We have studied the effect of blockade of mitochondrial respiration on the binding of human 125I-TNFα to L929 cell receptors. Specific TNFα binding was decreased to about 20–40% of controls by blocking mitochondrial respiration. This effect was dose- and time-related and was observed independently of the level at which the respiration was blocked (respiratory chain, proton backflow, ATPase, anaerobiosis). This blockade had no effect on the half-life of the specific TNFα binding, the internalization or degradation of TNFα-receptor complexes, or the number of TNFα-binding sites. Scatchard analysis of TNFα binding data indicated a 2–4-fold decrease in the affinity of these binding sites. These effects did not appear to be related to the protein kinase C activity or to reactive oxygen radicals, since they were not antagonized by pretreatment of cells with oxygen radical scavengers, deferoxamine, or inhibitors of protein kinase C. Decrease in TNFα binding capacity correlated significantly with cellular ATP content (r = 0.94; p < 0.01) and with the cytotoxic activity of TNFα against L929 cells. These findings suggest that blockade of mitochondrial respiration down-regulates the binding of TNFα to cells, most likely by changing the affinity of receptors for this cytokine. This down-regulation may increase the resistance of cells to TNFα cytotoxicity.

Tumor necrosis factor α (TNFα) is a polypeptide cytokine with a wide range of biological activities (1–4), including tumor cytotoxicity (5). TNFα initiates its biological effects by its binding to high affinity receptors (6). The expression of these receptors is necessary to determine responsiveness of target cells (7). It has also shown that some intracellular events, such as protein kinase C activity or intracellulal levels of cAMP may regulate the binding of TNFα to these specific receptors (8–14). Two TNFα receptors have been recognized: a type I receptor of 55–60 kDa and a type II receptor of 75–80 kDa. The cytotoxic effect of TNFα is believed to be transduced by the type I receptor (15), although the type II receptor may also contribute to this effect (16, 17). Unlike the species-specific type II receptor, the type I receptor in the murine L929 cell will bind heterologous (i.e. human) TNFα (18) and may initiate apoptotic cell death. In this paper, we show that mitochondrial function modulates the binding of TNFα to cell receptors, an effect that might influence the effects of this cytokine on cell cytotoxicity.

Mitochondrial dysfunction has been suggested to be an early event of TNFα cytotoxicity in tumor cells (19).

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant human TNFα was purchased from Genzyme Co. (Cambridge, MA). RPMI 1640 medium was from Biochrom (Berlin, Germany), and 125I-Bolton-Hunter-labeled human TNFα (specific activity, 48.8 Ci/μg) was from DuPont NEN. SDS, rotenone, thienyltrifluoracetone (TTFA),1 antimonials A, potassium cyanide (KCN), trifluoracetic acid, 2,4-dinitrophenol, dglucin, actinomycin D, cytochrome c, sodium orthovanadate, okadaic acid, staurosporine, and 1-(5-isoquinolinesulfonfyl)-2-methylpyperazine dihydrochloride (H-7) were purchased from Sigma. Fetal calf serum (FCS) was from Sera-lab (Sussex, United Kingdom), and phosphate buffered saline was from SCN Biomedicals, Inc. (Costa Mesa, CA). Plastic cell culture flasks and dishes were from Nunc (Roskilde, Denmark) and Falcon Division of Becton Dickinon Co. (Oxnard, CA).

Methods—L929 cells, a murine fibrosarcoma cell line, were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, glutamine, penicillin (100 units/ml), and streptomycin (0.1 mg/ml) in a humidified incubator in 5% CO2 in air.

**Binding Assays**—Binding assays were performed in confluent cultures in 35-mm plastic plates containing approximately 1 × 106 L929 cells, as described by Tsujimoto et al. (20). The effect of inhibitors of mitochondrial respiratory chain on the binding of TNFα to specific cell receptors was evaluated by the same assay after incubation of L929 cells with these agents for 1, 2 or 3 h at 37 °C. NADH-dehydrogenase complex (complex I) was inhibited with rotenone; succinate dehydrogenase (complex II) was inhibited with TTFA and malonic acid; b-cytochrome oxidase complex (cytochrome a-a3; complex III) was inhibited with antimonials A; cytochrome c (complex IV) was inhibited with KCN and NaF; and ATPase was inhibited with dglucin. As uncoupling agent, 2,4-dinitrophenol was used, and as phosphatase inhibitors, sodium orthovanadate and okadaic acid were employed. Anaerobiosis was induced by incubating cells in a nitrogen atmosphere, after replacing oxygen by nitrogen and sealing culture flasks tightly. All of these assays were carried out in triplicate. At the time of the TNF binding studies, no evidence of cytotoxicity was detected. Calculation of the dissociation constant (Kd) and the number of binding sites was done by Scatchard plot analysis of the binding (21), as described by Linge and Green (22), under control conditions and after incubation of cells with either 10 μg/ml antimonials A or 0.24 μg rotenone for 2 h at 37 °C. These assays were performed in duplicate.

Internalization of cell-bound TNFα was evaluated by the procedure described by Costlow and Hample (23) and modified by Tsujimoto et al. (20). Degradation of internalized TNFα was measured following the induction of Tsujimoto et al. (20).

Cytotoxicity—Fifty microliters of solution containing the inhibitory drug were added to L929 cells cultured in 35-mm plates at 1 × 106 cells in 2 ml RPMI 1640 medium. At the time indicated, 25 ng/ml human TNFα and 1 μg/ml antimonials D were added to the culture medium. Cytotoxicity was measured using the index of lactate dehydrogenase enzyme leakage from damaged cells and expressed as a percentage of total cellular activity, as described by Becker and Lohmann-Mathes (24).

1 The abbreviations used are: TTFA, thienyltrifluoroacetone; KCN, potassium cyanide; FCS, fetal calf serum; H-7, 1-(5-isoquinolinesulfonfyl)-2-methylpyperazine dihydrochloride.
Cellular Respiration Blockade Down-regulates TNF-α Receptors

Intracellular ATP Concentration—ATP content was measured using a modified bioluminescence assay as described by Wulff and Döpp (25). Cellular ATP levels were expressed in mmol/10⁶ cells.

Statistical Analysis—All results are expressed as mean ± S.D. unless otherwise mentioned. Student’s t test was used to evaluate the differences in means between groups, accepting p < 0.05 as level of significance (26). Pearson’s correlation coefficient was used for correlation analysis between variables that demonstrated statistically significant changes after treatment of cells.

RESULTS

Effect of Blocking the Mitochondrial Respiratory Chain on Specific TNF-α Binding to Cell Receptors—Complex I of the respiratory chain accepts electrons from NADH and transfers them to ubiquinone (27) (Fig. 1). The blockade of this transfer of electrons through complex I by incubating cells with 0.24 μM and 30 μM rotenone for 1–3 h led to a marked decrease in the binding of TNF-α to specific type I cell receptors. This effect increased in proportion to the incubation time, there being only about 19 ± 7% of the control binding after 3 h of incubation with 30 μM rotenone (Table I).

Ubiquinone passes these electrons to the b-c1 complex (complex III), which transfers them to cytochrome c (27). The function of complex III can be blocked with antimycin A (Fig. 1). Therefore, we incubated L929 cells either with 10 μg/ml or 26 μg/ml antimycin A for 1 to 3 h. Our study showed that the blockade of this complex decreased TNF-α binding to about 40 and 30%, respectively, of the control after 3 h of incubation (Table I).

Ubiquinone is involved in carrying electrons from the b-c1 complex to the cytochrome oxidase complex (cytochrome a-a3, or complex IV). This complex finally transfers these electrons to oxygen (27). KCN binds to this complex and thereby blocks the transport of electrons. Treatment of cells with either 250 μM or 500 μM KCN for 3 h reduced TNF-α binding by 47 and 37%, respectively, of controls (Table I). Likewise, 5 mM NaF, another inhibitor of cytochrome c oxidase, decreased specific binding of TNF-α to 18% of the control level after 3 h of incubation (Fig. 2A).

Transport of electrons through the respiratory chain is coupled to the pumping of protons from the mitochondrial matrix to the intermembrane space. ATPase synthesizes ATP when protons flow back from the intermembrane space into the matrix (27). Oligomycin inhibits ATPase activity, and lipidophilic weak acids uncouple electron transport from ATP synthesis. Incubation of L929 cells for 3 h with either 10 μg/ml oligomycin or 2 μg/ml 2,4-dinitrophenol, an uncoupling agent, decreased the binding of TNF-α to 27 and 57%, respectively, of control values (Table I and Fig. 2A).

Cytochrome c is involved in carrying electrons from the b-c1 complex to the cytochrome oxidase complex (cytochrome a-a3, or complex IV). This complex finally transfers these electrons to oxygen (27). KCN binds to this complex and thereby blocks the transport of electrons. Treatment of cells with either 250 μM or 500 μM KCN for 3 h reduced TNF-α binding by 47 and 37%, respectively, of controls (Table I). Likewise, 5 mM NaF, another inhibitor of cytochrome c oxidase, decreased specific binding of TNF-α to 18% of the control level after 3 h of incubation (Fig. 2A).

Effect of Inhibitors of the Respiratory Chain on Number, Affinity, Internalization, Degradation and Half-life of the TNF-α Cell Receptors—Scatchard analysis of the binding data showed that the dissociation constant of TNF-α receptors in L929 cells was 5.7 × 10⁻¹⁰ M and that the average number of specific binding sites was about 3372/cell. These results coincided with those reported by Tsujimoto et al. (20). Scatchard analysis after treatment of L929 cells for 2 h with either 10 μg/ml antimycin A or 0.24 μM rotenone, two powerful inhibitors of TNF-α binding, indicated a 2-4-fold decrease in the receptor affinity (rotenone-treated: Kd = 11.7 × 10⁻¹⁰ M; antimycin A-treated: Kd = 21.9 × 10⁻¹⁰ M). In rotenone-treated cells, the number of specific binding sites did not differ significantly (3308 binding sites) from those of control cells. However, this number decreased slightly, to 82% of the controls, in cells without inhibitors of the respiratory chain but otherwise processed in the same way. Data represent mean values from a single experiment, which was conducted in quadruplicate, r, correlation coefficient of Pearson.

Table I

| Time (h) | 1 | 2 | 3 | 5 |
|---------|---|---|---|---|
| Rotenone (0.24 μM) | 70 | 57 | 37 | −0.99 | <0.001 |
| Rotenone (30 μM) | 19 | 19 | 19 | 19 |
| Antimycin (10 μg/ml) (Exp. 1) | 87 | 64 | 45 | 45 |
| Antimycin (10 μg/ml) (Exp. 2) | 81 | 50 | 37 | 37 |
| Antimycin (26 μg/ml) | 56 | 44 | 27 | 27 |
| KCN (250 μM) | 91 | 68 | 47 | 47 |
| KCN (500 μM) | 37 | 37 | 37 | 37 |
| Oligomycin (10 μg/ml) | 56 | 44 | 27 | 27 |
| TTFA (250 μM) | 81 | 63 | 29 | 29 |
treated with antimycin A (2752 binding sites) (Fig. 3).

Neither 10 μg/ml antimycin A nor 250 μM KCN, the less effective blocker of TNF-α binding, modified the rate of internalization of human 125I-TNF-α. Intracellular radioactivity resistant to acid elution rose early after incubation of cells at 37 °C, but this rise was followed by a slow decline. No significant differences were seen in the kinetics of intracellular TNF-α between control cells and cells treated with either antimycin A or KCN (Fig. 4A). Analysis of 125I radioactivity in the TCA-soluble fraction of culture media collected before addition of the glycine buffer and in solubilized cells gave a measure of degradation and internalization, respectively, of TNF-α-receptor complex. Diagram is representative of two separate experiments. Panel B, L929 cells were incubated with or without 10 μg/ml antimycin A in the presence or absence of 0.1 mM cycloheximide at 37 °C. At the indicated times TNF-α binding capacity was determined as indicated in Table I and under “Experimental Procedures.” Specific human 125I-TNF-α binding is given as percentage of untreated cells (11,300 ± 635 cpm). Data represent the mean of triplicate samples. CHX, cycloheximide.
Treatment of cells with 10 μg/ml antimycin A decreased the specific binding of TNFα to 37 ± 7% of control cells (Fig. 5). However, the addition of 10 μg/ml superoxide dismutase, an enzyme that removes superoxide anion from the cells, as well as 100 mM mannitol, a scavenger of hydroxyl radicals, or 100 μM deferoxamine, a specific iron chelator, did not antagonize this effect of antimycin A (Fig. 5). We also studied the effect of the protein kinase C inhibitors H-7 and staurosporine on the decreased binding of TNFα to L929 cells induced by antimycin A (10 μg/ml). As Fig. 5 shows, neither H-7 (50 μM) nor staurosporine (0.5 μM) had any significant effect on the specific binding of TNFα to these cells, and neither of them antagonized the antimycin A-induced reduction of TNFα binding activity. Likewise, the addition of orthovanadate (1 μM) or okadaic acid (20 ng/ml), two phosphatase inhibitors, did not reverse the effect of antimycin A on TNFα binding to the cells (data not shown).

Effect of Blockade of the Mitochondrial Respiratory Chain on Intracellular ATP Levels—As expected, incubation of L929 cells for 2 h with any inhibitor of cellular respiration resulted in a significant decrease in the intracellular ATP content (Fig. 6A). These levels correlated significantly with the binding of TNFα to cells treated for 2 h with the inhibitor (r = 0.94; p < 0.01) (Fig. 6B).

Fig. 5. Effect of oxygen radical scavengers and protein kinase C inhibitors on antimycin A-induced reduction of TNFα binding capacity to L929 cells. L929 cells were pretreated for 3 h with 10 μg/ml antimycin A (A) in the presence or absence of one of the following agents: 0.5 μM staurosporine (St), 50 μM H-7 (H7), 10 μg/ml superoxide dismutase (SD), 100 μM deferoxamine (DF), or 100 mM mannitol (M). Afterward, specific TNFα binding was quantified as described in Fig. 2 and “Experimental Procedures.” Specific binding is expressed as percentage of radioactivity of untreated cells (C) cultured in RPMI 1640 medium without antimycin A but otherwise processed in the same way (15,187 ± 695 cpm). Values are means ± S.D. of triplicate samples.

Effect of Inhibition of the Mitochondrial Respiratory Chain on TNFα-induced Cytotoxicity—The effect of the pretreatment of cells for 2 h with various inhibitors of the respiratory chain on TNFα-induced cytotoxicity is shown in Fig. 7A. L929 cells are sensitive to the cytotoxic activity of 25 ng/ml human TNFα in the presence of 1 μg/ml actinomycin D. Cells treated with any inhibitor of the respiratory chain become more resistant to this effect of TNFα. In most cases, resistance to cytosis persisted 24 h after application of the cytokine. Blockade of complex I (0.24 μM rotenone) reduced cytotoxic activity of TNFα to 20% of controls after 9 h of incubation with TNFα. Likewise, inhibition of the b-Complex (10 μg/ml antimycin A), blockade of ATPase (10 μg/ml oligomycin) or treatment of cells with 2 μM 2,4-dinitrophenol decreased cytotoxicity of TNFα to 34, 25, and 11%, respectively, of controls. In contrast, blockade of cytochrome c oxidase with 250 μM KCN resulted in a moderate reduction in the cell death after 9 h of incubation with TNFα to 68% of control values. Treatment of cells with 25 ng/ml TNFα and 1 μg/ml actinomycin D under aerobic conditions for 12 h induced the cytosis of about 50% of the cells. However, only 10% of the cells died in the absence of oxygen. Anaerobiosis 4 h after the addition of TNFα did not significantly protect cells from TNFα (Table II). Likewise, resistance to cytosis decreased when the respiratory chain was inhibited by other agents 1-4 h after the addition of TNFα.

Cytotoxicity of TNFα on L929 cells pretreated with rotenone or antimycin A correlated closely with the binding of TNFα to these cells (Fig. 7B).

DISCUSSION

In this study, we show that treatment of L929 cells with a number of inhibitors of cellular respiration resulted in a significant decrease in the binding of human TNFα to cell surface receptors. Although there were some differences in the degree of this inhibition depending on the drug used for blocking the transport of electrons throughout the respiratory chain, this effect was observed independently of the level at which the inhibition was induced. Down-regulation of TNFα binding by blocking cellular respiration was intensified in proportion to the incubation time and to the concentration of inhibitor in the culture medium. After 3 h of inhibition, the binding of human 125I-TNFα to cell receptors was only 18-47% of the control. This effect on TNFα binding had biological implications, since
it was followed by a decrease in the cytoidal activity of TNFα in L929 cells, and both effects, TNFα binding and TNFα cytotoxicity, were closely correlated. Thus, the interpretation of changes in TNFα-mediated cytotoxicity induced by pretreatment of cells with inhibitors of the mitochondrial respiratory chain as well as by any other factor demands that the effect on TNFα binding be taken into account.

Several factors have been identified as modulators of TNFα-receptor function. These factors include protein kinase C and interleukin-1 (8–12), which are involved in the down-regulation of TNFα binding. In contrast, cAMP, protein kinase A (13, 14) and interferon-γ up-regulate this binding (14, 28, 29). Now we demonstrate that there is a close relationship between cellular respiration and the binding of TNFα to its specific type I receptors.

The mechanisms by which the blockade of the mitochondrial respiratory chain led to a down-regulation of the TNFα binding capacity of L929 cells are not known. This effect cannot be attributed to a reduced cell viability as a result of these treatments, since no cell death was detected at the time these experiments were done. On the contrary, these treatments protect the cells from TNF-induced cytotoxicity. This effect could be also due to changes in the number of receptors or in their affinity for the ligand but might also reflect modifications in the kinetics of receptor-ligand complex internalization and degradation or an increase in the shedding of TNFα receptor into the culture medium.

The binding of TNFα to specific cell receptors is usually followed by internalization of the receptor-TNFα complex into the cell and by degradation of TNFα by lysosomal hydrolases (30). However, our study detected no significant difference in the kinetics of internalization or degradation of human TNFα between untreated cells and cells treated with either antimycin A or KCN (Fig. 4). Moreover, determination of the half-life of TNFα binding sites showed that it is not affected by blocking of the respiratory chain, suggesting that it is unlikely that increased degradation or shedding of these receptors can account for the down-regulation of the TNFα binding capacity of cells. On the other hand, Scatchard analysis of antimycin A or rotenone-treated L929 cells indicated that the number of TNFα receptors did not change significantly, ruling out the possibility that a change in TNFα receptor synthesis may account for this down-regulation. Likewise, this result led to doubts about an enhanced shedding of TNFα receptors as the cause of the reduced binding of TNFα to these cells. Our data indicate that this effect is better explained by a decrease in receptor affinity. Scatchard analysis showed that the affinity of TNFα binding sites was markedly decreased in cells treated with either antimycin A or rotenone. Thus, while TNFα binding sites had a dissociation constant of $5.7 \times 10^{-10}$ M in control cells, this constant increased between 2- and 4-fold in cells treated with rotenone ($K_d = 11.7 \times 10^{-10}$ M) or antimycin A ($K_d = 21.9 \times 10^{-10}$ M).

Blockade of the respiratory chain has a number of consequences for cell metabolism and function. Mitochondria are the major source of reactive oxygen species (31–33), and, under certain conditions, including mitochondrial inhibition and TNFα treatment, generation of reactive oxygen radicals increases severalfold (19, 34–42). Our results do not support the involvement of these radicals as mediators for this relationship between mitochondrial respiration and TNFα binding to the cells. Neither superoxide dismutase nor mannitol prevented

**Table I**

| Time course of the effect of the blockade of cellular respiration on TNFα binding to L929 cells | HOURS PRIOR TO OR AFTER TREATMENT WITH TNFα/ACTINOMYCIN D |
|---|---|---|---|---|---|---|---|
| 0 | 2 | 4 | 6 | 8 | 10 | 12 | 14 |
| Control | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Antimycin A (10 µg/ml) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Olomoucycin | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Rotenone (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| DNP (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| KCN (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Antimycin A (10 µg/ml) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Olomoucycin | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Rotenone (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| DNP (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| KCN (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Antimycin A (10 µg/ml) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Olomoucycin | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Rotenone (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| DNP (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| KCN (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Antimycin A (10 µg/ml) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Olomoucycin | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Rotenone (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| DNP (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| KCN (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Antimycin A (10 µg/ml) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Olomoucycin | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Rotenone (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| DNP (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| KCN (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Antimycin A (10 µg/ml) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Olomoucycin | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Rotenone (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| DNP (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| KCN (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Antimycin A (10 µg/ml) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Olomoucycin | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Rotenone (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| DNP (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| KCN (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Antimycin A (10 µg/ml) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Olomoucycin | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Rotenone (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| DNP (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| KCN (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Antimycin A (10 µg/ml) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Olomoucycin | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Rotenone (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| DNP (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| KCN (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Antimycin A (10 µg/ml) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Olomoucycin | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Rotenone (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| DNP (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| KCN (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Antimycin A (10 µg/ml) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Olomoucycin | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Rotenone (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| DNP (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| KCN (250 µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

* ND, not determined.
the inhibitory effect of antimycin A on the TNFα binding capacity of cells (Fig. 6). Conversion of superoxide anion and hydrogen peroxide to hydroxyl radical can be catalyzed by iron ions. However, chelation of these ions by deferoxamine had no effect on TNFα binding either. Moreover, anaerobiosis, a condition under which no reactive oxygen radicals are generated, significantly decreased TNF binding to L929 cells (Fig. 7A).

It has been demonstrated that TNFα binding to the cell receptors can be significantly decreased by activation of protein kinase C (8–12, 43). The mechanism of this effect is unclear (8, 9, 11, 14). However, it has been shown that activation of protein kinase C enhances shedding of TNFα receptors into the medium (43–45). It has been suggested that the decrease in TNFα binding sites caused by phorbol myristate acetate is most likely mediated through enhanced phosphorylation and activation of a cellular protease, which cleaves the TNFα-receptors (43).

Protein kinase C has been shown to have similar down-regulating effects on other cell receptors (46–48), and Dowling et al. (49) reported that phorbol myristate acetate increased shedding of CSF-1 receptors through protein kinase C-mediated activation of a protease. The results of our study appear to indicate that the effect of blockade of the mitochondrial respiratory chain on TNFα binding activity is not likely to be mediated by a protein kinase C-phosphorylated protein. Neither the inhibition of this enzyme with H-7 or staurosporine nor the blockade of cellular phosphatase with orthovanadate or okadaic acid antagonized the effect of antimycin A on TNFα binding activity (Fig. 5). Moreover, blocking the mitochondrial transfer of electrons decreased TNFα receptor affinity markedly, with no significant effect on the number of these receptors (Fig. 3). As we have previously commented, receptor shedding also seems unlikely since the half-life of TNFα-receptors was not reduced.

In this study, we found that the binding capacity of L929 cells for TNFα was significantly correlated with the intracellular concentration of ATP. This finding suggests that cellular ATP content may have a direct or indirect modulatory role in the TNFα binding activity of TNFα receptors. As far as we are aware, no data regarding cellular ATP levels and TNFα receptors have been published so far. A series of studies have found some relationships between other hormone receptors and ATP content in cells (50–55). However, results from these studies are heterogeneous and frequently contradictory and thus fail to indicate a possible common mechanism of action. It is conceivable that the binding of TNFα to specific cell receptors is an energy-consuming process. However, further studies are required to explain this relationship.

This down-regulation could be simply a side effect of mitochondrial dysfunction, secondary to the loss of cellular energy and, therefore, lacking in teleologic meaning. However, we speculate that this mechanism might protect cells from being killed by TNFα. It has been shown that TNFα treatment leads to a dose-dependent inhibition of mitochondrial transport of electrons and to the formation of reactive oxygen species (19, 37–40, 42). Based on abundant evidence, it has been suggested that these reactive metabolites play a major role in the cytotoxicity induced by TNFα (19, 37, 38, 42, 56, 57). Thus, down-regulation of TNFα binding by blockade of the mitochondrial respiratory chain might reduce the biological effects of TNFα and, consequently, could diminish oxidative stress, hydroxyl radical generation, and cell death. Holtmann and Wallach (8), Wallach et al. (58) and Hahn et al. (59) showed that cells pretreated with TNFα for 1–12 h became more resistant to the cytotoxic effect of TNFα, and Aggarwal et al. (28) demonstrated that TNFα and TNFβ inhibit the binding of TNFα to the cells. Other mechanisms of self-protection have been shown to be induced by TNFα. This is the case for manganous superoxide dismutase, a mitochondrial enzyme involved in the scavenging of superoxide radicals. Wu et al. (56, 57) and Hirose et al. (50) demonstrated that treatment of cells with TNFα induces the expression of mRNA for this enzyme, and these investigators proposed manganous superoxide dismutase as one of the protective proteins whose synthesis is induced by TNFα.

We conclude that blockade of the mitochondrial respiratory chain down-regulates the binding of TNFα to L929 cells, most likely by decreasing the affinity of TNFα receptors for this cytokine. The mechanism of this down-regulation is not known, but it appears to be closely related to intracellular ATP levels. This down-regulation may increase the resistance of cells to TNFα cytotoxicity.

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Cellular Respiration Blockade Down-regulates TNFα Receptors

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