ROI [22,23]. Relative to human microvascular endothelial cells or human mucosal fibroblasts obtained from HIV^- donors, AIDS-KS cells contain significantly lower levels of GSH, as well as a key cofactor (NADPH) necessary for reduction of the disulfide GSSG [22,23]. Also, AIDS-KS cells show an impaired adaptation to oxidant stress as indicated by failure to upregulate catalase function [23] and modulate bioenergetic profiles [22] subsequent to oxidant challenge. Collectively, our data suggest that as a consequence of their perturbed thiol redox status, TNF-α-initiated ROI would persist longer in AIDS-KS cells, resulting in a more extensive signal transduction. In this study, we used cellular capacity to upregulate superoxide dismutase (SOD) activity to address the extent of cellular response to TNF-α. Functional SOD analyses and determination of the intracellular distribution (cytosolic vs mitochondrial) of this cytoprotective enzyme were used for the following reasons: (1) TNF-α is known to upregulate mitochondrial (Mn) SOD expression [31], (2) assessment of SOD functional activity addresses the efficacy of TNF-α-mediated signal transduction, and (3) AIDS-KS cellular survival in the sustained inflammatory states that characterize late AIDS is contingent on upregulation of cytoprotective enzymes like SOD. Concurrent with SOD analyses, nucleotide profiles were obtained to assess cellular bioenergetic responses during TNF-α challenge.

Proliferative growth levels of MnSOD activity showed an activity spectrum ranging from lowest activity in AIDS-KS cells, to intermediate levels in matched, nonlesional cells from the AIDS-KS donors, to highest activities in HIV^- normal fibroblasts. However, markedly different SOD results were obtained subsequent to TNF-α challenge. We determined that relative to either matched nonlesional fibro-

blasts from the AIDS-KS donors, or HIV$^-$ fibroblasts, AIDS-KS cells showed the most immediate and dramatic response to TNF-α, as reflected by their 24-h post challenge SOD profiles. Further, the GSH modulation studies substantiated the contribution of the cellular thiol redox status in TNF-α-mediated signal transduction in AIDS-KS cells. Subsequent to GSH depletion, the response of normal fibroblasts to TNF-α mimicked that of AIDS-KS cells. In addition, provision of the GSH precursor, N-acetylcysteine during TNF-α challenge diminished Mn-SOD activity and mitochondrial enzymatic compartmentalization in the AIDS-KS cells but did not affect normal or nonlesional cells.

This study describes for the first time a correlation between an existing thiol redox perturbation in AIDS-KS cells and their enhanced responsiveness to the AIDS relevant cytokine, TNF-α. Also, we determined that the internal control population; i.e., the matched nonlesional cells from the AIDS-KS donors behaves as a biochemically distinct population, which demonstrate intermediate levels of many of the cellular biochemical parameters evaluated in this study. We speculate that the AIDS-KS increased responsiveness to TNF-α facilitates lesional progression by upregulating pathways e.g. activation of NF-κB, conducive to the development of KS cellular growth autonomy.

MATERIALS AND METHODS

Isolation and Culture of Cells

All cellular strains used in this study had undergone <20 population doubling levels.

AIDS-KS cells. HIV$^+$ individuals that had clinical lesions suggestive of KS were referred for participation in this study by the Ohio State University Department of Infectious Disease. Prior to biopsy of the suspected KS lesion, an examination was conducted to determine the extent and clinical presentation of the individuals' lesion(s). A portion of each biopsy was submitted for light microscopic examination to confirm the diagnosis of AIDS-KS.

The AIDS-KS cells were isolated from the tissue specimen as previously described [23]. Prior to the TNF-α challenge assays, the AIDS-KS cells were cultured on human fibronectin-coated flasks (hFN) and grown in COMPLETE medium, which consisted of M-199 (GIBCO, Grand Island, NY), supplemented with 15 mM HEPES, 2.9 μg/ml l-glutamine, 1.1 μg/ml Na pyruvate, sodium heparin (Sigma, St. Louis, MO), 90 μg/ml) endothelial cell growth supplement (prepared in-house, from bovine brain, 150 μg/ml), 15% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 5% heat-inactivated pooled, male human serum (Sigma).

Nonlesional fibroblasts from AIDS-KS donors. At either the initial biopsy appointment, or the 1-week recall examination, nonlesional tissue was obtained from the AIDS-KS donors' clinically normal oral mucosa. A portion of the specimen was submitted for microscopic confirmation of "no pathologic change." Nonlesional fibroblasts were isolated and cultured in the same fashion as the AIDS-KS cells. Nonlesional cells from the AIDS-KS donors were included as the endogenous control in this study, as these cells have been exposed to the same in vivo factors, e.g., sustained high serum levels of TNF-α as the AIDS-KS cells, but have not undergone transformation.

Normal fibroblasts from HIV$^-$ gender and age-matched donors. Tissue explants were obtained from normal oral mucosa of HIV$^-$ men, and a portion of the tissue submitted for light microscopy to confirm a diagnosis of "no pathologic change." Normal fibroblasts were isolated and cultured in the same fashion as the AIDS-KS cells. HIV$^-$ fibroblasts were included as the overall control cellular population. These cells, which were obtained from clinically healthy age-matched men, would not have been exposed in vivo to the serum cytokine abnormalities characteristic of the HIV$^+$ state. Also, inclusion of normal fibroblasts provides a basis for enzymatic activity comparison with published values, as numerous studies on SOD activities have been conducted on human fibroblasts.

Overall Sample Handling for Conduction of Biochemical Assays

As complete of a biochemical profile as possible was obtained for each group of experiments; i.e., cells from a specific treatment or control group were pooled at harvest, and representative cellular aliquots taken to assess concurrently SOD, nucleotides, and, where applicable, GSH. To prevent the loss of SOD activity as a result of proteolytic enzymatic degradation, aprotinin (Sigma), 100 kallikrein inhibitory U/ml, was incorporated into the washing and buffer solutions used during cellular harvesting.

Evaluation of Cellular Response to TNF-α

We previously determined that COMPLETE medium contains an array of various cytokines [3]. To reduce the interference from these various cytokines and other serum factors, permitting a more precise assessment of TNF-α-mediated cellular effects, TNF-α challenge assays were conducted in BASE medium (M-199 medium supplemented with 0.5% heat-inactivated fetal bovine serum (FBS), 2.9 µg/ml l-glutamine, 1.1 µg/ml Na pyruvate, 15 mM HEPES). At 24 h prior to the introduction of human recombinant TNF-α (100 U/ml, Genzyme, Cambridge, MA), the cultures were rinsed twice with room temperature phosphate-buffered saline (PBS), and COMPLETE medium was replaced with BASE medium.

Cells were harvested, an aliquot taken for enumeration and viability assessment (trypan blue exclusion) the remaining sample resuspended to a volume of 400 µl in 50 mM phosphate buffer, pH 7.8 (SOD assay buffer), and the sample transferred to a microfuge tube. Two courses of liquid nitrogen freeze-thaw were then used to disrupt plasma and mitochondrial membranes, releasing MnSOD and cytosolic (CuZn)SOD into the supernate. Preliminary studies, using commercially available SOD (Sigma), showed that this enzymatic harvesting procedure did not result in loss of recoverable SOD activity. The cellular samples were then centrifuged at 4°C, 1,400 rpm for 3 min, the supernate carefully removed, and the supernate volume recorded. The SOD samples remained frozen at -80°C until assayed.

Assessment of Total, Cytosolic, and Mitochondrial Cellular SOD Activity

Cellular levels of SOD were determined during the following culture conditions: (1) proliferative growth in COMPLETE medium; (2) following a 24-h serum deprivation in BASE medium, then challenge with 100 U/ml TNF-α for a period of 24 h (treated cultures); (3) following a 24-h serum deprivation in BASE medium, then culture for a period of 24 h in BASE medium (control cultures); (4) following a 24-h serum deprivation in BASE medium, then GSH depletion, followed by a 24-h TNF-α challenge in BASE medium; and (5) following a 24-h serum deprivation in BASE medium supplemented with 30 mM N-acetylcysteine (NAC), then a 24-h TNF-α challenge in BASE medium plus 30 mM NAC.

Cellular SOD activity was determined, using a modification of the method of Flohe and Otting [12], by calculating the rate of inhibition of the reduction of cytochrome c. The assay was conducted on a DU 7400 Beckman spectrophotometer, equipped with a Peltier temperature regulating device, at 550 nm, 25°C, using a xanthine/xanthine oxidase system to generate $O_2^{\cdot -}$. Acetylated cytochrome c was used to increase the specificity of the assay, as this form of cytochrome c can still be reduced by $O_2^{\cdot -}$, but can no longer undergo redox enzymatic reactions [2]. A five-point SOD standard curve (0.05, 0.10, 0.50, 0.75, 1.00 U/ml) was conducted with each assay, with one unit of SOD activity defined as the amount of enzyme that results in a 50% inhibition of the reduction of acetylated cytochrome c. To determine mitochondrial (Mn)SOD activity, 2 mM (final concentration) of KCN was added to the MnSOD samples to inhibit cytosolic (CuZn)SOD activity. CuZn-SOD activity was then calculated by subtraction of the MnSOD levels from the total (samples without KCN) SOD activity. Cellular SOD activity was reported in units of activity/mg of cell protein.

Determination of NAD(P)H Oxidase Activation Subsequent to TNF-α Challenge

Previous reports have shown that TNF-α membrane interaction results in NAD(P)H oxidase activation in both phagocytic and some nonphagocytic cells [41]. Due to the importance of oxidative burst generated ROI in signal transduction, studies were conducted to determine whether or not NAD(P)H oxidase was activated, resulting in the generation of $O_2^{\cdot -}$ in our cellular populations as a consequence of TNF-α binding.

Cells were grown to confluence in COMPETE medium. At 24 h prior to TNF-α challenge, the cells were rinsed with room temperature PBS, and BASE medium added. On the day of assay, the cells were harvested, resuspended to a 1ml volume in PBS-85 µM cytochrome c, and placed in microfuge tubes. The SOD or comparable volume of PBS was placed in the sample tubes prior to the introduction of TNF-α. Experimental groups consisted of (1) 100 U/ml TNF-α, (2) 100 U/ml TNF-α plus 20 U/ml SOD, (3) reagent control (PBS without either TNF-α or SOD), and (4) enzymatic control (PBS without TNF-α

but including 20 U/ml SOD. The samples were then placed on a shaking platform in a 37°C, 5% CO_2 incubator for 30 min. Following incubation, an aliquot was taken for cell counts and viability, then the samples were centrifuged at 4°C, 1,400 rpm for 3 min, the supernate carefully removed, and the cellular pellet kept for protein determination. Samples were read at 550 nm to determine the extent of cytochrome c reduction, then a few crystals of sodium dithionite were added to completely reduce the cytochrome c. The data were expressed as the percentage of the total reduction of cytochrome c per milligram protein.

Modulation of Cellular GSH Status Prior to TNF-α Challenge

To address the contribution of the cellular thiol redox status in TNF-α-mediated cellular signaling, modifications of the cellular GSH status were conducted in these studies prior to TNF-α challenge.

GSH depletion. Cellular levels of GSH were decreased by a method previously employed [24]. Cellular synthesis of GSH was inhibited by a 2-h incubation in BASE medium containing 1×10^{-4} M buthionine sulfoximine, followed by a rinse with room temperature PBS. To decrease the existing levels of GSH, the cultures were then incubated for 1 h in BASE medium containing 100 μM 2-cyclohexene-1-one (2 CHX-1-one). We have previously shown that this method is highly effective at reducing cellular GSH levels without promoting intracellular retention of GSSG and is readily reversible [24]. The cultures were then rinsed with room temperature PBS, BASE medium containing 100 U/ml TNF-α added, and cells incubated at 37°C, 5% CO_2 for 24 h.

Augmentation of cellular thiol status. The BASE medium was supplemented with the membrane permeable GSH precursor, 30 mM NAC, during both the 24-h serum starvation and TNF-α challenge incubations.

Determination of Cellular GSH Levels

Cellular levels of GSH were determined according to the method of Eyer and Podradsky [11]. NADPH, GSH glutathione reductase (type IV), and DTNB were obtained from Sigma.

Rapid reaction kinetics were followed on a SLM-AMINCO (Urbana, IL) dual-wavelength spectrophotometer, with the following instrument settings: 412 nm vs 550 nm, 3-nm bandwidth, 0.05 Abs, 50 sec/in. Sample concentrations (nmol/ml) were determined by comparison with a 10- point (0.2- to 2-nmol/ml) standard curve conducted concurrently. The results are expressed as nmol GSH/mg protein.

Determination of Cellular Bioenergetic Status

Cellular levels of nucleotides and nucleosides were determined by high-performance liquid chromatography (HPLC), by a modification of the method of Geisbuhler et al. [13], as reported in our publications [22,23,25].

Protein Determination

Cellular protein levels were determined by the Lowry method, using bovine γ-globulins as the standard protein [21].

Statistical Analysis

Nonparametrical statistics (the Kruskal-Wallis One-Way ANOVA, followed by the two tailed Mann-Whitney U-test) were used to evaluate the nucleotide profiles and SOD results obtained during proliferative growth and the initial TNF-α challenge assays. Statistical evaluation of the thiol redox modulation studies was conducted using an ANOVA followed by a multiple t-test.

RESULTS

Culture Group Differences in Proliferative Growth SOD Activities and Bioenergetic Profiles

Initial studies were conducted to determine cellular SOD and bioenergetic profiles during proliferative growth in COMPLETE medium. The cellular strains from all three groups (AIDS-KS, nonlesional from the KS donors, healthy HIV^-) showed positive adaptations to in vitro culture in COMPLETE medium as demonstrated by high mitotic indices, possession of an average energy charge (e.c.) of >0.92:

$$\text{(e.c.} = [ATP] + \tfrac{1}{2}[ADP]/[ATP] + [ADP] + [AMP])$$

indicative of ongoing active oxidative metabolism, and retention of a constant, strain specific cellular phenotype (Table I).

Comparison of the proliferative growth SOD results showed similarities as well as differences among the three groups (Fig. 1). While all three groups contained comparable total levels of SOD, the mitochondrial (MnSOD) levels revealed an activity spectrum ranging from high-

TABLE I. Bioenergetic Profiles of Cellular Populations During Proliferative Growth, Control, and TNF-α Challenge Conditions[a]

Nucleotide	Normal	Nonlesional	AIDS-KS
ATP			
Proliferative	32.47 ± 6.85	34.64 ± 5.50	29.42 ± 14.54
Control	27.70 ± 5.15**	26.08 ± 6.38	17.11 ± 4.04**
TNF-α challenge	23.04 ± 4.82†	22.83 ± 6.19	15.93 ± 6.07†
Total adenine nucleotides			
Proliferative	35.31 ± 6.14	36.85 ± 5.87	31.96 ± 14.52
Control	30.03 ± 5.91*	28.34 ± 6.52	19.51 ± 4.31*
TNF-α challenge	25.77 ± 4.49	25.11 ± 4.70	18.01 ± 5.42
NAD^+			
Proliferative	6.88 ± 1.58	8.07 ± 2.09	6.78 ± 3.08
Control	4.74 ± 1.16	5.16 ± 1.31	4.54 ± 0.86
TNF-α challenge	4.09 ± 0.22	4.95 ± 1.14	4.69 ± 1.72
NADH			
Proliferative	2.90 ± 0.41	4.02 ± 2.24	3.04 ± 0.65
Control	3.63 ± 0.71	4.86 ± 1.55	3.60 ± 0.65
TNF-α challenge	3.21 ± 0.45	3.52 ± 0.95	3.82 ± 1.58
$NADP^+$			
Proliferative	0.20 ± 0.56	0.20 ± 0.08	0.12 ± 0.04
Control	0.18 ± 0.14	0.19 ± 0.15	0.17 ± 0.07
TNF-α challenge	0.13 ± 0.08	0.26 ± 0.11	0.14 ± 0.10
NADPH			
Proliferative	0.25 ± 0.06**	0.25 ± 0.08*	0.12 ± 0.04*,**
Control	0.32 ± 0.07†,‡	0.19 ± 0.05‡	0.19 ± 0.05†
TNF-α challenge	0.28 ± 0.06**	0.21 ± 0.07	0.14 ± 0.08**
GTP			
Proliferative	4.88 ± 0.89	5.18 ± 0.90	3.88 ± 1.89
Control	4.58 ± 0.93	4.17 ± 0.92	3.33 ± 1.16
TNF-α challenge	3.89 ± 0.56	3.85 ± 0.77	3.02 ± 1.15
UTP and CTP			
Proliferative	5.81 ± 0.66	6.61 ± 1.72	4.37 ± 2.26
Control	5.44 ± 0.99	4.49 ± 1.13	2.91 ± 1.26
TNF-α challenge	4.99 ± 1.15	4.64 ± 1.30	3.02 ± 1.46
Energy charge			
Proliferative	0.95 ± 0.03	0.97 ± 0.01	0.92 ± 0.06
Control	0.95 ± 0.01	0.96 ± 0.02	0.92 ± 0.02
TNF-α challenge	0.94 ± 0.04	0.94 ± 0.03	0.92 ± 0.03
ATP/ADP			
Proliferative	18.27 ± 8.52	19.93 ± 3.95	13.94 ± 7.13
Control	15.66 ± 3.02	14.52 ± 5.36	12.11 ± 4.77
TNF-α challenge	13.06 ± 5.82	15.32 ± 8.92	9.74 ± 4.63
Redox poise			
Proliferative	0.46 ± 0.10	0.52 ± 0.26	0.52 ± 0.15
Control	0.82 ± 0.10	0.95 ± 0.23	0.82 ± 0.13
TNF-α challenge	0.83 ± 0.11	0.76 ± 0.34	0.88 ± 0.29

[a]Cultured acquired immunodeficiency syndrome-related Kaposi's sarcoma (AIDS-KS), nonlesional cells from the AID-KS donors (nonlesional) and fibroblasts from HIV^- donors (normal) were harvested for nucleotide analyses during either proliferative growth in COMPLETE medium (M-199 supplemented with 15 mM HEPES, 2.9 μg/ml 1-glutamine, 1.1 μg/ml Na pyruvate, 90 μg/ml Na heparin, 150 μg/ml bovine brain extract, 15% heat-inactivated fetal bovine serum, 5% heat-inactivated male human serum), or during culture in BASE medium (M-199 supplemented with 1-glutamine, Na pyruvate, and HEPES, and 0.5% FBS) without (control) or with 100 U/ml of TNF-α. Number of cellular strains employed (normal, nonlesional, and AIDS-KS cultures, respectively) were as follows: proliferative: n = 5, 7, 5; control n = 6, 4, 4; TNF-α challenge n = 6, 4, 5. Values are depicted as mean levels ± SD. Statistical analyses were conducted by using the Kruskal-Wallis one-way ANOVA, followed by the Mann-Whitney U-test, two-tailed test.

*$P < 0.010$.

**$P < 0.020$.

†$P < 0.040$ (normal vs AIDS-KS).

‡$P \leq 0.040$ (normal vs nonlesional).

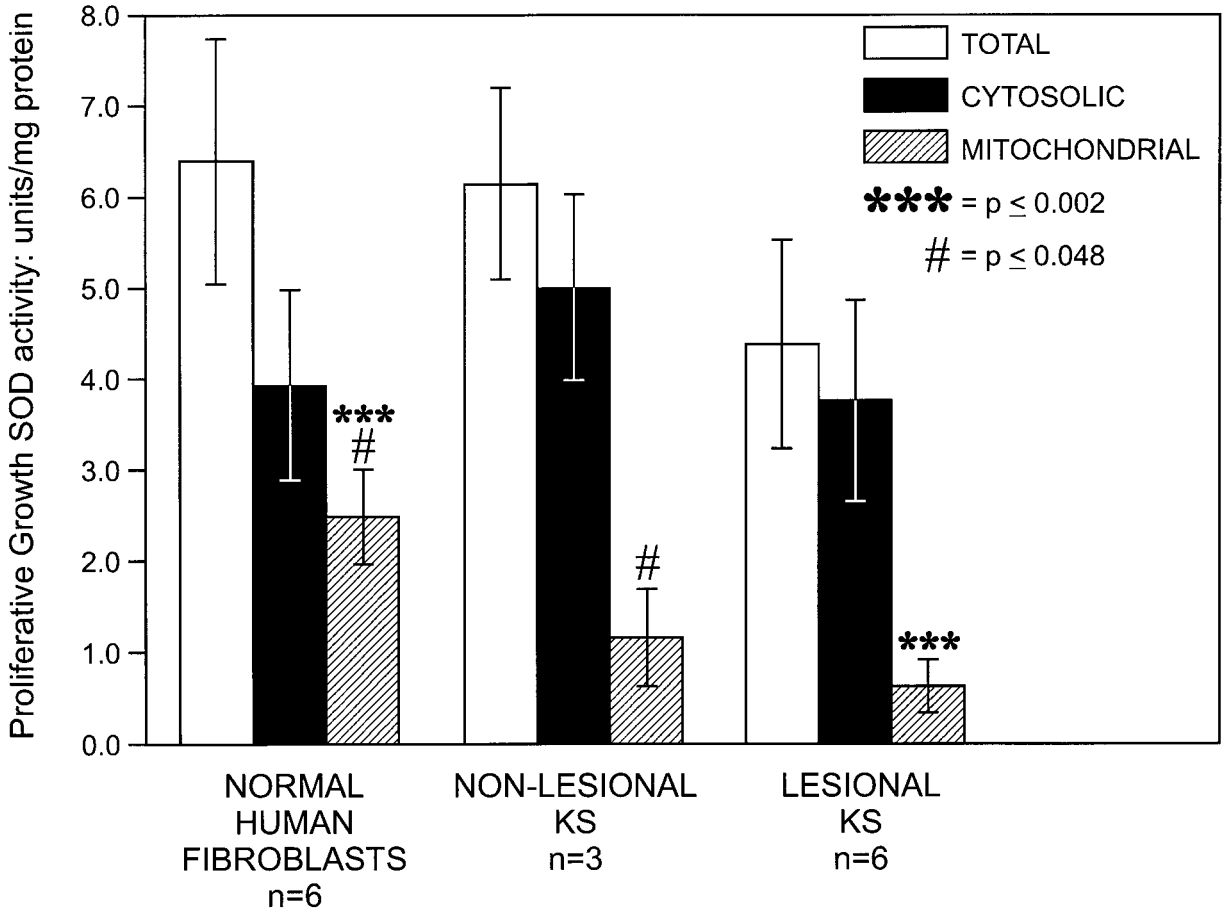

Fig. 1. Cellular levels of superoxide dismutase (SOD) activities determined during proliferative growth. Cultured AIDS-related Kaposi's sarcoma (AIDS-KS), nonlesional cells from the AIDS-KS donors (nonlesional) and fibroblasts from HIV⁻ donors (normal) were harvested for determination of SOD activity and cellular compartmentalization during proliferative growth in "COMPLETE" medium (M-199 supplemented with 15 mM HEPES, 2.9 µg/ml l-glutamine, 1.1 µg/ml Na pyruvate, 90 µg/ml Na heparin, 150 µg/ml bovine brain extract, 15% heat-inactivated fetal bovine serum, 5% heat-inactivated male human serum). Values are expressed as units of SOD activity/mg protein +SD, with one unit of SOD activity defined as the amount of enzyme that results in a 50% inhibition of the reduction of acetylated cytochrome c. Statistical analyses of cellular SOD activities were conducted using the Kruskal Wallis One Way ANOVA, followed by the two-tailed Mann-Whitney U-test.

est activities in HIV⁻ donors' cells, intermediate levels in nonlesional cells, and lowest MnSOD activities in AIDS-KS cells. AIDS-KS cells possessed significantly lower levels of both MnSOD activity (0.62 ± 0.29 SD U/mg, n = 6 AIDS-KS; 2.48 ± 0.52 units/mg, n = 6, HIV⁻ donors $P < 0.002$), as well as percentage mitochondrial distribution (KS = 14.80% ± 6.42, n = 6; HIV⁻ donors = 39.02% ± 5.54, $P < 0.002$) relative to HIV⁻ donors' fibroblasts. Relative to HIV⁻ donors' cells, the nonlesional cells also possessed significantly lower MnSOD activities (1.15 ± 0.53, n = 3, nonlesional; 2.48 ± 0.52, n = 6, HIV⁻ donors, $P < 0.048$), as well as a lower percentage of total SOD contained in the mitochondria (18.71% ± 5.55, nonlesional; 39.02% ± 5.54, HIV⁻, $P < 0.02$). While the nonlesional cells demonstrated both higher Mn-SOD function and mitochondrial distribution relative to the AIDS-KS cells, these differences were not statistically significant (Fig. 1).

Nucleotide analyses showed several findings common to all three groups during proliferative growth (Table I). ATP represented the primary high-energy phosphate, and NAD^+/NADH the primary redox pair. Also, comparable levels (nmol/mg protein) of total adenine nucleotides, high-energy phosphates and redox poise (ratio $NAD(P)H/NAD(P)^+$) were found in all three groups. However, significant differences were detected in levels of NADPH, the cofactor essential for GSSG reductase. Relative to either HIV⁻ donors' fibroblasts, or nonlesional cells, AIDS-KS cells contained significantly lower lev-

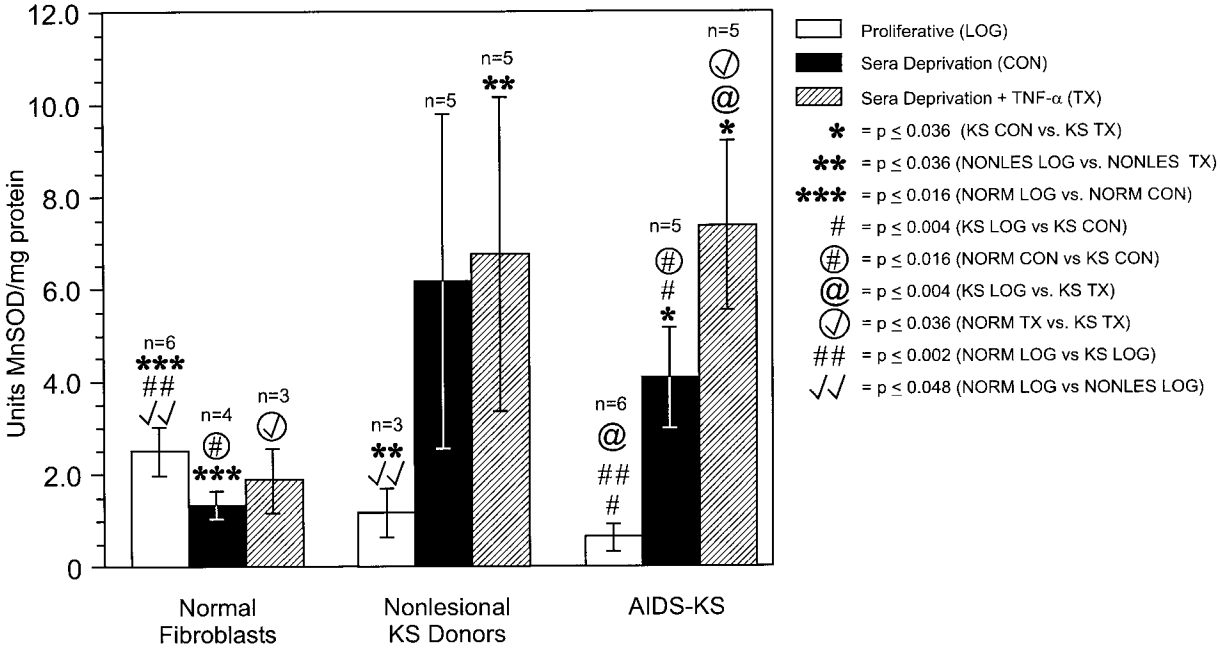

Fig. 2. Comparison of proliferative, control and TNF-α challenged MnSOD levels in our cellular populations. Cultured AIDS-KS cells (AIDS-KS), nonlesional cells from the AIDS-KS donors (nonlesional), and HIV⁻ donor fibroblasts (normal) were harvested during either proliferative growth in "COMPLETE" medium, or during culture in BASE medium (M-199 supplemented with l-glutamine, Na pyruvate, HEPES, and 0.5% heat-inactivated fetal bovine serum) without (CON) or with (TX) 100 U/ml TNF-α. Values are expressed as units of MnSOD activity/mg protein +SD. Statistical analyses conducted were the Kruskal-Wallis One-Way ANOVA, followed by the two-tailed Mann-Whitney U-test.

els of this thiol redox-regulating nucleotide [Table I nmol NADPH/mg±S.D.; AIDS-KS = 0.12±0.06, n = 5; HIV⁻ donors' = 0.25±0.06, n = 5; ($P < 0.020$); nonlesional = 0.25±0.09, n = 7 ($P < 0.010$)]

Cellular SOD Modulation in Response to Serum Deprivation and TNF-α Challenge

The TNF-α challenge experiments evaluated cellular adaptation capacities at numerous levels. As mentioned previously, the TNF-α/MnSOD induction assays were conducted in BASE medium to reduce interference from other serum factors. Marked differences were observed among the three groups in response to serum deprivation (Fig. 2, Table II). Commensurate with the removal of serum and its numerous metabolism-stimulating cytokines and mitogens, the HIV⁻ donors' control fibroblasts showed a decrease in both MnSOD activity and mitochondrial distribution when cultured in BASE medium. However, when TNF-α was included in the BASE medium, HIV⁻ donors cellular MnSOD levels and percentage of total SOD distributed in the mitochondria returned to levels approximating those detected during growth in the serum-enriched COMPLETE medium (Fig. 2, Table II).

In contrast, the AIDS-KS cells responded to serum deprivation by a statistically significant upregulation of both their MnSOD activity (Figure 2) and mitochondrial SOD ($\bar{x} \pm$ S.D.) distribution (proliferative n = 5, 14.80% ± 6.42 vs. AIDS-KS con n = 5, 61.45 ± 17.64, $P < 0.004$) While the MnSOD levels of the nonlesional cells also increased during serum deprivation, these differences were not statistically significant.

The most profound responses to TNF-α challenge, as assessed by increased MnSOD activity and mitochondrial compartmentalization, were shown by the AIDS-KS cells (Fig. 2, Table II). While both the nonlesional and KS lesional cells showed increases in MnSOD levels as a result of serum deprivation, only the AIDS-KS cells showed a statistically significant increase in MnSOD activity in comparison of TNF-α challenged vs. control cultures (Fig. 2, Table II). Also, under conditions of serum deprivation, both the AIDS-KS control and AIDS-KS TNF-α challenged cells contained statistically significantly higher levels of MnSOD activity and

TABLE II. 24-h Cellular SOD Enzymatic Activity and Cellular Compartmentalization[a]

Cell group	Total SOD	CuZn SOD	Mn SOD	% mitochondrial
Norm Con (n = 4)	6.99 ± 0.75	5.68 ± 0.97	1.31 ± 0.30†	19.25 ± 7.22†,‡
Norm TNF-α (n = 3)	8.32 ± 1.61	6.49 ± 1.64	1.84 ± 0.69*	22.41 ± 8.50*
Nonles Con (n = 5)	9.05 ± 3.92	2.89 ± 0.66	6.15 ± 3.62	59.81 ± 16.80‡
Nonles TNF-α (n = 5)	11.44 ± 5.96	4.71 ± 5.72	6.74 ± 3.40	66.26 ± 24.18
KS Con (n = 5)	7.36 ± 3.09	3.31 ± 2.05	4.05 ± 1.09**,†	61.45 ± 15.78†
KS TNF-α (n = 5)	10.61 ± 3.50	3.24 ± 2.47	7.37 ± 1.83*,**	71.72 ± 14.65*

[a]Post-confluent cultured AIDS-related Kaposi's sarcoma cells (KS), nonlesional cells from the AIDS-KS donors (Nonles), and fibroblasts from HIV⁻ donors (Norm) were rinsed with room temperature phosphate buffered saline, and then incubated for 24 h (37°C, 5% CO_2) in "BASE" medium (M-199 supplemented with 15 mM HEPES, 2.9 μg/ml 1-glutamine, 1.1 μg/ml Na pyruvate plus 0.5% heat inactivated fetal bovine serum. The cultures were then provided fresh "BASE" medium with 100 U/ml human TNF-α (TNF-α) or without TNF-α (Con). Following a second 24 hour incubation, the cells were harvested, and cellular superoxide dismutase (SOD) activities (mean units of activity + S.D. per mg cell protein) and cellular enzymatic compartmentalization determined. Statistical analyses were conducted using the Kruskal Wallis One Way ANOVA, followed by the Mann-Whitney U- (2-tailed) test.

*$P < 0.036$ KS TNF-α vs Norm TNF-α.

**$P < 0.032$ KS TNF-α vs KS Con.

†$P < 0.016$ KS Con vs Norm Con.

‡$P < 0.016$ Norm Con vs Nonles Con.

mitochondrial enzymatic distribution relative to the HIV⁻ donors' control and treated cultures, respectively (Fig. 2, Table II). Nonlesional cells from the KS donors demonstrated a transitional TNF-α responsiveness profile that more closely approximated the AIDS-KS cells (Table II, Fig, 2).

Release of ROI as a Consequence of TNF-α Binding

Cell strains from the HIV⁻ donors, AIDS-KS lesional and nonlesional cells were assayed to determine whether or not NAD(P)H oxidase was activated subsequent to TNF-α binding. Findings were similar in all three cellular populations and showed that (1) cellular harvesting and incubation alone resulted in a small amount of acetylated cytochrome c reduction (~30%), (2) the addition of TNF-α markedly increased cytochrome c reduction over levels obtained by harvesting alone (~75% cytochrome c reduction), and (3) the inclusion of SOD diminished cytochrome c reduction (decrease to 20% and 40% in the control harvest and TNF-α challenged cells, respectively). Collectively, these data imply that (1) NAD(P)H oxidase is activated to a low level by nonspecific membrane perturbing events such as cellular harvesting, and (2) introduction of specific stimuli such as TNF-α increases NAD(P)H oxidase activation and ROI generation in all three cellular populations.

Bioenergetic Profile Modulations During TNF-α Challenge

Several similar findings, consistent with decreased cellular proliferation subsequent to serum deprivation, were found in the TNF-α challenge HPLC nucleotide analyses (Table I). All three culture groups showed a decrease (relative to proliferative growth profiles) in high-energy phosphates, the ATP/ADP ratio, total adenine and nicotinamide nucleotides, and an alteration in the NADH/NAD^+ redox pair (decreased NAD^+ with a concurrent increase in NADH) resulting in a reduction in the overall cellular redox state. The energy charge of all three cellular populations remained >0.90, indicative of ongoing cellular oxidative metabolism.

Several significant intergroup differences, which reflect cellular capacities to maintain both ATP and adenine nucleotide levels and GSH in its functional, reduced state, were noted during culture in BASE medium. Relative to the HIV⁻ fibroblasts, AIDS-KS cells possessed significantly lower levels of ATP during both control and TNF-α conditions, and lower total adenine nucleotides during control growth (Table I). Intermediate levels of ATP and total adenine nucleotides, which more closely approximated normal fibroblasts, were detected in the nonlesional cells from the AIDS-KS donors during both control and TNF-α challenge conditions. In addition, significant differences

TABLE III. Responsiveness of SOD Activity and Cellular Enzymatic Compartmentalization to Growth State, GSH Levels, and TNF-α Challenge[a]

Cell group	Total SOD	Mn SOD	% mitochondrial	[GSH] nmol/mg
Norm pro	6.40 ± 1.35*	2.48 ± 0.52*,**	39.02 ± 5.54*	Not conducted
Norm con	10.92 ± 4.31	6.12 ± 3.60	49.95 ± 19.97	12.40 ± 1.66†
Norm TNF-α tx	15.78 ± 7.89	10.21 ± 5.50**	63.50 ± 4.45*	8.61 ± 1.67‡
Norm ↓GSH + TNF-α	18.73 ± 4.11*	14.61 ± 3.38*	78.31 ± 7.80*	7.19 ± 3.26
Norm NAC + TNF-α	16.66 ± 8.59	10.04 ± 4.94*	64.12 ± 14.21*	22.68 ± 6.06
Nonles. pro	6.15 ± 1.05	1.15 ± 0.53	18.71 ± 7.55	Not conducted
Nonles. con	11.12 ± 1.97	8.25 ± 2.16	70.99 ± 6.94	9.98 ± 2.50
Nonles. TNF-α tx	13.15 ± 3.48	10.43 ± 2.91	82.37 ± 20.16	5.87 ± 1.35
Nonles. ↓GSH + TNF-α	12.45 ± 0.25	8.52 ± 1.45	68.53 ± 9.23	2.11 ± 1.17
Nonles. NAC + TNF-α	16.58 ± 3.11	14.35 ± 4.43	84.99 ± 15.51	17.81 ± 4.11
KS pro n = 6	4.38 ± 1.15*	0.62 ± 0.29*	14.80 ± 6.42*,**	Not conducted
KS con	12.93 ± 6.74*	6.75 ± 3.01*	54.41 ± 4.93*	6.85 ± 1.75†
KS TNF-α tx	17.26 ± 5.44*	12.58 ± 6.35*	69.57 ± 14.35*	3.10 ± 0.41‡
KS ↓ GSH + TNF-α	15.45 ± 7.41*	14.29 ± 8.00*	87.89 ± 9.66*	3.40 ± 2.99
KS NAC + TNF-α	19.39 ± 12.38*	10.47 ± 4.92*	60.57 ± 16.57**	15.30 ± 9.50

[a]Post-confluent cultured AIDS-related Kaposi's sarcoma cells (AIDS-KS), nonlesional cells from the AIDS-KS donors (nonlesional), and fibroblasts from HIV⁻ donors (normal) were rinsed with room temperature phosphate buffered saline, and were incubated for 24 h (37°C, 5% CO_2) in BASE medium (M-199 supplemented with 15 mM HEPES, 2.9 μg/ml 1-glutamine, 1.1 μg/ml Na pyruvate plus 0.5% heat-inactivated fetal bovine serum. The cultures were then provided fresh BASE medium that either included 100 U/ml human TNF-α (tx) or excluded TNF-α (con). GSH levels were augmented by inclusion of 30 mM N-acetylcysteine during both the 24-h h serum deprivation and TNF-α challenge. (NAC). At 3 h prior to TNF-α challenge, levels of GSH were decreased by a 2-h incubation with 1×10^{-4} M buthionine sulfoximine, followed by a 1-h incubation with 100 μM 2-cyclohexene-1-one (↓GSH). Following a second 24-h incubation, the cells were harvested, and cellular superoxide dismutase (SOD) activities (U/mg protein) and cellular enzymatic distribution determined. Proliferative levels (pro) of SOD activity were obtained from cell harvested during log growth in COMPLETE medium (M-199 supplemented with HEPES, 1-glutamine, Na pyruvate, 15% heat-inactivated FBS, 5% heat-inactivated male human serum, 90 μg/ml Na heparin, 150 μg/ml endothelial cell growth supplement). Number of cell strains employed (normal, nonlesional, and AIDS-KS cultures, respectively) were as follows: proliferative 6, 3, 6; n = 3 for all the remaining groups. Statistical analyses for the SOD data (x ± SD) were conducted using the Kruskal–Wallis one-way ANOVA, followed by the Mann-Whitney U- (two-tailed) test.
*$P < 0.024$.
**$P < 0.048$.
GSH samples (nmol/mg protein): n = 3 for all groups: ANOVA, followed by multiple t-test
†$P < 0.05$.
‡$P < 0.01$.

were once again noted in the cellular NADPH levels (Table I). During control culture conditions (BASE medium only), HIV⁻ donor fibroblasts possessed significantly higher levels of NADPH relative to either nonlesional or AIDS-KS cells. Subsequent to TNF-α challenge, these differences in NADPH levels between normal and AIDS-KS cells were increased, whereas the nonlesional [NADPH] increased to levels similar to those detected in normal, HIV⁻ fibroblasts.

Contribution of the Cellular Thiol Redox Status in TNF-α-Mediated Signal Transduction

Our data, which showed a positive correlation between the significantly lower levels of the thiol redox regulating nicotinamide nucleotide NADPH and heightened TNF-α responsiveness in AIDS-KS cells, in conjunction with our previous findings which demonstrated that AIDS-KS cells possess significantly lower levels of GSH [22,23], prompted additional studies to investigate the contribution of the cellular thiol redox status in TNF-α-mediated signal transduction.

This second series of TNF-α challenge experiments were conducted using a new lot of TNF-α which apparently elicited a greater biological response per unit of activity. Consequently, following TNF-α challenge, all culture groups showed a marked increase in MnSOD activity and mitochondrial enzymatic distribution relative to levels present during proliferative growth (Table III). In addition, during this series of experiments, all culture groups responded to serum deprivation with an increase in both MnSOD activity as well as mitochondrial enzymatic distribution. The normal fibroblasts used

for these studies included strains obtained from different donors than those used in the initial studies.

Earlier studies conducted in our laboratory, using more than 20 strains of normal human fibroblasts, have shown the GSH depletion protocol to be both effective (levels of GSH immediately post-treatment are <1 nmol/mg protein), and readily reversible [24]. However, these current experiments revealed a cytopathic response, which was limited to one AIDS-KS strain and one nonlesional strain from a different KS donor, when GSH depletion was followed by TNF-α challenge. With the exception of these two strains, the cellular viability of the cultures used for the GSH modulation experiments was >94% at harvest.

Cellular GSH levels determined at the 24-h harvest (using intrastrain treatment group comparisons) showed pervasive findings among all the three culture groups for every cell strain evaluated: (1) the introduction of TNF-α decreased GSH levels (control vs TNF-α challenged); and (2) in a converse fashion, inclusion of the GSH precursor, NAC, increased same strain [GSH] relative to all other treatment groups. Whereas in both the normal and nonlesional cultures the 24-h harvest levels of GSH were lower in the GSH depletion/TNF-α challenge group relative to the corresponding group provided TNF-α only, levels of GSH were constant in the comparable AIDS-KS cultures irrespective of GSH depletion. Moreover, HIV^- donor fibroblasts possessed statistically significantly higher levels of GSH, relative to AIDS-KS cultures, during both control and TNF-α challenged growth conditions (Table III). The nonlesional cells from the KS donors contained levels of GSH which were intermediate in distribution relative to normal and AIDS-KS cells cultured under similar conditions.

The GSH modulation studies illustrated the thiol redox contribution to cellular responsiveness to TNF-α. Only in the normal fibroblasts was the reduction of GSH levels accompanied by a marked relative increase in MnSOD activity following TNF-α challenge (Fig. 3). In contrast, the MnSOD activity of TNF-α challenged nonlesional and AIDS-KS cells remained relatively constant regardless of GSH depletion (Fig. 3).

All three cellular groups tolerated the inclusion of NAC, with cellular viability remaining >95% in the NAC-treated cultures. While addition of NAC resulted in approximately a threefold GSH level increase in nonlesional and normal fibroblasts (TNF-α tx. vs NAC+TNF-α), the GSH levels of the AIDS-KS strains showed a higher magnitude (approximately fivefold) increase after addition of NAC. However, depending on the culture group, NAC inclusion either decreased, increased or had no effect on cellular response to TNF-α challenge (Fig. 3). Provision of NAC to AIDS-KS cultures significantly suppressed TNF-α-mediated increases in mitochondrial enzymatic distribution relative to either nonlesional or normal cells, and also resulted in significantly lower upregulation of MnSOD activity relative to both normal and nonlesional cultures (Fig. 3). In contrast to the AIDS-KS cells, nonlesional cells responded to the inclusion of NAC with moderate increases in both MnSOD activity and percentage of SOD localized in the mitochondria. The MnSOD activities and mitochondrial enzymatic distributions of normal fibroblasts were unresponsive to NAC and demonstrated comparable levels of MnSOD activity and mitochondrial enzymatic distribution subsequent to TNF-α challenge, regardless of the presence or absence of NAC.

DISCUSSION

Although clinical evidence suggests that AIDS-KS is a multifactorial disease, a consistent feature of most putative AIDS-KS etiologic agents is sustained serum elevations of proinflammatory cytokines such as TNF-α [18,30]. Serum TNF-α levels, which are initially increased by HIV^+ seroconversion, are further augmented by opportunistic infections which arise, like AIDS-KS, during the latter stages of AIDS [18,30]. Further, many of the clinical manifestations of late AIDS e.g. cachexia are sequelae of these aberrantly elevated serum cytokines [8,18,30]. Our data, which show that a perturbation in the cellular thiol redox status accentuates AIDS-KS cellular responsiveness to TNF-α, suggest a biochemical rationale for this recognized TNF-α AIDS-KS clinical correlation. In vivo, elevated serum TNF-α levels coupled with heightened responsiveness to TNF-α would promote AIDS-KS development by prolonged activation of signal transduction pathways conducive to the establishment of autologous growth loops [15].

As reflected by their energy charges, all three cellular groups showed positive adaptations to

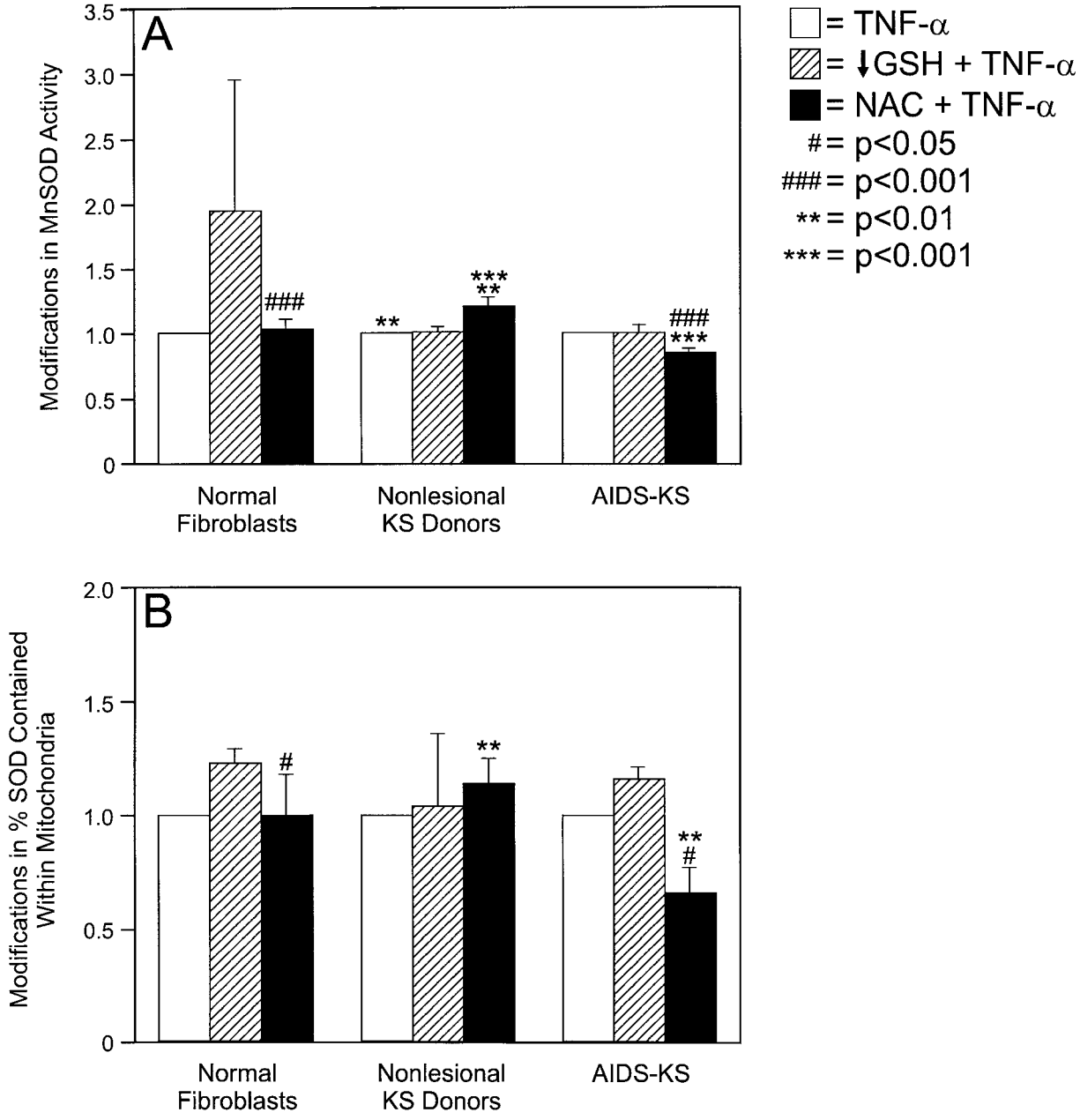

Fig. 3. Comparison of the relative efficacy of modification of the cellular glutathione (GSH) status to modify TNF-α response in cultured AIDS-related Kaposi's sarcoma (AIDS-KS), nonlesional cells from the AIDS-KS donors (nonlesional) and HIV$^-$ donor fibroblasts (normal). Post-confluent cultures were rinsed with room temperature phosphate-buffered saline and were incubated (37°C, 5% CO_2) for 24 h in BASE medium. The cultures were then provided fresh BASE medium that included 100 U/ml TNF-α (TX). GSH levels were augmented by inclusion of 30 mM N-acetylcysteine during both the 24-h sera deprivation and TNF-α challenge (NAC). At 3 h prior to TNF-α challenge, levels of GSH were decreased by a 2-h incubation with 1×10^{-4} M buthionine sulfoximine, followed by a 1-h incubation with 100 μM 2-cyclohexene-1-one (⇓GSH). Following a second 24-h incubation, the cells were harvested and levels of MnSOD activity and mitochondrial enzymatic compartmentalization determined. Data are represented as mean values +SD, n = 3 for every group. Statistical analyses were conducted using the ANOVA followed by the multiple t-test.

culture in COMPLETE medium. However, during proliferative growth, significant population specific differences were detected. Statistically significantly lower levels of NADPH were found in AIDS-KS cells relative to either nonlesional cells from the KS donors or normal fibroblasts. This finding, which is consistent with our previous results [22], suggests that NADPH generating pathways such as the hexose monophosphate shunt, isocitrate dehydrogenase, malic

enzyme and the energy dependent transhydrogenase may be compromised in AIDS-KS cells [27]. Reduced NADPH levels would impair the activities of enzymes such as GSSG reductase and thioredoxin, which are dependent on NADPH for the provision of reducing equivalents [29]. Previous studies from our laboratory, in which AIDS-KS cells retained GSSG subsequent to oxidant challenge, support this premise [22].

The SOD levels and cellular enzymatic compartmentalization detected during proliferative growth compare favorably with SOD studies reported by other investigators in human fibroblasts [26,46]. Further, the large standard deviations (which likely reflect the outbred human population) found during our enzymatic analyses are also consistent with the findings of other investigators [26,46]. During proliferative growth, HIV^- donor fibroblasts demonstrated the highest MnSOD activity and mitochondrial enzymatic distribution. These findings suggest that during optimal growth conditions, normal fibroblasts' MnSOD levels are maintained by a high level of mitochondrial oxidative metabolism and its accompaning increased oxygen intermediate generation by the electron transport system.

A consistent finding of this study was that nonlesional cells from the AIDS-KS donors behaved as a biochemically distinct population, with many of their assessed biochemical parameters determined to be in an "intermediate" position between the normal fibroblasts and AIDS-KS cells. A relevant question, but one that is difficult to address, is whether these biochemical changes were present prior to HIV infection, or developed as a consequence of the HIV^+ state. Ongoing studies in our laboratory on cells isolated from AIDS-KS negative, same risk group HIV^+ donors with comparable viral load and $CD4^+$ counts will help address this question.

Because of our interest in redox regulation of ROI-associated signal transduction, we determined whether or not TNF-α receptor occupancy resulted in a release of oxygen intermediates in our cultured cells. Consistent with results found by other investigators in nonphagocytic cells [41], we determined that TNF-α challenge resulted in generation of $O_2^{\cdot -}$ in all three cell populations. Subsequent to TNF-α binding, oxygen intermediates can be generated by at least two plasma membrane associated enzymes (NAD(P)H oxidase and cyclooxygenase) with the resultant ROI functioning as an intracellular signaling system [15,41].

The bioenergetic profile modulations observed during the TNF-α challenge assays were consistent with cells entering a quiescent state, and potentially initiating a stress response, as a consequence of serum deprivation [33]. Notably, during these experiments all culture groups maintained their energy charges, and although the ATP/ADP ratios did show a modest decrease, the resulting ratios remained high, suggesting that the mitochondria remained in state 4 (ADP limiting) respiration. Also, during serum deprivation, all culture groups showed a reduction of their redox state, a finding that may reflect coenzyme Q (ubiqinone) as a targeted site for induction of the environmental stress response [27,33]. These data that show that the bioenergetic status of the AIDS-KS cells was most affected by serum deprivation and TNF-α challenge, in conjunction with our previous studies [22,23] suggest that AIDS-KS cells are more dependent on glycolytic pathways of metabolism, an accommodation that may reflect impaired or reduced mitochondrial oxidative metabolism in AIDS-KS cells [27]. Also, the lower NADPH levels in the AIDS-KS cells may promote formation of protein mixed disulfides, as TNF-α exposure is likely to be accompanied by GSSG formation when GSH is used to quench the TNF-α generated ROI. Finally, an oxidized thiol status would compromise the activities of thiol-dependent enzymes such as the tyrosine phosphatases, which are essential in arresting the signal transduction cascade initiated by TNF-α receptor interaction [14,42,44].

Virtually every cell type responds to TNF-α with gene activation and expression of proinflammatory cytokines [5]. However, the extent of TNF-α-mediated cellular signaling is regulated by a delicate balance between activation (generation of ROI and activation of kinases) and arresting (quenching of ROI and phosphatase activation) events [14,15,42,44]. Although the exact mechanism by which TNF-α upregulates the activity of MnSOD has not been elucidated, it is accepted that both tyrosine kinase activation and the cellular redox state are integral in its regulation [39]. Recent studies show that as a downstream event TNF-α disrupts mitochondrial electron flow, resulting in production of $O_2^{\cdot -}$ at the ubiquinone site, with the released ROI serving as secondary messengers capable of activating the antioxidant re-

sponse genes [39]. Related studies by Anderson et al. suggest that ROI scavengers such as GSH are key modulators in this process and act to decrease the extent to which mitochondrial derived oxidants increase tyrosine phosphorylation [1]. Pertinent to our study, the speculative routes by which TNF-α increases mitochondrial ROI release all entail redox sensitive pathways including perturbations in Ca^{2+} homeostasis [39], phosphorylation events [14,39,44], and thiol sensitive target proteins at the ubiquinone site [39]. Further, although the mitochondria is a targeted organelle for both TNF-α's modulatory as well as cytotoxic actions, it is the overall cellular capacity to respond to the increased levels of ROI that determines whether cell death or gene induction ensues [39]. Apparently, those cellular strains from our study that demonstrated a cytotoxic response to GSH reduction followed by TNF-α challenge had diminished ROI degrading capacities relative to the other cultures.

It is well accepted that a large group of environmental stimuli such as nutrient deprivation, oxidant stress and cytokine exposure can evoke a spectrum of similar responses inclusive of activation of a wide variety of genes [33,47,48]. Therefore, it is not unanticipated that our results showed that the two forms of environmental stress used in our study—serum deprivation and cytokine challenge—were accompanied by MnSOD induction, which demonstrated an apparent agent additive effect, with the greatest induction found in those cultures that underwent both serum deprivation and TNF-α challenge. Because not every cell is equally endowed with the capacity to initiate a stress response [33,47], the failure of the normal fibroblast strains to upregulate MnSOD during serum deprivation during the initial experiments likely reflects population heterogeneity. The capacity of disparate environmental stimuli such as heat shock, oxidants, and cytokines to all elicit cytoprotective enzyme upregulation suggests a common signaling pathway [33,47,48]. Further, our data, which showed a reduction in the cellular redox state, MnSOD upregulation, and decreased levels of high-energy phosphates, suggest that the mitochondrion is a potential convergence site during mediation of the stress response. Biochemical features of ubiquinone that would promote its use in ROI-based signaling include lipid solubility, its large stoichiometric excess relative to other members of the mitochondrial respiratory chain, and its apparent mobility, which permits collection and transfer reducing equivalents from the more fixed respiratory chain components [27].

Our results show that an inherent perturbation in the AIDS-KS cellular thiol redox status augments cellular responsiveness to TNF-α, and that only in the normal fibroblasts did GSH depletion potentiate TNF-α-initiated upregulation of MnSOD. Therefore, while provision of reducing equivalents per se was not a problem (all culture groups showed a reduction of their redox poise), the modulation of signal transduction appeared much more closely aligned with cellular GSH status and its capacities to perform thiol disulfide interchange and quench ROI.

Only in the AIDS-KS cells did NAC supplementation curtail TNF-α-mediated upregulation of MnSOD and mitochondrial enzymatic compartmentalization. The ability of NAC to downregulate TNF-α responsiveness most probably reflects the inherently lower levels of reduced thiols in AIDS-KS cells. At the biochemical level, NAC has the abilities to serve both as a GSH precursor as well as the potential to directly scavenge ROI, thereby acting as a "sparing" agent for the cellular GSH stores [28,36]. Unlike the AIDS-KS cells, incorporation of NAC to either the nonlesional or normal cells did not suppress TNF-α responsiveness, a finding which likely results from adequate endogenous GSH stores in the nonlesional and normal cells. Also, our SOD upregulation data are in agreement with recent studies by Offerman et al., which show that thiol antioxidants suppress expression of inflammatory response genes in AIDS-KS cells [35]. We speculate that the lower levels of GSH and other molecules in close redox communication e.g. NADPH found in AIDS-KS cells are permissive for sustained activation of TNF-α-initiated signal transduction pathways. Also, we surmise that this increased sensitivity to TNF-α facilitates AIDS-KS lesional progression by initiating pathways, e.g., activation of NF-κB, conducive to enhanced autologous expression of growth promoting cytokines.

In conclusion, we present results that suggest a biochemical basis for the well-recognized clinical association between elevated TNF-α levels and AIDS-KS progression. Our findings from both this current and previous AIDS-KS studies suggest that AIDS-KS cells possess biochemical perturbations which impair cellular capacity to degrade oxidants, thereby resulting in

intracellular retention of ROI and subsequent oxidant stress [22,23]. Finally, our findings in which AIDS-KS cells are exquisitely sensitive to ROI are substantiated by data that have demonstrated the clinical efficacy of redox cycling drugs such as doxorubicin in the treatment of AIDS-KS.[34]

ACKNOWLEDGMENTS

The authors extend appreciation to our summer research students Greg Sabol and Omar Minshawi for their invaluable assistance on this project, and to Dr. Leona Ayers for her gracious support from the AIDS Malignancy Bank.

REFERENCES

1. Anderson MT, Staal FJT, Gitler C, Herzenberg LA, Herzenberg LA (1994): Separation of oxidant-initiated and redox-regulated steps in the NF-κB signal transduction pathway. Proc Natl Acad Sci USA 91:11527–11531.
2. Azzi A, Monticucco C, Richter (1975): The use of acetylated cytochrome c for the detection of superoxide radical produced in biological membranes. Biochem Biophys Res Commun 65:597–603.
3. Bailer RT, Ng Bautista CL, Ness GM, Mallery SR (1995): Comparison of constitutive cytokine release in high and low histologic grade AIDS-related Kaposi's sarcoma cell strains and in sera from HIV^+/KS^+ and HIV^+/KS^- patients. J Interferon Cytokine Res 15:473–483.
4. Baeuerle PA (1991): The inducible transcription activator NF-κB: regulation by distinct protein subunits. Biochim Biophys Acta 1072:63–80.
5. Beutler B (1995): TNF, Immunity and inflammatory disease: Lessons of the past decade. J Invest Med 43:227–235.
6. Blenis J (1993): Signal transduction via the MAP kinases: Proceed at your own RSK. Proc Natl Acad Sci USA 90:5889–5892.
7. Bours VE (1994): The NF-κB transcription factor and cancer: High expression of NF-κB and IκB-related proteins in tumor cell lines. Biochem Pharmacol 47:145–149.
8. Cotran RS, Kumar V, Robbins SL (1994): "Pathologic Basis of Disease." Philadelphia: WB Saunders, pp 219–231.
9. Dbaibo GS (1993): Tumor necrosis factor-α (TNF-α) signal transduction through ceramide. J Biol Chem 268:17762–17766.
10. Ensoli B, Gendelman R, Markham P, Fiorelli V, Colombini S, Raffeld M, Cafaro A, Chang H-K, Brady JN, Gallo RC (1994): Synergy between basic fibroblast growth factor and HIV-1 Tat protein in induction of Kaposi's sarcoma. Nature 371:674–680.
11. Eyer P, Podhradsky D (1986): Determination of glutathione using enzymatic cycling and Ellman's reagent. Anal Biochem 153:57–66.
12. Flohe L, Otting F (1984): Superoxide dismutase assays. Methods Enzymol 105:93–104.
13. Geisbuhler T, Altschuld RA, Trewyn RW, Ansel AJ, Lanka K, Brierley GP (1984): Adenine nucleotide metabolism and compartmentalization in isolated rat heart cells. Circ Res 54:536–546.
14. Guy GR, Cairns J, Ng SB, Tan YH (1993): Inactivation of a redox-sensitive protein phosphatase during the early events of tumor necrosis factor/interleukin-1 signal transduction. J Biol Chem 268:2141–2148.
15. Heller RA and Kronke M (1994): Tumor necrosis factor receptor-mediated signaling pathways. J Cell Biol 126: 5–9.
16. Hoeck WG, Ramesha CS, Chang DJ, Fan N, Heller RA (1993): Cytoplasmic phospholipase A_2 activity and gene expression are stimulated by tumor necrosis factor: Dexamethasone block the induced synthesis. Proc Natl Acad Sci USA 90:4475–4479.
17. Huang YQ, Li JJ, Kim KS, Nicolaides A, Zhang WG, Le J, Poiesz BJ, Friedman-Kien AE (1993): HIV-1 Infection and modulation of cytokine and growth factor expression in Kaposi's sarcoma-derived cell in vitro. AIDS 7:317–322.
18. Jacobson LP and Armenian HK (1995): An integrated approach to the epidemiology of Kaposi's sarcoma. Curr Opin Oncol 7:450–455.
19. Kalebic T, Kinter A, Poli G, Anderson ME, Meister A, Fauci AS (1991): Suppression of human immunodeficiency virus expression in chronically infected monocytic cells by glutathione, glutathione ester and N-acetylcysteine. Proc Natl Acad Sci USA 88:986–990.
20. Lander HM (1997): An essential role for free radicals and derived species in signal transduction. FASEB 11:118–124.
21. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951): Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275.
22. Mallery SR, Bailer RT, Hohl CM, Ng-Bautista CL, Ness GM, Livingston BE, Hout BL, Stephens RE, Brierley GP (1995): Cultured AIDS-related Kaposi's sarcoma cells demonstrate impaired bioenergetic adaptation to oxidant challenge: Implication for oxidant stress in AIDS-KS pathogenesis. J Cell Biochem 59:317–328.
23. Mallery SR, Ng-Bautista CL, Lantry LE, Ness GM, Hegtvedt AK, Lazo A, Bailer RT, Hout BL, Stephens RE, Brierley GP (1994): Cultured AIDS-related Kaposi's sarcoma cells retain a proliferative bioenergetic profile but demonstrate reduced cytoprotective capabilities. J Cell Biochem 56:568–581.
24. Mallery SR, Laufman HB, Solt CW, Stephens RE (1991): Association of cellular thiol redox status with mitogen-induced calcium mobilization and cell cycle progression in human fibroblasts. J Cell Biochem 45:82–92.
25. Mallery SR, Lantry LE, Laufman HB, Stephens RE, Brierley GP (1993): Modulation of human microvascular endothelial cell bioenergetic status and glutathione Levels during proliferative and differentiated growth. J Cell Biochem 53:360–372.
26. Marklund SL (1992): Regulation by cytokines of extracellular superoxide dismutase and other superoxide dismutase isoenzymes in fibroblasts. J Biol Chem 267: 6696–6701.
27. Martin DW, Mayes PA, Rodwell VW, Granner DK (1985): "Harper's Review of Biochemistry." Los Altos, CA: Lange Medical Publications, pp 91–100.
28. Meister A (1994): Glutathione, ascorbate and cellular protection. Cancer Res 54:1969s–1975s.
29. Meister A (1988): Glutathione metabolism and its selected modification. J Biol Chem 263:17205–17208.

30. Miles S (1994): Pathogenesis of HIV-related Kaposi's sarcoma. Curr Opin Oncol 6:497–502.
31. Mokuna K, Ohtani K, Suzumura A, Kiyosawa K, Hirose Y, Kawai K, Kato K (1994): Induction of manganese superoxide dismutase by cytokines and lipopolysaccharide in cultured mouse astrocytes. J Neurochem 63:612–616.
32. Moore PS, Gau SJ, Dominguez G, Ceasarman E, Lungu O, Knowles DM, Garber R, Pellett PE, McGeoch DJ, Chang Y (1996): Primary characterization of a herpesvirus agent associated with Kaposi's sarcoma. J Virol 70:549–558.
33. Morimoto R, Tissieres A, Georgopoulos C (eds) (1990): "Stress Proteins in Biology and Medicine." Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
34. Northfelt DW (1994): Treatment of Kaposi's sarcoma. Current guidelines and future perspectives. Drugs 48: 569–582.
35. Offerman MK, Lin JC, Mar EC, Shaw R, Yang J, Medford RM (1996): Antioxidant-sensitive regulation of inflammatory-response genes in Kaposi's sarcoma cells. J Acquir Immune Defic Syndr 13:1–11.
36. Roederer M (1993): N-Acetylcysteine: Potential for AIDS therapy. Pharmacology 46:121–129.
37. Roederer M, Staal FJT, Raju PA, Ela SW, Herzenberg LA, Herzenberg LA (1990): Cytokine-stimulated human immunodeficiency virus replication is inhibited by N-acetyl-L-cysteine. Proc Natl Acad Sci USA 87:4884–4888.
38. Schreck R, Rieber P, Bauerle PA (1991): Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. EMBO J 10:2247–2258.
39. Schultze-Osthoff K, Beyaert R, Vandevoorde V, Haegeman G, Fiers W. (1993) Depletion of the mitochondrial electron transport abrogates the cytotoxic and gene-inductive effects of TNF. EMBO J 12:3095–3104.
40. Schultze S, Potthoff K, Machleidt T, Berkovic D, Wiegmann K, Kronke M. (1992): TNF activates NF-κB by phosphatidyl-choline-specific phospholipase C-induced "acidic" sphingomyelin breakdown. Cell 71:1–20.
41. Sedor JR, Carey SW, Emancipator SN (1987): Immune complexes bind to cultured rat glomerular mesangial cells to stimulate $O_2^{\cdot-}$ release. J Immunol 138:3751–3757.
42. Sen CK, Packer L (1996): Antioxidant and redox regulation of gene transcription. FASEB J 10:709–720.
43. Smith CA, Farrah R, Goodwin RG (1994): The TNF receptor superfamily of cellular and viral proteins: activation, costimulation and death. Cell 76:959–962.
44. Staal FJT, Anderson MT, Staal GEJ, Herzenberg LA, Gitler C, Herzenberg LA (1994): Redox regulation of signal transduction: Tyrosine phosphorylation and calcium influx. Proc Natl Acad Sci USA 91:3619–3622.
45. Stall FJT (1990): Intracellular thiols regulate activation of nuclear factor κB and transcription of human immunodeficiency virus. Proc Natl Acad Sci USA 87: 9943–9947.
46. Stralin B and Marklund SL (1994): Effects of oxidative stress on expression of extracellular superoxide dismutase, CuZn-superoxide dismutase and Mn-superoxide dismutase in human fibroblasts. Biochem J 298:347–352.
47. Strandell E, Buschard K, Saldeen J, Welsh N (1995): Interleukin-1β induces the expression of HSP70, heme oxygenase and Mn-SOD in FACS-purified rat islet β-cells, but not in α-cells. Immun Letts 48:145–148.
48. Tacchini L, Pogliaghi F, Radice L, Anzon E, Bernelli-Zazzera A (1995): Differential activation of heat-shock and oxidation-specific stress genes in chemically induced oxidative stress. Biochem J 309:453–459.
49. Vietor I, Schwenger P, Li W, Schlissinger J, Vileck J (1993): Tumor necrosis factor-induced activation and increased tyrosine phosphorylation of mitogen-activated protein (MAP) kinase in human fibroblasts. J Biol Chem 268:18994–18999.
50. Wong GHW, Elwell JH, Oberley LW, Goeddel DV (1989): Manganous superoxide dismutase Is essential for cellular resistance to cytotoxicity of tumor necrosis factor. Cell 58:923–931.
51. Zheng CK and Guan KL (1993): Properties of MEKs, the kinases that phosphorylate and activate the extracellular signal-regulated kinases. J Biol Chem 268: 23933–23939.