Iranian Journal of Basic Medical Sciences
Vol. 11, No. 3, Autumn 2008, 190-196
Received: Sep 23, 2008; Accepted: Dec 4, 2008

IJBMS

Original article

Synergistic Effect of LPS, IFN-γ and Iron on Apoptosis of Balb/c Mice Macrophages Following Nitric Oxide Production

*[1]Leila Pirdel, [2]Ahmad Zavaran Hosseini

Abstract

Objective(s)
Previous studies have demonstrated that the nitric oxide (NO) dependent death of murine peritoneal macrophages activated *in vitro* with IFN-γ and LPS is mediated through apoptosis. In the present study, we investigated the synergistic effect of LPS, IFN-γ and iron on NO production and apoptosis.
Materials and Methods
After determination of iron cytotoxicity, the peritoneal macrophages of Balb/c mice were cultured with iron, LPS, and IFN-γ separately, or a mixture of these for 18 hr at 37 ˚C. Then after 18 hr incubation, the level of NO in supernatant was measured by the Griess method. At the same time, after incubation with ethidium bromide and acridine orange dye, the apoptotic macrophages were detected by fluorescence microscopy.
Results
NO production was significantly greater than the control group in macrophages exposed to iron, LPS, or IFN-γ alone (P=0.02), while no significant difference was detected in apoptosis rate in the presence of LPS (P=0.08). However, the differences were remarkable between NO production and apoptosis rate in the presence of iron, LPS and IFN-γ ($P\leq0.05$).
Conclusion
These findings indicate the immunostimulatory effect of iron on NO production by IFN-γ and LPS.

Keywords: Apoptosis, Interferon gamma, Iron, Lipopolysaccharide, Macrophage, Nitric Oxide

1- College of Medicine and Medical Sciences, Islamic Azad University, Ardebil Branch, Ardebil, Iran
* Corresponding author: Tel: + 98- 182 5267322, Fax: +98- 451 7727805; email: leilapirdel@yahoo.com
2- Department of Immunology, School of Medicine, Tarbiat Modares University, Tehran, Iran

Introduction

Nitric oxide (NO) is a messenger molecule with complex biological activities including vasodilatation, neurotransmission, immunoregulation, and inflammation (1). NO is synthesized enzymatically from L-arginine by at least three different Nitric Oxide Synthases (NOSs) The endothelial and the neuronal isoforms are constitutively expressed and released relatively low level of NO, while the third isoform, inducible NOS (iNOS, NOSII), has to be induced with stimuli that include lipopolysaccharide (LPS) and cytokines (2). Once expressed, NOSII synthesizes large amount of NO, which can lead to the inhibition of T-cell proliferation, has tumoricidal activity, suppresses the cellular protein synthesis, and lead to oxidative damage and apoptosis (1).

NO generated from the NO donor, or synthesized by NOS, induces cell death via apoptosis in a variety of different cell types. These include macrophages (3), dendritic cells (4), thymocytes (5), pancreatic islets (6), certain neurons (7), tumor cells (8), and mast cells (9). The factors affecting cell-specific sensivity to NO-mediated apoptosis can be associated with the redox state within the cells, activation of the apoptotic signaling cascade (such as caspases), the mitochondrial cytochrome c release, or regulation of cell survival and apoptotic gene expression (10).

A number of studies have also described the complex relationships between iron and NO. Iron modulates immune effector mechanisms, such as cytokine activity (IFN-gamma effector pathway towards macrophages), nitric oxide formation or immune cell proliferation, and thus hosts immune surveillance (11). In other experiments have been demonstrated that some forms of iron such as ferric nitrilotriacetic acid (Fe- NTA) are very toxic to renal proximal tubules due to free radical injury by increased NO production (12). Moreover, NO-dependent death of murine peritoneal macrophages activated *in vitro* with IFN-γ and LPS is mediated through apoptosis (4). Hence, we investigated the synergistic effect of LPS, IFN-γ and iron on apoptosis of macrophages of Balb/c mice following NO production *in vitro*.

Materials and Methods

Reagents

All chemicals obtained from Sigma Chemical Co. (St Louis, USA), unless otherwise indicated. Sterile tissue culture plasticwares purchased from NUNC (Roskilde, Denmark).

Iron cytotoxicity analysis

The murine peritoneal cells harvested from five of 6-8 weeks inbred female Balb/c mice without elicitation. The cells were washed, resuspended in complete culture medium (RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 μg/ml streptomycin and 100 IU/ml penicillin) and plated in 96-well (1×10^5 cell/100 μl) culture plates in triplicates.

To analyze iron cytotoxicity, after 2 hr of incubation, nonadherent cells removed by extensive washing. The adherent cells then cultured with 100, 150, 200, 250, 300, 350 and 400 μM of iron at 37 °C in a humidified atmosphere with 5% CO_2. After 1, 2 and 3 hr of incubation, the percentage of cell death determined by using trypan blue in the detached cells. This study approved by the Institutional Review Board (IRB) and the animal use protocol authorized by the Institutional animal care of Tarbiat Modares University.

Cell treatments

The harvested macrophages, as described previously, plated in 6-well (2×10^6 cell/2 ml) culture plates (for fluorescence microscopy investigation). After 2 hr incubation, nonadherent cells removed by extensive washing. The adherent cells then cultured with or without iron (250 μM), LPS and IFN-γ (20 ng/ml). After being cultured for 18 hr, the supernatants harvested for nitrite analysis and adherent cells prepared for fluorescence microscopy study.

Measurement of nitrite production

Cell-free culture fluids obtained by centrifugation and assayed for the stable end product of NO, nitrite, using the Griess method, as described else where. Briefly, in a 96-well plate, 100 µl of each supernatant in triplicate wells incubated with an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid/0.1% naphtylene–diamine hydrochloride) at room temperature for 10 min. Plates placed in an ELISA reader (Multiskan MS, England), at 540 nm. $NaNO_2$ in RPMI used to construct a standard curve for each plate reading (13, 14).

Fluorescence microscopy study of cell death (apoptosis)

To study cell death and morphological changes in the nucleus, we used a dye exclusion method in which viable (intact plasma membrane) and dead (damage plasma membrane) cells can be visualized by fluorescence microscopy (Axiophot, Zeiss, Germany) after staining with two DNA-fluorochromes (fluorescent DNA-binding dyes): ethidium bromide and acridine orange. Ethidium bromide does not penetrate the plasma membrane in viable cells and stains only the non-viable cells, but acridine orange penetrates the plasma membrane without permeabilisation and stains viable and non-viable cells. Apoptotic cells were identified by their morphologic features, such as nuclear fragmentation and chromatin condensation. Fluorescence microscopy with differential uptake of fluorescent DNA binding dyes was a method of choice for its simplicity, rapidity and accuracy.

Statistical analysis

Data analyzed by one-way analysis of variance (ANOVA) and unpaired student's t-test to determine differences between each group. Differences considered statistically significant when $P<0.05$.

Results

Kinetics of iron cytotoxicity

After incubation for 1, 2 and 3 hr, a gradual increase in the percentage of cell death observed. Based on these results, 250 µM of iron defined as an optimal concentration in the following experiments ($P=0.0001$) (Figure 1).

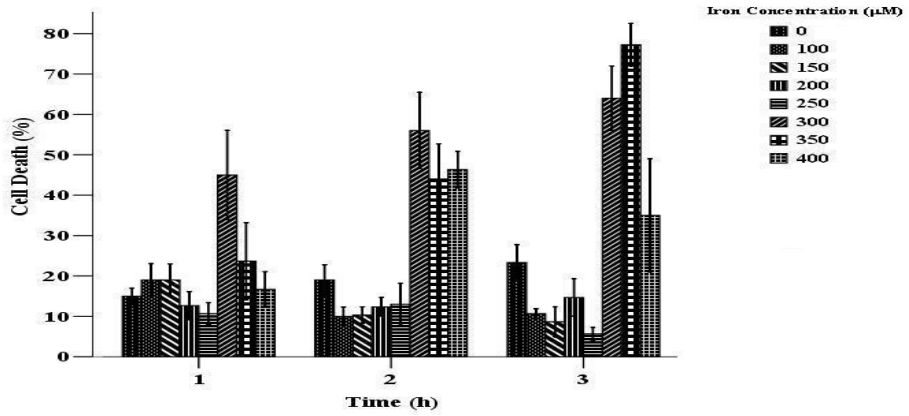

Figure 1. Effect of different concentrations of iron on cell death in murine peritoneal macrophages. Data are mean±SEM of triplicate experiments.

Effect of IFN-γ, LPS and Iron on NO production

There was a synergistic increase in the generation of NO when macrophages co-incubated with IFN-γ/LPS and iron following incubation for 18 hr ($P=0.02$) (Table 1, Figure 2).

Table 1. Effect of administration of LPS, IFN-γ and iron on NO production and the percentage of apoptosis after 18 hr incubation. Values represent mean± SEM (n = 4). *, significant difference ($P<0.05$).

Groups	Nitrite (µM)	Apoptosis (%)
Macrophage	33.09 ± 1.63	3.75 ± 0.75
Macrophage + Iron	31.58 ± 0.21	6.75 ± 0.25
Macrophage + LPS + Iron	56.58 ± 0.21	6 ± 0.70
Macrophage + IFN-γ + Iron	77.20 ± 3.48	9±0.40
Macrophage + LPS + IFN-γ	38.44±4.33	8.25 ± 1.18
Macrophage + LPS + IFN-γ + Iron	102.56 ± 5.62	10.75 ± 1.18

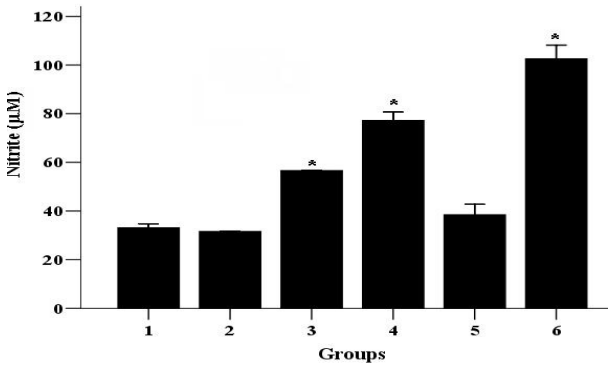

Figure 2. Effect of IFN-γ, or LPS and iron on NO production in peritoneal macrophages. Isolated macrophages were stimulated with IFN-γ, or LPS (each at 20 ng/ml), and iron (250 µM) and the amount of nitrite in the medium which serves as a measure of NO was determined at 18 hr. Data are expressed as mean±SEM (n=4). ***** Statistically different from corresponding control values (P<0.05).

Effect of IFN-γ, LPS and Iron on macrophage apoptosis

To determine the effect of IFN-γ, LPS and iron on macrophage apoptosis, macrophages cultured in medium alone or medium supplemented with either IFN-γ, or LPS, also, a mixture of IFN-γ, LPS and iron. Following incubation for 18 hr, medium collected for NO assay and macrophages undergoing apoptosis identified by fluorescence microscopy. The pictures presented in Figures 4-6 exemplify the nuclear and cytoplasmic changes that detected in the cells undergoing apoptosis. In the presence of IFN-γ/LPS and Iron, the increase in NO production (measured in µM) contributed to increase in the percentage of apoptotic cells. As shown in Table 1 and Figure 3, a significant difference between the increase in NO production and the enhancement of apoptosis, following IFN-γ/LPS and Iron co-stimulation could be observed (P<0.05).

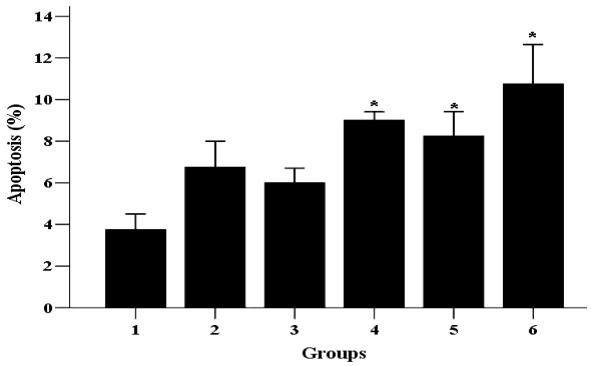

Figure 3. Apoptosis of macrophages exposed to IFN-γ, or LPS, and iron, 18 hr post exposure. Values are expressed as mean±SEM (n=4). ***** Statistically different from corresponding control values (P<0.05).

Figure 4. Viable and apoptotic macrophages in viable cell fluoresced green, whereas nuclear fragmentation are clearly seen in the apoptotic macrophage (arrow) (× 100).

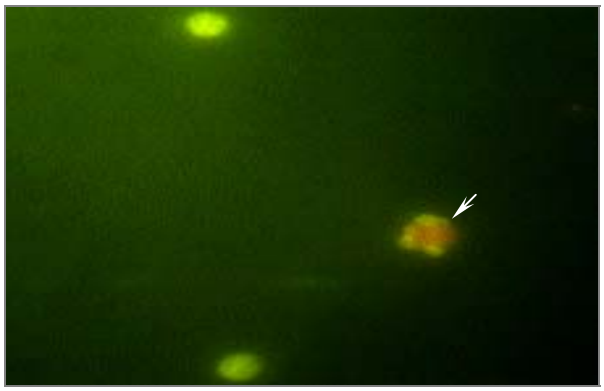

Figure 5. Apoptotic macrophage among two viable cells was identified by morphological features such as nuclear fragmentation (arrow) (× 100).

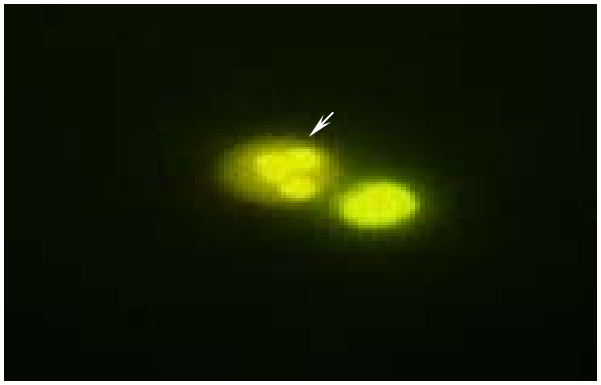

Figure 6. Apoptotic macrophages with nuclear fragmentation with a viable cell (arrow) (×100).

Discussion

NO and its related molecules exert double-edged effects on cell death, depending on its rate of production, redox state of the cells and cell types. NO activates the apoptotic signal cascade in some conditions, whereas it protects cells against spontaneous or induced apoptosis in other cases (10). In addition, iron

also, plays a critical role in macrophage-mediated cytotoxicity by production of highly toxic hydroxyl radical and nitric oxide after activation by immunogenic stimuli, especially IFN-γ and LPS (15).

Considering that LPS induces NO production and death induction of the murine peritoneal macrophages stimulated by LPS itself, it has been demonstrated that activated-macrophages recovered from LPS stimulated cultures, exhibited an intermediate NO resistance. The resistance to NO-induced apoptosis could result from up-regulation of key sets of auto-protective intracellular redox buffering systems such as glutathione, glutathione peroxidase, occurring simultaneously with modulation of expression of apoptotic molecules of the Bcl2-Bcl-XL/Bax-Bad family (16).

Moreover, treatment with IFN-γ in combination with iron were enhanced NO production and rate of apoptosis that might be attributed to the effect of IFN-γ. It has been reported that the IFN-γ induced increases of FasL expression and down-regulated Bcl-2 expression in *Neospora caninum*-infected cells are associated with apoptosis *in vitro* (17).

We found that there was an increase in NO production when macrophages were co-incubated with IFN-γ/LPS, together with iron compared with control cells. In contrast, we did not observe any significant increase in NO production following incubation of macrophages with either IFN-γ/LPS without the addition of iron. This suggests the NO-independent mechanisms of macrophages activated *in vitro* with IFN-γ/LPS, including competitive effects of LPS and IFN-γ on modulation of expression of anti-apoptotic molecules of Bcl-2 family should be evaluated. Further studies are therefore required to clarify this relationship.

Cytokines such as TNF-α and IFN-γ can make cells more susceptible to apoptosis but are insufficient to induce apoptosis (18). However, it has been reported that IFN-γ enhances the sensitivity of many cell types, such as colon carcinoma cells, human T cells, hepatocytes and the U937 leukemia cell line (19). The iNOS induction in macrophage cells by IFN-γ/LPS induces nitrosative stress by increasing the formation of S-nitrosylated proteins, which is a critical factor for the NO-mediated apoptotic cell death (2). We thus believe that the iron is required for generation of high No level in response to LPS and IFN-γ, hence making macrophages susceptible to undergo apoptosis.

In the present study, there was a significant difference between NO level and apoptosis rate in the presence of LPS and iron. This is likely to be NO affinity to iron decreases intracellular iron and ferric level, induced apoptotic cell death after incubation with LPS.

Some studies have reported that LPS can cause apoptosis through the NO production or more reactive peroxynitrite (4, 21), a potent oxidant that causes direct cellular injury and induces apoptosis (20). Kim *et al*. have recently suggested that non hem-iron content determined whether cytotoxic level of NO resulted in apoptosis in hepatic cells (1). The cytotoxicity of NO appears to result from the simultaneous production of NO and superoxide leading to formation of peroxynitrite. The major apoptotic pathway of NO may be associated with cytochrome C release through activation of JNK/SAPK, increases in the Ratio of pro-apoptotic Bax to anti-apoptotic Bcl-xl by the overexpression of p53, or the activation of caspase through mitochondrial cytochrome C release (2, 10, 21). NO also, suppresses the DNA-binding activity of NF-Kβ. It can then downregulate the expression of antiapoptotic genes, such as superoxide dismutase, TRAF1, TRAF2, c-AIP1, and c-AIP2 (2).

Iron or iron-induced oxidative stress have also been found to activate cell signaling cascades triggering apoptosis of hepatocytes via NF-Kβ and AP-1 dependent on gene products (22, 23). It has also, been recognized that induction of apoptosis occurs through poly (ADP-ribose) polymerase and some heat shock proteins (24). However, the upregulation of heme oxygenase (HO-1) and increased production of superoxides when exposed to LPS may play a role in response (25).

The addition of IFN-γ and iron did not induce apoptosis, although they partially lead to NO production. It may be attributable to limiting the availability of iron, because of decreased of expression of transferrin receptor and iron uptake (15). Other possibility is that the level of iron within the cell and the presence of molecular oxygen were insufficient for nitrosylation of caspases by NO (21).

Conclusion

The present study indicates that the apoptosis pathways elicited IFN-γ and LPS in peritoneal macrophages involve modification of cellular iron homeostasis through NO-dependent mechanisms. Then the iron can increase synergistically induction of apoptosis through IFN-γ/LPS-induced NOS2 expression. Furthermore, IFN-γ/LPS and iron not only increase NO production but can also lead to an increase in apoptosis.

Acknowledgment

We are thankful to Dr Taghi Tiraihi (Department of Anatomical Sciences, Tarbiat Modares University, Tehran, Iran) for his useful information. The authors declare that they have no conflict of interests.

References

1. Feger F, Ferry-Dunazet H, Matsuda MM, Bordenave J, Dupouy M, Nussler AK, *et al*. Role of iron in tumor cell protection from the pro-apoptotic effect of nitric oxide. Cancer Res 2001; 61:5289-5294.
2. Kim KK, Kim PK, Kwon YG, Bai SK, Nam WD, Kim YM. Regulation of apoptosis by nitrosative stress. J Biochem Mol Biol 2002; 35:127-133.
3. Albina JE, Cui S, Mateo RB, Reichner JS. Nitric oxide-mediated apoptosis in murine peritoneal macrophages. J Immunol 1998; 150:5080-5085.
4. Lu I, Bonham CA, Chambers FG, Watkins SC, Holfman RA, Simmons RI, *et al*. Induction of nitric oxide synthase in mouse dendritic cells by IFN-gamma, endotoxin, and interaction with allogenic T cells: nitric oxide production is associated with dentritic cell apoptosis. J Immunol 1998; 157:3577-3586.
5. Gordon SA, Abou-Jaoude W, Hoffman RA, MacCarthy SA, Kim YM, Zhou X, *et al*. Nitric oxide induces murine thymocyte apoptosis by oxidative injury and a p53-dependent mechanism. J Leukoc Biol 2001; 70:87-95.
6. Kaneto H, Fujii J, Seo HG, Suzuki K, Matsuoka T, Nakamura M, *et al*. Apoptotic cell death triggered by nitric oxide in pancreatic beta-cells. Diabetes 1995; 44:733-738.
7. Dawson VL, Kizushi VM, Huang PL, Synder SH, Dawson TM. Resistance to neurotoxicity in cortical cultures from neuronal nitric oxide synthase-deficient mice. J Neurosci 1996; 16:2479-2487.
8. Hirakava M, Oike M, Masuda K, Ito Y. Tumor cell apoptosis by irradiation-induced nitric oxide production in vascular endothelium. Cancer Res 2002; 62:1450-1457.
9. McCauley SD, Gilchrist M, Befus AD. Nitric oxide: a major determination of mast cell phenotype and function. Mem Inst Oswaldo Cruz 2005; 100:11-14.
10. Choi BM, Pae HO, Jane SI, Kim YM, Chung HT. Nitric oxide as a pro-apoptotic as well as anti-apoptotic modulator. J Biochem Mol Biol 2002; 35:116-126.
11. Huang H. Iron overload and its association with cancer risk in humans: evidence for iron as a carcinogenic metal. Mutat Res 2003; 533:153-171.
12. Hida AI, Kawabata T, Minamiyama Y, Mizote A,Okada S. Saccharated colloidal iron enhances lipopolysaccharide- induced nitric oxide production *in vivo*. Free Radic Biol Med 2003; 34:1426-1434.
13. Kharazi S, Zavaran HA, Tiraihi T. The role of overproduction of nitric oxide in apoptosis of BALB/C mice macrophages infected with leishmania major *in vitro*. IAARI 2003; 2(4):209-214.
14. Pirdel L, Zavaran HA, Tiraihi T. Role of iron overload in apoptosis of Balb/c mice macrophages infected with leishmania major in vitro. Yakhteh 2007; 9(3):196-201.
15. Mulero V, Brock JH. Regulation of iron metabolism in murine J774 macrophages: role of nitric oxide-dependent and independent pathways following activation with gamma interferon and lipopolysaccharide. Blood 1999; 94:2383-2389.
16. Ferret PJ, Soum E, Negre O, Fradelizi D. Auto-protective redox buffering systems in stimulated macrophages. BMC Immunol 2002; 3:1-13.
17. Nishikawa Y, Mishima M, Nagasawa H, Igarash I, Fujisaki K, Otsuka H, *et al*. Interferon-gamma-induced apoptosis in host cells infected with *Neospora caninum*. Parasitology 2001; 123: 25-31.
18. Meier P, Dayer E, Blanc E, Wauters JP. Early T cell activation correlates with expression of apoptosis markers in patients with end-stage renal disease. J Am Soc Nephrol 2002; 13:204-212.

19. Luttmann W, Dauer E, Schmidt S, Mark O, Hossfeld M, Matthys H, *et al*. Effects of interferon-γ and tumour necrosis factor-α on CD95/Fas ligand-mediated apoptosis in human blood eosinophils. Scand J Immunol 2000; 51:54-59.
20. Kim YM, Chung HT, Kim SS, Han JA, Yoo YM, Kim KM. Nitric oxide protects PC12 cells from serum deprivation-induced apoptosis by cGMP-dependent inhibition of caspase signaling. J Neurosci 1999; 19:6740-6747.
21. Gonzalez A, Lopez AS, Alegre E, Alcazar JL, Lopez-Moratalla N. Dose nitric oxide plays a role in maternal tolerance towards the foetus? J Physiol Biochem 2004; 60: 227-238.
22. Philippe MA, Ruddell RG, Ramm GA. Role of iron in hepatic fibrosis: one piece in the puzzle. World J Gastroentrol 2007; 13:4746-4754.
23. Liu TZ, Lee KT, Chern CL, Cheng JT, Stern A, Tasi LY. Free radical-triggered hepatic injury of experimental obstructive jaunide of rats involves overproduction activation of nuclear factor κB. Ann Clin Lab Sci 2001; 31:383-390.
24. Kawabata T, Ma Y, Yamador I, Okada S. Iron-induced apoptosis in mouse renal proximal tunules after an injection of renal carcinogen, iron-nitrilotriacetate. Carcinogenesis 1997; 18:1389-1394.
25. Hsieh CH, Jeng SF, Hsieh MW, Chen YC, Rau CS, Lu TH, *et al*. Statin-induced heme oxygenase-1 increases NF-κB activation and oxygen radical production in cultured neuronal cells exposed to lipopolysaccharide. J Toxicol Sci 2008; 102:150-159.