Liposomal NAD\(^+\) prevents diminished O\(_2\) consumption by immunostimulated Caco-2 cells

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Khan, Abrar U., Russell L. Delude, Yong Y. Han, Penny L. Sappington, Xianonan Han, Joseph A. Carcillo, and Mitchell P. Fink. Liposomal NAD\(^+\) prevents diminished O\(_2\) consumption by immunostimulated Caco-2 cells. Am J Physiol Lung Cell Mol Physiol 282: L1082–L1091, 2002.—Accumulating data support the view that sepsis is associated with an acquired intrinsic derangement in the ability of cells to consume O\(_2\), a phenomenon that has been termed “cytopathic hypoxia.” We sought to use an in vitro “reductionist” model system using cultured cells stimulated with proinflammatory cytokines to test the hypothesis that cytopathic hypoxia is mediated, at least in part, by depletion of intracellular levels of NAD\(^+/\)NADH secondary to activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP). We measured O\(_2\) consumption by Caco-2 enterocytes growing on microcarrier beads after cells were incubated for 24 h under control conditions or with cytokinx, a mixture of tumor necrosis factor-\(\alpha\), interleukin-1\(\beta\), and interferon-\(\gamma\). Immunostimulated cells consumed O\(_2\) at about one-half the rate of control cells, but this effect was largely prevented if any one of the following pharmacological agents was present during the period of incubation with cytokinx: 4,5-dihydroxy-1,3-benzene disulfonic acid, a superoxide radical anion scavenger; 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazololine-1-oxyl-3-oxide, a nitric oxide scavenger; 5,10,15,20-tetrakis-(6-tert-butyldimethylsilyl)porphyrinato-iron[III], a peroxynitrite (ONOO\(^-\)) decomposition catalyst; urate, an ONOO\(^-\) scavenger; 3-aminobenzamide, a PARP inhibitor; or N-(6-oxo-5,6-dihydrophenanthridin-2-yl)-N,N-dimethylacetamide HCl, a chemically dissimilar and more potent PARP inhibitor. The decrease in O\(_2\) uptake induced by cytokinx was completely reversible if liposome-encapsulated NAD\(^+\) was added to the cultures during immunostimulation. Empty liposomes also increased O\(_2\) uptake by immunostimulated Caco-2 cells, but much less effectively than liposomes containing NAD\(^+\). These data are consistent with the view that enterocytes exposed to proinflammatory cytokines consume less O\(_2\) due to NAD\(^+/\)NADH depletion secondary to activation of PARP by ONOO\(^-\) or other oxidants.

poly(ADP-ribose) polymerase; nitric oxide; peroxynitrite; enterocyte; epithelium; intestinal

ACCUMULATING DATA SUPPORT the view that sepsis is associated with an acquired intrinsic derangement in the ability of cells to consume O\(_2\). This phenomenon has been termed “cytopathic hypoxia” (15). Direct and indirect evidence exists for the development of cytopathic hypoxia in sepsis (or related inflammatory conditions, such as endotoxemia) in experimental animals. For example, tissue PO\(_2\) has been shown to increase in rats (33) and pigs (42) infused with purified lipopolysaccharide (LPS) and also in human patients with septic shock (5). In an indirect way, these data suggest that the ability of cells to extract O\(_2\) from arterial blood is impaired in these conditions, which are associated with systemic inflammation. More direct evidence comes from a study performed by Kantrow et al. (22), where it was shown that the rate of O\(_2\) consumption by hepatocytes isolated from septic rats was less than that by liver cells from control rats. More recently, King et al. (25), working in our laboratory, showed that ex vivo O\(_2\) consumption by ileal mucosa was decreased (relative to the normal value) when the tissue was obtained from rats injected 8 h earlier with a nonlethal dose of LPS. In this study, treatment with aminoguanidine, a drug that inhibits inducible nitric oxide synthase, ameliorated the decrease in ileal mucosal O\(_2\) consumption induced by LPS. These findings implicate increased production of nitric oxide (NO\(^+\)) in the pathogenesis of cytopathic hypoxia secondary to sepsis or endotoxemia. NO\(^-\) might impair cellular respiration through several different (and mutually compatible) mechanisms. At physiologically relevant concentrations (~1 \(\mu M\)), NO\(^-\) has been shown to be a rapid, potent, and completely reversible inhibitor of cytochrome \(a,a\), the terminal complex of the mitochondrial electron transport chain (6). Although it can reversibly inhibit cytochrome \(a,a\), NO\(^-\) itself is not a toxic molecule. However, under the right conditions, NO\(^-\) reacts rapidly with superoxide radical anion (\(O_2^-\)) to form peroxynitrite (ONOO\(^-\)), which is a potent oxidizing and nitrating agent (44). Appropriate conditions for the production of ONOO\(^-\) are present during a variety of acute inflammatory conditions, including endotoxemia and sepsis. In the

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laboratory, exposing mitochondria to ONOO\textsuperscript{−} from an exogenous source causes irreversible inhibition of mitochondrial respiration. Several mechanisms have been implicated as being important in this phenomenon. Specifically, ONOO\textsuperscript{−} has been shown to inhibit the mitochondrial Fe\textsubscript{3}F\textsubscript{4}ATPase ("complex V"), which carries out phosphorylation of ADP to form ATP (32). In addition, ONOO\textsuperscript{−} also inhibits three of the mitochondrial enzyme complexes, namely, complexes I–III, which are involved in electron transport (32). Finally, ONOO\textsuperscript{−} has been shown to inhibit the activity of acetyl-CoA, the essential tricarboxylic acid cycle enzyme that converts citrate to isocitrate (8). Endogenous production of ONOO\textsuperscript{−} secondary to inducible nitric oxide synthase induction plus O\textsubscript{2}• production has been implicated as the major factor leading to impaired mitochondrial respiration in some tissues, such as rat diaphragm, after in vivo challenge with LPS (4).

Activation of the enzyme poly(ADP-ribose) polymerase (PARP) is another way that ONOO\textsuperscript{−} might impair cellular O\textsubscript{2} consumption. PARP is a nuclear enzyme that participates in a variety of cellular functions, including the repair of single-strand breaks in nuclear DNA (34), DNA replication (37), and apoptosis (37). This enzyme catalyzes the cleavage of nicotinamide adenine dinucleotide (NAD\textsuperscript{+} and NADH) into ADP-ribose and nicotinamide and the polymerization of the resultant ADP-ribose units into branching poly(ADP-ribose) homopolymers (10). Simultaneously, poly(ADP-ribose) is degraded by various nuclear enzymes, especially poly(ADP-ribose) glycohydrolase (10). The concurrent actions of PARP and poly(ADP-ribose) glycohydrolase constitute the functional equivalent of an NADase. Under pathological conditions, such as those likely to be present during acute inflammation due to sepsis, ONOO\textsuperscript{−}-induced single-strand breaks in nuclear DNA might lead to intense activation of PARP and, hence, massive depletion of NAD\textsuperscript{+}/NADH. Because redox cycling of the NAD\textsuperscript{+}/NADH couple is intrinsic to the process of oxidative phosphorylation, activation of PARP could lead to a marked impairment in aerobic respiration.

In the present study, we used a "reductionist" in vitro model system to investigate the pathophysiological mechanisms responsible for the development of impaired cellular respiration in enterocytes exposed to an inflammatory milieu. We incubated Caco-2 enterocytes grown on microcarrier beads with a cocktail of proinflammatory cytokines ("cytomix") containing interferon-\gamma (IFN-\gamma), interleukin-1\beta (IL-1\beta), and tumor necrosis factor-\alpha (TNF-\alpha). O\textsubscript{2} consumption by Caco-2 cells was markedly decreased after 24 h of incubation with cytomix. If the cells were washed free of cytomix and incubated for 4 h in the absence of cytokines, normal O\textsubscript{2} consumption was restored. The cytomix-induced decrease in O\textsubscript{2} consumption also was ameliorated by using various pharmacological interventions to prevent NO-mediated damage and PARP activation. Cytokine-treated Caco-2 cells coinubated with NAD\textsuperscript{+}-containing liposomes, a maneuver designed to preserve NAD\textsuperscript{+}/NADH levels, despite PARP activation, consumed O\textsubscript{2} normally. Collectively, these data support the view that cytopathic hypoxia in this model system is mediated by NAD\textsuperscript{+}/NADH depletion secondary to activation of PARP.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma-Aldrich Chemical (St. Louis, MO) unless otherwise noted. DMEM and PBS were from BioWhittaker (Walkersville, MD), FBS (<0.05 endotoxin units/ml) from Hyclone (Logan, UT), and the ONOO\textsuperscript{−} decomposition catalyst 5,10,15,20-tetraakis-[4-sulfonatophenyl]-porphyrinato-iron[III] (FeTPPS) from CalBiochem-Novabiochem (San Diego, CA). The PARP inhibitor N-(6-oxo-5,6-dihydrophenanthridin-2-yl)-N,N-di- methylaceticamide HCI (PJ-34) was a generous gift from A. Salzman and C. Szabo (Inotek, Beverly, MA). Ultra-low-attachment six-well tissue culture plates were obtained from Costar (Cambridge, MA) and collagen 1-coated tissue culture dishes from Becton Dickinson (Bedford, MA). Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA).

Cell culture. Caco-2 cells were fed bweekly and grown in complete medium composed of DMEM supplemented with 10% FBS, penicillin G (100 U/ml), streptomycin (100 \mu g/ml), pyruvate (2 mM), l-glutamine (4 mM), and 2% (vol/vol) non-essential amino acid supplement. For measurements of O\textsubscript{2} consumption, cells were cultured on Cytodex-3 microcarrier beads (100–230 \mu m diameter; Amersham Pharmacia Biotech, Piscataway, NJ) to eliminate the need to harvest the cells mechanically or with trypsin before an experiment. One hundred milligrams of Cytodex-3 microcarrier beads were seeded with 2 × 10\textsuperscript{6} cells in a 125-ml Wheaton Magna Flex Spinner Flask (Fisher Scientific, Pittsburgh, PA) containing 20 ml of complete medium. The jar was incubated at 37°C in a humidified 5% CO\textsubscript{2} atmosphere for 5 h. The jar was gently swirled twice by hand during this incubation to briefly suspend the contents. The flask received an additional 30 ml of complete medium and was incubated overnight with the stirring mechanism set just fast enough to keep the beads suspended. On the next day, the culture received an additional 25 ml of complete medium to achieve a final volume of 75 ml. The medium was replaced with fresh medium every fourth day by letting the beads settle and changing 50 ml of medium. Cells growing on beads were used for experiments 2 wk after seeding.

For other studies, 1 × 10\textsuperscript{6} cells were seeded into collagen-coated six-well polystyrene tissue culture dishes and fed twice weekly with complete medium changes. These cells were maintained in a humidified 5% CO\textsubscript{2} incubator at 37°C. These cells were used for experiments 21–28 days after seeding.

Experimental design for O\textsubscript{2} consumption studies. Two milliliters of a suspension of microcarrier beads in medium were added to each well of Costar six-well polystyrene ultra-low-attachment cell culture dishes. These dishes are coated with a covalently bound hydrophilic and uncharged hydrogel layer that inhibits attachment of cells. Cytomix, consisting of IL-1\beta (1 ng/ml final concentration), IFN-\gamma (1,000 U/ml), and TNF-\alpha (10 ng/ml), in the presence or absence of various pharmacological agents, was added to some wells. The cells were incubated for 24 h at 37°C in a humidified 5% CO\textsubscript{2} incubator. At the end of the incubation period, aliquots of the beads were removed for determination of cellular O\textsubscript{2} consumption as described below.

To determine whether cytomix-induced repression of O\textsubscript{2} consumption was reversible, cells growing on microcarrier
beads were incubated for 24 h with cytomix in ultra-low-attachment cell culture dishes. The beads were then split into two wells of a six-well dish and washed three times with 2 ml of fresh medium. Two milliliters of fresh medium were added to each well, and some of the wells received cytomix. All the wells were incubated for an additional 4 h, and then the beads were removed for determination of O2 consumption as described below.

**Determination of O2 consumption by Caco-2 enterocytes.** O2 consumption by cells was determined by measuring the rate of decrease of O2 concentration in the fluid (tissue culture medium) contained within a gas-tight 400-μl temperature-jacketed polarographic chamber (Instech, Plymouth Meeting, PA) maintained at 37°C. O2 concentration was measured using a fiber-optic O2 sensor. The sensing end of this device is coated with a fluorophor. Binding of O2 to this material results in quenching of its fluorescence. Two-point calibration of the O2 sensor was carried out before each series of measurements: the low point on the calibration curve (Po2 = 0 mmHg) was established by making a reading with the chamber loaded with a freshly prepared saturated solution of sodium hydrosulphite, and the high point was determined by making a reading with the chamber loaded with tissue culture medium equilibrated with room air. To load the chamber with cells, the beads were well suspended with a 3-ml syringe, which was then used to transfer the sample to the polarographic chamber. The cells were allowed to settle in the chamber, and fresh medium was injected into the polarographic chamber, with great care taken to eliminate all air bubbles from the system and not to disturb the settled beads. The system was then sealed, and a magnetic stir bar within the chamber was used to suspend the beads in the fresh medium. O2 concentration was measured every 10 s for 10 min, and the results were used to calculate the slope of a line plotting O2 content of the chamber (in nmol) vs. time (in s). These calculations were made by assuming that the concentration of O2 in water equilibrated with air at 37°C is 0.223 mM. Final results are expressed as nanomoles of O2 per second per microgram of DNA.

To determine the DNA content of the cells loaded into the chamber, the suspension of beads was removed after measurements of O2 consumption were completed and placed into a microcentrifuge tube. The beads were permitted to settle, and the overlying medium was aspirated and discarded. The beads were washed once with PBS. The supernatant fluid was aspirated and discarded, and 1 ml of PBS containing 10 μg/ml mithramycin and 15 mM MgCl2 was added. The mixture was sonicated to disrupt the cells. Beads were removed by centrifugation, and the DNA content of the supernatant was measured spectrofluorometrically at an excitation wavelength of 440 nm (slit width = 2.5 nm), an emission wavelength of 540 nm (slit width = 10 nm), and an integration time of 10 s. Reference standards contained 0.2–16.0 μg/ml of DNA.

**Determination of total NAD+/NADH content of Caco-2 cells.** To determine the total NAD+/NADH content of Caco-2 enterocytes, 1 × 105 cells were plated per well in six-well dishes and incubated at 37°C for 3 wk in DMEM with additives as noted above. Medium was refreshed every 4–5 days. Postconfluent cells were incubated for 24 h with fresh medium, fresh medium plus cytomix, or fresh medium plus cytomix or one of several different pharmacological agents. To first reduce NO2 to NO3, supernatants from Caco-2 cell cultures, cadmium filings (0.4–0.7 g/tube; Fluka Chemicals, Milwaukee, WI) were loaded into 1.5-ml microcentrifuge tubes. The filtrings were washed twice with 1 ml of deionized water, twice with 1 ml of 0.1 M HCl, and twice with 1 ml of 0.1 M NH4OH. Ten microliters of 30% (wt/vol) ZnSO4 were added to 200 μl of culture supernatant, and the sample was vortexed, incubated at room temperature for 15 min, and centrifuged for 5 min. The resulting supernatant was added to a cadmium-containing microcentrifuge tube and incubated at room temperature overnight with constant rocking. The samples were transferred to fresh microcentrifuge tubes and centrifuged again. The supernatants were subsequently assayed for NO2 using a modified Griess assay as previously described (43). NaN3O2 was used to generate a standard curve.

**Preparation of liposomal NAD+.** Preparation of liposomal NAD+ was adapted from a modification of the thin-film method (28). Briefly, egg phosphatidycholine (PC) was obtained in chloroform solvent from Avanti Polar Lipids (Alabaster, AL). The PC solution was placed into a rotary vacuum evaporator, and the solvent was evaporated until a thin film of PC remained. NAD+ (50 mM) in 10 mM HEPES buffer (pH 7.5) was added, and the contents were vigorously vortexed until no PC was adherent to the glassware. Liposomes spontaneously formed as sheets of PC bilayers became dislodged from the glassware and encapsulated the NAD+ solution. To create a more homogeneous, intermediate-sized population of liposomes (41), this product was then serially extruded under pressured N2 gas, first through 0.6-μm- and then through 0.2-μm-membrane filters (Millipore, Bedford, MA). Encapsulated liposomal NAD+ was separated from nonencapsulated, free NAD+ by a modification of the centrifugation column chromatography method of Fry et al. (16) using Sephadex G-50 exclusion chromatography beads and 10 mM HEPES (pH 7.5) as elution buffer. NAD+ concentrations of eluate samples for these liposome preparations were measured by a colorimetric assay similar to that described above (3) after confirmation of reproducibility in earlier liposome preparations with radioisotope labeling. We obtained an encapsulation efficiency of ~3%, yielding a stock suspension of 1.5 mM liposomal NAD+.
Effect of liposomal NAD\(^+\) on NAD\(^+/\)NADH content, \(O_2\) consumption, and lactate production. To determine the effect of liposomal NAD\(^+\) on the NAD\(^+/\)NADH content of Caco-2 cells, Caco-2 enterocytes were plated at 1 \(\times\) 10\(^6\) cells per well in six-well dishes and incubated at 37°C for 3 wk in DMEM with additives as noted above. Medium was refreshed every 4–5 days. Postconfluent cells were incubated for 24 h with 2 ml of fresh medium (control), 2 ml of fresh medium plus cytomix, or 2 ml of fresh medium plus cytomix plus one of the following additives: 20 \(\mu\)l of a suspension of empty liposomes prepared as described above (without addition of NAD\(^+\)), 20 \(\mu\)l of a suspension of liposomes containing NAD\(^+\) prepared as described above, or free NAD\(^+\) (10 \(\mu\)M final concentration). NAD\(^+/\)NADH content was measured as described above. Lactate concentrations were measured using a blood-gas analyzer (model ABL 700, Radiometer, Copenhagen, Denmark). To determine the effect of liposomal NAD\(^+\) on \(O_2\) consumption by Caco-2 cells, 2 ml of a suspension of microcarrier beads in medium were added to each well of six-well polystyrene ultra-low-attachment cell culture dishes. The cells were incubated for 24 h with 2 ml of fresh medium, 2 ml of fresh medium plus cytomix, or 2 ml of fresh medium with or without cytomix plus one of the following additives: 20 \(\mu\)l of a suspension of empty liposomes, 20 \(\mu\)l of a suspension of liposomes containing NAD\(^+\), or 10 \(\mu\)M free NAD\(^+\). At the end of the incubation period, aliquots of the beads were removed for determination of cellular \(O_2\) consumption as described above.

Statistical methods. Values are means \(\pm\) SE. In general, data were analyzed using analysis of variance followed by Duncan’s multiple range test. \(P < 0.05\) was considered significant.

RESULTS

Enterocytes growing on microcarrier beads were incubated for 24 h in medium with and without cytomix (Cyto24 and Control24, respectively). As depicted in Fig. 1A, \(O_2\) consumption was significantly lower in cells incubated for 24 h with cytomix than in cells incubated under control conditions. We considered the possibility that the decrease in \(O_2\) consumption induced by cytomix was simply a reflection of a decrease in the number of viable cells covering the microcarrier beads. However, virtually all the Caco-2 cells growing on microcarrier beads excluded the vital dye trypan blue, irrespective of whether the cells had been incubated with cytomix for 24 h or incubated under control conditions for the same period of time (data not shown). To further investigate whether cell death was responsible for the decrease in \(O_2\) consumption induced by cytomix, we incubated Caco-2 cells growing on microcarrier beads for 24 h with cytomix. Subsequently, the cell cultures were split into two subgroups. In the first subgroup (Cyto24/Control4), the beads were washed three times with control (i.e., cytokine-free) medium and then incubated for another 4 h in control medium. At the end of this second incubation period, \(O_2\) consumption was measured. In the second subgroup (Cyto24/Cyto4), the beads were washed with control medium as described above but were then incubated for another 4 h in medium containing cytomix before measurement of \(O_2\) consumption. As shown Fig. 1B, cells in the Cyto24/Control4 group consumed significantly more \(O_2\) than cells in the Cyto24/Cyto4 group.

Because incubation with cytomix decreased \(O_2\) consumption by Caco-2 cells, we wondered whether this change would be accompanied by an increase in the rate of anaerobic metabolism. Accordingly, Caco-2 cells growing as confluent monolayers on six-well dishes were incubated for 24 h with control medium or medium containing cytomix. At the end of this period, the medium was removed by aspiration and replaced with fresh control or cytokine-containing medium. Lactate concentrations in samples of the media were measured at the start of this period and 7 h later. Control monolayers released lactate into the medium at 1.57 \(\pm\) 0.12 \(\mu\)mol·h\(^{-1}\)·well\(^{-1}\). In contrast, cytokine-stimulated monolayers released lactate at 1.80 \(\pm\) 0.04 \(\mu\)mol·h\(^{-1}\)·well\(^{-1}\) (\(P = 0.037, n = 5–6\) per condition).
To investigate the mechanism(s) responsible for diminished O$_2$ consumption induced by cytomix, we incubated Caco-2 cells growing on microcarrier beads under control conditions or with cytomix in the presence or absence of a variety of pharmacological agents. As noted above, exposure of Caco-2 cells to cytomix caused a significant decrease in O$_2$ consumption. However, if the cells were coincubated with 10 mM 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron), a scavenger of O$_2^•$ (11), the decrease in O$_2$ consumption induced by cytomix was significantly ameliorated (Fig. 2). Coincubation with 100 µM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (C-PTIO), an NO$^+$ scavenger (1), had a similar effect. Significant protection against the decrease in O$_2$ consumption induced by cytomix was also afforded by 1 mM uric acid, an ONOO$^-$ scavenger (26), and 50 µM FeTPPS, an ONOO$^-$ decomposition catalyst (14). Finally, two chemically distinct PARP inhibitors, 3-aminobenzamide (3-AB, 3 mM) (40) and PJ-34 (5 µM) (17), also significantly ameliorated the decrease in O$_2$ consumption induced by incubating Caco-2 cells with cytomix for 24 h.

Accumulating data suggest that immune stimulation of various cell types by LPS and/or cytokines depends, in some poorly understood way, on activation of PARP (30). Accordingly, experiments using pharmacological agents that inhibit PARP, or even using cells from PARP-deficient mice, could be confounded by failure of the cells to develop an immunostimulated phenotype in response to incubation with cytomix. Because one marker of this phenotype is increased production of NO, we measured NO$_2$/NO$_3$ concentrations in cell supernatants from control cells, cytomix-stimulated cells, cytomix-stimulated Caco-2 cells incubated with 3-AB or PJ-34, and cytomix-stimulated cells incubated with several other pharmacological agents (Fig. 3). Incubating Caco-2 cells in the presence of cytomix for 24 h resulted in a large increase in NO$^+$ production (Fig. 3). Incubation with the superoxide scavenger Tiron had no apparent effect on cytomix-induced NO biosynthesis. As expected, the NO$^+$ scavenger C-PTIO markedly decreased the measured concentrations of NO$_2$/NO$_3$ in immunostimulated Caco-2 supernatants. Incubation with FeTPPS, a compound that catalyzes

![Graph](image-url)
the decomposition of ONOO⁻ into NO₃⁻, slightly, but significantly, decreased NO₃⁻/NO₂⁻ levels in Caco-2 supernatants. Both of the PARP inhibitors evaluated (i.e., 3-AB and PJ-34) also decreased NO⁻ production by cytomix-stimulated Caco-2 cells to a slight, but nevertheless statistically significant, extent.

The data from the previous experiments are consistent with the notion that cytomix caused a decrease in cellular respiration on the basis of NAD⁺/NADH depletion secondary to ONOO⁻-dependent PARP activation. If this idea is correct, then one would expect to observe a decrease in the NAD⁺/NADH content of Caco-2 cells after exposure to cytomix for 24 h. Moreover, this effect should be reversed by pharmacological inhibition of PARP or by agents that interfere with the formation of ONOO⁻. Prompted by this reasoning, we measured the total NAD⁺/NADH content of Caco-2 cells incubated for 24 h with control medium, cytomix-containing medium, or cytomix-containing medium supplemented with Tiron, C-PTIO, or 3-AB. As shown in Fig. 4, incubation with cytomix significantly decreased NAD⁺/NADH content to ~60% of the baseline level. The cytomix-induced decrease in NAD⁺/NADH content was partially but significantly ameliorated by Tiron and C-PTIO and was almost completely blocked by 3-AB.

As noted above, the decrease in O₂ consumption induced by cytomix was reversible if the cells were washed free of the cytokine cocktail (Fig. 1B). If the observed decrease in O₂ consumption were due to depletion of NAD⁺/NADH, then one would predict that washing cells free of cytomix should lead to restoration of normal intracellular NAD⁺/NADH content. To test this hypothesis, we incubated Caco-2 cells for 24 h in control medium or medium containing cytomix. Some of the cytomix-treated cultures were washed extensively and then incubated for another 4 h in fresh medium devoid of cytomix (Cyto24/Control4). Values are means ± SE (n = 5). Data were analyzed by ANOVA followed by Duncan’s multiple range test: *P < 0.05 vs. Control24; †P < 0.05 vs. Cyto24.

If NAD⁺/NADH depletion is responsible for the decrease in cellular respiration induced by cytomix, then replenishing cellular levels of this catalytically essential nucleotide should tend to restore normal rates of O₂ consumption. NAD⁺, however, is a bulky and highly charged molecule that would not be expected to diffuse across the cytosolic membrane. Furthermore, extracellular NAD⁺/NADH is a substrate for the cell-surface enzyme NAD glycohydrolase (CD38), which converts NAD⁺/NADH to cyclic ADP-ribose (24). Thus simply adding NAD⁺ or NADH to the incubation medium would not be expected to have much of an effect on cellular levels of NAD⁺/NADH. In an effort to circumvent this problem, we incubated cytomix-stimulated Caco-2 cells with liposome-encapsulated NAD⁺. We also examined the following control conditions: unstimulated cells incubated without liposomes, cytomix-stimulated cells incubated without any other agents, cytomix-stimulated cells incubated with free NAD⁺, and cytomix-stimulated cells incubated with empty liposomes. As expected from previous experiments, exposing Caco-2 cells to cytomix for 24 h resulted in a decrease in NAD⁺/NADH content to ~60% of the control value (Fig. 6). Adding free NAD⁺ to the cytomix-containing culture medium had no effect on the NAD⁺/NADH content of Caco-2 cells. In contrast, adding empty liposomes was associated with a small, albeit statistically significant, increase in cellular NAD⁺/NADH content.
followed by Duncan’s multiple range test: *P < 0.05 vs. cytomix alone.

Cells with NADH content. However, when cytomix-stimulated Caco-2 cells were incubated with liposome-encapsulated NAD⁺, the cellular NAD⁺/NADH level was increased to ~85% of the control value. The effect of NAD⁺-containing liposomes was significantly greater than the effect of empty liposomes (P < 0.05).

As shown in Fig. 7, incubation of control cells with NAD⁺-loaded or empty liposomes did not affect O₂ consumption. Consistent with our observations in prior experiments, incubation of Caco-2 cells with cytomix resulted in a marked decrease in O₂ uptake. Adding free NAD⁺ to the culture medium had no effect on O₂ consumption by cytomix-stimulated Caco-2 cells. In contrast, coincubation of cytomix-stimulated cells with empty liposomes resulted in a small but statistically significant increase in O₂ consumption compared with cells incubated with cytomix in the absence of liposomes. However, coincubation of cytomix-stimulated cells with NAD⁺-containing liposomes completely restored O₂ utilization to a rate that was not different from that observed in control cells not exposed to cytokines.

**DISCUSSION**

For the studies reported here, we employed a reductionist in vitro system to study the effects of proinflammatory cytokines on cellular respiration by intestinal epithelial cells. The cell line chosen for these studies, Caco-2, has been widely used by our laboratory to investigate the pathophysiology of intestinal epithelial barrier dysfunction (9, 27). When fully differentiated, Caco-2 cells recapitulate many of the microanatomic and physiological features of absorptive epithelial cells (31). The cells were stimulated with a cocktail of proinflammatory cytokines (cytomix) containing TNF-α, IL-1β, and IFN-γ. We previously showed that incubating Caco-2 monolayers with cytomix increases epithelial permeability to a hydrophilic macromolecular solute via a mechanism that is, at least partially, dependent on formation of ONOO⁻ (9).

In the present study, incubating Caco-2 enterocytes for 24 h with cytomix markedly decreased the rate of O₂ consumption by these cells. This phenomenon was entirely reversible: if the cells were washed free of cytomix and incubated for 4 h in cytomix-free medium, normal O₂ consumption was restored. This observation supports the view that the effect of cytomix on cellular respiration was caused by a sublethal metabolic derangement in the cells, rather than simply a decrease in the number of viable cells studied. This view is further supported by the observation that cytomix-stimulated Caco-2 cells excluded trypan blue. Our goal in the experiments reported here was to explore the hypothesis that decreased O₂ utilization by immunostimulated Caco-2 cells depends on increased NO⁻ and ONOO⁻ production and, on this basis, downstream PARP activation and NAD⁺/NADH depletion.

The notion that inflammation impairs cellular respiration via an NO⁻-dependent mechanism is not a novel concept. Even before the importance of NO⁻ as a signaling and effector molecule in mammalian biology was widely appreciated, Granger et al. (19) reported that cellular respiration in tumor target cells is inhibited when the tumor cells are cocultured with activated murine macrophages. Drapier and Hibbs (12, 13) and Hibbs et al. (21) subsequently obtained data indicating that this phenomenon was mediated by an L-arginine-dependent effector mechanism that inhibited several mitochondrial enzymes containing catalytically active iron-sulfur clusters. A few years later, Geng et al. (18)
extended this line of investigation still further by showing that mitochondrial respiration is inhibited via an NO-dependent process when vascular smooth muscle cells are incubated in the presence of the proinflammatory cytokines TNF-α and IFN-γ. According to current thinking, NO is capable of reversibly inhibiting mitochondrial respiration by binding to cytochrome \(a, a_3\) (6). In contrast, ONOO\(^{-}\), a potent oxidizing and nitrating species that results from the rapid reaction of NO\(^{-}\) with \(O_2^{-}\), exerts its effects on respiratory complexes I–III (32) as well as the tricarboxylic acid cycle enzyme aconitase (8).

Activation of PARP is another way that inflammatory mediators could impair cellular respiration. About 15 years ago, Schraufstatter et al. (35, 36) presented evidence that a major pathway of injury in cells exposed to the potent oxidizing agent hydrogen peroxide was metabolic inhibition due to NAD\(^+\)/NADH depletion secondary to activation of PARP. More recently, Szabó et al. (39) showed that exposure of cultured cells to physiologically relevant concentrations of another potent oxidant, ONOO\(^{-}\), also activated PARP and, on this basis, resulted in impaired mitochondrial respiration. Szabó and co-workers further showed that endogenously generated ONOO\(^{-}\) was capable of activating PARP and, thereby, inhibiting mitochondrial respiration in cultured immunostimulated macrophages (45) and vascular smooth muscle cells (40). In these studies, alterations in cellular respiration were detected by measuring the reduction of MTT (40, 45). However, the MTT assay reflects the activity of a number of different dehydrogenases, particularly succinate dehydrogenase (20) and, therefore, is only an indirect measure of mitochondrial respiration.

In the present series of experiments, O\(_2\) consumption was directly measured, rather than inferred. Incubation of Caco-2 cells with cytomix for 24 h decreased O\(_2\) consumption to about one-half of the basal rate. The evidence presented here supports the view that this phenomenon was, at least partially, dependent on the formation of ONOO\(^{-}\). ONOO\(^{-}\) results from the rapid reaction of NO\(^{-}\) and \(O_2^{-}\). We observed that pharmacologically scavenging NO\(^{-}\) (with C-PTIO) or \(O_2^{-}\) (with Tiron) significantly ameliorated the decrease in cellular O\(_2\) consumption induced by cytomix, thereby implicating ONOO\(^{-}\) as an upstream mediator of this phenomenon. Furthermore, we showed that the decrease in cellular O\(_2\) consumption induced by cytomix was also ameliorated by coinubcation with urate, an ONOO\(^{-}\) scavenger, or FeTPPS, an ONOO\(^{-}\) decomposition catalyst.

Consistent with the hypothesis articulated by Szabó et al. (38), we believe that decreased O\(_2\) consumption in cytomix-stimulated epithelial cells was caused, at least in part, by ONOO\(^{-}\)-dependent DNA damage, leading to activation of the nuclear enzyme PARP. Although we did not obtain direct evidence of PARP activation in our system, previous studies using similar in vitro models have documented that PARP is indeed activated in immunostimulated cells (40, 45). Furthermore, in our studies, we showed that O\(_2\) utilization was significantly increased when cytomix-stimulated Caco-2 cells were incubated with either of two chemically dissimilar PARP inhibitors, 3-AB and PJ-34. We recognize that blocking activation of PARP can interfere with expression of the immunostimulated phenotype in various cell types studied in vitro or in animals (30). Indeed, we observed that incubating Caco-2 cells with 3 mM 3-AB or 5 \(\mu M\) PJ-34 partially downregulated NO\(^{-}\) biosynthesis, an important marker of the immunostimulated phenotype in these cells. Thus interpretation of the O\(_2\) consumption results from the studies using the PARP inhibitors is not straightforward. However, because the effects of 3-AB or PJ-34 on NO\(^{-}\) production were more modest than the effects of these agents on O\(_2\) consumption, the data obtained using these compounds support, but do not prove, the contention that PARP activation directly impairs O\(_2\) consumption in immunostimulated Caco-2 cells.

We observed that the NAD\(^+\)/NADH content of Caco-2 cells decreased after incubation for 24 h with cytomix. This finding is consistent with previously reported results from a number of related studies (38, 39, 45), including a series of experiments wherein Caco-2 cells were incubated with exogenous ONOO\(^{-}\) (23). Consistent with the hypothesis that NAD\(^+\)/NADH depletion was mediated by ONOO\(^{-}\)-induced PARP activation, we observed that pharmacological approaches that blocked ONOO\(^{-}\) production or PARP activity tended to restore cellular NAD\(^+\)/NADH levels toward normal. Furthermore, we showed that NAD\(^+\)/NADH levels were restored toward normal when Caco-2 cells were washed free of cytokines and permitted 4 h to recover in cytokine-free medium. Most importantly, however, we showed that incubation of Caco-2 cells with liposome-encapsulated NAD\(^+\) abrogated the decrease in O\(_2\) consumption induced by exposure to cytomix. Adding free NAD\(^+\) to cultures had no effect on O\(_2\) utilization by immunostimulated Caco-2 cells. This observation was entirely expected, since extracellular NAD\(^+\)/NADH is a substrate for the cell-surface enzyme NAD glycohydrolase (CD38), which converts NAD\(^+\)/NADH to cyclic ADP-ribose (24). Indeed, it was the existence of this enzyme that prompted us to package NAD\(^+\) into liposomes to facilitate entry of this compound into cells.

Although free NAD\(^+\) had no effect on O\(_2\) consumption by the cells studied here, O\(_2\) consumption was slightly, but significantly, improved by incubating immunostimulated Caco-2 cells with empty liposomes. We are not prepared to offer an explanation for this effect but would speculate that the liposomes had an immunomodulatory effect, as has been reported previously in some other systems (2, 7).

In summary, we used an in vitro reductionist model to show that immunostimulation decreases O\(_2\) consumption by transformed human enterocytes. The data presented here are insufficient to rule out the possibility that inactivation of various mitochondrial enzymes, such as aconitase or complexes I–III, contributes to this phenomenon. Nevertheless, our findings suggest that the predominant mechanism is activation of
PARP, leading to NAD⁺/NADH depletion on this basis. With respect to previous work in this general area of research, our findings are novel, in that we directly measured O₂ utilization, rather than inferring it on the basis of a somewhat nonspecific biochemical assay, and we showed for the first time that normal O₂ utilization can be preserved if the immunostimulated cells are incubated with liposome-encapsulated NAD⁺. These findings provide key experimental support for the role of NAD⁺/NADH depletion as an important factor contributing to impaired O₂ consumption by immunostimulated epithelial cells. Moreover, these data suggest that liposomal NAD⁺ might warrant further evaluation as a therapeutic agent for certain inflammatory conditions associated with alterations in O₂ uptake and energy metabolism.

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