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Decreased Release of Nitric Oxide (NO) by Alveolar Macrophages after *In Vivo* Loading of Rats with Either Iron or Ethanol

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ABSTRACT. Alveolar macrophages were isolated by pulmonary lavage from rats which had been either chronically overloaded with iron by intraperitoneal injections of iron dextran for four weeks, or rendered alcoholic by administration of increasing concentrations of alcohol vapour, also for four weeks. Although the hepatic iron content increased in both groups of animals, only the macrophages isolated from the iron-loaded animals showed a significant increase in iron content ($P = < 0.05$). Furthermore, in these macrophages there was a significant increase in oxidative tone as demonstrated by a six fold increase in superoxide dismutase activity. In both the iron-loaded and chronically alcoholised macrophages, there was a significant diminution in nitric oxide release after stimulation with lipopolysaccharide and/or interferon-γ, which impaired the ability of both of these groups of macrophages to inhibit the germination of spores from the fungus *Rhizopus*, a nitric oxide-dependent process. Such an alteration in nitric oxide release reduces the macrophage's microbicidal activity. BIOCHEM PHARMACOL 55;1:21–25, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. alveolar macrophages; iron-loading; chronic alcoholisation; oxidative stress; nitric oxide

Macrophages are phagocytic cells which play a central role in cell-mediated immunity. After stimulation by a variety of pathogens, macrophages can release numerous mediators which include cytokines and arachidonic acid metabolites in addition to the generation of a wide number of reactive oxygen species, (ROS), including superoxide, (O_2^-) and nitric oxide, (NO) [1, 2]. Arginine is the substrate for NO production in this cell via its inducible enzyme, nitric oxide synthase, (iNOS), with citrulline as a byproduct [2]. This enzyme is induced only after activation of the macrophages. The transcription of iNOS is induced by cytokines and by lipopolysaccharides, a component of gram-negative bacteria, while interferon-γ (IFN-γ) works synergistically with other cytokines to increase levels of gene transcription [3]. NO decomposes into other nitrogen oxides such as nitrite (NO_2^-) and nitrate (NO_3^-), and in the presence of the superoxide anion O_2^- forms the highly toxic reactive species, peroxynitrite ($ONOO^-$). A decreased production of such mediators, particularly if NO release is compromised, may increase the host's susceptibility to subsequent infections.

One of the biological functions of macrophages is to catabolise exhausted erythrocytes and recycle the iron for haemoglobin synthesis [4]. In certain iron-loading syndromes where there is excessive red cell destruction, e.g. thalassaemia [5, 6], in the anaemia of chronic disease, e.g., which occurs in conditions such as rheumatoid arthritis [7] or in advanced HIV infection [8], macrophages accumulate excessive amounts of iron which may decrease their microbiocidal ability. *In vitro* studies of iron-loaded macrophages have indicated decreased iNOS activity with a diminution of NO release after stimulation with interferon-γ plus lipopolysaccharide (IFN-γ/LPS). By contrast, addition of the iron chelator desferrioxamine at the time of the stimulation with IFN-γ/LPS increased iNOS activity in J774 cells [9], possibly reflecting a contribution of iron to the down regulation of iNOS. Alcohol may also increase the iron content within the reticuloendothelial system in certain susceptible alcohol misusers [10], which could contribute to the suppression of various aspects of the immune system, including the microbicidal activity of the macrophages [11].

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; H_2O_2, hydrogen peroxide; IFN-γ, interferon gamma; iNOS, inducible nitric oxide synthase; IRE, iron responsive element; IRF, iron responsive factor; LPS, lipopolysaccharides; NO, nitric oxide; NO_2^-, nitrite; NO_3^-, nitrate; O_2^-, superoxide; OH^-, hydroxyl; $ONOO^-$, peroxynitrite; ROS, reactive oxygen species.

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In the present study, we have assayed the iron content of rodent macrophages after chronic loading, *in vivo*, with iron or ethanol to ascertain whether the oxidative tone of these cells is altered. Macrophages have then been stimulated *ex vivo* by IFNγ/LPS to assess their ability to release NO and to inhibit the germination of the spores of the zygomycete fungus *Rhizopus*, as this inhibition is known to be NO-mediated [2].

MATERIALS AND METHODS

Animal Models

ANIMAL MODEL OF RETICULOENDOTHELIAL IRON LOADING. Male Wistar rats (70–80 g at the commencement of the study), $n = 6$, were overloaded with iron by intraperitoneal injection of iron dextran (Imferon), 3 times/week for 4 weeks (total amount of iron administered = 120 mg). Control rats were injected with saline at a comparable time to that of the iron injections.

ANIMAL MODEL OF CHRONIC ALCOHOLISATION. Male Wistar rats (70–80 g at the commencement of the study), $n = 6$, were individually housed in cages and administered ethanol vapour over 28 days [12]. This was achieved by a mixture of alcohol vapour and air being pulsed into the chamber via a mixing system, the ethanol content gradually being increased every 2 days (+5 U of ethanol) relative to the air over the time-course of the alcoholisation from 100:20 to 100:80 air:ethanol vapour. The control group was housed under similar conditions but without the alcohol vapour.

Isolation and Culture of Alveolar Macrophages

ISOLATION OF RAT ALVEOLAR MACROPHAGES [13]. Rats were killed with intraperitoneally administered nembutal, sodium pentabarbitone, 0.6 mg/kg. The trachea of the rat was exposed and a midline incision was made to expose the heart and lungs. After insertion of a tube via the trachea into the lungs, the alveolar macrophages were recovered by five lavages, each with 10 mL phosphate-buffered saline, (PBS). The lungs were observed during this procedure to ensure their enlargement and that there was no leakage of PBS. An aliquot of the cells was taken for the *ex vivo* stimulation experiments, while the remainder of the cellular suspensions was frozen at −20° prior to the analyses of iron content and superoxide dismutase activity.

CULTURE OF RAT ALVEOLAR MACROPHAGES. Rat alveolar macrophages were washed with PBS-Dulbecco's medium, without calcium and magnesium, and the cells were suspended in Dulbecco's modified Eagle's medium without phenol red (DMEM) supplemented with 5% fetal calf serum (FCS) and penicillin-streptomycin. The macrophage populations were enriched by adherence to plastic on 24-well sterile dishes (Costar) with (calculated) 5×10^5 macrophages added to each well. Cells were allowed to adhere for 90 min at 37°, after which non-adherent cells were removed by 3 washes with prewarmed DMEM. Subsequently, 1 mL of medium (either with 84 mg (4×10^{-4} M) of L-arginine per litre or without L-arginine), with or without appropriate stimuli of fungi, was added to duplicate wells which were incubated for 24 hr at 37° in 5% CO_2. The adherent population was verified to contain more than 95% macrophages by morphological (May Grunwald–Giemsa staining) and biochemical (nonspecific esterase staining) criteria.

ISOLATION OF SPORES OF THE RHIZOPUS STRAIN MICROSPORES. The *Rhizopus* strain, *Rhizopus microsporus* var. rhizopodiformis (ATCC 66276) was maintained on Sabouraud dextrose agar (Difco). Spongiospores (spores) were prepared by growing fungi for 10 days. Mycelia were scraped into sterile deionised water and the sporulated mycelial suspension was vigorously agitated for 10 min in the presence of sterile glass beads (min. diameter, *ca.* 2 mm) and then filtered through gauze. Eluate, containing spores, was washed twice with water and concentrated by centrifugation to obtain a suspension that was free of hyphal fragments and contained 10^7 spores per milliliter, as estimated by hemocytometer. Spores were stored at 4° in the presence of penicillin and streptomycin at 30 and 70 μg/mL, respectively.

MEASUREMENT OF THE INHIBITION OF GERMINATION. Fungal growth was studied by turbidimetric measurement after coculture with macrophages. Fungal spores (0.5 mL) at a concentration of 10^6/mL were added to each macrophage culture (0.5×10^6 rat alveolar macrophages). Macrophage controls were cultivated in media without the addition of *Rhizopus* spores. Macrophage-fungus co-cultures were incubated in humidified 5% CO_2–95% air at 37° for 24 hr. At the appropriate time points, the supernatant was aspirated, a macrophage-free supernatant was harvested by centrifugation for 15 min at $2000 \times g$. The hypha-free supernatant was stored at −20° prior to nitrite assay. Pellets were harvested in PBS and sonicated (50 W, 1 min). The A_{400} was read, and background absorption due to the medium was subtracted [14]. The absorbance obtained with macrophage *Rhizopus* co-cultures is expressed as percent *Rhizopus* absorbance (growth) without alveolar macrophages added.

Assays

ASSESSMENT ON NO PRODUCTION BY THE MACROPHAGE. Nitrite (NO_2^-) was assayed in the cell-free supernatant of the macrophages by a spectrophotometric method based on the Griess reaction, a sensitive technique for measuring nitrite, but does not detect nitrate [13]. Ninety microlitres of 6.5 M HCl and 40 μL of 37.5 M sulfanilic acid was added to 400 μL of supernatant. After 10 min, 40 μL of 12.5 mM *N*(1-naphtyl)ethylene diaminehydrochloric acid was added and 30 min later, the absorbance was measured at 540 nm. The nitrite concentration was calculated from a standard

TABLE 1. Hepatic and macrophage iron content after administration of either iron dextran or chronic alcoholisation for 4 weeks

	Control ($n = 6$)	Iron-loaded ($n = 6$)	Chronic alcoholisation ($n = 6$)
Liver Fe content (μg/g tissue)	206 ± 72.2	4795 ± 991.0[a]	375 ± 149[a]
Macrophage Fe content (ng/μg protein)	3.61 ± 1.8	6.28 ± 1.3[a]	3.28 ± 0.76

Results are mean ± SD.
[a]$P < 0.05$.

curve in the same medium as used in the experiments and expressed as $\mu M/10^6$ cells. The nitrite content of the DMEM alone or supplemented with FCS and the stimuli was always less than 0.5 nmol/mL, both after fresh addition and after 24 hr in culture in plastic wells without cells (detection limit 0.5 μM).

SUPEROXIDE DISMUTASE ASSAY. Superoxide dismutase activity was assayed in macrophages, after freezing and thawing 3 times, by the method of Beauchamp and Fridovich [15]. Aliquots (100 μL) of suitably diluted samples were mixed with the buffered substrate (1275 μL) containing nitroblue tetrazolium, 2.5×10^{-5} M, xanthine 1×10^{-4} M, EDTA 1×10^{-4} M, sodium carbonate 0.05 M, pH 10.2, in a disposable cuvette at 25°. Xanthine oxidase, 0.625 U/mL, (25 μL) was added to generate superoxide, and the reduction of nitroblue tetrazolium by the radical species was recorded in a spectrophotometer at 560 nm during the first 4 min. A blank of 0.25 M sucrose was assayed in each analytical run together with bovine SOD in the standard range 0.25–2.0 U. One unit of enzyme activity was equivalent to 50% inhibition of the reduction rate of nitroblue tetrazolium.

IRON ANALYSES. The iron status of each animal was assessed by the assay of liver iron content. A liver homogenate was prepared, 10% w/v, in 0.25 M sucrose and its iron concentration assayed by electrothermal atomic absorption (Perkin-Elmer, Zeeman/3030, Uberliner, Germany) as previously described [16]. Iron was similarly analysed in the macrophages after dilution of the samples with water to the standard range 10–50 ng/liter.

PROTEIN ANALYSIS. The protein content of the macrophage preparations used for the analysis of iron content and activity of superoxide dismutase was assayed by the Bio-Rad method (Bio-Rad) with bovine albumin as standard.

STATISTICAL ANALYSIS. The results are presented as mean ± standard deviation. Significance is set at $p < 0.05$ and analysed by paired t-test.

RESULTS

As shown in Table 1, the hepatic iron content increased 20-fold in the rats administrated iron dextran for 4 weeks, while the alcoholised rats showed a 2-fold increase. The alveolar macrophage (AM) iron content increased 2-fold after iron loading, but no significant change occurred in the macrophages isolated from the chronically alcoholised rats.

Superoxide dismutase activity was assayed before stimulation of the cells. Its activity increased significantly in the iron-loaded macrophages by approximately seven-fold: 13.6 ± 5.5 mU/mg protein by comparison to control macrophages 1.94 ± 0.64 mU/mg protein. There was no significant change in its mean activity in macrophages isolated from the chronically alcoholised rats: 2.31 ± 1.58 mU/mg protein.

The decreased ability of the macrophages, whether exposed to excessive iron or ethanol, to release nitrite (NO_2^-) after challenge with INF-γ, LPS or INF-γ together with LPS is shown in Fig. 1. As can be clearly seen, each stimulus induced an approximate 50% decrease in nitrite release after immunological stimulation, when compared to normal macrophages.

Control rat alveolar macrophages incubated with the *Rhizopus* strain and LPS and IFN-γ were capable of inhibiting *Rhizopus* spore germination as shown by a significant decrease in absorbance (A_{400} 20.3 ± 8.0; $n = 3$, mean ± SD) as compared to the absorbance recorded when no macrophages were added to the incubation media, (A_{400} 88.1 ± 1.1; $n = 3$). After incubation of either iron-loaded or chronically alcoholised macrophages with the spores of *Rhizopus*, there was little change in the absorbance assayed (A_{400} 73.1 ± 7.3; $n = 3$) and (A_{400} 93.0 + 6.0, $n = 3$), respectively, the results being comparable to the control value, indicating normal *Rhizopus* germination.

DISCUSSION

In the present study, we have clearly shown that intraperitoneal injection of iron dextran for 4 weeks induces significant increases in the iron content of the alveolar macrophages, which may be due to the up regulation of both ferritin and transferrin surface receptors on the macrophage [17]. In contrast, although the iron status of the chronically alcoholised rats significantly increased, there was no change in macrophage iron content. Despite the different effects on the iron content of alveolar macrophages after iron loading or chronic alcoholisation, both treatments induced a significant decrease in nitrite release, after *ex vivo* stimulation with either LPS and/or

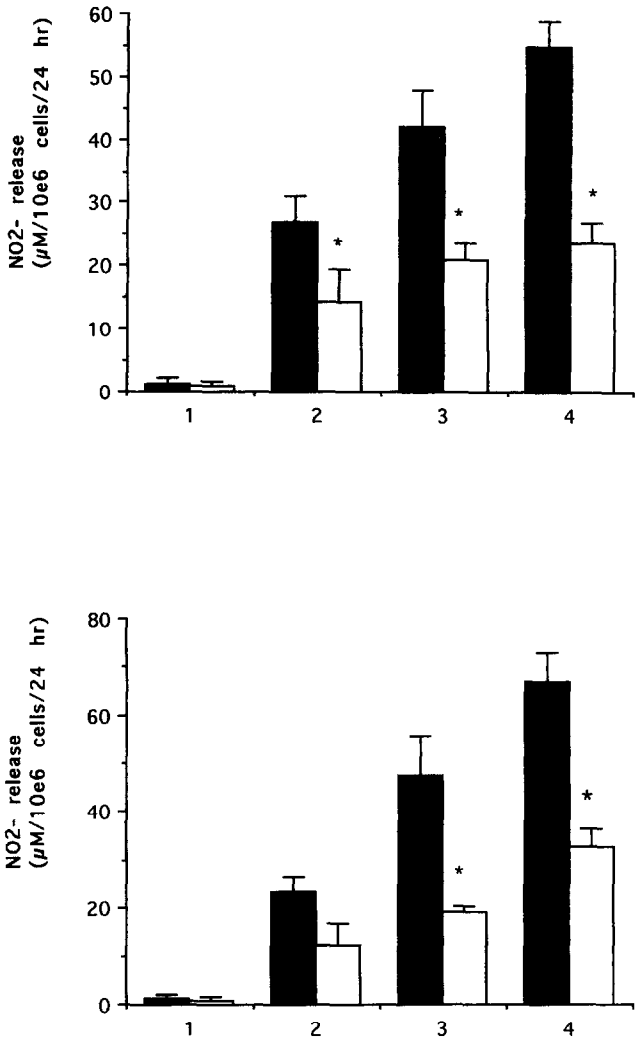

FIG. 1. Release of NO_2^- from alveolar macrophages isolated from untreated (black column), iron-loaded (white column, top half) and chronically alcohol-vapourised (white column, bottom half) rats after *in vitro* stimulation with 1) no stimuli, 2) lipopolysaccharide, 1 μg/mL, 3) interferon-γ, 50 U/mL, and 4) lipopolysaccharides, 1 μg/mL + interferon γ, 50 U/mL. Results are presented as mean ± SED for three rats. *P − < 0.05.

interferon-γ. It remains unclear whether the mechanism by which these two procedures alter NO release may be similar. However, both iron-loaded and chronically alcoholised macrophages exhibited a decreased ability to inhibit the germination of spores from the fungus *Rhizopus*, which is an NO-mediated process [2].

As far as is known, ours is the first study to examine iNOS activity and its antifungal effect after iron loading of the animal *in vivo* rather than examining a cell line *in vitro*. Previous *in vitro* studies of iron-loaded J774 identified decreased NO release caused by a reduction in transcription of iNOS [9]. Such a reduction in NO release could be both advantageous and disadvantageous. On the one hand, it will limit further cellular iron accumulation, through down-regulation of transferrin receptors. NO-induced binding of iron regulatory factor (IRF) to iron-responsive elements specifically represses the translation of transfected iron regulatory element (IRE)-containing indicator mRNAs as well as the biosynthesis of the cellular iron storage protein ferritin [18]. On the other hand, it might impair a release of NO, which is well established as a major microbicidal mechanism, thus partly explaining the enhanced severity of infection caused by intracellular pathogens in cases of experimental iron overload [19].

One previous study has investigated the ability of *in vivo* ethanol-loaded macrophages to release NO after stimulation with LPS or IFN-γ [20]. In this study, however, NO was already produced in the macrophages before stimulation, due to the presence of gram-positive bacteria within the feed, initiating a low-grade infection within the animals. In the present study NO_2^- was not present at time 0 in the cell culture media. Each treatment, iron or ethanol loading, resulted in a 50% decrease in NO release after stimulation, while in the study of D'Souza et al. [20], the change in NO release reported after stimulation with LPS and/or interferon-γ was much smaller, i.e. a decrease of 14%, 19% and 14%, respectively. Whether ethanol acts directly upon iNOS transcription or translation or indirectly through intermediates such as cytokines remains to be ascertained. Indeed, acute and chronic ethanol administration have differing effects on iNOS in alveolar macrophages. Acute ethanol decreases iNOS mRNA levels in alveolar macrophages with a diminution of NO release [21], whereas chronic ethanol administration may not adversely alter iNOS mRNA [22]. Another explanation for the inhibition of NO release in such experiments may be a decrease in arginine availability.

Alveolar macrophages have been used as the source of macrophages in this present study. Although they are proteolytically less active than their counterparts from the bone marrow [23], they are readily available in large numbers and in a relatively pure state. Macrophages are well equipped for their biological role, containing as they do three major classes of enzymes, lysosomal acid hydrolases, neutral proteases and lysozymes in addition to being able to generate various activated oxygen metabolites, e.g. O_2^-, H_2O_2, $OH^\bullet$ as well as NO. Changes in the balance of the two reactive oxygen species O_2^- and NO have been implicated in various pathological states [24]. Superoxide ions present in the macrophage during the resting state will be derived primarily from mitochondrial leakage, so that the increase in intracellular superoxide dismutase which was assayed only in the nonstimulated iron-loaded macrophages may be attributable to iron-induced changes in the mitochondrial membranes. It is unknown whether the activity of superoxide dismutase is altered after stimulation. Antony et al. [25] showed that alveolar macrophages, chronically exposed to ethanol, either *in vivo* or *in vitro*, showed a decrease in both O_2^- and hydrogen peroxide release after stimulation with *Staphylococcus aureus*, immune complexes or phorbol myristate acetate.

Alveolar macrophages play a critical role in the prevention and control of pulmonary infection, with resident

activated macrophages engulfing and digesting bacteria and fungi. Acute ethanol has been shown to attenuate *Mycobacterium tuberculosis*-induced increase in mRNA for iNOS and reactive nitrogen intermediates [26]. In the present study, we have clearly shown that macrophages where the iNOS is compromised by iron or ethanol loading have an impaired ability to limit the transformation of *Rhizopus* spores into hypha.

Our results indicate that the macrophage's ability to release NO and possibly other oxygen intermediates may be compromised after iron or chronic alcohol loading, which may diminish the ability of these cells to respond to toxic external stimuli. Our observations may have important connotations for a variety of diseases where the iron status is elevated or its distribution altered, as well as for subjects consuming excessive quantities of alcohol, where levels of exhaled NO may be diminished [27], resulting in an increased susceptibility to pulmonary infections.

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