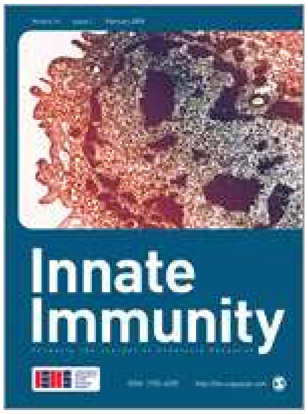


Lactoferrin decreases LPS-induced mitochondrial dysfunction in cultured cells and in animal endotoxemia model

Journal:	*Innate Immunity*
Manuscript ID:	INI-09-0003.R1
Manuscript Type:	Original Manuscript
Date Submitted by the Author:	
Complete List of Authors:	Kruzel, Marian Actor, Jeffrey Radak, Zsolt Bacsi, Attila Saavedra-Molina, Alfredo Bolodgh, Istvan
Keywords:	SIRS, sepsis, lactoferrin, LPS, oxidative stress, mitochondria, DNA damage
Abstract:	Lactoferrin (LF) is a non-heme iron-binding glycoprotein, produced by mucosal epithelial cells and granulocytes in most mammalian species. It is involved in regulation of immune responses, possesses anti-oxidant, anti-carcinogenic, anti-inflammatory properties, and provides protection against various microbial infections. In addition, LF has been implicated in protection against the development and progression of insult-induced systemic inflammatory response syndrome (SIRS) into septic conditions in vivo. Here we show a potential mechanism by which LF lessens oxidative insult at cellular and tissue levels after lipopolysaccharide (LPS)-exposure. LF pre-treatment of cells decreased LPS-mediated oxidative insults in a dose-dependent manner. LPS-induced oxidative burst was found to be of mitochondrial origin, and release of reactive oxygen species (ROS) was localized to the respiratory

	complex III. Importantly, LF nearly abolished LPS-induced increases in mitochondrial ROS generation and accumulation of oxidative damage to DNA. In vivo, pretreatment of experimental animals with LF significantly ($p < 0.05$) lowered LPS-induced mitochondrial dysfunction as shown by both decreased release of H_2O_2 and DNA damage in the mitochondria. In contrast, deferoxamine, an iron chelating compound, provided only partial protection in LPS-treated animals. Together these data suggest that LF protects against oxidative insult at the mitochondria level, and indicate a potential utility of LF in prevention and treatment of SIRS.

Lactoferrin decreases LPS-induced mitochondrial dysfunction in cultured cells and in animal endotoxemia model

Marian L. Kruzel[1], Jeffrey K. Actor[1], Zsolt Radak[3], Attila Bacsi[2], Alfredo Saavedra-Molina[4], Istvan Boldogh[2]

[1]The University of Texas, Health Science Center at Houston, Texas, USA, [1]University of Texas Medical Branch at Galveston, Texas, USA, [2]School of Medicine, University of Debrecen, Debrecen Hungary, [3]Institute of Sport Science, Faculty of Physical Education and Sport Science, Semmelweis University, Budapest, Hungary, [4]Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, México

Running title: Prevention by lactoferrin of oxidative stress and DNA damage

Key words: Lactoferrin, oxidative stress, mitochondria, DNA damage

Correspondence:
Istvan Boldogh, Ph.D.
Department of Microbiology and Immunology
University of Texas Medical Branch,
Galveston, Texas 77555-1077
Telephone: (409) 772-9414
Fax: (409) 747-6869
Email: sboldogh@utmb.edu

ABSTRACT

Lactoferrin (LF) is a non-heme iron-binding glycoprotein, produced by mucosal epithelial cells and granulocytes in most mammalian species. It is involved in regulation of immune responses, possesses anti-oxidant, anti-carcinogenic, anti-inflammatory properties, and provides protection against various microbial infections. In addition, LF has been implicated in protection against the development and progression of insult-induced systemic inflammatory response syndrome (SIRS) into septic conditions *in vivo*. Here we show a potential mechanism by which LF lessens oxidative insult at cellular and tissue levels after lipopolysaccharide (LPS)-exposure. Lactoferrin pre-treatment of cells decreased LPS-mediated oxidative insults in a dose-dependent manner. Lipopolysaccharide-induced oxidative burst was found to be of mitochondrial origin, and release of reactive oxygen species (ROS) was localized to the respiratory complex III. Importantly, LF nearly abolished LPS-induced increases in mitochondrial ROS generation and accumulation of oxidative damage to DNA. *In vivo*, pretreatment of experimental animals with LF significantly ($p < 0.05$) lowered LPS-induced mitochondrial dysfunction as shown by both decreased release of H_2O_2 and DNA damage in the mitochondria. In contrast, deferoxamine, an iron chelating compound, provided only partial protection in LPS-treated animals. Together these data suggest that LF protects against oxidative insult at the mitochondria level, and indicate a potential utility of LF in prevention and treatment of SIRS.

Abbreviations: 3-NPA, 3-nitropropionic acid: AA, Antimycin A: H_2Et; dihydryethidium; DFO, deferoxamine; LF, lactorferrin; LPS, lipopolysaccharide; $O_2^{\cdot -}$, superoxide anion; ROS, reactive oxygen species; Rot, Rotenone; SDH; succinate dehydrogenase; SOD, superoxide dismutase; Stigm, Stimatellin;

INTRODUCTION

Lactoferrin (LF) is an 80 kD, non-heme iron-binding glycoprotein that belongs to the transferrin family [1]. It is found in most mucosal sites and secondary granules of neutrophils in mammals [2-4]. Several functions have been attributed to LF as a key component in the host's first line of defense, contributing to a variety of physiological changes at both the cellular and organ level [4,5]. Lactoferrin is a major pleiotropic mediator that plays an important role in the development of inflammatory responses [6-8]. It was recently demonstrated that LF, has the ability to inhibit progression of systemic inflammatory response syndrome (SIRS) into sepsis in endotoxemic mice [9,10]. Lactoferrin is well documented as having direct antimicrobial activity, including an iron-dependent bacteriostatic property and a non-iron-dependent bactericidal action on LPS-bearing Gram-negative bacteria [11,12]. While suppressing microbial growth, LF also exerts its first-line defense activity with significant impact on the development of adaptive immune responses [13]. Sequestration of iron by LF not only inhibits microbial growth but also reduces oxidative stress. In addition, LF induces very potent anti-allergic responses in animals challenged with ragweed pollen and dust mite proteins as shown by decreased accumulation of inflammatory cells in airways and subepithelium of sensitized experimental animals [14]. Results also show that LF decreased pollen-induced cellular ROS levels in bronchial epithelial cells and prevented both development of mucin producing cells as well as levels of mucin production [14].

There is growing evidence showing that progression of systemic inflammatory response syndrome into sepsis is due to the cellular damage and death induced by acute inflammatory responses. The cell death depends in part upon mitochondrial dysfunction, which is often characterized by increased production of reactive oxygen species (ROS), increased membrane permeability and eventual release of cell death mediators from mitochondria [15]. Extensive mitochondrial damage leads to loss of cellular ATP pools, which can be linked to necrotic cell death, to further inflammatory responses. Consequently, mitochondrial dysfunction contributes to a wide range of human pathologies including SIRS and sepsis. It has been suggested that therapies aimed at improving cellular redox state and energy production, may be applicable for treatment of sepsis [16].

The goal of this study was to show that LF modulates lipopolysaccharide (LPS)-induced changes in cellular redox state and identify potential subcellular targets of LF. Here we report for the first time that LF protects against oxidative stress-induced mitochondrial dysfunction and DNA damage, both in cell culture and within an animal model of endotoxemia. Data derived from these studies provide an on-going mechanistic understanding by which LF decreases intensity of LPS-induced systemic inflammation. These results are also in support of LF potential therapeutic utility for treatment of SIRS and sepsis.

MATERIALS AND METHODS

Cell cultures

The AML12, nontumorigenic parenchymal liver cells with features characteristic of matured hepatocytes were cultured in Dulbecco's minimal essential medium (DMEM)-Ham's F-12 containing ITS solution and 0.1 nM dexamethasone. The U937 (established from histiocytic lymphoma and displaying many monocytic characteristics) cells were maintained in RPMI-1640. A549 bronchial epithelial cells were cultured in Ham's F-12 medium. All cell lines were obtained from American Type Culture Collection and they are periodically tested for mycoplasma contamination. All media (Invitrogen, Carlsbad, CA) were supplemented with 2 mM L-glutamine, 1.0 mM sodium pyruvate, 100 units/ml penicillin, 100 µg/ml streptomycin (Sigma Aldrich, St. Louis, MO) and 10% fetal bovine serum (FBS, Atlanta Biological),. The cells were routinely subcultured using trypsin-EDTA and incubated under a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Animals and treatment

BALB/c mice were purchased from Harlan Sprague-Dawley (San Diego, CA, USA). All animal experiments were performed according to the National Institutes of Health Guide for Care and Use of Experimental Animals and approved by UTMB Animal Care and Use Committee (# 0807042). To investigate the effects of LF on mitochondrial dysfunction and DNA damage induced by LPS were determined using previously established treatment and animal model [8]. Briefly, 9 to 11 mice (8-10 weeks old, 18 to 20 g per mouse) per group were given 5 mg LF per mouse intra peritoneally (ip). Twelve h later LPS (2.5 mg per body weight kg) was added via intra peritoneal route. Control groups of animals received only LF, LPS or solvent injections.

Reagents

Low endotoxin human milk lactoferrin (< 0.2 E.U. $mg^{-1}$by LAL assay; < 20% iron saturated, >95% purity) was provided by PharmaReveiw Corporation, Houston, TX. Rotenone (R-8875), cytochrome c (C-3131), decylubiquinone (D7911), antimycin A (A8674), oligomycin (O-4876), pyruvate, malate, succinate, 3-nitropropionic acid, catalase were purchased from

Sigma Aldrich (St. Louis, MO). 2',7'-dichlorodihydro-fluorescein diacetate; dihydroethidium, MitoTracker Red and Amplex® Red (10-acetyl-3,7-dihydroxyphenoxazine are from Molecular Probes. Lipopolysaccharide from *E. coli* serotype O111:B4 (3×10^6 E.U. mg^{-1}) was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Establishment of respiration-deficient cells

Mitochondrial DNA-deficient cells were developed as we described previously [17]. Both cell cultures AML-12 and A549 were maintained in the presence of 100 ng/ml ethidium bromide for >60 population doublings. Depletion of mitochondrial DNA (mtDNA) was confirmed by Southern blot hybridization [17,18]. Respiration-deficient cells became pyrimidine auxotrophs, and media were supplemented with uridine (50 µg/ml) and sodium pyruvate (120 µg/ml) [19]. For verification of the absence of mtDNA in p^0MH-S cells, DNA isolated, treated with DNase-free RNase then digested with *Bam*HI. After the gel electrophoresis DNA was transferred onto nitrocellulose membrane (Schleicher and Schuell BioScience, Keene, NH), blocked and hybridized with a PCR-generated DNA probe for the mitochondrial genome. The forward and reverse primer sequences were as follow: 5'-GCAGGAACAGGATGAACAGTCT-3' and 5'-GTATCGTGAAGCACGATGTCAAGGG ATGTAT-3', respectively. The 725-bp product recognized a 10.8-kb restriction fragment when hybridized to MH-S mtDNA digested with *Bam*HI as described previously [17,18].

Mitochondria isolation

Mitochondria were isolated from mock and LPS-treated cells as we described previously [17]. Briefly, cell pellets were incubated in 10X volume of hypotonic buffer (10 mM KCl, 20 mM MOPS, and 1 mM EGTA (ethylene glycol-bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid) for 20 min then Dounce-homogenized. The homogenate was centrifuged at 800 × *g* and the supernatants re-centrifuged at 10,000 × *g* to collect mitochondria. Mitochondrial pellets were washed, and resuspended in 10 mM KCl, 20 mM MOPS, and 1 mM EGTA containing 200 mM sucrose and 50 mM mannitol. In selected experiments, fresh mitochondrial suspensions were purified on a continuous sucrose gradient (0.25 M to 1.5 M).

Mitochondria were isolated from the livers of 4-6 months old Balb/c mice under anesthesia with pentobarbital (100 mg/kg i.p.) in compliance with the UTMB's Animal Care and Use Committee-approved protocol. Organs of sacrificed animals were excised and rinsed in buffer A (100 mM KCl, 20 mM MOPS, 1 mM EGTA, 5 mM $MgSO_4$, and 1 mM ATP; pH 7.6) at 4°C. Livers were homogenized in buffer A, containing 200 mM sucrose, 50 mM mannitol, 0.2% bovine serum albumin, using a Dounce homogenizer. Isolation of mitochondria was done as described above. Fresh mitochondrial suspensions from cultured cells or organs were purified on a continuous sucrose gradient (0.1M to 1.5M) and used immediately for determining the site(s) of superoxide anion formation or stored at –80 °C for further studies.

Measurement of mitochondrial and intracellular ROS

The intracellular site of ROS generation was identified by fluorescence microscopy [17,20]. Cells were loaded with 2 µM dihydroethidium (H_2Et; Molecular Probes, Eugene, OR) for 10 min after which the cells were treated with 100 µg/ml LPS (pH 7.4) and placed in a thermo-controlled microscopic chamber. MitoTracker Red (Molecular Probes, Eugene, OR) a cell-permeable fluorescent probe that accumulates in active mitochondria was used to stain mitochondria at a final concentration of 10 nM. Fluorescent images were captured just after 60 min incubation with LPS using a Zeiss LSM510 META System driven by Metamorph™ Version 6.09 software (Universal Imaging, Downingtown, Pennsylvania).

A redox-sensitive probe, 2',7'-dichlorodihydro-fluorescein diacetate (H_2DCF-DA; Molecular Probes), was used to determine changes in overall cellular ROS levels [20,21]. Mock- or LPS-treated cell suspensions were loaded with 2', 7'-Dichlorodihydro-fluorescein diacetate (H_2DCF-DA; Molecular Probes, Eugene, OR) at 5 µM concentration for 15 min at 37°C. The change in fluorescence (excitation 485 nm; emission 530 nm) was measured using a FLX800 microplate reader (Bio-Tek Instruments, Winooski, VT, USA). In confirmatory studies changes in DCF fluorescence of LPS-treated versus mock-treated cells were determined by FACSaria (Becton Dickinson, Mountain View, CA). Each data point represents the mean fluorescence for 15,000 cells, from three or more independent experiments.

8-OxoG assays

The 8-oxoG in nuclear DNA was quantified as we previously described [22]. Briefly, cells on microscope slides were air-dried, and fixed in acetone-methanol (1:1), rehydrated in PBS for 15 min, then sequentially treated with 100 µg/ml pepsin in 0.1 N HCl for 15 to 30 min at 37°C, 1.5 N HCl for 15 min, and sodium borate for 5 min. The cells were incubated with nonimmune IgG (100 µg/ml) for 30 min and washed in PBS containing 0.5% bovine serum albumin, 0.1% Tween 20 (PBS-T). After incubation with anti-8-oxoG antibody (Trevigen, Gaithersburg, MD; 1:200 dilutions) [23] for 30 min, the cells were washed three times with PBS-T for 15 min then exposed to fluorescein-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 60 min. Cells were washed with PBS-T for 15 min (3-times) and their DNA stained with DAPI (10 ng/ml) for 15 min. The cells were air dried and mounted in anti-fade medium (Dako North America, Inc., Carpinteria, CA) on a microscope slide. The fluorescence intensities of a minimum of 40 cells per plate were determined using a Zeiss LSM510 META system, operated via MetaMorph software version 6.06r (Universal Imaging Corporation, Downingtown, PA).

Oxygen consumption

The oxygen consumption rates of mitochondria were determined at 30°C with a Clark-type oxygen electrode (Strathkelvin Oxygen System Model 782, Strathkelvin Instruments, United Kingdom) as we previously described [17,24]. Purified mitochondria (0.2 mg) were suspended in 1 ml respiration medium (125 mM KCl, 20 mM HEPES; pH 7.4), 5 mM potassium phosphate, ±substrates (5 mM pyruvate plus 5 mM malate as well as 10 mM succinate). The signal from the oxygen sensor was recorded on a computer at sampling intervals of 0.5 seconds with the aid of software from Strathkelvin Instruments (782 System V3.0). Respiration was measured without ADP (state IV) and with 0.5 mM ADP (state III) [17]. Mitochondria suspensions showing higher than 3 respiratory control ratios were used in Amplex red assays.

Amplex Red assay

Amplex® Red (10-acetyl-3,7-dihydroxyphenoxazine; Molecular Probes, Eugene, OR) reacts with H_2O_2 in the presence of horseradish peroxidase (HRP) to generate a stable product, resorufin [25]. Briefly, mitochondria (100 µg/ml) were suspended in 100 µl (per well) reaction buffer and incubated at room temperature (25°C) for 30 min with 0.25 U per ml (determined in preliminary studies) of Amplex® Red and 0.5 U/ml of HRP [17,24]. The increase in fluorescence (with excitation and emission wavelengths of 563 and 587 nm, respectively) was measured using a microplate reader (SpectraMass M2, Molecular Devices Inc). The rate of H_2O_2 production was linear with mitochondrial protein concentration. Reactions were carried out with exogenously added superoxide dismutase (SOD). The addition of catalase (400 U/ml, Sigma Chemical Co., St. Louis, MO, USA) decreased H_2O_2 levels by ~90 percent. As a positive control, increasing concentrations of H_2O_2 (0 to 400 pmol) were used.

Estimation of mitochondrial and nuclear DNA damage

About 3 × 10^6 cells (U937) were plated and treated with LPS (100 ng per ml). DNA was extracted using a genomic DNA extraction kit (Qiagen, Chatsworth, VA) using the protocol supplied with the kit (Qiagen, Chatsworth). DNA concentration was determined using the PicoGreen® dsDNA Quantitation Kit (Molecular Probes). Free PicoGreen dye is essentially nonfluorescent and exhibits >1000-fold fluorescence enhancement upon binding to dsDNA at an excitation and emission wavelength of 480 and 530, respectively. The assay displays a linear correlation between dsDNA quantity and fluorescence, being extremely sensitive (detection range extending from 25 pg/ml to 1 µg/ml). Q-PCR was performed using a protocol described previously [26,27], and the quantitation of PCR products was done using PicoGreen dye as we previously described [28].

The primer nucleotide sequences for U937 were as follows: for the 17.7-kb 5′ flanking region of the β-globin gene 5′-TTGAGACGCATGAGACGTGCAG-3′ (forward), and 5′-GCACTGGCTTAGGAGTTGGACT-3′ (reverse) and for the 16.2-kb fragment of the mitochondrial genome, 5′-TGAGGCCAAATATCATTCTGAGGGGC-3′ (forward) and 5′-TTTCATCATGCGGAGATGTTGGATGG-3′ (reverse) [29].

The primer nucleotide sequences for AML-12 were as follows for 7.2 kb fragment of b-globin gene 5’-GGA GCA AGG TCC AGG GTG AAG AA-3’ (forward) and 5’-TTT GCA TCC AGA TCA TGG TCC CT-3’ (reverse). The 10.4 kb mitochondrial fragment 5’-GCC AGC CTG ACC CAT AGC CAT AAT AT-3’ (forward) and 5’-GAT GGT TTG GGA GAT TGG TTG ATG T-3’ (reverse) [30]. The PCR was initiated with a 75°C hot-start addition of the polymerase and allowed to undergo the following thermocycler profile: an initial denaturation for 1 min at 94°C followed by 25 cycles of 94°C denaturation for 15 sec and 68°C primer extension for 12 min. A final extension at 72°C was performed for 10 min at the completion of the profile. To ensure quantitative conditions, a control reaction containing 7.5 ng of template DNA were included in amplification reactions. For quality control and specificity of primers an aliquot of each PCR product was resolved on a 1% agarose gel and electrophoresed in TBE (90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3) at 80 V (5 V/cm) for 4 h. DNA lesion frequencies were calculated as described previously [28,31]. Briefly, the amplification of damaged samples (A_D) was normalized to the amplification of a nondamaged control (A_O), resulting in a relative amplification ratio. Assuming a random distribution of lesions and using the Poisson equation --$fx = e^{-\lambda}\lambda^x/x$, where λ = the average lesion frequency for the nondamaged template (i.e., the zero class; $x = 0$), the average lesion per DNA strand was determined as: $\lambda = -\ln A_D/A_0$.

Statistical analysis

Results were analyzed for significant differences using ANOVA procedures and Student's t-tests (Sigma Plot 6.0). Data are expressed as the mean ±SE. Results were considered significant at $p<0.05$. (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$).

RESULTS

Effect of LF on LPS-induced cellular ROS levels

To investigate the changes in cellular ROS levels, U937 (and AML12) cells were treated with increasing concentrations of LPS and 45 min later cells were loaded with H_2DCF-DA (50 µM, for 15 min). The changes in LPS-induced ROS levels were dose dependent from 25 ng/ml to 100 ng/ml, higher doses (200, 400 and 800 ng/ml) did not further increase fluorescent levels (Fig. 1A). Similar results were obtained using both U937 (Fig. 1A) and AML12 cells (Fig. 1A inset). In additional studies, LPS was used at a concentration of 100 ng/ml. As shown in Fig. 1B, LPS induces a biphasic increase in ROS levels. The first wave of ROS was observed at ~1 h and another was seen ~6 h after LPS addition (Fig 1B). Treatment of cells with H_2O_2 (50 µM) or glucose oxidase (GO; 100 ng/ml), an enzymatic oxidative stress generator [20], resulted in a single wave change in DCF fluorescence (Fig. 1B, inset).

There are number of hypothesis for LF' antioxidant properties; however, the most obvious one is linked to its iron sequestering capability [32]. To test LF' antioxidant potential, cells (U937, AML12) were pre-treated for 3 h with increasing concentrations of LF, and 100 ng/ml LPS was then added. LF, beginning at 8 µg/ml and at higher concentrations, significantly ($p<0.05$) decreased LPS-induced ROS levels; 64 and 128 µg/ml were the most effective doses for use in U937 cells (Fig. 2A). Similar results were obtained for AML12 cells (data not shown). In controls, 64 µg/ml LF also decreased H_2O_2 and GO-induced increase in ROS levels in both U937 (not shown) and AML12 (Fig. 2A, inset). Deferroxamine (DFO), an iron sequestering compound [33] decreased LPS-induced ROS levels but not significantly (Fig. 2B). Substantial decrease by DFO in ROS levels were obtained after H_2O_2 and GO-treatment of cells as shown previously [14]. The level of DCF-mediated fluorescence was partially decreased when cells were pre-incubated with diphenyleneiodonium (DPI, Fig. 2B), a NADPH oxidase inhibitor [34]. To test if LPS-induced increase in DCF signal was specific for ROS, antioxidants were used. It is noteworthy that 64 µg/ml LF decreased ROS-mediated DCF fluorescence similar to *N*-acetyl-L-cysteine (NAC, 10 mM, Fig. 2A,B), an antioxidant and a free radical-scavenging agent that increases intracellular GSH [35] and Ebselen (10 µg per ml) a thioredoxin reductase-dependent catalyst for alpha-tocopherol quinone reduction in the mitochondria [36].

Lipopolysaccharide increases cellular ROS levels via mitochondria

Lipopolysaccharide increases intracellular levels of ROS, via NADPH oxidases in macrophages and mitochondria in neutrophils [37,38], however, there are no reports for these effects in monocytic cells (U937) or hepatocytes (AML12). To identify the intracellular site of ROS production, AML12 cells were LPS-treated at 50 % confluence, and then loaded thirty minutes later with dihydroethidium (2 μM) for 15 min and changes in fluorescence were recorded by microscopy. The green fluorescence mediated by dihydroethidium+superoxide reaction products [39] was co-localized with MitoTracker Red suggesting that the mitochondria are the primary sites of ROS generation (Fig. 3A). Intensity of fluorescence by dihydroethidium+superoxide reaction products in LF or NAC pre-treated-LPS-exposed cells was decreased nearly to levels seen in mock-treated controls cells. Similar intensity of mitochondria-mediated dihydroethidium+superoxide fluorescence was also observed in U937 cells (data not shown).

To test further whether or not LPS increases cellular ROS levels from mitochondria we utilized mtDNA depleted *p0*AML-12 and *p0*U937 cells [17]. When *p0* cells were exposed to LPS, increase in ROS levels were significantly ($p< 0.05$) lower compared to wild type cells (Fig. 3B). In control experiments, addition of H_2O_2 to *p0* cells increased DCF-signals similar to wild type cells. Addition of DPI, nearly abolished LPS-induced increase in ROS levels in mtDNA depleted cells (Fig. 3B, inset). On the other hand, DPI only partial inhibited LPS-induced ROS generation in wild type cells (Fig. 2B). Together these data suggest that mitochondria are the primary source of ROS generation, in LPS-treated cells.

Decreased mitochondrial release of H_2O_2 by LF

Next, mitochondria were isolated from ±LPS-exposed cells to determine the amount of organelle specific H_2O_2 (originating from $O_2^{.-}$ dismutation by superoxide dismutase) released. First, LF was investigated to determine its effect on LPS-induced release of H_2O_2; and second, experiments were performed to define the site of ROS generation in the mitochondrial respiratory chain. Mitochondria from LPS-treated AML-12 (and U937 cells; data not shown) cells release approximately 3-times more H_2O_2 (compared to mitochondria from mock-treated cells) (Fig. 4A). When LPS-exposed cultures were pre-treated with LF, mitochondrial release of H_2O_2 was not significantly different from those of mock-treated cells (Fig. 4A). DFO did not significantly decrease H_2O_2 levels in mitochondrial isolated from LPS-treated cells (Fig. 4A).

To identify the site of ROS generation, specific inhibitors of electron transport chain (located in the respiratory complexes) were applied to mitochondrial suspensions respiring from pyruvate/malate plus succinate (complex I and II substrates, respectively). Both Rotenone (an inhibitor of NADH-decylubiquinone reductase activity of complex I) and 3-NPA (an inhibitor of succinate dehydrogenase), individually decreased while together they lowered LPS induced H_2O_2 release nearly to background levels (Fig. 4B). Antimycin A (an inhibitor of cytochrome *b* reoxidation in complex III) significantly ($p< 0.05$) increased H_2O_2 release when added to mitochondria from LPS-exposed cells. Stigmatellin, an inhibitor of entry of electrons into complex III, inhibited H_2O_2 production from mitochondria isolated from LPS-exposed cells, when used at a concentration of 0.6 μM. Stigmatellin also showed inhibition when antimycin A was added to mitochondria from LPS-exposed cells (Fig. 4B). Addition of catalase decreased H_2O_2 to an undetectable levels under our assay conditions (Fig. 4B). Although additional studies are required to precisely identify the primary site of LPS-induced mitochondrial ROS generation these result indicate that respiratory complex III is the major site of superoxide anion generation.

To obtain information if mitochondria-generated ROS can diffuse into other subcellular compartments, such as the nucleus, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxoG) levels were determined in nuclear DNA as a marker for oxidative cellular stress [40]. AML-12 cells were pre-treated with LF (for 3h) and subjected to oxidative stress by addition of LPS or H_2O_2. Data showing that LPS, in a similar manner to H_2O_2, increases 8-oxoG levels in nuclear DNA (Fig. 5) inline with their pro-oxidant capacity shown in DCF assays. Importantly, in the presence of LF a significant ($p<0.05$) decrease in 8-oxoG levels was observed. As a control, NAC (3 h pre-treatment prior to LPS or H_2O_2 addition) decreased 8-oxoG formation (Fig. 5), and DFO significantly decreased 8-oxoG levels only in H_2O_2-treated cells (Fig. 5). These data provide additional support for the specific nature of LPS-mediated ROS generation and help to clarify LF antioxidant properties (as shown in DCF assays).

LF attenuates mitochondrial dysfunction in liver of LPS-treated mice

Next, we sought to determine whether the observed protection of mitochondrial dysfunction by LF occurs in LPS-treated animals. Balb/c mice were LF (or mock)-treated (for 12 h) and then injected with LPS as previously described [10]. Twelve h later, livers were excised and mitochondria were isolated. As shown in Fig. 6A, mitochondria from LPS-treated animals released significantly ($p<0.05$) higher amounts of H_2O_2 than those isolated from LF only or saline-injected animals. Importantly, mitochondria from LF-pre-treated plus LPS-challenged animals produced significantly ($p<0.05$) less H_2O_2. In addition, mitochondria isolated from heart, skeletal muscle, and brain of LF-pretreated-LPS-treated mice released significantly lower amounts of H_2O_2 compared to mitochondria isolated from LPS-challenged mice in the absence of LF pretreatment (Fig. 6B). When animals were injected with DFO (5 mg per mouse), only partial reduction in H_2O_2 levels released were observed from the mitochondria isolated from LPS-challenged animals (Fig. 6A). On the other hand, NAC significantly inhibited mitochondrial H_2O_2 release ($p<0.05$). Together these results strongly suggest that LF attenuates mitochondrial dysfunction *in vivo* and support the hypothesis that cellular effects of LF are more complex than those based on iron sequestration.

Low levels of LPS-mediated oxidative damage to mtDNA in the presence of LF

The release of H_2O_2 to the extra-mitochondrial space is a good indication for mitochondrial dysfunction; however, it does not provide evidence for release of ROS into mitochondrial matrix where mtDNAs reside. Therefore, the changes in mtDNA damage levels were further investigated by quantitative PCR, utilizing AML-12 (and U937) cells treated with LPS. As shown in Fig. 7A, the PCR-amplifiable levels of mtDNA are decreased significantly ($p< 0.05$) in LPS treated U937 cells. Damage to a nuclear (β-globin) gene was used as control. LPS-treatment significantly ($p< 0.05$) increased levels of both mtDNA and nuclear DNA damage. Time course experiments showed that mtDNA damage persisted for longer time period compared to damage to control β-globin gene sequences (Fig. 7A). Persistence of mtDNA damage is consistent with sustained ROS generation, as shown in the DCF assays above (Fig. 1B). Remarkably, levels of damaged DNAs were less in LF-pre-treated cells. While NAC significantly ($p<0.05$) lowered damage to DNA, DFO pre-treatment of cells had detectable but not significant effects on mtDNA damage after LPS addition (Fig. 7B).

Next, the changes in levels of mtDNA damage were determined in liver of animals treated with LPS and/or LF. As shown in Fig. 7C, at 12 h after LPS administration the levels of undamaged mtDNA decreased in the liver compared to time 0. These results are inline with levels of released H_2O_2 (Fig. 4). Lactoferrin, DFO, or NAC alone did not change mtDNA levels during the pre-treatment period (Fig. 7C) thus it was similar to saline-injected control animals (data not shown). However, quite strikingly, LF was able to increase levels of intact (non-damaged) mtDNA in the LPS-treated mice (Fig. 7C). While NAC showed significant protection, treatment of mice with DFO only partially decreased mtDNA damage after LPS challenge (Fig. 7C)**.** Together, these result show that LF decreases mitochondrial ROS generation, and has the potential to lower LPS-mediated damage to mtDNA. Because DFO did not provide significant protection, this suggests that iron sequestration itself is not the major mechanism by which LF protects mtDNA. These data also imply that ROS generated by the respiratory complexes are released both to the extra mitochondrial space and mitochondrial matrix, where they can overwhelm existing antioxidant oxidant machineries.

DISCUSSION

Lactoferrin is a natural immunomodulator and antimicrobial agent. It is also known to reduce toxic effects of LPS by both direct and indirect mechanisms *in vivo*. As such LF is considered for a therapeutic use in systemic inflammatory response syndromes (reviewed in [4]. Data supporting the potential clinical utility of LF are overwhelming; however, the exact cellular mechanism(s) by which LF demonstrates preventive and/or therapeutic potential is not yet known. Here we demonstrate that LF decreases intracellular oxidative stress levels induced by LPS via reduced mitochondrial dysfunction in cultured cells. Importantly, LF attenuated mitochondrial dysfunction in liver, and other organs of LPS-treated animals, as demonstrated by reduced release of H_2O_2 from mitochondria and significantly lower mitochondrial DNA damage. In contrast, deferoxamine, an iron chelator, provided only marginal protection of mitochondria after LPS treatment. Thus, LF protects against oxidative insults at the cellular level via a more complex mechanism than simple iron sequestration.

There is growing evidence to show that progression of systemic inflammatory response syndrome into sepsis is due to cellular damage and death induced by acute inflammatory responses. Both apoptotic and necrotic cell death are tightly associated with mitochondrial dysfunction, often characterized by increased production of ROS, increased membrane permeability, loss of mitochondrion integrity and alterations in cellular ATP levels [15]. Mitochondrial dysfunction and attendant bioenergetic defects are indeed increasingly becoming recognized as important role players in both chronic and acute disorders, including sepsis [41]. The results presented here revealed that pre-treatment of cultured cells and animals with LF led to decreased LPS-induced elevation in ROS levels, lessened damage to nuclear and mtDNA, and overall sustained protection against mitochondrial dysfunction. These findings are supported by previous outcomes demonstrating protective capabilities of LF in experimental mice treated with lethal doses of bacteria or LPS [9]. In these studies prophylactic or therapeutic administration of LF significantly decreased levels of proinflammatory mediators (TNF-α, IL-6 and IL-10) and nitric oxide levels after LPS injection [10].

Evidence supports that LPS-induced mitochondrial dysfunction is linked to mtDNA depletion and results in inhibition of mitochondrial transcription, although the precise molecular mechanism for this function has not yet been clearly established [38,42,43]. In our studies, cultured U937 (cells of monocytic origin) and AML12 cells (immortalized hepatocytes) were used as an *in vitro* link to identify potential liver related dysfunction. The later was important relative to findings identified within livers of experimental animals. In vitro, a consistent biphasic increase in ROS levels was observed. We propose that the second ROS wave is a consequence of initial oxidative insult/damage to mitochondrial respiratory complex proteins and mtDNA after LPS exposure. Ebselen, an antioxidant that exerts a primary effect within the mitochondria [36], decreased the level of initial oxidative insult, and LPS did not induce a second stress wave although it remained present in the medium. Data generated by the use of mitochondrial depleted *p0* cells further support the mitochondrial origin of cellular ROS. mtDNA depleted *p0* cells maintain mitochondrial membrane potential via utilization of ATP generated by glycolysis to generate low levels of ROS [44,45]; thus these cells respond poorly to agents that induce mitochondrial ROS via receptor mediated signal transduction pathways (such as LPS). Indeed, in our *p0* cells, LPS-induced ROS levels were significantly lower than in wild type cells, indicating that the majority of ROS are generated via mitochondria after LPS exposure.

In an attempt to identify the mitochondrial site of ROS generation, it was shown here that the initial oxidative insult did not have detectable effect on function of respiratory complex I and II and electron transport to ubiquinol pool. Specifically, rotenone (inhibit ubiquinone reduction site of complex I, while its NADH oxidoreductase activity remain unaffected, [46]) and 3-NPA (a succinate dehydrogenase inhibitor, [47]) individually only partially decreased speroxide levels, while together they lowered release of H_2O_2 from mitochondria to nearly background levels. These observations imply that electron flow into respiratory complex III is required for ROS generation. In support, stigmatellin (acts at the QO center of the bc1 complex, binds to the heme b-566 domain of cytochrome b as well as to the Fe2-S2 protein in respiratory complex III) [48], alone significantly lowered H_2O_2 release from mitochondria in LPS-treated cells. Antimycin A (binds to matrix side of complex III and inhibit the Qi site of cytochrome c oxidoreductase, in the cytochrome b subunit; [49]) alone increased mitochondrial H_2O_2 release; however, when it was added to mitochondria from LPS-treated cells the H_2O_2 concentrations were synergistically increased. Although it requires further investigations, these data together imply that mitochondrial site of ROS release occurs at complex III in LPS-exposed cells. Indeed, complex III is one of the major sources of electrons that reduce molecular oxygen to superoxide anion in patho-physiological conditions [50]. Previous studies have suggested that inhibition of complex I by rotenone increased intracellular levels of ROS in neutrophils in response to LPS [51]. Another study show that LPS-induced activation of redox-sensitive MAPK occurs via the mitochondrial uncoupling protein, UCP2 in macrophages [52]. These observations are inline with our data while it is possible that there are cell type specific variations in responses to

LPS. Nonetheless further studies are in progress to define the precise mitochondrial site of ROS generation and its biological significance.

To investigate if mitochondrial ROS change the redox conditions in the nucleus and also in the mitochondrial matrix, specific studies were undertaken to determine oxidative DNA damage levels in subcellular compartments. Among DNA bases, guanine is the most susceptible to oxidation forming 8-oxoG a two electron oxidation product found in the RNA, DNA and GTP pool of pro- and eukaryotic cells [40]. Significant increase in 8-oxoG levels in nuclear DNA were demonstrated, suggesting that mitochondrial superoxide anion via enzymatic and/or iron-mediated dismutation converted into H_2O_2 that diffuse into nuclei of cells. When it is in the nucleus, directly or via iron-mediated dismutation of H_2O_2 hydroxyl radical (most reactive specie) are formed that cause damage DNA bases, such as guanine, which has the lowest redox potential among DNA bases. Most importantly, LF lowered 8-oxoG levels to nearly background levels.

Changes in levels of mtDNA damage were determined by the well-accepted quantative PCR method [27-29]. The increase in damage to mtDNA provides evidence that LPS-induced ROS overwhelms antioxidant capacity of mitochondrial matrix. Time-course analysis showed that of mtDNA damage persisted for a substantial period of time, compared to accumulated nuclear DNA damage. Mitochondria have efficient and abundant machineries for oxidative DNA damage repair [53], and the explanation for persisting DNA damage as related to mitochondrial ROS generation has yet to be defined relative to LPS-treatment. We propose that excessive damage to mitochondrial respiratory chain proteins and mtDNA by the initial oxidative stress is a relative cause for increased ROS levels, which feed back and induce additional damage to DNA and proteins that sustain ROS generation both in cultured cells or LPS-challenged animals. Indeed, after a single intraperitoneal injection of LPS, liver mtDNA copy number decreased, as determined by Southern analysis, within 24 hours [42]. This provides new insight into the physiologic significance of mtDNA mutagenesis and perhaps into natural mechanisms to offset the pathological consequences of environmental insults.

An important question of how LF may protect against mitochondrial dysfunctions has been addressed in both cultured cells and in mice treated with LPS. A possibility is that LF attenuates cellular responses to LPS exposure which possibly involves iron binding, while yet undefined events may not be excluded. DFO, a naturally occurring iron-chelating agent has been successfully used for clinical indications of iron overload [54,55]. In our studies, we compared the effects of DFO to LF on preventing mitochondrial dysfunction (release of

H_2O_2, mtDNA damage in cultured cells and liver). DFO decreased the levels of released H_2O_2 from mitochondria and lessened mtDNA damage, but not significantly, indicating that iron chelation itself is not sufficient for prevention of LPS-induced mitochondrial dysfunction. Inline with our data, LF decreased cellular oxidative stress levels and airway inflammation, while DFO' effect was insignificant both in cultured cells and in a mouse model of allergic inflammation [14].

Together these data suggest that iron chelation by LF is not the only explanation for its beneficial effects on mitochondrial function. In support, our transcriptomal network analysis showed regulated changes at RNA levels during cellular response to LF-exposure (unpublished data by Boldogh and colleagues). Finally, these data also support the significance of LF in the resolution or progression of the immune responses during the development of insult-induced metabolic imbalance, including the initial reactions to infectious assault, trauma, and injury. These findings may be critically important in the development of clinical protocols to limit pathological damage by sepsis.

In conclusion, systemic inflammatory response syndrome is a progressive and life-threatening condition that remains a cause of high mortality. The majority of current treatments against sepsis fail to demonstrate significant clinical benefit. Our results support use of LF in prevention and therapy, as a direct therapeutic to combat ensuing mitochondrial dysfunction and generation of ROS that culminates in ultrastructural mitochondrial abnormalities and signals for cell destruction within affected tissue [56,57]. Overall, LF has a potential to protect against a cellular based organ failure in systemic inflammatory response syndrome and sepsis [42,43,58].

FIGURE LEGENDS

Fig. 1 Dose dependent changes in LPS-induced ROS levels. A. Parallel cultures of cells were treated with increasing concentrations of LPS and 45 min later cells were loaded with H_2DCF-DA (50 µM, 15 min). B. Cells were treated with LPS (100 ng per ml) for increasing time periods as indicated in the Fig. Inset: Changes in ROS levels by H_2O_2 (50 µM, in serum free medium) or glucose oxidase (GO, 100 ng/ml) an enzymatic ROS generator. ■, GO; ♦, H_2O_2; ▲, mock-treated. Fifteen min before assessment of ROS levels cells were loaded with H_2DCF-DA (50 µM). The changes in fluorescence intensities were assessed in an FLx800 microplate reader (Bio-Tek Instruments, Winooski, VT, USA) at 488 nm excitation and 530 nm emission. Results are means ±SEM (n=4-7).

Fig. 2 Lactoferrin decreased LPS-induced ROS levels in a dose-dependent manner. **A**. Parallel cultures of U937 cells were treated for 3h with increasing concentrations of LF as indicated in the Fig. As control, cells were treated with 10 mM of N acetyl-L-cysteine (NAC) for 3 h. LPS (100 ng per ml) was added for 45 min and cells were loaded with H_2DCF-DA (50 µM, 15 min). Inset: AML-12 cells were treated with 64 µg/ml LF and H_2O_2 or GO was added. B. Parallel cultures of U937 and AML-12 cells were treated with LF (64 µg/ml), NAC, (10 mM), DFO (64 µg/ml) or ebselen (10 µM) for 3 h and LPS was added for 45 min as in legend to A. The changes in fluorescence intensities were assessed in an FLx800 microplate reader (Bio-Tek Instruments, Winooski, VT, USA) at 488 nm excitation and 530 nm emission (488 nm excitation and 530 nm emission). Results are means ±SEM (n=4-7) independent experiments $^{**}p<0.01$, $^{***}p<0.001$. LF, lactoferrin, NAC, *N*-acetyl-L-cysteine; GO, glucose oxidase, DFO, deferoxamine, DPI, diphenyleneiodonium.

Fig. 3 LPS increases ROS levels via mitochondria. A. Parallel cover-slip cultures of AML-12 cells were treated with LF (64 µg/ml) or NAC (10 mM) for 3 h and LPS (100 ng/ml) was added. Forty-five min later cells were loaded with MitoTracker red (10 nM) and dihydroethidium (2 µM) for 10 min. Fluorescent images were captured just after 60 min incubation with LPS using a Zeiss LSM510 META System driven by Metamorph™ Version 6.09 software. B. mtDNA-depleted (*p0* cells) and wild type cells were treated with LPS (100 ng per ml) and 45 min later cells were loaded with H_2DCF-DA (50 µM, for 15 min). Inset: p0AML-12 cells were treated with H_2O_2 or LPS ±DPI (10 µM) and 1 h later changes in DCF fluorescence was assessed. Changes in fluorescence intensities were assessed in an FLx800 microplate reader [24]. Results are means ±SEM (n=4-7) independent experiments. DPI, diphenyleneiodonium.

Fig. 4 Lactoferrin decreases LPS-induced mitochondrial release of H_2O_2. **A.** Cells (AML-12) were propagated to large quantities, ±LF (64 µg/ml), ±DFO (64 µg/ml) or ±NAC (10 mM) for 3h and then LPS-challenged for 1 h. Cells were harvested and collected by centrifugation, homogenized to isolate and purify mitochondria. H_2O_2 release during 30 min incubation period from mitochondrial suspension (100 µg protein per ml per sample) respiring on pyruvate+malate+succinate were determined by Amplex red assays (Materials and Methods). Results are expressed as means ±SEM values of at least three independent experiments (n=4-5) ***p=0.001. B. Tentative site(s) of superoxide anion formation in mitochondrial respiratory chain was identified by use of inhibitors of electron transport. Mitochondria (100 µg protein per ml per sample; respiring on pyruvate+malate+succinate) from LPS-treated cells were isolated, purified and the released H_2O_2 (during 30 min) in the presence or absence of inhibitors was determined as in legend to A. Rot, rotenone (10 µM); 3-NPA, 3-nitropropionic acid (1 mM); AA; Antimicyn A (3 µM); Stigm, Stigmathellin (0.6 µM). Inhibitory concentrations were determined in preliminary studies (not shown). In panel B, the addition of catalase to mitochondrial suspension decreased H_2O_2 levels by ~95 percent. Results are expressed as means ±SEM values of at least three independent experiments (n=3-5), ***p=0.001. LF, lactoferrin, NAC, N-acetyl-L-cysteine; DFO, deferoxamine.

Fig. 5 Lactoferrin prevents LPS-increased 8-oxoG levels. Parallel cover-slip cultures of AML-12 cells were treated for 3 h with LF (64 µg per ml) and 100 ng per ml LPS was added for 1 h. In controls, the cells were pretreated with NAC (10 mM) or DFO (64 µg/ml) and then were exposed to LPS (100 ng/ml) for 1h. Similar studies were undertaken using 50 µM H_2O_2 (in serum free medium) as controls. After treatment cells were washed, dried and fixed in aceton-methanol for 10 min and processed for staining with antibody to 8-oxoG (as in Material and Methods). After staining, cells were air dried and mounted in anti-fade medium. The fluorescence intensities of minimum of 40 cells per sample were determined using a Zeiss LSM510 META system, operated via MetaMorph software version 6.06r. Results are expressed as means ±SEM values of at least three independent experiments (n=4-5) **p>0.01, ***p=0.001. LF, lactoferrin; NAC, N-acetyl-L-cysteine; DFO, deferoxamine.

Fig. 6 Lactoferrin decreases mitochondrial H_2O_2 release in LPS-treated mice. A. Balb/c mice were LF- (5 mg per mouse) (or mock)-treated (for 12 h) and then they were injected with LPS (2.5 mg/kg) as in Materials and Methods. Twelve h later livers were excised and mitochondria were isolated. In controls, animals were pre-treated with NAC (50 mg/kg) and DFO (5 mg per mouse) intra-peritoneally then LPS-treated. Livers were excised and mitochondria were isolated. H_2O_2 released from purified mitochondria was determined by Amplex Red assay (Materials and Methods). Results are expressed as means ±SEM values from 6-9 animals in independent experiments, $^{**}p>0.01$, $^{***}p=0.001$. B. Decreased release of H_2O_2 from mitochondria isolated from heart, skeletal muscle and brain LF-treated mice. Animals were pretreated with LF or saline and LPS-challenged. Heart, skeletal muscle, brains and lives were collected and releases of H_2O_2 from purified mitochondria were determined. Results are expressed as means ±SEM from 4-6 animals, $^{**}p>0.01$, $^{***}p=0.001$. LF, lactoferrin; NAC, *N*-acetyl-L-cysteine; DFO, deferoxamine.

Fig. 7 Lactoferrin decreases mitochondrial DNA damage after LPS challenge. **A.** Kinetic changes in LPS-induced damage to mitochondrial and nuclear DNA. Parallel cultures of AML-12 cells were treated with LPS (100 ng/ml) for 60 min and genomic DNAs were isolated at 0, 1, 2, 4, 6, 12, and 18h. DNA concentrations were determined using PicoGreen (dsDNA Quantitation Kit) as in Material and Methods. Q-PCR was undertaken using primer pairs for 16 kb mitochondrial DNA (16 kb) and the nuclear β-globin DNA (17 kb) as described previously [26,27]. The quantification of PCR products was determined by fluorescence intensities of PicoGreen-mediated fluorescence we previously described [28]. Results are expressed as ±SEM fluorescence in tensities from 3-5 independent experiments. B, Parallel cultures of AML-12 cells were pre-treated with LF (64 µg per ml), DFO (64 µg per ml) and NAC (10 mM) for 3 h and LPS (100 ng/ml) was added for 60 min. Genomic DNAs were isolated (at 2 and 6 h after LPS addition) and DNA concentrations, levels of intact mitochondrial DNA were determined as described in legend to A. Results are expressed as ±SEM fluorescence intensities from 3-4 independent experiments, $^{***}p<0.001$. n.s, not significant. C. Mice (6-9 per group) were pre-treated with LF, DFO, or NAC for 3h and were intraperitoneally injected with LPS (2.5 mg per kg). Livers were excised 12 h afer LPS challenge and genomic DNAs were quantified (as in legend to A). Levels of intact mitochondrial DNAs were determined by quantitative PCR. In A, B and C thermocycler profile: an initial denaturation for 1 min at 94°C followed by 25 cycles of 94°C denaturation for 15 sec and 68°C primer extension for 12 min. The quantification of PCR products was done using PicoGreen dye (Materials and Methods). Results are expressed as ±SEM fluorescence intensities from PCRs of 6 to 9 liver per treatment. $^{**}p<0.01$ $^{***}p<0.001$. LF, lactoferrin; NAC, N-acetyl-L-cysteine; DFO, deferoxamine.

Acknowledgement: This work was supported by the National Institute of General Medical Sciences (1R41GM079810-01), NIAID AI062885-01 (IB) and NIEHS Center Grant, EOS 006677 (IB).

REFRENCES

1 Schanbacher FL, Goodman RE, Talhouk RS: Bovine mammary lactoferrin: Implications from messenger ribonucleic acid (mrna) sequence and regulation contrary to other milk proteins. *J Dairy Sci* 1993;**76**:3812-3831.

2 van der Strate BW, Beljaars L, Molema G, Harmsen MC, Meijer DK: Antiviral activities of lactoferrin. *Antiviral Res* 2001;**52**:225-239.

3 Bennett RM, Kokocinski T: Lactoferrin content of peripheral blood cells. *Br J Haematol* 1978;**39**:509-521.

4 Caccavo D, Pellegrino NM, Altamura M, Rigon A, Amati L, Amoroso A, Jirillo E: Antimicrobial and immunoregulatory functions of lactoferrin and its potential therapeutic application. *J Endotoxin Res* 2002;**8**:403-417.

5 Conneely OM: Antiinflammatory activities of lactoferrin. *J Am Coll Nutr* 2001;**20**:389S-395S; discussion 396S-397S.

6 Baveye S, Elass E, Mazurier J, Spik G, Legrand D: Lactoferrin: A multifunctional glycoprotein involved in the modulation of the inflammatory process. *Clin Chem Lab Med* 1999;**37**:281-286.

7 Baynes RD, Bezwoda WR: Lactoferrin and the inflammatory response. Adv Exp Med Biol 1994;**357**:133-141.

8 Kruzel ML, Zimecki M: Lactoferrin and immunologic dissonance: Clinical implications. *Arch Immunol Ther Exp (Warsz)* 2002;**50**:399-410.

9 Kruzel ML, Harari Y, Chen CY, Castro GA: Lactoferrin protects gut mucosal integrity during endotoxemia induced by lipopolysaccharide in mice. *Inflammation* 2000;**24**:33-44.

10 Kruzel ML, Harari Y, Mailman D, Actor JK, Zimecki M: Differential effects of prophylactic, concurrent and therapeutic lactoferrin treatment on lps-induced inflammatory responses in mice. *Clin Exp Immunol* 2002;**130**:25-31.

11 Bellamy W, Takase M, Yamauchi K, Wakabayashi H, Kawase K, Tomita M: Identification of the bactericidal domain of lactoferrin. *Biochim Biophys Acta* 1992;**1121**:130-136.

12 Zagulski T, Lipinski P, Zagulska A, Broniek S, Jarzabek Z: Lactoferrin can protect mice against a lethal dose of escherichia coli in experimental infection in vivo. *Br J Exp Pathol* 1989;**70**:697-704.

13 Kruzel ML, Actor JK, Boldogh I, Zimecki M: Lactoferrin in health and disease. *Postepy Hig Med Dosw (Online)* 2007;**61**:261-267.

14 Kruzel ML, Bacsi A, Choudhury B, Sur S, Boldogh I: Lactoferrin decreases pollen antigen-induced allergic airway inflammation in a murine model of asthma. *Immunology* 2006;**119**:159-166.

15 Exline MC, Crouser ED: Mitochondrial mechanisms of sepsis-induced organ failure. *Front Biosci* 2008;**13**:5030-5041.

16 Crouser ED: Mitochondrial dysfunction in septic shock and multiple organ dysfunction syndrome. *Mitochondrion* 2004;4:729-741.

17 Bacsi A, Woodberry M, Widger W, Papaconstantinou J, Mitra S, Peterson JW, Boldogh I: Localization of superoxide anion production to mitochondrial electron transport chain in 3-npa-treated cells. *Mitochondrion* 2006;**6**:235-244.

18 Dobson AW, Grishko V, LeDoux SP, Kelley MR, Wilson GL, Gillespie MN: Enhanced mtdna repair capacity protects pulmonary artery endothelial cells from oxidant-mediated death. *Am J Physiol Lung Cell Mol Physiol* 2002;**283**:L205-210.

19 King MP, Attardi G: Human cells lacking mtdna: Repopulation with exogenous mitochondria by complementation. *Science* 1989;**246**:500-503.

20 Das A, Hazra TK, Boldogh I, Mitra S, Bhakat KK: Induction of the human oxidized base-specific DNA glycosylase neil1 by reactive oxygen species. *J Biol Chem* 2005;**280**:35272-35280.

21 Boldogh I, Roy G, Lee MS, Bacsi A, Hazra TK, Bhakat KK, Das GC, Mitra S: Reduced DNA double strand breaks in chlorambucil resistant cells are related to high DNA-pkcs activity and low oxidative stress. *Toxicology* 2003;**193**:137-152.

22 Bhakat KK, Mokkapati SK, Boldogh I, Hazra TK, Mitra S: Acetylation of human 8-oxoguanine-DNA glycosylase by p300 and its role in 8-oxoguanine repair in vivo. *Mol Cell Biol* 2006;**26**:1654-1665.

23 Bespalov IA, Bond JP, Purmal AA, Wallace SS, Melamede RJ: Fabs specific for 8-oxoguanine: Control of DNA binding. *J Mol Biol* 1999;**293**:1085-1095.

24 Bacsi A, Chodaczek G, Hazra TK, Konkel D, Boldogh I: Increased ros generation in subsets of ogg1 knockout fibroblast cells. *Mech Ageing Dev* 2007;**128**:637-649.

25 Zhou M, Diwu Z, Panchuk-Voloshina N, Haugland RP: A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: Applications in detecting the activity of phagocyte nadph oxidase and other oxidases. *Anal Biochem* 1997;**253**:162-168.

26 Ayala-Torres S, Chen Y, Svoboda T, Rosenblatt J, Van Houten B: Analysis of gene-specific DNA damage and repair using quantitative polymerase chain reaction. *Methods* 2000;**22**:135-147.

27 Santos JH, Mandavilli BS, Van Houten B: Measuring oxidative mtdna damage and repair using quantitative pcr. *Methods Mol Biol* 2002;**197**:159-176.

28 Mandavilli BS, Boldogh I, Van Houten B: 3-nitropropionic acid-induced hydrogen peroxide, mitochondrial DNA damage, and cell death are attenuated by bcl-2 overexpression in pc12 cells. *Brain Res Mol Brain Res* 2005;**133**:215-223.

29 Yakes FM, Van Houten B: Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. *Proc Natl Acad Sci U S A* 1997;**94**:514-519.

30 Bennetts LE, Aitken RJ: A comparative study of oxidative DNA damage in mammalian spermatozoa. *Mol Reprod Dev* 2005;**71**:77-87.

31 Pang Z, Geddes JW: Mechanisms of cell death induced by the mitochondrial toxin 3-nitropropionic acid: Acute excitotoxic necrosis and delayed apoptosis. *J Neurosci* 1997;**17**:3064-3073.

32 Zak O, Aisen P, Crawley JB, Joannou CL, Patel KJ, Rafiq M, Evans RW: Iron release from recombinant n-lobe and mutants of human transferrin. Biochemistry 1995;**34**:14428-14434.

33 Rodgers SJ, Raymond KN: Ferric ion sequestering agents. 11. Synthesis and kinetics of iron removal from transferrin of catechoyl derivatives of desferrioxamine b. *J Med Chem* 1983;**26**:439-442.

34 Aldieri E, Riganti C, Polimeni M, Gazzano E, Lussiana C, Campia I, Ghigo D: Classical inhibitors of nox nad(p)h oxidases are not specific. *Curr Drug Metab* 2008;**9**:686-696.

35 Arakawa M, Ito Y: N-acetylcysteine and neurodegenerative diseases: Basic and clinical pharmacology. *Cerebellum* 2007:1-7.

36 Fang J, Zhong L, Zhao R, Holmgren A: Ebselen: A thioredoxin reductase-dependent catalyst for alpha-tocopherol quinone reduction. *Toxicol Appl Pharmacol* 2005;**207**:103-109.

37 Rada B, Leto TL: Oxidative innate immune defenses by nox/duox family nadph oxidases. *Contrib Microbiol* 2008;15:164-187.

38 Yamada H, Arai T, Endo N, Yamashita K, Fukuda K, Sasada M, Uchiyama T: Lps-induced ros generation and changes in glutathione level and their relation to the maturation of human monocyte-derived dendritic cells. *Life Sci* 2006;**78**:926-933.

39 Zhao H, Kalivendi S, Zhang H, Joseph J, Nithipatikom K, Vasquez-Vivar J, Kalyanaraman B: Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: Potential implications in intracellular fluorescence detection of superoxide. *Free Radic Biol Med* 2003;**34**:1359-1368.

40 Loft S, Hogh Danielsen P, Mikkelsen L, Risom L, Forchhammer L, Moller P: Biomarkers of oxidative damage to DNA and repair. *Biochem Soc Trans* 2008;**36**:1071-1076.

41 Miller SW, Trimmer PA, Parker WD, Jr., Davis RE: Creation and characterization of mitochondrial DNA-depleted cell lines with "Neuronal-like" Properties. *J Neurochem* 1996;**67**:1897-1907.

42 Suliman HB, Carraway MS, Piantadosi CA: Postlipopolysaccharide oxidative damage of mitochondrial DNA. *Am J Respir Crit Care Med* 2003;**167**:570-579.

43 Suliman HB, Welty-Wolf KE, Carraway M, Tatro L, Piantadosi CA: Lipopolysaccharide induces oxidative cardiac mitochondrial damage and biogenesis. *Cardiovasc Res* 2004;**64**:279-288.

44 Chandel NS, Schumacker PT: Cells depleted of mitochondrial DNA (rho0) yield insight into physiological mechanisms. *FEBS Lett* 1999;**454**:173-176.

45 Buchet K, Godinot C: Functional f1-atpase essential in maintaining growth and membrane potential of human mitochondrial DNA-depleted rho degrees cells. *J Biol Chem* 1998;**273**:22983-22989.

46 Seo BB, Kitajima-Ihara T, Chan EK, Scheffler IE, Matsuno-Yagi A, Yagi T: Molecular remedy of complex i defects: Rotenone-insensitive internal nadh-quinone oxidoreductase of saccharomyces cerevisiae mitochondria restores the nadh oxidase activity of complex i-deficient mammalian cells. *Proc Natl Acad Sci U S A* 1998;**95**:9167-9171.

47 Coles CJ, Edmondson DE, Singer TP: Inactivation of succinate dehydrogenase by 3-nitropropionate. *J Biol Chem* 1979;254:5161-5167.

48 von Jagow G, Ohnishi T: The chromone inhibitor stigmatellin--binding to the ubiquinol oxidation center at the c-side of the mitochondrial membrane. *FEBS Lett* 1985;**185**:311-315.

49 Kluepfel D, Sehgal SN, Vezina C: Antimycin a components. I. Isolation and biological activity. *J Antibiot (Tokyo)* 1970;**23**:75-80.

50 Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ: Production of reactive oxygen species by mitochondria: Central role of complex iii. *J Biol Chem* 2003;**278**:36027-36031.

51 Zmijewski JW, Lorne E, Zhao X, Tsuruta Y, Sha Y, Liu G, Siegal GP, Abraham E: Mitochondrial respiratory complex i regulates neutrophil activation and severity of lung injury. A*m J Respir Crit Care Med* 2008;**178**:168-179.

52 Emre Y, Hurtaud C, Nubel T, Criscuolo F, Ricquier D, Cassard-Doulcier AM: Mitochondria contribute to lps-induced mapk activation via uncoupling protein ucp2 in macrophages. *Biochem J* 2007;**402**:271-278.

53 Larsen NB, Rasmussen M, Rasmussen LJ: Nuclear and mitochondrial DNA repair: Similar pathways? *Mitochondrion* 2005;**5**:89-108.

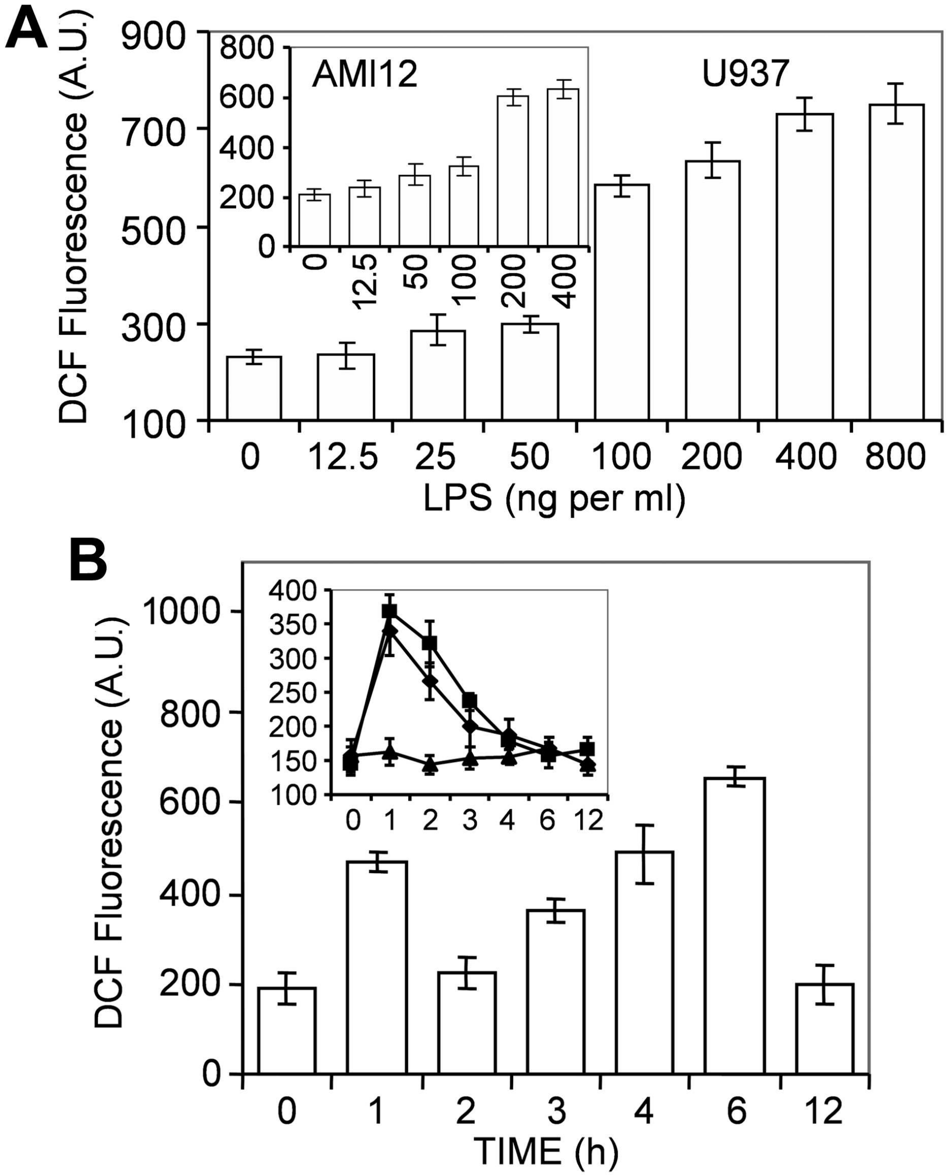
54 Brittenham GM, Griffith PM, Nienhuis AW, McLaren CE, Young NS, Tucker EE, Allen CJ, Farrell DE, Harris JW: Efficacy of deferoxamine in preventing complications of iron overload in patients with thalassemia major. *N Engl J Med* 1994;**331**:567-573.

55 Cohen A, Martin M, Schwartz E: Depletion of excessive liver iron stores with desferrioxamine. *Br J Haematol* 1984;**58**:369-373.

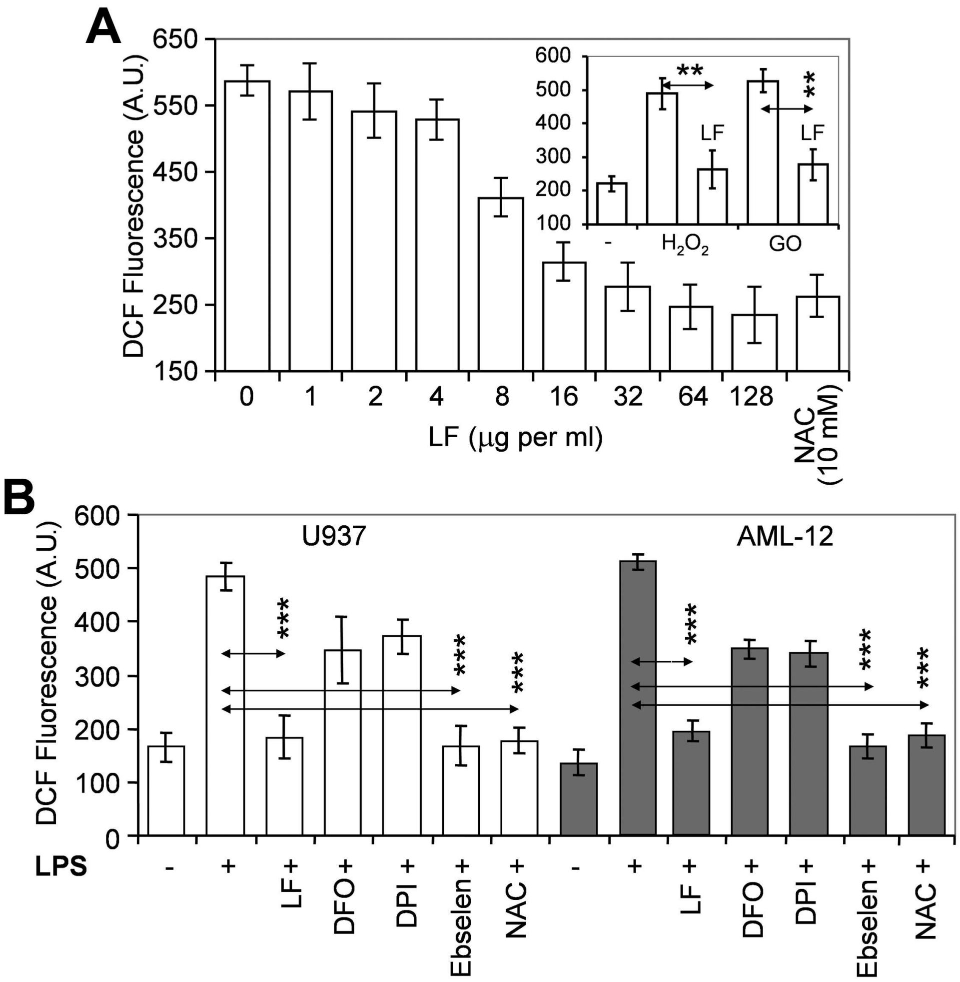
56 Crouser ED, Julian MW, Blaho DV, Pfeiffer DR: Endotoxin-induced mitochondrial damage correlates with impaired respiratory activity. *Crit Care Med* 2002;**30**:276-284.

57 Gellerich FN, Trumbeckaite S, Opalka JR, Gellerich JF, Chen Y, Neuhof C, Redl H, Werdan K, Zierz S: Mitochondrial dysfunction in sepsis: Evidence from bacteraemic baboons and endotoxaemic rabbits. *Biosci Rep* 2002;**22**:99-113.

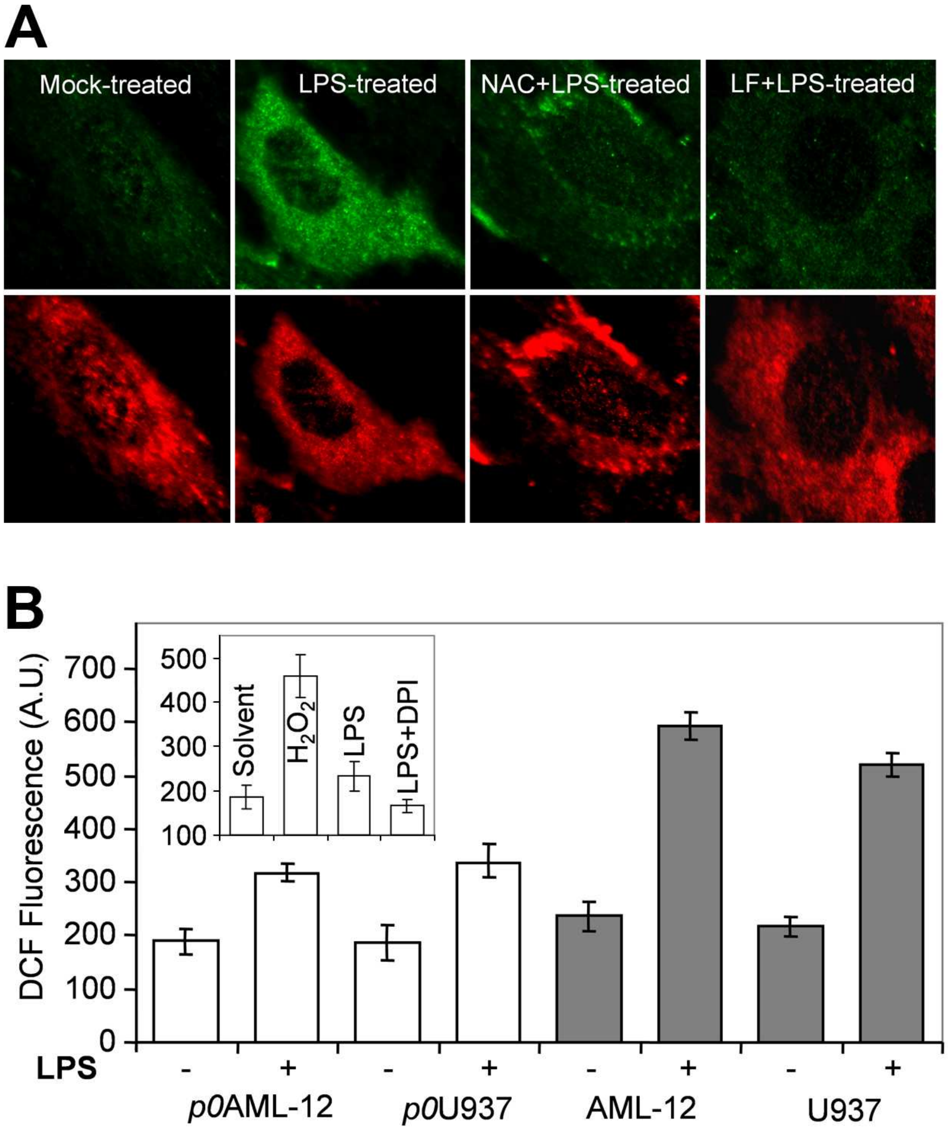
58 Flierl MA, Rittirsch D, Huber-Lang MS, Sarma JV, Ward PA: Molecular events in the cardiomyopathy of sepsis. *Mol Med* 2008;**14**:327-336.



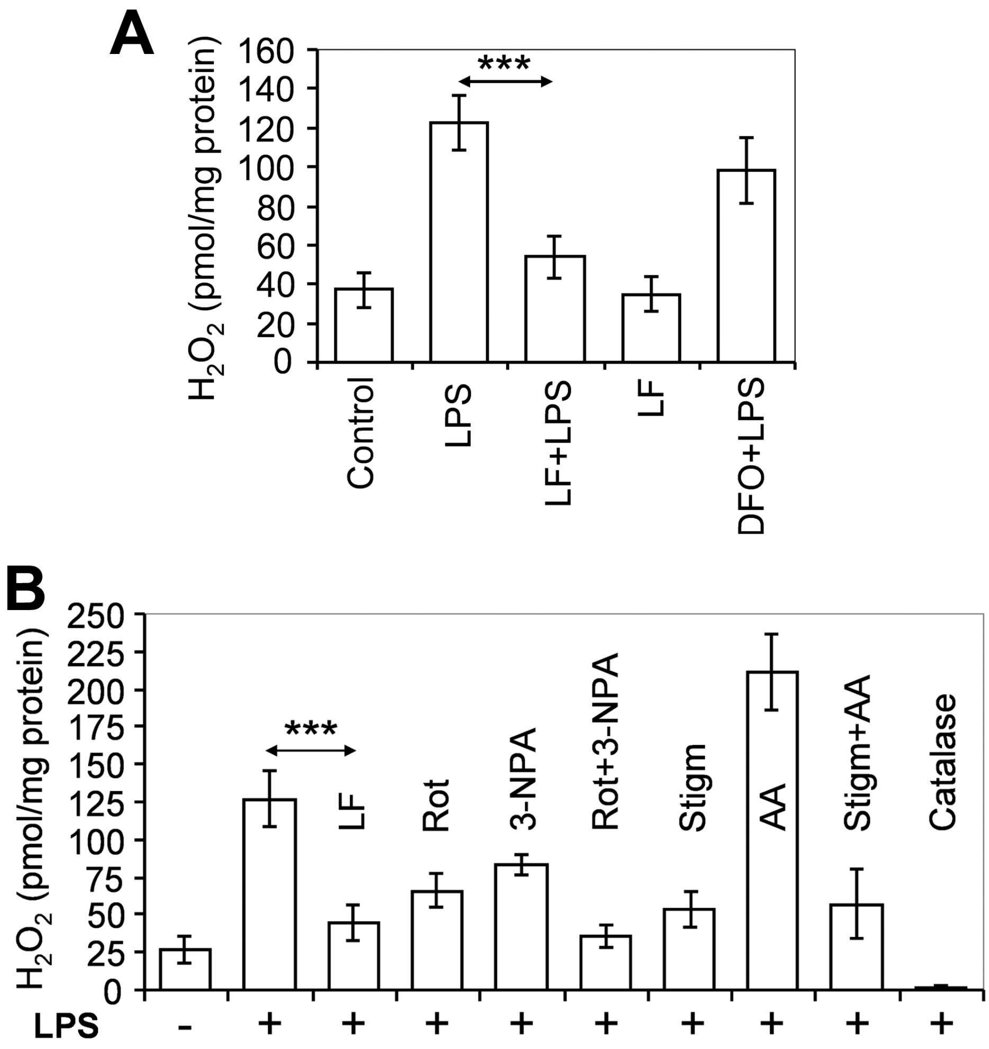
80x100mm (600 x 600 DPI)



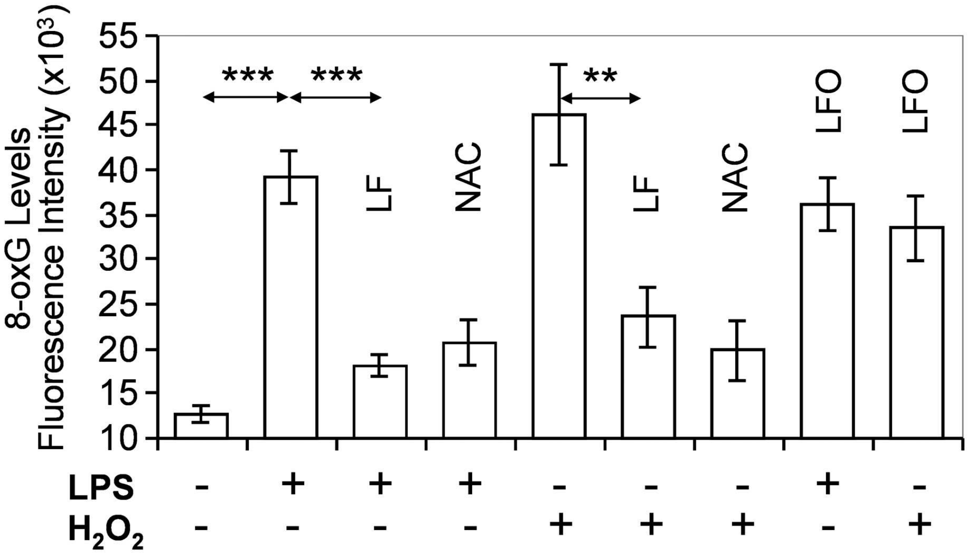
99x99mm (600 x 600 DPI)



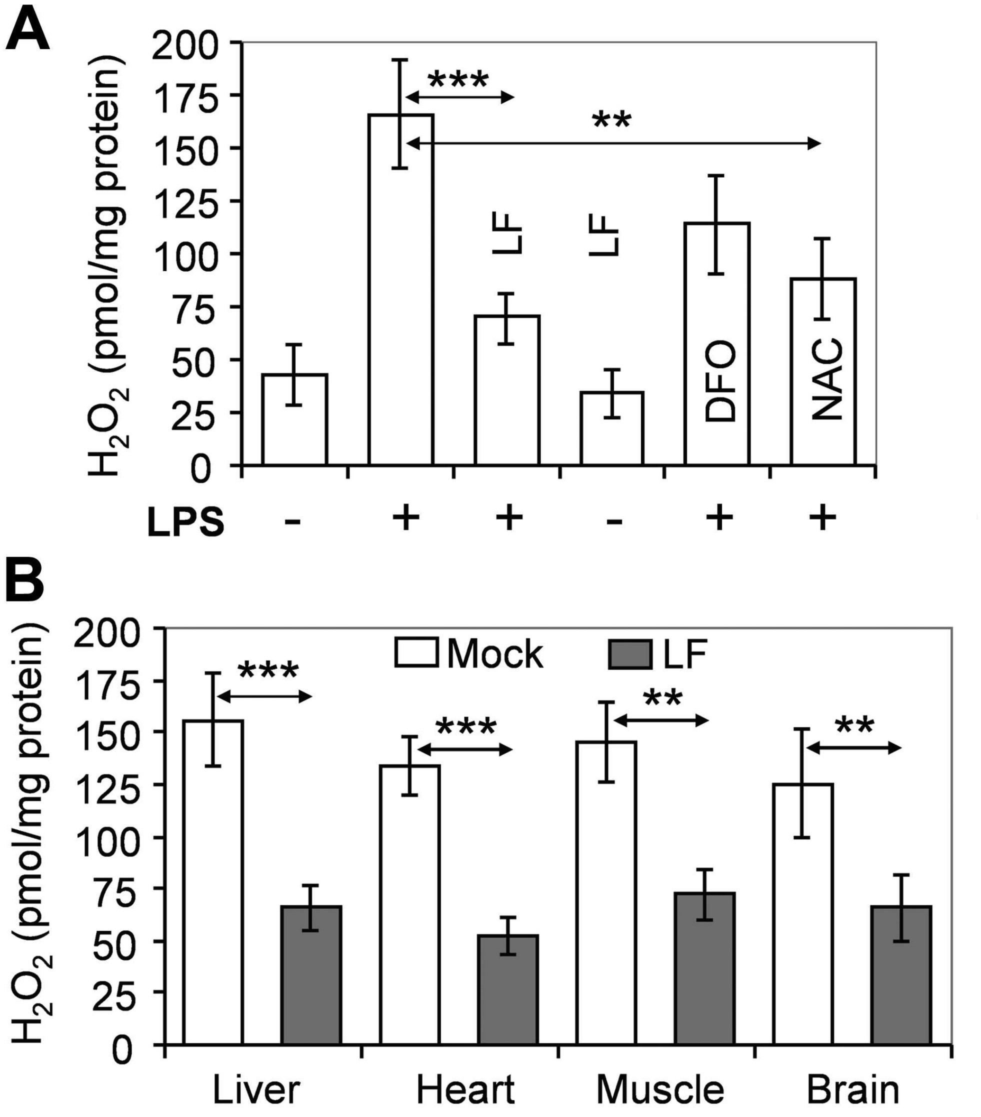
80x100mm (400 x 400 DPI)



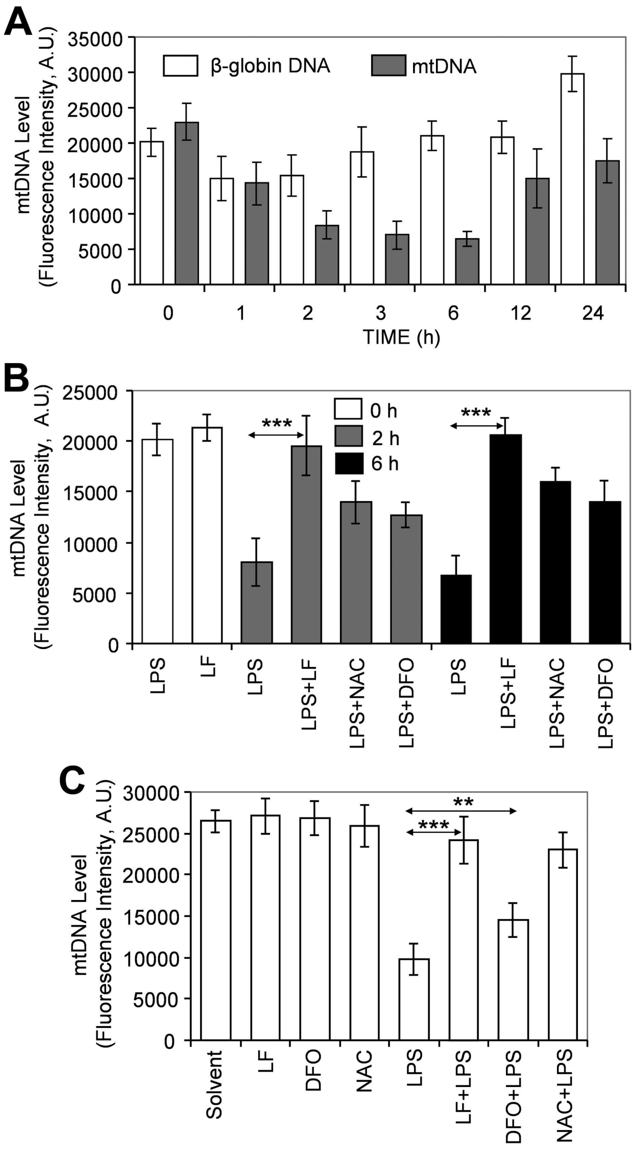
80x90mm (600 x 600 DPI)



80x48mm (600 x 600 DPI)



80x90mm (600 x 600 DPI)



80x150mm (600 x 600 DPI)