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Nitric Oxide Regulates Immune Cell Bioenergetic: A Mechanism to Understand Immunomodulatory Functions of Nitric Oxide-Releasing Anti-Inflammatory Drugs

Stefano Fiorucci,† Andrea Mencarelli,‡ Eleonora Distrutti,§ Monia Baldoni,¶ Piero del Soldato,† and Antonio Morelli*†

The 2-(acetyloxy)benzoic acid 3-(nitrooxymethyl)phenyl ester (NCX-4016) is a NO-releasing derivative of aspirin. In this study, we provide evidence that NCX-4016 delivered to PMBC-derived T lymphocytes and monocytes causes a transitory inhibition of cell respiration and ~50% reduction of cellular ATP, which translates in a time-reversible inhibition of cell proliferation and IL-2, IL-4, IL-5, and IFN-γ secretion. Exposure of lymphocytes and monocytes to aspirin, 2-(acetyloxy)benzoic acid 3-(hydroxymethyl)phenyl ester (NCX-4017), a non-NO-releasing analog of NCX-4016, and cyclooxygenase inhibitors, reduced PG formation, but has no effect on cytokine/chemokine release. In contrast, delivering NO with (z)-1-[2-(2-aminoethyl)amino]diazene-1-i um-1,2 diolate (DETA-NO) reproduced most of the metabolic and anti-cytokine activities of NCX-4016. Scavenging NO with hemoglobin or adding selective substrates of complex II, III, and IV of the mitochondrial respiratory chain reverses NCX-4016 inhibitory activities. Exposure to DETA-NO and NCX-4016 enhances glucose uptake, glycolytic rate, and lactate generation in CD3/CD28-costimulated lymphocytes, while reduced citric acid cycle intermediates. These effects were not reproduced by selective and nonselective cytochrome oxidase 2 inhibitors. In summary, we demonstrated that exposure of lymphocytes to NCX-4016 causes a metabolic hypoxia that inhibits lymphocyte reactivity to costimulatory molecules, providing a potential counterregulatory mechanism to control activated immune system.

Ener
gy metabolism of immune cells is subject to drastic changes during cell activation by Ags and mitogens and plays a central role in regulating cell response in innate and adaptive immunity (1–3). A rapid and sustained increase of energy expenditure is required to support proliferation, differentiation, and effector functions of immune cells, including the increased synthesis of cytokines, chemokines, and surface adhesion molecules. However, in contrast to hepatocytes and myocytes, resting lymphocytes and monocytes do not have large internal glycogen stores and are highly dependent on the import of extracellular glucose and glycolysis for the production of ATP (4–8). Regulation of glucose uptake in lymphocytes is only partially defined. Indeed, while many studies suggest that glucose uptake and glycolytic regulation are homeostatically controlled by cellular ATP and metabolite levels, a growing body of evidence indicates that bioenergetic functions are the selective target of costimulatory molecule, such as CD28 (9, 10). Thus, it has been shown that costimulation of TCR of human peripheral blood cell with anti-CD28 mAb leads to PI3K/Akt-dependent up-regulation of glucose transporter-1 (GLUT-1)2 expression, glucose uptake, and glycolytic flux, allowing T cells to anticipate energetic and biosynthetic needs associated with an immune stimulation (10).

During aerobic glycolysis, ATP is generated by mitochondria as a function of O2 consumption in a chain of reactions that involves a group of enzymes clustered at the inner mitochondrial membrane and collectively known as mitochondrial respiratory chain (11). Microenvironmental conditions found in injured tissues, however, are characterized by low levels of O2 and glucose, as well as high concentrations of lactate and reductive metabolites (12). In those areas, inflammation and vasodilation are often maintained by activation of the high output NO synthase (i.e., the inducible NO synthase (NOS)), NO, a physiological messenger that regulates many aspects of cell physiology, is progressively emerging as a possible modulator of O2 consumption (13–15) and energy expenditure in many cell types (16–18). Thus, exposure to NO causes the S-nitrosylation/inhibition of cytochrome c oxidase, i.e., the terminal component (complex IV) of the mitochondrial respiratory chain responsible for almost all O2 consumption (15), switching cell bioenergetic functions from an aerobic to anaerobic set, thus making NO a potential mediator of immune cell function (19–21).

Nonsteroidal anti-inflammatory drugs (NSAIDs) modulate mitochondrial respiratory chain in nonimmune cells (3). Disruption of mitochondrial function caused by NSAIDs, however, is irreversible and has been mechanistically linked to gastrointestinal side effects rather than to the anti-inflammatory activity (3). The 2-(acetyloxy)benzoic acid 3-(nitrooxymethyl)phenyl ester (NCX-4016), an anti-thrombotic and anti-inflammatory agent (reviewed in Ref. 22) that consists of an acetylsalicylic acid (ASA)-like moiety linked to a nitroxybutyl moiety, represents the prototype of a new family of NSAIDs that inhibits cyclooxygenase (COX) activities and releases NO (NO-NSAIDs). Thus, while NCX-4016

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*Abbreviations used in this paper: GLUT, glucose transporter; Δψm, mitochondrial membrane potential; ASA, acetylsalicylic acid; COX, cyclooxygenase; DCFDA, 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate; DET A-NO, (z)-1-[2-(2-aminoethyl)amino]diazene-1-ium-1,2 diolate; FB, hemoglobin; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide; NCX-4016, 2-(acetyloxy)benzoic acid 3-(nitrooxymethyl)phenyl ester; NCX-4017, 2-(acetyloxy)benzoic acid 3-(hydroxymethyl)phenyl ester; NOS, NO synthase; NSAID, nonsteroidal anti-inflammatory drug; PI, propidium iodide.
inhibits COX-1 and COX-2, it also exerts COX-independent, NO-mediated activities on platelets, lymphocytes, and monocytes (22–28). Because modulation of cell respiration might represent an important mechanism for controlling immune cell reactivity, we have designed a study to investigate whether NCX-4016 regulates bioenergetic functions in lymphocytes and monocytes. The results of the present study demonstrate that regulation of mitochondrial functions represents a new anti-inflammatory mechanism of NO-releasing NSAIDs.

**Materials and Methods**

The (2-aminooethyl)-N-(2-ammonioethyl)aminoo dien-1-ium-1,2 diolate (DETA-NO) was obtained from Alexis Biochemicals (San Diego, CA). NCX-4016 and 2-(acetoxy)benzoic acid 3-(hydroxyethyl)phenyl ester (NCX-4017), a non-NO-releasing analog of NCX-4016, were from Nicox SA (Sophia Antipolis, France). The 2,7-dichlorodihydrofluorescein diacetate was from Alexis Biochemicals, Malate, pyruvate, succinate, rotenone, antimycin A, myxothiazol, oligomycin, and BSA were obtained from Sigma-Aldrich (St. Louis, MO).

**PBMC preparation and monocytes and T lymphocyte isolation**

Human PBMCs were obtained from normal individual donors to the Blood Bank Service of Perugia University Hospital. PBMCs were isolated by density gradient centrifugation (400 × g for 30 min at room temperature) through a Ficoll-Hypaque gradient (Pharmacia Biotech, Uppsala, Sweden), as described (27). The major band, containing the mononuclear cells, was harvested with a yield typically of 1.5–2 × 10^9 cells per isolation. PBMCs were washed by centrifugation in RPMI 1640 (Life Technologies Italia Srl, Milan, Italy) supplemented with 10% FCS, 2 mM t-glutamine, and 1% penicillin/streptomycin (Life Technologies), henceforth called culture medium. and 5 × 10^7 PBMCs were allowed to adhere in 75-cm² cell culture flasks for 45 min in a 37°C humidified 5% CO₂ atmosphere. The nonadherent cells were removed, and adherent cells were washed with sterile PBS, harvested, and stained with monocyte-specific anti-CD14 mAb to assess the purity of the preparation. Ninety percent of the isolated cells expressed CD14. Cell viability, evaluated by trypan blue exclusion, was always >95%. In some experiments, highly purified monocytes (>97%) were obtained by positive selection of CD14+ cells. Highly purified T cells (>98%) were obtained from the plastic-nonadherent cell population by negative selection of T lymphocytes using anti-CD19-coated magnetic cell separation microbeads (Miltenyi Biotec, Florence, Italy).

**Cell proliferation**

Cell proliferation was assessed by measuring [3H]thymidine incorporation. A total of 1 µCi of [3H]thymidine was added for 16 h to the cultures. At the end of incubation, cells were harvested onto glass-fiber filters, and radioactivity was counted in a Beckman scintillation counter (Beckman Coulter, Milan, Italy). Data are represented as the mean disintegrations per minute ± SD from triplicate cultures.

**NO generation and oxygen consumption**

NO formation was measured using a 2-mm NO-sensitive electrode, as described previously (16, 18, 26), connected to the ISO-NO Mark II meter (World Precision Instruments, Sarasota, FL). The NO electrode was calibrated by addition of known concentrations of NaNO₂ under reducing conditions (K/H₂SO₄). O₂ consumption was measured using a Clark-type oxygen electrode (World Precision Instruments) in a magnetically stirred, thermostatically regulated chamber at 30°C (26). Briefly, cells were suspended in a volume of 0.5 ml of air-satureted isometric buffer composed of 220 mM mannitol, 70 mM sucrose, 10 mM HEPES (pH 7.2), 5 mM KCl, 40 mM NaCl, 1 mM MgCl₂, 0.9 mM CaCl₂, pH 7.4) to deplete intracellular glucose stores. Triplicate samples of 1 × 10⁶ cells were incubated with 1 µM of [14C]-deoxyglucose (New England Nuclear, Boston, MA) in glucose uptake buffer for 2 min at room temperature and immediately spun through a layer of bromododecane (Sigma-Aldrich) into 20% perchloric acid/98% sucrose, stopping the reaction and separating the cells from unincorporated [14C]-deoxyglucose. The perchloric acid/sucrose/T-cell layer was removed and analyzed by liquid scintillation using a 1450 Beckman (Beckman Coulter) scintillation counter.

**GLUT-1 mRNA assay**

In mammalian nonepithelial cells, facilitated transport of glucose is mediated by a family of stereospecific transport proteins known as GLUT proteins (29). To examine GLUT-1 expression, lymphocytes were incubated with the appropriate agent and then lysed. RNA was isolated with the RNeasy kit (Qiagen, Valencia, CA), and cDNA was prepared with SuperScript II RNase H− reverse transcriptase (Life Technologies, Rockville, MD). The sequence of sense primers used in RT-PCR for GLUT-1 was TCCAGGAGCATCTTCGAGA, and the antisense was ATACTGGAAGCACATGCC. For GAPDH, the sense primer was TGGTATCGTGGA and the antisense was ATACTGGAAGCACATGCC. For GAPDH, the sense primer was TGGTATCGTGGA and the antisense was ATACTGGAAGCACATGCC.

**ATP and lactate assay**

ATP and lactate concentrations were measured in the same experiments (16–18). Thus, aliquots of the cell suspensions (monocytes and T lymphocytes) were acidified with HClO₄ and neutralized with KHCIO₄. Glucose and lactate levels in culture supernatants were measured using enzymatic diagnostic kits (Sigma-Aldrich), following the manufacturer’s instructions. ATP concentrations were measured by chemiluminescence using a commercially available kit (Sigma-Aldrich) following the manufacturer’s instructions.

**Detection of enzymatic activities**

For determinations of total aconitate and succinate dehydrogenase activity, the cells were collected and sonicated with Tris buffer (50 mM, pH 7.4) containing protease inhibitor mixture tablet (Boehringer Mannheim, Mannheim, Germany), and the cell lysate was separated from cellular debris by centrifugation. After 15 min at 200 × g, the supernatant was mixed with reaction buffer (50 mM Tris, pH 7.4, 100 mM KCl, 10 mM NaCl, 1 mM MgCl₂, 0.9 mM CaCl₂, pH 7.4) and stored at −20°C until measurement. The OD 550 was measured at 550 nm. For succinate dehydrogenase determination, 50 µg of protein from the supernatant was mixed with a different reaction buffer (50 mM Tris, pH 7.4, 20 mM sodium succinate, 1 mM potassium cyanide, 1 mM phenazine methosulfate, and 1 mM cytochrome c), and the OD was measured at 550 nm after 5 min at 25°C (16–18).

**Detection of cell death and apoptosis**

Apopotic cells were detected by flow cytometry after staining with FITC-conjugated annexin V and propidium iodide (PI) by using a commercially

**Flow cytometric measurement of mitochondrial membrane potential (ΔΨm)**

The ΔΨm was measured, as previously described, in cells loaded with 5,5′,6,6′-tetrachloro-1′,3′,3′-tetraethylbisimidazolocarbocyanine iodide (JC-1) (26). Briefly, after exposure to different agents, monocytes and lymphocytes (5 × 10⁶/ml) were incubated with 5 µg/ml JC-1 (26). This cyanine dye accumulates in the mitochondrial matrix under the influence of the ΔΨm and forms 3 aggregates that have characteristic absorption and emission spectra. Flow cytometry was performed on an Epics XL instrument (Beckman Coulter). After gating out small sized (i.e., noncellular) debris, 50,000 events were collected for each analysis. Results are expressed as either the mean aggregate fluorescence (red) alone or as the ratio of aggregate/monomer (red/green).

**Peroxynitrite assay**

Levels of peroxynitrite were measured with the fluorescent probe 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate (DCFDA), which can be hydrolyzed by cellular nonspecific esterases and oxidized to form the fluorescent product, 2′,7′-dichlorofluorescein, after reactions with peroxynitrite (26). After loading with 10 µM DCFDA at 37°C for 30 min, lymphocytes were washed twice to remove unloaded dye and incubated with 100 µM ASA, NCX-4016, DETA-NO, and NCX-4017 for 3 h, and fluorescence was determined at 502 nm excitation and 523 nm emission for DCFDA.

**Glucose uptake**

Glucose uptake was measured using a modified protocol of Whetten et al. (4). Briefly, stimulated T cells and monocytes were incubated for 15 min at 37°C in 10 mM Na₂HPO₄, 10 mM KH₂PO₄, 2.6 mM KCl, 136 mM NaCl, 0.5 mM MgCl₂, 0.9 mM CaCl₂ pH 7.4) to deplete intracellular glucose stores. Triplicate samples of 1 × 10⁶ cells were incubated with 1 µCi of [14C]-deoxyglucose (New England Nuclear, Boston, MA) in glucose uptake buffer for 2 min at room temperature and immediately spun through a layer of bromododecane (Sigma-Aldrich) into 20% perchloric acid/98% sucrose, stopping the reaction and separating the cells from unincorporated [14C]-deoxyglucose. The perchloric acid/sucrose/T-cell layer was removed and analyzed by liquid scintillation using a 1450 Beckman (Beckman Coulter) scintillation counter.
available kit (annexin V-FITC kit; Immunotech, Marseille, France), as described (26). Cells were considered apoptotic when they were annexin V positive and PI negative. Staining of cells by PI was an indicator of the loss of plasma membrane integrity and therefore of necrosis (16, 26).

Cytokine and chemokine assay

RANTES, IL-1β, IL-6, IL-8, IL-12p40, IL-18, TNF-α, and IFN-γ concentrations in cell supernatants were measured using commercial ELISA kits (Endogen, Woburn, MA), using the standard procedure recommended by manufacturers. Cytokine concentrations were calculated from the standard curves using the GraphPad Prism software (GraphPad, San Diego, CA), and results were expressed as pg/ml. Each kit was specified (26). Cells were considered apoptotic when they were annexin V positive and PI negative. Staining of cells by PI was an indicator of the loss of plasma membrane integrity and therefore of necrosis (16, 26).

Statistics

Data were analyzed with two-tailed Student’s t test using Prism 3 (GraphPad). Values are given as mean ± SEM. A p value <0.05 was considered to be statistically significant.

Results

Effect of DETA-NO and NCX-4016 on energy metabolism in resting T cells

As illustrated in Fig. 1, exposure of resting lymphocytes to DETA-NO and NCX-4016 resulted in a time- and concentration-dependent formation of NO. At the steady state (3 h of incubation), exposure of PMBC-derived lymphocytes to 100 μM DETA-NO and NCX-4016 generated ∼800 and ∼400 nM NO, as assessed by a NO-sensitive electrode (n = 8; p < 0.001 vs medium alone). Adding nitro-l-arginine-methyl ester, 100 μM, to inhibit the constitutive and the inducible form of NOS (data not shown) had no effect on NO formation caused by DETA-NO and NCX-4016 (n = 4; p > 0.05 vs DETA-NO and NCX-4016). Exposure of resting lymphocytes to DETA-NO and NCX-4016, 100 μM, for 3 h resulted in a time- and concentration-dependent increase of ΔΨm (Fig. 1, b and c; n = 8; p < 0.01 vs baseline). However, not only the ΔΨm hyperpolarization induced by NCX-4016 was more pronounced than that caused by DETA-NO, but the effect was sustained over time and maintained for up to 12 h (Fig. 1e). Furthermore, DETA-NO exerts a biphasic effect on ΔΨm, i.e., an early hyperpolarization, followed by a subsequent depolarization (Fig. 1e).

Exposure of PMBC-derived lymphocytes to NCX-4016 and DETA-NO associates with a profound decline in O2 consumption and ATP generation (Fig. 1d; n = 8; p < 0.01 vs medium). Indeed, as shown in Fig. 1e, measurement of the steady state ATP levels indicated that lymphocytes treated with 100 μM NCX-4016 and DETA-NO for 24 h have nearly half of the level of ATP of the cells exposed to the medium alone (n = 8; p < 0.05 vs medium). As shown in Fig. 1f, exposure to DETA-NO, but not to NCX-4016, resulted in 3- to 4-fold increase of peroxynitrite concentrations (n = 7; p < 0.01 vs medium). In contrast to NCX-4016 and DETA-NO, ASA and NCX-4017, a non-NO-releasing derivative of NCX-4016, did not induce NO formation and failed to modulate O2 consumption, ΔΨm, and ATP levels, nor did they induce peroxynitrite formation (n = 6–7; p > 0.05 vs medium alone). Exposure to NCX-4016 and DETA-NO had no effect on cell survival rates, resulting in 24-h apoptotic rates of 2.0 ± 0.3, 2.4 ± 0.5, and 2.6 ± 0.9%, respectively, in cell treated with medium alone or 100 μM NCX-4016 and DETA-NO.

FIGURE 1. Effect of DETA-NO and NCX-4016 on resting lymphocytes. a, NO generation in response to NCX-4016 and DETA-NO in resting lymphocytes. NO formation was monitored continuously for 3 h using a NO-sensitive electrode. Data are mean ± SE of eight independent measures. *p < 0.01 vs control cells. b, Changes in ΔΨm as measured by JC1 relative red fluorescence intensity. The mitochondrial potential was assessed after 3 h of incubation with each agent. Data are mean ± SE of eight independent measures. *p < 0.01 vs control cells. c, Time course of ΔΨm in lymphocytes incubated with 100 μM NCX-4016, DETA-NO, ASA, and NCX-4017. Data are mean ± SE of eight independent measures. *p < 0.01 vs control cells. d, DETA-NO and NCX-4016 inhibit O2 consumption in resting lymphocytes. Data are mean ± SE of eight independent measures. *p < 0.01 vs control cells. e, ATP levels were measured in resting lymphocytes incubated for 24 h. Data are mean ± SE of eight independent measures. *p < 0.05 vs control cells. f, DETA-NO, 100 μM, increases peroxynitrite formation. Data are mean ± SE of eight independent measures. *p < 0.05 vs control cells.
Effect of DETA-NO and NCX-4016 on lymphocyte effector functions

Because proliferation and production of chemokine and cytokines are hallmarks of lymphocyte function, we then investigated whether NCX-4016 modulates cytokine release from lymphocytes challenged with anti-CD3/anti-CD28 mAbs. As shown in Fig. 2, a and b, CD3/CD28 cross-linking triggers lymphocyte proliferation

FIGURE 2. Effect of DETA-NO and NCX-4016 on effector functions of lymphocytes stimulated with anti-CD3/anti-CD28. a, DETA-NO and NCX-4016 inhibit cell proliferation induced by costimulatory molecules. Data are mean ± SE of eight independent measures. *, p < 0.01 vs anti-CD3/anti-CD28 alone. b, DETA-NO and NCX-4016 inhibit cytokine production induced by costimulatory molecules. Data are mean ± SE of eight independent measures. *, p < 0.01 vs anti-CD3/anti-CD28 alone. c and d, Inhibition of IL-2 secretion caused by DETA-NO and NCX-4016 is time reversible. Data are mean ± SE of six independent measures. *, p < 0.01 vs anti-CD3/anti-CD28 alone.

Effect of DETA-NO and NCX-4016 on monocyte effector functions

Because proliferation and production of chemokine and cytokines are hallmarks of monocyte function, we then investigated whether NCX-4016 modulates cytokine release from monocytes stimulated with bacterial endotoxin.

FIGURE 3. Effect of DETA-NO and NCX-4016 on effector functions of PMBC-derived monocytes stimulated with bacterial endotoxin, 10 μg/ml. a and b, DETA-NO and NCX-4016 inhibit cytokine and chemokine production induced by LPS. Data are mean ± SE of eight independent measures. *, p < 0.01 vs LPS alone. c and d, Inhibition of IL-1β and IL-6 secretion caused by DETA-NO and NCX-4016 is time reversible. Data are mean ± SE of eight independent measures. *, p < 0.01 vs LPS alone.
and cytokine release, an event that was significantly attenuated by coincubating the cells with 100 μM NCX-4016 (n = 8; p < 0.01 vs anti-CD3/anti-CD28 alone), but not with ASA. NCX-4016 did not induce cell toxicity because only 4.0 ± 0.3% of anti-CD3/anti-CD28-stimulated lymphocytes stained positively to annexin V after exposure to 100 μM NCX-4016 compared with 2.6 ± 0.4% of control cells and 2.9 ± 0.8% of lymphocytes treated with anti-CD3/anti-CD28 alone (n = 6; p > 0.05 vs medium).

To evaluate whether inhibition of cytokine release induced by NCX-4016 was time reversible, lymphocytes were rechallenged with a second addition of anti-CD3/anti-CD28 mAbs 24 h after starting the first incubation with NCX-4016. As shown in Fig. 2, c and d, the second addition of costimulatory mAbs fully restored the ability of lymphocytes to generate cytokine in response to CD3/CD28 cross-linking (n = 8; p > 0.05 vs LPS in the second incubation).

Similarly to NCX-4016, DETA-NO, 100 μM, reduced lymphocyte proliferation and IL-2, IL-4, and IL-5 release induced by lymphocyte stimulation with anti-CD3/anti-CD28 (n = 8; p < 0.05 vs LPS). However, in contrast to NCX-4016, DETA-NO failed to modulate IFN-γ secretion (n = 8; p > 0.05 vs LPS). Similarly to NCX-4016, inhibition of IL-2 release caused by DETA-NO was time reversible (Fig. 2, c and d).

To investigate whether the effect of NCX-4016 extended to other immune cells, we tested whether this compound inhibits cytokine/chemokine release from LPS-stimulated monocytes and found that, similarly to lymphocytes, exposure to NCX-4016 and DETA-NO, 100 μM, inhibited cytokine/chemokine secretion induced by exposure of cells to 10 μg/ml LPS for 24 h (Fig. 3, a and b; n = 6; p < 0.01 vs LPS). This effect was not reproduced by ASA or NCX4017 (data not shown). Inhibition of cytokine secretion caused NCX-4016 in LPS-primed monocytes to be time reversible. Thus, as shown in Fig. 3, c and d, inhibition of IL-1β and IL-6 secretion caused by NCX-4016 in monocytes, fully reversed after a second addition of LPS (n = 8; p < 0.01 vs LPS). DETA-NO was significantly less effective than NCX-4016 in modulating cytokine and chemokine release in response to LPS, and while it inhibited IL-1β, IL-6, IL-12p40, and IL-18 (n = 8; p < 0.05 vs LPS alone), it had no effect on secretion of RANTES, IL-8, IFN-γ, and TNF-α induced by LPS (n = 8; p > 0.05 vs LPS alone).

Because NCX-4016 has two active moieties and both contribute to its pharmacological effects, we have then explored whether inhibition of COX-1 and COX-2 is involved in cytokine/chemokine regulation exerted by NCX-4016 on lymphocytes (data not shown) and monocytes. As illustrated in Fig. 4, however, despite the fact that incubation with maximally effective concentrations of ASA and selective and nonselective COX-2 inhibitors (indomethacin and celecoxib) abrogated PGE2 and 6-keto-PGF1α release from LPS-stimulated monocytes (n = 6; p < 0.01 vs medium), these agents failed to reduce IL-1β, IL-18, and IFN-γ secretion (Fig. 4b; n = 8).

**Effect of DETA-NO and NCX-4016 on ATP and glycolysis in stimulated T cells**

Measurement of the steady state ATP levels indicated that lymphocytes (Fig. 5, a and b) and monocytes (Fig. 5, c and d) exposed

**FIGURE 4.** a, NCX-4016 abrogated PGE2 and 6-keto-PGF1α release induced in monocytes by LPS, 10 μg/ml. Data are mean ± SE of eight independent measures. *p < 0.01 vs LPS alone. b, Selective and nonselective COX-2 inhibitors fail to inhibit cytokine secretion induced by LPS. Data are mean ± SE of eight independent measures. *p < 0.01 vs anti-CD3/anti-CD28 alone, but not with ASA. NCX-4016 did not induce cell toxicity because only 4.0 ± 0.3% of anti-CD3/anti-CD28-stimulated lymphocytes stained positively to annexin V after exposure to 100 μM NCX-4016 compared with 2.6 ± 0.4% of control cells and 2.9 ± 0.8% of lymphocytes treated with anti-CD3/anti-CD28 alone (n = 6; p > 0.05 vs medium).

**FIGURE 5.** Effect of DETA-NO and NCX-4016 on ATP and glycolysis in stimulated T cells. Measurement of the steady state ATP levels indicated that lymphocytes (Fig. 5, a and b) and monocytes (Fig. 5, c and d) exposed
to NCX-4016 and DETA-NO (100 μM) have nearly half the level of the cells treated with anti-CD3/anti-CD28 or LPS (n = 8; p < 0.01). The ATP levels in cells treated with DETA-NO and NCX-4016 were the same as control cells. Although exposure to anti-CD3/anti-CD28 and LPS markedly increased O2 consumption, NCX-4016 and DETA-NO (Fig. 5; n = 8; p < 0.01) abrogate these changes.

As shown in Fig. 6, stimulation of lymphocytes with anti-CD3/ anti-CD28 for as little as 6 h was sufficient to produce a significant increase in glucose uptake (n = 6; p < 0.001 vs medium), while either Ab alone had no effect on the glucose uptake rate (data not shown). Addition of NCX-4016 and DETA-NO, 100 μM, resulted in a further stimulation of glucose uptake (Fig. 6a; n = 6; p < 0.05 vs CD3/CD28 alone). Stimulation of glucose uptake by CD3/CD28 costimulation correlated with increased expression of the GLUT-1 mRNA (Fig. 6, b and c; n = 6; p < 0.05 vs control cells) (4, 10). This effect was maintained in cells exposed to DETA-NO and NCX-4016, 100 μM, for 18 h (n = 6–8).

Consistent with these data, exposure of anti-CD3/anti-CD28-stimulated lymphocytes to NCX-4016 and DETA-NO resulted in a significant enhancement of the glycolytic rate, as assessed by measuring the activity of 6-phosphofructokinase (Fig. 6d; n = 6; p < 0.05 vs control cells). However, data shown in Fig. 6e demonstrated that the major product of increased glycolysis was lactate. Lactate generation was even further increased by the exposure of CD3/CD28-costimulated lymphocytes to NCX-4016 (and DETA-NO) (Fig. 6; n = 8; p < 0.05). Consistent with these changes, exposure of lymphocytes to NCX-4016 and DETA-NO decreased...
the activity of succinate dehydrogenase and aconitase, two of the enzymes involved in the citric acid cycle \((n = 8; p < 0.01)\).

Scavenging NO with Hb, 10 \(\mu\)M, fully restored the ability of lymphocytes to respond to costimulatory mAbs by increasing the rate of \(O_2\) consumption and ATP formation as well as IL-2 secretion (Fig. 7; \(n = 8; p < 0.05\) vs DETA-NO and NCX-4016). Furthermore, despite the fact that an increased energy demand was placed, coinubation with Hb reduced the rate of glucose uptake (Fig. 1b; \(n = 8; p < 0.05\) vs NCX-4016 and DETA-NO) and lactate formation (data not shown).

**Effect of DETA-NO and NCX-4016 on mitochondrial respiration**

The mechanism through which NCX-4016 and DETA-NO inhibit \(O_2\) was further investigated by analyzing the effect these agents exert on oxidative phosphorylation intermediates. Results shown in Table I demonstrate that exposure to DETA-NO and NCX-4016 inhibits the activity of complexes I, II, III, and IV. This effect was fully reversible by Hb, 10 \(\mu\)M, after 3 h of incubation with NCX-4016 and DETA-NO \((n = 8; p < 0.01\) vs cells incubated without Hb).

**Discussion**

The central finding of this study is that NCX-4016, a NO-releasing derivative of ASA, reduces ATP formation and \(O_2\) consumption in immune cells, resulting in a transitory impairment of the ability of lymphocytes and monocytes to release cytokines and chemokines in response to costimulatory molecules or bacterial endotoxin. Although NCX-4016 has two active moieties and both contribute to its pharmacological activity (22), present results indicate that modulation of mitochondrial functions exerted by NCX-4016 is due to the release of NO. Support to this view comes from the following observations: first, exposure of resting lymphocytes (and monocytes) to NCX-4016 leads to NO formation; second, NCX-4016, a non-NO-releasing analog of NCX-4016, ASA, and selective and nonselective COX-2 inhibitors reduce prostanoid generation, but fail to inhibit cytokine release induced by anti-CD3/anti-CD28 and LPS (27). In contrast, we observed a slight, although significant increase of IL-1\(\beta\) and TNF-\(\alpha\) release from monocytes incubated with ASA, suggesting that endogenous prostanoid contributes to limit cytokine/chemokine release in immune cells (30, 31); third, inhibition of cytokine release and mitochondrial perturbation caused by NCX-4016 was, at least partially, reproduced by incubating lymphocytes and monocytes with the NO donor DETA-NO; and, finally, scavenging NO with Hb greatly attenuated inhibition of cytokine secretion and mitochondrial effects of NCX-4016 and DETA-NO.

Despite the fact that, similarly to NCX-4016, DETA-NO greatly impairs ATP formation and \(O_2\) consumption in resting and CD3/CD28-costimulated lymphocytes, it failed to fully reproduce the inhibitory effect NCX-4016 exerts on cytokine secretion. Several of our results might contribute to explain these discrepancies: first, at the concentration used in this study, i.e., 100 \(\mu\)M, the amount of NO released by DETA-NO approaches that of inducible NOS (i.e., \(\approx 1\) \(\mu\)M), being significantly higher than that released by the equimolar concentration of NCX-4016 (32). Second, most likely due to the large amount of NO released, and in contrast to NCX-4016, exposure to DETA-NO causes peroxynitrite formation. Because peroxynitrites activate NF-\(\kappa\)B (33, 34), and directly stimulate cytokine secretion (33-35), it is likely that failure of DETA-NO to attenuate cytokine/chemokine secretion in monocytes is due to formation of these radical species. Third, as a consequence of peroxynitrite formation and high NO release, DETA-NO exerts biphasic effect on \(\Delta\psi_m\), i.e., an early hyperpolarization, followed by a late depolarization (16–18). Fourth, we have previously shown that, in contrast to DETA-NO, NCX-4016 at the concentration of 100 \(\mu\)M inhibits nuclear translocation of NF-\(\kappa\)B (36). Finally, NCX-4016 and DETA-NO seem to release NO with a different mechanism. Indeed, while DETA-NO releases NO spontaneously, NCX-4016 requires the intervention of cellular esterases. By confocal microscopy analysis, we have previously shown that NO formation in cells exposed to DETA-NO associates

**FIGURE 7.** a–d. NO scavenging with Hb reverses the effects of DETA-NO and NCX-4016 on \(O_2\) (a), glucose uptake (b), ATP levels (c), and IL-2 secretion (d). Data are mean \pm\ SE of eight independent measures. *\(p < 0.01\) vs medium; **\(p < 0.01\) vs anti-CD3/anti-CD28; \(\psi\), \(p < 0.05\) vs DETA-NO and NCX-4016 alone.
with the appearance of diffuse cytosolic fluorescence, while NCX-4016 induces a characteristic pattern of hot spots of activity localized at specific subcellular compartments near to the plasma membrane (26). Although the pharmacological relevance of this observation remains unclear, it suggests that NCX-4016 releases low amount of NO with a different kinetic of DETA-NO.

NO is increasingly recognized as a physiological regulator of O2 use (13, 14, 37). At least two components of the mitochondrial respiratory chain have been shown to interact with NO, resulting in a profound inhibition of O2 consumption (15, 37). Clementi et al. (16) have shown that at low cellular concentrations, 1–10 μM, NO donors interact in a reversible manner with cytochrome c oxidase (complex IV), causing a transitory inhibition of cell respiration and oxidative phosphorylation. In cells exposed to higher concentrations of a NO donor (50–100 μM), inhibition of cell respiration becomes irreversible and appears largely dependent on the S-nitrosylation/inhibition of complex I (16, 37). Although O2 consumption by mitochondria is mainly regulated by cellular ATP levels, interaction of O2 with NO might lead to a situation in which, even in the presence of an adequate supply of O2, cytochrome c oxidase exerts a considerable control over the rate of respiration through changes in its affinity for O2 (15, 37). In this situation, NO might generate a situation of metabolic hypoxia in which the available O2 cannot be adequately used by cells (14). Because O2 consumption is essential for oxidative phosphorylation, the major metabolic consequence of these interactions will be a reduction of oxidative breakdown of glucose, leading to further limitation of ATP formation in immune cells (1–3). Indeed, the rate of O2 consumption required to maintain ATP production rate is significantly higher than those required for inhibition of the activity of cytochrome c oxidase (~60 nM) (14, 18, 42).

Arachidonic acid and its metabolites are considered to be important regulators of facilitated glucose transport in insulin-sensitive cells such as adipocytes (43). In these cells, arachidonic acid stimulates glucose uptake and GLUT-1 membrane incorporation (43, 44). However, the finding that ASA and NCX-4017 do not modulate GLUT-1 expression in anti-CD3/anti-CD28-stimulated lymphocytes suggests that COX inhibition is not involved in the effect exerted by NCX-4016. Supporting this view, it has previously been demonstrated that facilitation of glucose transport induced by arachidonic acid is COX independent, while it involves lipoxygenase and peroxisome proliferator-activated receptor-γ pathways (44).

The results of present study might have clinical relevance. Indeed, an increased rate of O2 consumption has been described in PMBC of patients with systemic lupus erythematosus (45) and active rheumatic diseases (46). Because high levels of ATP are required to sustain energy consuming by immune processes, it seems likely that interfering with lymphocyte’s bioenergetic might help to reduce inflammatory reaction. Consistent with this view, it has been shown that antirheumatic/immunosuppressive drugs such as chloroquine (47), auranofin (48), glucorticoid (45, 48), and cyclosporine (49) inhibit mitochondrial respiration and/or decrease ATP formation in immune cells (1–3).

In conclusion, this study suggests that NO might exert a regulatory function on lymphocytes and monocytes, providing a potential counterregulatory signal in communication between the innate and acquired immunity. The demonstration that NCX-4016 inhibits immune cell bioenergetic suggests that NO-releasing NSAIDs have the potential to exert anti-inflammatory effects that extend behind COX inhibition.

References